Veterinary and Animal Science 30 (2025) 100509

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

Effect of dietary wine lees on oxidative stress, egg quality and yolk lipid stability in laying hens fed with a diet rich in n-3 polyunsaturated fats

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ARTICLE INFO

Keywords:
Wine lees
Antioxidant activity
Egg quality
Fatty acid composition
Oxidative stability

ABSTRACT

Wine lees (WLs) are a by-product of the winemaking process, but the valorization of WLs as poultry feed has not yet been achieved. Accordingly, the aim of the present study was to evaluate the potential benefits of using WLs as a feed ingredient. To this end, we investigated their effects on serum and hepatic markers of oxidative stress and egg quality in laying hens susceptible to lipid peroxidation by feeding them diets enriched in n-3 PUFA. In addition, lipid profile and oxidative stability of the yolk during storage were evaluated. A total of 128 30-week-old Tetra SL laying hens were divided into four groups (n = 32): the negative control (C–) received a standard diet, while the three other groups (positive control, C+; WL2.5; and WL5) received the standard diet supplemented with 5 % linseed oil rich in n-3 PUFA. In the WL2.5 and WL5 groups, WLs were introduced into the diet at levels of 2.5 % and 5 %, respectively. The increased intake of n-3 PUFA induced oxidative stress, which was evidenced by increased malondialdehyde (MDA) and reduced serum and liver antioxidant enzyme activity. However, these parameters were significantly improved in hens supplemented with WLs. The reduction in n-3 PUFA content in stored eggs was < 19 % in hens fed with WLs, while the reduction exceeded 33 % in the C+ group. In conclusion, WLs can improve egg production and egg quality (yolk color and cholesterol content), the antioxidant status of hens and the oxidative stability of eggs, with the possibility of extending their shelf life.

1. Introduction

Supplementing the diet of laying hens with n-3 PUFA has become an accepted practice for the nutritional improvement of the lipid fractions in egg yolk because it increases the concentration of n-3 PUFA, which has positive effects (cardioprotective, antioxidant, immunomodulatory, etc.) on human health (Irawan et al., 2022; Radanovic et al., 2023).

Flaxseed and linseed oils (*Linum usitatissimum*) are most commonly used to enrich eggs with n-3 PUFA (Hosseini et al., 2023). However, PUFA and especially n-3 PUFA in poultry feed represent a group of pro-oxidants causing increased oxidative stress in hens (Cherian & Hayat, 2009), and increasing the content of n-3 PUFA in egg yolk increases the susceptibility of the yolk to oxidative damage during storage (Mierlita et al., 2024a, 2024b; Romero et al., 2022).

Numerous studies have highlighted that diets supplemented with n-3 PUFA increase levels of oxidative stress in laying hens, thus affecting ovarian function (egg production), egg quality, and the productive lifespan of hens (Gao et al., 2022; Lee et al., 2021; Xu et al., 2025). Oxidative stress is often measured by the activity of antioxidant enzymes (SOD, CAT and GSH-Px) and the concentration of malondialdehyde (MDA), which is a by-product of lipid peroxidation and reflects the degree of oxidation in the body (Oke et al., 2024).

In this context, there is a particular interest in natural plant-derived antioxidants that can ensure oxidative balance by scavenging reactive oxygen species (ROS) or increasing the activity of antioxidant enzymes in blood and tissues (Papadopoulos et al., 2023), thus improving the antioxidant status of birds during oxidative stress, as well as oxidative stability and fatty acid profile in egg yolk (Oke et al., 2024). The quality of eggs is improved by enriching them with natural antioxidants (Al-Khalaifah et al., 2024; Mierlita et al., 2024a, 2024b; Vlaicu et al., 2022).

WLs could be a natural alternative to the synthetic antioxidant products used in the feed of laying hens. Numerous studies have shown that grape by-products (grape pomace, grape seeds and grape skins) improve antioxidant status in animals (D'Alessandro et al., 2024; Selim et al., 2023), but no research has yet evaluated the antioxidant potential

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https://doi.org/10.1016/j.vas.2025.100509

Available online 15 September 2025

and the effect on egg production and quality of the dietary inclusion of WLs.

WLs are the residue (heterogeneous matrix) that settles at the bottom of the vessel during wine fermentation, which is often not properly managed (it is typically discarded). This wine by-product is composed of mainly yeast cells (Saccharomyces cerevisiae), ethanol, tartaric acid, carbohydrates, phenolic compounds, inorganic matter, pulp and other parts of the grapes (Caponio et al., 2024; Chioru et al., 2023; De Iseppi et al., 2020). This waste is considered to be highly polluting, as it contains antibacterial phenolic substances that are resistant to biological degradation (Devesa-Rey et al., 2011). Recent studies have shown that WLs have a high content of phenolic compounds with antioxidant, antimicrobial, and anti-inflammatory properties (Caponio et al., 2024; Chioru et al., 2023; De Iseppi et al., 2020). The composition of WLs depends on numerous parameters such as the grape variety, the lees used and the winemaking method, resulting in a wide compositional heterogeneity (De Iseppi et al., 2020). For example, wine yeast contains between 1.9 and 16.3 g of polyphenols/kg, depending on the type and cultivar of grapes, the degree of ripeness of the berries and the winemaking practice (Bustamante et al. 2008). The phenolic compounds in wine yeast are mainly phenolic acids, flavanols, flavonols and anthocyanins (De Iseppi et al., 2020). An analysis of WLs using the HPLC method showed that quercetin is among the most abundant polyphenols, with a concentration exceeding almost three times the amount of any other individual polyphenolic compound detected (Landeka Jurčević et al., 2017). However, although they are a common by-product in winemaking, wine lees are still one of the least studied and most underexploited residues.

Therefore, we hypothesized that this by-product could be used as a dietary supplement in poultry production to mitigate the effects of oxidative stress induced by diets high in unsaturated fats. To the best of our knowledge, this is the first study investigating the use of WLs as a dietary supplement in the diets of laying hens. The study evaluated the effects of dietary supplementation with WLs on serum and hepatic markers of oxidative stress and egg quality in laying hens (susceptible to lipid peroxidation) by feeding them diets enriched in n-3 PUFA. In addition, the productive performance, fatty acid profile and oxidative stability of the egg yolks were also evaluated. Our hypothesis is that the use of WLs in laying hens fed a diet rich in n-3 polyunsaturated fats could modify the laying rate, egg quality, antioxidant potential and shelf life of eggs enriched in n-3 PUFA. This research may help to reduce the research gap regarding the use of WLs in poultry feed, encouraging the promotion of an environmentally friendly economy.

2. Materials and methods

2.1. Ethical statement

Experimental procedures were approved by the Ethics Committee of the University of Oradea, Romania (decision 8/23-05-2025), ensuring that all procedures followed strict animal welfare guidelines (Law 206/2004, Directive 2010/63/EU, Law 43/2014).

2.2. Wine lees collection and analysis

In this study, we used the yeast sediments obtained after the first alcoholic fermentation of wine obtained from black grapes (Cabernet Sauvignon and Băbească Neagră). Fresh WLs (the thick semi-liquid precipitate, decanted in barrels), provided by a local winery (Casa Vinului Fumeni, Romania), were initially filtered through three layers of gauze to reduce the moisture content. The obtained yeast biomass was packed in dark plastic bags and stored at -20 °C until the start of the experiment. The yeast was dried in an oven at 40 °C for 72 h, after which it was ground with an electric coffee grinder, vacuum-packed in dark foil bags and stored at 4 °C until use (Mierlita et al., 2024a).

Before being introduced into the feed of laying hens, WLs were analyzed to determine their proximal composition, contents of major FAs, phenolic content, and antioxidant activity (DPPH and ABTS test, expressed as TE (Trolox equivalent)).

2.3. Experimental design and dietary treatments

A total of 128 30-week-old Tetra SL laying hens (initial body weight: 1934.2 g ± 79.43 g), purchased from local producers, were randomly assigned to four groups (8 replicates per group of 4 laying hens/replicate) and offered the following diets for 8 weeks: the negative control (C–) received a standard diet, while the other three groups (positive control, C+; WL2.5 and WL5) received the standard diet supplemented with 5 % linseed oil (FO) rich in n-3 PUFA. In the WL2.5 and WL5 groups, WLs were introduced into the diet at levels of 2.5 % and 5 % (% of diet), respectively. The C– group was used to evaluate the effect of oxidative stress caused by dietary supplementation with FO. The positive control (C+) was used as a reference to evaluate the effect of WLs introduced in increasing doses (2.5 % or 5 %) in the FO-supplemented diet. The experimental diets (C+, WL2.5 and WL5) were prepared with 5.0 % FO to induce dietary oxidative stress, which was confirmed in previous studies on broiler chickens (Voljč et al., 2013; Pirman et al., 2021). The amount of WLs in the two experimental diets was designed while taking into account the phenolic content of the analyzed WL samples and the phenolic content of diets supplemented with different plant by-products to improve the antioxidant status of the laying hens (Romero et al., 2022; Selim et al., 2023; Vlaicu et al., 2021).

For the formulation of the diets (Table 1), appropriate software (HYBRIMIN® Futter 5) was used, according to the Commercial Layer Management Guide Tetra SL (2019). The diets were prepared weekly and stored in black plastic containers, at 12–15 °C, to minimize PUFA

Table 1
Ingredients and chemical composition of the diets used in the study.

Item	Experimental diets[a]			
	C-	C+	WL2.5	WL5
Ingredient (% of feed)				
Corn	65.483	49.503	47.504	47.035
Wheat bran	-	11.51	11.00	10.00
Soybean meal	24.20	23.70	23.70	22.65
Flaxseed oil	-	5.00	5.00	5.00
Wine lees (WLs)	-	-	2.50	5.00
Lysine	-	-	0.02	0.03
DL-Methionine 99 %	0.087	0.087	0.086	0.085
Ronozyme W	0.02	0.02	0.02	0.02
Choline chloride	0.03	0.03	0.03	0.03
Limestone	8.10	8.10	8.10	8.10
Dicalcium phosphate	1.43	1.40	1.40	1.40
Salt	0.25	0.25	0.25	0.25
Vitamin–mineral premix*	0.40	0.40	0.40	0.40
Nutrient composition (calculated unless noted)**				
Metabolizable energy (kcal/kg)	2751	2765	2751	2750
Crude protein (CP) (analyzed, %)	17.15	17.35	17.20	17.08
Ether extract (EE) (analyzed, %)	3.37	7.44	7.52	7.50
Crude fiber (analyzed, %)	2.93	3.72	4.37	4.93
Lysine (%)	0.90	0.90	0.90	0.90
Methionine + Cystine (%)	0.70	0.70	0.70	0.70
Threonine (%)	0.67	0.67	0.67	0.66
Tryptophan (%)	0.18	0.17	0.17	0.17
Calcium (%)	3.85	3.85	3.85	3.85
Total phosphorus (%)	0.65	0.66	0.66	0.65
Available phosphorus (%)	0.42	0.42	0.42	0.42

[a] C-: standard diet, C+: standard diet containing flaxseed oil, WL2.5 and WL5: C+ diets containing 2.5 % and 5 % WLs, respectively.

* Contents per 1 kg diet: vit A, 12 000 IU; vit D3, 4000 IU; vit E, 45 IU; vit K, 2.5 mg; pantothenic acid, 12.0 mg; riboflavin, 6.0 mg; niacin, 50.0 mg; thiamine, 3.0 mg; pyridoxine, 8.0 mg; vit B12, 0.03 mg; biotin, 0.2 mg; iodine, 0.4 mg; manganese, 50.0 mg; copper, 17.0 mg; zinc, 60.0 mg; selenium, 0.1 mg; iron, 80.0 mg.

** Calculated values according to NRC (1994).

auto-oxidation and the depletion of natural antioxidants.

The birds were placed in 60 cm x 60 cm cages, at a density of 4 hens/cage (900 cm^2/hen), with ad libitum access to feed and water. The microclimate conditions were based on the Commercial Layer Management Guide Tetra SL (2019). Throughout the experiment, the hens had a 16 h light: 8 h dark schedule.

2.4. Data collection

2.4.1. Laying performance

Egg production was recorded daily. The egg production rate was determined as the daily percentage of eggs laid per replicate. Feed consumption was documented weekly, and the average egg weight was documented weekly by weighing all eggs produced each week. The average daily feed intake, average egg weight, egg mass (g/hen/d), and feed conversion ratio (FCR; g of feed per g of egg mass) were calculated using established calculation methods (Anene et al., 2021).

2.4.2. Egg quality characteristics

In weeks 4 and 8 of the study, 24 eggs (3 eggs from each replicate) were randomly collected from each group for an egg quality assessment. The measured parameters included eggshell weight and yolk weight (using an electronic scale, Balance Mettler-To-111 ledo LLC, USA), and the albumen weight was calculated according to the difference. Eggshell thickness was determined at three different points, using a thickness gauge (Mitutoyo 547 Thickness Gauge, ±3 μm Accuracy, 0.01 mm Resolution, Japan). Albumen height was evaluated using a micrometer tripod, and the Haugh unit was calculated based on albumen height and egg weight. Yolk color was measured based on the Roche Yolk Color Fan (RYCF) value (1–15 points).

In week 8 of the study, 24 eggs (3 eggs/replicate) were randomly collected from each group on two consecutive days, half of which (i.e., 24 eggs/treatment) were stored at room temperature (22 ± 2 °C) for 30 days, while the other half were analyzed as fresh eggs. The yolk was separated and then the samples were stored at -80 °C for the determination of the FA profile, antioxidant content (TPC and α-tocopherol) and lipid oxidative status of the yolk.

2.4.3. Serum and liver preparations

At the end of the experiment, one laying hen from each replicate was randomly selected for blood and liver sampling (n= 8 per treatment). Blood samples were obtained from the brachial vein, after a 12 h food deprivation period. Serum was obtained by centrifugation at 3500 rpm for 10 min at 4 °C and then divided into 4 aliquots (approximately 0.5 ml each), which were stored at −80 °C until analysis.

After blood collection, the birds were electrically stunned and exsanguinated to obtain the liver. After removal, the liver was rinsed with phosphate-buffered saline and then divided into two pieces: the left lobe was stored at −20 °C for FA profile analysis, and the right lobe was cut into small pieces (approximately 0.1 g). Approximately 0.5 g of liver sample was homogenized with pre-cooled phosphate-buffered saline (1:9; w/v). The homogenate was centrifuged at 3500 rpm for 10 min at 4 °C (Zhu et al. 2020). After separation, the supernatant was transferred to Eppendorf tubes and stored at -80 °C until biochemical analyses were performed.

2.5. Chemical analysis

2.5.1. Proximate chemical composition of feed

Wine lees (WLs) and the tested diets were analyzed using standard procedures (AOAC, 1990) for dry matter (DM) content (gravimetric method) and crude protein (CP) (N x 6.25; Kjeltec auto 1030; Tecator Instrumente, Sweden). Crude fat (EE) content was determined via petroleum ether extraction according to method 920.39 (SOXTHERM, C. Gerhardt GmbH, Königswinter, Germany). For the WLs, the starch (STA) and sugar (SUG) content was determined (AOAC, 1990) and the AMEn (apparent metabolizable energy corrected for nitrogen balance) was calculated. The AMEn of the WLs was estimated according to the equation proposed by the World's Poultry Science Association as follows:

$$AMEn\ (kJ/kg\ DM) = 15.51\ (CP) + 34.31\ (EE) + 16.59\ (STA) + 13.01\ (SUG).$$

2.5.2. Wine lees chemical analysis

2.5.2.1. Determination of phenolic content. The extraction of polyphenols from the WLs was carried out as described by Costa et al. (2023) and Caponio et al. (2024). In brief, 3 g of ground sample was treated with a mixture of methanol/water (80:20, v/v) in a ratio of 1:10 (v/w) and shaken continuously for 30 minutes in an orbital shaker (GFL 3005, GEMINI, Apeldoorn, Netherlands). Then, the samples were centrifuged at 12000 g for 15 min at 4 °C (Sigma 2-16KL Refrigerated Centrifuges, Berlin, Germany); finally, the supernatants obtained were passed through a nylon filter with a pore diameter of 0.45 μm.

The phenolic content was determined via spectrophotometric methods, using 96-well microplates (Frilabo, Milheirós, Portugal) and microplate readers (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Vantaa, Finland) for absorbance measurement, according to Gouvinhas et al. (2018).

The TPC of the WL extracts was determined according to the Folin–Ciocalteu Method as described by Caponio et al. (2024), by adding 100 μL of Folin-Ciocalteu reagent to 20 μL of the sample. Then, 80 μL of sodium carbonate solution (7.5 %) was added and kept at room temperature in the dark for 60 min. After incubation (40-45 °C) for 30 min, the absorbance was measured at 750 nm and the results were expressed as mg gallic acid equivalents (GAE)/g WLs, using gallic acid as a standard.

The ortho-diphenol (ODC) content was determined following the procedure described by Yu et al. (2021). Briefly, 160 μL of the sample was added to each well of the microplate, followed by 40 μL of sodium molybdate solution. The mixtures were vortexed and then incubated for 15 min, protected from light. The absorbance was measured at 375 nm, and gallic acid was used as a standard. The ODC content was expressed as mg gallic acid equivalents (GAE) per g WLs.

The flavonoid content (FC) was assessed using the colorimetric method described by Yu et al. (2021). Briefly, 24 μL of the sample was mixed with 28 μL of sodium nitrate solution (50 g/L) in a microplate. After 5 min of incubation, 28 μL of aluminum chloride solution (100 g/L) was added. The mixture was allowed to react for 6 min, after which 120 μL of sodium hydroxide solution (1 M) was added, and the microplate was gently shaken for 30 s. The absorbance was measured at 510 nm, and catechin was used as the standard solution. FC was expressed in mg of catechin equivalent per gram of sample (mg CAT/g WL).

2.5.2.2. Determination of antioxidant capacity. The antioxidant capacity was determined spectrophotometrically using two radical scavenging methods: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH).

For the ABTS assay, 12 μL of WL extract was mixed with 188 μL of ABTS•+ solution, previously prepared according to the method described by Paié-Ribeiro et al. (2024), in each well of the microplate. A separate well served as a control, to which 12 μL of distilled water and 188 μL of ABTS•+ solution were added. After incubation in the dark at room temperature for 2 h, the absorbance was measured at 734 nm and quantified using Trolox solutions (1 mM) of different concentrations, and the results are expressed as μmol Trolox equivalents (TE)/g WLs.

For the DPPH radical scavenging assay, 10 μL of WL extract and 190 μL of DPPH working solution (previously prepared according to the method described by Brito et al. (2018) were added to each well of the

microplate. The mixture was allowed to react for 30 min in the dark and at room temperature, and then the absorbance was read at 520 nm, using 70 % hydroethanol as a control. The scavenging capacity of the WL extracts was calculated using the calibration curve generated with different concentrations of Trolox solutions (5 mM), and the results were expressed in μmol Trolox/g WLs.

2.5.3. Serum and liver biochemical parameters

Total lipids were determined spectrophotometrically at 600 nm after oxidation with concentrated sulfuric acid and potassium dichromate (Chiang et al., 1957).

Total cholesterol (TC) and triglycerides (TG) were assessed using an automated analyzer with Roche test kits (Roche Cobas Mira Plus CC). For the liver samples, the results were normalized to the total protein concentration in the supernatant.

Superoxide dismutase (SOD) activity was estimated using the Sigma Aldrich SOD assay kit (cat. no. 19160), following the manufacturer's instructions. Glutathione peroxidase (GSH-Px) levels were estimated using the colorimetric method described by Habig et al. (1974). Catalase activity (CAT) was determined after H2O2 reduction at 240 nm (Beers & Sizer, 1952).

Commercial kits (Sigma-Aldrich, MAK187) were used to determine the total antioxidant capacity (T-AOC).

Lipid peroxidation in the serum and liver supernatants was measured as thiobarbituric acid reactive substances (TBARS) using a Biodiagnostic Kit (Cat. No. MD 2529, Biodiagnithitionc, Egypt) according to the manufacturer's manual.

2.5.4. Antioxidant status of egg yolk

The TPC content of the egg yolks was measured spectrophotometrically using the Folin–Ciocalteu assay, according to the procedures previously described by Mierlita et al. (2024a). Absorbance was measured at 750 nm and the results were expressed in mg gallic acid equivalent (GAE) per g of yolk.

The α-tocopherol content of egg yolk was measured according to the HPLC procedure, using a PDA-UV detector (Finningan Surveyor Plus, Thermo-Electron Corporation, Waltham, MA) at a wavelength of 292 nm. A HyperSil BDS C18 column, 250 × 4.6 nm (particle size 5 μm) (Thermo-Electron Corporation, Waltham, MA) was used as the stationary phase and methanol/water (96 % methanol, 4 % ultrapure water) as the mobile phase, at a flow rate of 1.5 ml/min.

Total antioxidant activity was assessed spectrophotometrically using an ABTS assay, according to the procedure previously described by Mierlita et al. (2024a). Absorbance was measured at 734 nm, and the results were expressed as Trolox equivalents (TE) μmol/g yolk.

The MDA content in egg yolk was measured using the TBARS assay, according to the procedures previously described by Mierlita et al. (2024a). After sample processing, the absorbance of the product formed by the reaction of lipid peroxidation products with thiobarbituric acid was measured in a spectrophotometer at 532 nm. The MDA concentration (μg MDA/g yolk) in the yolk samples was calculated relative to the standard curve prepared using standard MDA tetrabutylammonium salt (Sigma-Aldrich, Switzerland).

2.5.5. Cholesterol content in egg yolk

This metric was determined according to AOAC (method 994.10) using a Perkin-Elmer GC (Shelton, MA, USA) equipped with an HP-5 GC fused silica capillary column (30 m × 0.32 mm ID, 0.1 μm film thickness, Agilent J&W GC Columns, Santa Clara, CA, USA) and a flame ionization detector (FID), as previously described (Mierlita et al., 2024a).

2.5.6. Fatty acid analysis

Lipid extraction was achieved using the standard method (AOAC, 1990), and FA methyl esters (FAMEs) were prepared according to the method described by Joseph and Ackman (1992). Chromatographic analysis of FAMEs was performed with a gas chromatograph (Shimadzu GC-2010 Plus, Tokyo, Japan) equipped with FID and an HP-88 column (100 m × 0.25 mm ID), following the procedures previously described by Mierlita et al. (2024a). External standards (Supelco 37 Component FAME mix; Supelco Bellefonte, PA, USA) were used for the quantification of fatty acids, and the results are expressed as the % FA of total FA.

2.6. Statistical analysis

Data were analyzed via one-way analysis of variance (ANOVA). Multiple comparisons among different groups were evaluated using Tukey's post hoc test with the SPSS 26.0 software package (IBM Inc., Chicago, IL, USA). Data analyses of the performance and egg quality, serum and liver biochemical parameters, FA profiles, and oxidative status of serum and liver were tested only for the effect of diet, and yolk FA profile, yolk antioxidant content and lipid oxidative status of yolk were tested for "type of diet" (C-, C+, WL2.5 and WL5) and "type of eggs" (fresh or stored). The replicates were considered the experimental unit for all analyses. Differences were considered statistically significant when $p < 0.05$ (95 % confidence interval). Mean values and SEM (standard error of the means) are reported.

3. Results

3.1. Compositions of WLs and experimental diets

The analyzed WL samples were characterized by a high content of crude fiber (28.97 %) and crude protein (21.62 %), while the crude fat content was modest (4.59 %) (Table 2). The analyzed WLs showed high antioxidant activity, measured by both the ABTS test (245.53 μmol Trolox/g) and DPPH (184.72 μmol Trolox/g); this result is in agreement with the TPC (36.51 mg GAE/g).

The major fatty acids identified in FO were ALA (α-linolenic acid; 51.35 %) and OA (oleic acid; 17.58 %), while, in WLs, LA (linoleic acid; 30.88 %) and PA (palmitic acid; 22.19 %) predominated (Table 3). The addition of FO to the basal diet increased the content of n-3 PUFA as a percentage of total FA from 3.78 % to 31.84 %, which is an increase of more than 18 times, i.e., from 1.16 g/kg to 20.91 g/kg diet.

3.2. Laying performance and egg quality traits

The effects of dietary treatments on the laying hen performance and egg quality are presented in Table 4. The inclusion of FO in the diets of laying hens did not modify the laying rate or egg quality, while WLs had a positive effect on egg production, egg mass, and FCR ($p < 0.05$). The different dietary treatments had no significant effects on the egg weight,

Table 2
Chemical composition, phenolic content and antioxidant activity of wine lees (WLs).

Chemical composition		Phenolic content	
Moisture (g/100 g fresh)	62.26 ± 1.07	TPC (mg GAE/g WLs)	36.51 ± 1.47
Moisture (%)	9.81 ± 0.22	ODC (mg GAE/g WLs)	22.96 ± 0.78
Crude protein (CP) (%)	21.62 ± 0.44	FC (mg CAT/g WLs)	16.72 ± 0.94
Crude fat (EE) (%)	4.59 ± 0.36		
Crude fiber (CF) (%)	28.97 ± 0.72		
Starch (STA) (%)	3.23 ± 1.78	Antioxidant activity	
Sugars (SUG) (%)	2.41 ± 0.22	DPPH (μmol TE/g WLs)	184.72 ± 8.46
Ash (%)	16.61 ± 0.94	ABTS (μmol TE/g WLs)	245.53 ± 16.12
AMEn (kcal/kg WL)	2414		

AMEn: apparent metabolizable energy corrected for nitrogen balance.
TPC: total phenolic content; ODC: orto-diphenol; FC: flavonoid content; GAE: gallic acid equivalent; TE: Trolox equivalent.

Table 3
Major fatty acid (FA) content in flaxseed oil (FO), wine lees (WLs) and experimental diets.

FO		WLs	Experimental diets[1]				SEM[2]	*p*-value
			C-	C+	WL2.5	WL5		
Major FA content (% of total FA)								
C16:0 (PA)	6.27	22.19	18.12	17.15	18.22	17.67	0.453	0.187
C18:0 (SA)	3.96	12.08	4.77	5.48	6.24	6.21	0.128	0.062
C18:1 c9 (OA)	17.58	17.45	18.42	16.62	16.80	17.07	0.523	0.121
C18:2n-6 (LA)	14.91	30.88	50.66^{a}	24.15^{b}	24.21^{b}	24.60^{b}	0.318	<0.001
C18:3n-3 (ALA)	51.35	11.34	3.34^{b}	31.62^{a}	31.43^{a}	31.59^{a}	0.275	<0.001
SFA	11.59	36.67	25.31	24.63	25.34	24.17	0.641	0.198
MUFA	20.06	18.92	18.86	17.21	17.03	17.58	0.178	0.324
PUFA	67.15	42.87	56.25	56.16	55.97	56.52	0.681	0.159
n-6 FA	15.34	31.45	52.47^{a}	24.32^{b}	24.43^{b}	24.85^{b}	0.204	<0.001
n-3 FA	51.81	11.42	3.78^{a}	31.84^{b}	31.54^{b}	31.67^{b}	0.932	<0.001
n-6/n-3 FA	0.30	2.75	13.74^{a}	0.76^{b}	0.77^{b}	0.78^{b}	0.407	<0.001
FA content of the diets (g/kg, as is basis)								
PUFA (g/kg)	-	-	16.62^{b}	36.87^{a}	37.35^{a}	37.77^{a}	0.512	<0.001
n-6 FA (g/kg)	-	-	14.91	15.96	16.30	16.61	0.223	0.371
n-3 FA (g/kg)	-	-	1.16^{b}	20.91^{a}	21.05^{a}	21.16^{a}	0.474	<0.001

[1] C-: standard diet, C+: standard diet containing flaxseed oil, WL2.5 and WL5: C+ diet containing 2.5 % and 5 % WLs, respectively.
[2] SEM: standard error of the mean. ^{a,b}Means within a row with different superscripts are different ($P < 0.05$). PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid, ALA: α-linolenic acid.

Table 4
Effect of dietary treatments on the performance and egg quality of laying hens.

	Dietary treatment[1]				SEM[2]	p-values
	C-	C+	WL2.5	WL5		
Body weight change (g/56 d)	+67.2	+85.4	+73.5	+51.8	7.262	0.137
Laying rate (%)	88.74^{b}	89.24^{b}	91.57^{a}	92.03^{a}	0.673	0.031
Egg mass (g/hen/d)	53.89^{b}	54.79^{b}	55.33^{ab}	56.66^{a}	0.642	0.042
Feed intake (g/hen/d)	114.11	112.17	112.88	113.34	0.498	0.103
Feed conversion ratio (FCR)*	2.11^{a}	2.04^{ab}	2.04^{ab}	2.00^{b}	0.046	0.047
Egg weight (g)	60.73	61.40	60.42	61.57	0.529	0.107
Albumen ratio (%)	64.37	65.11	63.77	64.02	0.708	0.267
Yolk ratio (%)	24.12	23.71	24.42	24.89	0.391	0.168
Shell ratio (%)	11.51	11.18	11.81	11.09	0.403	0.135
Haugh unit value	85.16	83.54	83.63	85.21	0.875	0.155
Eggshell thickness (mm)	0.386^{a}	0.378^{a}	0.327^{b}	0.314^{b}	0.008	0.007
Roche yolk color fan score (1-15 scale)**	6.57^{b}	6.21^{b}	7.28^{a}	7.42^{a}	0.357	0.038

[1] C-: standard diet, C+: standard diet containing flaxseed oil, WL2.5 and WL5: C+ diet containing 2.5 % and 5 % WLs, respectively.
[2] SEM: standard error of the mean. ^{a,b}Means within a row with different superscripts are different ($P < 0.05$). *FCR: g of feed/g of egg mass
** 1: pale yellow, 15: deep orange.

relative weight of the yolk, albumen or shell, or Haugh units ($p > 0.05$). Yolk coloration was improved in the groups fed WLs (2.5 % and 5 %), as indicated by the higher yolk score ($p < 0.05$), while eggshell thickness was reduced ($p < 0.01$).

3.3. Serum and hepatic lipid and antioxidant parameters

Total lipids, cholesterol and serum and hepatic triglycerides decreased ($p < 0.05$) in laying hens fed FO-supplemented diets compared to the negative control (C-), while laying hens fed WL diets had lower serum and hepatic cholesterol and triglycerides compared to the positive control (C+) ($p < 0.05$) (Table 5).

The inclusion of FO in the hens' diet (C+ group) emphasized lipid oxidation in the body, causing an increase in MDA concentration and a decline in antioxidant enzyme activities (SOD, CAT and GSH-Px) and T-AOC, as compared to the negative control ($p < 0.05$). The introduction of WLs into the n-3-PUFA-enriched diet reduced both serum and hepatic MDA concentrations and improved antioxidant enzyme activity (SOD, CAT and GSH-Px) and T-AOC ($p < 0.05$). It is noteworthy that the modification of these serum and hepatic parameters was not dose-dependent with the inclusion of WLs (2.5 % or 5 %) in the hens' diet. An analysis of the FA composition in blood and liver showed that linoleic acid (C18:2n6) and total and individual n-3 FA (ALA, EPA and DHA) increased ($p < 0.001$) in the FO-supplemented group (group C+), while oleic acid (C18:1c9) and palmitic acid (C16:0) decreased ($p < 0.01$), as compared to the negative control (Table 6). The introduction of WLs into the diet of the hens at a high dose (5 % of diet) led to an increase in the concentration of PUFA in blood serum and liver compared to the C+ group, while oleic acid decreased in blood serum and palmitic acid decreased in the liver ($p < 0.05$). The ratio of n-6/n-3 FA in the serum and liver improved ($p < 0.001$) after the introduction of FO into the diet of hens, but it was not influenced by the presence of WLs.

3.4. Antioxidant status, cholesterol content and yolk fatty acid profile

There were no significant differences in yolk TPC content between the C- and C+ treatments, while α-tocopherol content was higher ($p < 0.001$) in the FO-supplemented groups (C+, WL2.5 and WL5) ($p < 0.01$). The introduction of WLs into the laying hens' diet had a positive effect on yolk TPC but did not change the α-tocopherol content (Fig. 1).

Yolk MDA content was higher ($p < 0.01$) in the FO-supplemented groups (C+, WL2.5 and WL5) compared to the negative control, but lower ($p < 0.05$) in the WL-supplemented groups compared to the positive control (C+). These differences were found in both fresh (F) and stored (S) eggs (Fig. 1). Remarkably, the levels of TPC, α-tocopherol and ABTS radical scavenging activity in WL eggs remained elevated even after 30 days of storage compared to the control (C- and C+). Furthermore, when WL levels were increased in the laying hens' diet, the antioxidant content and ABTS radical scavenging activity in the egg yolks increased numerically, and the MDA concentration decreased numerically in a dose-dependent manner, without the differences being statistically significant.

The level of yolk cholesterol, measured in fresh eggs, decreased ($p < 0.05$) when supplementing the diet with FO. WLs had a reducing effect ($p < 0.05$) on yolk cholesterol content compared to the diet supplemented with FO alone (Fig. 2).

Supplementation of the diet with FO resulted in a significant increase ($p < 0.01$) in the content of ALA and EPA + DHA in the yolk of F and S eggs, while the content of C18:1c9 and C16:0 decreased ($p < 0.01$) compared to the negative control (Table 7). Thus, the sum of

Table 5
Effect of dietary treatments on serum and liver lipid and antioxidant parameters in laying hens.

	Dietary treatment[1]				SEM[2]	*p*-values
	C	WL0	WL2.5	WL5		
Serum parameters						
Total lipids (mg/dL)	18.72^{a}	15.81^{b}	15.34^{b}	14.41^{c}	0.770	0.027
Total cholesterol (mg/dL)	184.6^{a}	159.5^{b}	148.2^{bc}	130.7^{c}	8.46	0.041
Triglycerides (mg/dL)	396.6^{a}	351.1^{b}	294.7^{c}	308.4^{c}	27.91	0.035
SOD (U/mL)	259.2^{b}	217.4^{c}	270.8^{a}	288.9^{a}	0.047	0.008
CAT (U/mL)	83.50^{b}	51.24^{c}	94.91^{a}	92.40^{a}	0.423	0.043
GSH-Px (U/mL)	189.3^{a}	148.8^{b}	193.2^{a}	217.4^{a}	11.352	0.021
T-AOC (U/mL)	5.60^{b}	5.13^{c}	7.32^{a}	7.18^{a}	0.104	0.004
TBARS (MDA, nmol/mL)	6.23^{b}	10.34^{a}	6.92^{b}	7.01^{b}	0.354	0.007
Liver parameters						
Total lipids (of DM)	28.65^{a}	24.12^{b}	24.71^{b}	22.88^{c}	3.654	0.005
Total cholesterol (nmol/g prot)	16.23^{a}	15.71^{a}	12.34^{b}	10.84^{c}	1.381	<0.001
Triglycerides (nmol/g prot)	13.41^{a}	9.18^{b}	6.06^{c}	5.88^{c}	1.853	<0.001
SOD (U/mg prot)	156.61^{a}	134.42^{b}	140.72^{ab}	151.38^{a}	3.072	0.040
CAT (U/mg prot)	57.64^{b}	53.81^{b}	79.34^{a}	74.97^{a}	0.832	0.038
GSH-Px (mg/g prot)	6.79^{a}	5.81^{b}	6.77^{a}	7.56^{a}	0.431	0.023
T-AOC (U/mL)	4.55^{b}	3.82^{c}	5.07^{a}	5.23^{a}	0.181	0.044
TBARS (MDA, nmol/mg prot)	0.58^{b}	0.89^{a}	0.46^{b}	0.43^{b}	0.032	<0.001

[1] C-: standard diet, C+: standard diet containing flaxseed oil, WL2.5 and WL5: C+ diet containing 2.5 % and 5 % WLs, respectively.
[2] SEM: standard error of the mean. $^{a-c}$Means within a row with different superscripts are different (P <0.05).
SOD: superoxide dismutase, CAT: catalase, GSH-Px: glutathione peroxidase, T-AOC: total antioxidant capacity, MDA: malondialdehyde.

Table 6
Major fatty acid composition of plasma and liver of laying hens (% of total FA).

	Dietary treatment[1]				SEM[2]	*p*-value
	C-	C+	WL2.5	WL5		
Plasma						
C16:0 (PA)	22.91^{a}	19.05^{b}	19.18^{b}	18.36^{b}	0.427	0.007
C18:0 (SA)	7.34^{b}	8.51^{ab}	10.12^{a}	10.73^{a}	0.264	0.043
C18:1c9 (OA)	44.81^{a}	39.58^{b}	38.12^{bc}	37.76^{c}	2.78	0.005
C18:2n-6 (LA)	13.40^{c}	16.81^{b}	17.64^{ab}	18.22^{a}	1.27	0.008
C18:3n-3 (ALA)	0.52^{b}	6.45^{a}	6.34^{a}	6.62^{a}	0.287	<0.001
C20:5n-3 (EPA)	0.09^{b}	0.30^{a}	0.35^{a}	0.36^{a}	0.121	<0.001
C22:6n-3 (DHA)	0.14^{c}	0.54^{b}	0.51^{b}	0.63^{a}	0.091	<0.001
SFA	32.52^{a}	28.31^{b}	29.88^{b}	29.69^{b}	0.215	0.004
MUFA	49.52^{a}	43.70^{b}	41.95^{bc}	40.17^{c}	0.571	0.009
PUFA	14.78^{c}	24.63^{b}	25.36^{ab}	26.52^{a}	0.178	<0.001
n-6 FA	14.02^{c}	17.32^{b}	18.14^{ab}	18.88^{a}	0.227	0.006
n-3 FA	0.76^{b}	7.31^{a}	7.22^{a}	7.64^{a}	0.084	<0.001
n-6/n-3 FA	18.45^{a}	2.37^{b}	2.51^{b}	2.47^{b}	0.058	<0.001
Liver						
C16:0 (PA)	21.86^{a}	19.98^{b}	20.54^{ab}	18.39^{c}	0.279	0.007
C18:0 (SA)	12.17	12.78	12.24	13.53	0.472	0.121
C18:1cis-9 (OA)	45.32^{a}	39.52^{b}	39.87^{b}	40.53^{b}	0.561	0.003
C18:2n-6 (LA)	12.10^{b}	15.83^{a}	16.22^{a}	16.55^{a}	0.218	0.008
C18:3n-3 (ALA)	0.47^{b}	3.05^{a}	2.87^{a}	2.61^{a}	0.165	<0.001
C20:5n-3 (EPA)	0.14^{b}	0.73^{a}	0.75^{a}	0.76^{a}	0.083	<0.001
C22:6n-3 (DHA)	0.21^{c}	1.03^{b}	1.35^{ab}	1.78^{a}	0.208	<0.001
SFA	34.31	33.98	33.58	32.71	1.243	0.097
MUFA	48.63^{a}	41.60^{b}	40.78^{b}	41.22^{b}	0.782	0.007
PUFA	14.44^{c}	22.57^{b}	23.50^{ab}	24.30^{a}	0.652	<0.001
n-6 FA	13.62^{b}	17.73^{a}	18.52^{a}	19.15^{a}	0.544	0.006
n-3 FA	0.82^{b}	4.84^{a}	4.98^{a}	5.15^{a}	0.307	<0.001
n-6/n-3 FA	16.61^{a}	3.66^{b}	3.72^{b}	3.72^{b}	0.046	<0.001

[1] C-: standard diet, C+: standard diet containing flaxseed oil, WL2.5 and WL5: C+ diet containing 2.5 % and 5 % WLs, respectively.
[2] SEM: standard error of the mean. $^{a-c}$Means within a row with different superscripts are different ($P < 0.05$). PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid, ALA: α-linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

polyunsaturated FA (PUFA) in the yolk of F and S eggs was higher ($p < 0.01$) in the experimental groups (C+, WL2.5 and WL5) as compared to hens fed the standard diet (group C-), which had a very high content of SFAs and MUFAs ($p < 0.01$).

The introduction of WLs into the diet of laying hens did not significantly influence the FA profile of fresh eggs compared to the group supplemented with FO alone. The reduction in n-3 PUFA content in WL5 eggs (hens fed with FO and WL supplements) after 30 days of storage at room temperature was < 19 %, while the reduction in C+ eggs (hens fed only FO) was over 33 % (Fig. 2). The most strongly affected FAs during storage were EPA+DHA, which decreased by 29 % in WL5 eggs and by over 42 % in C+ eggs.

Thus, the highest content of antioxidants and n-3 PUFA in the yolk and the best oxidative stability of yolk lipids was obtained by supplementing the diet with FO in association with WLs, independent of the WL dose.

4. Discussion

To our knowledge, this research is the first study on the effects of introducing WLs into the diet of laying hens. In addition, the WLs used in this study underwent minimal processing (filtration, drying and grinding). In contrast to our study, other studies have used complex and expensive methods for the extraction of polyphenols for their use in food fortification (Caponio et al., 2024; Costa et al., 2023). Therefore, similar studies using other wine by-products, such as grape pomace, were taken into account in the discussion of the results (Herranz et al., 2024; Reis et al., 2019; Romero et al., 2022; Selim et al., 2023; Sun et al., 2018; Tufarelli et al., 2021).

The use of diets enriched with n-3 PUFA in poultry nutrition to improve the quality of dietary fats has been associated with increased lipid peroxidation in the plasma, liver and yolk (Gladine et al., 2007; Mierlita, 2019). In this context, supplementing diets with natural antioxidants from different plant wastes represents a nutritional strategy of interest to effectively limit lipid oxidation (D'Alessandro et al., 2024; Lioliopoulou et al., 2024; Mierlita et al., 2024a, 2024b; Vlaicu et al., 2022). As a result, the present experiment aimed to evaluate the capacity of WLs to limit lipid peroxidation in plasma, liver, and egg yolk in hens fed a diet enriched with n-3 PUFAs.

4.1. Chemical compositions of feed

The analyzed WLs exhibited a crude protein content (21.62 %) similar to that previously reported by Rivas et al. (2021) for red wine lees, but much lower than that reported by Chioru et al. (2023), who found a protein content of 42.62 % in wine lees biomass. The high protein content of WLs is related to the residues of yeast cell autolysis (Caponio et al., 2024), and the differences between different studies can

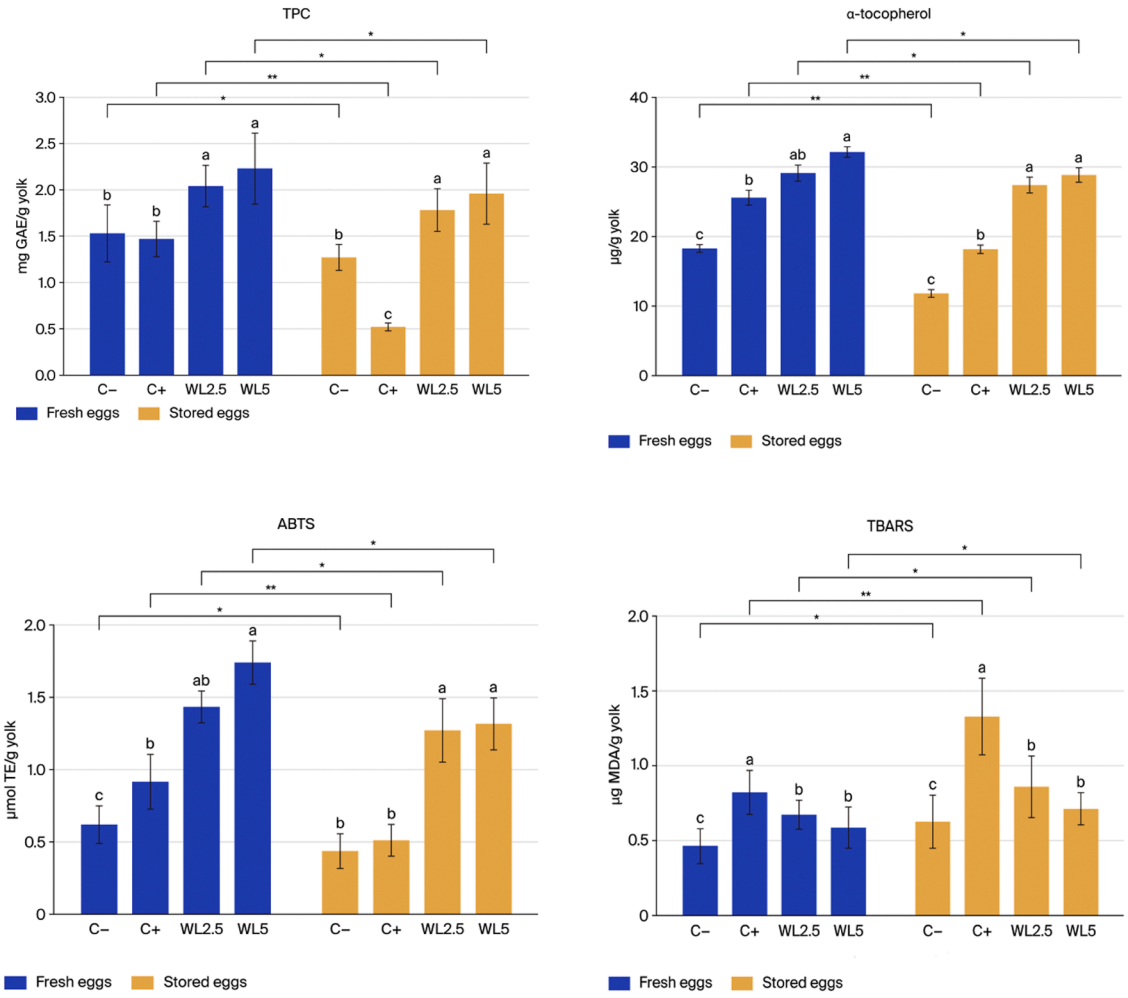

Fig. 1. Egg yolk antioxidant content and lipid oxidative status before (fresh: F) and after storage (stored: S) for 30 days at room temperature. Different lowercase letters indicate significant differences ($p < 0.05$) between groups for the same trait and for the same egg type (F or S). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant differences between F vs. S eggs for the same trait and for the same dietary treatment. Values are represented as the mean and SEM. Abbreviations: C-: standard diet; C+: standard diet containing flaxseed oil; WL2.5 and WL5: C+ diet containing 2.5 % and 5 % wine lees, respectively; GAE: gallic acid equivalents; TPC: total phenolic content; TE: Trolox equivalent; MDA: malondialdehyde.

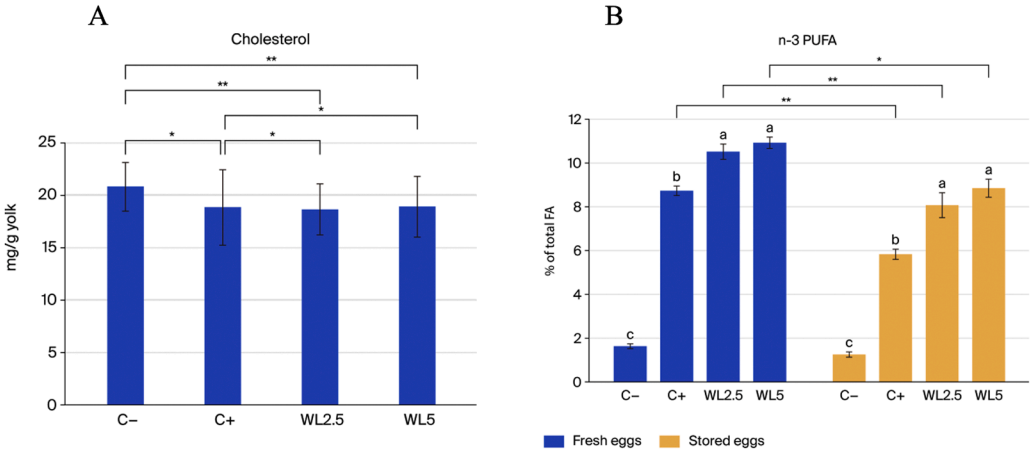

Fig. 2. Egg yolk cholesterol content (A) and n-3 PUFA concentration of fresh (F) egg yolks and egg yolks stored (S) for 30 days at room temperature (B). * ($p < 0.05$) and ** ($p < 0.01$) indicate significant differences between groups for yolk cholesterol content (A). Different lowercase letters indicate significant differences ($p < 0.05$) between groups for the same egg type (F or S). * ($p < 0.05$) and ** ($p < 0.01$) indicate significant differences between F and S eggs for the n-3 PUFA content for the same dietary treatment (B). Values are represented as the mean and SEM. Abbreviations: C-: standard diet; C+: standard diet containing flaxseed oil; WL2.5 and WL5: C+ diet containing 2.5 % and 5 % wine lees, respectively.

Table 7
Egg yolk FA composition (% of total FA) before and after storage for 30 days at room temperature (temperature adjusted to 22 ± 2 °C).

		Dietary treatment[1]				SEM[2]	p-value
		C-	C+	WL2.5	WL5		
C16:0	F	24.68[a]	20.31[b]	21.03[b]	20.18[b]	0.271	0.007
	S	24.17[a]	21.42[b]	21.88[b]	21.37[b]	0.453	0.036
	SEM[2]	0.215	0.267	0.305	0.283	-	-
	p-value	0.254	0.172	0.084	0.208	-	-
C18:0	F	8.93	9.87	8.44	8.63	0.195	0251
	S	9.72	10.21	9.15	8.86	0.182	0.183
	SEM[2]	0.146	0.312	0.275	0.186	-	-
	p-value	0.075	0.102	0.067	0.122	-	-
C18:1c9	F	38.06[a]	34.65[b]	35.74[b]	36.29[b]	0.387	0.005
	S	40.18[a]	37.71[b]	38.65[b]	37.41[b]	0.215	0.008
	SEM[2]	0.342	0.318	0.304	0.336	-	-
	p-value	0.045	0.007	0.005	0.024	-	-
C18:2n-6	F	20.01	22.45	21.07	20.73	0.676	0.056
	S	17.39[b]	19.68[a]	19.09[a]	20.65[a]	0.402	0.042
	SEM[2]	0.836	0.586	0.398	0.715	-	-
	p-value	0.036	0.040	0.039	0.171	-	-
C18:3n-3	F	0.63[c]	6.36[b]	7.56[a]	7.89[a]	0.408	<0.001
	S	0.56[c]	4.40[b]	5.91[ab]	6.66[a]	0.397	0.006
	SEM[2]	0.316	0.259	0.421	0.375	-	-
	p-value	0.072	0.008	0.042	0.031	-	-
EPA + DHA	F	0.71[b]	1.87[a]	2.43[a]	2.44[a]	0.187	<0.001
	S	0.55[c]	1.07[b]	1.68[a]	1.73[a]	0.132	0.003
	SEM[2]	0.218	0.159	0.124	0.267	-	-
	p-value	0.064	0.005	0.007	0.043	-	-
SFA	F	34.05[a]	30.38[b]	29.72[b]	29.21[b]	0.415	0.009
	S	34.12	32.09	31.27	30.53	0.689	0.004
	SEM[2]	0.672	0.781	0.590	0.488	-	-
	p-value	0.224	0.076	0.058	0.075	-	-
MUFA	F	41.24[a]	35.21[b]	36.33[b]	36.72[b]	0.482	0.005
	S	44.07[a]	39.82[b]	39.12[b]	37.99[c]	0.518	0.007
	SEM[2]	0.419	0.625	0.425	0.397	-	-
	p-value	0.045	0.004	0.029	0.072	-	-
PUFA	F	23.99[b]	32.54[a]	32.83[a]	33.20[a]	0.491	0.003
	S	20.58[c]	26.04[b]	28.58[a]	30.90[a]	0.357	0.002
	SEM[2]	0.360	0.421	0.298	0.355	-	-
	p-value	0.047	<0.001	0.032	0.023	-	-
n-6 FA	F	22.36	23.81	22.32	22.28	0.615	0.159
	S	19.33[b]	20.21[b]	20.51[b]	22.05[a]	0.452	0.008
	SEM[2]	0.543	0.350	0.369	0.422	-	-
	p-value	0.038	0.046	0.029	0.136	-	-
n-6/n-3 FA	F	13.72[a]	2.72[b]	2.12[c]	2.04[c]	0.162	<0.001
	S	15.46[a]	3.46[b]	2.54[c]	2.49[c]	0.239	<0.001
	SEM[2]	0.486	0.243	0.281	0.212	-	-
	p-value	0.007	0.004	0.032	0.021	-	-

[1] C-: standard diet, C+: standard diet containing flaxseed oil, WL2.5 and WL5: C+ diet containing 2.5 % and 5 % WLs, respectively.
[2] SEM: standard error of the mean. [a-c]Means within a row with different superscripts are different (P <0.05). F: Fresh, S: Stored, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid, ALA: α-linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

be attributed to the type of grape, the type of yeast used for fermentation and the winemaking method (Rivas et al., 2021).

The TPC of WLs in this study is consistent with the values reported in previous studies (Duarte et al. 2023; Reis et al., 2016) for wine lees from red wine production (36–44 mg GAE/g). Higher values for TPC (254 mg GAE/g) have been reported for yeast obtained after 12 months of aging wine in American oak barrels (Romero-Díeza et al., 2018). In our study, the WLs were obtained after the alcoholic fermentation phase (after approx. 10 days) of a red wine produced in stainless steel tanks. These very different values between studies can also be attributed to the extraction process (solvent and extraction method) of phenolic compounds (Jara-Palacios, 2019). However, wine lees has a lower TPC than grape pomace (30.86 mg GAE/g vs. 90.21 mg GAE/g) (Reis et al., 2016).

The phenolic content and antioxidant activity measured via the DPPH test were lower in our study than in the data reported by Ye et al. (2022) for yeasts obtained from the fermentation of Pinot Noir wine; this is probably due to the different ways the grapes were harvested. In our study, the grapes were harvested mechanically, which caused the berries to break and oxidation to occur, while in the aforementioned study, the grapes were harvested manually and SO2 was added during grape processing.

The results of this study regarding phenolic content and antioxidant activity justify the initial hypothesis that WLs are a rich source of bioactive substances with antioxidant activity suitable for mitigating the effects of oxidative stress induced by diets rich in n-3 PUFA.

4.2. *Laying performance and egg quality*

Phenolic compounds in plants can increase estrogen and progesterone levels, which together stimulate follicular maturation and ovulation; this contributes to improving egg production (Ross & Kasum, 2002). In addition, the antioxidant and anti-inflammatory properties of phenolic compounds can protect the intestine from oxidative damage and improve nutrient utilization, thus increasing the egg laying rate (Sun et al., 2018). These findings are consistent with the results of our study; hens fed WLs achieved significantly higher egg production than those in the C+ group. In addition, hens in group WL5 had a better FCR than the positive control, suggesting that WLs have the ability to mitigate the effects of oxidative stress on metabolism, thus ensuring the better utilization of nutrients in the feed.

Our study indicated that the use of WLs in the diet of laying hens did not alter egg weight. This could be because egg weight is directly related to nutrient intake (Sun et al., 2018). In agreement with our results, Tufarelli et al. (2021) demonstrated that feeding laying hens grape pomace rich in phenolics did not affect daily feed intake, but it did improve egg production and FCR.

The results of the present study are partially in agreement with those of Selim et al. (2023), who observed that supplementing the diet of laying hens with ostrich pomace (60 or 90 g/kg diet) increased egg production and egg weight. These inconsistent results can be attributed to the different phenolic content of the two wine by-products, different levels of inclusion in the diet, the grape variety, the diet composition, the age of the hens and the duration of the experiment.

The introduction of WLs into the hens' diet resulted in a deterioration in eggshell thickness ($p < 0.01$), as phenolic compounds have the ability to chelate calcium ions, which could have reduced calcium absorption, thus preventing the proper formation of the mineral shell in the uterus (Herranz et al., 2024).

The reduction in eggshell thickness due to dietary supplementation with WLs can have a negative impact on poultry producers by increasing losses due to cracking and shell breakage and increasing the risk of bacterial contamination (such as Salmonella), which would lead to significant economic losses. Studies by Zhu et al. (2020) demonstrated that dietary supplementation with tea polyphenols reduces the shell thickness and strength due to decreased calcium availability, while the dirty and broken egg rates increased, thus reducing the saleable egg rate. In addition, recently, Herranz et al. (2024) found that dietary supplementation with grape pomace (50 g/kg of diet) reduced mineral shell thickness, similar to our study; however, this change was not reflected in the mechanical parameters of the eggs.

Although shell thickness is of major importance in the production of laying hens, with direct and substantial economic implications for producers, we believe that improving oxidative stability outweighs the harm to eggshell quality caused by dietary supplementation with WLs,

contributing to increasing the nutritional value and shelf life of consumer eggs. Furthermore, even though shell thickness is often associated with eggshell strength and the percentage of cracked eggs (Kibala et al., 2018), in our study, we did not measure this shell quality parameter, so we cannot formulate a clear conclusion.

It is known that the nutrition of laying hens does not seem to have a large effect on albumin quality (Williams, 1992), a finding also confirmed in the present study. However, some studies have reported that dietary supplementation with rosehips, flax seeds, and olive leaf extracts can improve Haugh units (Papadopoulos et al., 2023; Vlaicu et al., 2022).

In agreement with the present study, Romero et al. (2022) and Selim et al. (2023) reported that supplementing the diets of laying hens with red grape pomace increased the egg yolk color score. This is because grapes are a source of β-carotene, lutein (Sayago-Ayerdi et al., 2009) and anthocyanins (Romero et al., 2022), which play an essential role in egg yolk pigmentation. The decrease in egg yolk color in C+ hens compared to the C- hens is likely a consequence of replacing corn kernels, which contain high amounts of pigments (e.g., xanthophyll) for egg yolk, with wheat bran and FO.

4.3. Serum, hepatic and yolk cholesterol contents

The reduction in serum, liver and egg yolk cholesterol in hens fed WLs could be explained by the ability of the phenolic compounds in WLs to inhibit cholesterol absorption in the intestine and its production by the liver (D'Alessandro et al., 2024), as well as to stimulate the conversion of cholesterol into bile acids that are then excreted from the body through enterohepatic circulation (Elazab et al., 2022). These findings are supported by Al-Khalaifah et al. (2024), who demonstrated that quercetin, which is the main flavonoid present in WLs, can form insoluble complexes with cholesterol in the digesta, thereby reducing intestinal cholesterol absorption in laying hens and increasing cholesterol excretion in feces. Furthermore, previous studies have shown that grape seed polyphenols inhibited cholesterol biosynthesis by inhibiting HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) synthesis in hens, causing a reduction in plasma and yolk cholesterol (Akbarian et al., 2011). In addition, similar to our studies, Landeka Jurčević et al. (2017) found that introducing WLs into a high-cholesterol diet had a reducing effect on serum and hepatic cholesterol and triglycerides in mice.

4.4. Serum and hepatic biochemical indices and antioxidant capacity

In the present study, total serum and liver lipid content decreased significantly in laying hens fed FO, confirming the results of previous studies (Tarek et al., 2015), which reported that flaxseed (100 g/kg diet) reduces serum and liver lipids and attenuates fatty liver syndrome in laying hens. The linear reduction in serum and hepatic lipid levels as a function of the dose of WLs in the diet suggests that polyphenols affect the digestion and absorption of dietary fat, probably due to condensed tannins, which can bind to bile salts and thus limit fat utilization (Krogdahl, 1985). Similarly, Brenes et al. (2008) reported a decrease in fat digestibility in chickens when fed high amounts of grape pomace concentrate (60 g/kg of diet).

The significant decrease in antioxidant enzyme activity and T-AOC and the increase in serum and liver MDA in C+ hens, compared to the negative control (group C-), indicates the onset of oxidative stress (Iskender et al., 2016). Oxidative stress induced by a diet rich in unsaturated fats causes a reduction in antioxidant enzyme activity and T-AOC, which is correlated with increased MDA concentration in the serum and liver in rats (Lasker et al., 2019), pigeons (Hosseinian & Hasanzadeh, 2021) and laying hens (Mi et al., 2024), consistent with our results.

In the present study, we found that the inclusion of WLs in hens' diets contributed to the restoration of oxidative balance by increasing the activity of endogenous antioxidant enzymes (SOD, CAT and GSH-Px) and consequently reducing the level of MDA. This suggests that WLs may reduce oxidative stress by neutralizing free radicals in the hen's body and protecting lipids from oxidative damage (Li et al., 2023) and may protect liver tissues from oxidative damage (Vasilopoulou et al., 2023). In agreement with the present study, Selim et al. (2023) indicated that phenolic compounds in grape pomace can reduce the production of reactive free radicals and prevent lipid peroxidation, thus leading to an increase in the concentrations of antioxidant metabolites (SOD, CAT, GSH-Px and T-AOC) in the bodies of laying hens. A significant decrease in SOD, CAT and GSH-Px activities was observed in mice fed a high-cholesterol diet; in contrast, dietary supplementation with WLs prevented oxidative stress by restoring hepatic and serum antioxidant enzyme activities to values similar to the control group (Landeka Jurčević et al., 2017).

In line with our findings, Huang et al. (2020) reported a significant increase in the concentrations of ALA (C18:3n-3), EPA (C20:5n-3) and DHA (C22:6n-3) in the liver and blood serum for laying hens fed additionally with extruded flaxseed. The changes in the antioxidant system induced by dietary supplementation with WLs led to an increase in the concentration of PUFAs in serum and liver, as compared to C+.

4.5. Yolk antioxidant content

Although WLs contain considerable amounts of phenolic compounds (Table 2), their deposition in egg yolk is limited because some phenols have poor solubility in aqueous solutions and are highly susceptible to degradation by intestinal microbes and liver enzymes, as suggested by Lioliopoulou et al. (2024). The reduced bioavailability of polyphenols found in our study suggests that intestinal bacteria metabolize polymeric polyphenols from WLs, giving rise to new smaller phenolic compounds that can be absorbed and transferred to egg yolk (Chamorro et al., 2019). On the other hand, Nimalaratneet et al. (2011), after investigating the potential transfer of polyphenols from food to egg yolk, found only trace amounts of polyphenols, mainly ferulic acid.

A similar result was reported by Aziza et al. (2010), who found that increasing polyphenols in the diet of chickens by 28.4 %, 31.7 % and 46.4 % did not significantly alter the concentrations of phenolic compounds in the breast and thigh muscles, but instead reduced the feed utilization efficiency (the FCR value increased from 1.67 in the control to 1.95, 1.91 and 2.0, respectively). However, Herranz et al. (2024) demonstrated that only gallic acid exhibited a better bioavailability when the diet of laying hens was supplemented with grape pomace (50 g/kg), contributing to an increase in the oxidative stability of yolk lipids.

Flaxseed oil is known to have a high content of α-tocopherol (Bernacchia et al., 2014), which, in the present study, was efficiently transferred to the yolk, significantly improving the antioxidant status of the yolk in fresh and stored eggs (Fig. 1). Dietary supplementation with WLs increased the α-tocopherol content in the yolk compared to C+, probably because phenolic compounds in WLs protected tocopherol from oxidative damage during digestion, improving the absorption of tocopherol from feed and its deposition in the yolk (Loetscher, et al., 2014; Papadopoulos et al., 2023).

4.6. Fatty acid content and antioxidant status of yolks

The fatty acid profiles of egg yolk followed similar patterns to those in serum and liver. The increase in EPA and DHA validated the capacity of the desaturation and elongation enzyme systems that converted dietary ALA to its long-chain metabolites (Mierlita, 2019).

Previous studies have shown that there is a limit to the conversion of ALA to long-chain n-3 FA such as EPA and DHA (Mierlita, 2019; Tadesse et al., 2023). However, the use of natural antioxidants extracted from various plants has been reported to increase the conversion rate of ALA to EPA and DHA in egg yolk (Tadesse et al., 2023; Vlaicu et al., 2021). These findings are consistent with our studies, which found higher concentrations of EPA and DHA in the yolk of eggs laid by hens

supplemented simultaneously with FO and WLs compared to the diet supplemented with FO alone. The increase in EPA and DHA in egg yolks observed in WL hens could be explained by the prevention of n-3 PUFA degradation by phenolic compounds in WLs, but also by an increase in the efficiency of ALA conversion to EPA and DHA by activating hepatic desaturation enzymes (Huang et al., 2017).

Similarly to the current study, the dietary inclusion of grape pomace (Romero et al., 2022; Selim et al., 2023) allowed for an increase in the proportion of n-3 PUFA and a decrease in the n-6/n-3 FA ratio in egg yolk.

The inclusion of FO in the hens' diet improved the n-3 PUFA content in egg yolks, but it caused a significant decrease in the oxidative stability of eggs, especially in stored eggs. The higher MDA concentration found in hens fed the C+ diet reflects the increased intensity of yolk lipid peroxidation (Tadesse et al., 2023). The introduction of WLs into the diet significantly reduced the MDA content in egg yolk by up to 28.7 % in fresh eggs and by up to 46.4 % in stored eggs compared to the positive control. It follows that the WLs used in this study had the ability to reduce yolk lipid oxidation due to their phenolic and flavonoid content. However, phenolics and flavonoids can also have a pro-oxidant effect when present in high amounts, which could explain the non-dose-dependent effect of WLs (Papadopoulos et al., 2023).

Indeed, the dual role of polyphenols (a phenomenon known as hormesis), though which is produces either beneficial or toxic effects, depends mainly on the dose and/or the type of experimental cell (Scicchitano et al., 2023). There are a number of scientific works that support the idea that polyphenols, when added to poultry feed in small quantities, can act as antioxidants, improving the antioxidant status and quality of eggs and meat. When added in large quantities, they are ineffective or even pro-oxidants (harmful) (Bešlo et al., 2023). Studies investigating the activity of polyphenols have confirmed that when the diet contains a high dose of polyphenols, their oxidation by polyphenol oxidase occurs, resulting in highly reactive free radicals, and this can be associated with the pro-oxidant activity of polyphenols due to their tendency for auto-oxidation accompanied by the formation of ROS and H2O2 (Rodrigo et al., 2011). On the other hand, low doses of polyphenols increase the amount of antioxidant enzymes, which capture ROS, causing a constant reduction in mitochondrial ROS and an attenuation of the effects of oxidative stress (Scicchitano et al., 2023).

The ineffectiveness of a higher dose of WLs in hen diets found in the present study suggests that a higher dose of WLs is associated with stimulation of antioxidant defense systems by generating low levels of ROS and inducing mild oxidative stress, causing an adaptive cellular response (Lambert et al., 2010; Moskaug et al., 2005). It is likely that the toxic effect of WLs resulting from the pro-oxidant properties of polyphenols, manifesting in decreased egg performance and quality, may occur at higher dietary doses than the one tested in this study.

Our findings regarding the lack of a dose-dependent effect of WLs in the diet of laying hens are consistent with other previous studies, which reported that increasing dietary supplementation of grape pomace from 3 % to 6 % (Romero et al., 2022) or from 6 % to 9 % (Selim et al., 2023) had no significant effect on egg production, egg weight or FCR and yolk MDA concentration in fresh and stored eggs. In addition, the lack of a difference between WL2.5 and WL5 eggs regarding MDA accumulation in the yolk may be related to the fact that dietary polyphenols are not well absorbed or transferred to the egg yolk (Fig. 1) (Surai, 2014).

In agreement with our study, it has been shown that increasing the grape pomace concentration in the diet of broiler chickens from 30 mg to 60 mg/kg had no effect on growth performance and lipid peroxidation (Sayago-Ayerdi et al., 2009). On the other hand, after supplementation of olive leaf extract (rich in phenols and flavonoids) in the diet of broiler chickens at two levels (1 % and 2.5 %), it was demonstrated that the lower dose was more beneficial for growth performance and reduced meat lipid oxidation during storage (Vasilopoulou et al., 2023).

Reducing lipid peroxidation by supplementing the diet with WLs is considered beneficial because the accumulation of MDA in the yolk decreases the shelf life of eggs. Furthermore, MDA can be absorbed and accumulated in human plasma, affecting the health of consumers (Gorelik et al., 2008).

In agreement with this study, a reduction in lipid oxidation and an improvement in antioxidant capacity were found in the egg yolk of laying hens fed grape pomace (Reis et al., 2019; Romero et al., 2022; Selim et al., 2023).

The use of WLs in the diet of laying hens significantly increased the accumulation of TPC and tocopherol in the egg yolk, which contributed to the reduced lipid peroxidation and the preservation of n-3 PUFA in eggs during storage, thus ensuring a superior stored egg quality, in accordance with the purpose of the research.

5. Conclusions and future perspectives

Wine lees are effective in reducing oxidative stress induced by a diet rich in n-3 PUFA and in improving the oxidative stability of egg yolk lipids, being a natural source of antioxidants beneficial for the nutrition of laying hens. In addition, the addition of WLs to the diet improved egg production and yolk color and reduced its cholesterol content.

Regarding the dose of WLs that should be used in the diet of laying hens, this is not easy to determine, since, between the two WL concentrations (2.5 % vs. 5 %), no significant differences were found in terms of performance, egg quality, or oxidative stress parameters. However, taking into account the cost–benefit ratio, we recommend adding 2.5 % WLs to the diet of laying hens enriched with n-3 PUFA.

Future studies should investigate biomarkers of oxidative damage in mitochondria, Nrf2 activation, and the expression of genes related to antioxidant enzymes under the influence of dietary WLs.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The publication of this manuscript was funded by the University of Oradea, Romania.

Data availability statement

Data are contained within the article.

CRediT authorship contribution statement

Daniel Mierlita: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Stelian Dărăban:** Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Adrian Macri:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ioan Mircea Pop:** Writing – review & editing, Visualization, Validation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors confirm to have no conflicts of interest.

Acknowledgments

The authors would like to thank the poultry farm and laboratory of analyzed technicians who participated in the research.

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