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Effects of phytogenic feed on productive performance, egg quality, antioxidant activity and lipid metabolism of laying hens

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ABSTRACT. The objective of this study was to investigate the effect of different proportions of *Moringa oleifera* leaf powder (MOLP) and mulberry leaf powder (MLP) on the performance of laying hens. The use of MOLP and MLP as animal feed can meet the nutritional requirements of livestock and improve antioxidant status. Combinations of different feeds could produce a synergistic effect in animals. Two hundred and ten birds were randomly divided into three groups. The control group was fed a basal diet and the treatment groups received a basal diet supplemented with 2.5% MOLP + 2.5% MLP and 5% MOLP + 2.5% MLP, respectively. The results showed that the supplementation of 5% MOLP + 2.5% MLP significantly decreased the laying rate, albumen height and Haugh unit. Yolk colours were significantly more intense after MOLP and MLP supplementation. Significant decreases in abdominal fat index were observed in the 5% MOLP + 2.5% MLP group. The levels of superoxide dismutase 2 (*SOD2*) mRNA expression were significantly increased, while those of apolipoprotein B (*APOB*) were significantly reduced, as determined by qRT-PCR analysis. The combined administration of MOLP and MLP could have regulated the antioxidant status and lipid metabolism by affecting *SOD2* and *APOB* gene expression. A supplementation level of 2.5% MOLP + 2.5% MLP was recommended.

Introduction

The concept of "phytogenic feed additives" refers to natural medicinal products derived from herbs that are used in livestock nutrition to enhance performance (Pliego et al., 2020). *Moringa oleifera* and mulberry can be applied as a source of plant protein for livestock and poultry (Wu and Li, 2006; Wang et al., 2018). A series of studies have been carried out concerning the effect of *M. oleifera* or mulberry in poultry production. Lu et al. (2016) proposed that supplementation with 5% *M. oleifera* leaf (MOL) could improve laying performance and egg quality of Hy-Line Grey hens. Cui et al. (2018) stated that 1.56% MOL supplementation improved muscle quality, including polyunsaturated fatty acid contents, oxidative stability and breast muscle colour in Arbor Acres broilers. Ashour et al. (2020) findings indicated that the inclusion of *M. oleifera* seeds in the diet of Japanese quail significantly increased egg production and quality, while reducing certain blood biochemical components. A study in pigs showed that supplementation levels of mulberry leaf powder lower than 12% improved meat quality (Liu et al., 2019). The application of mulberry was also suggested to improve performance, quality of products and oxidant activity in poultry. Olteanu et al. (2015) found that dietary supplementation

of mulberry leaves could improve breast meat quality in broilers. A study by Lin et al. (2017) suggested that mulberry leaf extract-based dietary supplementation modulated the antioxidant activity in laying hens. Collectively, the literature shows that *M. oleifera* or mulberry has been widely used in poultry as a feed or feed additive, and have been reported to significantly improve performance and product quality.

Addition of various feed supplement combinations to the basal diet can induce a synergistic effect in animals. A study by Rofiq and Gorgulu (2014) demonstrated that the combination of cloves and orange peel in a dairy total mixed ration played an antagonistic role in reducing digestion. Martono et al. (2016) reported that combining different feed supplements could increase feed efficiency in dairy cattle. However, no relevant literature has been found regarding the combined effect of *M. oleifera* and mulberry supplementation. The purpose of this study was to determine the appropriate ratio of *M. oleifera* and mulberry in the diet of Chinese local chicken breeds and to provide a reference for utilisation of these plants in poultry.

Material and methods

This study was performed in strict accordance with the the guidelines established by the Ministry of Agriculture of China. All procedures were approved by the Institutional Animal Care and Use Committee of Jiangsu University of Science and Technology (SYXK(Su) 2016-2020).

M. oleifera leaf powder (MOLP) was obtained from Yunnan Dayaoshan Trading Co., Ltd. (Yunnan, China), and mulberry leaf powder (MLP) was obtained from Danyang Tianyuan Shengshu Ecological Park Co., Ltd. (Zhenjiang, JS, China). M. oleifera and mulberry leaves were picked and dried naturally in a sunny and ventilated place. The dried leaves were ground using an FFC45A pulveriser (Yuxing Factory, Qingdao, SD, China), and stored at ambient temperature (15–25 °C) before mixing with diets. The chemical compositions of M. oleifera and mulberry leaves were determined according to the Official Methods of Analysis (AOAC International, 2012). Metabolisable energy was calculated according to the United States Department of Agriculture (USDA) database (https://ndb.nal.usda.gov/ndb/) based on the Atwater factor system. Total phenolics and total flavonoids were determined according to Meda et al. (2005). The basal diet was a corn-soybean meal feed provided by China Oil and Foodstuffs Corporation. The ingredients and chemical composition of the feed are shown in Table 1.

Table 1. Proportion (%) of ingredients used in the formulation of experimental diets

Ingredient, %	Groups		
	Control	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5
Corn	64.420	63.092	62.605
Soybean meal	23.520	21.440	19.975
Shell powder	6.700	6.700	6.700
NaCl	0.300	0.300	0.300
Calcium hydrogen phosphate	0.878	0.810	0.780
Limestone powder	2.107	1.75	1.635
Zeolite powder	1.632	0.459	0.053
Choline chloride	0.170	0.170	0.170
Methionine	0.116	0.122	0.125
Trace mineral premix[1]	0.100	0.100	0.100
Vitamin premix	0.050	0.050	0.050
Phytase	0.007	0.007	0.007
Total %	100	100	100
Nutrient levels[2]	Control	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5
Metabolisable energy, MJ/kg	11.0874	11.0870	11.0872
Crude protein, %	16.00	16.00	16.00
Crude fiber, %	2.418	3.378	3.765
Lysine, %	0.785	0.785	0.793
Methionine, %	0.370	0.370	0.370
Calcium, % DM	3.35	3.35	3.35
Phosphorus, % DM	0.32	0.32	0.32

Control – basal diet, MOLP2.5 + MLP2.5 – basal diet supplemented with 2.5% moringa leaf powder and 2.5% mulberry leaf powder, MOLP5 + MLP2.5 – basal diet supplemented with 5% MOLP and 2.5% MLP; CHP – calcium hydrogen phosphate, DM – dry matter; [1] premix provided per kilogram of diet: IU: vit. A (retinyl palmitate) 7 715, vit. E 8.8; international chick units: vit. D_3 (cholecalciferol) 2 755; mg: vit. K (menadione sodium bisulfate complex) 2.2, vit. B_{12} (cobalamin) 0.01, menadione (menadione sodium bisulfate complex) 0.18, riboflavin 4.41, pantothenic acid (D-Calcium pantothenate) 5.51, niacin 19.8, folic acid 0.28, pyridoxine (pyridoxine hydrochloride) 0.55, Mn (manganese sulfate) 50, Fe (ferrous sulfate) 25, Cu (copper sulfate) 2.5, Zn (zinc sulfate) 50, iodine (calcium iodate) 1.0, selenium (sodium selenite) 0.15; [2] calculated value

Our experimental animals were provided by the Jiangsu Institute of Poultry Science. Two hundred and ten Chinese local strain chickens, aged 37 weeks, were randomly allocated to 3 groups, each group included 5 replicates, with 14 hens per replicate. Hens in the control group were fed the basal diet, while those in the test groups were fed the basal diet supplemented with 2.5% moringa leaf powder and 2.5% mulberry leaf powder (MOLP2.5 + MLP2.5), and 5% MOLP and 2.5% MLP (MOLP5 + MLP2.5), respectively. The experiments lasted 7 weeks, including 1 week of adaptation. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Jiangsu University of Science and Technology. All hens were raised in three-tiered stair-step caging.

Each replicate consisted of 14 cages with hens that were housed in the upper two layers of cages. Hens were reared with water and food ad libitum, with the photoperiod regime of 16L:8D throughout the study.

Daily egg production was monitored during the trial; the average egg weight and feed intake were recorded weekly. The laying rate was expressed as an average hen-day production, calculated from the total number of eggs divided by the total number of days. The intake of feeds was recorded weekly, and their conversion rate was determined.

Freshly laid eggs were collected at 2, 4, and 6 weeks. The internal and external egg quality of 30 randomly selected eggs per group (6 eggs/replicate) was assessed. Eggs were stored at room temperature before measurements. The length and width of the eggs were measured using an electronic digital calliper, and the egg shape index was calculated (length/width × 100). Eggshell thickness was measured using an ESTG-1 eggshell thickness tester (ORKA Food Technology Ltd., Ramat HaSharon, Israel) at the blunt, equatorial, and sharp regions to obtain an average value. Eggshell colour was determined using a CM-2300D spectrophotometer (Konica Minolta, Inc., Tokyo, Japan) and the following parameters were recorded: lightness of eggshell (L*), redness of eggshell (a*) and yellowness of eggshell (b*). Eggshell strength was evaluated using an EFR-01 EggShell Force Gauge (ORKA Food Technology Ltd., Ramat HaSharon, Israel). Egg weight, albumen height, Haugh unit (HU), and yolk colour were measured using an EA-01 Egg Multi Tester (ORKA Food Technology Ltd., Ramat HaSharon, Israel). Subsequently, the yolk weight was measured, and the yolk rate was calculated. Eggshell weight was determined after natural drying.

Thirty hens (2 hens/per replicate, 10 hens per group) were randomly selected after 12 h of fasting at the end of week 6. Blood was collected from the wing vein and subsequently serum was obtained by centrifuging the blood at 4000 rpm for 10 min and stored at −20 °C. The hens were then humanely sacrificed by intravenous barbiturate overdose, followed by cervical dislocation. Internal organs, including heart, liver, spleen, lung, kidney, and abdominal fat were removed and measured, and the internal organ index was calculated using the following formula: internal organ index, % = (internal organ weight/body weight) × 100. Liver tissues were collected and stored at −80 °C until assayed for antioxidant or lipid indices and their related gene expression profiles.

Liver samples were homogenised in saline to obtain a 10% homogenate with 0.9% sodium chloride buffer in tubes placed on ice, and subsequently centrifuged at 4000 rpm at 4 °C C for 10 min. The serum and liver supernatants were used to determine malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), glutathione (GSH), triglyceride (TG), total cholesterol (T-CHO), high-density lipoprotein (HDLC) and low-density lipoprotein (LDLC) levels by ELISA, with commercial kits purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, JS, China.

Total liver RNA was extracted using the Trizol reagent (TaKaRa Biotechnology, Dalian, LN, China). Quality and integrity of RNA was assessed using a Nanodrop ND-2000c spectrophotometer (Thermo Scientific, Camden, NJ, USA) and gel electrophoresis. The OD260/OD280 ratio ranged from 1.8 to 2.1, indicating that RNA could be used in the next experiment. Reverse transcription was carried out according to the Takara reverse transcription kit protocol (Perfect Real Time, PrimeScriP™ TaKaRa Biotechnology, Dalian, LN, China). The reverse transcription reaction conditions were as follows: reaction at 37 °C for 15 min, denaturation at 85 °C for 15 s, and finally cooling to 4 °C. Real-time quantitative polymerase chain reaction was carried out using the SYBR Premix Ex Taq II kit (TaKaRa Biotechnology, Dalian, LN, China) in an ABI 7300 (Applied Biosystems, Foster City, CA, USA). The reaction mixture (20 µl) contained 10 µl of SYBR Premix Ex Taq buffer, 0.4 µl of each of primer and ROX, 1 µl of cDNA template, and 7.8 µl of distilled water. The real-time polymerase chain reaction cycling conditions were as follows: 30 s at 95 °C, 40 cycles at 95 °C for 5 s, and 60 °C for 31 s. Relative mRNA expression was determined using actin, beta (*ACTB*) as an internal reference gene. The significance and correlation of quantitative results were analysed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). All gene accession numbers are listed in Table 2. The *ACTB* primer sequences was designed and maintained in our laboratory. Other primers were designed using Primer 3.0 to amplify an intron-spanning region.

All data were analysed using SPSS 20.0 statistical software and are represented as arithmetic means and standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was used to evaluate differences between groups. Data were assumed to be statistically significant at $P < 0.05$.

Table 2. Primer sequences used in the current study

Gene	Primer	Primer sequence, 5'→3'	Product size, bp	Tm, °C	Accession no.
ACTB	Forward	CAGCCATCTTTCTTGGGTAT	169	59.1	NM_205518.1
	Reverse	CTGTGATCTCCTTCTGCATCC		59.1	
CAT	Forward	TGCAAGGCGAAAGTGTTTGA	158	58.9	NM_001031215.2
	Reverse	CCCACAAGATCCCAGTTACCT		59.1	
NRF2	Forward	AACGCACCAAAGAAAGACCC	147	58.9	NM_205117.1
	Reverse	ACTGAACTGCTCCTTCGACA		58.9	
SOD1	Forward	TACCGGCTTGTCTGATGGAG	172	59.1	NM_205064.1
	Reverse	TCCTCCCTTTGCAGTCACAT		58.9	
SOD2	Forward	AGAGGAGAAATACAAAGAGGCG	245	57.8	NM_204211.1
	Reverse	AGCCTGATCCTTGAACACCA		58.9	
ACC	Forward	AGACGAGCTCCTTGGTGAAA	217	58.9	XM_046929959.1
	Reverse	GAAGCCACAGTGAAATCCCG		59.2	
APOB	Forward	ACGGGAACAGCAGATTCTCA	225	59	NM_001044633.2
	Reverse	TGTTCCATCCTGAGTGCTGA		58.6	

ACTB – actin beta, *CAT* – catalase, *NRF2* – nuclear factor erythroid 2-related factor 2, *SOD* – superoxide dismutase, *ACC* – acetyl coenzyme A carboxylase, *APOB* – apolipoprotein B

Results and discussion

The chemical composition of *M. oleifera* leaves and mulberry leaves on a dry matter basis is summarised in Table 3.

Table 3. Chemical composition of *Moringa oleifera* and mulberry leaves (on dry matter basis)

Item, %	*M. oleifera* leaf	Mulberry leaf
Metabolic energy, MJ/kg	7.96	7.15
Crude protein	27.60	13.79
Crude fibre	19.26	24.89
Ether extract	5.76	1.98
Crude ash	6.19	5.75
Calcium	2.20	4.0
Phosphorus	0.40	0.45
Lysine	1.83	0.65
Methionine	0.25	0.13
Phenolic	44.37 GAE mg/g	30.57 GAE mg/g
Total flavonoids	23.78 QE mg/g	55.42 QE mg/g

GAE – gallic acid equivalents, QE – quercetin equivalents

No sick or dead chickens were found in any of the groups during the experiment. As shown in Table 4, the laying rate of MOLP5 + MLP2.5 group decreased significantly ($P < 0.05$) compared to the control group and the MOLP2.5 + MLP2.5 group. There was no significant difference in the average egg weight between the groups ($P > 0.05$). The average daily feed intake was 91.22, 84.62 and 82.07 g per hen in the control, MOLP2.5 + MLP2.5, and MOLP5 + MLP2.5 groups, respectively. The feed conversion was 2.51, 2.42 and 2.47 in the control, MOLP2.5 + MLP2.5, and MOLP5 + MLP2.5 groups, respectively. A significant difference in feed conversion was observed between the groups ($P < 0.05$).

Table 4. Effect of *Moringa oleifera* leaf and mulberry leaf powder on productive performance of laying hens

Item	Supplementation, %			SEM	*P*-value
	0	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5		
Laying rate, %	78.91^{a}	76.94^{a}	73.64^{b}	0.618	0.002
Average egg weight, g	46.06	45.61	44.93	0.981	0.342
Average daily feed intake, g/bird/day	91.22^{a}	84.62^{b}	82.07^{b}	1.127	<0.001
Feed conversion, g of feed/g of egg	2.51^{a}	2.42^{c}	2.47^{b}	0.037	<0.001

MOLP2.5 + MLP2.5 – basal diet supplemented with 2.5% *M. oleifera* leaf powder and 2.5% mulberry leaf powder, MOLP5 + MLP2.5 – basal diet supplemented with 5% MOLP and 2.5% MLP; SEM – standard error of the mean; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

Egg weight, eggshell colour, eggshell strength, eggshell weight, eggshell thickness, yolk weight and yolk rate did not differ significantly between the groups at three time points (Table 5). Notably, the yolk colour value significantly increased with raising MOLP and MLP supplementation levels in the diets at week 3 ($P < 0.05$). However, albumen height and HU decreased with the supplementation of MOLP and MLP; there was also a significant decrease in albumen height in the MOLP5 + MLP2.5 group at weeks 4 and 6 ($P < 0.05$). With respect to the eggshell index, a significant difference between the control group and the treatment groups was recorded only at week 4 ($P < 0.05$).

Table 6 shows that there were no significant differences between the groups in terms of the serum antioxidant index and lipid indicators such as SOD, MDA, T-AOC, T-CHO, HDLC, TG, and LDLC levels ($P > 0.05$).

Table 5. Effect of *Moringa oleifera* leaf and mulberry leaf powder supplementation on egg quality

Item		Supplementation, %			SEM	*P*-value
		0	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5		
2 week						
egg weight,g		45.59	44.37	44.69	0.33	0.058
eggshell colour	L	75.67	77.08	76.23	0.43	0.406
	a	9.12	8.73	8.16	0.26	0.064
	b	20.23	18.58	20.25	0.39	0.135
eggshell weight g		4.40	4.48	4.34	0.05	0.545
eggshell thickness, mm		0.39	0.38	0.40	0.03	0.058
yolk weight, g		14.62	14.15	14.28	0.13	0.308
eggshell strength, kgf		3.49	3.75	3.76	0.09	0.405
albumen height, mm		3.93	3.81	4.03	0.09	0.656
Haugh unit		64.64	64.04	66.25	0.91	0.573
yolk colour		7.42[a]	9.48[b]	9.95[c]	0.15	<0.001
eggshell index		1.36	1.33	1.33	0.005	0.123
yolk rate		0.31	0.32	0.32	0.01	<0.001
4 week						
egg weight, g		46.34	45.74	44.99	0.35	0.290
eggshell colour	L	74.24	75.42	75.22	0.40	0.437
	a	9.24	8.13	8.47	0.27	0.241
	b	21.30	19.20	20.18	0.35	0.142
eggshell weight, g		4.56	4.55	4.53	0.04	0.942
eggshell thickness, mm		0.40	0.40	0.39	0.004	0.689
yolk weight, g		14.67	14.04	14.76	0.15	0.098
eggshell strength, kgf		3.82	4.09	4.01	0.07	0.306
albumen height, mm		4.22	4.13	3.91	0.08	0.508
Haugh unit		69.64[a]	69.55[a]	65.40[b]	2.43	0.027
yolk colour		7.73[a]	9.91[b]	10.47[b]	0.17	<0.001
eggshell index		1.34[a]	1.30[b]	1.31[b]	0.005	0.014
yolk rate		0.32	0.31	0.32	0.003	0.101
6 week						
egg weight, g		45.68	46.25	45.58	0.35	0.695
eggshell colour	L	74.09	73.63	74.64	0.36	0.519
	a	9.09	9.23	8.79	0.24	0.744
	b	21.11	20.94	20.13	0.34	0.460
eggshell weight, g		4.30	4.52	v4.35	0.06	0.256
eggshell thickness, mm		0.36	0.37	0.37	0.003	0.605
yolk weight, g		14.71	14.85	14.73	0.13	0.890
eggshell strength, kgf		3.52	3.77	3.72	0.07	0.375
albumen height, mm		4.23[a]	4.24[a]	3.74[b]	0.08	0.011
Haugh unit		69.17[a]	67.74[a]	62.74[b]	0.77	0.001
yolk colour		7.27[a]	9.92[b]	10.70[c]	0.20	<0.001
eggshell index		1.31	1.31	1.29	0.005	0.093
yolk rate		0.32	0.32	0.32	0.002	0.943

MOLP2.5 + MLP2.5 – basal diet supplemented with 2.5% *M. oleifera* leaf powder and 2.5% mulberry leaf powder, MOLP5 + MLP2.5 – basal diet supplemented with 5% MOLP and 2.5% MLP; SEM – standard error of the mean; [abc] – means within a row with different superscripts are significantly different at $P < 0.05$

As presented in Table 7, no significant effects on antioxidant activity in the liver were detected in any of the groups ($P > 0.05$). There was no significant difference in TG, T-CHO, HDLC and LDLC lipid indices of between the groups.

Table 6. Effect of *Moringa oleifera* leaf and mulberry leaf on antioxidant and lipid indicators in hen serum

Item	Supplementation, %			SEM	*P*-value
	0	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5		
MDA, nmol/ml	12.39	12.31	12.05	0.242	0.227
SOD, U/ml	16.04	15.79	16.12	0.36	0.948
T-AOC, U/ml	0.54	0.54	0.57	0.03	0.913
GSH, U/ml	15.11	11.19	14.10	1.60	0.602
TG, nmol/gprot	14.14	13.60	10.42	0.701	0.06
T-CHO, nmol/gprot	5.28	4.72	4.45	0.26	0.44
HDLC, nmol/gprot	2.47	2.08	1.73	0.18	0.266
LDLC, nmol/gprot	0.86	0.77	0.61	0.046	0.077
Glucose, nmol/gprot	18.01	16.69	17.92	0.701	0.715

MOLP2.5 + MLP2.5 – basal diet supplemented with 2.5% *M. oleifera* leaf powder and 2.5% mulberry leaf powder, MOLP5 + MLP2.5 – basal diet supplemented with 5% MOLP and 2.5% MLP; MDA – malondialdehyde, SOD – superoxide dismutase, T-AOC – total antioxidant capacity, GSH – glutathione, TG – triacylglycerol, T-CHO – total cholesterol, HDLC – high-density lipoprotein, LDLC – low-density lipoprotein, SEM – standard error of the mean; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$

Table 7. Effect of *Moringa oleifera* leaf and mulberry leaf on antioxidant capacity and lipid liver indices in hens

Item	Supplementation, %			SEM	*P*-value
	0	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5		
MDA, nmol/ml	4.72	4.38	4.97	0.34	0.801
SOD, U/ml	9.37	10.56	9.45	0.73	0.766
T-AOC, U/ml	3.22	2.96	2.95	0.11	0.515
GSH, U/ml	123.45	124.70	140.41	6.45	0.519
TG, nmol/gprot	4.70	4.74	3.42	0.30	0.123
T-CHO, nmol/gprot	0.76	0.65	0.59	0.032	0.078
HDLC, nmol/gprot	0.08	0.06	0.06	0.006	0.288
LDLC, nmol/gprot	0.41	0.38	0.38	0.012	0.641

MOLP2.5 + MLP2.5 – basal diet supplemented with 2.5% *M. oleifera* leaf powder and 2.5% mulberry leaf powder, MOLP5 + MLP2.5 – basal diet supplemented with 5% MOLP and 2.5% MLP; MDA – malondialdehyde, SOD – superoxide dismutase, T-AOC – total antioxidant capacity, GSH – glutathione, TG – triacylglycerol, T-CHO – total cholesterol, HDLC – high-density lipoprotein, LDLC – low-density lipoprotein, SEM – standard error of the mean; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$

The expression levels of catalase (*CAT*), nuclear factor erythroid 2-related factor 2 (*NRF2*), *SOD1, SOD2,* acetyl coenzyme A carboxylase (*ACC*) and apolipoprotein B (*APOB*) mRNA in the liver are shown in Figure 1. *CAT, NRF2, SOD1* and *ACC* mRNA showed no significant differences between the groups. Treatment with MOLP and MLP significantly reduced the *APOB* mRNA expression levels ($P < 0.05$), and significantly increased the expression level of *SOD2* mRNA.

As shown in Table 8, dietary supplementation with MOLP and MLP had no significant effect on the heart, liver, spleen, lung, kidney, muscular stomach, and glandular stomach indices ($P > 0.05$).

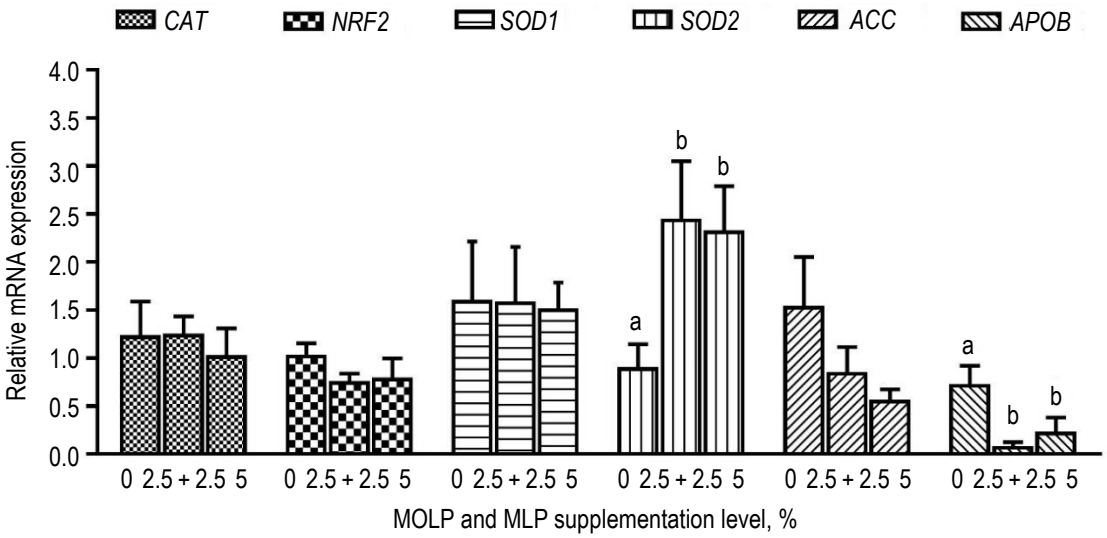

Figure 1. Expression levels of antioxidant- and lipid-related genes after dietary supplementation with *Moringa oleifera* leaves (MOLP) and mulberry leaves (MLP)

CAT – catalase, *NRF2* – nuclear factor erythroid 2-related factor 2, *SOD* – superoxide dismutase, *ACC* – acetyl coenzyme A carboxylase, *APOB* – apolipoprotein B; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$

Table 8. Effect of *Moringa oleifera* leaves and mulberry leaves on hen internal organ indices

Internal organ index, %	Supplementation, %			SEM	*P*-value
	0	MOLP2.5 + MLP2.5	MOLP5 + MLP2.5		
Heart	0.530	0.446	0.518	0.016	0.053
Liver	1.939	1.873	1.880	0.041	0.785
Spleen	0.110	0.087	0.102	0.004	0.065
Lung	0.400	0.394	0.441	0.013	0.306
Kidney	0.480	0.465	0.464	0.019	0.936
Abdominal adipose	5.50[a]	3.73[a]	2.95[b]	0.412	<0.001
Muscular stomach	0.364	0.361	0.363	0.007	0.984
Glandular stomach	0.200	0.211	0.206	0.008	0.931

MOLP2.5 + MLP2.5 – basal diet supplemented with 2.5% *M. oleifera* leaf powder and 2.5% mulberry leaf powder, MOLP5 + MLP2.5 – basal diet supplemented with 5% MOLP and 2.5% MLP; SEM – standard error of the mean; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$

The abdominal adipose index was significantly reduced in the MOLP5 + MLP2.5 group compared to the control group and the MOLP2.5 + MLP2.5 group ($P < 0.05$).

The aim of our study was to search for an appropriate supplementation ratio of MOLP and MLP in Chinese local strain chickens. Our previous studies showed that the optimal supplementation level of MLP should be less than 4% in the basal diet of Yangzhou goose (Zhao et al., 2019) and Blue eggshell chickens (Wu et al., 2014). Considering that the chicken used in the current experiment were a local Chinese breed, we set the supplemented MLP level to 2.5%. Previous studies showed that a high supplementation level of *M. oleifera* leaves caused side effects in hen laying performance (Lu et al., 2016; Cui et al., 2018); therefore, the final highest total supplementation level of MOLP and MLP in the basal diet was set to 7.5%. Our study demonstrated that MOLP and MLP proportions affected the productive performance of the local Chinese strain. Supplementation with 5% MOLP and 2.5% MLP caused side effects associated with laying performance, which was consistent with previous studies (Lu et al., 2016), which showed that the higher MOLP supplementation in hens, the greater the adverse effect. A study by Cui et al. (2018) reported that the recommended MOLP supplementation to broiler feed was 1.56%. Fibre content in MOLP and MLP is 19.26 and 24.89%, respectively. Generally, the dietary fibre is considered a diluent of poultry diet and the optimum supplementation should not exceed 3% in broiler feed (Jha et al., 2019). However, dietary fibre has also been proven to increase gizzard weight, amylase activity, and bile acid, which are beneficial for intestinal health (Mahmood and Guo, 2020). A healthy gut indirectly affects the laying performance by saving host's energy, which translates into increased egg production (Diaz Carrasco et al., 2019). In the current study, the MOLP2.5 + MLP2.5 group had increased feed conversion, without any adverse effects on laying performance. Therefore, supplementing feed with an appropriate level of MOLP and MLP could be helpful in supporting bird health and productivity.

Yolk colour was significantly intensified in both the MOLP2.5 + MLP2.5 and MOLP5 + MLP2.5

groups, which was consistent with previous works (Abou-Elezz Fouad Mohammed et al., 2012; N'Nanle et al., 2020). Yolk colour is mainly dependent on α-carotene, β-carotene, lutein and carotenoids (Yin et al., 2014), and influenced by many factors such as breed, age, management and feed, the latter having the greatest impact. Both *Moringa oleifera* and mulberry are rich in β-carotene, and its content in leaves has been estimated at 13.48–18.50 mg/100 g (Anwar et al., 2007; Falowo et al., 2018) and 7.44 mg/100 g (Yang et al., 2019), respectively. Eggs with a darker yolk colour are highly popular among Chinese consumers, indicating that MOLP and MLP supplementation can have a beneficial effect on yolk colour and egg market.

Of all lipid metabolism trends, MOLP and MLP supplementation decreased lipid index values. Furthermore, MOLP and MLP administration significantly decreased *APOB* mRNA expression levels. A report by Alnidawi et al. (2016) showed that the supplementation of *M. oleifera* decreased chicken serum T-CHO, TG, HDL and LDL levels. Krauss et al. (2004) reported that certain antioxidant substances could reduce the breakdown of *APOB*. In chicken, *APOB* plays a role in lipid transport to the follicle for yolk deposition (Nimpf et al., 1988). The obtained values of serum and liver lipid indices showed that MOLP and MLP treatment reduced these markers, but did not reach statistical significance. A longer experimental observation period may be required in the future. It has been verified that plant-derived feed can activate the *SOD2* signalling pathway to eliminate reactive oxygen species (ROS). Our study showed that supplementing different ratios of MOLP and MLP had no significant effect on antioxidant enzyme activities in serum and liver. However, MOLP and MLP treatment significantly increased *SOD2* mRNA expression level in the liver, while only slight increasing *SOD2* activity in this organ. *SOD2* has been shown to play a role in eliminating ROS under oxidative stress and has been implicated in many downstream signalling pathways, such as *FOXO3*, *SIRT3*, and *STAT* (Kim et al., 2017; Li et al., 2020). The reason for the difference between oxidative activity and mRNA expression may be the short supplementation time, which did not affect the synthesis of antioxidant enzymes. Further treatment studies with long-term MOLP and MLP supplementation are therefore recommended.

A study by Wen et al. (2020) showed that flavonoid-rich mulberry leaf could prevent ROS production and upregulate the expression of antioxidant-related genes, including *SOD2* and *NRF2*. A report concerning *M. oleifera* leaves indicated that phenolic-rich leaf tissue had potential antioxidant activity and provided protection against oxidative damage (Sreelatha and Padma, 2009). A series of works on nutrition demonstrated that *M. oleifera* largely contributed to the proper antioxidant status in different animal species (Verma et al., 2009; Oinam et al., 2012; Abdulkadir et al., 2018). Previous studies analysed the combination of mulberry and other phytogenic additives and suggested that the combined administration provided better outcomes. Another study showed that combining 30% mulberry leaf meal and 0.4% bamboo charcoal additive improved blood lipid metabolism and antioxidant activity in juvenile tilapia (Miao et al., 2020). The ratio of mulberry leaf extract to mulberry fruit of 2:1 in the meal fed to obese mice alleviated obesity and obesity-related metabolic stressors by reducing oxidative stress (Lim et al., 2013; Nova et al., 2020).

Conclusions

From the findings of our and previous studies, it can be concluded that the combination of MOLP and MLP improves antioxidant status and exerts no adverse effect on productive performance during late laying peak period in hens.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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