

Article

Effects of Degossypolized Cottonseed Protein on the Laying Performance, Egg Quality, Blood Indexes, Gossypol Residue, Liver and Uterine Histopathological Changes, and Intestinal Health of Laying Hens

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Abstract

This experiment aimed to investigate the appropriate level of degossypolized cottonseed protein (DGCP) in the diet of laying hens. A total of 600 49-week-old Lohmann pink laying hens were allocated to five treatments, with six replicates per treatment and 20 birds per replicate. The control group was fed a corn-soybean meal basal diet. Four experimental diets were formulated by replacing 25%, 50%, 75%, and 100% of the soybean meal protein-equivalent capacity with DGCP, where 100% replacement corresponded to the maximum safe inclusion of DGCP. The study period lasted for 8 weeks. The results showed that the feed intake, average egg weight, egg mass, laying rate, and the albumen percentage were significantly reduced in the 100% DGCP group ($p < 0.05$). Plasma uric acid (UA), total cholesterol (TC), triglyceride (TG), and potassium (K) levels were significantly lower ($p < 0.05$), and depth of crypt (CD) was significantly higher ($p < 0.05$) in the 100% DGCP group. The DGCP diet linearly increased the relative abundance of Bacteroidota and Bacteroides and significantly increased the relative abundance of Desulfobacterotas in the cecum contents compared to the control group ($p < 0.05$). The ACE and Chao1 indices in both the control group and the 100% DGCP group were significantly decreased ($p < 0.05$). In conclusion, the dietary addition of DGCP can reach up to 114.6 g/kg.

Keywords: degossypolized cottonseed protein; laying performance; egg quality; gossypol residue; blood indexes; intestinal health



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1. Introduction

Soybean meal (SBM) is widely acknowledged as a significant protein source for poultry feeds due to its high protein content. However, in recent years, the sharp increase in SBM prices has created an urgent need to identify alternative protein sources. Cottonseed meal (CSM), a high-quality plant protein derived from the oil extraction process of cotton seeds, presents a promising substitute for protein in poultry diets [1] and can serve as a sustainable protein source [2,3].

Free gossypol (FG) is the primary anti-nutritional factor present in CSM, which is known to be toxic to the reproductive system, heart, and liver of monogastric animals [4]. During cottonseed oil extraction, FG binds to the amino group of lysine due to high temperatures, baking, pressing, and other factors, thereby reducing the availability of lysine. Consequently, the binding-induced lysine deficiency establishes it as the most limiting amino acid in CSM [5,6]. CSM as poultry feed is limited by FG and amino acid imbalance. Studies have shown that the balance of amino acids can be adjusted by supplementing synthetic lysine [7]. FG remains the primary factor restricting the application of CSM. It can influence the laying performance of hens [8], leading to egg yolk discoloration [9,10] and negatively impacting the blood biochemistry of livestock and poultry [11]. However, some studies indicate that FG does not adversely affect the laying performance of laying hens within a specific range. Nagalakshmi et al. found that feeding DGCP (20 mg/kg or 59 mg/kg FG) had no effect on the performance and egg quality of laying hens [4]. Reid et al. observed that 10% CSM (61 mg/kg FG) had no significant effect on egg production rate, whereas 15% CSM (101.5 mg/kg FG) reduced it [1]. Wang et al. found that a CSM diet with varying levels of FG (0, 120, and 170 mg/kg) did not significantly affect laying performance, but the 170 mg/kg FG level was associated with a reduction in the levels of secretory immunoglobulin A (SIgA) in the jejunum and ileum mucosa of laying hens [12]. In recent years, with the advancement of cottonseed oil extraction technology and degossypolization technology, the production of new DGCP [13] has greatly reduced the content of FG and improved the nutritional value of CSM. The nutritional value of DGCP is much higher than that of traditional CSM, but there are few reports on the appropriate addition of DGCP with high protein content in laying hens.

This experiment was conducted to investigate the effects of DGCP (CP 65%) as a replacement for SBM on the laying performance, egg quality, blood indices, egg yolk score, intestinal morphology, pathological changes in the liver, magnum fallopian tube, ovary, and gossypol residue of 49-week-old Lohmann pink laying hens. The aim was to determine the suitable dietary level of DGCP.

2. Materials and Methods

This study was approved by the Animal Care and Use Committee, Sichuan Agricultural University (Ethical Approval Code: SICAUAC202110-2; Chengdu, China).

2.1. Experimental Design and Diets

The conventional nutritional components and amino acid composition of DGCP from Xinjiang Taikun Co., Ltd, Wujiaqu, China. are shown in Table 1. A total of 600 49-week-old Lohmann pink laying hens were allocated to one of five dietary treatments with 6 replicates of 20 birds each, 5 hens per cage, and 4 consecutive cages per replicate. The control group was fed a corn-soybean meal basal diet. Four experimental diets were formulated by replacing 25%, 50%, 75%, and 100% of the soybean meal protein-equivalent capacity with DGCP, where 100% replacement corresponded to the maximum safe inclusion of DGCP (providing 9.97% CP). Soybean meal was reduced proportionally, while maintaining iso-nitrogenous and iso-energetic conditions through amino acid balancing. DGCP was supplemented at 38.2, 76.4, 114.6, and 152.8 g/kg diet for each respective level. The nutritional level was based on the Chinese chicken feeding standard (NY/T33-2004) [14] and the New Lohmann Pink Laying Hens Feeding Manual. The dietary-free gossypol content for each treatment group was measured at 0, 13.9, 27.81, 41.71, and 55.62 mg/kg, respectively, based on the analyzed values of free gossypol in DGCP. Feed formulations and nutrient levels are shown in Table 2. The experimental period was 8 weeks. Layers were maintained on a 15 h light schedule and provided with ad libitum access to both

diets and water. All diets were provided in mash form, and the room temperature was consistently maintained between 20 °C and 25 °C.

Table 1. Composition (DM basis) of DGCP used in the study.

Composition	DGCP
Dry matter, %	97.22
GE, MJ/kg	18.2
Crude protein, %	65.43
Crude fat, %	1.74
Crude fiber, %	1.87
Ash, %	8.21
NDF, %	7.17
ADF, %	1.46
FG, mg/kg	311.64
Essential amino acids, EAA %	
Lysine	2.55
Methionine	0.57
Threonine	1.98
Histidine	1.76
Leucine	3.51
Isoleucine	1.84
Phenylalanine	3.36
Arginine	7.88
Valine	2.61
Glycine	2.51
Serine	2.72
Nonessential amino acids, NEAA %	
Aspartic acid	5.67
Glutamic acid	12.9
Alanine	2.3
Cysteine	0.76
Tyrosine	1.86
Proline	2.33
Total AA	57.11

Table 2. The composition and nutrient levels of the experimental diets (air-dry basis, %).

Ingredients	DGCP Replacement Levels, %				
	0	25	50	75	100
Corn	56.52	58.77	61.01	63.26	65.50
Soybean meal (43% CP)	26.95	20.21	13.48	6.74	0.00
Degossypolized cottonseed protein	0.00	3.82	7.64	11.46	15.28
Wheat bran	2.00	2.69	3.38	4.06	4.75
Soybean oil	2.85	2.50	2.14	1.79	1.43
Powdery lithopone	2.50	2.50	2.50	2.50	2.50
Grainy lithopone	7.03	7.08	7.13	7.17	7.22
CaHPO ₄	1.15	1.11	1.08	1.04	1.00
L-Lysine Sulphate	0.00	0.15	0.29	0.44	0.58
DL-Methionine	0.09	0.09	0.10	0.10	0.10
L-Threonine	0.00	0.03	0.07	0.10	0.14
L-Tryptophan	0.00	0.01	0.01	0.02	0.03
NaCl	0.40	0.40	0.40	0.40	0.40
Choline chloride (60%)	0.10	0.10	0.10	0.10	0.10
Mineral premix ¹	0.15	0.15	0.15	0.15	0.15
Vitamin premix ²	0.15	0.15	0.15	0.15	0.15

Table 2. Cont.

Ingredients	DGCP Replacement Levels, %				
	0	25	50	75	100
Other ³	0.11	0.25	0.39	0.53	0.67
Total	100.00	100.00	100.00	100.00	100.00
Calculated value of nutrition level, % ⁴					
Metabolizable energy, MJ/kg	11.40	11.40	11.40	11.40	11.40
Crude protein, %	16.15	16.15	16.15	16.15	16.15
Calcium, %	4.00	4.00	4.00	4.00	4.00
Nonphytate phosphorus, %	0.30	0.30	0.30	0.30	0.30
Digestible lysine, %	0.749	0.749	0.749	0.749	0.749
Digestible methionine, %	0.316	0.316	0.316	0.316	0.316
Digestible threonine, %	0.530	0.530	0.530	0.530	0.530
Digestible tryptophan, %	0.158	0.158	0.158	0.158	0.158
Free gossypol, mg/kg	0.00	13.90	27.81	41.71	55.62

¹ The mineral premix provided the following per kg of diets: Cu (CuSO₄·5H₂O) 15 mg, Fe (FeSO₄·H₂O) 120 mg, Mn (MnSO₄·H₂O) 150 mg, Zn (ZnSO₄·H₂O) 120 mg, and Se (Na₂SeO₃) 0.3 mg. ² The vitamin premix provided the following per kg of diets: h, VD₃ 4950 IU, VE 69 IU, VK₃ 2.4 mg, VB₁ 2.4 mg, VB₂ 6.3 mg, VB₆ 4.2 mg, VB₁₂ 0.025 mg, niacin acid 30 mg, pantothenic 10 mg, biotin 0.05 mg, and folic acid 0.5 mg. ³ Other parts contain pigment and bentonite. ⁴ Nutrient levels were calculated.

2.2. Laying Performance and Egg Quality

Daily records were maintained for total egg numbers, total egg weight, and qualified eggs, which excluded sand-shell, soft, broken, malformed, dirty, large (above 70 g), and small (below 50 g) eggs. Additionally, qualified egg weight and the number of dead chickens were documented each day. Feed consumption was recorded weekly, along with the determined average daily feed intake (ADFI), hen-housed laying rate, hen-day laying rate, average egg weight, feed conversion ratio (FCR), number of hen-housed eggs, and egg mass, which were calculated as per the following:

$$\text{Hen} - \text{housed laying rate} = \text{total egg numbers} / (\text{number of housed hens} \times \text{days}) \times 100$$

$$\text{hen} - \text{day laying rate} = \text{total egg numbers} / (\text{number of hens in stock} \times \text{days}) \times 100$$

$$\text{average egg weight} = \text{total egg weight} / \text{total egg numbers}$$

$$\text{ADFI} = \text{feed consumption} / (\text{number of housed hens} \times \text{days})$$

$$\text{FCR} = \text{total feed intake} / \text{total egg weight}$$

$$\text{number of hen} - \text{housed eggs} = \text{total egg numbers} / (\text{number of housed hens} \times \text{days})$$

$$\text{egg mass} = \text{total egg weight} / (\text{number of housed} \times \text{days})$$

At weeks 0, 4, and 8 of the experiment, 18 eggs per treatment (3 per replicate) were collected for egg quality assessment. The evaluation included measurements of egg weight, yolk color, albumen height, and Haugh unit, all conducted using an automatic egg quality analyzer (EMT-7300, Robotmation Co., Ltd., Tokyo, Japan). Additionally, eggshell strength was measured using an eggshell force gauge (model II, Robotmation Co., Ltd., Tokyo, Japan). Additionally, eggshell thickness was measured at three locations (equator, small end, and large end) using an eggshell thickness gauge (Robotmation Co., Ltd., Tokyo, Japan). Egg yolk and shell weights were recorded with a 0.01 g precision electronic scale, while horizontal and vertical egg diameters were determined using vernier calipers. Egg white quality index: The egg white was separated and subsequently passed through a 40-

mesh test sieve. The thin albumen was filtered through the sieve, while the thick albumen remained on the sieve and was weighed separately.

$$\text{Thick albumen proportion} = \text{thick albumen weight} / \text{egg weight} \times 100$$

$$\text{Thick to thin ratio} = \text{thick albumen weight} / \text{thin albumen weight}$$

2.3. Sample Collection and Determination

At the conclusion of the 8th week, one laying hen was randomly selected from each replicate. We collected 9 mL of blood from the jugular vein, of which approximately 2 mL was loaded into an EDTA anticoagulant tube for measuring blood routine indicators. The remaining 7 mL of blood was divided into two non-anticoagulant blood collection tubes (approximately 3.5 mL per tube), centrifuged at 3500 rpm for 10 min to obtain plasma for measuring plasma biochemical indicators. After collecting blood samples, the chickens were euthanized using carbon dioxide. Liver, magnum fallopian tube, and ovarian tissue samples were collected and fixed in 4% neutral formaldehyde, and pathological sections were made to observe tissue pathological changes. Additionally, ileal segments approximately 2 cm in length were harvested and fixed in 4% paraformaldehyde for histological analysis.

2.4. Egg Yolk Sensory Score

In the 4th and 8th weeks of the experiment, 90 eggs were collected each time (3 eggs/replicate, 18 eggs/treatment) and stored at 4 °C for 0, 2, 4, and 6 weeks, respectively. The egg yolk was subjected to sensory evaluation according to the proportion of spots or plaques on the surface of the egg yolk and the degree of discoloration provided by Davis et al. [10]. A sensory score of ≥ 3 was deemed unacceptable (OBJ).

2.5. Blood Indexes

A fully automatic animal hematology cell analyzer (Vitagas 5E, Cornley Hi-Tech, Shenzhen, China) was utilized to measure various parameters, including white blood cell count (WBC), red blood cell count (RBC), platelet count (PLT), hematocrit (HCT), hemoglobin concentration (HGB), and platelet volume (PCT). Additionally, a fully automatic blood biochemistry analyzer (HATACHI 7180, Tokyo, Japan) was employed to assess aspartate aminotransferase (AST) activity, total protein (TP), albumin (ALB), uric acid (UA), creatinine (CR), potassium (K), chlorine (Cl), calcium (Ca), sodium (Na), total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

2.6. Free Gossypol Residue

Gossypol residues in egg yolk samples treated at 4 and 8 weeks and liver samples treated at 8 weeks were determined. The determination of free gossypol in egg yolk and liver was based on Nomeir et al. [15].

2.7. Liver and Uterine Histopathological Changes and Intestinal Morphology

The ileal segment, liver, magnum fallopian tube, and ovary samples, which were soaked in 4% paraformaldehyde, were removed and subsequently dehydrated using ethanol. After dehydration, the tissues were cleared with xylene and embedded in paraffin wax. Sections were then cut to a thickness of 4 μm using a Leica CM1860 microtome and transferred onto glass slides. The sections underwent hematoxylin-eosin staining. Then, the histopathological changes in liver, magnum fallopian tube, and ovary were assessed using a light microscope (DM500, Leica, Wetzlar, Germany), and intestinal morphology

was observed using Image Pro Plus 6.0 (Media Cybernetics, Ltd., Bethesda, MD, USA). A total of 10 intact and straight villi were selected from each sample to measure CD and villus heights (VH). The CD denotes the depth of invagination between adjacent villi, while VH represents the distance from the top of the villus to the crypt-villus junction. The ratio of VH to CD was defined as VH/CD.

2.8. Analysis of Bacterial Microbiota by 16S RNA

Cecum was collected and cecal chow was collected in EP tubes, snap frozen with liquid nitrogen, and stored at -80°C until analysis. 16s rRNA analyses of cecum microorganisms, including α diversity of microorganisms and relative abundance of species, were performed by Beijing Nuohe Bioinformatics Technology Company.

2.9. Statistical Analysis

Data were analyzed using SPSS 27.0. A one-way analysis of variance (ANOVA) was used for the primary analysis. Polynomial orthogonal contrasts were used to determine the linear and quadratic effects of DGCP level. Duncan's method was used for multiple comparisons. Data were expressed as the mean and standard error of the mean. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Production Performance and Egg Quality

As presented in Table 3, the hen-day laying rate (weeks 5–8 and 1–8), average daily feed intake (ADFI), egg mass, and average egg weight in the 100% DGCP group were significantly lower than those in other replacement groups ($p < 0.05$), exhibiting linear decreases and quadratic effects with increasing DGCP levels. As shown in Table 4, the quadratic regression equation for the production performance indicators reveals that the optimal substitution level for DGCP is 25%, while comprehensively, the substitution level can be as high as 75%.

Table 3. Effect of replacing SBM with DGCP on the production performance of laying hens.

Items	DGCP Replacement Levels, %					SEM	<i>p</i> -Value		
	0	25	50	75	100		Anova	Linear	Quadratic
Hen-housed laying rate/%									
weeks 1–4	93.99	94.73	93.45	93.57	92.00	1.330	0.362	0.086	0.152
weeks 5–8	93.78	94.61	92.95	93.75	90.03	1.812	0.138	0.050	0.059
weeks 1–8	93.89	94.67	93.20	93.66	91.01	1.418	0.144	0.042	0.056
Hen-day laying rate/%									
weeks 1–4	93.99	95.23	93.80	93.57	92.00	1.235	0.170	0.050	0.060
weeks 5–8	93.78 ^a	96.23 ^a	94.17 ^a	93.75 ^a	90.03 ^b	1.519	0.008	0.012	0.002
weeks 1–8	93.89 ^a	95.73 ^a	93.99 ^a	93.66 ^a	91.01 ^b	1.195	0.012	0.011	0.003
ADFI/g									
weeks 1–4	119.7 ^a	120.0 ^a	119.5 ^a	119.6 ^a	116.6 ^b	0.474	<0.001	<0.001	<0.001
weeks 5–8	115.8 ^a	119.2 ^a	117.1 ^a	115.7 ^a	102.0 ^b	2.217	<0.001	<0.001	<0.001
weeks 1–8	117.7 ^a	119.6 ^a	118.3 ^a	117.7 ^a	109.3 ^b	1.281	<0.001	<0.001	<0.001
FCR									
weeks 1–4	2.00	2.00	2.01	2.04	2.08	0.318	0.071	0.008	0.012
weeks 5–8	1.91	1.91	1.92	1.96	1.86	0.042	0.283	0.713	0.295
weeks 1–8	1.95	1.95	1.96	2.00	1.97	0.033	0.639	0.281	0.528

Table 3. Cont.

Items	DGCP Replacement Levels, %					SEM	<i>p</i> -Value		
	0	25	50	75	100		Anova	Linear	Quadratic
Average egg weight/g									
weeks 1–4	63.73 ^a	63.14 ^a	63.61 ^a	62.81 ^a	61.04 ^b	0.584	<0.001	<0.001	<0.001
weeks 5–8	64.83 ^a	64.88 ^a	64.74 ^a	63.14 ^a	60.88 ^b	0.822	<0.001	<0.001	<0.001
weeks 1–8	64.27 ^a	64.01 ^a	64.17 ^a	62.98 ^a	60.96 ^b	0.682	<0.001	<0.001	<0.001
Number of hen-housed eggs									
weeks 1–4	26.32	26.53	26.17	26.20	25.76	0.373	0.362	0.086	0.152
weeks 5–8	26.26	26.49	26.03	26.25	25.21	0.507	0.138	0.050	0.059
weeks 1–8	52.58	53.02	52.19	52.45	50.97	0.794	0.144	0.042	0.056
Qualified rate/%									
weeks 1–4	97.81	98.62	97.42	97.95	98.23	0.535	0.260	0.896	0.897
weeks 5–8	96.42	98.13	98.12	97.28	97.77	0.665	0.085	0.262	0.122
weeks 1–8	97.12	98.37	97.77	97.61	98.00	0.515	0.196	0.414	0.483
Egg mass/(kg/bird)									
weeks 1–4	1.68 ^a	1.67 ^a	1.67 ^a	1.65 ^a	1.57 ^b	0.030	0.013	0.002	0.002
weeks 5–8	1.70 ^a	1.72 ^a	1.69 ^a	1.66 ^a	1.53 ^b	0.038	<0.001	<0.001	<0.001
weeks 1–8	3.38 ^a	3.39 ^a	3.35 ^a	3.31 ^a	3.11 ^b	0.066	0.001	<0.001	<0.001

Data are expressed as the mean and SEM. ^{a,b} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$).

Table 4. The quadratic regression equation of production performance indicators.

Items	Equation	R ²	Optimal Dosage
Hen-housed laying rate	$y = 93.93571 + 0.02926x - 0.00056x^2$	$r^2 = 0.83065$	26.125
Hen-day laying rate	$y = 94.14057 + 0.05519x - 0.00087x^2$	$r^2 = 0.89145$	31.72
ADFI	$y = 117.41714 + 0.15263x - 0.00227x^2$	$r^2 = 0.93617$	33.62
Average egg weight	$y = 64.11229 + 0.02506x - 0.00056x^2$	$r^2 = 0.97028$	22.38
Egg mass	$y = 3.37200 + 0.00232x - 0.00005x^2$	$r^2 = 0.96520$	23.2

According to Table 5, at the 4th week of the experiment, with the increase in DGCP level, the egg weight and albumen percentage showed linear and quadratic decreases ($p < 0.05$). The 100% DGCP group was significantly lower than the control group ($p < 0.05$); the yolk percentage showed a linear increase ($p < 0.05$). The thick albumen proportion showed a quadratic decrease ($p < 0.05$), and the 25% DGCP group was significantly higher than the control group and the 100% DGCP group ($p < 0.05$). At the 8th week of the experiment, with the increase in DGCP level, the egg shape index, egg weight, albumen percentage, and thick albumen proportion showed linear and quadratic decreases ($p < 0.05$), while the yolk percentage showed a linear increase ($p < 0.05$).

Table 5. Effect of replacing SBM with DGCP on the egg quality of laying hens.

Items	DGCP Replacement Levels, %					SEM	<i>p</i> -Value		
	0	25	50	75	100		Anova	Linear	Quadratic
Egg weight/g									
4 weeks	65.19 ^a	66.93 ^a	66.41 ^a	65.21 ^a	62.88 ^b	1.065	0.003	0.016	<0.001
8 weeks	65.56 ^{bc}	67.88 ^a	66.35 ^{ab}	63.97 ^{cd}	62.16 ^d	1.085	<0.001	<0.001	<0.001
Yolk percentage/%									
4 weeks	28.83b ^c	27.73 ^c	29.34 ^b	29.07 ^b	31.11 ^a	0.610	<0.001	<0.001	<0.001
8 weeks	28.36 ^b	28.13 ^b	28.06 ^b	28.93 ^b	30.05 ^a	0.551	0.002	0.001	<0.001

Table 5. Cont.

Items	DGCP Replacement Levels, %					SEM	<i>p</i> -Value		
	0	25	50	75	100		Anova	Linear	Quadratic
Eggshell percentage/%									
4 weeks	11.01	10.78	11.14	11.03	11.07	0.130	0.095	0.213	0.442
8 weeks	10.87	10.47	11.14	11.02	10.88	0.240	0.216	0.381	0.645
Albumen percentage/%									
4 weeks	60.08 ^b	61.49 ^a	59.54 ^b	59.89 ^b	57.82 ^c	0.640	<0.001	<0.001	<0.001
8 weeks	60.77 ^{ab}	61.40 ^a	60.80 ^a	60.05 ^{bc}	59.06 ^c	0.851	<0.001	<0.001	<0.001
Egg shape index									
4 weeks	1.29	1.30	1.32	1.31	1.29	0.130	0.149	0.800	0.052
8 weeks	1.32 ^{ab}	1.34 ^a	1.31 ^b	1.31 ^b	1.3 ^b	0.107	0.016	0.015	0.037
Shell color									
4 weeks									
L*	80.46	80.55	79.76	78.91	80.46	0.670	0.073	0.325	0.211
a*	4.65	4.73	5.03	5.42	4.44	0.400	0.139	0.715	0.163
b*	17.82	17.37	17.85	19.1	17.60	0.640	0.077	0.452	0.614
8 weeks									
L*	79.42	77.60	77.97	77.81	78.04	0.740	0.120	0.132	0.063
a*	5.52	6.42	6.24	6.44	6.23	0.470	0.281	0.173	0.133
b*	17.54	19.49	18.39	18.68	18.04	0.710	0.086	0.908	0.144
Yolk color									
4 weeks	13.06	13.63	13.43	13.56	13.27	0.250	0.149	0.575	0.127
8 weeks	12.68	12.38	12.35	12.57	12.68	0.252	0.524	0.723	0.253
Albumen height/mm									
4 weeks	8.04	8.73	8.07	8.24	8.01	0.320	0.140	0.487	0.361
8 weeks	8.19	8.62	8.01	8.09	7.90	0.291	0.123	0.099	0.250
Haugh unit									
4 weeks	88.21	91.46	87.82	89.28	88.72	1.770	0.274	0.846	0.815
8 weeks	89.07	90.67	86.69	88.77	88.28	1.572	0.165	0.332	0.568
Thick albumen proportion/%									
4 weeks	31.56 ^c	35.67 ^a	34.54 ^{ab}	34.26 ^{abc}	32.33 ^{bc}	0.130	0.015	0.960	0.008
8 weeks	36.33 ^{ab}	38.18 ^a	37.94 ^a	35.90 ^{ab}	34.00 ^b	1.235	0.007	0.017	0.001
Thick-to-thin ratio									
4 weeks	1.33	1.61	1.57	1.52	1.5	0.150	0.404	0.502	0.238
8 weeks	1.73	1.99	1.91	1.89	1.64	0.215	0.471	0.565	0.198
Eggshell thickness/ μ m									
4 weeks	380.65	380.96	377.52	371.22	375.74	5.020	0.288	0.107	0.250
8 weeks	377.17	378.06	376.56	373.5	360.06	8.451	0.191	0.042	0.051
Eggshell strength/(kg/cm ²)									
4 weeks	4.41	4.50	4.66	4.56	4.25	0.200	0.299	0.460	0.164
8 weeks	4.44	4.70	4.46	4.32	4.35	0.235	0.523	0.292	0.487

Data are expressed as the mean and SEM. ^{a–d} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$).

3.2. Egg Yolk Score

The eggs from the 4th and 8th week of the experiment were stored at 4 °C for 0, 2, 4, and 6 weeks without any obvious spots, with a score of 0, and no unacceptable eggs were found. The eggs from the 4th and 8th week of the experiment were stored at 4 °C for 0 and 6 weeks, as shown in Figures 1 and 2, respectively.

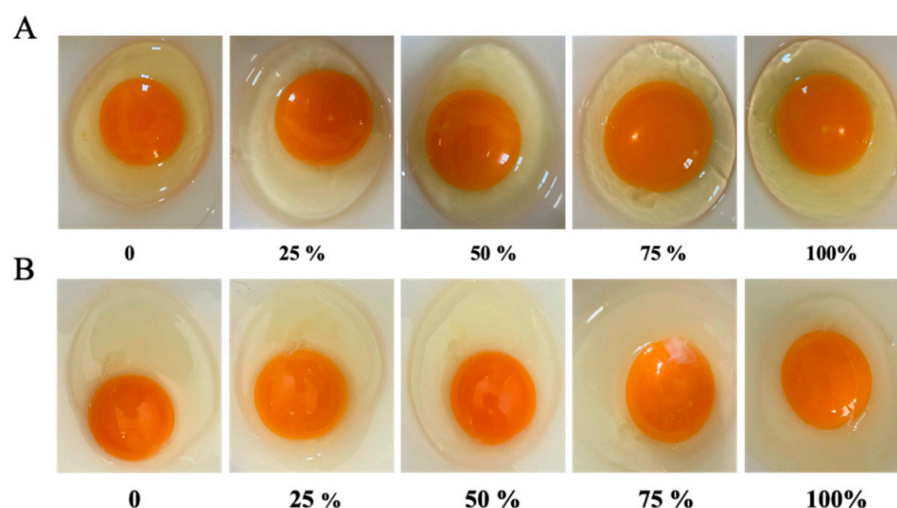


Figure 1. Eggs from the 4th week were stored for 0 weeks and 6 weeks. (A) Eggs from the 4th week, when stored for 0 weeks. (B) Eggs from the 4th week, when stored for 6 weeks.

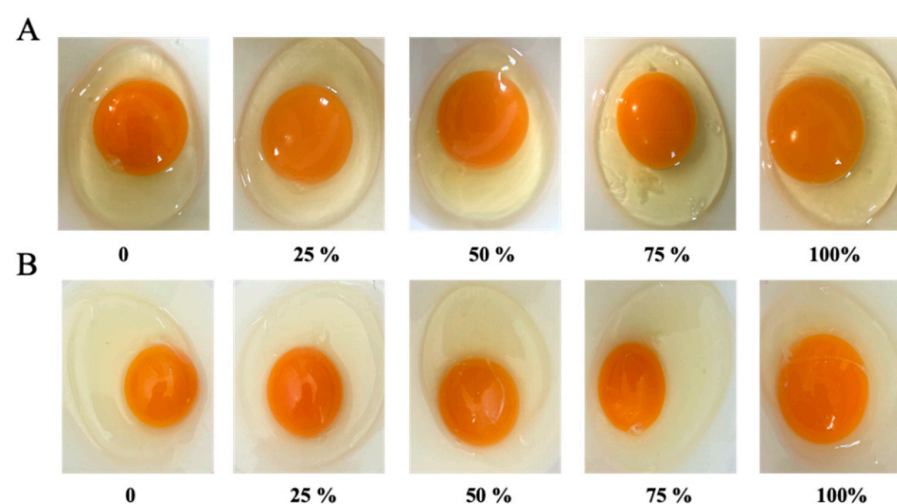


Figure 2. Eggs from the 8th week were stored for 0 weeks and 6 weeks. (A) Eggs from the 8th week, when stored for 0 weeks. (B) Eggs from the 8th week, when stored for 6 weeks.

3.3. Blood Routine Parameters

As shown in Table 6, with the increase in DGCP level, there was no significant effect on the WBC, RBC, PLT, HCT, HGB, and PCT in the whole blood of laying hens ($p > 0.05$).

Table 6. Effect of replacing SBM with DGCP on blood routine parameters of laying hens.

Items ¹	DGCP Replacement Levels, %					SEM	p-Value		
	0	25	50	75	100		Anova	Linear	Quadratic
WBC/(10^9 /L)	37.82	31.84	39.05	35.39	37.25	4.578	0.567	0.815	0.902
RBC/(10^{12} /L)	2.23	2.30	2.31	2.33	2.33	0.112	0.895	0.352	0.582
PLT/(10^9 /L)	31.67	39.33	36.00	33.33	23.00	7.51	0.285	0.179	0.084
HCT/%	28.72	29.30	29.50	30.02	29.52	1.248	0.886	0.391	0.59
HGB/(g/L)	139.0	138.2	138.5	140.8	137.5	6.407	0.988	0.981	0.983
PCT/%	0.02	0.02	0.02	0.02	0.01	0.004	0.169	0.084	0.044

Data are expressed as the mean and SEM. ¹ WBC, white blood cell count; RBC, red blood cell count; PLT, platelet count; HCT, hematocrit; HGB, hemoglobin concentration; PCT, platelet volume.

3.4. Plasma Biochemistry Indices

According to Table 7, with the increase in DGCP level, there was no significant effect on the plasma AST activity and TP, ALB, CR, Ca, Cl, and Na contents ($p > 0.05$). The plasma UA and K contents of laying hens showed a linear and quadratic decrease ($p < 0.05$) with the increase in DGCP level. The plasma UA content of the 100% DGCP group was significantly lower than that of the other groups ($p < 0.05$). The plasma K content in the 25% to 100% DGCP groups was significantly lower than that in the control group ($p < 0.05$).

Table 7. Effect of replacing SBM with DGCP on plasma biochemistry indices of laying hens.

Items ¹	DGCP Replacement Levels, %					SEM	p-Value		
	0	25	50	75	100		Anova	Linear	Quadratic
AST/(U/L)	160.3	168.7	154.4	158.7	180.9	10.93	0.153	0.231	0.417
TP/(g/L)	44.45	47.20	46.00	46.52	44.34	3.240	0.848	0.825	0.939
ALB/(g/L)	17.97	19.28	18.23	19.86	17.75	1.596	0.613	0.863	0.793
CR/(umol/L)	49.29	73.36	57.12	69.35	47.92	20.99	0.640	0.883	0.470
UA/(umol/L)	175.8 ^a	171.3 ^{ab}	153.8 ^{ab}	138.0 ^b	98.27 ^c	16.20	<0.001	<0.001	0.002
Ca/(mmol/L)	5.19	5.85	4.27	6.20	4.04	0.931	0.116	0.094	0.236
Cl/(mmol/L)	110.30	109.26	110.17	110.26	111.12	1.125	0.584	0.618	0.836
Na/(mmol/L)	31.87	31.43	32.25	32.38	32.73	0.463	0.075	0.018	0.061
K/(mmol/L)	6.42 ^a	5.50 ^b	4.44 ^c	5.66 ^b	5.11 ^{bc}	0.369	<0.001	0.086	0.005

Data are expressed as the mean and SEM. ^{a-c} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$). ¹ AST, aspartate amino transferase; TP, total protein; ALB, albumin; CR, creatinine; UA, uric acid; Ca, calcium; Cl, chlorine; Na, sodium; K, potassium.

3.5. Plasma Lipid Metabolism Indices

As shown in Table 8, with the increase in DGCP level, there was no significant effect on the plasma LDL-C and HDL-C contents of laying hens ($p > 0.05$). The plasma TC and TG contents decreased linearly and quadratically ($p < 0.05$) with the increase in DGCP level. The plasma TC and TG contents in the 100% DGCP group were significantly lower than those in the other groups ($p < 0.05$).

Table 8. Effect of replacing SBM with DGCP on plasma lipid metabolism indices of laying hens.

Items ¹	DGCP Replacement Levels, %					SEM	p-Value		
	0	25	50	75	100		Anova	Linear	Quadratic
TC/(mmol/L)	2.46 ^a	2.85 ^a	2.44 ^a	2.50 ^a	1.66 ^b	0.289	0.007	0.011	0.002
TG/(mmol/L)	14.65 ^a	17.16 ^a	15.9 ^a	15.27 ^a	7.70 ^b	2.626	0.012	0.033	0.025
LDL-C/(mmol/L)	0.91	1.14	1.07	1.05	0.60	0.246	0.224	0.128	0.265
HDL-C/(mmol/L)	0.89	1.22	1.06	1.06	0.69	0.277	0.412	0.158	0.353

Data are expressed as the mean and SEM. ^{a,b} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$). ¹ TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein.

3.6. Free Gossypol Residue in Liver and Yolk

At the end of the 8th week of the experiment, no residual free gossypol was detected in the liver and yolk of each treatment of laying hens.

3.7. Pathological Changes in Liver, Magnum Fallopian Tube, and Ovary

Histological lesions were not observed in the liver, magnum fallopian tube, or ovary tissues of hens among the treatment groups (Figure 3A–C).

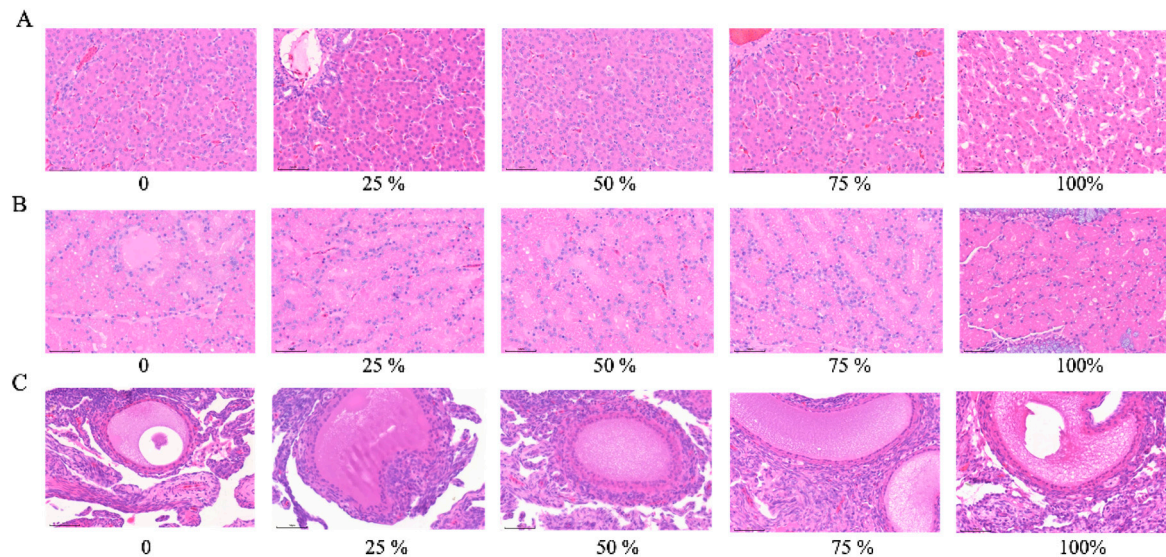


Figure 3. Effect of DGCP replacement level on liver, magnum fallopian tube, and ovary histopathology of layers fed the experimental diets (40×). (A) liver histopathology; (B) magnum fallopian tube histopathology; (C) ovary histopathology.

3.8. Intestinal Morphology

According to Table 9, the VH of the 75% DGCP group was significantly higher than that of the control group and the 100% DGCP group ($p < 0.05$), and there was no significant difference between the 25% and 50% DGCP groups ($p > 0.05$). With the increase in DGCP level, the CD showed a linear and quadratic increase ($p < 0.05$), with the 100% DGCP group being significantly higher than the other groups ($p < 0.05$). The VH/CD showed a linear and quadratic decrease ($p < 0.05$), and the 100% DGCP group was significantly lower than the other groups ($p < 0.05$).

Table 9. Effect of replacing SBM with DGCP on the ileal tissue morphology of laying hens.

Items ¹	DGCP Replacement Levels, %					SEM	<i>p</i> -Value		
	0	25	50	75	100		Anova	Linear	Quadratic
VH/(μm)	1048 ^b	1141 ^{ab}	1104 ^{ab}	1239 ^a	1024 ^b	67.84	0.039	0.872	0.095
CD/(μm)	94.53 ^b	91.48 ^b	102.6 ^b	104.3 ^b	127.5 ^a	8.673	0.003	<0.001	<0.001
VH/CD	11.14 ^a	12.66 ^a	11.02 ^a	12.05 ^a	8.050 ^b	0.889	<0.001	0.008	<0.001

Data are expressed as the mean and SEM. ^{a,b} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$).

¹ VH, villus height of jejunum; CD, crypt depth of jejunum; VH/CD, the ratio of VH to CD of jejunum.

3.9. Gut Microbiota Diversity

Figure 4A shows the rate of emergence of new ASVs under continuous sampling, with the box plot position leveling off and species in the environment not increasing significantly with sample size, indicating that sampling was sufficient for data analysis. Figure 4B shows that there were 808 common ASVs in the cecum microbiota in each of the five treatment groups, with different specific ASVs in each group. There were 324, 490, 344, 306, and 390 unique ASVs in the control, 25%, 50%, 75%, and 100% groups, respectively, suggesting that sweet DGCP affects gut microbial composition. Figure 4C shows the effect of DGCP on microbial α diversity in the cecum of laying hens. Chao 1, ACE, and the he Shannon index were significantly lower in the control and 100% replacement groups than in the 25% and 75% replacement groups ($p < 0.05$) and were not significantly different from those in the 50% replacement groups ($p > 0.05$).

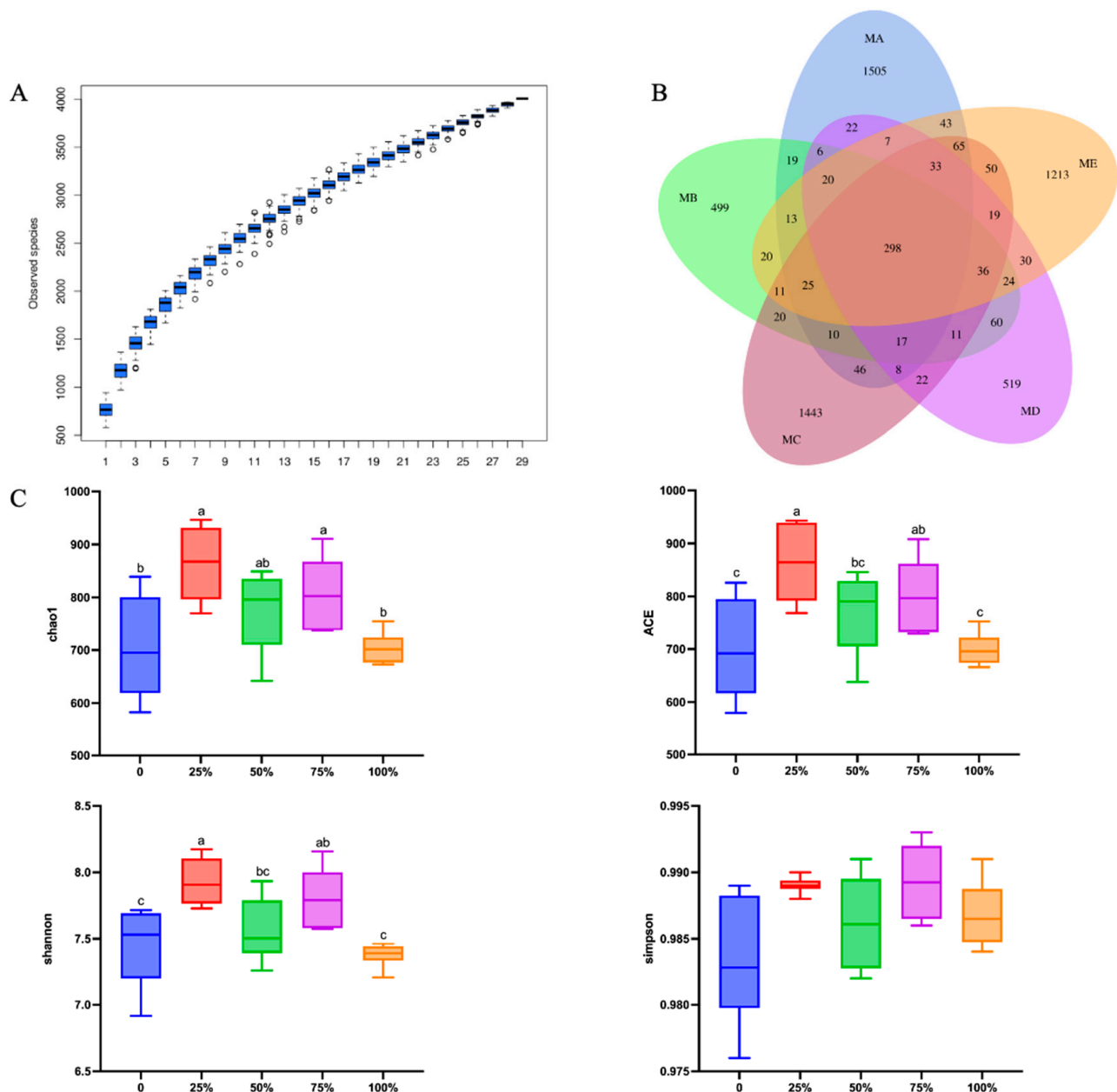


Figure 4. (A) Cumulative box plot of species. Abscissa: sample size; ordinate: ASV count. (B) Venn diagram of ASV distribution. Each circle represents a group; the overlapping areas indicate shared ASVs; non-overlapping areas indicate unique ASVs. (MA) Control group. (MB) 25% DGCP group. (MC) 50% DGCP group. (MD) 75% DGCP group. (ME) 100% DGCP group. (C) Alpha diversity index. ^{a-c} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$).

Figure 5A displays the top 10 clades by maximum relative abundance, with the remainder grouped as Others. Bacteroidota showed the highest relative abundance, followed by Campylobacterota and Firmicutes. Figure 5B shows the 10 genera with the highest relative abundance of species, with Bacteroides, Campylobacter, and Fusobacterium as the dominant genera. Both Bacteroidota and Bacteroides increased linearly with the amount of DGCP. As can be seen in Figure 5C,D, Fusobacteriota was significantly lower ($p < 0.05$) and Desulfobacterota was significantly higher ($p < 0.05$) in the 100% replacement group compared to all other treatment groups.

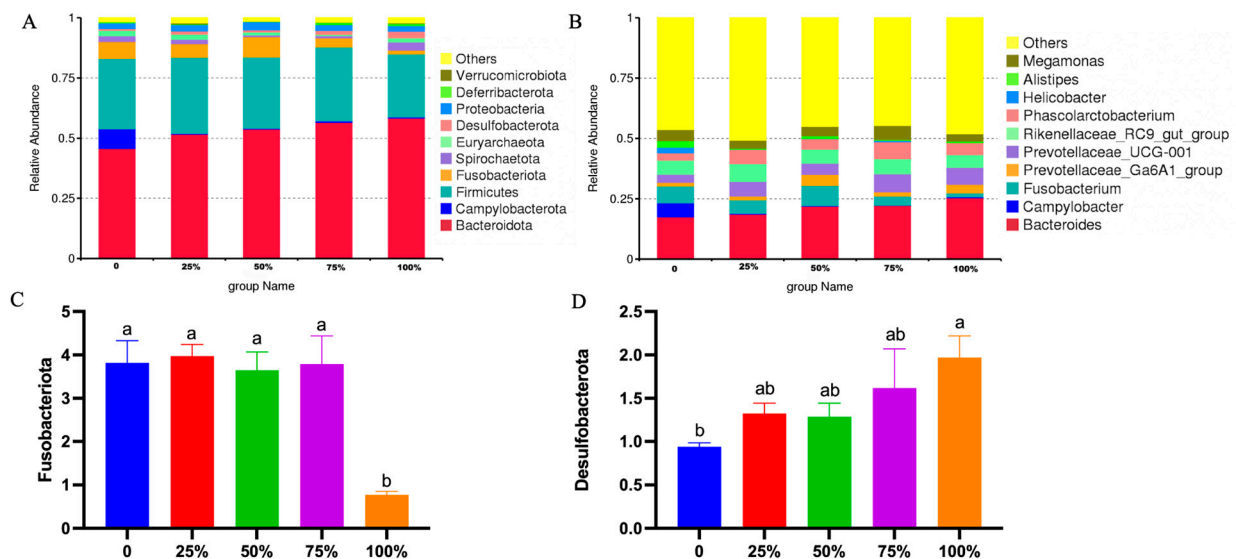


Figure 5. (A) Histogram of relative abundance of species at the phylum level. (B) Histogram of relative abundance of species at the genus level. (C,D) Differences in microbiota at the phylum level. ^{a,b} Value differences in the same row differ significantly ($p < 0.05$) ($n = 6$).

4. Discussion

Previous studies have reported the effects of DGCP on laying hen performance. Reid et al. found that including 50 g/kg of CSM (containing 30 mg/kg FG) in the diet significantly reduced the body weight of laying hens [1]. The results indicated that replacing soybean meal with 100% DGCP (FG 28.35 mg/kg) in the diet of 40-week-old Hy-Line brown laying hens led to a significant reduction in both egg weight and feed intake at 46–51 weeks of age [16]. Mu et al. examined diets containing 0%, 6%, and 12% CSM (FG of 0, 41.63 mg/kg, and 83.26 mg/kg, respectively) in laying hens and found that the 12% group had a significantly reduced egg weight and feed intake [17]. Additionally, Lordelo et al. found that adding 200 mg/kg FG to the diet of 34-week-old Hy-Line laying hens significantly decreased both laying rate and feed intake [8]. The results of this experiment also indicated that the 100% DGCP group significantly reduced the laying rate, feed intake, average egg weight, and egg mass of laying hens. This reduction may be attributed to the poor palatability of DGCP, which diminishes the feed intake of laying hens and fails to meet their nutritional requirements, consequently leading to a decline in egg production performance [18]. Additionally, this issue may be related to FG levels. He et al. reported that FG concentrations reaching 28.35 mg/kg significantly reduced egg weight and feed intake [16]. In this experiment, FG levels reached 55.62 mg/kg with 100% replacement, potentially exceeding the maximum FG tolerance of laying hens, which could adversely affect their health and, in turn, their laying performance. In addition, previous studies have reported the antagonistic effects of excessive arginine and lysine in feed [19]. The high arginine content in DGCP leads to an antagonistic effect that disrupts the amino acid balance in the diet, resulting in decreased production performance in the 100% replacement group [16].

Egg quality is an important indicator in the production process of laying hens, significantly influencing the sensory, structural, and functional characteristics of eggs. The albumen is composed of two parts: thick albumen and thin albumen. The protein content in egg white is extremely high, making it a valuable nutrient. Wang et al. found that when 32-week-old laying hens were fed a diet containing 19.5% DGCP (FG 56.5 mg/kg), the proportion of albumen significantly decreased compared to the control group [20]. He et al. reported that a 100% replacement of soybean meal (FG 28.35 mg/kg) with DGCP led

to a significant reduction in albumen weight [16]. The results of this experiment showed a significant decrease in the albumen percentage within the 100% replacement group, aligning with previous research findings. The proportion of thick albumen is regarded as an important parameter for assessing egg freshness, and consumers show a greater preference for eggs with a higher thick albumen proportion [21]. Additionally, Zhang et al. reported that feeding a diet containing 16.76% DGCP significantly reduced the thick albumen proportion compared to the control group [22]. In this study, it was also found that the proportion of thick albumen in the 100% replacement group was significantly reduced. This reduction may be related to the ovomucin content in the protein, which plays a key role in maintaining the viscoelasticity and gel structure of the protein, thus affecting the albumen height, Haugh unit, and the proportion of thick albumen [23,24]. Omana et al. reported that the ovalbumin content in thick albumen is two–three times higher than that in thin albumen [25]. Zhang et al. reported that DGCP significantly reduced the content of ovalbumin in proteins [22]. In this study, the 100% replacement group showed a significant decrease in both egg weight and albumen percentage compared with other treatment groups. He et al. also found that a 100% substitution of soybean meal (FG 28.35 mg/kg) with DGCP significantly reduced the albumen percentage [16]. Penz et al. demonstrated that a decrease in egg weight was mainly caused by a decrease in albumen [26]. Previous studies have indicated that CSM has no effect on the egg shape index [16,22]. In this study, the egg shape index of the 100% replacement group exhibited a decreasing trend, which may be attributed to the breed of laying hens and the hatching rate of the eggs [27]. The nutrients in eggs are primarily concentrated in the yolk, and eggs with a higher yolk proportion tend to possess relatively higher nutrient content. Therefore, the yolk proportion can serve as an intuitive indicator of the nutritional quality of eggs. The results of this experiment revealed that as the replacement level of DGCP increased, the yolk proportion also increased linearly, indicating that DGCP enhances the nutritional value of eggs. Furthermore, the egg yolk score is an important factor affecting consumer senses and purchasing decisions, and a higher score correlates with more pronounced discoloration and spots on the egg yolk. Gilani et al. found that when laying hens were fed cottonseed meal (FG 142 mg/kg) in their diet, the discoloration of eggs significantly increased with the duration of refrigeration [28]. The results of this experiment indicated that as the replacement level of DGCP, feeding time, and egg refrigeration time increased, no obvious spots were observed in the egg yolks of any treatment. This suggests that the DGCP, after dephenolization, did not cause spots in the egg yolks. Schaible et al. reported that the addition of 1% ferrous sulfate to cotton meal rations was able to inhibit the formation of yolk spots and effectively improve egg quality [29]. The addition of Fe^{2+} to cotton meal rations at 4:1 and 8:1 ratios of Fe^{2+} to cotton phenol by mass showed that cotton phenol partially alleviated the inhibition of egg-laying performance, and that the negative effect of cotton phenol on egg weight was completely eliminated by the addition of Fe^{2+} [30]. The ferrous sulfate detoxification method is easy to operate, has a low cost of investment, and has been recognized as a better detoxification method.

Blood parameters can reflect physiological indicators of animal metabolism, health, and nutritional status to some extent [31]. In the present study, the replacement level of DGCP had no significant effect on routine blood indicators, such as WBC, RBC, HGB, and HCT, in laying hens. This finding is consistent with a previous study [16]. The results indicate that the effect of DGCP on the health of laying hens is relatively minimal. UA is produced by the breakdown of proteins and nucleic acids and is the main form of amino acid excretion in poultry [32]. It is a major indicator of kidney health. The results of this study indicated that as the replacement level of DGCP increased, the plasma UA content significantly decreased. The reason for the inconsistency with previous results may be

due to different egg-laying cycles, DGCP causing purine metabolism disorders, or kidney damage in laying hens. K has important physiological functions in animal bodies and can participate in protein and sugar metabolism. Hypokalemia is the main adverse reaction of gossypol [33]. In this study, the plasma K content of laying hens showed a linear decrease with the increase in DGCP replacement level. This may be due to gossypol damaging mitochondria, interfering with other enzyme systems related to oxidative phosphorylation, and thus, affecting the active transport of K [34]. TG content is an important indicator of liver function [35], which reveals the development and deposition of fat and also reflects fat metabolism activity [36]. TG and TC synthesized by the liver are transported from the liver to peripheral tissues in the form of lipoproteins through the blood. This study showed that the plasma TG and TC contents in the 100% replacement group were significantly reduced, possibly due to the inhibition of lipid synthesis by gossypol, leading to a decrease in lipoprotein secretion [4]. It may also be due to a decrease in fatty acid synthesis in the liver [37].

The intestine is an important site for animal digestion and absorption [38]. Intestinal morphology is an important indicator for evaluating intestinal health [39,40], and VH, CD, and VH/CD reflect the digestion and absorption of nutrients [41]. The higher the VH, the larger the contact area of feed digestion and absorption, which is more conducive to the digestion and absorption of nutrients. The depth of the crypt reflects the rate of morphological development of villous epithelial cells. Shallow crypts show accelerated cell maturation and enhanced secretory function [42]. The VH/CD mainly reflects the net absorption of the intestine [43]. Wang et al. found that adding DGCP (FG 56.50 mg/kg) to the basal diet of laying hens significantly reduced the VH compared to the SBM group [20]. Similarly, in this study, the 100% replacement group showed a significant decrease in VH, a significant increase in CD, and a significant decrease in VH/CD, which reduced the digestive and absorptive capacity of laying hens.

The liver is the main organ for FG metabolism and also a sensitive target organ for anti-nutritional factors [20,44]. The integrity of liver tissue structure is the basis for maintaining normal liver function. Liver steatosis and cell swelling are typical pathological symptoms of animal gossypol poisoning. In this study, we did not observe significant pathological changes in the liver in all groups. This is consistent with previous reports. Wang et al. found that adding DGCP (FG 56.50 mg/kg) to the diet of laying hens did not create significant changes in the liver [20]. He et al. found that adding 189.0 g/kg DGCP to the diet of laying hens did not affect the histological characteristics of the liver after 12 weeks of feeding [16]. The chicken fallopian tubes are composed of five parts: the infundibulum, magnum, isthmus, uterus, and vagina. They are important reproductive organs and the main site for egg formation. The magnum is the longest component of the fallopian tube, which is the site for protein formation and secretion during egg formation. He et al. found that adding 189.0 g/kg DGCP to the diet of laying hens did not affect the histological characteristics of the magnum of the fallopian tubes after 12 weeks of feeding [16]. This is consistent with the results of this study, in which no significant pathological changes were observed in the magnum of the fallopian tubes in each group. When FG reaches a certain amount, it can damage the testicles of roosters, inhibit the secretion of estradiol and progesterone from mature follicles in hens, cause ovarian atrophy, egg rupture, and even loss of ovulation function and reproductive ability. The results of this study showed that no significant pathological changes were observed in the ovaries of each group, indicating that the FG content in this experiment did not reach the level that causes ovarian damage.

The toxicity of FG is mainly produced by active aldehyde groups and hydroxyl groups. When ingested excessively or for a long time in monogastric animals, it can lead to a large accumulation of FG in the animal body, resulting in poisoning and even death [45]. He

et al. showed that no residual FG was detected in the liver and eggs of laying hens in each treatment group (FG content of 7.5, 14.75, 21.63, and 28.35 mg/kg, respectively) where DGCP replaced SBM [16]. The results of this study are consistent with previous studies, and no residual FG was detected in the liver and egg yolk of laying hens in all groups. However, Yuan et al. added 6%, 8%, and 10% puffed CSM instead of SBM to the diet of 40-week-old laying hens, where the FG in the liver and egg yolk significantly increased with the increase in substitution ratio [46]. The inconsistency with the results of this study may be due to the fact that the gossypol content in the diet of this study is within the tolerance range of the laying hens, which is ultimately metabolized by the body and therefore not accumulated.

The balance of intestinal microflora is very important to maintain the health of the body; the cecum microorganisms are the key components of the intestinal microflora, and food intake, body development, and the immune system have an important regulatory role [47,48]. The microflora of the poultry cecum is of interest because of its highest microbial cell density and diversity and the longest retention time of digestive juices in the gastrointestinal tract [49]. Alpha diversity is commonly used to analyze the diversity of microbial communities within a sample and reflects the richness, evenness, and diversity of microbial communities [50]. The Chao1 and ACE indices reflect community richness, and the Shannon and Simpson indices reflect community diversity. In this study, we found that Chao 1, ACE, and the Shannon index were significantly increased in the 25% and 75% treatment groups and were not significantly different from the 50% treatment group, indicating that DGCP increased the species richness and diversity of cecum microorganisms. A similar result was reported by Tegtmeier et al., who found that feeding black gadfly larvae with cottonseed press cake showed higher α -diversity than larvae reared on chicken feed [51]. The low α -diversity in the control and 100% treatment groups is speculated to be due to the naturally occurring anti-nutritional factors in the control soybean meal and the residues of cotton phenol in the 100% treatment group, limiting the colonization of certain microorganisms. There are fewer studies on the effect of CSM on the intestinal flora of egg-laying poultry, and in the present study, Bacteroidota and Firmicutes were the dominant flora, which is consistent with previous studies in poultry [52]. The DGCP diet increased the relative abundance of Bacteroidota and Bacteroides in the cecum compared to the control, speculating that it may be that the more complex and polymerized fiber structure of DGCP compared to soybean meal may be a better fit for the substrate preference of the phylum Bacteroidota [53]. Fusobacteriota was significantly reduced and Desulfobacteriota was significantly increased in the 100% treatment group; Zhang et al. showed that Fusobacteriota metabolizes carbohydrates into short-chain fatty butyrate, which provides many benefits to the host, including supplying energy to gastrointestinal cells, exerting anticancer effects, and providing anti-inflammatory properties [54]. It is speculated that the significant increase in Desulfobacteriota may be due to the higher sulfur-containing amino acids (e.g., methionine and cysteine) in DGCP that provide key substrates for Desulfobacteriota. Combined with the results of this study and those of previous researchers, it is suggested that the safe additive limit of DGCP in laying hens' diets should be controlled at 114.6 g/kg (based on $\text{FG} \leq 400$ ppm diet), and this limit needs to be dynamically adjusted according to the source of DGCP, the breed of the poultry, and the age of the day.

5. Conclusions

In conclusion, the 100% DGCP group had a negative impact on the laying performance, egg quality, and intestinal morphology of laying hens. The dietary addition of DGCP can reach up to 114.6 g/kg without adverse effects on egg production performance, egg quality, or the health of laying hens.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest: Authors Sharina Qi, Xiaojuan Bi, Chao He and Xuanming Chen were employed by the company Sichuan Sundaily Farm Ecological Food Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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