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Effect of cinnamon supplementation on the gut environment and ESR gene expression in the ovaries of laying quail

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Abstract: This study aimed to analyze the effects of adding cinnamon powder to the diet on the expression of genes related to the antioxidant defense system in the intestine, the expression of alpha estrogen receptor (*ESRα*) gene in the ovary, and the intestinal histomorphometry in laying quails. We used 144 quails distributed in a completely randomized design with two treatments: without cinnamon supplementation (NSC) and the supplementation of 9 g/kg of cinnamon powder (CPS) added to the diet for 84 days. At the end of the experimental period, animals were sacrificed and the ovarian and duodenal tissues were collected for the expression analysis of the tumor necrosis factor alpha (*TNFα*), glutathione peroxidase 7 (*GPX7*), catalase (*CAT*), superoxide dismutase (*SOD*), and estrogen receptor alfa (*ESRα*) genes. Fragments of the central portions of the duodenum and jejunum were also collected for intestinal histological analysis. It was observed that quails fed with CPS diet had greater expression of the *ESRα* gene ($P = 0.0004$) than the ones from NCS diet. The animals from the CPS treatment also presented a higher height of villi ($P = 0.004$) and greater depth of crypts ($P = 0.013$) in the duodenum, and a higher height of villi and villus:crypt ratio ($P = 0.005$) in the jejunum. This can improve the efficiency of nutrient absorption in the intestine, resulting in a better productive performance of the birds in the phase of posture.
Keywords: Eggs, eugenol, free radicals, ovary, histomorphometry.

1. Introduction

The high demand related to continuous egg production in laying quails may result in increased production of reactive oxygen species (ROS). These increased ROS act by damaging the cellular components of tissues, which can lead to cell death, bleeding, and serious infections (Beattie and Jimenez, 2019). Studies have shown that the action of free radicals can impair the fertility of birds, causing cell apoptosis and follicular atresia in the ovary and impairing the synthesis of sex hormones (Rafieian-Naeini et al., 2021; Zhu et al., 2021). In males, oxidative stress is also related to the infertility, mainly due to impaired sperm motility (Khalil-Khalili et al., 2021). Thus, it is important counting on the action of efficient antioxidant mechanisms.

However, besides the efficiency of the reproductive system, egg production also depends on the proper use of the nutrients from the diet, which is directly related to the birds' intestinal health (Mehaisen et al., 2019). Supplementation using plant extracts that enhance the body's antioxidant defense, whether by modulating the action of antioxidant enzymes or by contributing to a healthy intestinal environment, can increase the productive efficiency of poultry (Mustafa and Wasman, 2020). Eugenol, a compound present in cinnamon bark (*Cinnamomum zeylanicum L.*) in concentrations of up to 5.7 mg/g (Liyanage et al., 2021), was described to have potential antioxidant, antibacterial and antimicrobial properties (Abd El-Hack et al., 2020; Mohamed et al., 2020). Mehdipour et al. (2013) found that dietary supplementation with cinnamon oil (200 mg/kg) significantly improved the feed conversion rate of quails. Mehdipour and Afsharmanesh (2018) showed that the ileal coliform count was decreased and the ileal Lactobacillus count was increased in quails fed on cinnamon oil (200 ppm/kg of diet). According to Chowdhury et al. (2018), supplementation of 300 mg/kg of cinnamon bark oil reduced the number of *Escherichia coli* in the pre-cecal content of chickens. According to Krauze et al. (2021), the inclusion of a phytobiotic containing cinnamon oil in the proportion of 0.25 mL/L of water in the quail diet improved the bird performance, which may be associated with the beneficial effect of the preparation on the microbiome and gut morphometry.

Previous data from our research group has observed that the supplementation of 9 g/kg of powdered cinnamon in the laying quail diet contributed to better efficiency in feed conversion and egg mass (2.42 vs 2.49g/g, Bastos et al., 2017). We suggest that this result is related to the antioxidant activity and expression of genes linked to lipid metabolism in the liver of these birds. In view of this, we observed the importance of knowing the action of cinnamon in the intestinal environment of quails, since intestinal health and adequate absorption of nutrients can also influence egg production (Abad et al., 2020; El-Sabrout et al., 2023).

This research was carried out with the objective of evaluating the effect of powdered cinnamon bark supplementation on intestinal histomorphometry and on the expression of *TNFα, GPX7, CAT* and *SOD* genes in the duodenum and *ESRα* in the ovary of laying quails. Our hypothesis is that the supplementation of powdered cinnamon bark in the diet of laying quails could contribute to the increase the expression of genes related to the antioxidant defense systems in the intestine and improve the intestinal environment.

2. Material and methods

This experiment was approved by the animal production research ethics committee of the Federal University of Sergipe (Protocol No. 09/2015).

2.1. Animals and experimental design

One hundred forty-four Japanese quail (*Coturnix japonica*) obtained from a commercial hatchery (VICAMI, Assis, Brazil) were used in this experiment. The quails aged 15 weeks old and with an average weight of 133 g were housed in a masonry shed, uniformly distributed in laying cages (0.50 x 0.15 x 0.35 m).

Birds with a laying rate of 85% and 18 weeks of age were distributed in a completely randomized design with two treatments: no cinnamon supplementation (NCS - control group) and cinnamon powder supplementation of 9 g/kg (CPS) to replace the inert filler (Kaolin). Each treatment consisted of six replicates (6 subgroups) with 12 birds each, totaling 72 quails per treatment.

The experimental diets (Table 1) were formulated based on corn and soybean meals according to the nutritional recommendations found in the Brazilian Tables for Poultry and Swine (Rostagno et al., 2017). The experiment lasted for 84 days. The birds received water and feed *ad libitum* and a 16-hour daily light program. At the end of the experimental period, six animals from each treatment were weighed and euthanized by cervical dislocation. The animals were eviscerated to evaluate the relative weight of the intestine, along with intestine length. The relative weight was calculated as (organ weight/bird weight) x100.

Diet	(%)
Corn	57.792
Soybeanmeal 45%	30.450
Soyoil	1.561
Dicalciumphosphate	1.091
Limestone	6.802
Salt	0.323
L-LysineHCl	0.261
DL-Methionine	0.396
L-Threonine	0.024
Vitaminand mineral mixture1	0.100
Kaolin (Inertfiller)	1.200
Total	100.000
Nutritionalcomposition	
Metabolizableenergy (kcal/kg)	2807
Crudeprotein (%)	18.80
Fat (%)	4.101
Calcium (%)	2.922
Availablephosphorus (%)	0.304
Sodim (%)	0.146
Chlorine (%)	0.242
Potassium (%)	0.725
Digestible amino acids (%)	
Methionine	0.642
Methionine + Cysteine	0.900
Lysine	1.097
Threonine	0.658
Tryptophan	0.206

[1]Vitamin and mineral misture (guaranteed levels per kg of product): Ac. Folic (min.) 200mg; ac. pantothenic (min.) 5.350 mg; copper (mín.) 4.000 mg; iron (mín) 20 g; iodine (mín.) 1.500 mg; manganese (mín.) 75 g; niacin (mín.) 19,9 g; selenium (mín.) 250 mg; Vit. A (mín.) 8.000.000 IU; Vit.B12 (mín.) 10.000 mcg; Vit.B2 (mín.) 4.000 mg; Vit.B6 (mín.) 1.000 mg; Vit. D3 (mín) 2.000.000 IU; Vit. E (mín.) 15.000 IU; Vit. K3 (mín.) 2.000 mg; zinc (mín.) 50 g.

Table 1 – Percent composition and nutritional values of basal diet.

2.2. Gene expression

At the end of the experimental periodtissue samples were collected from the duodenum and ovary of 4 birds from each treatment and stored in RNA Holder (Bio Agency Biotecnologia, SãoPaulo, Brasil) at -20°C until total RNA was extracted.

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions (1 mL per 100 mg of tissue). All of the materials used were pre-treated with the RNase inhibitor RNase AWAY (Invitrogen, Carlsbad, USA). The total RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. RNA integrity was analyzed by 1% agarose gel electrophoresis stained with ethidium bromide (10 mg/mL), and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions to remove potential genomic DNA contamination.

A GoScrip Reverse Transcription System (Promega Corporation, Fitchburg, USA) kit was used for cDNA synthesis from 1 µg of DNase-treated total RNA, according to the manufacturer's instructions. The cDNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. The cDNA samples were diluted to 40 ng/µL and stored at -20°C until further use

as a template in the amplification reaction. Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN PCR Master Mix (Applied Biosystems, Waltham, USA). The amplification reaction consisted of 5 µL of diluted cDNA, 0.5 µL of each primer (forward and reverse) at 10 µM (final concentration: 200 nM), 12.5 µL of SYBR GREEN PCR Master Mix, and water to a total volume of 25 µL. To measure the efficiency of each primer/gene set, a series of 25 µL reactions was analyzed as described above using 5 µL of a serial dilution of pooled cDNA as the template. The thermal cycling parameters for all genes were as follows: hot-start at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min, and ending with a melt curve from 65–95°C. All of the analyses were performed in duplicate.

The primers used for *SOD, GPx 7, CAT, TNFα* and *ESRα* were designed according to the gene sequences deposited at www.ncbi.nlm.nih.gov (accession numbers, NM_205064.1, NM_001163245.1, XM_015863594.1, NM_204267.1, and XM_015858163.1, respectively) using the site www.idtdna.com. ß-actin (accession number L08165) was selected as endogenous control because its amplification efficiency was similar to the amplification efficiency of the target genes. The amplification efficiencies (90% to 110%) were similar for the genes of interest (Table 2). Analysis of the dissociation curves did not reveal any non-specific PCR products, such as the formation of primer dimers. Thus, demonstrating the reliability of the data for estimating the mRNA expression of the evaluated genes. The endogenous control, β-actin, did not show any significant differences between treatments, which confirmed its suitability as a control.

Genes	Amplicom (Pb)	AT (°C)	Sequences of primers (5'-3')1
SOD2	126	60	TGGACCTCGTTTAGCTTGTG ACACGGAAGAGCAAGTACAG
GPX7	140	60	TTGTAAACATCAGGGGCAAA TGGGCCAAGATCTTTCTGTAA
CAT	76	60	TTGGGTTGGCTCGTTGAGG CGGAGCTACAGAAGCACGAT
TNFα	64	60	GAGCGTTGACTTGGCTGTC AAGCAACAACCAGCTATGCAC
ESRα	103	60	AAGGGAAATGTGTAGAGGGC ACACCAGAATTGAGCAGGATG
ß-actina	136	60	ACCCCAAAGCCAACAGA CCAGAGTCCATCACAATACC

[1]Pb, amplicom size in base pairs; AT, annealing temperature
[2]*SOD*, superoxidedismutase; *GPX7*, glutathioneperoxidase 7; *CAT*, catalase; *TNFα*, tumor necrosisfactor alpha; *ESRα*, estrogen receptor alfa.

Table 2 – Primers used for qRT-PCR.

2.3. Histomorphometry

Fragments of approximately 2.0 cm in length were collected from the middle portions of the duodenum and jejunum segments of ten animals from each treatment. These fragments were fixed in Formol 10%, according to the methodology described by Sun et al. (2005). Subsequently, the samples were dehydrated, diaphanized, embedded in paraffin, and cut at 5.0 µm, then the slides were prepared and stained with hematoxylin-eosin. Two slides of each segment and of each animal were photographed and analyzed in Motic Images Plus 3.0 image analyzer. The lengths of 15 villi and 15 well-oriented crypts of each intestinal region per animal were selected and measured according to the unit adopted (µm). Measurements of villus height were taken from the basal villi, coinciding with the upper portion of the crypts, to their apex, and the depth of the crypts was measured from their base to the crypt:villus transition. The relation between villi height and depth of crypts (height of villi/depth of crypt) was also calculated. The histology results were log-transformed to standardize the data.

2.4. Statistical analysis

The Shapiro-Wilk test was used to verify the normality of the expression data of the genes under study (expressed as $2^{-\Delta Ct}$) and the other data evaluated. One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$) between the treatments (SAS Inst. Inc., Cary, USA). The results are presented as means and standard error.

3. Results

3.1. Gene expression

Table 3 shows the results of the expression of genes related to antioxidant activity in the duodenum of laying quails fed with or without cinnamon powder supplementation. There was no effect of the cinnamon on the expression of the evaluated genes.

Gene	NCS	CPS	*P* value
TNFα1	0.403 (±0.043)	0.481±0.086	0.3977
GPX7	0.860±0.107	0.815±0.166	0.3021
CAT	0.119±0.016	0.090±0.006	0.1679
SOD	2.194±0.794	2.040±0.853	0.0927

[1]*TNFα*, tumor necrosis factor alpha; *GPX7*, glutathione peroxidase 7; *CAT*, catalase; *SOD*, superoxide dismutase

Table 3 – Mean and standard error of gene expression in the duodenum of laying quail receiving a diet with no cinnamon supplementation (NCS) and with cinnamon powder supplementation (CPS).

The treatments evaluated significantly influenced estrogen receptor alfa gene expression (P = 0.0004); birds that received cinnamon inclusion had higher ESRα expression than birds that consumed basal diet (35.3 vs 8.27AU) (Figure 1).

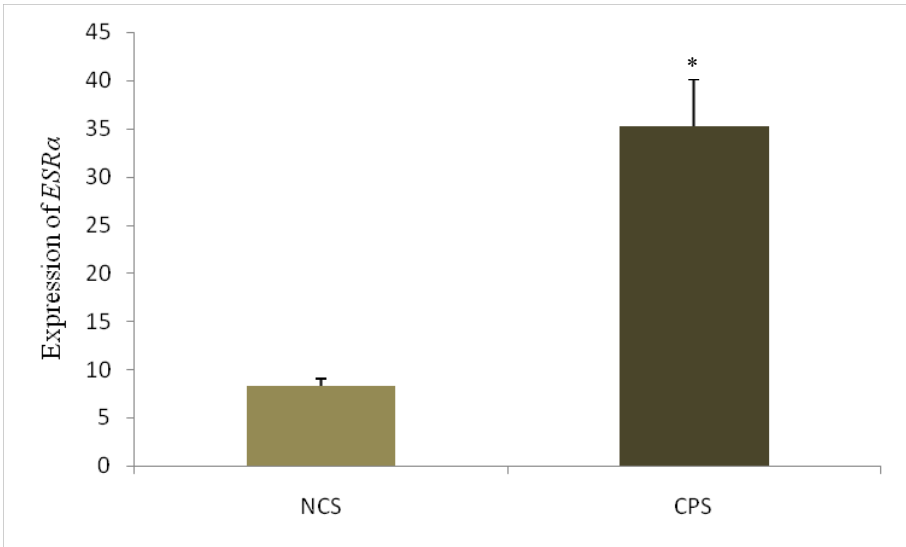

Figure 1 – Average and standard errors of the expression of ESRα (AU) mRNA in the laying quail's ovary receiving diets with (CPS) and without cinnamon powder (NCS). * Significant by the F test P = 0.0004.

3.2. Histomorphometry

The results of histomorphometry can be observed in Table 4. There was a significant effect *P* = 0.004 of cinnamon supplementation on villus height, crypt depth, but not on villus:crypt relation in the duodenum. Quails fed CPS diet presented the highest values of villus height (150.14µM), crypts depth (13.41µM), and villus:crypt relation (11.18 µM). In the jejunum, higher villus height and villus:crypt relation of 102.98µM and 9.14µM, respectively were observed in quails fed with the CPS diet.

We observed a significant effect of the inclusion of 9 g/kg of powdered cinnamon in the diet for laying quails on the relative weight of the intestine (*P* = 0.039): quails fed CPS diet presented lower intestine relative weight than quails fed NCS diet (2.849 vs 3.653, respectively).

Duodenum	NCS	CPS	*P* value
Villus height	104.00 (±0.04)	150.14±0.030	0.004*
Crypt depth	11.85 (±0.01)	13.41±0.014	0.013*
Villus:crypt relation	8.91 (±0.05)	11.18±0.0241	0.051*
Jejunum			
Villus height	65.21±0.0607	102.98±0.0319	0.005*
Crypt depth	10.30±0.0282	11.28±0.0247	0.313
Villus:crypt relation	6.31±0.0536	9.14±0.022	0.005*
Intestine			
Intestine relative weight (g)	3.65 ± 0.256	2.85± 0.113	0.039*
Intestine length (cm)	44.28 ± 1.963	42.35 ± 2.169	0.714

Table 4 – Means and standard error (SE) of histomorphometry (µm) results of laying quails receiving a diet with no cinnamon supplementation (NCS) and with cinnamon powder supplementation (CPS).

4. Discussion

The intestinal health of the birds is directly related to their productive performance (Diaz Carrasco et al., 2019) since maintaining the integrity of the intestinal structures and controlling the microorganisms present in this segment can improve the absorption of the nutrients provided through the diet (Adedokun and Olojede, 2019; Alagawany et al., 2021), and thus, improve egg production.

The intestinal lining of birds is composed of microscopic structures (villi) that increase the contact surface, providing greater efficiency in nutrient absorption (Rezaei et al., 2018; Shini et al., 2021). When compared to other species, these villi play a crucial role in the metabolism, since it is through the cells at the apex of these structures that transeptelial transport of nutrients from food digestion occurs (Wickramasuriya et al., 2022).

According to Pluske et al. (1997), villus height is an important feature that determines the intestinal health of birds, as well as crypt depth is an indicative of cell proliferative activity, which ensures tissue renewal when other cells are lost at apex of villus. In our study, cinnamon supplementation increased the villi height, the depth of the crypts and the villi:crypt relationship in the duodenum of the birds, as well as increased villi height and the villi:crypt ratio in the jejunum. These results may indicate that the cinnamon may have improved the intestinal environment favoring better development of the structures that compose it. Researchers point out that the best efficiency in nutrient utilization coupled with better productive performance is directly related to the height of the birds' intestinal villi (Oso et al., 2019; Yadav and Jha, 2019).

The integrity of intestinal epithelial microstructures is strongly influenced by factors such as nutritional management, sanity and oxidative stress (Escobar et al., 2020), and when there is imbalance in the production and elimination of free radicals, the cells at the intestinal lining can be damaged with structure and function changes and even that cell death (Ifeanyi, 2018; Chen et al., 2021). Thus, studies have shown the importance of the balance between the production and elimination of free radicals for a good productive performance of the laying birds (Surai et al., 2019; Abdel Moneim et al., 2020). In our study, unlike our initial hypothesis, we did not observe a significant effect of cinnamon supplementation on any of the genes evaluated in the duodenum. However, based on the positive effect observed in the results of morphology, we can suggest that in conditions where there is no environmental challenge, there is no change in the expression of genes related to the antioxidant activity, but even so, cinnamon supplementation contributes to greater efficiency in egg production (Bastos et al., 2017) because it contributes to a greater development of the intestinal structures related to the efficiency in the absorption of nutrients.

In addition to the health in the intestinal environment, the fertility of the laying birds also depends on the action of reproductive hormones, such as estrogen that has its biological functions manifested through the binding with high-affinity receptors in the ovary, such as estrogen receptor alfa (*ESRα*), which is essential in the egg synthesis process (Çiftci, 2017; Jiang et al., 2020). In our study, besides the positive effect on the intestinal morphology, we also observed a higher expression of *ESRα* in the ovary of birds that received cinnamon in the diet, indicating that cinnamon supplementation may contribute to greater balance in the body and act on the expression of genes related to reproduction in the ovary of birds at laying phase.

5. Conclusion

The results show that cinnamon powder supplementation in the diet of laying quail increased villus height, crypt depth and improved the villus:crypt ratio in intestine segments. This may be a reflection of the beneficial effects provided by cinnamon to the intestinal health of birds. This may also favor the absorption of nutrients and improve the performance of laying hens, since we observed greater expression of the *ESRa* gene in the ovary of quails supplemented with powdered cinnamon.

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