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Addition of grape pomace flour in the diet on laying hens in heat stress: Impacts on health and performance as well as the fatty acid profile and total antioxidant capacity in the egg

João H. Reis[a], Roger R. Gebert[a], Mauricio Barreta[b], Marcel M. Boiago[a,b], Carine F. Souza[c], Matheus D. Baldissera[d], Ingrid D. Santos[e], Roger Wagner[e], Luciane V. Laporta[f], Lenita M. Stefani[a,b], Aleksandro S. Da Silva[a,b,c,*]

[a] Department of Animal Science, Universidade do Estado de Santa Catarina, Chapecó, SC, Brazil
[b] Graduate Program of Animal Science, Universidade do Estado de Santa Catarina, Chapecó, SC, Brazil
[c] Graduate Program in Toxicological Biochemistry, Universidade Federal de Santa Maria, Santa Maria (UFSM), Brazil
[d] Graduate Program in Pharmacology, Universidade Federal de Santa Maria, Santa Maria (UFSM), Brazil
[e] Graduate Program in Food Science, Universidade Federal de Santa Maria, Santa Maria (UFSM), Brazil
[f] Universidade Franciscana, Santa Maria (UFSM), Brazil

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ABSTRACT

The aim of this study was to evaluate whether the addition of grape pomace flour (GPF) in the diet of laying hens at the end of the productive cycle and on heat stress could exert benefits on their health and performance, as well as egg quality. For this, 74-week-old laying hens (n = 64) were divided into four groups with four repetitions each, as follow: T0 (the control group; without GPF), T1 (1% GPF), T2 (2% GPF) and T3 (3% GPF) during 35 days. Percentage of laid eggs was higher in the group T1 compared to T0, and the feed intake was higher in the groups T1, T2 and T3 compared to T0. There was no difference regarding the chemical-physical composition of fresh eggs; however, eggs from GPF-fed chickens showed changes after storage regarding specific gravity, yolk index, pH of yolk, albumen and Haugh unit compared to T0. Fresh or stored egg yolk from GPF groups showed higher antioxidant capacity and lower lipid peroxidation compared to T0. GPF (3%) prevented the reduction of monounsaturated fatty acids in the yolk of stored eggs compared to T0. Glutathione peroxidase and superoxide dismutase activities, as well as total antioxidant capacity against peroxyl radicals were higher in the serum of laying hens that received GPF compared to T0, while lipid peroxidation was lower. In summary, the addition of GPF in the diet for laying hens at the end of the productive cycle can be beneficial for animal health and exerted positive effects in their performance and egg quality.

1. Introduction

The chronic heat is a major stress factor in laying hens and many studies on the effect of heat stress have been published (Mignon-Grasteau et al., 2015), and the age of the birds can be an aggravating factor. The end of the productive cycle of egg-laying hens is associated with some difficulties problems, mainly linked to increased egg size (Jardim Filho et al., 2005), in addition to lower deposition of calcium as a consequence of increased shell surface, which impairs egg-peel strength (Roll et al., 2009). It is important to highlight that the quality of fresh eggs is not affected by chicken´s age (Nak et al., 2015), but lower calcium intake and/or eggshell deposition are considered a problem due to the increased risk of eggshell breakage (Vellasco et al., 2016). According to Alleoni and Antunes (2001), after seven days of storage under room temperature (25 °C), egg quality was significantly impaired compared to fresh eggs, since stored eggs are unable to maintain their chemical-physical composition. Therefore, it is important to find alternatives to minimize these issues at the end of the egg-production cycle, as well as to maintain egg quality. In this sense, the use of natural products has been considered an interesting approach to improve egg quality and animal health. In this context, the use of curcumin was able to maintain egg quality after 21 days of storage (Galli et al., 2018). Also, the use of diet supplemented with essential oils and vitamin E was able to improve egg quality by reducing its oxidant levels (Kaya et al., 2013).

Grape pomace flour (GPF) extracted from skins and seeds is

* Corresponding author at: Department of Animal Science, Universidade do Estado de Santa Catarina, Chapecó, SC, Brazil.
E-mail address: aleksandro.silva@udesc.br (A.S. Da Silva).

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obtained from the grape industry as a residue rich in antioxidants (Stevenson and Hurst, 2007), reason by which it is sold to be consumed by humans. GPF is composed by phenols, flavonoids, anthocyanins and catechins, as well as other compounds well-known by its antioxidant potential, such as resveratrol, luteolin, quercetin and kaempferol (Ferreira, 2010). In this sense, the use of resveratrol in the diet of laying hens exerted positive effects on their performance and in enzymes linked to the antioxidant defense system (Feng et al., 2017).

The use of isolated components present in grapes is considered unfeasible due to high extraction costs of its pure molecules. However, the use of GPF can be considered advantageous, mainly in countries with significant wine production, where these residues can be acquired by lower prices. Our hypothesis is that GPF added in the diet of laying hens may increase the serum levels of antioxidants, and consequently, improve egg quality and aid egg conservation during storage. Therefore, the aim of this study was to evaluate whether the addition of GPF in the diet of laying hens at the end of their productive cycle could exert benefits on their health, performance and egg quality.

2. Material and methods

2.1. GPF and resveratrol quantification

The flour used in this study was obtained from grape pomace (seeds and skins) at a local store (Chapecó city, Southern Brazil). Its concentration of resveratrol was determined using 25 mg diluted in 15 mL of ethanol and, heated at 40 °C for 30 min under agitation. After this, the content was transferred to a volumetric flask and completed with ethanol up to 25 mL. The sample was diluted at 10 µg/mL and filtered using a regenerated cellulose acetate membrane (0.45 µm), in triplicate. In the sequence, the standard of resveratrol was prepared at theoretical concentration of 10 µg/mL. Resveratrol quantification was performed using the method described by Bender et al. (2016) with a chromatograph (Shimadzu) LC system, with bomb LC-20AT, automatic injector SIL-20, oven for column CTO-20AC, diode array detector SPD-M20A (PDA) and software CBM-20 A. A chromatography column C18 (4.6 mm × 150 mm) and column temperature at 25 °C was used. The mobile phase was composed by acetonitrile and acidified water pH 3.0 (20:80 v/v), injector volume of 20 µL, flow of the mobile phase at 1 mL/min and wavelength of 280 nm. The average of resveratrol content on the GPF was 5.85% $\pm$ 0.01, i.e., 58.5 mg of resveratrol per g of GPF.

2.2. Animals and experimental design

Sixty-four laying hens (Hy-Line lineage; 74-week-old) were used as the experimental model. The animals were maintained in an experimental house, allocated in cages with four animals each in a completely randomized design with four treatments of four repetitions. A basal diet of corn and soybean meal (Table 1) was used. The treatments were: group T0 (basal diet; without GPF), T1 (basal diet supplemented with 1% of GPF), T2 (basal diet supplemented with 2% of GPF), and T3 (basal diet supplemented with 3% of GPF). All animals received water *ad libitum.*

The experiment was carried out in the south of Brazil, in a shed without air conditioning, which used to handle curtains daily in the summer. During the experiment the temperature was measured inside the installation during the afternoon (01:00 P.M.), with oscillation between 29 °C and 37 °C during the experimental period.

2.3. Performance

Daily, the number of eggs per repetition was recorded to calculate the percentage of laid eggs per treatment. To evaluate performance, the eggs were weighted using a digital balance of the end productive cycle (days 33, 34 and 35 of experiment), and average egg weight was determined. The egg mass (g/animal/day) was obtained by the percentage of laid eggs in relation to the average egg weight. Feed consumption was *ad libitum* daily; however, feed consumption was determined at the end of the experiment. Thus, feed conversion per kg of eggs and per dozen were also determined.

Table 1
Ingredients and composition of laying hens diet with grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Ingredients	T0	T1	T2	T3
Corn	62.330	62.330	62.330	62.330
Soybean meal	20.720	20.720	20.720	20.720
Soybean oil	2.000	2.000	2.000	2.000
L-lysine 99	1.100	1.100	1.100	1.100
DL-methionine	0.175	0.175	0.175	0.175
L-threonine 99	0.055	0.055	0.055	0.055
NaCl	0.410	0.410	0.410	0.410
Phosphate bicalcium	1.100	1.100	1.100	1.100
Calcitic limestone	9.720	9.720	9.720	9.720
Premix vitamin/mineral	0.300	0.300	0.300	0.300
Vehicle	3.000	2.000	1.000	0.000
Grape pomace flour	0.000	1.000	2.000	3.000
Ccomposition				
Metabolizable energy Mcal/Kg	2.762	2.762	2.762	2.762
Total protein (%)	14.567	14.635	14.703	14.770
Available phosphorus (%)	0.293	0.293	0.293	0.293
Calcium (%)	4.095	4.095	4.095	4.095
Sodium (%)	0.196	0.196	0.196	0.196
Digestive Lysine (%)	0.710	0.710	0.710	0.710
Digestive Methionine (%)	0.392	0.392	0.392	0.392
Digestive Lysine + Methionine (%)	0.607	0.607	0.607	0.607

Premix vitaminic per kg of feed: folic acid (200 mg/kg); pantothenic acid (min 4.33 mg/kg); copper (min 2.66 mg/kg); choline (min 78.12 mg/kg); iron (min 16.7 mg/kg); phytase (min 166.66 ftu/kg); iodine (min 400 mg/kg); manganese (min 23.3 g/kg); niacin (min 10 g/kg); selenium (min 66.7 mg/kg); vitamin A (min 2.333.333 UI/kg); vitamin B1 (min 666.7 mg/kg); vitamin B12 (min 3.333 mcg/kg); vitamin B2 (min 1.666 mg/kg); vitamin B6 (min 1000 mg/kg); vitamin D3 (min 733.333 UI/kg); vitamin E (min 3.666 UI/kg); vitamin K3 (min 533.33 mg/kg); zinc (min 16.7 g/kg); colistin sulfate (min 3.333 mg/kg).

2.4. Sample collection

In order to evaluate egg quality, two eggs per repetition/day (corresponding at eight eggs per group/day were collected on days 33, 34 and 35 of the experiment. The eggs from day 35 were processed soon after laid, i.e., the fresh egg. The eggs from days 33 and 34 were processed in two moments, i.e., after 21 and 30 days of storage in paper trays in a controlled environment (25 ± 2 °C).

Blood sampling (2 mL) was performed on days 0 and 35 of the experiment from the brachial vein of two laying hens per repetition (totalizing eight animals per treatment). These samples were allocated in tubes without anticoagulant to obtain serum (after centrifugation at 3500 rpm during 10 min). The serum was stored at –20 °C until analyses.

2.5. Chemical-physical egg composition

Specific gravity was measured according to the method described by Freitas et al. (2004), peel strength was measured using a texture analyzer (TA.XT. plus), and albumen height was evaluated using a tripod micrometer (Galli et al., 2018). After the determination of albumen height and egg mass (W), the Haugh unit was calculated by the equation: HU = 100 log (H (mm) + 7.57 – 1.7 W (g) 0.37), following the technique described by Haugh (1937). Yolk index was estimated using a digital pachymeter as a relation between yolk height (mm) and diameter (mm). A colorimetric matrix (DSM-Yolk Color Fan) in a colorimeter (Minolta CR-400) was used to measure: luminosity (L*), intensity of red (a*) and intensity of yellow (b*). To quantify the percentage of shell, eggshells were manually separated, washed and dried at room temperature for 48 h. The percentage of yolk, albumen and dried shells was calculated. Yolk and albumen pH were obtained using a digital pHmeter.

2.6. Fatty acid profile of feed and egg

Fatty acid extraction was carried out by Bligh and Dyer (1959) method with some modifications to egg yolk and feed. Each sample (0.5 g) was diluted in 1.3 mL of water, 4 mL of methanol and 2 mL of chloroform using a 15 mL polypropylene tube and gentle shaking for 30 min. Then, 2 mL of chloroform and 1.5% of $NaSO_4$ solution were added to promote a biphasic system. This mixture was shaken for 2 min, and then centrifuged for 5 min at 3000 rpm. Lipids obtained from the chloroform phase were submitted to fatty acid analysis. Fatty acid (FA) methylation was performed by a transesterification method proposed by Hartman and Lago (1973), where 20 mg of lipids was mixed to 1 mL of 0.4 M of KOH methanolic solution in a test tube and shaken in a vortex for 1 min. Samples were kept in a water bath for 10 min at boiling point. Subsequently, they were cooled at room temperature and 3 mL of 1 M of H_2SO_4 methanolic solution was added and shaken in vortex and maintained in a water bath for 10 min. After cooling, 2 mL of hexane was added and centrifuged at 5000 rpm for 5 min. Lastly, hexane with the fatty acid methyl esters (FAME) were submitted to chromatography analysis.

For the FAME determination, a gas chromatograph model 3400CX equipped with a flame ionization detector (Varian, Palo Alto, CA) and automated injection system (Autosampler, Varian 8200) was employed. One microliter of samples was injected in a split/splitless injector, operated in a split mode (20:1) at 250 °C. Hydrogen was used as carrier gas at a constant pressure of 15 psi and initial flow rate of 2.17 mL/min. FAME separation was carried out using a DB-WAX (30 m × 0.25 mm × 0.25 µm, J & W, Folson, CA, USA) chromatography column. The initial oven temperature was set at 50–180 °C (increasing 15 °C/min) and to 220 °C (increasing 2 °C/min) up to 230 °C (increasing 20 °C/min), and maintained for 3 min in an isothermal condition. The temperature detector was kept at 240 °C. The FAME compounds were identified by comparison of the experimental retention time with those from an authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were expressed as the percentage of each FA identified in the lipid fraction. Results of feed FA profile were shown in Table 2.

Table 2
Fatty acid and fat levels on laying hens diet with grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Composition	T0	T1	T2	T3
Fatty acids on diet (%)				
C14:0	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
C16:0	14.1 (0.1)	13.9 (0.2)	14.1 (0.1)	13.9 (0.4)
C16:1	0.2 (0.0)	0.1 (0.0)	0.2 (0.0)	0.2 (0.0)
C17:0	0.1 (0.0)	0.1 (0.0)	0.1(0.0)	0.1 (0.0)
C17:1	0.1 (0.0)	0.1 (0.0)	0.1(0.0)	0.1 (0.0)
C18:0	3.9 (0.2)	3.9 (0.1)	4.1 (0.1)	3.9 (0.1)
C18:1n9c	26.8 (0.4)	27.0 (0.2)	27.0 (0.3)	26.9 (0.4)
C18:2n6c	48.9 (0.3)	49.1 (0.2)	48.9 (0.1)	49.2 (0.4)
C18:3n3	3.8 (0.0)	3.7 (0.1)	3.6 (0.1)	3.7 (0.0)
C20:0	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)
C20:1	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)
C22:0	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)	0.6 (0.1)
C24:0	0.4 (0.0)	0.4 (0.0)	0.4 (0.0)	0.4 (0.1)
Fat in the diet (g/100 g)	4.19 (0.2)	4.57 (0.1)	4.48 (0.5)	4.49 (0.3)

Myristic acid (C14:0); palmitic acid (C16:0); margaric acid (C17:0); heptadecanoic acid (C17:1); stearic acid (C18:0); oleic acid (C18:1n9c); linoleic acid (C18:2n6c); alpha-linoleic (C18:3n3); arachidic acid (C20:0); behenic acid (C22:0); lignoceric acid (C24:0). Note: There was no significant difference between groups regarding fatty acid profile and fat in the diet.

2.7. Egg yolk lipid peroxidation and antioxidant capacity against peroxyl radical (ACAP)

Lipid peroxidation was determined according to the methodology described by Giampietro et al. (2008), which measured the content of thiobarbituric acid reactive substances (TBARS) in egg yolk. The decomposition of lipid peroxides was measured via spectrophotometer (532 nm) using 1,1,3,3-tetramethoxypropane (TMP) as the standard. The results were expressed as mg TPM/kg of yolk.

ACAP was determined according to the method described by Amado et al. (2009) with modifications for eggs yolks. This method consists of finding the antioxidant capacity of tissues using a fluorescent substrate (2′.7′ dichlorofluorescein diacetate - H2DCF-DA) and the production of peroxyl radicals by thermal decomposition of ABAP (2.2′-azobis 2 methylpropionamidine dihydrochloride). The fluorescence was determined through a microplate reader (Spectramax I3) at 37 °C (excitation:485 nm; emission: 530 nm) with readings after every 5 min during 30 min. The results were expressed as units/g of egg yolk.

2.8. Serum biochemistry

Serum levels of total proteins, albumin, triglyceride, cholesterol and uric acid were evaluated using the semi-automated BioPlus (Bio-200) and commercial kits (Analisa®). Serum globulin levels were calculated by the difference between serum levels of total protein and albumin.

2.9. Serum levels of oxidants and activity of antioxidant enzymes

The activity of glutathione peroxidase (GPx) was measured indirectly by monitoring the oxidation rate of NADPH at 340 nm using cumene hydroperoxide (CuOOH), according to Wendel (1981). The glutathione S-transferase (GST) was determined spectrophotometrically according to the method described by Habig et al. (1974). The activity of superoxide dismutase (SOD) was determined according to the autooxidation principle of pyrogallol, inhibited in the presence of SOD. The optical density change was determined kinetically for two minutes at 420 nm, at ten second intervals according to methodology described by Beutler (1984). All enzymatic activities were expressed as U/mg of protein.

ACAP in serum was determined according to the method described by Amado et al. (2009) with modification for mammals, as described in the previous section of this paper for egg yolk, and the results were expressed as fluorescence units/mg of protein. The levels of lipid peroxidation were evaluated by seric malondialdehyde (MDA) concentration, using the measurement of TBARS according to the protocol stablished by Jentzsch et al. (1996), and the results were expressed as nmol MDA/mg of protein.

The protein concentration in serum was determined by the Coomassie Blue method, following the methodology described by Read and Northcote (1981) using bovine serum albumin as a standard.

2.10. Statistical analyses

The data were subjected to a normality test (Shapiro-Wilk test). The biochemical analyses, as well as the ACAP and LPO variables did not show normality, and they were transformed to logarithm. After all variables showed normal distribution, the data was subjected to the parametric one-way analysis of variance (ANOVA), followed by Tukey post hoc test considering $P < 0.05$. All results were shown as mean ± standard deviation.

3. Results

3.1. Animal performance

For most of the day, laying hens exhibited behavior of suffering

Table 3
Ration consumption (RC), feed conversion (FC) per mass and per dozen eggs, and laying percentage using different levels of grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Groups	RC (g/animal/day)	FC per mass of eggs (kg kg^{-1})	FC per dozen of eggs (kg per dozen)	% laying
T0	116.57 ± 5.82[b]	2.23 ± 0.38[ab]	1.90 ± 0.26	72.50 ± 9.04[b]
T1	121.16 ± 9.07[a]	1.99 ± 0.39[b]	1.72 ± 0.27	85.95 ± 11.62[a]
T2	125.29 ± 10.00[a]	2.42 ± 0.46[ab]	1.93 ± 0.60	81.31 ± 12.65[ab]
T3	125.30 ± 19.76[a]	2.78 ± 0.77[a]	2.06 ± 0.57	74.82 ± 9.17[ab]
P value	0.001*	0.001 *	0.258	0.042*

Note: P < 0.05 indicate significant difference. Different letters in the same column indicate significant difference between groups (Tukey post hoc test).

from thermal stress. Laying hens from groups T1, T2 and T3 showed higher daily feed consumption when compared to the group T0. Also, the group T3 showed higher feed conversion per mass of eggs compared to the group T0, while the group T1 showed higher percentage of laid eggs (Table 3).

3.2. Chemical-physical egg composition

No difference was observed between groups regarding the chemical-physical composition of fresh eggs. Similarly, there were no differences between groups regarding peel strength, luminosity (L+), red intensity (a+), yellow intensity (b+), eggshell thickness, and percentage of albumin, yolk and shell in stored eggs (Table 4). Specific gravity was lower in groups T1, T2 and T3 compared to the control group after 30 days of storage. Yolk index was lower on day 21 (groups T2 and T3) and 30 (groups T1, T2 and T3) of storage compared to the group T0. The pH of yolk egg was lower in groups T1, T2 and T3 compared to the group T0 on day 30 of storage, as well as for albumen pH on days 21 and 30 of storage. Haugh unit was lower in groups T2 and T3 compared to the group T0 on days 21 and 30 of storage (Table 4).

3.3. Fatty acids profile in egg yolk

No significant difference was observed between groups regarding the content of myristic (C14:0), myristylic (C14:1), pentadecylic (C15:0), palmitic (C16:0), heptadecanoic (C17:1), stearic (C18:0), oleic (C18:1n9c), alpha-linoleic (C18:3n3) and arachidonic (C20:4n6) fatty acids in egg yolk. After 21 days of storage, butyric acid (C4:0) content

Table 4
Chemical and physical composition of fresh (day 1) and stored eggs under environmental temperature during 21 and 30 days from laying hens fed with different levels of grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Variable	Day	T0	T1	T2	T3	P
Specific gravity	1	1.083 ± 0.008	1.084 ± 0.004	1.085 ± 0.006	1.086 ± 0.005	0.958
	21	1.024 ± 0.033	1.026 ± 0.013	1.022 ± 0.013	1.03 ± 0.011	0.087
	30	1.024 ± 0.016[a]	0.893 ± 0.354[b]	0.927 ± 0.232[b]	0.912 ± 0.277[b]	0.001 *
Peel strength ($x10^3$)	1	4.56 ± 1.10	4.06 ± 1.20	4.95 ± 1.46	4.48 ± 0.76	0.258
	21	4.36 ± 0.88	4.57 ± 0.85	4.62 ± 0.86	4.18 ± 1.03	0.365
	30	5.43 ± 0.67	4.38 ± 2.03	4.38 ± 1.70	4.37 ± 1.37	0.198
Color range (sub)	1	8.6 ± 0.547	8.0 ± 2.16	8.5 ± 1.41	7.2 ± 1.72	0.098
	21	8.0 ± 1.63	7.29 ± 2.49	8.86 ± 0.9	8.57 ± 1.72	0.425
	30	8.0 ± 1.67	6.93 ± 3.21	8.33 ± 1.97	7.26 ± 3.07	0.147
L*	1	58.54 ± 4.63	58.28 ± 2.16	58.48 ± 2.19	58.23 ± 2.71	0.960
	21	61.60 ± 2.51	63.97 ± 4.64	62.57 ± 3.58	63.18 ± 3.40	0.745
	30	62.72 ± 2.75	59.77 ± 6.29	63.21 ± 1.71	59.92 ± 6.97	0.568
a*	1	1.96 ± 0.68	2.34 ± 0.90	1.60 ± 0.78	1.80 ± 0.70	0.365
	21	0.82 ± 1.30	1.86 ± 1.37	0.92 ± 0.57	1.01 ± 0.85	0.456
	30	0.42 ± 1.06	0.36 ± 1.47	0.17 ± 0.75	0.06 ± 0.96	0.196
b*	1	40.97 ± 3.68	41.02 ± 3.08	44.36 ± 2.30	43.19 ± 3.03	0.745
	21	56.62 ± 6.81	53.12 ± 6.57	57.93 ± 4.81	58.68 ± 2.53	0.365
	30	56.77 ± 2.48	57.29 ± 5.22	59.31 ± 5.64	58.07 ± 3.74	0.825
Yolk index	1	0.46 ± 0.03	0.46 ± 0.2	0.43 ± 0.07	0.44 ± 0.04	0.145
	21	0.32 ± 0.05 [a]	0.28 ± 0.3 [ab]	0.27 ± 0.04 [b]	0.27 ± 0.04 [b]	0.035 *
	30	0.29 ± 0.05 [a]	0.22 ± 0.10 [b]	0.26 ± 0.03 [ab]	0.21 ± 0.10 [b]	0.024 *
pH of yolk	1	5.99 ± 0.02	6.00 ± 0.04	6.02 ± 0.043	6.08 ± 0.04	0.854
	21	6.35 ± 0.08	6.34 ± 0.10	6.26 ± 0.09	6.31 ± 0.03	0.924
	30	7.25 ± 0.51 [a]	5.72 ± 0.93 [b]	6.01 ± 0.85 [b]	5.67 ± 0.60 [b]	0.021 *
pH of albumen	1	7.93 ± 0.18	7.89 ± 0.06	7.91 ± 0.14	7.97 ± 0.11	0.974
	21	9.01 ± 0.16 [a]	7.17 ± 0.04 [b]	7.18 ± 0.03 [b]	7.16 ± 0.03 [b]	0.001 *
	30	9.06 ± 0.25 [a]	7.37 ± 0.98 [b]	8.10 ± 0.30 [b]	7.18 ± 0.29 [b]	0.001 *
Eggshell thickness	1	0.40 ± 0.06	0.40 ± 0.05	0.38 ± 0.05	0.39 ± 0.02	0.887
	21	0.35 ± 0.04	0.38 ± 0.02	0.37 ± 0.03	0.35 ± 0.04	0.654
	30	0.39 ± 0.06	0.35 ± 0.14	0.38 ± 0.05	0.35 ± 0.05	0.598
Haugh unit	1	87.85 ± 4.66	88.54 ± 5.44	90.26 ± 9.79	84.87 ± 4.63	0.568
	21	39.01 ± 12.75 [a]	36.24 ± 7.93 [a]	25.70 ± 8.50 [b]	24.98 ± 7.92 [b]	0.041 *
	30	40.81 ± 15.79 [a]	28.90 ± 11.36 [ab]	23.20 ± 11.03 [b]	22.11 ± 7.27 [b]	0.024 *
Yolk (%)	1	24.2 ± 2.33	25.3 ± 1.67	25.65 ± 2.37	25.41 ± 2.24	0.935
	21	28.72 ± 4.11	27.17 ± 1.65	28.01 ± 3.22	27.78 ± 1.51	0.901
	30	28.00 ± 2.12	28.79 ± 2.60	29.50 ± 1.13	28.97 ± 2.19	0.914
Albumen (%)	1	67.04 ± 2.15	65.50 ± 1.67	65.07 ± 2.30	65.16 ± 2.72	0.235
	21	61.85 ± 4.53	62.99 ± 1.93	62.12 ± 3.60	62.69 ± 2.03	0.821
	30	61.84 ± 2.61	61.96 ± 3.42	60.68 ± 1.98	60.68 ± 1.65	0.701
Shell (%)	1	8.76 ± 1.17	9.20 ± 0.40	9.28 ± 0.75	9.43 ± 0.76	0.136
	21	9.43 ± 1.40	9.84 ± 0.88	9.87 ± 0.77	9.53 ± 0.90	0.835
	30	10.16 ± 1.52	9.24 ± 0.82	9.82 ± 1.31	10.36 ± 1.82	0.235

Note: P < 0.05 indicate significant difference. Different letters in the same column indicate significant difference between groups (Tukey post hoc test).

Table 5
Egg fatty acid profile of fresh (day 1) and stored eggs in environmental temperature during 21 and 30 days of laying hens fed with different levels of grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Fatty acid	Day	T0	T1	T2	T3	P value
	1	0.27 ± 0.20	0.22 ± 0.04	0.21 ± 0.03	0.22 ± 0.05	0.087
C4:0	21	0.23 ± 0.02[a]	0.21 ± 0.02[b]	0.22 ± 0.03[b]	0.20 ± 0.04[b]	0.001*
	30	0.35 ± 0.13	0.28 ± 0.02	0.29 ± 0.03	0.33 ± 0.07	0.245
	1	0.38 ± 0.06[a]	0.24 ± 0.04[b]	0.24 ± 0.01[b]	0.21 ± 0.06	0.001*
C6:0	21	0.29 ± 0.05	0.29 ± 0.02	0.26 ± 0.05	0.26 ± 0.04	0.075
	30	0.37 ± 0.06[a]	0.27 ± 0.02[b]	0.29 ± 0.01[b]	0.25 ± 0.10[b]	0.024*
	1	0.30 ± 0.02	0.27 ± 0.03	0.28 ± 0.01	0.26 ± 0.03	0.423
C14:0	21	0.28 ± 0.05	0.28 ± 0.03	0.27 ± 0.01	0.27 ± 0.03	0.856
	30	0.27 ± 0.05	0.26 ± 0.03	0.27 ± 0.01	0.28 ± 0.04	0.703
	1	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.9878
C15:0	21	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.962
	30	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.954
	1	24.70 ± 1.08	24.38 ± 1.20	24.78 ± 0.77	24.62 ± 0.94	0.856
C16:0	21	23.56 ± 1.28	24.55 ± 0.65	23.62 ± 0.01	24.02 ± 0.42	0.745
	30	23.31 ± 1.19	23.24 ± 0.66	24.07 ± 0.68	23.50 ± 1.53	0.687
	1	0.17 ± 0.03	0.18 ± 0.03	0.19 ± 0.02	0.18 ± 0.03	0.903
C17:0	21	0.23 ± 0.03[a]	0.19 ± 0.03[b]	0.21 ± 0.22[ab]	0.22 ± 0.02[ab]	0.035*
	30	0.21 ± 0.03	0.21 ± 2.19	0.21 ± 0.02	0.22 ± 0.05	0.845
	1	9.47 ± 0.55	10.01 ± 0.64	10.50 ± 0.42	10.80 ± 2.60	0.569
C18:0	21	11.27 ± 0.91	10.10 ± 0.40	10.73 ± 0.66	11.08 ± 1.85	0.620
	30	10.23 ± 1.59	11.24 ± 0.06	10.57 ± 0.60	10.68 ± 0.93	0.423
	1	**35.34 ± 2.4**	**35.35 ± 2.0**	**36.25 ± 1.1**	**36.34 ± 2.1**	**0.854**
[&]**Σ SFA**	**21**	**35.88 ± 1.6**	**35.67 ± 1.7**	**35.36 ± 0.9**	**36.11 ± 0.7**	**0.887**
	30	**34.80 ± 1.9**	**35.56 ± 1.2**	**35.77 ± 1.2**	**35.32 ± 0.9**	**0.526**
	1	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.02	0.895
C14:1	21	0.03 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.935
	30	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.00	0.05 ± 0.02	0.914
	1	2.21 ± 0.21	1.97 ± 0.31	2.03 ± 0.26	2.00 ± 0.73	0.785
C16:1	21	1.59 ± 0.42[b]	2.14 ± 0.10[a]	1.75 ± 1.86[ab]	1.58 ± 0.41[b]	0.048
	30	1.81 ± 0.55	1.59 ± 1.56	1.96 ± 0.28	1.84 ± 0.70	0.335
	1	0.10 ± 0.00	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.02	0.954
C17:1	21	0.10 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.947
	30	0.11 ± 0.00	0.11 ± 0.04	0.12 ± 0.01	0.11 ± 0.01	0.968
	1	41.67 ± 1.54	40.80 ± 2.28	41.09 ± 1.73	41.82 ± 1.58	0.879
C18:1n9c	21	39.98 ± 2.03	41.16 ± 1.42	42.01 ± 2.77	40.34 ± 0.87	0.198
	30	39.42 ± 1.11	41.70 ± 1.56	40.98 ± 1.12	41.88 ± 1.97	0.695
	1	0.23 ± 0.01[a]	0.25 ± 0.02	0.23 ± 0.03[a]	0.06 ± 0.02[b]	0.001*
C20:1	21	0.08 ± 0.02	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.542
	30	0.07 ± 0.04	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.795
	1	**44.26 ± 1.3**	**43.16 ± 2.1**	**43.49 ± 1.4**	**44.02 ± 0.7**	**0.895**
[&]**Σ MUFA**	**21**	**41.78 ± 0.9** [b]	**43.51 ± 2.1**[ab]	**43.96 ± 2.5** [ab]	**44.12 ± 1.6**[a]	**0.001***
	30	**41.45 ± 1.1** [b]	**43.49 ± 1.7**[ab]	**43.16 ± 1.2**[ab]	**43.94 ± 1.7**[a]	**0.041***
	1	15.53 ± 1.02[b]	17.79 ± 1.08[a]	16.56 ± 0.99[ab]	15.83 ± 2.14[ab]	0.031*
C18:2n6c	21	17.98 ± 0.85	16.78 ± 1.40	16.73 ± 1.60	17.75 ± 0.83	0.298
	30	17.57 ± 0.61	16.79 ± 2.19	16.86 ± 1.30	17.07 ± 2.96	0.504
	1	0.11 ± 0.03	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	0.756
C18:3n6	21	0.16 ± 0.02	0.13 ± 0.01	0.15 ± 0.02	0.15 ± 0.04	0.803
	30	0.14 ± 0.02[b]	0.17 ± 0.03[ab]	0.16 ± 0.03[ab]	0.19 ± 0.05[a]	0.001*
	1	0.48 ± 0.10	0.54 ± 0.12	0.52 ± 0.07	0.43 ± 0.14	0.085
C18:3n3	21	0.45 ± 0.09	0.49 ± 0.02	0.47 ± 0.07	0.45 ± 0.11	0.120
	30	0.47 ± 0.12	0.44 ± 0.06	0.52 ± 0.04	0.47 ± 0.07	0.073
	1	0.20 ± 0.01[b]	0.20 ± 0.02[b]	0.17 ± 0.03[b]	0.24 ± 0.02[a]	0.034*
C20:2	21	0.20 ± 0.03	0.23 ± 0.02	0.24 ± 0.01	0.20 ± 0.03	0.097
	30	0.24 ± 0.03	0.21 ± 0.05	0.25 ± 0.00	0.23 ± 0.03	0.245
	1	0.15 ± 0.02[b]	0.19 ± 0.01[a]	0.16 ± 0.02[b]	0.16 ± 0.00[b]	0.015*
C20:3n6	21	0.18 ± 0.02	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.02	0.423
	30	0.19 ± 0.02	0.17 ± 0.03	0.18 ± 0.02	0.19 ± 0.01	0.687
	1	1.87 ± 0.24	1.76 ± 0.12	1.82 ± 0.05	0.16 ± 0.05	0.085
C20:4n6	21	0.20 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	0.19 ± 0.04	0.062
	30	0.18 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	0.21 ± 0.05	0.114
	1	0.11 ± 0.06[c]	0.88 ± 0.05[b]	0.92 ± 0.03[b]	1.81 ± 0.14[a]	0.001*
C22:2	21	2.01 ± 0.20	1.84 ± 0.13	1.83 ± 0.11	1.83 ± 0.17	0.426
	30	1.83 ± 0.11	1.86 ± 0.09	1.86 ± 0.13	2.12 ± 0.17	0.347
	1	0.97 ± 0.15[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.91 ± 0.09[a]	0.023*
C22:6n3	21	1.10 ± 0.11	1.01 ± 0.10	0.91 ± 0.07	1.05 ± 0.11	0.456
	30	0.07 ± 0.03[a]	0.04 ± 0.04[b]	0.05 ± 0.01[b]	0.08 ± 0.02[a]	0.028*
	1	**20.42 ± 1.1**	**21.49 ± 0.9**	**20.28 ± 1.3**	**19.67 ± 1.6**	**0.168**

(*continued on next page*)

Table 5 (*continued*)

Fatty acid	Day	T0	T1	T2	T3	P value
[&]Σ PUFA	**21**	**20.28 ± 0.8**	**20.82 ± 1.4**	**20.67 ± 1.2**	**21.78 ± 0.7**	**0.095**
	30	**20.69 ± 0.6**	**19.86 ± 1.8**	**20.07 ± 1.7**	**20.56 ± 0.9**	**0.412**

Butyric acid (C4:0); caproic acid (C6:0); myristic acid (C14:0); myristylic acid (C14:1); pentadecylic acid (C15:0); palmitic acid (C16:0); palmitolytic acid (C16:1); margaric acid (C17:0); heptadecanoic acid (C17:1); stearic acid (C18:0); oleic acid (C18:1n9c); linoleic acid (C18:2n6c); alpha linoleic acid (- C18:3n6); linolelaidic acid (C18:3n3); gadoleic acid (C20:1); eicosadienoic acid (C20:2); dihomo-y-linolenic acid (C20:3n6); arachidonic acid (C20:4n6); docosadienoic acid (C22:2); docosahexaenoic acid (C22:6n3).
Note: $P < 0.05$ indicate significant difference. Different letters in the same column indicate significant difference between groups (Tukey post hoc test).
[&] Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA).

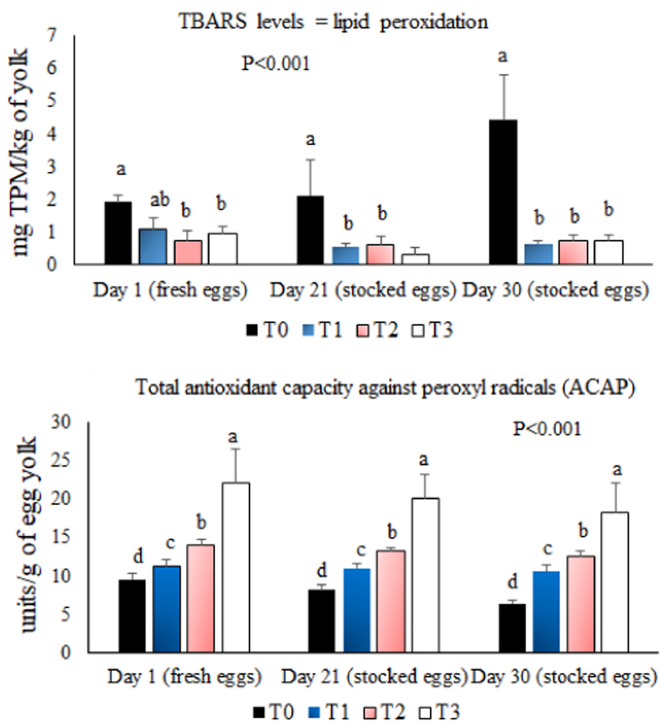

Fig. 1. Levels of lipid peroxidation (thiobarbituric acid reactive substances – TBARS) and antioxidant capacity against peroxyl radicals (ACAP) in egg yolk of laying hens fed with different levels of grape pomace flour (GPF) (fresh egg (day 1) and stored egg under environmental temperature during 21 and 30 days. T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF). Letters (a, b) different in the same graph, at each moment (Days 1, 21, 30), differ statistically between treatments (Tukey post hoc test).

was lower in groups T1, T2 and T3 compared to the group T0, while the content of caproic acid (C6:0) was lower in the same groups after 30 days of storage. Content of palmitolytic acid (C16:1) after 21 days of storage was higher in the group T1 compared to the group T0, while content of margaric acid (C17:0) was lower in groups T1, T2 and T3 compared to the group T0 after 21 days of storage. No significant difference was observed between groups regarding the sum of saturated fatty acids.

Content of linoleic acid (C18:2n6c) in fresh eggs was higher in the group T1 compared to the group T0, while the content of gadoleic acid (C20:1) was lower in the group T3 compared to T0. It is important to highlight that the sum of monounsaturated fatty acid content was higher after storage in the group T3 compared to T0, which was not observed in fresh eggs.

Content of eicosadienoic acid (C20:2) and docosadienoic acid (C22:2) in fresh eggs were higher in the group T3 compared to the group T0, while the content of dihomo-y-linolenic acid (C20:3n6) was higher in the group T1 compared to the group T0. On the other hand, the content of docosahexaenoic acid (C22:6n3) in fresh eggs was lower in the groups T1 and T2 compared to T0. No significant difference was observed between groups regarding the sum of polyunsaturated fatty acids.(Table 5)

3.4. Lipid peroxidation and antioxidant capacity against peroxyl radical (ACAP) in egg yolk

TBARS in fresh (groups T2 and T3) and stored (groups T1, T2 and T3) egg yolk were lower when compared to the group T0. On the other hand, ACAP levels were higher in fresh and stored egg yolk in the groups T1, T2 and T3 compared to T0 (Fig. 1).

Table 6
Serum biochemistry of laying hens fed with different levels of grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Variable	Day	T0	T1	T2	T3	P value
Total protein (mg/dL)	1	6.15 ± 0.83	6.07 ± 0.29	5.77 ± 0.58	6.22 ± 0.34	0.465
	35	8.67 ± 1.27^{a}	6.0 ± 1.26 ^{b}	6.17 ± 1.96 ^{b}	6.6 ± 1.15 ^{b}	0.035*
Albumin (mg/dL)	1	1.95 ± 0.27	2.08 ± 0.21	1.8 ± 0.28	1.68 ± 0.25	0.521
	35	2.25 ± 0.84	1.82 ± 0.20	1.98 ± 0.32	1.97 ± 0.31	0.258
Globulin (mg/dL)	1	4.2 ± 1.02	3.98 ± 0.46	3.97 ± 0.75	4.53 ± 0.39	0.687
	35	6.42 ± 2.44 ^{a}	4.18 ± 1.32 ^{b}	4.18 ± 1.82 ^{b}	4.63 ± 1.61 ^{b}	0.001*
Triglycerides (mg/dL)	1	613 ± 428.39	524.5 ± 221.58	375.33 ± 172.7	330.8 ± 191.11	0.247
	35	1038.2 ± 627.2	985.3 ± 557.76	925.5 ± 472.98	1053 ± 560.3	0.562
Uric acid (mg/dL)	1	4.97 ± 2.45	6.52 ± 2.28	5.5 ± 2.85	5.45 ± 3.55	0.543
	35	7.28 ± 1.01 ^{a}	5.92 ± 2.06 ^{ab}	5.48 ± 2.38 ^{ab}	5.03 ± 2.15 ^{b}	0.041*
Cholesterol (mg/dL)	1	62.67 ± 25.08^{a}	72.0 ± 28.34^{a}	65.0 ± 29.13	48.67 ± 22.67	0.187
	35	100.67 ± 27.2^{a}	98.0 ± 24.66^{a}	91.5 ± 22.42^{ab}	67.17 ± 2.90^{b}	0.049*

Note: $P < 0.05$ indicate significant difference. Different letters in the same column indicate significant difference between groups (Tukey post hoc test).

Table 7
Serum glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione S-transferase (GST) activities, and levels of thiobarbituric acid reactive substances (TBARS) and antioxidant capacity against peroxyl radicals (ACAP) in laying hens fed with different levels of grape pomace flour (GPF). T0 (the control group; without GPF), T1 (1% of GPF), T2 (2% of GPF) and T3 (3% of GPF).

Variable	Day	T0	T1	T2	T3	P value
GPx	1	1.43 ± 1.50	1.59 ± 0.96	1.87 ± 1.32	1.79 ± 1.28	0.450
	35	4.66 ± 1.97^{d}	9.16 ± 6.60^{c}	16.42 ± 12.8^{bc}	32.38 ± 9.28^{a}	0.001*
SOD	1	3.13 ± 2.32	4.48 ± 2.67	5.09 ± 1.59	4.45 ± 0.88	0.365
	35	3.88 ± 1.22^{b}	5.48 ± 2.18^{ab}	7.31 ± 0.87^{a}	5.21 ± 1.46^{ab}	0.013*
GST	1	3.71 ± 1.91	3.71 ± 2.73	4.54 ± 3.24	4.35 ± 3.02	0.536
	35	3.05 ± 1.43	4.43 ± 2.86	2.89 ± 1.02	4.27 ± 1.53	0.197
TBARS	1	0.25 ± 0.07	0.54 ± 0.23	0.65 ± 0.70	0.36 ± 0.26	0.385
	35	1.26 ± 0.60^{a}	1.59 ± 1.28^{ab}	0.64 ± 0.36^{b}	0.54 ± 0.20^{b}	0.001*
ACAP	1	2.00 ± 1.05	1.97 ± 1.15	2.92 ± 1.58	3.04 ± 1.68	0.412
	35	1.42 ± 1.31 ^{b}	1.76 ± 1.3 ^{ab}	4.89 ± 2.96 ^{a}	5.71 ± 1.08 ^{a}	0.001*

Note: $P < 0.05$ indicate significant difference. Different letters in the same column indicate significant difference between groups (Tukey post hoc test). GPx, SOD and GST (U/mg of protein); TBARS (nmol MDA/mg of protein); ACAP (fluorescence units/mg of protein).

3.5. Serum biochemistry

Serum levels of total protein and globulins were lower in the groups T1, T2 and T3 compared to the group T0 on day 35 of the experiment. No significant difference was observed between groups regarding serum levels of albumin, triglyceride, uric acid and cholesterol (Table 6).

3.6. Serum levels of oxidants and activities of antioxidant enzymes

Serum GPx (groups T1, T2 and T3) and SOD (the group T2) activities were higher compared to the group T0 on day 35 of the experiment, while ACAP levels were higher in the groups T3 and T4 at the same moment of the experiment. On the other hand, serum TBARS levels were lower in the groups T3 and T4 compared to the group T0 on day 35 of the experiment. No significant difference was observed between groups regarding serum GST activity (Table 7).

4. Discussion

The use of GPF in laying hens diet stimulated feed consumption in all tested concentrations, as already observed by Ragni et al. (2014) in lambs fed a diet supplemented with 10%, 20% and 30% of grape seed flour, and by Lichovnikova et al. (2015) in broilers chickens fed a diet containing 1.5% of red grape pomace. Also, the percentage of laid eggs was higher in animals that received 1% of GPF, which can be considered a positive result, since eggs commercialization is based on the unitary value and sold per dozen. On the other hand, it is also important to highlight that 4% and 6% of dried pomace supplementation did not affect live weight, feed intake, egg production and feed efficiency, i.e., was unable to improve laying hens performance (Kara et al., 2016). Moreover, a significant reduction on lipid peroxidation and increased antioxidant capacity in egg yolk were found in laying hens fed with GPF, as observed by Kara et al. (2016) and Galli et al. (2018) in laying hens fed grape pomace and curcumin, respectively. These results can be considered beneficial, since yolk oxidation leads to MDA production (De Oliveira et al., 2015), a toxic substance with negative effects on human and animal health (Pearson et al., 1983).

Eggs of laying hens fed with GPF and stored for 30 days showed lower specific gravity, which reflects higher rates of water loss and increased cell air size during storage (Santos et al., 2009). A reduction on yolk index was verified in animals that received GPF, which can be considered a negative impact of supplementation, since this index reflects egg quality (Qu et al., 2018). The use of GPF decreased yolk and albumen pH, in disagreement to Kara et al. (2016), that did not observe such differences in eggs of laying hens that received 4% and 6% of grape dried pomace. These alterations might be a consequence of proteic denaturation which occurs in the yolk, affecting its consistence (Shang et al., 2004). Albumen pH stability of animals GPF-fed may lead to lower metabolization of nutrients, CO_2 production and release to the environment, which could affect negatively egg taste (Leandro et al., 2005). Moreover, doses of 2% and 3% of GPF reduced the Haugh unit in stored eggs, which affects internal egg quality. It is very important to emphasize that a study conducted by Kara et al. (2016) using grape pomace (4% and 6%) unaltered several factors linked with egg quality: albumen index, Haugh unit, egg yolk index, yolk color, eggshell ratio and eggshell thickness, egg-specific gravity after 90 days of storage, concluding that this supplementation did not affect egg quality. We believe that this difference can be linked to different time and concentrations used for each treatment, as well as different productive cycle between studies. Therefore, the use of GPF exerted negative and positive effects on chemical-physical composition of eggs, but positive effects overcame the negatives, mainly by the stability of monounsaturated fatty acids, which is a positive factor to human nutrition.

The use of GPF was able to alter the composition of fatty acid in fresh and stored eggs. Lower contents of caproic, butyric and margaric fatty acid can be considered a benefit to consumers, since these saturated fatty acids are linked with human cardiovascular disease (Santos et al., 2013). However, there was no significant difference on total saturated fatty acids between groups, as observed by Omidi et al. (2015) in egg yolk of laying hens fed with a diet containing 3% of grape seed oil. There was a decrease in gadoleic acid (C20:1), considered also a negative effect of GPF-addition; however, taking into account the sum of yolk monounsaturated fatty acids, it was noted stability of them even after storage, which did not occur in the group T0. This effect is desirable, and it can be related to the reduction on lipid peroxidation and increased antioxidant levels in the egg, since the lipid peroxidation process affects the fatty acid profile (Mancini et al., 2017). Although the content of polyunsaturated fatty acid differed between groups in fresh and stored eggs, the sum of polyunsaturated fatty acids did not differ between groups, in disagreement with Omidi et al. (2015) in laying hens fed with a diet containing 3% of grape seed oil. These authors demonstrated that the use of grape seed oil reduced the content of palmitolytic acid, elaidic acid and alpha-linolenic acid, but increased the total content of polyunsaturated fatty acids, and also decreased the total content of monounsaturated fatty acids.

Serum levels of total protein were lower in all groups treated with GPF in consequence to decreased globulin content, as observed by Magbolah and Wahba (2016) in serum of rats treated with white grape seed. According to these authors, these results can be linked to improved immune and anti-inflammatory responses elicited by resveratrol, since increased serum globulin levels generally occur in response to pathogens and stress conditions that impairs the immune system (Fernández-Cruz et al., 2009). In this sense, a study conducted by Fu et al. (2018) demonstrated that the use of resveratrol during 14 days was able to improve the immune and anti-inflammatory responses of piglets, being considered an adjuvant to enhance the immune responses to vaccines, as well as a dietary additive for animals to enhance humoral and cellular immunity.

The use of GPF in laying hens diet stimulated their antioxidant responses, and consequently, it reduced the levels of oxidants in serum and egg yolk, as reported by Kara et al. (2016) while testing grape pomace at concentrations of 4% and 6% in laying hens-feed. According to Surai (2014), the antioxidant characteristics of grape and grape products (e.g. grape juice, wine and molasses), and their by-products (e.g. grape pomace and seed) may be associated with to phenolic compounds that they contain, scavenging free radicals to form complexes with metal ions and to prevent or reduce the development of singlet oxygen. In the serum, the GPx and SOD activities were stimulated, in accordance to Feng et al. (2008) using laying hens supplemented with 0.5 g/kg of fed, indicating an improvement on the antioxidant defense mechanism system. The augmentation of the antioxidant system is corroborated by the augmentation in serum ACAP level, a variable linked to improved total antioxidant capacity, which might be directly linked to the presence of polyphenols, as resveratrol, and its action mechanism is related to scavenging oxidant species that initiate peroxidation, reduction of excessive O_2 concentrations and inhibition of peroxide formation (Brewer, 2011). We point out that the augmentation of seric antioxidant levels contributed to reduce egg yolk lipid peroxidation, since these antioxidants can accumulate in the egg yolk. This result is important, because peroxidation leads to a rancid flavor, and consequently, decreased sensorial and nutritional egg quality (Belitz et al., 2009). Moreover, it is worth mentioning that the GPF preserved eggs quality even when stored for 30 days, increasing their shelf life. Therefore, the GPF in the laying hens diet has practical application in the production of eggs of better quality and greater durability, as well as the health benefits of hens in non-climatized environments with daily temperature fluctuations.

Based on these evidences, the use of GPF in the diet of laying hens promoted enhanced productive efficiency and stimulated the antioxidant defense mechanism system, exerting beneficial properties on animal health, under conditions of heat stress. Also, GPF was able to reduce egg lipid peroxidation and to increase antioxidant levels, contributing to improved internal egg quality, as well as to maintain yolk pH of stored eggs similarly to fresh eggs. Overall, the use of GPF for laying hens at the end of the productive cycle exerted benefits to development, egg quality and animal health. Defining the best dose of GPF to be used is not an easy task, since lower concentrations of it (1%) led to better animal performance, and higher doses (2% and 3%) caused similar results to 1%, thus considering the cost-benefit we recommend 1% GPF to feed laying hens.

4.1. Ethical note

This experiment was approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC) under protocol number 9936300717.

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