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Hormetic dose-response of pure hydroxytyrosol in laying hens: Optimal dose for egg shelf-life extension and yolk docosahexaenoic (DHA) and eicosapentaenoic (EPA) acid concentration

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ABSTRACT

Hydroxytyrosol (HT) is a potent olive-derived antioxidant, but its dose-response effects on laying hen performance, gut health, and egg quality remain poorly defined. This study evaluated the effects of dietary supplementation with pure HT (0, 5, 10, 20, and 50 mg/kg) on productive performance, intestinal morphometry, oxidative stability, and egg quality in ISA Brown hens at 80 weeks of age. Performance traits and serum biochemical indicators were not influenced by HT supplementation ($P > 0.05$). Regarding intestinal morphometry, villus height decreased linearly with increasing HT ($P < 0.001$), suggesting a physiological threshold at higher doses. For egg quality, HT reduced yolk TBARS linearly after 30 days at 22 ± 4°C ($P < 0.001$), while Haugh units showed a quadratic, hormetic response with best preservation at 10–20 mg/kg ($P < 0.001$) and lower efficacy at 50 mg/kg. Docosahexaenoic acid (DHA) content in yolk was highest at 20 mg/kg ($P = 0.032$), with a quadratic response for eicosapentaenoic acid (EPA) concentration (Quadratic effect: $P = 0.016$). Multivariate analysis (PCA/HCPC) confirmed distinct metabolic profiles and identified 20 mg/kg as the optimal biological threshold. In conclusion, 20 mg/kg pure HT is the optimal dose to extend egg shelf life and enrich eggs with n-3 PUFAs, whereas 50 mg/kg exceeds the physiological threshold, limiting intestinal morphology and egg quality. These results provide a clear dose recommendation for producing functional eggs with enhanced oxidative stability and DHA content under non-refrigerated storage and are particularly relevant for egg production systems in tropical and subtropical regions where non-refrigerated storage is common.

Introduction

Eggs are a highly nutritious food, rich in high-quality proteins and bioactive lipids that contribute to cardiovascular health, cognitive function, and physiological homeostasis (Puglisi and Fernandez, 2022; Xiao et al., 2020). Enriching eggs with n-3 polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA), is a widely adopted strategy to enhance their nutritional value and consumer health benefits (Maina et al., 2023). However, the high PUFA content in yolk makes eggs prone to lipid oxidation during storage, leading to rancidity,

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off-flavors, and reduced shelf life (De Leon and Borges, 2020; Matumoto-Pintro et al., 2017). This challenge is particularly acute in tropical and subtropical regions, such as Brazil, where eggs are commonly stored at room temperature due to logistical and economic constraints (Malfatti et al., 2021). Therefore, alternative strategies to maintain egg quality during storage, such as in-feed antioxidants, are of great interest to the poultry industry.

Hydroxytyrosol (HT), a phenolic compound derived from olives, is a powerful antioxidant with proven benefits in broilers, including enhanced performance, gut health, and anti-inflammatory properties (Dias et al., 2024; Shan and Miao, 2022). HT effectively shields low-density lipoprotein from oxidation even at low doses and possesses a high safety margin, with a no-observed-adverse-effect level (NOAEL) of 500 mg/kg/day in toxicological assessments (Auñon-Calles et al., 2013a, 2013b; Rietjens et al., 2007). Nonetheless, most poultry research has utilized crude olive extracts or by-products, which differ in composition and HT content (Al-Harthi, 2015; Dedousi et al., 2022; Papadopoulos et al., 2023). Data on the dose-response effects of pure HT in laying hens, particularly regarding gut morphology and egg shelf life, remain limited. To date, most available studies have focused on broiler or mixed olive by-products, leaving a critical gap in understanding how pure HT modulates gut health and egg functional quality in late-phase laying hens under non-refrigerated storage conditions.

In poultry nutrition, many bioactive compounds exhibit a hormetic dose-response, in which moderate doses improve performance and poultry quality, whereas high doses become detrimental (Anagnostopoulos and Mountzouris, 2025; El-Hack et al., 2023). For antioxidants, this often manifests as improved oxidative stability and product quality at intermediate doses, but pro-oxidant or cytotoxic effects at supra-physiological levels (Lambert and Elias, 2010; Viveros et al., 2011). Therefore, identifying the optimal inclusion level is critical for both efficacy and safety.

The objective of this study was to evaluate the dose-dependent effects of dietary pure hydroxytyrosol (0, 5, 10, 20, and 50 mg/kg) on productive performance, intestinal morphometry, serum biochemistry, and egg quality (fresh and after 30 days of storage at room temperature) in ISA Brown laying hens. We hypothesized that HT would exhibit a hormetic dose-response, improving egg shelf life and DHA concentration at moderate doses (10 to 20 mg/kg) but impairing gut health and egg quality at high doses (50 mg/kg). The results provide a clear dose recommendation for using pure HT to produce functional eggs with extended shelf life and enhanced n-3 PUFA content under non-refrigerated storage conditions.

Materials and methods

Ethical approval and experimental design

The experimental protocol was approved by the Animal Use Ethics Committee of Santa Catarina State University (CEUA UDESC) under registration number 9735210622. The trial was conducted in the experimental poultry house for commercial laying hens of the Department of Animal Science at Santa Catarina State University – UDESC (Chapecó, SC, Brazil) using 150 ISA Brown semi-heavy laying hens (64 to 80 weeks of age; initial body weight 1.85 ± 0.12 kg; baseline egg production 88 ± 3%). The birds were randomly allocated to cages based on similar initial body weight and egg production to minimize variation. To evaluate long-term effects on performance and egg quality, the experiment lasted 112 days and was divided into four 28-day cycles. Environmental conditions were maintained at 22 ± 3°C, 65 ± 5% relative humidity, 16L:8D photoperiod, and ad libitum access to feed and water.

Hens were housed in cages (80 × 60 × 50 cm; 5 birds/cage) in a completely randomized design with five treatments (0, 5, 10, 20, 50 mg/kg hydroxytyrosol) and six replicates (cages) per treatment ($n = 30$ cages total). The cage (groups of five hens) was considered the experimental unit for all statistical analyses. For variables measured on individual birds or eggs, values from birds/eggs within the same cage were averaged so that each cage contributed a single observation to the analysis, thereby avoiding pseudoreplication.

Diets and treatments

Five isoenergetic and isonitrogenous corn-soybean-based diets were formulated to meet the nutrient requirements of late-phase laying hens according to the Brazilian Tables for Poultry and Swine (Rostagno et al., 2017). Treatments consisted of increasing concentrations of pure hydroxytyrosol (HT): 0 (control), 5, 10, 20, and 50 mg/kg diet. Hydroxytyrosol was provided as 1-HT powder (25%HT at ≥98% purity, 75% chicory root inulin excipient; Nova Mentis Ltd., Dublin, Ireland).

The specific concentration of hydroxytyrosol in the experimental diets was calculated from the additive's declared purity (25% HT with ≥ 98% purity), because no validated analytical method was available for reliable ppm-level HT quantification in complex feed matrices. To promote dosage accuracy and homogeneity, the commercial HT additive was first premixed with ground corn and then incorporated into the basal diet using a horizontal ribbon blender for 15 minutes, in accordance with standard feed manufacturing practices. The diet composition and the fatty acid profile are presented in Tables 1 and 2.

Productive performance and egg quality

Feed intake, egg production (%), egg weight, egg mass, and feed conversion ratio (FCR; kg feed/kg egg and kg feed/dozen eggs) were recorded weekly. On the last day of each cycle and at the end of the experiment, 12 fresh eggs per cage were evaluated for quality, and 12 eggs were also assessed for quality after 30 days of storage at 22 ± 4°C and 65 ± 5% RH. Specific gravity was determined by flotation (Freitas et al., 2004). Shell strength (kgf) was measured using a texturometer (TA.XT Plus, Stable Micro Systems, Godalming, UK). Haugh unit was calculated from albumen height (tripod micrometer) and egg weight

Table 1
Composition and calculated nutrient levels of the experimental diet for ISA Brown laying hens (64 to 80 weeks of age).

Ingredients	Composition (%)
Corn	65.70
Soybean meal (45% Crude Protein)	21.80
DL- Methionine (99%)	0.200
Limestone	8.90
Dicalcium phosphate	1.50
Soy oil	1.10
Sodium chloride	0.500
Vitamin and Mineral Premix*	0.300
Total	100.00
Calculated nutrient levels	
Metabolizable energy (kcal/kg)	2.850
Crude protein (%)	15.60
Calcium (%)	3.88
Available phosphorus (%)	0.37
Sodium (%)	0.23
Potassium (%)	0.60
Digestible lysine (%)	0.68
Digestible methionine (%)	0.43
Digestible methionine + cystine (%)	0.65
Digestible threonine (%)	0.52
Digestible tryptophane (%)	0.16

* Vitamin and mineral premix composition per kg of product: Vitamin A (as retinyl acetate) – 4,000,000 IU; Cholecalciferol – 400,000 IU; Vitamin E (as dl-α-tocopheryl acetate) – 4,000 IU; Vitamin B1 (Thiamine mononitrate) – 3.0 g; Vitamin B2 (Riboflavin) – 1.0 g; Vitamin B6 (Pyridoxine HCl) – 1.0 g; Vitamin B12 (Cyanocobalamin) – 40 mg; Nicotinic acid – 6 g; Pantothenic acid – 2 g; Vitamin K3 (Menadione) – 1.0 g; Folic acid – 0.25 g, Manganese (MnSO4) – 15 g; Iron (FeSO4) – 20 g; Zinc (ZnSO4) – 12 g; Copper (CuSO4) – 2.5 g; Cobalt – 0.5 g; Iodine – 0,25 g; Selenium – 60 mg; excipient – 500 g.

Table 2
Analyzed concentrations of fatty acids and total fat in the feed (%).

Fatty acid	%	Fatty acid	%
C4:0	0.127	C18:1 N-9T	0.1025
C10:0	0.0145	C18:1 N-9C	33.218
C13:0	0.0229	C18:2 N-6C	43.841
C14:0	0.0234	C20:0	0.3598
C14:1	0.0175	C18:3 N-6	0.0287
C15:0	0.0149	C20:1 N-9	0.2574
C16:0	14.142	C18:3 N-3	2.478
C16:1	0.1351	C20:2 CIS	0.0134
C17:0	0.1112	C20:4 N-6	0.0429
C17:1	0.0503	C24:0	0.2629
C18:0	4.329	Total fat	4.68

using the expression: HU = 100 x log (H + 7.57 – 1.7 x $W^{0.37}$), in which HU = Haugh unit, h = albumen height (mm) and w = egg weight (g) (Haugh, 1937). Yolk color (L*, a*, b*) was assessed using DSM color fan (DSM Nutritional Products, Basel, Switzerland) and Minolta CR-400 colorimeter (Konica Minolta Sensing Inc., Tokyo, Japan).

Biochemical parameters and lipid oxidation

At the end of the experiment, blood was collected from the ulnar vein of 2 birds per cage ($n = 12$/treatment), pooled per cage, and centrifuged (3,000 x g, 10 min, 4°C) to separate serum. Commercial kits analyzed albumin, total protein, glucose, cholesterol, triglycerides, uric acid, AST, ALT, and alkaline phosphatase. Antioxidant enzymes included glutathione peroxidase (Lawrence and Burk, 1976), superoxide dismutase (Gao et al., 1998), and reactive oxygen species (Ali et al., 1992). Yolk lipid peroxidation from stored eggs (2 yolks/cage pooled) was quantified as TBARS (De Leon and Borges, 2020).

Fatty acid profile

Total lipids for yolk (3 yolks/cage pooled) were extracted (Bligh and Dyer, 1959), methylated by transesterification (Hartman and Lago, 1973), and analyzed by gas chromatography (TRACE 1310, Thermo Fisher Scientific, Waltham, MA, USA; RT-2560 column, Restek, Bellefonte, PA, USA, 100 m x 0.25 mm x 0.20 μm). Fatty acids were identified against standards and expressed as a percentage of the total identified fatty acids (Visentainer and Franco, 2006).

Intestinal morphometry

Two birds per cage ($n = 12$/treatment) were randomly selected based on the average body weight of the experimental unit (cage) and euthanized (CO_2 chamber) to ensure a representative sample for morphometric analysis. A 3-cm jejunal segment (mid-portion, 2 cm from Meckel's diverticulum) was collected, flushed with PBS, fixed in 10% formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin-eosin. Villus height (base to tip), crypt depth (base to opening), and villus:crypt ratio were measured (x40 magnification, 10 measurements/well-oriented villi - ImageJ software, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data analysis was conducted using R (v 4.4.1; R Core Team, 2024). Normality (Shapiro-Wilk) and homogeneity (Levene) were verified. Data were analyzed by one-way ANOVA. Since dietary treatments consisted of graded levels of HT, the dose-response relationships were evaluated using orthogonal polynomial contrasts (linear and quadratic). Tukey's HSD test was used only for qualitative comparisons where polynomial trends were not informative. Principal Component Analysis (PCA) and Hierarchical Clustering on Principal Components (HCPC) were performed using the R packages FactoMineR (Le et al., 2008) and factoextra (Kassambara and Mundt, 2020) and integrated Haugh unit, villus height, villus:crypt ratio, TBARS, DHA, and yolk color (a* and b*). These variables were preselected a priori based on their biological relevance to gut function, oxidative stability, and functional egg quality. Tendencies were considered when $0.05 < P < 0.10$.

Results

Productive performance and intestinal morphometry

The productive performance parameters, including egg production, feed intake, and feed conversion ratio, were not influenced by the dietary inclusion of hydroxytyrosol (HT) ranging from 0 to 50 mg/kg ($P > 0.05$, Table 3).

Dietary HT supplementation did not affect feed intake, egg production, egg weight, egg mass, or feed conversion ratio ($P > 0.05$; Table 3). In contrast, HT inclusion affected intestinal morphometry (Table 4). Villus height decreased linearly with increasing HT inclusion ($P < 0.001$), with the lowest values observed at 50 mg/kg. Crypt depth was reduced at 20 mg/kg compared with other treatments. Although the villus:crypt ratio was similar between the control and 20 mg/kg groups, both villus height and crypt depth were proportionally smaller at this dose.

Serum biochemical parameters

Serum biochemical indicators, including AST, ALT, glucose, cholesterol, triglycerides, total protein, albumin, and uric acid, were not affected by HT supplementation ($P > 0.05$; Table 5). Antioxidant enzyme activities remained stable across treatments ($P > 0.05$), showing no significant deviations from the control group.

Egg quality

Haugh Units of fresh eggs did not differ among treatments ($P = 0.860$). After 30 days of storage, dietary HT affected internal egg quality ($P = 0.009$; Table 6). The quality of fresh eggs was not affected ($P > 0.05$; Supplementary Table S1). Polynomial contrasts indicated a quadratic response ($P < 0.001$), with the highest Haugh Units recorded at 10 and 20 mg/kg and the lowest values observed at 50 mg/kg (Fig. 1). Other egg quality parameters were not affected by dietary treatment ($P > 0.05$).

Oxidative stability and fatty acid profile

Dietary HT supplementation affected yolk oxidative stability after 30 days of storage (Table 7; Fig. 2). TBARS values decreased linearly with increasing HT inclusion up to 20 mg/kg ($P < 0.001$), with all HT-supplemented treatments exhibiting lower lipid peroxidation than the control group. Fatty acid composition remained stable across treatments ($P > 0.05$; Table 7). However, DHA concentration was highest in the 20 mg/kg group ($P = 0.032$). For EPA concentration, although the overall treatment effect was not significant ($P = 0.079$), pre-planned orthogonal polynomial contrasts revealed a significant quadratic response to HT inclusion ($P = 0.016$).

Multivariate analysis

Principal Component Analysis explained 58.2% of the total variance among variables (Fig. 3). Hierarchical Clustering on Principal Components separated dietary treatments into three distinct clusters: the 50 mg/kg treatment formed an isolated group, the 10 and 20 mg/kg treatments clustered together, and the 0 and 5 mg/kg treatments were grouped together (Fig. 4).

Table 3
Effects of dietary hydroxytyrosol supplementation on productive performance parameters of ISA Brown laying hens from 64 to 80 weeks of age.

Variable[2]	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg	SEM[1]	*P*-value
Feed Intake (g/day)	111.6	112.9	113.5	112.3	111.7	1.32	0.822
Egg Production (%)	90.5	91.3	90.1	89.7	92.4	1.36	0.697
Egg Weight (g)	63.5	64.5	64.3	64.7	63.1	0.94	0.763
Egg Mass (g/day)	57.5	58.8	57.8	58.0	58.3	0.98	0.893
FCR (kg/kg)[3]	1.96	1.92	1.97	1.95	1.92	0.03	0.893
FCR (kg/dz)[3]	1.48	1.47	1.49	1.48	1.45	0.02	0.690

[1] Pooled Standard Error of the Mean ($n = 6$ replicates per treatment).
[2] Treatments: 0 (Control), 5, 10, 20, and 50 mg/kg of pure hydroxytyrosol.
[3] FCR = Feed Conversion Ratio.

Table 4
Effects of dietary hydroxytyrosol supplementation on the intestinal morphometry of ISA Brown hens at 80 weeks of age.

Variable[2]	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg	SEM[1]	*P*-value
Villus (μm)[3]	759.4	730.7	667.9	625.0	562.2	8.24	< 0.001
Crypt (μm)[3]	80.1	78.1	81.3	63.1	69.0	1.35	< 0.001
Villus: Crypt[3]	9.52	9.36	8.21	9.95	8.15	0.28	< 0.001

[1] Pooled Standard Error of the Mean ($n = 6$ replicates per treatment).
[2] Treatments: 0 (Control), 5, 10, 20, and 50 mg/kg of pure hydroxytyrosol.
[3] Polynomial contrasts: Villus height (Linear effect: $P < 0.001$); Crypt depth (Quadratic effect: $P < 0.001$); Villus:Crypt ratio (Quadratic effect: $P < 0.001$).

Table 5
Effects of dietary hydroxytyrosol supplementation on serum biochemical parameters of ISA Brown laying hens at 80 weeks of age.

Variable[2]	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg	SEM[1]	*P*-value
Albumin (g/dL)	2.09	2.15	2.09	2.23	2.13	0.10	0.828
Cholesterol (mg/dL)	110.8	110.1	119.8	117.5	112.4	6.42	0.819
Alk. Phosphatase (U/L)	322.2	473.0	402.1	363.3	258.9	82.5	0.540
Glucose (mg/dL)	223.1	241.3	251.7	247.0	239.3	7.35	0.066
Uric Acid (mg/dL)	4.31	4.96	4.96	5.34	5.27	0.36	0.297
Total Protein (g/dL)	6.11	4.84	5.35	5.80	5.39	0.30	0.051
AST (U/L)[3]	352.8	315.6	321.1	322.6	315.7	20.1	0.654
ALT (U/L)[3]	25.7	23.7	26.5	26.5	18.1	2.95	0.235
Triglycerides (mg/dL)	643.3	654.4	640.4	664.1	653.6	66.8	0.999

[1] Pooled Standard Error of the Mean ($n = 6$ replicates per treatment).
[2] Treatments: 0 (Control), 5, 10, 20, and 50 mg/kg of pure hydroxytyrosol.
[3] AST = Aspartame Aminotransferase; ALT = Alanine Aminotransferase.

Table 6
Effects of dietary hydroxytyrosol supplementation on the quality of ISA Brown hen eggs stored for 30 days at room temperature (22 ± 4°C).

Variable[2]	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg	SEM[1]	*P*-value
Haugh Unit[3]	22.40	30.95	32.82	27.87	19.03	3.25	0.009
Yolk Index	0.26	0.25	0.26	0.28	0.27	0.01	0.365
Specific Gravity (g/cm^3)	1.038	1.035	1.029	1.036	1.039	0.003	0.171
Shell Strength (kgf)	4.24	3.72	3.89	3.63	4.56	0.32	0.175

[1] Pooled Standard Error of the Mean ($n = 6$ replicates per treatment).
[2] Treatments: 0 (Control), 5, 10, 20, and 50 mg/kg of pure hydroxytyrosol.
[3] Polynomial contrasts: Haugh unit (Quadratic effect: $P < 0.001$).

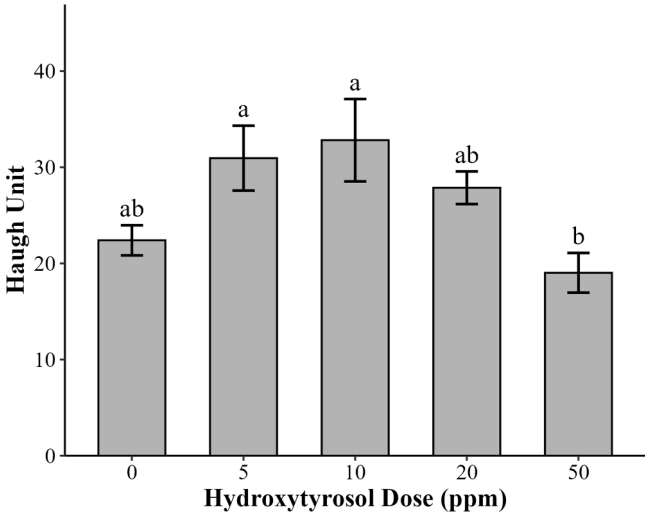

Fig. 1. Haugh Unit values obtained across the treatments after 30 days of storage. Means with different letters differ ($P < 0.05$) by Tukey's test.

Discussion

Productive performance and intestinal morphometry

Hydroxytyrosol supplementation (0 to 50 mg/kg) did not affect feed intake, egg production, egg weight, or FCR, confirming its tolerance in late-phase laying hens under the specific conditions of this trial. This aligns with meta-analyses showing that polyphenols rarely impair productivity unless they exceed 1 to 2% inclusion (Abd El-Hack et al., 2023). However, intestinal morphometry revealed dose-dependent effects absent in performance metrics, highlighting gut sensitivity as an early biological limit indicator.

Villus height decreased linearly ($P < 0.001$; 759 → 562 μm at 50 mg/kg; −26%), contrasting with broiler studies, where 25 mg/kg HT increases villus height by 15% (Dias et al., 2024). This discrepancy likely reflects species and age differences and cannot be attributed to HT alone: broilers benefit from antimicrobial and growth-promoting effects, whereas mature hens (80 weeks) may exhibit greater tissue sensitivity to high-polyphenol exposure in an alkaline intestinal milieu (Lambert and Elias, 2010). It is important to note that this pro-oxidant shift at high concentrations is a well-documented class effect shared by many phenolic compounds, such as quercetin and catechins, and it is not specific to hydroxytyrosol. Similar linear villus reductions (−30%) were

Table 7
Effects of dietary hydroxytyrosol supplementation on oxidative stability and yolk fatty acid profile of ISA Brown hen eggs stored for 30 days at room temperature.

Variable[2]	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg	SEM[1]	*P*-value
TBARS (mg MDA/kg)[3, 4]	2.41	1.95	1.76	1.59	1.64	0.11	< 0.001
Palmitic (C16:0)	27.65	27.39	26.84	27.46	27.82	0.26	0.134
Stearic (C18:0)	8.18	8.54	8.73	8.36	8.18	0.22	0.231
Oleic (C18:1 n9)	39.43	39.65	40.37	39.53	40.57	0.46	0.279
Linoleic (C18:2 n6)	18.12	17.63	17.81	18.22	16.78	0.42	0.138
ALA (C18:3 n3)[3]	0.45	0.46	0.47	0.48	0.35	0.02	< 0.001
Arachidonic (C20:4 n6)	1.72	1.78	1.61	1.70	1.67	0.05	0.311
EPA (C20:5 n3)[3, 4]	0.01	0.01	0.01	0.01	0.01	0.00	0.079
DHA (C22:6 n3)[3, 4]	0.48	0.48	0.43	0.51	0.43	0.02	0.032
SFA (%)[3]	36.34	36.43	36.05	36.32	36.47	0.22	0.771
MUFA (%)[3]	42.44	42.76	43.19	42.31	43.89	0.28	0.136
PUFA (%)[3]	21.21	20.81	20.75	21.37	19.64	0.45	0.084
Total n-3[4]	0.95	0.95	0.91	1.00	0.79	0.02	< 0.001
Total n-6	20.08	19.66	19.65	20.17	18.67	0.43	0.122
n-6:n-3 Ratio	21.27	20.66	21.60	20.16	23.82	0.45	< 0.001

[1] Pooled Standard Error of the Mean ($n = 6$ replicates per treatment).
[2] Treatments: 0 (Control), 5, 10, 20, and 50 mg/kg of pure hydroxytyrosol.
[3] TBARS = Thiobarbituric Acid Reactive Substances; MDA = Malondialdehyde; ALA = Alpha-linolenic acid; EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; SFA = Saturated Fatty Acids; MUFA = Monounsaturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids.
[4] Polynomial contrasts: TBARS (Linear effect: $P < 0.001$); EPA (Quadratic effect: $P = 0.016$); DHA (Quadratic effect: $P = 0.025$); Total n-3 (Quadratic effect: $P < 0.001$).

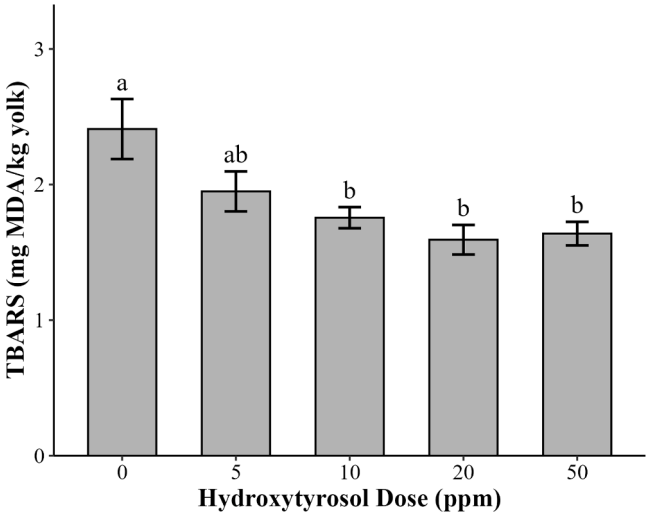

Fig. 2. TBARS values obtained across the treatments. Means with different letters differ ($P < 0.05$) by Tukey's test.

observed with 2% grape pomace polyphenols (Viveros et al., 2011), supporting direct epithelial interaction via protein binding or ROS generation. The mechanisms underlying the reduced villus height are not fully elucidated by our data. Future research should investigate whether the high dose (50 mg/kg) induces local inflammation, alters the redox state in the intestinal epithelium, or modulates the gut microbiome.

The 20 mg/kg villus:crypt ratio (9.95) matched control (9.52) due to proportional crypt depth reduction (63.1 μm), masking absolute absorptive loss. The maintenance of performance and enhanced DHA deposition despite reduced villus area at 20 mg/kg suggests compensatory mechanisms. HT might enhance nutrient utilization through pathways independent of gross morphology, such as modifying enterocyte membrane fluidity or upregulating specific fatty acid transport proteins. Late-phase hens exhibit reduced epithelial turnover (Wingching-Jones et al., 2023), amplifying subtle damage. Performance resilience suggests digestive redundancy, but prolonged exposure may manifest as nutrient deficits (Abdel-Moneim et al., 2020).

Serum biochemistry and systemic oxidative status (SOD)

Serum markers (AST, ALT, glucose, lipids, uric acid) remained unchanged ($P > 0.05$), confirming systemic safety up to 50 mg/kg. This matches HT's NOAEL (500 mg/kg/d; Auñon-Calles et al., 2013a, 2013b), with hepatic/renal clearance preventing metabolite accumulation. Although not statistically significant at the 5% level, numerical trends in total protein ($P = 0.051$) and glucose ($P = 0.066$) were noted. However, all values remained within physiological ranges (4.5 to 6.5 g/dL; 200 to 260 mg/dL). These results indicate that systemic safety is preserved even at 50 mg/kg, suggesting that the adverse effects observed in this study were restricted to local intestinal morphology.

The numerical reduction in SOD at 10 to 20 mg/kg aligns with the concept of "antioxidant sparing" observed in other studies (Shan and Miao, 2022; Surai et al., 2016). However, this remains a hypothesis that requires confirmation through more sensitive models or larger sample sizes.

Internal egg quality

It is noteworthy that in fresh eggs (Supplementary Table S1), no differences were observed for internal quality parameters, such as Haugh Unit and Yolk Index ($P > 0.05$). This indicates that the beneficial effects of HT observed in this study are primarily associated with the mitigation of oxidative degradation during storage rather than an immediate enhancement of egg synthesis. Although high doses (50 mg/kg) increased shell thickness ($P = 0.003$) and altered instrumental yolk yellowness (b*, $P < 0.001$), these changes did not compromise shell strength or the visual yolk color score (DSM Fan), suggesting limited practical impact on the fresh product.

After 30 days of storage, polynomial contrasts indicated a quadratic effect of HT dose on Haugh Units ($P < 0.001$), with the highest values at 10 mg/kg (32.8 HU), consistent with a hormetic pattern. The low Haugh unit values reflect extreme degradation after 30 days of storage at 22 ± 4°C, a condition representative of non-refrigerated supply chains in tropical regions, and are not directly comparable to values from fresh or refrigerated eggs. Moderate HT scavenges storage-generated ROS, degrading ovomucin-lysozyme (Kirunda and McKee, 2000), whereas 50 mg/kg accelerates proteolysis via pro-oxidant shift. Yolk index numerical superiority at 20 mg/kg (0.37) suggests vitelline membrane protection, delaying albumen-yolk water migration. Shell traits were unaffected, indicating intact calcification independent of intestinal effects.

Oxidative stability and nutritional enhancement

Linear TBARS reduction ($P < 0.001$; 2.41 to 1.59 mg MDA/kg at 20 mg/kg; –34%) is consistent with HT's peroxyl radical scavenging via its ortho-dihydroxy structure (Martínez et al., 2018). This magnitude of protection is at least comparable to, and in some cases appears to exceed, the efficacy reported for vitamin E in similar egg storage trials

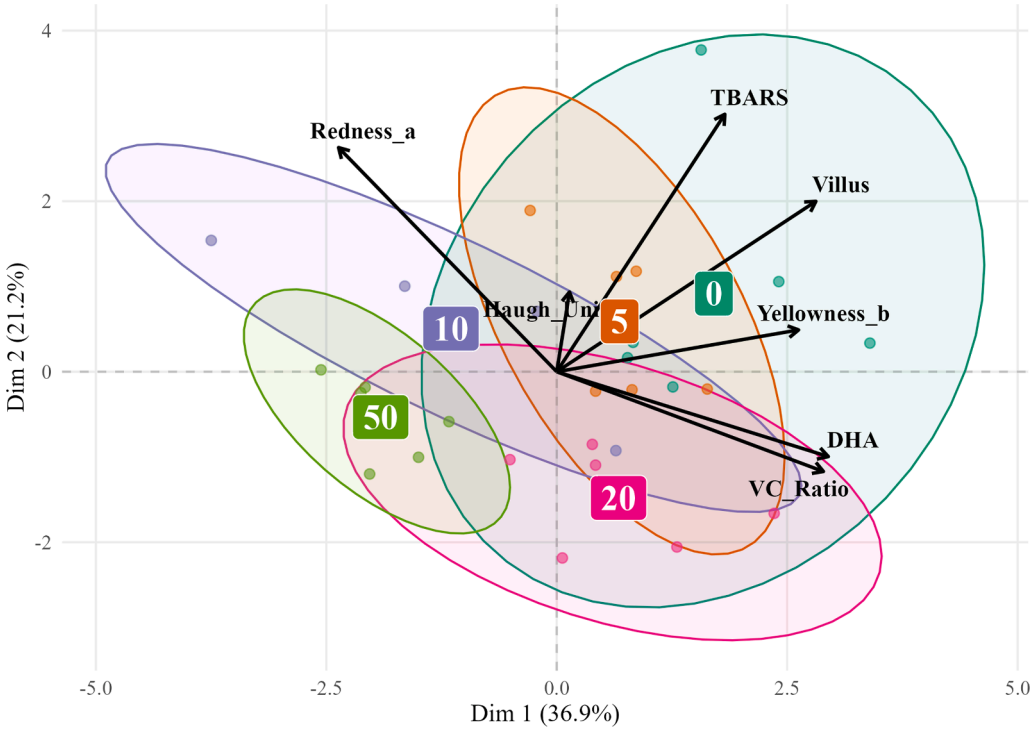

Fig. 3. PCA biplot correlating treatments (HT doses) and selected variables.

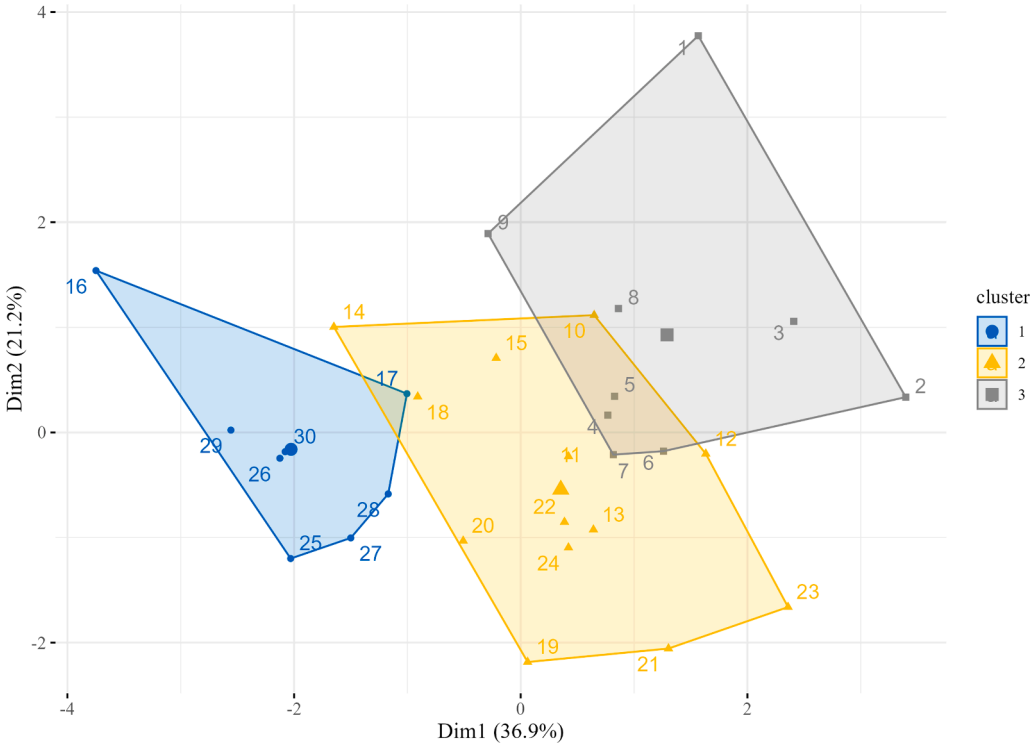

Fig. 4. HCPC factor map showing the three clusters obtained during analysis.

(Matumoto-Pintro et al., 2017).

DHA concentration peaked at 20 mg/kg (0.51%; $P = 0.032$), slightly higher than values reported for algal oil supplementation (≈ 0.45%; Maina et al., 2023), suggesting that HT based strategies may complement existing n-3 enrichment approaches. The significant quadratic contrast observed for EPA ($P = 0.016$) suggests a potential sparing effect on desaturation-elongation pathways (Tocher et al., 2019), despite the non-significant overall ANOVA result ($P = 0.079$). DHA decline at 50 mg/kg (−10.4%) correlates with villus damage. Furthermore, the reduction in total n-3 fatty acids at 50 mg/kg ($P < 0.001$) reinforces the hypothesis of malabsorption or oxidative loss of PUFAs at supra-physiological doses (Papadopoulos et al., 2023).

Integrated dose-response pattern

The multivariate analyses support a non-linear, dose-dependent response. Treatments at 10 and 20 mg/kg consistently clustered together, reflecting enhanced internal quality, better oxidative stability, and moderate intestinal morphology. Conversely, the 50 mg/kg treatment clustered separately, indicating distinct physiological responses at the highest dose. This dose-response pattern aligns with observations that phenolics often display non-monotonic biological activity (Lambert and Elias, 2010).

Practical implications

However, while 20 mg/kg HT significantly enhances chemical stability, future applied research should include sensory evaluation to confirm acceptance and ensure that reduced lipid oxidation translates into superior organoleptic quality during non-refrigerated storage. Furthermore, as this study identified the optimal dose for late-phase ISA Brown hens, this inclusion level should be validated across different genetic lines and production stages to confirm its universal applicability across the laying hen industry. These results provide clear evidence-based recommendations for producing functional eggs with enhanced oxidative stability and nutritional value, which are particularly relevant for egg production systems in tropical and subtropical regions where non-refrigerated storage is a common logistical constraint.

Moreover, the use of room-temperature storage at 22 ± 4°C, which reflects tropical market conditions but does not capture refrigerated chains, and the reliance on calculated, rather than analytically confirmed, HT concentrations in feed, due to the lack of a validated ppm-level assay, remains a limitation. Future trials would benefit from the development and application of robust analytical methods to confirm dietary HT concentrations, thereby strengthening the quantitative precision of dose-response recommendations.

It is essential to note that these findings are limited to late-phase hens (80 weeks) and room-temperature storage. While 20 mg/kg of HT showed the best results for the parameters measured, further research is needed to validate these biological thresholds in younger flocks or different environmental challenges to avoid overgeneralizing the benefits of HT supplementation.

Conclusion

The supplementation of pure hydroxytyrosol exhibits a distinct hormetic biological profile. Although it is systemically safe at all tested levels, its efficacy is dose-dependent. 20 mg/kg is identified as the optimal inclusion level, providing maximum antioxidant protection (lowest TBARS), preserving albumen quality (highest HU), and enhancing nutritional value (highest DHA). Doses above this threshold (50 mg/kg) exceed the physiological antioxidant threshold, leading to pro-oxidant effects on intestinal morphology and compromise egg quality. Therefore, 20 mg/kg represents a practical target dose for the poultry industry to produce functional eggs with extended shelf life and improved n-3 PUFA content.

Data availability statement

The data obtained in this study can be provided upon reasonable request to the corresponding author.

Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Gemini (Google) in order to improve the readability, clarity, and grammatical accuracy of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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CRediT authorship contribution statement

Marcel Manente Boiago: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Nauan Lima da Silva:** Methodology, Investigation. **Andressa Vilani:** Methodology, Investigation. **Aleksandro Schafer da Silva:** Writing – review & editing, Writing – original draft, Conceptualization. **Diovani Paiano:** Writing – review & editing, Writing – original draft. **Reeta Davis:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition. **Margaret Walsh:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition. **Enrico Angelo Altieri:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition. **Kevin Edward O'Connor:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition. **Alessandro Cazonatto Galvão:** Writing – review & editing, Writing – original draft, Formal analysis. **Bruna Klein:** Methodology, Investigation. **Weber da Silva Robazza:** Writing – review & editing, Writing – original draft, Formal analysis.

Disclosures

Authors R. Davis, M. Walsh, E. A. Altieri, and K. E. O'Connor are employees of Nova Mentis Ltd., the company that supplied the hydroxytyrosol used in this study. However, the company had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2026.106982.

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