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# Dietary Application of Grape Waste in Laying Hens Reared Under Two Stocking Densities

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## Abstract
A total of 224 69-week-old Nick Chick laying hens were allocated to eight treatments with eight replicate cages to investigate the effects of stocking density (SD) and dietary inclusion of different levels of grape waste (GW) on performance, some egg quality traits and yolk oxidative status of laying hens from 69 to 76 weeks of age. The experiment was conducted as a 2×4 factorial arrangement, with two levels of stocking density (3 and 4 hens/cage as normal (NSD) and high stocking density(HSD), respectively) and four dietary levels of GW (0, 1.5, 3 and 4.5 % of diet, as a corn replacement). The results indicated that rearing hens under HSD conditions resulted in a worse feed conversion ratio (FCR) and less egg production than NSD ($P < 0.05$). Dietary inclusion of GW had no detrimental effect on performance or egg quality traits. Increasing density had deleterious consequences on FCR, egg production, egg weight and egg mass ($P < 0.05$), while dietary addition of GW, particularly at the level of 4.5% alleviated those negative effects. Egg quality traits (shell weight, resistance and thickness, yolk weight, albumen height and Hague Unit) were significantly not influenced by GW, SD or their interaction. Stocking density did not affect malondialdehyde (a peroxidation indicator) level in egg yolk lipid, while all hens fed GW had lower levels of malondialdehyde than those fed diet without GW ($P < 0.05$). The interaction between SD and GW level significantly influenced egg albumen pH at 3 and 30 d room storage ($P < 0.05$). In conclusion, dietary inclusion of up to 4.5 % GW had no deleterious effect on laying hen's egg quality and improved some performance traits and oxidative stability of egg yolk, particularly in hens reared under HSD. Thus, it could be used as a corn replacement in laying hens' diet.

## Introduction
Due to animal and human health concerns, the application of natural feed additives including herbs and their products in the animal industry has received more attention and interest (Mahfuz *et al*., 2021). One group of natural compounds with beneficial effects on animal and human health, known as polyphenols, are available in different fruits, vegetables, herbs, spices, tea and coffee (Abd El-Hack *et al*., 2022). Numerous biological activities of polyphenols including anti-microbial, anti-inflammatory, antioxidant, anti-mutagenic and anti-allergic, have been reported (Abdel-Moneim *et al*., 2020). Additionally, they can act as detoxifiers, growth-promoter and immunomodulator substances. Considering these positive effects, different studies have been conducted on the effects of polyphenols-rich products on animals and poultry. In laying hens, application of phenolic compounds can cause in beneficial consequences on performance, egg quality and storage life of eggs (Abd El-Hack *et al*., 2022).

One of the most important fruits is grape, which is produced in huge amounts (77 million tons) annually in the world (Ebrahimzadeh *et al*., 2018). Industrial

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processing of grape for producing different products results in large amounts of grape by-products, which is regarded as a main challenge for waste management and causing environmental and economic concerns (Costa *et al*., 2022). Hence, applying these materials is important to reduce environmental pollution (Ebrahimzadeh *et al*., 2018).

Grape products are rich in phenolic compounds (8.08% total extractable polyphenols) (Romero *et al*., 2022). The effects of grape products on the laying hens have been investigated in different studies. Recently, it has been shown that dietary inclusion of grape pomace or extract had beneficial consequences on some egg quality characteristics and improved antioxidant stability of egg yolk during storage (Romero *et al*., 2022). In another study, dietary addition of 4 and 6 % dried grape pomace had no effect on performance and egg quality traits of laying hens but improved egg lipid stability (Kara *et al*., 2016).

As a stress factor, high stocking density has deleterious consequences on immunity, health, welfare and performance of laying hens (Kang *et al*., 2016, 2018; Hofmann *et al*., 2021). Thus, different strategies, such as dietary supplementation with organic chromium and vitamin C, have been used to alleviate the negative impacts of this stressor in laying hens (Mirfendereski and Jahanian, 2015; Jahanian and Mirfendereski, 2015). Considering the extensive beneficial effects of phenolic compounds, particularly their antioxidant activity and positive effects on gut health (Nm *et al*., 2018) as well as egg quality (Galli *et al*., 2018), it could be expected that feeding hens with a phenolic-rich feedstuff for instance grape products can beneficially influence stressed-hens such as those reared under high stocking density. For example, it was reported that dietary supplementation with grape pomace had beneficial effects on the performance and egg quality of laying hens under heat stress conditions (Reis *et al*., 2019).

On the other hand, corn, as the most important energy source in poultry diets, is an expensive feedstuff. Thus, poultry nutritionists have made different efforts to find cheaper replacements for this feed ingredient. It is expected that grape–byproducts could be used as a replacement for conventional feeds. In addition to their beneficial compounds, such as polyphenols, they have low prices and contain appropriate levels of crude protein, metabolizable energy, lipids, minerals and vitamins (Costa *et al*., 2022). Taking together, it could be supposed that GW can be used as a corn replacement in laying hens' diet, particularly at late stages of the production cycle. It is expected that dietary inclusion of polyphenol-rich by-products such as GW results in several advantages including reduction in environmental pollution, lower diet cost, improving the antioxidant capacity of egg lipids and alleviating the adverse effects of HSD in laying hens. On the best of our knowledge, there is no report on the possible effects of replacing dietary corn with grape by-products in laying hens reared under different SD. Thus, the objective of the current study was to determine the effects of varying levels of GW, as a possible replacement for corn, on performance, yolk lipid oxidation and some egg quality traits of hens reared under normal or high stocking density.

## Materials and methods

A total of 224 69-week-old Nick Chick laying hens at the late stage of the production cycle were assigned to the experimental treatments in 2×4 factorial arrangements with two levels of rearing stocking density (3 or 4 hens/cage) and four levels of dietary GW (0, 1.5, 3 and 4.5 %). Each treatment had eight replicate cages. In normal SD, three hens were kept in each cage, while under high SD, this number increased to 4 hens per cage. Different levels of GW as a corn replacement were included in the basal diet (Table 1), without any change in percentage of other feed ingredients.

A sufficient amount of black grape waste was obtained from a big greengrocery store in Shiraz, Fars province of Iran. The collected grape waste included unsaleable fruit, which is conventionally considered as grape waste and is usually thrown out by sellers. Intact fruits were separated from branches, dried then ground and included in the basal diet depending on the experimental treatment.

The proximate composition of GW was determined using the Official Methods of Analyses (AOAC, 2000) procedure. It contained 92.04 % dry matter, 8.91 % CP, 7.01 % EE, 4.22 % ash and 28.12 % CF. The energy content of GW was not measured in the current study. However, there is some data on the energy value of grape products. For example, grape pomace was reported to contain 4397.63±164.22 (Ebrahimzadeh *et al*., 2018) or 4539±9.9 Kcal/kg (Romero *et al*., 2022) Gross energy. Additionally, the metabolizable energy value (on a dry weight basis) of grape pomace was in the range of 1220-2080 Kcal/kg (mean value was 1602 Kcal/kg) (Costa *et al.,* 2022).

All hens were reared in cages under the same housing management conditions. The length, width and height of each cage were 43, 34 and 41 cm, respectively. The first week was considered for hen's adaptation and the remaining 7 weeks were for data recording. Hens had free access to feed and water throughout the experimental period. A lighting program of 16h L: 8h D was imposed during the experiment. Feed intake was recorded weekly. Egg production and egg weight were measured daily for each cage. Feed conversion ratio was calculated as feed consumption (kg) to egg weight (kg).

**Table 1.** Feed ingredient and nutrient composition of the basal diet.

| Feed ingredient | Amount (kg/ton) |
|---|---|
| Corn | 529.00 |
| Barley | 50.00 |
| Soybean oil | 31.00 |
| Soybean meal (44 % CP) | 262.00 |
| Oyster | 90.00 |
| Dicalcium phosphate | 13.20 |
| Wheat bran | 12.70 |
| Vitamin premix[1] | 2.50 |
| Mineral premix[2] | 2.50 |
| Common salt (NaCl) | 2.50 |
| Sodium bicarbonate | 1.00 |
| DL-Methionine | 1.80 |
| L- Lysine | 0.70 |
| L-Threonine | 1.10 |
| Calculated Nutrient composition | |
| ME (Kcal/kg diet) | 2747 |
| Crude protein (%) | 17.0 |
| Calcium (%) | 3.71 |
| Available phosphorus (%) | 0.37 |
| Sodium (%) | 0.16 |
| Methionine (%) | 0.44 |
| Lysine (%) | 0.91 |

[1]The vitamin premix supplied the following per kilogram of diet: 11,000 IU vitamin A, 2300 IU vitamin $D_3$, 121 IU vitamin E, 2.3 mg vitamin $K_3$, 4 mg vitamin $B_1$, 4 mg vitamin $B_2$, 4 mg vitamin $B_6$, 0.03 mg biotin, 1 mg folic acid, 0. 02 mg vitamin $B_{12}$, 840 mg choline chloride.

[2]The mineral premix supplied the following per kilogram of diet: 100 mg Mn, 50 mg Fe, 169. 4 mg Zn, 100 mg Cu, 0.2 mg Se, 1 mg I, 0. 47 mg Co

At the end of the experiment, 6 eggs from each cage (48 eggs/treatment) were randomly sampled to evaluate egg quality traits ,including shell weight, shell resistance, shell thickness, yolk weight, albumen height and Hague Unit. An Egg Multi Tester (EMT-5200, Japan) was used to measure and calculate those parameters automatically. Concentration of malondialdehyde (as a yolk lipid peroxidation indicator) in egg yolk lipids was evaluated by measuring the TBARS (thiobarbituric acid reactive substances) (Botsoglou *et al*., 1992). Eggs were broken and their albumen carefully separated to measure albumen pH. Egg albumen pH was measured at 3 and 30 days after storage in room and refrigerator conditions. Statistical analysis of experimental data was done using the General Linear Models procedure of SAS software (2005). Duncan's multiple range test was used to compare the means. The level of statistical significance was set at $P < 0.05$.

## Results

### Performance traits

The findings indicated that SD, GW level, and their interaction had no significant effect on feed intake (Table 2). As expected, HSD had deleterious influences on FCR and egg production ($P < 0.05$). Thus, hens reared in HSD had an inferior FCR and lower egg production when compared to those in NSD ($P < 0.05$).

Dietary inclusion of different levels of GW had no negative effect on traits even though higher levels of GW caused in better FCR (4.5 % GW) and heavier eggs (3 and 4.5% GW) compared to the 0 % GW ($P < 0.05$), suggesting that up to 4.5 % GW could be included in laying hens' diet as an alternative feed for corn, without adverse effect on their performance. Significant interactions were observed between SD and GW level for FCR, egg production, egg weight and egg mass ($P < 0.05$). Under NSD, GW had no significant effect on FCR or egg production, but in HSD, improved FCR was observed in hens fed with 4.5% GW, when compared to the other levels of GW ($P < 0.05$). Also, feeding hens with different levels of GW increased the percentage of egg production ($P < 0.05$). Indeed, GW had a beneficial effect on egg weight in both NSD and HSD. Under NSD, egg mass remained unaffected, but hens fed diet without GW and reared under HSD had the lowest egg mass among the experimental treatments ($P < 0.05$). Dietary supplementation with different levels of GW improved egg mass compared to this treatment ($P < 0.05$) which indicates the beneficial effect of GW on egg mass under HSD conditions.

**Table 2.** The main effect of stocking density and grape waste level and their interaction on the performance traits of laying hens

| Item | | Feed intake (g) | Feed conversion ratio | Egg production (%) | Egg weight (g) | Egg Mass |
|---|---|---|---|---|---|---|
| Main effect of stocking density | | | | | | |
| Normal | | 109 | 1.90[b] | 92.14[a] | 64.03 | 58.99 |
| high | | 108 | 2.15[a] | 83.02[b] | 64.02 | 53.14 |
| *SEM* | | 0.98 | 0.03 | 1.20 | 0.22 | 1.01 |
| *P*-value | | 0.401 | 0.035 | 0.022 | 0.804 | 0.315 |
| Main effect of grape waste level (% diet) | | | | | | |
| 0 | | 108 | 2.08[a] | 85.49 | 64.62[b] | 55.17 |
| 1.5 | | 111 | 2.07[a] | 88.07 | 64.21[b] | 56.54 |
| 3 | | 109 | 2.00[ab] | 88.14 | 65.26[a] | 57.54 |
| 4.5 | | 109 | 1.96[b] | 88.47 | 65.60[a] | 57.94 |
| *SEM* | | 0.96 | 0.05 | 1.10 | 0.16 | 1.02 |
| *P*- value | | 0.322 | 0.017 | 0.133 | 0.0001 | 0.160 |
| Interaction | | | | | | |
| stocking density | Grape waste level | | | | | |
| Normal | 0 | 108 | 1.90[bc] | 91.86[a] | 64.24[c] | 59.08[a] |
| | 1.5 | 113 | 1.91[bc] | 92.62[a] | 64.99[b] | 59.34[a] |
| | 3 | 108 | 1.92[bc] | 91.98[a] | 65.81[a] | 60.58[a] |
| | 4.5 | 108 | 1.88[c] | 91.49[a] | 65.52[ab] | 60.01[a] |
| High | 0 | 108 | 2.25[a] | 79.08[c] | 63.99[c] | 51.26[d] |
| | 1.5 | 108 | 2.23[a] | 83.53[b] | 64.42[c] | 53.47[c] |
| | 3 | 108 | 2.09[a] | 84.30b | 64.71[b] | 54.50[bc] |
| | 4.5 | 108 | 2.03[bc] | 85.45[b] | 65.67[ab] | 55.88[b] |
| *SEM* | | 0.88 | 0.02 | 1.21 | 0.21 | 1.04 |
| *P*- value | | 0.400 | 0.0009 | 0.008 | 0.026 | 0.026 |

Means in each column with different letters are significantly different at $P \leq 0.05$.

**Egg quality traits**

The results (Table 3) indicated that eggshell traits (weight, thickness and resistance) as well as yolk weight, albumen height and Hugh Unit were significantly not influenced by SD, GW level, or their interaction which means that rearing hens in HSD or feeding them with diets containing up to 4.5 % GW had no deleterious effect on above-mentioned parameters. Stocking density did not affect yolk color. In contrast, hens fed with the highest level of GW (4.5 %) had higher yolk color than 0 and 1.5 % GW ($P < 0.05$).

**Albumen pH and yolk lipid oxidation**

Our results (Table 4) indicate that SD had no significant effect on albumen pH at different storage days. At 3 d room storage, dietary addition of GW decreased albumen pH in a dose-dependent manner, hence, the lowest pH was observed at 4.5 % GW ($P < 0.05$). At 30 d room storage, 3 and 4.5 % GW had lower pH than the 0 % GW ($P < 0.05$). At 30 d refrigerated storage, pH of 3 % GW was lower than other levels ($P < 0.05$).

The interaction between SD and GW level significantly influenced Albumen pH at 3 and 30 d room storage. For 3 d room storage, GW significantly reduced albumen pH, in both SD ($P < 0.05$). For 30 d room storage, adding 4.5 % GW to diet of hens reared in NSD resulted in lower albumen pH than the 0 % level ($P < 0.05$). Stocking density did not affect the MDA level in egg yolk, while GW significantly affected this parameter. All hens fed GW had lower levels of MDA than those fed a diet without GW ($P<0.05$).

**Table 3.** The main effect of stocking density and grape waste level and their interaction on egg quality traits of laying hens

| Item | Shell weight (g) | Yolk weight (g) | Shell thickness (mm) | Shell resistance (kg/cm$^2$) | Albumen height (mm) | Haugh unit | Yolk Color |
|---|---|---|---|---|---|---|---|
| Main effect of stocking density | | | | | | | |
| Normal | 6.52 | 18.42 | 0.305 | 3.13 | 5.70 | 71.87 | 5.68 |
| high | 6.43 | 18.37 | 0.308 | 3.25 | 5.80 | 72.31 | 5.57 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *SEM* | | 0.05 | 0.13 | 0. 01 | 0.08 | 0.10 | 0.97 | 0.06 |
| *P*-value | | 0.055 | 0.363 | 0.329 | 0.480 | 0.136 | 0.185 | 0. 230 |
| Main effect of grape waste level (% diet) | | | | | | | | |
| 0 | | 6.59 | 18.65 | 0.308 | 3.248 | 5.77 | 72.54 | $5.53^{b}$ |
| 1.5 | | 6.53 | 18.58 | 0.309 | 3.179 | 5.80 | 71.31 | $5.43^{b}$ |
| 3 | | 6.54 | 18.18 | 0.309 | 2.99 | 5.44 | 69.53 | $5.48^{ab}$ |
| 4.5 | | 6.25 | 18.17 | 0.301 | 3.348 | 6.10 | 74.99 | $5.91^{a}$ |
| *SEM* | | 0.04 | 0.12 | 0.01 | 0.09 | 0.12 | 0.94 | 0.05 |
| *P* -value | | 0.353 | 0.826 | 0.379 | 0.456 | 0.136 | 0.083 | 0.015 |
| Interaction | | | | | | | | |
| stocking density | Grape waste level | | | | | | | |
| Normal | 0 | 6.53 | 18.27 | 0.304 | 3.28 | 5.47 | 70.25 | $5.50^{ab}$ |
| | 1.5 | 6.61 | 18.32 | 0.314 | 3.38 | 5.85 | 52.51 | $5.68^{a}$ |
| | 3 | 6.50 | 18.50 | 0.305 | 2.94 | 5.30 | 68.11 | $5.62^{ab}$ |
| | 4.5 | 6.09 | 18.70 | 0.303 | 3.18 | 6.20 | 76.58 | $5.91^{a}$ |
| High | 0 | 6.65 | 23.18 | 0.310 | 3.51 | 6.09 | 74.84 | $5.62^{ab}$ |
| | 1.5 | 6.45 | 18.44 | 0.304 | 2.97 | 5.55 | 70.06 | $5.18^{b}$ |
| | 3 | 6.58 | 18.55 | 0.311 | 3.02 | 5.57 | 70.94 | $5.62^{ab}$ |
| | 4.5 | 6.41 | 18.67 | 0.304 | 3.15 | 6.01 | 73.34 | $5.91^{a}$ |
| *SEM* | | 0.05 | 0.15 | 0.02 | 0.08 | 0.10 | 0.77 | 0.05 |
| *P*- value | | 0.137 | 0.193 | 0.240 | 0.397 | 0.242 | 0.118 | 0.029 |

Means in each column with different letters are significantly different at $P \leq 0.05$.

**Table 4.** The main effect of stocking density and grape waste level and their interaction on egg yolk malondialdehyde level and albumen pH of eggs stored for 3 and 30 days under room and refrigerator conditions

| Item | | Albumen pH | | | MDA level (ng/g) |
|---|---|---|---|---|---|
| | | 3 d room storage | 30 d room storage | 30 d refrigerated storage | |
| Main effect of stocking density | | | | | |
| Normal | | 8.44 | 9.55 | 8.76 | 0.00058 |
| high | | 8.44 | 9.56 | 8.74 | 0.00055 |
| *SEM* | | 0.02 | 0.03 | 0.02 | 0.00007 |
| *P*- value | | 0.831 | 0.623 | 0.385 | 0.788 |
| Main effect of grape waste level (% diet) | | | | | |
| 0 | | $8.56^{a}$ | $9.66^{a}$ | $8.80^{a}$ | $0.00097^{a}$ |
| 1.5 | | $8.48^{b}$ | 8.58ab | $8.74^{a}$ | $0.00054^{b}$ |
| 3 | | $8.39^{c}$ | $8.51^{bc}$ | $8.69^{b}$ | $0.00038^{c}$ |
| 4.5 | | $8.34^{d}$ | $9.46^{c}$ | $8.77^{a}$ | $0.00035^{c}$ |
| *SEM* | | 0.02 | 0.01 | 0.02 | 0.00007 |
| *P* -value | | 0.0001 | 0.001 | 0.056 | 0.003 |
| Interaction | | | | | |
| stocking density | grape waste level | | | | |
| Normal | 0 | $8.55^{a}$ | $9. 65^{a}$ | 8.80 | 0.00021 |
| | 1.5 | $8.48^{b}$ | $9.58^{ab}$ | 8.72 | 0.00037 |
| | 3 | $8.39^{c}$ | $9.52^{ab}$ | 8.76 | 0.00027 |
| | 4.5 | $8.34^{cd}$ | $9.43^{b}$ | 8.76 | 0.00040 |
| High | 0 | $8.56^{a}$ | $9.66^{a}$ | 8.80 | 0.00080 |
| | 1.5 | $8.48^{b}$ | $9.58^{ab}$ | 8.77 | 0.00071 |
| | 3 | $8.39^{c}$ | $9.50^{ab}$ | 8.61 | 0.00043 |
| | 4.5 | $8.35^{c}$ | $9.49^{ab}$ | 8.77 | 0.00037 |
| *SEM* | | 0.03 | 0.02 | 0.01 | 0.00004 |
| *P*- value | | 0.0001 | 0.011 | 0.060 | 0.232 |

Means in each column with different letters are significantly different at $P \leq 0.05$.

**Discussion**

Our findings indicated that HSD caused poorer performance (worse FCR and less egg production) in laying hens, which agree with previous results (Saki *et al*., 2012; Kang *et al*., 2016, 2018). Findings of performance traits indicate that substitution of dietary corn with up to 4.5 % GW improved hens' performance in some cases. Certainly, this substitution could be important to reduce concerns about environmental pollution and diet costs.

There is little information on the effects of grape products on laying hens (Romero *et al*., 2022). Dietary supplementation with grape by-products in laying hens has not resulted in constant findings. In a very recent study, when different levels of grape pomace (30 or 60 g/kg diet) or grape extract (0.5 or 1.0 g/kg) were added to laying hens diets, egg production and egg mass were not influenced while using both levels of pomace and 0.5 g/kg grape extract decreased the average egg weight. Also, all groups fed grape by-products consumed less feed and had better FCR than the control group (Romero *et al*., 2022). In another study, feed intake, FCR and egg production of laying hens fed diets supplemented with 4 and 6 % grape pomace were not changed, while 4 % grape pomace increased egg weight, which could be attributed to the “augmentative effect” of grape pomace on intestinal probiotic bacteria (Kara *et al*., 2016). Kaya *et al.* (2014) reported that supplementation with grape seed or grape seed extract had no significant effect on feed intake and FCR of laying hens but resulted in higher egg production.

Based on our results, dietary inclusion of GW had the potential to reduce or prevent the negative consequences of HSD on laying hen’s performance. No report was found in the literature on the effect of grape by-products on the laying hens reared under HSD. However, it is reported that the antioxidant activities of polyphenol compounds can attenuate the deleterious effects of heat stress in poultry (Hu *et al*., 2019). In a previous study, the addition of different levels (1, 2 and 3%) of grape pomace flour to the diet of heat-stressed-laying hens had beneficial consequences on their performance, egg quality, antioxidant capacity and health (Reis *et al*., 2019). In another study, dietary inclusion of 4 % grape seeds increased the activity of several enzymes (superoxide dismutase and glutathione peroxidase) with antioxidant activity in the plasma and reduced the oxidative stress in broilers (Abu Hafsa & Ibrahim, 2018). Moreover, the finding of Hajati *et al*. (2018) indicated that supplementation with grape seed extract decreased the adverse effects of heat stress in broilers, even though better than vitamin C and resulted in better performance, immunity and antioxidant activity.

The beneficial effects of phenols may be related to the following mechanisms: reduction in population of gut pathogens and positive effects on gut morphology due to their anti-inflammatory and antioxidant activity. Also, they can change surface area in the intestines and improve gut digestive enzyme activity, resulting in better absorption of nutrients. On the other hand, increase in digestive system secretions, including endogenous enzymes, mucus, bile and saliva are other reasons for those effects (Valenzuela-Grijalva *et al*., 2017; Mountzouris *et al*., 2011; Viveros *et al*., 2011). Indeed, phenols can improve and maintain the health of the gastrointestinal tract by optimizing the balance between beneficial and harmful bacteria (Franz *et al*., 2010; Gadde *et al*., 2017). Intestinal mucosa is negatively influenced by reactive oxygen radicals, resulting in an impaired absorption of nutrients. Antioxidant compounds such as phenols can neutralize these harmful radicles and thereby cause a better intestinal environment and absorption (Valenzuela-Grijalva *et al*., 2017). These extensive positive consequences may explain the reasons for improving the performance of hens fed with grape waste in the current experiment.

Our findings indicated that egg quality traits were significantly not influenced by SD, GW, or their interaction, which means that up to 4.5 % GW could be included in laying hens' diet without negative effect on egg quality. Different previous findings have been reported on the effect of SD on the egg quality of laying hens. In the study of Kang *et al*. (2018), rearing Hy-line Brown hens at high SD (19 birds/$m^2$) resulted in lower eggshell strength than other SD (13, 15 and 17 birds/$m^2$). Geng *et al*. (2020) reported that most of the quality traits of egg in a native chicken in China (Beijing You Chicken) were not influenced by different SDs (5, 6, 7 and 8 birds/$m^2$). In another study on egg-laying ducks, different stocking densities (4, 5, 6, 7 and 8 birds/$m^2$) did not affect some egg quality traits such as egg weight, yolk weight, albumen weight, eggshell weight, percent of yolk and egg shell, egg shape index, yolk color and Haugh Unit, but HSD impaired egg shell strength and thickness (Xiong *et al*., 2020).

In agreement with the current study, Kara *et al*. (2016) reported that feeding hens with 4 and 6 % grape pomace had no effect on Hagh unit, egg yolk index, eggshell ratio and eggshell thickness. In the study of Kaya *et al*. (2014), egg shape index, shell strength, shell thickness, shell weight, yolk index and albumen index were not influenced by grape seed or seed extract, while in contrast with our results, higher Haugh unit was observed in hens fed diets containing grape seed or extract. Another finding indicated that feeding hens with grape pomace (30 or 60 g/kg diet) or grape extract (0.5 or 1.0 g/kg) resulted in a higher yolk color score. Also, the Haugh unit increased in groups fed diets containing two levels of grape pomace or 1 g/kg grape extract. Dietary treatments did not affect shell thickness (Romero *et al*., 2022). Additionally, dietary supplementation with different levels of grape pomace flour (1, 2 and 3 %) to the diet of heat-stressed-laying hens did not change the physical and chemical composition of fresh eggs (Reis *et al*., 2019).

Higher yolk color in 4.5 % GW group compared to the 0 and 1.5 % found in our study indicates that GW could be considered a rich source of egg yolk pigments ,even though it can provide more pigments than corn. Previously, Nobakht (2015) reported that grape green leaf contained high levels of beta carotene and increased egg yolk color in laying hens.

In the current study, SD did not influence egg yolk MDA which was not in line with a previous report when increasing SD negatively influenced the antioxidant capacity of laying ducks as measured by higher plasma and liver MDA (Xiong *et al*., 2020).

Based on our findings, supplementation with GW reduced the MDA level in egg lipid. As MDA is a toxic compound with harmful effects on animal and human health (Romero *et al*., 2022), a lower level of this compound indicates that GW can reduce the yolk oxidation process showing a beneficial effect of GW. In a previous study, feeding heat-stressed-laying hens with 1, 2 and 3 % grape pomace flour resulted in higher antioxidant capacity against peroxyl radicles and lower lipid peroxidation of egg yolk in both fresh and stored eggs at room temperature for 21 and 30 days (Reis *et al*., 2019). On the other hand, the dietary addition of grape pomace (30 or 60 g/kg diet) or grape extract (0.5 or 1.0 g/kg) had no significant effect on the level of MDA in fresh eggs, but 60 g/kg grape pomace decreased level of MDA in stored eggs (Romero *et al*., 2022).

Similarly, in the study of Kaya *et al*. (2014), yolk MDA in fresh eggs was not influenced by dietary grape seed or seed extract but after 14 d of refrigerated storage, lower MDA was observed in eggs of hens fed with grape by-products. Kara *et al*. (2016) reported that feeding with 4 and 6 % grape pomace lowered the level of MDA in plasma and egg yolk of laying hens, resulting in a longer shelf life.

Grape by-products can reduce the lipid peroxidation of animal products and thereby increase their shelf life. This activity is perhaps associated with the presence of phenolic compounds, with the ability to scavenge free radicals, inhibit or decrease the development of singlet oxygen and form complexes with metal ions (Kara *et al*., 2016). As natural antioxidants, phenols can protect biomolecules such as polyunsaturated lipids, proteins, sugars and nucleic acids against oxidative damage mediated by free radicals (Mahfuz *et al*., 2021).

In the current study, albumen pH was not influenced by SD. Supplementation with GW particularly its higher levels, resulted in the lower albumen pH under 3 and 30 d room storage conditions which is in agreement with a previous report when dietary supplementation with different levels of grape pomace flour (1, 2 and 3 %) to the diet of heat stressed-laying hens, decreased the pH of albumen on days 21 and 30 of storage (Reis *et al*., 2019). The pH of yolk and albumen in commercial eggs is influenced by storage time. Deterioration of protein can increase the pH of albumen (Abd El-Hack *et al*., 2022). In a recent study, using pumpkin seed meal in diet of laying hens lowered the albumen pH of eggs after 28 days of storage at room (21ºC) and refrigerator (5ºC) temperature. It was concluded that antioxidant and polyphenol compounds in pumpkins can delay adverse changes, including protein denaturation and lipid oxidation, thereby increasing the shelf life of storage eggs (Vlaicu and Panaite, 2022).

It should be mentioned that factors such as rearing conditions and bird's breed can influence the response of poultry to SD and thus result in different results (Geng *et al*., 2020). Also, the response of laying hens to the grape by-products can vary depending on the content of polyphenols in these products, grape type, dietary inclusion level, process method used to produce by-products and presence of other compounds in feed. However, different factors such as animal species and stage, length of experimental period, pharmacokinetics, and composition and dose of phenolic compounds can influence the action mechanism of these substances (Mahfuz *et al*., 2021).

In conclusion, the current finding indicates that up to 4.5 % of dietary corn could be replaced with GW. This substitution has beneficial effects on performance traits and oxidative stability of egg yolk lipids, particularly when fed under HSD. Hence, it could be used as a corn replacement in laying hens' diet, leading to lower feed costs and fewer environmental concerns.

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