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Dietary Passion Fruit Seed Oil Supplementation for Health and Performance of Laying Hens

Laís Garcia Cordeiro [1,*], Paola Aparecida Damázio Rodrigues [1], Gabrieli Andressa de Lima [1], Elis Omar Figueroa Castillo [1], Joyce Andrade da Silva [1], Júlia de Lima Lopes [1], Anna Luísa Lang [1], Samir Moura Kadri [2], Antônio Celso Pezzato [1] and José Roberto Sartori [1]

[1] Department of Animal Breeding and Nutrition, School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu 18618-681, SP, Brazil; paola.damazio@unesp.br (P.A.D.R.); gabrieli.lima@unesp.br (G.A.d.L.); e.castillo@unesp.br (E.O.F.C.); joyce.silva@unesp.br (J.A.d.S.); jl.lopes@unesp.br (J.d.L.L.); anna.lang@unesp.br (A.L.L.); celso.pezzato@unesp.br (A.C.P.); jose.sartori@unesp.br (J.R.S.)

[2] Department of Animal Production, School of Technology and Agricultural Sciences, São Paulo State University (UNESP), Dracena 17915-899, SP, Brazil; samir.kadri@unesp.br

* Correspondence: lais.cordeiro@unesp.br; Tel.: +55-(14)-3880-2962

Abstract: Passion fruit seed oil (PFSO) is rich in bioactive compounds, which can enhance laying hens' health and performance. The current study was conducted to investigate the effect of increasing PFSO supplementation in laying hens' productive performance, egg quality, relative weight and length of organs, plasma lipid oxidation, antioxidant status, and gene expression of SOD, GPx, CAT, and NRF2 in the liver. One hundred ninety-two 25-week-old Lohmann Whites were randomly divided into three treatments (n = 8 replicates/diet, 8 hens/replicate). The groups were fed a corn–soybean basal diet containing 0.00%, 0.45%, and 0.90% PFSO for 16 weeks. The results indicated that increasing supplementation of PFSO decreased plasma lipid oxidation (n = 8; linear, p = 0.012) and increased CAT gene expression (n = 8; linear, p = 0.001). SOD and NRF2 genes tended to increase linearly, and GPx was not affected (n = 4; $p > 0.05$). The CAT activity tended to decrease linearly and the SOD and GPx were not affected (n = 8; $p > 0.05$) by diets. Performance and most egg quality, relative weight, and length of organs did not differ among treatments (n = 8; $p > 0.05$). Therefore, increasing the supplementation of PFSO in the diet may have positive effects on the laying hens' health by decreasing oxidative stress, stimulating the antioxidant defense system, and sustaining egg production and quality.

Keywords: functional feed; gene expression; laying hens; malonaldehyde; oxidative stress; *Passiflora*; poultry nutrition; sustainability

Academic Editors: Petru Alexandru Vlaicu, Alexandra Gabriela Oancea and Mihaela Dumitru

Received: 12 March 2025
Revised: 5 April 2025
Accepted: 7 April 2025
Published: 16 April 2025

Citation: Cordeiro, L.G.; Rodrigues, P.A.D.; Lima, G.A.d.; Castillo, E.O.F.; Silva, J.A.d.; Lopes, J.d.L.; Lang, A.L.; Kadri, S.M.; Pezzato, A.C.; Sartori, J.R. Dietary Passion Fruit Seed Oil Supplementation for Health and Performance of Laying Hens. *Agriculture* **2025**, *15*, 864. https://doi.org/10.3390/agriculture15080864

1. Introduction

Passion fruit seed oil (PFSO) is a vegetal oil rich in bioactive compounds, such as tocopherol, carotenoids, phytosterols and phenolic compounds, and unsaturated fatty acids (ω6, linoleic acid; ω9, oleic acid; and ω3, linolenic acid), which are important for the immune system and inflammatory response, and antioxidant system [1–4]. These compounds can modulate animals' performance, gene expression, and final product quality [5,6].

Poultry rearing and handling conditions, even following the breeder recommendations, have a stressful effect [7]. Stress factors can lead to oxidative stress (OS), which negatively affects the performance of hens due to damage to macromolecules, immunosuppression, metabolic disorders, and reduced growth rate [8–10]. Egg production is also affected, as OS

causes disturbances in calcium metabolism, resulting in inefficient deposition of this mineral in the eggshell, which leads to the formation of thin and fragile shells, compromising gas exchange and defense against pathogens [11,12]. Additionally, lipid and protein oxidation alters the consistency and composition of the albumen and yolk. These factors directly compromise egg quality, increasing susceptibility to breakage and shortening shelf life [13].

Given the prevalence of these agents, thought should be focused on nourishing the immune and antioxidant systems by using ingredients or additives rich in bioactive compounds and unsaturated fatty acids, such as PFSO, the properties of which can minimize the effects of oxidative stress by reducing lipid oxidation in plasma and meat, and stimulating the antioxidant enzymes activity [4,14]. The inclusion of PFSO in the diet of broilers under heat stress revealed protein modulation indicating a probable adaptive mechanism by reducing oxidative stress, activating neuroprotective mechanisms by the downregulation of neurofilament medium polypeptide subunit (NF-M), protecting against apoptosis, reducing inflammatory responses, and regulating energy metabolism [15]. All these mechanisms appear to be related to the PFSO content, especially the tocopherol, carotenoids, and phenolic compounds. Among the phenolic compounds, the flavonoids may have directly influenced the NF-M downregulation by the β-secretase activity reduction, thereby inhibiting β-amyloid formation, preventing the aggregation of toxic oligomers, and mitigating neuronal inflammation and cell death [16,17].

As PFSO has successfully been tested in broilers' diets to improve their health and meat quality, its inclusion in laying hens' diets may provide additional advantages for egg production and oxidative stress resistance. However, up until now, there have been few studies focused on the use of PFSO in poultry feed. Therefore, the current study was designed to assess the effect of PFSO inclusion in diets for laying hen performance, egg quality, plasma lipid oxidation, relative organ weight, antioxidant status, and relative gene expression of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and erythroid nuclear factor 2 (NRF2).

2. Materials and Methods

All the procedures described below were approved by the Ethics Committee on Animal Use from the São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Botucatu, SP, Brazil, under protocol number 0097/2022.

2.1. Experimental Design and Diets

The experiment was conducted in the Poultry Nutrition Laboratory at UNESP, School of Veterinary Medicine and Animal Science, Botucatu. One hundred ninety-two, 25-week-old, Lohmann White commercial laying hens with similar laying rates (98.44%) and body weight (1548.80 g) were randomly divided into three treatments. Laying hens in each treatment were designated to eight replicates with eight hens per replicate.

Mash feed and fresh water were available ad libitum during the 16 weeks of the experiment, from 25 to 41 weeks of age. The basal diet was formulated following the hen's nutritional requirements and nutritional values of Rostagno et al. [18]. Passion fruit seed oil (PFSO) was extracted by cold pressing and purchased from Extrair-Óleos Naturais (Boa Esperança, ES, Brazil), with its composition described in Table 1.

Table 1. Characterization and fatty acid profile of passion fruit seed oil based on the literature.

Antioxidant Compounds [3]	
Parameter	Quantity
Total carotenoids, mg of β-carotene/100 g of oil	75.63
Total phenolic compounds, g GAE/100 g of oil	1.47
Fatty acid profile [4]	
Name	Quantity, %
Myristic acid	0.09 ± 0.01
Palmitic acid	10.99 ± 0.03
Palmitoleic acid	0.17 ± 0.01
Margaric acid	0.07 ± 0.00
Stearic acid	2.89 ± 0.02
Oleic acid	14.89 ± 0.06
Vaccinium acid	0.96 ± 0.04
Linoleic acid	69.14 ± 0.01
Linolenic acid	0.39 ± 0.01
Eicosanoic acid	0.12 ± 0.02
Eicosenoic acid	0.11 ± 0.04
Docosanoic acid	0.06 ± 0.01
Erucic acid	0.05 ± 0.01
Lignoceric acid	0.06 ± 0.00
Saturated	14.28 ± 0.01
Monounsaturated	16.19 ± 0.02
Polyunsaturated	69.53 ± 0.02

PFSO was added to the treatments considering its apparent metabolizable energy (9378 kcal/kg) [14]. The experimental diet composition and nutritional levels are shown in Table 2.

Table 2. Ingredients and calculated nutritional levels of the experimental diets.

Ingredients, %	**Passion Fruit Seed Oil**		
	0.00%	**0.45%**	**0.90%**
Corn	58.49	58.49	58.49
Soybean meal	28.90	28.90	28.90
Soy oil	2.00	1.52	1.04
Passion fruit seed oil	0.00	0.45	0.90
Limestone (fine)	1.60	1.60	1.60
Limestone (coarse)	5.40	5.40	5.40
Dicalcium phosphate	0.45	0.45	0.45
Sodium chloride	0.08	0.08	0.08
DL-Methionine	0.08	0.08	0.08
Supplements [1]	3.00	3.00	3.00
Inert	0.00	0.03	0.06
Nutrient composition			
Metabolizable energy, kcal/kg	2804	2804	2804
Crude protein, %	17.51	17.51	17.51
Total Methionine, %	0.410	0.410	0.410

Table 2. *Cont.*

Ingredients, %	Passion Fruit Seed Oil		
	0.00%	**0.45%**	**0.90%**
Total Methionine + Cystine, %	0.699	0.699	0.699
Total Lysine, %	0.966	0.966	0.966
Total Tryptophan, %	0.212	0.212	0.212
Total Threonine, %	0.670	0.670	0.670
Total Isoleucine, %	0.772	0.772	0.772
Digestible Lysine, %	0.840	0.840	0.840
Digestible Methionine, %	0.390	0.390	0.390
Digestible Methionine + Cystine, %	0.632	0.632	0.632
Digestible Tryptophane, %	0.201	0.201	0.201
Digestible Threonine, %	0.594	0.594	0.594
Sodium, %	0.173	0.173	0.173
Calcium, %	3.688	3.688	3.688
Total Phosphorus, %	0.641	0.641	0.641
Digestible Phosphorus, %	0.418	0.418	0.418
Choline, mg/kg	0.110	0.110	0.110

[1] provided per kg of diet: Folic acid 0.45 mg; Pantothenic acid 10.5 mg; BHT 30 mg; Biotin 0.045 mg; calcium 7.5 mg; Copper 6.75 mg; Choline 180 mg; Iron 45 mg; Phytase 0.5001 ftu/g; Fluorine 13.8 mg; Phosphorus 1.47 g; Iodine 0.6 mg; Manganese 84 mg; Methionine 0.703 g; Selenium 0.18 mg; Sodium 1.35 g; Niacin 24 mg; vitamin A 6990 IU; vitamin B1 1.05 mg; vitamin B12 10.5 µg; vitamin B2 3 mg; vitamin B6 3 mg; vitamin D3 2400 IU; vitamin E 9.99 IU; vitamin K3 1.8 mg; zinc 60 mg; Virginiamycin 19.99 mg.

The laying hens were placed in the two-tier battery cage with four hens per cage at 562.5 cm^2/bird density. Two sequential cages with the same diet were arranged as a replicate and were equipped with trough feeders and nipple drinkers. The lighting program was applied according to the management guide [19]. The temperature and relative humidity were recorded daily using a digital thermo hygrometer (HOBO®) during the experimental period (Figure 1).

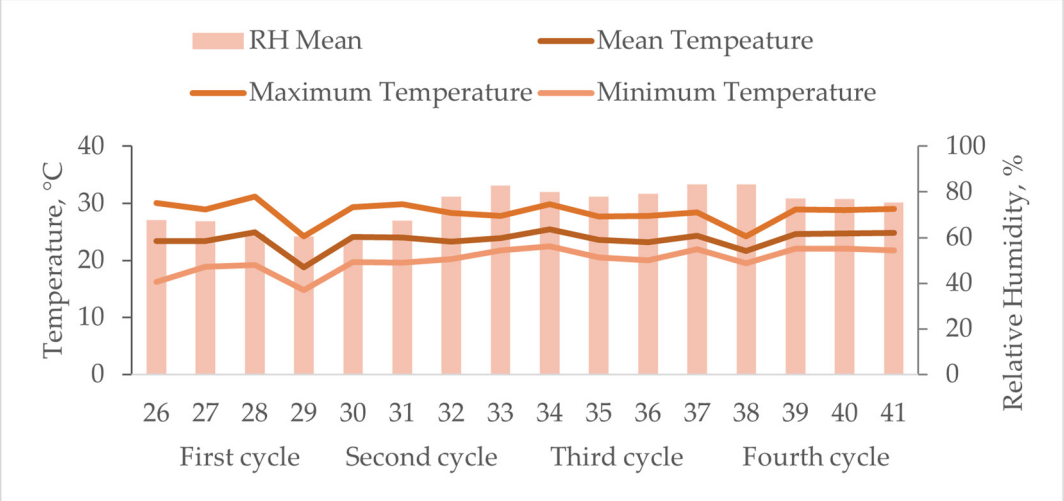

Figure 1. Temperature and relative humidity (RH) per week: first cycle (26, 27, 28, and 29 weeks), second cycle (30, 31, 32, and 33 weeks), third cycle (34, 35, 36, and 37 weeks), and fourth cycle (38, 39, 40, and 41 weeks).

2.2. Effects of PFSO on Performance

During the experimental period, total egg weight, egg production, shell-less eggs, cracked eggs, and broken eggs were recorded daily on a replicate cage basis. Feed intake (g) was also measured and recorded based on a replicate weekly. After each 4-week cycle, the egg production (%), viable eggs (%), total egg weight (g), average egg weight (g), daily egg mass (g/bird/day), daily feed intake (g/bird/day) and feed conversion ratio (kg/dz and kg/kg) were calculated during the experimental period. Body weight was recorded at the beginning and at the end of the experiment to calculate body weight gain (g). The results were calculated and expressed as means of accumulated cycles at the end of the experiment.

2.3. Effects of PFSO on Egg Quality

To analyze egg quality, three eggs per replicate were collected with a maximum interval of three hours after laying, excluding those with fissures or cracks and just two were analyzed at the end of each cycle in three consecutive days. The eggs from each replication were identified and individually weighed on a precision scale. Thereafter, eggs were submitted to a specific gravity test through immersion in saline solution with different densities (1.060, 1.065, 1.070, 1.075, 1.080, 1.085, 1.090, 1.095, and 1.100 g/mL), measured with a densimeter, dipping the eggs from the lowest to the highest density, according to Sleigh [20].

The eggshell strength (N) and deformity (mm) were obtained with the texture analyzer (Model TA-XT2i, Stable Micro Systems LTDA., Goldalming, UK). The samples were placed horizontally on the support with a pre-test velocity of 2.0 mm/s, test speed of 1.0 mm/s, and post-test velocity of 4.0 mm/s, adapted from Montenegro et al. [21].

The eggs were broken on a flat glass surface and the heights and diameters of yolk and albumen were obtained with a digital caliper. The yolk index was determined by the ratio between the average yolk height and the diameter. Haugh unit [22] was calculated with the following formula: $HU = 100 \times \log (H\ 7.57 - 1.7W^{0.37})$, where H refers to albumen height (mm) and W represents the egg weight (g). The yolk percentage was calculated by dividing the yolk weight by the egg weight. Albumen percentage was determined by the difference between the egg weight and the sum of the eggshell and yolk. The yolk color was measured by the DSM Yolk Color Fan and by a digital colorimeter, both on a scale from 0 to 16.

Eggshells were washed and dried in a dry oven at 55 °C for 24 h, weighed on a precision scale and the eggshell thickness was measured with the assistance of a digital micrometer at three points in the central region of the eggshell, after cooling. The eggshell percentage was calculated by dividing the eggshell weight by the egg weight. The shell weight per surface area (mg/cm^2) was obtained with following the equation: $SWUSA = \{SW/[3.9782 \times (EW^{0.7056})]\} \times 1000$, where SW = eggshell weight, EW = egg weight; according to Abdallah et al. [23].

2.4. Internal Organs

At the end of the experimental period (112 days), eight hens per treatment were randomly selected and slaughtered by cervical dislocation after eight hours of fasting to access internal organs. The oviduct, ovary, liver, spleen, pancreas, gizzard, and total intestines were removed and weighed. Abdominal fat was also weighed. The length of the total intestines was measured and recorded considering the beginning of the duodenum from the gizzard up to the cloaca, including the length of the cecum. Oviduct length was also measured. The relative organ weight was calculated in relation to the final hen body weight.

2.5. Lipid Oxidation in Plasma

At the end of the last cycle (41-week-old), one hen per replicate (n = 8) was selected and 5.0 mL of blood was collected via brachial vein and drawn into EDTA-containing tubes. The obtained plasma samples were divided into 2.0 mL microtubes and stored at −80 °C for testing. Lipid oxidation was achieved from the results of thiobarbituric acid reactive substances (TBARS) analysis using the methodology adapted from Vyncke [24].

Hence, with 600 µL of plasma, 1.500 µL of extractor solution (TBA 0.67% + TCA 15% + HCl 0.25 N) was added into a Falcon tube, being homogenized in the vortex. The sample was centrifuged for 15 min at 3500× g at 4 °C. When the sample was cleared, the supernatant was taken, boiled for 45 min, and cooled for 5 min on ice. Afterward, the supernatant was added to a cuvette to read the absorbance in a spectrophotometer at 532 nm. A standard curve of 1,1,3,3-tetra-ethoxypropane was used to calculate the results and the data was expressed in mg of malonaldehyde (MDA) per liter of plasma.

2.6. Antioxidant Enzyme Activity in the Liver

Half of the liver's left lobe was obtained from the same hens that were slaughtered by cervical dislocation. The samples were collected after removing all the internal organs. Livers were handled on top of ice-covered trays, preventing the enzyme's denaturation, and immediately packed in a 5 mL microtube, immersed, and stored in liquid nitrogen, until enzyme activity determination. Briefly, 0.5 g of the liver samples were placed in a 50 mL falcon tube and homogenized in 2.5 mL of phosphate buffer (pH 7.8). The homogenate was centrifuged at 5000 rpm for 30 min at 4 °C. Supernatant was used for enzyme activity and protein content analysis.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed according to Beauchamp and Fridovich [25] based on the enzyme's ability to convert superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen. This method measures one unit of SOD required to inhibit 50% of nitroblue tetrazolium, obtained by spectrophotometry at 560 nm. Catalase (CAT; EC 1.11.1.6) activity was assayed according to Sinha [26], in which dichromate in acetic acid is reduced to chromic acetate in the presence of H_2O_2 when heated, forming perchromic acid as an unstable intermediate. The absorbance reading was performed in a spectrophotometer at 610 nm. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed according to Flohé and Günzler [27]. For all the antioxidant enzyme assays, the total protein concentration was determined according to Bradford's [28] method with Coomassie brilliant blue G-250, using bovine serum albumin as a standard. The absorbance reading was performed in a spectrophotometer at 320 nm. The results were expressed as U/mg.

2.7. RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted from a 50 mg liver sample from four hens per treatment. The extraction was performed with the sample being minced with 1.000 µL of Trizol® reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer standards, homogenized, and incubated for 5 min. Following the rupture and cell content release, 400 µL of chloroform was added, homogenized in the vortex for one minute, incubated, and centrifuged for 15 min at 12,000× g at 4 °C, to separate the phases. Afterward, the upper phase was collected and transferred to a clean tube, with the addition of 500 µL of isopropanol 100%, and homogenized.

The same process was repeated (incubated and centrifuged again), with the supernatant discarded and the precipitate washed with 1 mL of ethanol 75% in H_2O DEPC 0.1%. Another centrifugation at 7500× g for 8 min at 4 °C was performed and the supernatant was discarded. The pellet was diluted in 20 µL ultrapure water (treated with

DEPC 0.1%), RNAse free. The samples were heated for 10 min at 56 °C and stored at −80 °C. The extracted RNA was used for cDNA synthesis using the High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Analysis for SOD, GPx, CAT, and NRF2 gene expression was performed with the quantitative real-time PCR technique using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and a SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). The samples were evaluated in triplicates, using the β-actin gene as an internal control to normalize the expression of the target genes. The results were obtained with the Pfaffl [29] formula: $(EFgoi^{\Delta CtGOI})/(EFact^{\Delta CtAct})$ where *EFgoi* = gene of interest efficiency; $^{\Delta CtGOI}$ = cycle threshold from the gene of interest; *EFact* = β-actin efficiency; and $^{\Delta CtAct}$ = cycle threshold from β-actin.

The procedures were conducted in the Biotechnology Institute (IBTEC) at UNESP, Botucatu, SP, Brazil. Primer sequences obtained from a commercial company (Thermo Fisher Scientific, Waltham, MA, USA) were tested for primer efficiency and their characteristics are described in Table 3.

Table 3. Primer sequences for the target and reference genes.

Gene [1]		Primers Sequence (5′–3′) [2]	Base Pairs	References
β-actin	F:	TGCTGTGTTCCCATCTATCG	136	[30]
	R:	TTGGTGACAATACCGTGTTCA		
GPx	F:	GACCAACCCGCAGTACATCA	205	[30]
	R:	GAGGTGCGGGCTTTCCTTTA		
NRF2	F:	GATGTCACCCTGCCCTTAG	215	[30]
	R:	CTGCCACCATGTTATTCC		
SOD2	F:	CTGACCTGCCTTACGACTATG	131	[31]
	R:	CGCCTCTTTGTATTTCTCCTCT		
CAT	F:	GAAGCAGAGAGGTTCCCATTTA	142	[31]
	R:	CATACGCCATCTGTTCTACCTC		

[1] GPx, glutathione peroxidase; SOD2, superoxide dismutase 2; NRF2, nuclear factor erythroid 2-related factor 2; CAT, catalase. [2] F: forward primer; R: reverse primer.

2.8. Statistical Analysis

Data were submitted to the UNIVARIATE procedure and, whether the residual distribution was normal, the results were analyzed with simple regression by the REG procedure in the software SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) [32], to verify which regression model (linear or quadratic) best describes the results. The means were compared using the Tukey test at the $p < 0.05$ value.

3. Results

3.1. Performance

In Table 4, the PFSO effect in laying hens' productive performance is shown as means of four cycles (28 days each). No effect of dietary treatment was found in egg production (%), viable eggs (%), total egg weight (kg), daily egg weight (g/hen/day), daily egg mass (g/hen/day), daily feed intake (g), feed conversion ratio per kilograms, and body weight gain (g) ($p > 0.05$). There was no mortality during the trial, from weeks 26 to 41.

Table 4. Laying hens performance fed different passion fruit seed oil concentrations during the experimental period.

Item	Passion Fruit Seed Oil			SEM [1]	*p*-Value	
	0.00%	0.45%	0.90%		Linear	Quadratic
Egg production, %	99.14	99.04	99.24	0.107	0.537	0.284
Viable eggs, %	98.79	99.05	98.47	0.253	0.426	0.395
Total egg weight, kg	54.25	53.40	53.95	0.525	0.690	0.285
Average egg weight, g/hen/day	61.25	60.79	60.85	0.543	0.603	0.701
Daily egg mass, g/hen/day	60.63	60.03	60.30	0.540	0.660	0.519
Daily feed intake, g/hen/day	115.37	115.99	116.14	1.191	0.646	0.877
Feed conversion ratio, kg/kg	1.933	1.983	1.958	0.021	0.412	0.152
Body weight gain, g	208.73	218.70	171.13	23.990	0.279	0.339

[1] SEM, standard error of the mean.

3.2. Egg Quality

The percentage of broken eggs calculated during the collection period for egg quality analysis was 1.05% (0.00% PFSO), 2.10% (0.45% PSFO), and 1.32% (0.90% PFSO), with no linear ($p = 0.726$) or quadratic ($p = 0.117$) effect. Egg quality data from the laying hens fed a control diet and PFSO levels are listed in Table 5. The results revealed that albumen height (mm) was reduced in linear regression ($p = 0.046$) with the PFSO inclusion, and there was also a trend to Haugh unit reduction ($p = 0.054$). The egg weight (g), albumen (%), yolk (%), eggshell (%), yolk fan color, yolk digital color, yolk index, albumen diameter (mm), eggshell strength (N), eggshell deformity (mm), eggshell thickness (mm) and SWUSA (mg/cm^2) were not affected ($p > 0.05$) from week 26 to 41.

Table 5. Egg quality from laying hens fed different passion fruit seed oil concentrations during the experimental period.

Item	Passion Fruit Seed Oil			SEM [1]	*p*-Value	
	0.00%	0.45%	0.90%		Linear	Quadratic
Egg weight, g	62.76	61.88	62.61	0.464	0.825	0.171
Albumen, %	63.45	63.15	63.05	0.280	0.314	0.771
Yolk, %	26.59	26.84	27.00	0.244	0.237	0.882
Eggshell, %	9.96	10.02	9.96	0.061	0.965	0.464
Yolk fan color	7.36	7.35	7.34	0.051	0.821	0.977
Yolk digital color	7.49	7.39	7.38	0.053	0.148	0.490
Yolk index	0.44	0.44	0.43	0.003	0.378	0.399
Haugh unit	93.19	92.26	91.78	0.497	0.054	0.709
Albumen diameter, mm	68.89	68.73	69.42	0.440	0.396	0.438
Albumen height, mm	8.85	8.62	8.57	0.094	0.046 [a]	0.459
Eggshell strength, N	46.10	46.19	47.46	0.923	0.303	0.611
Eggshell deformity, mm	0.88	0.88	0.92	0.038	0.480	0.728
Eggshell thickness, mm	0.399	0.400	0.400	0.002	0.754	0.965
Specific gravity, g/mL	1.096	1.096	1.096	0.000	0.465	0.890
SWUSA, mg/cm^2 [2]	84.70	84.79	84.58	0.477	0.853	0.802

[1] SEM, standard error of the mean. [2] SWUSA, shell weight per surface area. [a] $y = -0.3086x + 8.8167$, $R^2 = 0.17$.

3.3. Relative Internal Organ Weights

Table 6 summarizes the relative organ weights (%) and the oviduct and intestine (cm) measures of the hens fed diets containing different PFSO levels. The relative abdominal fat ($p = 0.081$) and ovary weights ($p = 0.098$) presented a quadratic regression response to the PFSO supplementation. There was no difference ($p > 0.05$) in the body weight and relative weight of the liver, spleen, pancreas, gizzard, oviduct, and total intestines. The oviduct and intestine lengths also showed no difference ($p > 0.05$).

Table 6. Relative internal organ weights and lengths from hens fed diets containing PFSO for 16 weeks.

Item	Passion Fruit Seed Oil			SEM [1]	*p*-Value	
	0.00%	0.45%	0.90%		Linear	Quadratic
Body weight, g	1768.25	1806.50	1726.63	51.82	0.575	0.363
Oviduct and intestine lengths						
Oviduct, cm	71.00	71.63	70.88	1.894	0.962	0.770
Intestines, cm	167.50	168.50	174.13	3.809	0.224	0.625
Relative internal organ weights						
Liver, %	2.02	2.15	2.11	0.092	0.500	0.471
Spleen, %	0.09	0.09	0.09	0.004	0.367	0.190
Pancreas, %	0.19	0.21	0.20	0.011	0.746	0.264
Gizzard, %	1.69	1.77	1.79	0.089	0.391	0.820
Abdominal Fat, %	4.79	5.42	4.35	0.376	0.440	0.081 [a]
Ovary, %	3.25	3.45	3.11	0.129	0.495	0.098 [b]
Oviduct, %	4.36	4.55	4.50	0.199	0.631	0.632
Intestines, %	4.31	4.36	4.54	0.155	0.314	0.723

[1] SEM, standard error of the mean. [a] $y = -5.3061x^2 + 4.193x + 4.8721$, $R^2 = 0.15$. [b] $y = -2.0468x^2 + 1.6029x + 3,2739$, $R^2 = 0.23$.

3.4. Lipid Oxidation in Plasma and Antioxidant Activity in the Liver

Lipid oxidation in plasma was evaluated considering the MDA concentration (mg/L) and the results are presented in Table 7. The responses showed that compared with the control group, the diet PFSO inclusion decreased lipid peroxidation in a linear regression ($p = 0.012$). The antioxidant enzyme activity in the liver is also described in Table 7. The CAT enzyme activity presented a trend ($p = 0.078$) to reduce linear regression. There was no effect of PFSO in the activity of SOD and GPx ($p > 0.05$).

Table 7. Effect of dietary passion fruit seed oil levels in 41-week-old laying hens plasma lipid oxidation and antioxidant enzyme activity in the liver.

Description [1]	Passion Fruit Seed Oil			SEM [2]	*p*-Value	
	0.00%	0.45%	0.90%		Linear	Quadratic
Plasma						
MDA (mg/L)	0.0984	0.0747	0.0643	0.00887	0.012 [a]	0.549
Liver						
SOD (U/mg)	71.36	70.18	68.49	1.88	0.302	0.920
GPx (U/mg)	2.90	2.72	2.52	0.31	0.384	0.975
CAT (U/mg)	3.34	2.96	2.97	0.13	0.078 [b]	0.270

[1] MDA, malonaldehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase. [2] SEM, standard error of the mean. [a] $y = -0.0379x + 0.0962$, $R^2 = 0.29$. [b] $y = -0.4211x + 3.2745$, $R^2 = 0.19$.

3.5. Gene Expression in the Liver

The effect of PFSO supplementation on the relative gene expression of CAT, SOD, NRF2, and GPx is shown in Figure 2. The PFSO supplementation increased CAT relative gene expression in a linear regression ($p = 0.001$). For the SOD ($p = 0.051$) and NRF2 ($p = 0.075$) genes, a positive trend was observed in the linear regression analysis. Nevertheless, the relative gene expression of GPx was not affected ($p > 0.05$) by PFSO inclusion.

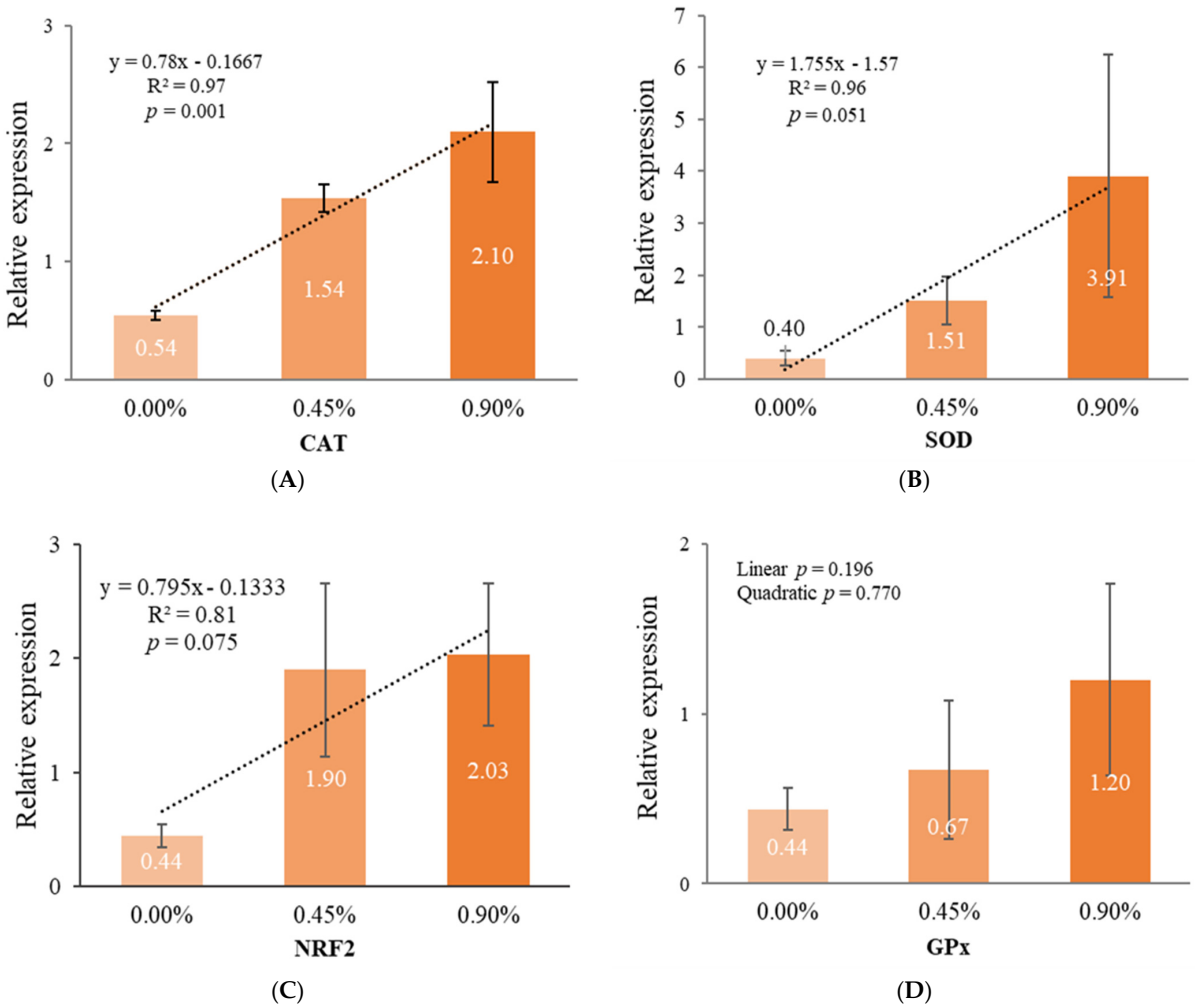

Figure 2. Effect of passion fruit seed oil on (**A**) catalase (CAT), (**B**) superoxide dismutase (SOD), (**C**) erythroid nuclear factor 2 (NRF2), and (**D**) glutathione peroxidase (GPx) relative expression in 41-week-old laying hens' liver. Each bar represents the mean ± SEM (n = 4).

4. Discussion

The inclusion of passion fruit seed oil (PFSO) in the diet stimulated the expression of antioxidant enzymes and reduced plasma oxidation in Lohmann White laying hens, even without enhancing the enzyme activity in the liver. PFSO has also sustained performance and most parameters from egg quality and relative organ weights.

Gene expression of CAT, SOD, and NRF2 linearly increased as PFSO levels were supplemented. Although the GPx gene expression was not significant, it was 52.27% (0.45% PFSO) and 172.70% (0.90% PFSO) higher than the control group. In addition, antioxidant analysis in the liver revealed that CAT enzymes tended to linearly reduce, and even without the other enzymes presenting a significative effect, the SOD was 1.65% (0.45% PFSO) and 4.02% (0.90% PFSO) lower than the control group, and GPx was 6.21% (0.45% PFSO) and 13.1% (0.90% PFSO) lower than the control group. These results indicate that the bioactive compounds of the PFSO acted in two different ways by directly acting as non-enzymatic antioxidants and by indirectly stimulating the enzymatic antioxidants gene expression. Cruvinel et al. [6], evaluating broiler chickens subjected to heat stress and fed diets containing 0.6% pequi oil, observed an increase in the NRF2 gene expression, but no effect of the enzyme activity in the liver. The NRF2 gene is linked to the antioxidant system by regulating numerous genes, such as CAT, SOD, and GPx, which is consistent with the increased mRNA expression of these enzymes in this study [30,33–35].

PFSO has positive effects on oxidative stress since studies evaluating broilers reared under heat stress conditions have shown an increase in the CAT enzymatic activity, along with the upregulation of proteins involved in the catabolic process of hydrogen, such as hemoglobin subunit epsilon. Additionally, PFSO influences the regulation of PGRMC1 (membrane-associated progesterone receptor component 1), which modulates the activity of cytochrome P450 which induces an increase in reactive oxygen species [36]. Assunção et al. [15] also reported that PFSO reduces the Ca^{2+} release in the cytosol and, consequently, the occurrence of cellular apoptosis; recovers and protects neuronal structures; inhibits NF-κB activation, as it reduces ROS with its antioxidant properties (tocopherol, phenolic compounds, and carotenoids); and reduces the expression of proteins from the glycogenolysis and gluconeogenesis pathways, as it controls oxidative stress and reduces energy demand to meet the demands of the antioxidant system. The PFSO also presents anti-inflammatory action when applied topically in rabbits [37], in hairless mice [38], in horses and Wistar rats [39], and in mice [40].

Although the bioactive compounds in PFSO, mainly tocopherol and carotenoids, have stimulated the transcription of hepatic enzymes responsible for protecting the body against oxidative stress, their action mechanisms are not yet well established [30,41,42]. Several studies have indicated that even after mRNA transcription, it is not guaranteed that these genes will be translated into proteins, depending on post-transcriptional regulation [43,44]. In addition, the protein can also be regulated at the level of translation and turnover [43]. NRF2 activity, for example, can be regulated by Keap1-NRF2 protein stability, transcriptional regulators (NF-B, ATF3, and ATF4), and post-transcription regulators (miRNAs) [45–47]. The CAT, beyond post-transcriptional regulation, can also be regulated by specific proteins or degraded through pexophagy, selective autophagy directed at peroxisomes, or through the ubiquitin–proteasome system [44]. In this regard, some stressors can act by reducing the target gene expression and, in the meantime, the supplementation of antioxidant substances can positively modulate their post-transcriptional process [44]. In this study, although the gene expression increases, the enzymatic activity is only slightly affected and, however, can be compared to those reported by [48], who supplemented exogenous enzymes in aged laying hens and found a lower expression of pancreatic amylase mRNA without altering its enzymatic activity, suggesting post-transcriptional regulation. Therefore, we speculate that the bioactive compounds in PFSO could indirectly regulate specific genes, through post-transcriptional mechanisms, to delay the activation of the corresponding antioxidant pathways, potentially avoiding excessive responses and maintaining balance in the organism [49].

The linear reduction in plasma lipoperoxidation due to PFSO inclusion is favorable to layers' health, considering their predisposition to hepatic lipidosis [50–52]. The antioxidant system is responsible for redox balance in the body and includes enzymatic antioxidants, such as CAT, SOD, and GSPx, and non-enzymatic antioxidants including carotenoids, phenolic compounds, and vitamins (A, C, and E) [53,54]. Vitamin E is an important antioxidant molecule composed of the α, β, and γ isoforms of tocopherols and tocotrienols, with emphasis on the alpha-tocopherol action [55]. The dietary vitamin E is absorbed with the lipids and then is preferentially adsorbed in the hepatic tissue, increasing its concentration [56], which is consistent with our results of increased gene expression of antioxidant enzymes in the hepatic tissue stimulated by tocopherol [41]. From the liver, the tocopherol is secreted into plasma, especially with LDL, acting to suppress lipoperoxidation [57] and scavenging the molecules resulting from oxidative processes, such as malonaldehyde [58–60]. Furthermore, several studies reported that tocopherol supplementation improved antioxidant capacity by enhancing GSH level [60], GSPx [61–63], SOD [62,63], and CAT activity [60]. In addition, polyunsaturated fatty acids could also be associated with the reduction in LDL

oxidation, as their hypocholesterolemic action results in larger chylomicrons, which appear to be eliminated more quickly than saturated fatty acids [64]. This result corroborates with [65], who found a reduction in the level of triglycerides, total cholesterol, and low-density lipoprotein-cholesterol in *Wistar* rats, and suggested that the PFSO hypolipidemic effect is associated with its rich content in linoleic acid.

The carotenoids (CAR) also play a crucial role in deactivating reactive species, such as peroxyl radicals ($ROO^{\bullet}$) and singlet oxygen ($^1O_2$). This protective mechanism occurs through different pathways: CAR can be oxidized by a radical to form CAR radical cations, reduced to form a CAR radical anion, or donate hydrogen to form a stable radical. In addition, reactive species from lipid oxidation, e.g., peroxyl lipid ($LOO^{\bullet}$), can also interact with CAR and form stable products [66,67]. Among the carotenoids, ß-carotene stands out [66] as a precursor of vitamin A that is significantly present in PFSO (75.63 mg/100g) [3], when compared to other sources of ß-carotene [68]. The improvement of lipid peroxidation can also be associated with the ß-carotene content present in the PFSO. The result corroborates the data presented by Zanetti [14] evaluating the PFSO inclusion in broiler diets. Cruvinel et al. [6], evaluating pequi oil, rich in β-carotene, also reported a malonaldehyde reduction. The author of [36] found results indicating that the use of PFSO reduced the percentage of DPPH and differentially expressed the protein spot Alcohol dehydrogenase 1 (ADH1) in broiler chickens, confirming the presence of retinol in the PFSO.

The present study aimed to improve the performance and egg quality of the layers fed with PFSO, since different oil sources can affect production variables according to the bioactive compounds concentration and the fatty acid ratio, modifying nutrient digestibility and absorption rate and the immune and antioxidant systems activity [69–71]. The PFSO inclusion, however, linearly reduced the albumen height, which is used to calculate the Haugh unit and explains its downward trend. In addition, the relative albumen weight decreased 0.47% (0.45% PFSO) and 0.63% (0.90% PFSO) compared to the control, while the yolk increased 0.94% (0.45% PFSO) and 1.54% (0.90% PFSO) compared to the control. These results corroborate studies that report that the lipid composition of the diet affects egg weight, especially yolk weight, with the concentration of linoleic acid being a limiting factor for yolk development [72,73]. There are also reports that Vitamin E can facilitate the release of vitellogenin, a lipoprotein specialized in transporting lipids from the liver to the ovary [74]. Thus, the high concentration of linoleic acid (69.14%) in the PFSO was used by the liver in the synthesis of lipoproteins, probably vitellogenins, which had their release stimulated by vitamin E, favoring an increase in the relative weight of the yolk. In addition, the HU results of this experiment (0.00% PFSO: 93.19; 0.45% PFSO: 92.26; and 0.90% PFSO: 91.78) exceed the minimum value determined for consumption (HU = 60), indicating quality eggs [75,76].

Despite the lack of studies using PFSO, especially in commercial layers, tests evaluating the use of oils have been carried out for years [51,77]. The studies vary from the use of soybean, canola, linseed, fish, sunflower, and rapeseed oils, among others, finding results that vary between no effect, positive effect, or negative effect on performance and egg quality [51]. The unaltered variables of this study, however, corroborate Lelis et al. [78], Ceylan et al. [79], Reddy et al. [80], and Costa et al. [81], who did not observe any effect of dietary inclusion of different oil sources on performance and egg quality. Several factors could affect oil sources, such as the vegetation stage, the climate, and the crops' production levels, causing inconsistency in the oil's effects [82].

The inclusion of PFSO in layers' diet did not affect the most relative organ weights, as indicated by other phytogenic additives [83,84], which may be related to the tested product and its composition, and by the poultry rearing conditions [6]. Although oxidative

stress can affect the relative weight of immunologic (spleen, bursa, and intestines) and important metabolic organs, such as the liver, the present study did not have enough challenge to affect their development [85], even with the probable increase in liver activity to produce vitellogenin, which will be deposited in the yolk, stimulated by vitamin E and the omega 6 content [72–74]. However, the quadratic trend observed for the relative weight of abdominal fat and ovary shows an increase in hens receiving 0.45% PFSO and a decrease in the 0.90% PFSO dose. This trend goes along with body weight gain and feed conversion ratio, which resulted in the final body weight (BW), with the hens in the 0.45% PFSO treatment (BW: 1,806.50) being 2.16% higher and those in the 0.90% PFSO treatment (BW: 1,726.63) being 2.35% lower than the average weight of the control group (BW: 1,768.25). Therefore, although this behavior can be seen in the other organs, the difference in these hens' weight was more markedly represented by the accumulation of abdominal fat and the development of the ovary, a common characteristic in laying hens, since lipid metabolism and the development of the organ that generates the yolk are correlated [52].

The supplementation levels of PFSO used in this study did not affect the most variables analyzed. However, the PFSO bioactive compounds have been widely studied, highlighting the potential use of this oil as an additive due to its antioxidant, immunomodulatory, and anti-inflammatory properties. The results presented in this paper provide a starting point for PFSO evaluation in laying hens' nutrition and suggest the need for further research under different conditions, such as heat stress or pathogenic challenges, to also assess the effects of PFSO on the immune system. Additionally, in-depth analyses using omics approaches are recommended to elucidate the PFSO impact on the post-transcriptional regulation of antioxidant genes.

5. Conclusions

In conclusion, PFSO at a level up to 0.90% provides beneficial responses to laying hens' health by acting as natural antioxidants and indirectly activating the defense mechanisms to reduce the effects of molecular oxidative damage, expressed by plasma lipid oxidation decrease and antioxidant enzyme gene expression increase, even without activating the enzymatic antioxidant system in the liver. Overall, the PFSO compounds were not enough to enhance laying hens' performance and egg quality but were able to sustain performance and most parameters from egg quality and relative organ weights and lengths.

Author Contributions: Conceptualization, L.G.C. and J.S; methodology, L.G.C., S.M.K. and J.R.S.; validation, L.G.C.; formal analysis, L.G.C.; investigation, L.G.C., P.A.D.R., G.A.d.L., E.O.F.C., J.A.d.S., J.d.L.L., A.L.L., S.M.K., A.C.P. and J.R.S.; resources, J.R.S.; data curation, L.G.C., P.A.D.R., G.A.d.L., E.O.F.C., J.d.L.L., A.L.L., S.M.K., A.C.P. and J.A.d.S.; writing—original draft preparation, L.G.C. and J.R.S.; writing—review and editing, L.G.C., P.A.D.R., G.A.d.L. and J.R.S.; visualization, L.G.C. and J.R.S.; supervision, J.R.S.; project administration, L.G.C. and J.R.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Council for Scientific and Technological Development—CNPq, grant number 163518/2021-1.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (protocol code 0097/2022) on 11 May 2022.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CAR	carotenoids
CAT	catalase
GPx	glutathione peroxidase
HCl	Hydrochloric acid
HU	Haugh unit
IBTEC	Biotechnology Institute
MDA	malonaldehyde
NRF2	erythroid nuclear factor 2
PFSO	passion fruit seed oil
RH	relative humidity
SOD	superoxide dismutase
SWUSA	shell weight per surface area
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
UNESP	São Paulo State University

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