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Effect of dietary polyphenol rich grape (*Vitis vinifera*) seed extract supplementation on production performance, egg quality, plasma MDA, reproductive performance and faecal microbiota of golden laying hens

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ABSTRACT

The study investigated the effects of grape seed extract (GSE) supplementation on laying hens aged 30–35 weeks. Hens were assigned to four groups: a control group with only basal diet and three experimental groups with basal diet supplemented with 250 (GSE 250), 500 (GSE 500) and 750 (GSE 750) mg/kg GSE. GSE-supplemented groups showed significantly lower feed intake in week 1 and 3 in GSE 750. No significant differences were observed in feed efficiency, egg weight, shell thickness, yolk weight, albumin weight, or Haugh Unit, suggesting GSE did not significantly impact these parameters. Egg production significantly increased in groups supplemented with 500 and 750 mg/kg GSE compared to the control group. Additionally, GSE-supplemented birds exhibited significantly lower levels of malondialdehyde (MDA), a marker of lipid peroxidation, indicating potential antioxidant effects. The study further revealed a significant increase in Lactobacillus at GSE500 and GSE 750 levels and a significant reduction in *E. coli* levels with GSE 750 supplementation, suggesting potential benefits on gut microbiota. In conclusion, GSE positively influenced egg production, promoted Lactobacillus growth, and reduced lipid peroxidation and *E. coli* populations. However, reproductive efficiency in golden laying hens was not significantly affected. These findings contribute valuable insights into the potential benefits of GSE in poultry nutrition.

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Polyphenol; laying hens; reproductive capacity; egg quality; grape seed extract

Introduction

The utilization of feed additives to influence the gut functions and microbial environment of domestic animals has gained recognition as a pivotal strategy for enhancing growth performance and feed efficiency (Khan et al. 2023a; Viveros et al. 2011). The surge in antimicrobial resistance among pathogens found in both humans and animals, coupled with the prohibition of antibiotic use as feed additives, has expedited the exploration of alternative approaches for more effective antimicrobials in animal husbandry (Hafeez et al. 2023). The elimination of antibiotic growth promoters from feed is anticipated to lead to a decline in feed efficiency and a rise in cases of intestinal disorders due to the proliferation of harmful gut pathogens (Saleh et al. 2021; Khan et al. 2023b). In response, various researchers have delved into the antimicrobial properties of diverse plant extracts against specific pathogens.

The scientific community has demonstrated a growing interest in utilizing environmentally friendly materials, particularly herbal-based extracts, as evidenced by recent studies (Aslam et al. 2023; Khan et al. 2023b). Phytobiotics have been documented to be rich in bioactive compounds known for their anti-inflammatory and antioxidant properties, which have shown potential benefits for reproduction (Hasan et al. 2022; Khan et al. 2023c). Grape seeds, primarily contain major compounds such as gallic acid, proanthocyanidins, epicatechin and various forms of proanthocyanidins as their principal components have been extensively studied (Viveros et al. 2011; Chand et al. 2021; Hafeez et al. 2023). Grape seeds exhibit superior antioxidant potential when compared to conventional antioxidant molecules (Bagchi et al. 2014). It has been documented that hens supplemented grape seed proanthocyanidin extract potentially mitigated ovarian aging by reducing oxidative stress (Liu et al. 2018). Furthermore, this compound also provides a degree of protection against heavy metal induced endocrine disruption in the reproductive system in the chicken embroyo (Hou et al. 2016).

Indeed, GSE has been shown to exert an influence on microbiota composition (Viveros et al. 2011). Polyphenols have the potential to alter the microbial composition within the intestines by selectively impeding the growth of pathogenic

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bacteria while concurrently fostering the growth of probiotic strains (Raza et al. 2023). The study by Grandhaye et al. (2020) underscores that a relatively short supplementation period of just two weeks with 2% GSE (Grape Seed Extract) can lead to notable improvements in the metabolic and laying parameters of breeder hens. This positive effect is attributed to a modification in the composition of the gut microbiota. These findings highlight the substantial impact that (poly)phenols can have on the delicate balance of the gut microbiota.

However, there is a dearth of investigations examining the in vivo effects of GSE on egg performance (both quantity and quality) and fertility parameters. This highlights the need for further research in this area to better understand the potential benefits of GSE in poultry production. The objective of this study was to assess the effects of supplementation of varying levels of grape seed extract on growth and production performance, egg quality, malondialdehyde (MDA) levels, reproductive performance and the composition of faecal microbiota in golden laying hens. This investigation aims to shed light on the potential benefits and impacts of GSE supplementation in poultry production.

Materials and methods

Grape seed extract

The process began by procuring red grapes of the *Vitis vinifera* var. Cencibel variety, ensuring they were at an optimal level of ripeness from the nearby market. Subsequently, the seeds were subjected to a drying period of 3–4 days in shaded conditions. Following the drying process, the seeds were finely ground into a powder with the help of electric metallic hammer mill. Around 100 g of this powder was then placed in a 1000 ml flask, and an extraction procedure was carried out using 100% ethanol (500 ml) over a duration of 48 h. The resulting solution, which contained the extracted compounds, was then separated from the residual seed material through the use of a filter. Maintaining a temperature range of 50–60°C, using a rotary evaporator, the ethanol content was then removed from the solution. The final extract obtained from this process was stored at a temperature of 4°C for subsequent use in the experiment. The final product was a red-brown powder.

Animal groups and diets preparation

A total of 480 golden laying hens (age, 30 weeks) were randomly allocated into a control and three supplemental groups on the basis of body weight. Each group contained 120 hens, with six replicates. In two-tier battery cages, the birds were individually housed measuring $45 \times 50 \times 60$ cm, located in an open-sided brick house. The temperature of the house was maintained approximately at 25°C and 61% humidity. A 16 h lighting programme was employed per day. Water was provided through nipple drinkers fitted within the cages for fresh water supply. The overall trial started between 30 and 35 weeks of age and lasted for a total period of 5 weeks.

Table 1. Composition and chemical analysis of basal diet.

Ingredients	Basal diet (%)
Corn	52.0
Soybean 48%	22.0
Rice husk	18.0
Monocaclium phosphate	1.5
Calcium carbonate	5.5
Vitamin-minerals premix[a]	1.0
Analyzed composition	
Crude protein %	16.70
ME (MJ/kg)	9.5
Crude fibre (%)	5.6
Neutral-detergent fibre (%)	12.3
Calcium, %	2.9
Total phosphorous, %	0.55
Methionine, %	0.25
Lysine, %	0.75

[a]The premix provided the following per kilogram of diet: Vitamin A 180,000 IU, Vitamin E 200 IU, Vitamin D3 50,000 IU, Vitamin B1 50 mg, Vitamin K3 10 mg, Vitamin B6 60 mg, Vitamin B2 120 mg, Vitamin B12 0.25 mg, Pantothenic acid 260 mg, Niacinamide 650 mg, Biotin 1.5 mg, Folic Acid 15 mg, Iron 1.4 g, Choline Chloride 8 g, Manganese 1.2 g, Copper 0.18 g, Se 2.5 mg, Zinc 1.2 g, Calcium 8%, iodine 20 mg, Phosphorus 2.0 mg.

The birds were fed a corn-soybean basal diet as shown in Table 1. The experimental rations were developed using feed win interactive software and Excel, tailored to meet the layer's metabolic energy and crude protein requirements. The chemical composition of the experimental feeds was determined following the guidelines stipulated in the AOAC (1990). The experimental groups were structured as follows: (1) a control group, (2) basal diet supplemented with GSE at a rate of 250 (GSE1), 500 (GSE2) and 750 mg/kg (GSE3) feed. Proximate composition analysis of GSE was determined according to the method outlined in AOAC (2000) as given in Table 2. In addition, total phenolics, flavan-3-ols and total soluble polyphenols were determined through HPLC (HP 1090 with diode array detector). The proximate analysis also revealed that these inclusion levels had no significant effect on final composition of the offered feed.

Laying performance

Starting from 30 weeks of age, the weights of the birds were documented till the end of the study (35 weeks of age). Eggs collected on daily basis were quantified as a percentage of hens-day egg production, representing the number of eggs/day. Similarly, feed intake was recorded on a daily basis per cage. The feed conversion ratio (FCR) was calculated by dividing the total feed intake by the hens for each treatment over the five-week period by the dozens of eggs produced for each respective treatment over the same duration.

Table 2. proximate composition (g/100 g) of grape seed.

Nutrients	Grape seed composition
Humidity	6.5 ± 0.04
Crude protein	11.4 ± 1.3
Ether extract	6.5 ± 0.12
Crude fibre	41 ± 4.56
Neutral detergent fibre	28.5 ± 2.4
Acid detergent fibre	Not detected
Grape seed extract composition (g/100 ml)	
Total extracted polyphenol	7.8 ± 0.15
Flavan-3-ols	1.15 ± 0.02
Total phenolics	0.49 ± 0.01

Several egg quality parameters were evaluated, including albumin height, yolk weight, albumin weight, albumin ratio and Haugh Unit. For the assessment of egg quality, 60 eggs laid between 06:00 and 22:00 h were randomly selected from each experimental treatment on the conclusion of the study (35 weeks age). Individual eggs underwent a thorough evaluation of various quality attributes. Using an EQM (Egg Quality Measurement) plate, the eggs were cracked open, and the weights of the yolk and albumen were determined. Eggshell weight was estimated by removing any attached albumen and the thin membrane. The eggshells were subsequently air dried at room temperature for 24 h. The albumen weight and eggshell thickness were recorded by the method described by Saleh et al. (2019b). For the determination of eggshell thickness, average measurements were randomly carried out from three (the air cell, equator and pointed end) distinct places on the egg. Eggshell thickness was measured using a micrometer calliper (Mitutoyo, 0.01–20 mm, Tokyo, Japan) after the removal of the eggshell membranes. The Haugh Unit was calculated using the provided equation.

$$\text{Haugh Unit} = 100 \times \log(\text{Albumin height} - 7.57 - 1.7 \times \text{Egg weight}^{0.37}).$$

Measurement of fertility and hatchability

The percentages for fertility and hatchability were determined as described by Hafeez et al. (2023) using artificial insemination. At the conclusion of each week, about 30 eggs were collected per replicate and immediately incubated for hatching. The number of hatched chicks was recorded following the incubation period. Unhatched eggs were carefully tested to compute fertility and hatchability percentage.

Serum malondialdehyde (MDA) measurement

Blood samples were obtained from three hens per replicate for the assessment of serum MDA. Blood samples were collected in and subsequently centrifuged at 700 g for a period of 20 min and stored at −20°C. Using commercial kit (Biocheck, USA), plasma MDA was determined as per methodology given in the kit.

Faecal microbiota

Faecal microbial population of *Lactobacillus* and *E. coli* were measured by the methods outlined by Raza et al. (2023). Briefly, on the final day of the experiment, fresh faecal samples (1 g) were directly collected from two birds in each replicate. These faecal samples were then thoroughly mixed with 9 mL of 1% peptone broth. Subsequently, ten-fold serial dilutions were prepared and plated onto MacConkey agar plates for isolation of *E. coli* and *Lactobacilli* medium III agar plates for *Lactobacillus*. The MacConkey agar plates were incubated at 37°C for 24 h and *Lactobacilli* medium III agar plates were then placed in an anaerobic incubator at 39°C for 48 h. After incubation, the colonies of *Lactobacillus* and *E. coli* were promptly counted, and expressed as $\log_{10}$ CFU/ g of faeces.

Statistical analysis

Statistical analysis was carried out using the general linear models procedure in SPSS following a one-way analysis of variance (ANOVA). A post hoc test, specifically the Newman–Keuls test, was performed at $P < 0.05$ for the statistical significance of the observed variations among the treatments.

Results

Table 3 illustrates the impact of GSE on the feed intake of laying hens at various levels. In week 1, the control group exhibited significantly ($P < 0.05$) higher feed intake (799.9) compared to the GSE500 (798.2) and GSE 750 (798.7). However, there was no significant difference in feed intake in week 2 ($P > 0.05$). In week 3, the control group again demonstrated significantly ($P < 0.05$) higher feed intake (800.1) compared to the GSE750 (797.6), GSE500 (798.5) and GSE250 (799.2). Feed intake in weeks 4 and 5 was not significantly affected by the use of grape seed extract ($P > 0.05$).

Table 4 shows the body weight gain of laying hens during in response to grape seed extract at various concentrations. In weeks 1, 2 and 3, body weight gain was not significantly affected ($P > 0.05$). However, in week 4, the GSE750 group exhibited significantly ($P < 0.05$) higher body weight gain (5.75) compared to the control group (4.75) and GSE250 (4.75). Body weight gain in week 5 showed no significant differences ($P > 0.05$) with the use of grape seed extract at different levels.

Table 5 illustrates the Feed Conversion Ratio (FCR) of laying hens during their peak production phase, when supplemented with varying levels of GSE. The results indicate no significant impact on the FCR of the laying hens. However, in week 1, the FCR was notably higher ($P < 0.05$) in the GSE1 group (2.17) in comparison to the GSE2 group (2.04). Nevertheless, the feed conversion ratio in weeks 2, 3, 4 and 5 showed no

Table 3. Mean feed intake in laying hens supplemented with different levels of grape seeds extract.

Group	Control	GSE250	GSE500	GSE750	SEM	*P*-value
Week 1	799.9^{a}	799.1^{ab}	798.2^{b}	798.7^{b}	0.229	0.04
Week 2	798.6	799.0	799.3	798.6	0.238	0.74
Week 3	800.1^{a}	799.2^{b}	798.4^{b}	797.6^{b}	0.334	0.03
Week 4	799.3	798.8	798.5	799.1	0.239	0.70
Week 5	799.2	799.3	798.7	799.1	0.223	0.87
Overall mean	799.42	799.08	798.62	798.62	0.25	0.47

Mean values bearing different superscripts in a row differ significantly ($P < 0.05$).

Table 4. Body weight gain in layer birds supplemented with different levels of grape seeds extract.

Group	Control	GSE250	GSE500	GSE750	SEM	*P*-value
Week 1	5.50	5.75	6.00	6.00	0.10	0.25
Week 2	6.00	5.75	5.75	5.00	0.16	0.10
Week 3	4.75	5.25	5.00	5.50	0.13	0.17
Week 4	4.75^{b}	4.75^{b}	5.50^{ab}	5.75^{a}	0.16	0.04
Week 5	5.00	5.50	5.75	5.50	0.27	0.84
Overall mean	5.2	5.4	5.6	5.5	0.16	0.28

Means with different superscripts in same row are significantly ($P < 0.05$).
GSE (Grape Seed Extract) at 250, 500 and 750 mg/kg, respectively.

Table 5. Feed conversion ratio in laying hens supplemented with different levels of grape seeds extract.

Group	Control	GSE250	GSE500	GSE750	SEM	*P*-value
Week 1	2.08^{ab}	2.17^{a}	2.04^{b}	2.10^{ab}	0.017	0.01
Week 2	2.11	2.01	2.18	2.15	0.029	0.19
Week 3	2.03	2.13	2.12	1.99	0.030	0.31
Week 4	2.05	2.04	2.09	2.10	0.022	0.75
Week 5	2.07	2.09	2.01	1.97	0.031	0.54
Overall mean	2.06	2.08	2.08	2.06	0.025	0.36

Means with different superscripts in same row are significantly different at $P < 0.05$.
GSE (Grape seed extract) at 250,500 and 750 mg/kg, respectively.

Table 8. Faecal microbial population in golden laying hens (Log_{10} CFU/g) supplemented with different levels of grape seeds extract.

Groups	*Lactobacillus*	*E. coli*
Control	7.91^{b}	4.21^{a}
GSE250	8.10^{b}	4.12^{a}
GSE500	8.21^{a}	4.06^{a}
GSE750	8.43^{a}	3.98^{b}
SEM	0.39	0.34
P-value	0.034	0.021

Means with different superscripts in same column are significantly different ($P < 0.05$).
GSE (Grape seed extract) at 250,500 and 750 mg/kg, respectively.

significant differences ($P > 0.05$) with the use of varying inclusion levels of GSE.

Table 6 illustrates the effects of grape seed extract supplementation on various egg quality parameters, including egg weight, yolk weight, shell thickness, albumin weight and Haugh Unit in laying hens. The results indicate that egg weight remained consistent ($P > 0.05$) across different inclusion levels of grape seed extract in the layer feed. Shell thickness, on the other hand, did not significantly ($P > 0.05$) changed in both the GSE1 group (0.327) and GSE2 group (0.327) compared to the Control group (0.322) and GSE3 group (0.326). Similarly, both albumin height and Haugh Unit showed no significant variations among the groups.

Table 7 presents the data on reproductive performance and MDA levels in laying hens, illustrating the impact of GSE supplementation. The findings revealed a noteworthy increase ($P < 0.05$) in hen day egg production for both the GSE500 and GSE750 groups when compared to the control group. The results show that different levels of GSE had no significant effect on reproductive performance in laying hen. The MDA level was significantly ($P < 0.05$) lower in GSE supplemented birds compared to the control.

Table 8 provides an overview of the impacts of varying levels of GSE on the faecal microbiota in both the control and treatment groups. The outcomes demonstrated a significant increase ($P < 0.05$) in the abundance of *Lactobacillus* with the administration of GSE500 and GSE750. Moreover, GSE750 exhibited a notable decrease ($p < 0.05$) in the presence of *E. coli* compared to the control group.

Discussion

The widespread benefits of antibiotics in promoting animal performance have been well-documented (Saleh et al. 2023). Despite a growing concern regarding their overuse, leading to various detrimental effects including the presence of environmental pollution, antimicrobial resistance, residues and potential implications for human health (Hafeez et al. 2021). In this scenario, many countries have implemented prohibitions on the use of antibiotics as growth promoters in poultry feed (Ahmad et al. 2020). This has prompted a surge in scientific experimentation to identify phytobiotics based alternatives for enhanced animal production performance and reduce reliance on antibiotics as growth promoters (Ali et al. 2019). In recent years, a multitude of such 'natural' additives have been discovered, showcasing their potential not only in improving productivity but also in contributing to the reduction of antibiotic usage and associated risks (Khan et al. 2012a, 2012b).

While there is limited research on the dietary use of grape products specifically in laying hens, there are more studies available that have investigated the effects of grape by-products on the growth performance of broiler chickens. These studies have explored the potential benefits of incorporating grape by-products into the diets of broiler chickens, examining factors such as growth rate, feed efficiency and overall performance. However, further research is needed to specifically evaluate the dietary effects of grape products in laying hens on the reproductive performance. In the current study, feed intake was

Table 6. Egg quality parameters in laying hens supplemented with different levels of grape seeds extract.

Groups	Egg weight (g)	Shell Thickness (mm)	Yolk Weight (g)	Shell weight (g)	Albumin Weight (g)	Haugh Unit
Control	52.85	0.322	15.8	5.26	31.7	87.3
GSE250	53.29	0.327	16.0	5.32	32.0	87.1
GSE500	52.97	0.327	15.9	5.29	31.8	87.1
GSE750	53.02	0.326	15.9	5.30	31.8	87.2
SEM	0.204	0.001	0.06	0.023	0.12	0.125
P-value	0.90	0.44	0.85	0.88	0.89	0.96

Table 7. Reproductive performance and MDA level in laying hens supplemented with different levels of grape seeds extract.

Groups	Hen Day egg production (%)	Fertility (%)	Hatchability* (%)	Hatchability** (%)	MDA (nmol/ml)
Control	66.5^{c}	90	80	77	9.8^{a}
GSE250	65.8^{b}	89	78	79	8.68^{b}
GSE500	68.3^{a}	88	80	81	7.77^{b}
GSE750	69.6^{a}	91	81	80	7.12^{b}
SEM	0.97	7.54	2.33	3.67	0.32
P-value	0.61	0.07	0.81	0.55	0.00

Means with different superscripts in same column are significantly different at = 0.05.
*From the total eggs set.
**From the fertile eggs.

significantly decreased at the provided inclusion level of GSE. In a study conducted by Sayago-Ayerdi et al. (2009) inclusion of GE byproduct at levels exceeding 6% in the feed exerted a negative impact on feed intake. Earlier studies have reported similar outcomes, demonstrating either negligible or detrimental impacts of grape byproducts in different animal modules (Chamorro et al. 2013; Romero et al. 2021; Hafeez et al. 2023). Grape polyphenols containing high level of condensed tannins, acting as antinutritional factors, which form complexes with proteins, leading to reduced protein digestibility (Romero et al. 2022). This compromised protein digestion, in turn, can lead to adverse repercussions on the overall productivity of laying hens (Sun et al. 2018). These findings underscore the significance of considering condensed tannins, which hinder protein digestion, when incorporating grape byproducts into animal diets. Egg laying process is controlled by the intricate workings of the neuroendocrine system in hens (Elkomy et al. 2023). Grape seed extract is known to contain flavonoids that, while not strongly oestrogenic, possess some oestrogenic activity. This leads to an elevation in oestrogen and progesterone levels, subsequently triggering the release of reproductive hormones from the pituitary gland (Saleh et al. 2019a, 2019c). These hormones, in turn, facilitate the maturation of follicles and the process of ovulation, ultimately resulting in an increased rate of egg production in layers (Sun et al. 2018).

In this study, hen day egg production exhibited a noteworthy increase in the supplemented groups compared to the control. This finding aligns with the observations of Kaya et al. (2014), who also noted a linear rise in egg production with the inclusion of GSE in laying hens' diet. Likewise, in our study, egg quality parameters remained consistent among hens fed varying levels of GSE. These results partially coincide with those reported by Kaya et al. (2014). Moreover, our study unveiled that blood Malondialdehyde (MDA) levels were significantly lower in the experimental groups compared to the control group. Kara et al. (2016) similarly observed a reduction in blood MDA level in layers. Additionally, GSE supplementation led to a decrease in free radical production in the egg yolk of layers (Barbe et al. 2020).

The antioxidant activity in GSE has been attributed to its overall phenolic content (Brenes et al. 2010). Due to its abundant polyphenolic compounds, GSE stands out as a robust reservoir of antioxidants within chicken diets, indicating that its inclusion in the poultry diet can lead to advantageous antioxidant effects. Considering that GSE supplementation has been shown to reduce reactive oxygen species (ROS) levels in the yolk, which can negatively affect fertility, it was expected that supplemented animals would exhibit improved fertility. Additionally, it was anticipated that hatchability would also improve, as increased oxygen levels during hatching can lead to higher production of free radicals through oxidative processes, potentially harming the chicks (Surai et al. 2016). While Hajati et al. (2014) showcased the potential of GSE supplementation to improve hatchability, our current study, in contrast, did not reveal any notable impacts on fertility and hatchability subsequent to GSE supplementation. According to Barbe et al. (2020), there could be several reasons to explain these findings. Firstly, the fertility rate of the birds in the control group may have already been high enough, making it difficult to observe any additional beneficial effects. Secondly, the levels of ROS in the yolk, which play a role in fertility regulation, may not have been high enough in the control group to negatively impact the fertilization process or embryo development. Lastly, the dosage and duration of GSE supplementation may not have been optimal or properly determined in this study. Further investigations are necessary to explore these factors and determine the potential effects of GSE supplementation on fertility parameters.

The existing literature on utilizing polyphenols as antibiotic alternatives in broiler chicken production is limited. The results demonstrated a significant increase in *Lactobacillus* levels with GSE500 and GSE750 ($P < 0.05$), whereas GSE750 led to a notable decrease in *E. coli* levels compared to the control. A prior study involving broilers revealed that feeding grape pomace or grape seed extract resulted in heightened counts of beneficial ileal bacteria like *Enterococcus,* while diminishing counts of potential pathogens like *Clostridium* (Viveros et al. 2011). Consistent with these findings, our study provides novel evidence that polyphenol-rich plant products can exert an influence on the microbial population within the intestines of laying hens. Although *Lactobacilli* are recognized as beneficial for intestinal health, *E. coli* can have detrimental effects on the intestinal mucosa. Our research substantiates that polyphenolics induce a notable shift in the bacterial composition within the intestinal tract. Although the precise mechanism through which polyphenols exert their antimicrobial effects remains elusive, it is conceivable that they may operate in either a bacteriostatic or bactericidal manner, or potentially impede the adhesion of pathogenic bacteria to intestinal cells, as proposed by Viveros et al. (2011). In our specific study, the differential stimulatory effects observed from GSE on *Lactobacilli* in the caecum could be attributed to the phenolic compounds. Another plausible explanation for the elevated level of Lactobacillus is that these bacteria utilize these phenolic compounds as nutritional substrates, exhibiting the ability to metabolize phenolic compounds extracting energy for their growth.

Conclusion

The results of the present study showed that grape seed extract supplementation at level of 750 mg/kg had a significant impact on egg production enhancing *Lactobacilli*, reducing lipid peroxidation and *E. coli* population with no effect on reproductive efficiency and egg quality parameters in golden laying hens.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

The Committee on Animal Rights and Welfare, The University of Agriculture, Peshawar, Pakistan approved this study (PS/25/2022)

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