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REDUCING THE INCIDENCE OF HEMORRHAGIC FATTY LIVER SYNDROME IN LAYING HENS FED RAISIN JUICE BYPRODUCTS AND RESVERATROL AND THEIR IMPACT ON NRF2 AND TNFA GENE EXPRESSION AND ANTIOXIDANT STATUS

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Abstract

This study investigated the effects of raisin juice byproducts (RJ) and resveratrol (RSV) as antioxidants on oxidative stress, gene expression (TNFα and NRF2), productive performance and liver histopathology in 72 Lohmann Brown laying hens are administered a high-energy, low-protein (HELP) diet that precipitates fatty liver haemorrhage syndrome. The treatments were allocated as follows: Treatment 1 (D1T1) included a standard diet without any additives; Treatment 2 (D1T2) was a standard diet plus 2% raisin juice byproducts; Treatment 3 (D1T3) included a standard diet plus 4% raisin juice byproducts; and Treatment 4 (D1T4) included a standard diet plus 500 mg/kg resveratrol. Treatment 5 (D2T1) consisted of a HELP diet devoid of additives, while Treatment 6 (D2T2) incorporated a HELP formulation supplemented with 2% sultana juice, Treatment 7 (D2T3) included a HELP ration + 4% raisin juice byproducts, and Treatment 8 (D2T4) included a HELP diet with 500 mg/kg of resveratrol. The results indicated significant superiority in productive performance for all supplementation treatments compared to the control. A significant reduction in ALT and AST enzyme levels and an elevation in ALP, GSH-PX, and SOD enzymes were seen in response to regular meals and supplemental treatments compared to the control treatment. The

results indicated a significant decrease in NRF2 and TNFα gene expressions for all supplementation treatments. Histological examination showed the liver tissues were free from pathological injury due to the RJ (4%) and resveratrol addition treatments.

Keywords: Hemorrhagic fatty liver, Raisin juice byproducts, Resveratrol, Laying hens, Oxidative stress.

تقليل حالة متلازمة الكبد الدهني النزفي في الدجاج البياض المضاف في عليقته مخلفات عصير الزبيب والريسيفرلترول وتاثيرها في التعبير لجيني NRF2 و TNFα وحالة مضادات الأكسدة

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الخلاصة

هدفت هذه الدراسة إلى تقييم تأثيرات مخلفات عصير الزبيب (RJ) والريسفيراترول (RSV) كمضادات أكسدة على الإجهاد التأكسدي، وتعبير الجينات (TNFα و NRF2)، والأداء الإنتاجي، وتركيب نسيج الكبد في الدجاج البياض المُغذى على عليقة عالية الطاقة منخفضة البروتين، والتي تُسبب متلازمة الكبد الدهني النزفي. استخدمت 72 دجاجة (Lohmann Brown). وزعت المعاملات وتوزعت المعاملات على النحو التالي: المعاملة الأولى D1T1 تضمنت عليقة اعتيادية وبدون أي إضافة، والمعاملة الثانية D1T2 تضمنت عليقة اعتيادية + مخلفات عصير الزبيب 2% /كغم علف، المعاملة الثالثة D1T3 كانت عبارة عن عليقة اعتيادية + مخلفات عصير الزبيب 4% /كغم علف. وتضمنت المعاملة الرابعة D1T4 عليقة اعتيادية + رسيفيراترول 500 ملغم/كغم علف، بينما المعاملة الخامسة D2T1 كانت عليقة عالية الطاقة ومنخفضة البروتين دون اي اضافة. المعاملة السادسة D2T2 تضمنت عليقة عالية الطاقة ومنخفضة البروتين + مخلفات عصير الزبيب 2% /كغم علف، بينما المعاملة السابعة D2T3 تضمنت عليقة عالية الطاقة ومنخفضة البروتين + مخلفات عصير الزبيب 4 /كغم علف. وأخيراً المعاملة الثامنة D2T4 تضمنت عليقة عالية الطاقة ومنخفضة البروتين + ريسيفيراترول 500 ملغم/كغم علف. اشارت نتائج التجربة الى حصول تفوق معنوي في الاداء الانتاجي لجميع معاملات الاضافة بالمقارنة مع معاملة السيطرة، اشارت النتائج الى حصول انخفاض معنوي في تركيز انزيمي ALT و AST، وارتفاع انزيمات GSHPX،ALP و SOD لصالح معاملة العليقة الاعتيادية ومعاملات الاضافة بالمقارنة مع معاملة السيطرة، كذلك اشارت النتائج

في المعاملة الرابعة والثامنة الى حصول انخفاض معنوي في التعبير الجيني لجيني NRF2 وTNFα لجميع معاملات الاضافة. اوضحت نتائج الفحص النسيجي سلامة النسيج الكبدي من الاصابة المرضية لصالح معاملات اضافة مخلفات عصير الزبيب 4 % /كغم علف والريسيفراترول.

كلمات مفتاحية: فروج اللحم، السلالة، وزن الجسم، الصفات الشكلية.

Introduction

Hemorrhagic fatty liver syndrome (HFLS) is among the most serious non-communicable metabolic diseases affecting poultry, particularly caged laying hens. It causes significant economic losses due to decreased egg production and increased mortality (3 and 26). The condition is marked by excessive lipid deposition in the liver and abdominal cavity, haemorrhaging into internal organs, and a delicate, pale-yellow liver. This leads to liver rupture and sudden death in affected birds (32). The causes of this syndrome include genetic, environmental, and hormonal factors, but the main reason is believed to be a high-energy, low-protein (HELP) diet (10).

Recent research has focused on using agricultural by-products in poultry feed (5). These can be used as natural antibiotics as an alternative to antibiotics, protecting cells from oxidative stress (14). Raisin juice is an essential agricultural by-product due to its high content of phenolic compounds, crude fiber, nutrients, and biologically active substances in its various parts, such as seeds, stems, and peels (21). These compounds possess multiple properties, including antioxidant, anti-inflammatory, anticancer, antibacterial, cardioprotective, and neuroprotective elements (19). Resveratrol is a polyphenolic compound that protects cells from oxidative stress and inflammation and may improve chronic liver diseases. These substances are extracted from grape skins, berries, and red wine (27). Resveratrol's antioxidant potential is attributed to its high regulatory capacity to defend cells and protect the liver from damage caused by oxidative stress by preventing lipid accumulation in the liver and regulating apoptosis pathways (16).

This study investigated the effect of raisin juice byproducts and resveratrol as natural antioxidants in eliminating oxidative damage by hydrogen donation and free radical binding in chickens fed an unbalanced diet (high energy, low protein), that induces oxidative stress in laying hens and causing hemorrhagic hepatitis. Additionally, it evaluated the impact of diets on productive and physiological performance, hepatic histology, and gene expression of TNFα and NRF2 genes during the production period. It also examined the effect of diet composition used in the study for preventing hemorrhagic fatty liver syndrome in cage reared hens.

Materials and Methods

The study was carried out over eight weeks at the Department of Animal Production, College of Agriculture, University Anbar's poultry farm and involved 72 28-week-old Lohmann Brown laying chickens. At a pace of three replicates (3 hens/replicate), the cages housing the birds were divided into eight treatments and randomly assigned to 24 homogeneous replicates. The treatments were as follows: Treatment 1 (D1T1)

included a standard diet without any additives; Treatment 2 (D1T2) included a standard diet plus 2% raisin juice byproducts; Treatment 3 (D1T3) included a standard diet plus 4% raisin juice byproducts; and Treatment 4 (D1T4) included a standard diet plus 500 mg/kg resveratrol. Treatment 5 (D2T1) consisted of a high-energy, low-protein diet devoid of additives; Treatment 6 (D2T2) comprised a high-energy, low-protein ration supplemented with 2% raisin juice byproducts; Treatment 7 (D2T3) included a high-energy, low-protein ration supplemented with 4% raisin juice byproducts; and Treatment 8 (D2T4) included a high-energy, low-protein ration enhanced with 500 mg/kg resveratrol. The elements of the ratio and their determined chemical makeup are presented in Table 1.

Table 1: Components and chemical formulations of the diet used in the trial.

Ingredients	Percentage
Crushed Corn	33.5
Crushed Wheat	34
Soybean meal	21
*Protein Concentrate (40%)	2.5
$CaHPO_4$	1.1
$CaCO_3$	7.8
NaCl	0.1
Total	100
** Chemical composition calculated	
DE (kcal/kg)	2768
CP (%)	17.3
Lys (%)	0.87
Met+Cys (%)	0.74
Arg (%)	0.93
Ca (%)	3.89
AvP (%)	0.41

* DE (kcal/kg), dietary energy in kilocalories per kilogram; CP (%), crude protein; Lys (%), lysine; Met+Cys (%), methionine plus cysteine; Arg (%), arginine; Ca (%), calcium; AvP (%), available phosphorus.

**The chemical composition values of the feed materials in the diet composition were used according to NRC, 1994.

The second ratios, where the energy percentage was increased to 3000 (kcal/kg feed) and the protein percentage reduced to 13%, are shown in Table 2.

Table 2: Ingredients and chemical compositions of the secondary diet were used in the experiment.

Feed Material	Percentage
Crushed Corn	36.65
Crushed Wheat	37
Soybean meal	11.4
*Protein Concentrate (40%)	2.5
$CaHPO_4$	1.2
$CaCO_3$	7.8
NaCl	0.1
Oil	2.9
Lysine	0.34
Methionine	0.11
Total	100
** Chemical composition calculated	
DE (kcal/kg)	2994
CP (%)	13.04
Lys (%)	0.87
Met+Cys (%)	0.74
Arg (%)	0.93
Ca (%)	3.89
AvP (%)	0.41

* DE (kcal/kg), dietary energy in kilocalories per kilogram; CP (%), crude protein; Lys (%), lysine; Met+Cys (%), methionine plus cysteine; Arg (%), arginine; Ca (%), calcium; AvP (%), available phosphorus.

**The chemical composition values of the feed materials in the diet composition were used according to NRC, 1994.

Egg production, egg weight, and feed consumption rates were recorded weekly during the experiment. At the end of the trial, blood samples (5 per treatment) were obtained from the wing vein of laying hens for measuring serum metabolites. Serum was taken by centrifugation at 3000 rpm for 5 min and stored at −20 °C till further analysis. As for antioxidant status, it included determining the activity of the GSH-PX and SOD enzymes according to (18). Furthermore, malondialdehyde (MDA) levels were determined according to (7), while the ALT, AST, and ALP enzymes were determined according to the method indicated by (22). For histological sectioning of liver samples, liver tissue samples were washed with normal saline and then fixed in formalin. A week later, the samples in formalin were processed and embedded in paraffin. Subsequently, the tissues were sliced into 5-mm sections and stained with hematoxylin and eosin (HandE). Electron microscopy was then used to view pathological sections, and photographs taken according to the method indicated by (22). Gene expression determination of the TNFα and NRF2 genes was performed using a single-step gene expression assay.

The QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from blood serum samples in accordance with the manufacturer's instructions. The ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to evaluate the quality and amount of the extracted RNA. Primers were created using the Primer3Plus program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and Gallus gallus sequences from the GenBank

database (NCBI, Bethesda, MD, USA) for three target genes, i.e., TNFα, NRF2, and GAPDH, which served as the housekeeping gene. The primers are produced by Macrogen Inc., a firm based in Seoul, the Republic of Korea.

Data analyses were conducted using the completely randomized design (CRD) with eight treatments and three replications. All values were subjected to one-way analysis of variance (ANOVA). Statistical analyses were conducted using the SAS software package (24) to investigate the impact of diet type and treatments. Duncan's multiple range test (9) was used to compare treatments at a level of significance of $P \leq 0.05$.

Results and Discussion

Table 3 demonstrates a significant ($P \leq 0.01$) advantage in egg production and egg weight for the usual treatment compared to the HELP ration treatment. All supplementation treatments outperformed the control treatment. The T1 and T2 intervention treatments outperformed the others, while no significant differences were observed in feed consumption rates across all experimental treatments. This superiority in egg production and egg weight is attributed to the role of raisin juice byproducts and resveratrol in improving production performance. Raisin juice byproducts possess numerous properties, including stimulating estrogenic hormones by binding to the same site as the steroidal estrogenic hormone. This association enhances the activity of estrogen and progesterone, which stimulates the pituitary gland to secrete FSH and LH.

The presence of FSH and LH enhances follicle maturation and ovulation, leading to increased egg production (25). (12) indicated that using grape seed extract in laying hens' diets at 250, 500, and 750 mg/kg feed increased egg production and egg weight. Resveratrol protects against oxidative stress in liver and ovarian cells caused by increased energy in the diet, thereby raising oocyte count and egg production (29). (28) indicated that its addition to the diets of laying hens improved performance by increasing egg production and egg weight.

Table 3: Effect of the studied treatments on the characteristics of cumulative production performance.

Factors		Egg production rate (%)	Egg weight rate (%)	Feed consumption (g/bird)
Diet: D	Normal: D1	79.16 ±1.98 a	4666.27 ±140.61 a	3147.08 ±9.19
	High energy and low protein: D2	73.50 ±0.91 b	4284.49 ±67.89 b	3144.25 ±7.18
	Significance Level	**	**	NS
Treatment: T	Control: T1	71.83 ±0.79 c	4129.27 ±86.19 b	3161.33 ±16.69
	RJ 2 % /kg –feed: T2	78.33 ±2.74 a	4694.42 ±181.58 a	3144.50 ±10.25
	RJ 4 % /kg –feed: T3	82.17 ±2.13 a	4823.86 ±139.15 a	3150.67 ±7.60
	RSV 500 mg/kg –feed: T4	73.00 ±0.89 c	4253.98 ±95.01 b	3126.17 ±5.37
	Significance Level	**	**	NS
Interaction: D * T	D1-T1	72.67 ±1.45 c	4201.51 ±150.09 bc	3160.67 ±29.49
	D1-T2	84.33 ±0.88 a	5095.04 ±22.79 a	3152.67 ±18.85
	D1-T3	86.33 ±1.76 a	5116.57 ±47.10 a	3146.67 ±14.97
	D1-T4	73.33 ±1.76 c	4251.96 ±150.34 bc	3128.33 ±11.25
	D2-T1	71.00 ±0.58 c	4057.03 ±96.93 c	3162.00 ±22.85
	D2-T2	72.33 ±0.88 c	4293.79 ±61.94 bc	3136.33 ±10.17
	D2-T3	78.00 ±1.52 b	4531.24 ±94.43 b	3154.67 ±6.98
	D2-T4	72.66 ±0.88 c	4255.99 ±150.08 bc	3124.00 ±3.60
	Significance Level	**	**	NS

NS = Not statistically significant difference, **P<0.01. ^{a,b,c} means in the same row with different superscripts differ significantly at P<0.05.

Table 4 shows the effect of the different treatments on liver enzyme status. A significant decrease (P≤0.01) was seen in ALT and AST enzyme concentrations in favor of the regular feed treatment, compared to the HELP treatment. The concentration of these enzymes diminished in all supplementation treatments relative to the control treatment, however the ALP enzyme concentration increased in the supplementation treatments and the normal feed. As for the interaction treatments, a significant decrease (P≤0.01) in ALT and AST enzyme concentrations was observed in favor of the T2, T3, and T4 treatments, while that for the ALP enzyme increased in favor of the T2 and T3 treatments. Changes in liver enzyme (ALT, AST, and ALP) concentrations act as an indicator for diagnosing liver diseases, as these enzymes spread throughout the liver tissue. If the liver is exposed to stress or damage, this leads to leakage.

These enzymes are present in the bloodstream, thus increasing their activity in the blood serum. The decrease in ALT and AST enzyme concentrations and the increase in ALP enzyme concentration may be attributed to the role played by raisin juice byproducts and resveratrol in improving the health of birds by reducing oxidative stress and eliminating free radicals (14). Raisin juice byproducts contain a group of phenolic compounds characterized by their ability to eliminate free radicals by forming complexes containing metal ions to eliminate reactive oxygen species (ROS) (2). Resveratrol eliminates free radicals and ROS by possessing a phenyl group capable of donating an electron to free radicals thus removing oxidation which, in turn, contributes to reducing ALT and AST enzymes in the liver (6). (31) indicated that adding resveratrol to laying hens' diets at a rate of 400 and 800 mg/kg feed led to a significant decrease and increase in AST and ALP enzyme concentrations, respectively.

Table 4: The effect of studied treatments on the oxidative AST, ALT, and ALP (U/L) enzymes.

	Factors	AST	ALT	ALP
Diet: D	Normal: D1	19.50 ±1.86 b	274.00 ±15.38 b	1115.58 ±36.48 a
	High energy and low protein: D2	29.17 ±1.09 a	332.33 ±10.02 a	928.17 ±21.46 b
	Significance Level	**	**	**
Treatment: T	Control: T1	30.33 ±1.67 a	351.83 ±15.40 a	908.83 ±24.65 c
	RJ 2 % /kg –feed: T3	23.50 ±2.78 b	287.67 ±21.42 bc	1077.00 ±52.79 ab
	RJ 4 % /kg –feed: T3	20.33 ±3.48 b	262.17 ±16.68 c	1098.00 ±66.32 a
	RSV 500 mg/kg –feed: T4	23.7 ±2.40 b	311.00 ±17.86 b	1003.67 ±52.19 b
	Significance Level	**	**	**
Interaction: D * T	D1-T1	28.66 ±2.60 a	340.00 ±22.14 ab	957.00 ±17.47 d
	D1-T2	17.67 ±1.85 b	249.33 ±24.31 de	1179.33 ±5674 ab
	D1-T3	13.67 ±1.76 b	266.33 ±7.79 e	1232.67 ±58.17 a
	D1-T4	18.00 ±1.15 b	280.33 ±20.67 cde	1093.33 ±25.77 cd
	D2-T1	32.00 ±2.08 a	363.66±23.58 a	860.67 ±20.33 d
	D2-T2	29.33 ±1.20 a	326.00 ±15.30 abc	974.67 ±15.60 cd
	D2-T3	27.00 ±3.60 a	298.00 ±6.80 bcd	963.33 ±21.92 d
	D2-T4	28.33 ±0.88 a	341.67 ±15.10 ab	914.00 ±70.11 d
	Significance Level	**	**	**

**P<0.01. [a,b,c] means in the same row with different superscripts differ significantly at P<0.05.

Table 5 demonstrates a substantial (P≤0.01) superiority in the concentrations of GSH-PX and SOD enzymes associated with the conventional feed treatment, in comparison to the HELP feed treatment. These enzymes surpassed all supplementation interventions in comparison to the control therapy. MDA levels diminished in the supplements and conventional feed treatments relative to the control and HELP feed groups. The T3 and T4 intervention treatments outperformed the others in GSH-PX and SOD enzyme concentrations. MDA concentrations decreased in favor of the T2, T3, T4, and T3 intervention treatments. The higher concentrations of GSH-PX and SOD may be attributed to the polyphenol content in the raisin juice byproducts, which increases the activity of antioxidant enzymes in the liver.

These chemicals are distinguished by their capacity to diminish oxidation rates through the scavenging of free radicals and the binding of oxidative metal ions. They contain hydroxyl groups, which contribute to donating hydrogen atoms to free radicals, thus halting the oxidation process. The primary function of phenolic compounds, which include raisin juice byproducts catechins, and epicatechin, is to inhibit reactive oxygen species (ROS). They protect the intestinal mucosa from oxidation and pathogens and remove free radicals (1). The increased concentrations of GSH-PX and SOD and the decreased concentrations of MDA may be attributed to the role of resveratrol in protecting cells from oxidative stress caused by hydrogen peroxide by inhibiting the activity of hydroxyl radicals and free radicals, thus reducing inflammation (34). The study indicated that adding resveratrol to laying hens' diets increased the concentrations of GSH-PX and SOD enzymes, with a significant decrease in MDA concentration (12). (12) indicated that using grape seeds at 500 and 750 mg/kg feed significantly decreased MDA concentration compared to the control treatment.

Table 5: Effect of the studied treatments on antioxidant status.

Factors		GPx (U/g)	SOD (U/g)	MDA (nmol/mL)
Diet: D	Normal: D1	32.74 ±2.31 a	77.35 ±7.79 a	45.72 ±2.66 b
	High energy and low protein: D2	23.32 ±1.03 b	40.73 ±3.63 b	59.58 ±2.95 a
	Significance Level	**	**	**
Treatment: T	Control: T1	21.10 ±1.11 c	30.41 ±4.54 c	66.01 ±3.59 a
	RJ 2 % /kg –feed: T2	28.50 ±1.70 b	63.33 ±8.80 b	52.01 ±3.31 b
	RJ 4 % /kg –feed: T3	34.67 ±4.31 a	76.86 ±11.64 a	42.20 ±3.28 c
	RSV 500 mg/kg –feed: T4	27.84 ±2.18 b	65.57 ±10.97 b	50.39 ±3.84 b
	Significance Level	**	**	**
Interaction: D * T	D1-T1	23.08 ±1.21 cd	36.97 ±7.41 cd	58.56 ±1.52 b
	D1-T2	32.07 ±0.81 b	81.34 ±5.71 c	45.61 ±3.12 c
	D1-T3	43.62 ±2.76 a	102.17 ±5.92 a	35.93 ±1.44 d
	D1-T4	32.18 ±1.42 b	88.93 ±3.98 ab	42.77 ±2.63 cd
	D2-T1	19.12 ±0.89 d	23.85 ±2.34 d	73.46 ±2.62 a
	D2-T2	24.94 ±1.05 c	45.31 ±5.49 c	58.40 ±2.08 b
	D2-T3	25.72 ±2.34 c	51.56 ±1.44 c	48.47 ±3.53 c
	D2-T4	23.49 ±1.72 cd	42.20 ±6.38 c	58.02 ±2.9 b
	Significance Level	**	**	**

**P<0.01. [a,b,c] means in the same row with different superscripts differ significantly at P<0.05.

Table 6 shows a significant rise in NRF2 gene expression associated with the HELP treatment compared to the usual feed treatment. The results indicate a significant

decrease in all supplemental therapies compared to the control therapy. The results indicated a significant reduction in all interaction treatments relative to the T5 interaction treatment. The NRF2 gene is a redox-sensitive transcription factor that confers cellular protection from oxidative damage. It regulates antioxidant genes such as SOD1, CAT, and GSH PX (17) and induces defense mechanisms when the cells of organisms are exposed to oxidative stress. NRF2 is present in the cytoplasm under normal conditions and is isolated by the inhibitor (Keap 1).

Cells that face oxidative stress undergo degradation, resulting in a conformational change in Keap 1, which subsequently loses its capacity to attach to NRF2 molecules. In this instance, NRF2 accumulates, translocates to the nucleus, and initiates the transcription of the target gene (20).

The decreased NRF2 gene expression in the addition and interference treatments may be attributed to the raisin juice byproducts content of flavonoids and phenolic compounds such as catechin, luteolin, quercetin, and anthocyanins (8). These compounds reduce oxidative stress in birds and possess powerful antioxidant properties. The beneficial effects of these antioxidants are harnessed by directly neutralising free radicals through the elimination of reactive oxygen species (ROS) and enhancing the activity of antioxidant enzymes, or by indirectly augmenting the efficacy of intracellular antioxidants via the regulation of NRF2 gene signalling (14).

Table 6: Effect of diet type and treatments in fold change of the NRF2 expression of the TNFα gene.

Factors		GAPDH	NRF2	DCT	DDCT	FOLDING
Diet: D	Normal: D1	22.78	31.73	8.952	-3.405	28.29 ±10.15 b
	High energy and low protein: D2	24.11	29.48	5.375	-6.985	133.44 ±24.21 a
	Significance Level.	NS	NS	NS	NS	**
Treatment: T	Control: T1	22.56	31.32	8.565	-3.795	96.69 ±21.69 a
	RJ 2 % /kg –feed: T2	22.79	29.72	6.925	-5.435	79.73 ±17.01 b
	RJ 4 % /kg –feed: T3	23.82	29.80	5.985	-6.370	82.97 ±5.27 b
	RSV 500 mg/kg –feed: T4	24.61	31.78	7.180	-5.180	64.06 ±22.86 b
	Significance Level	NS	NS	NS	NS	**
Interaction: D * T	D1-T1	21.08	33.44	12.36	0.00	1.00 ±0.00 e
	D1-T2	21.94	30.63	8.69	-3.67	12.72 ±4.39 e
	D1-T3	24.08	29.98	5.89	-6.46	88.25 ±14.82 d
	D1-T4	24.00	32.87	8.87	-3.49	11.20 ±3.29 e
	D2-T1	24.05	28.82	4.77	-7.59	192.39 ±35.07 a
	D2-T2	23.65	28.81	5.16	-7.20	146.74 ±17.22 b
	D2-T3	23.56	29.63	6.08	-6.28	77.70 ±5.02 d
	D2-T4	25.21	30.69	5.49	-6.87	116.93 ±11.48 c
	Significance Level	NS	NS	NS	NS	**

NS = Not statistically significant difference, **P<0.01. ^{a,b,c} means in the same row with different superscripts differ significantly at P<0.05.

Table 7 illustrates the notable reduction in TNFα gene expression under the standard feed treatment in comparison to the HELP feed treatment. Furthermore, all

interventions resulted in substantial reductions relative to the control treatment. The results indicated a significant decrease in favor of the T1, T2, and T4 intervention treatments compared to the rest. TNFα is one of the pro-inflammatory cytokines. It is a protein characterized by multiple functions responsible for various metabolic disorders, including obesity and abdominal fat deposition (4). Macrophages control the production and secretion of TNFα. Other groups of cells can participate in synthesizing this gene, such as T cells, monocytes, and smooth muscle cells (13). TNFα has many bodily functions, including stimulating apoptosis and lysis of cancer cells (15).

The decreased expression of the TNFα gene in the standard compared to the HELP diet may be attributed to the latter increasing oxidative stress on cells, negatively affecting the bird's metabolism (11). This, in turn, leads to increased gene expression of pro-inflammatory cytokines such as TNFα (28). The decreased expression of the addition and interaction factors may be attributed to the raisin juice byproducts content of phenolic compounds such as raisin juice byproducts and epicatechin. These possess powerful antioxidant properties that eliminate free radical damage by forming complexes with metal ions and withdrawing hydrogen from the hydroxyl group thereby eliminating free radical oxidation and preventing the development of singlet oxygen (23). Resveratrol eliminates oxidative stress by protecting DNA from oxidative damage (30).

Table 7: Effect of diet type and treatments in gold change of TNFα gene.

Factors		GAPDH	NRF2	DCT	DDCT	FOLDING
Diet: D	Normal: D1	22.78	28.33	5.550	-3.085	18.57 ±6.61 b
	High energy and low protein: D2	24.11	26.73	2.620	-6.015	67.13 ±8.35 a
	Significance Level	NS	NS	NS	NS	**
Treatment: T	Control: T1	24.61	28.11	3.510	-5.120	52.92 ±14.79 a
	RJ 2 % /kg –feed T2	22.79	27.04	4.240	-4.390	33.11 ±10.57 c
	RJ 4 % /kg –feed T3	23.82	26.86	3.045	-5.595	48.40 ±4.21 b
	RSV 500 mg/kg –feed: T4	22.56	28.10	5.545	-3.095	36.98 ±15.98 bc
	Significance Level	NS	NS	NS	NS	**
Interaction: D * T	D1-T1	21.08	29.72	8.64	0.00	1.00 ±0.00 d
	D1-T2	21.94	27.67	5.72	-2.91	7.54 ±0.85 d
	D1-T3	24.08	27.00	2.92	-5.72	52.62 ±9.71 c
	D1-T4	24.00	28.92	4.92	-3.71	13.13 ±2.08 d
	D2-T1	24.05	26.49	2.45	-6.19	72.96 ±8.36 b
	D2-T2	23.65	26.42	2.76	-5.87	58.68 ±6.97 bc
	D2-T3	23.56	26.73	3.17	-5.47	44.19 ±5.04 c
	D2-T4	25.21	27.31	2.10	-6.53	92.72 ±11.53 a
	Significance Level	NS	NS	NS	NS	**

NS = Not statistically significant difference, **P<0.01. ^{a,b,c} means in the same row with different superscripts differ significantly at P<0.05.

In regard to the effect of different treatments on histological changes in the liver, the results in the first treatment showed necrosis of liver tissue cells, with dilated blood vessels and infiltration of inflammatory cells, as indicated by the arrow. The second treatment showed inflammation of the blood vessels (Images C and D) representing the addition of raisin juice byproducts and resveratrol treatments. The results demonstrated

the liver tissue's safety from pathological injury and inflammation compared to the other experimental treatments. Image E shows inflammation of the lymphatic ducts with necrosis of tissue cells, congestion of blood vessels, and hemorrhage. Image F shows vascular congestion with hemosiderosis, a term used to describe excessive iron accumulation. Image G shows congested blood vessels with hemorrhage while Image H shows vasodilation with inflammatory cell accumulation around the vessels.

The improvement in liver tissue in the third and fourth treatments may be attributed to the raisin juice byproducts content of phenolic compounds and flavonoids. These are biologically active compounds that can enhance antioxidant capacity, increase resistance to oxidative stress, prevent platelet aggregation, and reduce inflammation, thus protecting liver tissue from damage. Resveratrol protects the liver from various diseases and damage due to its positive association with the activities of betatrophin and antioxidants. The deterioration of liver tissue in the fifth treatment may be attributed to nutritional stress resulting from increased energy and decreased protein in the feed, leading to increased liver fat and, consequently, free radicals, oxidative stress, and liver tissue damage.

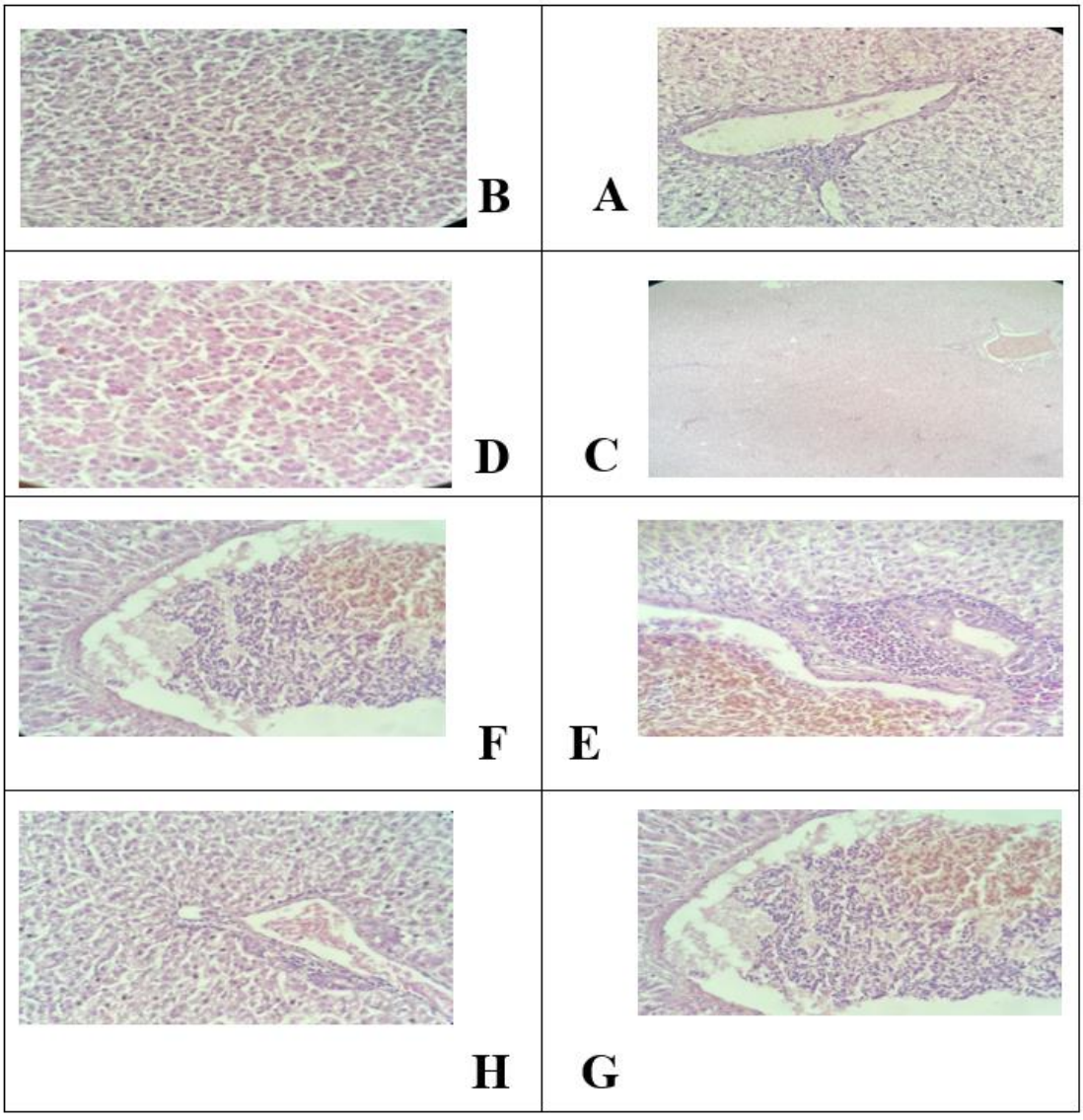

Figure 1: Effect of the different treatments on histological changes in the liver.

Liver tissue: **A** - first treatment (regular feed without supplementation or control group); **B** - second treatment (regular feed + RJ 2 % /kg feed); **C** - third treatment (regular feed + RJ 4 % /kg feed); **D** - fourth treatment (regular feed + 500 mg/kg resveratrol); **E** - fifth treatment (HELP feed without supplementation); **F** - sixth treatment (HELP feed + RJ 2 % /kg feed); **G** - seventh treatment (HELP feed + RJ 4 % /kg feed); and **H** - eighth treatment (HELP feed + 500 mg/kg resveratrol).

Conclusions

Supplementing raisin juice byproducts (RJ 2 % /kg feed and RJ 2 % /kg feed) and resveratrol (500 mg/kg feed) to the diets of the laying hens significantly improved production performance and the antioxidant status of GSH-PX, SOD, MDA, as well as the ALT, AST, and ALP oxidative enzymes in both T4 and T8. These diet supplementation treatments led to decreased NRF2 and TNFα gene expressions with improved hepatic protein status.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

O. K. Attallah: study conception and design; Th. T. Mohammed, S. M. Farhan and A. F. Al-Enzy: data collection; S. M. Abdulateef, H. M. Alnori and O. A. Saeed: analysis and interpretation of results; Th. T. Mohammed: draft manuscript preparation. All authors reviewed the results and approved the final version of the manuscript.

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References

1. Abbasi-Parizad, P., De Nisi, P., Scaglia, B., Scarafoni, A., Pilu, S., and Adani, F. (2021). Recovery of phenolic compounds from agro-industrial by-products: Evaluating antiradical activities and immunomodulatory properties. Food and Bioproducts Processing, 127: 338-348. https://doi.org/10.1016/j.fbp.2021.03.015.
2. Aditya, S., Ohh, S. J., Ahammed, M., and Lohakare, J. (2018). Supplementation of grape pomace (Vitis vinifera) in broiler diets and its effect on growth performance, apparent total tract digestibility of nutrients, blood profile, and meat quality. Animal Nutrition, 4(2): 210-214. https://doi.org/10.1016/j.aninu.2018.01.004.
3. Ahmed, A. Kh., Mehdi, N. Kh., Melak, S., and Peng, Z. (2025). Effect of selenium supplementation and expression of some genes on testis tissues in roosters. Anbar Journal of Agricultural Sciences, 23(1): 455-469. DOI: 10.32649/ajas.2025.154898.1464.
4. Al-Zghoul, M. B., and Saleh, K. M. M. (2020). Effects of thermal manipulation of eggs on the response of jejunal mucosae to posthatch chronic heat stress in broiler chickens. Poultry science, 99(5): 2727-2735. https://doi.org/10.1016/j.psj.2019.12.038.
5. Aslam, B., Hussain, A., Sindhu, Z. U. D., Nigar, S., Jan, I. U., Alrefaei, A. F., ... and Khan, R. U. (2023). Polyphenols-rich polyherbal mixture attenuates hepatorenal impairment, dyslipidaemia, oxidative stress and inflammation in alloxan-induced diabetic rats. Journal of Applied Animal Research, 51(1): 515-523. https://doi.org/10.1080/09712119.2023.2230754.
6. Attallah, O. K., Mohammed, T. T., and Al-Anbari, N. N. (2022). Effect of adding grape pomace and resveratrol on some physiological traits and gene expression to prevent hemorrhagic fatty liver syndrome in laying hens. In IOP Conference Series: Earth and Environmental Science, 1060(1): 012076. https://doi.org/10.1088/1755-1315/1060/1/012076.
7. Buege, J. A., and Aust, S. D. (1978). [30] Microsomal lipid peroxidation. In Methods in enzymology, 52: 302-310. https://doi.org/10.1016/S0076-6879(78)52032-6.
8. Dawod, M. S., and Al-Bayar, M. A. (2024). The effect of adding 25-hydroxycholecalciferol to the diet on some biochemical characteristics of Japanese quail. Anbar Journal of Agricultural Sciences, 22(2): 868-881. https://doi.org/10.32649/ajas.2024.142721.1071.
9. Duncan, D. B. (1955). Multiple range and multiple F tests. biometrics, 11(1): 1-42. https://doi.org/10.2307/3001478.
10. Gao, X., Liu, P., Wu, C., Wang, T., Liu, G., Cao, H., ... and Guo, X. (2019). Effects of fatty liver hemorrhagic syndrome on the AMP-activated protein kinase signaling pathway in laying hens. Poultry science, 98(5): 2201-2210. https://doi.org/10.3382/ps/pey586.
11. Gu, X., Xie, Z., Wang, Q., Liu, G., Qu, Y., Zhang, L., ... and Zhang, Q. (2009). Transcriptome profiling analysis reveals multiple modulatory effects of Ginkgo

biloba extract in the liver of rats on a high-fat diet. The FEBS Journal, 276(5): 1450-1458. https://doi.org/10.1111/j.1742-4658.2009.06886.x.

12. Hafeez, A., Hassni, S. F., Naz, S., Alonaizan, R., Al-Akeel, R. K., Sifa, D., ... and Ullah Khan, R. (2023). Impact of grape (Vitis vinifera) seed extract on egg production traits, nutrients digestability, lipid peroxidation and fertility of golden laying hens (Gallus gallus) during early stage of production. Veterinary Quarterly, 43(1): 1-7. https://doi.org/10.1080/01652176.2023.2262543.
13. Hirata, B. K., Cruz, M. M., De Sá, R. D., Farias, T. S., Machado, M. M., Bueno, A. A., ... and Telles, M. M. (2019). Potential anti-obesogenic effects of Ginkgo biloba observed in epididymal white adipose tissue of obese rats. Frontiers in Endocrinology, 10: 284. https://doi.org/10.3389/fendo.2019.00284.
14. Jonathan, O., Mnisi, C. M., Kumanda, C., and Mlambo, V. (2021). Effect of dietary red grape pomace on growth performance, hematology, serum biochemistry, and meat quality parameters in Hy-line Silver Brown cockerels. PloS One, 16(11): e0259630. https://doi.org/10.1371/journal.pone.0259630.
15. Karasawa, K., Tanigawa, K., Harada, A., and Yamashita, A. (2019). Transcriptional regulation of acyl-CoA: glycerol-sn-3-phosphate acyltransferases. International journal of molecular sciences, 20(4): 964. https://doi.org/10.3390/ijms20040964.
16. Li, L., Hai, J., Li, Z., Zhang, Y., Peng, H., Li, K., and Weng, X. (2014). Resveratrol modulates autophagy and NF-κB activity in a murine model for treating non-alcoholic fatty liver disease. Food and Chemical Toxicology, 63: 166-173. https://doi.org/10.1016/j.fct.2013.08.036.
17. Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., and Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cellular and molecular life sciences, 73: 3221-3247. https://doi.org/10.1007/s00018-016-2223-0.
18. Marklund, S., and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European journal of biochemistry, 47(3): 469-474.
19. Marruchella, G., Mosca, F., Hattab, J., Trachtman, A. R., and Tiscar, P. G. (2021). In vitro effects of non-steroidal anti-inflammatory drugs (Meloxicam and Flunixin Meglumine) and phytochemical (Harpagoside) on the respiratory burst of porcine polymorphonuclear neutrophils (PMNs). Ger J Vet Res, 1(2): 8-12. https://doi.org/10.51585/gjvr.2021.2.0008.
20. Niture, S. K., Khatri, R., and Jaiswal, A. K. (2014). Regulation of Nrf2—an update. Free Radical Biology and Medicine, 66: 36-44. https://doi.org/10.1016/j.freeradbiomed.2013.02.008.
21. Raut, A. R., Bhole, S. S., Mote, G. V., Prathapan, K., and Khot, J. A. (2024). Comparative study on extraction of concentrate from raisins and its application in jelly. Acta Scientiae, 7(2): 630-647.
22. Reitman, S., and Frankel, S. (1957). A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. American journal of clinical pathology, 28(1): 56-63. https://doi.org/10.1093/ajcp/28.1.56.

23. Romero, C., Arija, I., Viveros, A., and Chamorro, S. (2022). Productive performance, egg quality and yolk lipid oxidation in laying hens fed diets including grape pomace or grape extract. Animals, 12(9): 1076. https://doi.org/10.3390/ani12091076.
24. SAS. (2018). Statistical Analysis System, User's Guide (Version 9.6). SAS Institute Inc.
25. Sun, P., Lu, Y., Cheng, H., and Song, D. (2018). The effect of grape seed extract and yeast culture on both cholesterol content of egg yolk and performance of laying hens. Journal of Applied Poultry Research, 27(4): 564-569. https://doi.org/10.3382/japr/pfy035.
26. Trott, K. A., Giannitti, F., Rimoldi, G., Hill, A., Woods, L., Barr, B., ... and Mete, A. (2014). Fatty liver hemorrhagic syndrome in the backyard chicken: a retrospective histopathologic case series. Veterinary pathology, 51(4): 787-795. https://doi.org/10.1177/0300985813503569.
27. Wang, J., Jia, R., Gong, H., Celi, P., Zhuo, Y., Ding, X., ... and Zhang, K. (2021). The effect of oxidative stress on the chicken ovary: Involvement of microbiota and melatonin interventions. Antioxidants, 10(9): 1422. https://doi.org/10.3390/antiox10091422.
28. Wang, J., Qiu, L., Gong, H., Celi, P., Yan, L., Ding, X., ... and Zhang, K. (2020). Effect of dietary 25-hydroxycholecalciferol supplementation and high stocking density on performance, egg quality, and tibia quality in laying hens. Poultry Science, 99(5): 2608-2615. https://doi.org/10.1016/j.psj.2019.12.054.
29. Wang, X., Xing*, C., Yang, F., Zhou, S., Li, G., Zhang, C., ... and Hu, G. (2020). Abnormal expression of liver autophagy and apoptosis-related mRNA in fatty liver haemorrhagic syndrome and improvement function of resveratrol in laying hens. Avian Pathology, 49(2): 171-178. https://doi.org/10.1080/03079457.2019.1698712.
30. Xing, C., Wang, Y., Dai, X., Yang, F., Luo, J., Liu, P., ... and Hu, G. (2020). The protective effects of resveratrol on antioxidant function and the mRNA expression of inflammatory cytokines in the ovaries of hens with fatty liver hemorrhagic syndrome. Poultry Science, 99(2): 1019-1027. https://doi.org/10.1016/j.psj.2019.10.009.
31. Zhang, C., Kang, X., Zhang, T., and Huang, J. (2019). Positive effects of resveratrol on egg-laying ability, egg quality, and antioxidant activity in hens. Journal of Applied Poultry Research, 28(4): 1099-1105. https://doi.org/10.3382/japr/pfz072.
32. Zhang, S., Yan, Y., Meng, G., Zhang, Q., Liu, L., Wu, H., ... and Niu, K. (2023). Protein foods from animal sources and risk of nonalcoholic fatty liver disease in representative cohorts from North and South China. Journal of internal medicine, 293(3): 340-353. https://doi.org/10.1111/joim.13586.
33. Zhang, W., Wang, D., Hao, E., Shi, L., Chen, H., Zhang, W., and Chen, Y. (2024). Positive effects and mechanism of mulberry leaf extract on alleviating fatty liver hemorrhagic syndrome in laying hens. Poultry Science, 103(9): 103998. https://doi.org/10.1016/j.psj.2024.103998.

34. Zhao, X., Zhang, Y., He, W., Wei, Y., Han, S., Xia, L., ... and Cui, C. (2022). Effects of small peptide supplementation on growth performance, intestinal barrier of laying hens during the brooding and growing periods. Frontiers in Immunology, 13: 925256. https://doi.org/10.3389/fimmu.2022.925256.