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Responses of intestinal morphology, immunity, antioxidant status and cecal microbiota to the mixture of glycerol monolaurate and cinnamaldehyde in laying hens

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ABSTRACT This study was to determine the effects of the mixture of glycerol monolaurate and cinnamaldehyde (**GCM**) supplementation on the intestinal morphology, immunity, antioxidant status and cecal microbiota of laying hens. A total of 1,120 healthy laying hens (Jingfen-1 strain) at the age of 14 wk were randomly divided into 4 groups with 10 replicates of 28 layers in each and layers were fed diets containing 0 (control group), or 250, 500, and 1,000 mg/kg GCM for 12 wk. The results showed that dietary supplementation with GCM significantly increased intestinal villus height and villus height/crypt depth, duodenal villus area, total superoxide disumutase activities in the liver and jejunum, jejunal glutathione peroxidase activities while decreased duodenal and jejunal crypt depth, hydrogen peroxide content in the liver and jejunal malondialdehyde content of laying hens aging 28 wk ($P < 0.05$). Meanwhile, GCM addition significantly increased serum immunoglobulin A and immunoglobulin M concentration of layers at the age of 20, 24, and 28 wk ($P < 0.05$). Moreover, it was observed in the 16S rRNA sequencing that the addition of GCM elevated the abundance and diversity of gut microbiota in laying hens. The predominant bacteria from each group were *Bacteroidota* and *Firmicutes* at the phylum level and *Bacteroides* and *Lactobacillus* were the dominant genera. The composition and structure of cecal microflora were changed by the addition of GCM to the diet of laying hens. In conclusion, the addition of GCM (500−1,000 mg/kg diet) can improve intestinal morphology, immune function, intestinal and liver antioxidant status and intestinal flora of laying hens, thereby improving intestinal digestion and absorption capacity. These findings provide a new way to further explore the mechanism of GCM improving intestinal health.

Key words: glycerol monolaurate, cinnamaldehyde, intestinal morphology, cecal microbiota, laying hen

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INTRODUCTION

Because drug residues and antimicrobial resistance caused by misuse and abuse of antibiotics are harmful to human health (Lindberg, 2014; Kurjogi et al., 2019), the use of antibiotics for growth promotion purposes has been explicitly banned in China in 2020. The substitute of antibiotics, natural plant substitutes, have become a research hotspot, as a green additive to improve the production performance of animals (Hafeez et al., 2023).

Glycerol laurate (**GML**), synthesized from lauric acid and glycerol (Marta et al., 2002), is a plant-derived food additive permitted by the United States Food and Drug Administration (Jiang et al., 2018; Welch et al., 2020). GML has excellent antiviral, antioxidant, antibacterial and immune enhancement effects (Seleem et al., 2016; Jackman et al., 2020). Dietary GML supplementation can alleviate inflammation, promote immunity and intestinal barrier function, and regulate the abundance of cecal flora (Cui et al., 2023). Cinnamaldehyde is one of the active components in *Cinnamomum cassia*, which has a series of pharmacological effects such as antifungal, antibacterial and anti-tumor (Kaur et al., 2019). Dietary

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supplementation with oregano essential oil (200 mg/kg) and cinnamaldehyde (20 mg/kg) could improve production performance and reduce feed conversion efficiency of laying hens in later period by selectively regulating cecal microbial community (Gao et al., 2022).

It has been reported that intestinal microbes are closely related to intestinal morphology, structure, nutrient absorption and homeostasis maintenance (Subramanian et al., 2014), and play an important regulatory role in improving intestinal health, barrier function and growth performance in the host (Wang et al., 2016). However, the gut microbiota is also influenced by various factors, such as nutritional factors, genetic factors, management factors, and environmental factors (Wang et al., 2020). Studies have shown that GML or cinnamaldehyde supplemented in feed can increase the abundance of intestinal flora and improve gut microbiota of layers or broilers (Gao et al., 2022; Cui et al., 2023; Zheng et al., 2023). However, the effect of GML and cinnamaldehyde complex on intestinal flora of laying hens remains unclear.

It was found in our previous study that glycerol monolaurate and cinnamaldehyde mixture (**GCM**) addition can significantly improve egg quality and serum antioxidant status in laying hens (Li et al., 2024). Therefore, the purpose of this research was to evaluate the responses of intestinal morphology, immunity, antioxidant status and cecal microbiota to the GCM addition in laying hens, and to provide a new way to further explore the mechanism of GCM improving intestinal health.

MATERIAL AND METHODS

Animals, Diets, and Experimental Design

A total of 1,120 healthy laying hens (Jingfen-1 strain; body weight: 1,191.31 ± 42.17 g; egg production rate at the age of 16 wk: 16.09 ± 5.70 %) at the age of 14 wk were randomly divided into 4 groups with 10 replicates of 28 layers in each and layers were fed diets containing 0 (control group), or 250, 500, and 1,000 mg/kg GCM (powder, 80% GML, 5.4% cinnamaldehyde, and 0.6% thymol, Calid biotech (Wuhan) Co., Ltd., Wuhan, China), according to the studies (Zhao et al., 2019; Cui et al., 2023). The experiment lasted 14 wk and included a 2-wk acclimation period and a 12-wk experimental period. The trial used a corn-soybean meal based diet (Table 1) formulation that met or exceeded the nutritional requirements of the NRC (1994). During the trial, all layers were provided with an enclosed, ventilated and conventional house that maintained a stable temperature (25 ± 2 °C), relative humidity (60−70 %) and lighting program with the rule of increasing 0.5 h/wk from 12 h of light (17 wk of age) to 16 h of light (25 wk of age) (Liu et al., 2023). Feed and water were offered *ad libitum*.

Table 1. Composition of the experimental basal diet.

Items	Value
Ingredient (%)	
Corn (7.8% CP)	39.25
Wheat	30.00
Soybean meal (43.0% CP)	15.50
Cottonseed meal (43.0% CP)	5.00
Fish meal	0.50
Soy oil	0.50
Dicalcium phosphate	0.70
Limestone	8.00
Sodium chloride	0.20
L-Lys-HCl (78.0%)	0.05
DL-Met (99.8%)	0.10
Premix[1]	0.20
Total	100
Nutrient levels[2] (%)	
Metabolism energy (MJ/kg)	11.26
Crude protein	16.40
Calcium	3.49
Available phosphorus	0.30
Lysine	0.91
Methionine	0.41
Threonine	0.66

[1]The premixes provided the following per kg of diet: Vitamin A 7,500 IU, Vitamin D_3 2,500 IU, Vitamin E 25 mg, Vitamin K_3 2.5 mg, Vitamin B_1 1.5 mg, Vitamin B_2 4.5 mg, Vitamin B_6 3 mg, Vitamin B_{12} 0.02 mg, niacin 25 mg, folic acid 1.1 mg, calcium pantothenate 8 mg, biotin 0.12 mg, choline chloride 400 mg, Cu 20 mg, Mn 60 mg, Zn 80 mg, Fe 90 mg, I 0.8 mg, Se 0.3 mg.

[2]Metabolism energy was calculated value. Others were analyzed values.

Sample Collections

At the age of 20, 24, and 28 wk, 10 hens per group were randomly selected. Blood samples from wing vein were collected in a coagulant tube and centrifuged for 10 min (3,000 × *g*, 4°C). Serum was collected and stored at −80 °C for immunoglobulin level analysis. At the 28 wk of age, the laying hens (10 birds per group) were randomly selected to be weighed after fasting for 12 h and slaughtered by cervical dislocation method (Li et al., 2022a). The liver, spleen and abdominal fat were separated and weighed, and the liver index, spleen index and abdominal fat index were calculated according to the method reported by Shi et al. (2024). Duodenum, jejunum and ileum were separated and washed with PBS. Parts of the duodenum, jejunum and ileum (1 cm) isolated were fixed in 4% paraformaldehyde (Kong et al., 2021). Another part of the jejunum (1 cm) isolated and 2 g liver were collected and placed in liquid nitrogen for antioxidant status determination.

Intestinal Morphology

The small intestine segment fixed with paraformaldehyde was dehydrated with ethanol and xylene, then immersed in paraffin, cut into 5 μm thick sheets, and finally stained with hematoxylin and eosin for histomorphological observation. According to the previously reported method (Li et al., 2019), 10 intact villi were randomly selected under a DM3000 microscope (Leica, Wetzlar, Hessen), villi height (**VH**), crypt depth (**CD**), and villi area were measured, and villi height/crypt depth (**VH/CD**) was calculated.

Immunity and Antioxidant Status

According to the manufacturer's instructions, serum levels of immunoglobulin A (**IgA**), immunoglobulin G

(**IgG**) and immunoglobulin M (**IgM**) were measured by ELISA kit to estimate the immune status of laying hens. The tissue homogenates of liver and jejunum samples were prepared with PBS at the ratio of 1:9 (W [g]: V [mL]), and then the supernatant was precipitated by centrifugation at 1,000 g at 4 °C for 10 min to detect the antioxidant capacity of liver and jejunum of layer hens. The antioxidant status was determined by the content of malondialdehyde (**MDA**), hydrogen peroxide ($\mathbf{H_2O_2}$) and the activity of total superoxide dismutase (**T-SOD**), glutathione peroxidase (**GSH-Px**) and catalase (**CAT**) using a commercial kit (Nanjing Institute of Jiancheng Bioengineering, Nanjing, China). Both immune and antioxidant status were measured using a microplate reader (Bio-Tek ELX800, Biotek Instruments, Inc., Winooski, VA).

Microbial Sequencing and Analysis

The cecum chyme was collected, and sequenced and analyzed according to the method of Zhang et al. (2018). According to the manufacturer's instructions, the QIAamp fecal rapid DNA test kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) was used for the extraction of total DNA from cecal chyme samples from all groups. Nanodrop 2000 (Thermo Scientific, Wilmington, DE) and 1% agrose gel electrophoresis were used to measure DNA concentration and quality. Eligible DNA samples were amplified using 16S rRNA gene variable region V3-V4 specific bacterial primers. The amplification primers used were 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′). The purified amplicons were pooled in equimolar and then sequenced on the Hiseq 2500 PE250 at the Beijing Nuohe Zhiyuan Bio-Information Technology Co., Ltd., China. The α diversity (Chao1, Shannon index, Simpson index) and β diversity (weighted_unifrac) are analyzed using Qiime software (Qiime2-2019.7, Nature Biotechnology). Relevant materials were uploaded to NCBI (accession: PRJNA1062743).

Statistical Analysis

The results of intestinal morphology, organ indexes, serum immunity, antioxidant status and α diversity were all tested by one-way analysis of variance (**ANOVA**) using SPSS 20.0, followed by the Turkey test for multiple comparisons. The results were presented as means ± SEM. Values of $P < 0.05$ were considered significant. The GraphPad Prism 8.0 were used to make all the graphs.

RESULTS

Intestinal Morphology and Organ Index

As shown in Figure 1 and Table 2, duodenal and jejunal VH and VH/CD significantly increased ($P < 0.05$) in the all GCM groups compared with the control group

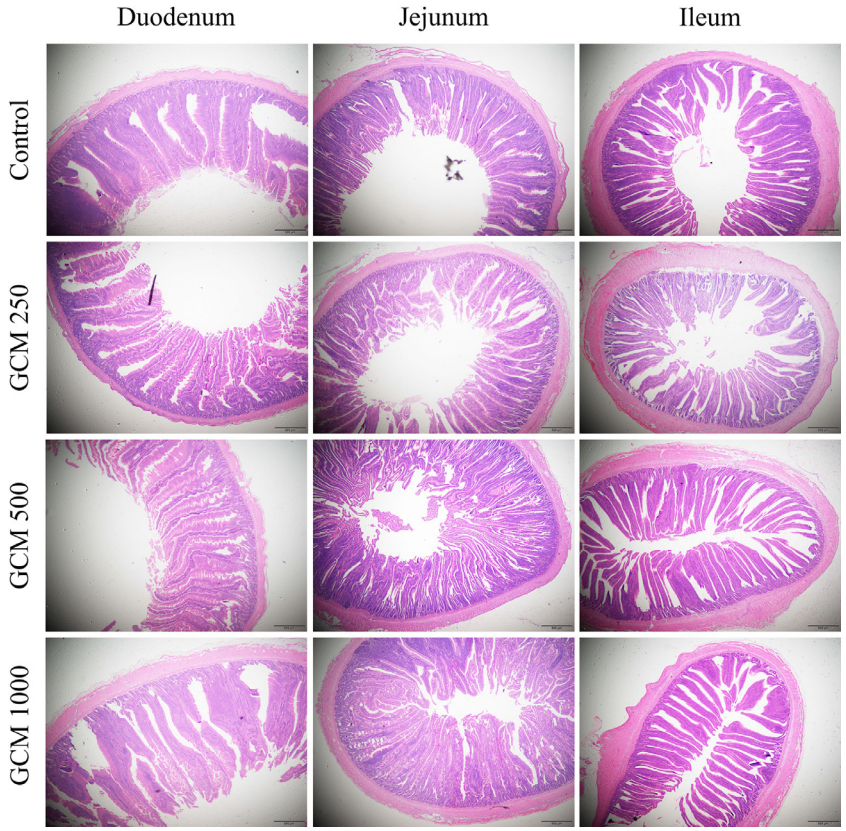

Figure 1. The morphology of duodenum, jejunum and ileum in laying hens fed with basal diets supplemented with 0 (control), 250, 500, and 1,000 mg/kg GCM (Scale bar: 500 mm). GCM, the mixture of glycerol monolaurate and cinnamaldehyde.

Table 2. The effects of GCM supplementation on intestinal morphology of laying hens at the age of 28 wk.[1]

Items	Control	GCM[2], mg/kg			SEM	*P*-value
		250	500	1,000		
Duodenum						
VH[2], μm	1,022.75^{c}	1,188.16^{b}	1,291.87^{ab}	1,418.54^{a}	29.81	0.000
CD[2], μm	257.60^{a}	189.61^{b}	203.31^{b}	223.60^{ab}	6.37	0.000
VH/CD[2]	4.25^{b}	6.53^{a}	7.02^{a}	6.98^{a}	0.27	0.000
Villus area, μm^2	0.17^{b}	0.28^{ab}	0.40^{a}	0.28^{ab}	0.03	0.039
Jejunum						
VH, μm	853.04^{b}	1,062.71^{a}	1,050.51^{a}	1,059.47^{a}	23.87	0.001
CD, μm	192.55^{a}	130.38^{b}	123.61^{b}	108.49^{b}	7.01	0.000
VH/CD	4.60^{b}	8.11^{a}	9.64^{a}	10.47^{a}	0.60	0.000
Villus area, μm^2	0.12	0.16	0.15	0.16	0.01	0.092
Ileum						
VH, μm	622.85^{c}	676.76^{bc}	826.60^{ab}	877.61^{a}	30.94	0.005
CD, μm	141.04	135.26	131.98	110.75	5.39	0.215
VH/CD	4.63^{b}	5.15^{b}	6.24^{b}	8.31^{a}	0.36	0.000
Villus area, μm^2	0.08	0.08	0.11	0.09	0.00	0.159

[1]Data are means and SEM (n = 10). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ($P < 0.05$).
[2]GCM: the mixture of glycerol monolaurate and cinnamaldehyde; VH: villus height; CD: crypt depth; VH/CD: villus height/crypt depth.

Table 3. Effects of GCM supplementation on organ indexes of laying hens at the age of 28 wk.[1]

Items	Control	GCM[2], mg/kg			SEM	*P*-value
		250	500	1,000		
Liver index	18.90	18.96	18.98	17.09	0.47	0.406
Spleen index	1.34	1.04	1.06	1.01	0.03	0.972
Abdominal fat index	26.56	26.31	26.90	27.46	1.34	0.992

[1]Data are means and SEM (n = 10). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ($P < 0.05$).
[2]GCM: the mixture of glycerol monolaurate and cinnamaldehyde.

for laying hens aged 28 wk. Compared with the control group, 500 mg/kg GCM groups significantly reduced ($P < 0.05$) duodenal and jejunal CD and increased ($P < 0.05$) duodenal villus area. Moreover, the group supplemented with 1,000 mg/kg GCM had the increased VH and VH/CD ($P < 0.05$) in ileum of laying hens. However, compared with the control group, GCM supplementation had no significant effect on liver index, spleen index and abdominal fat index of laying hens aged 28 wk (Table 3).

Immunity

As shown in Table 4, serum IgA concentration of 20-wk-old laying hens was significantly increased ($P < 0.05$) in the 500 and 1,000 mg/kg GCM groups. At 24 wk of age, 500 mg/kg GCM significantly increased ($P < 0.05$) serum IgA and IgM concentrations. At 28 wk of age, the serum IgM concentration in the 1,000 mg/kg GCM group was the highest, which was significantly higher than that in the control group ($P < 0.05$).

Antioxidant Status

Compared with the control group, laying hens fed the diets with 500 mg/kg GCM at the age of 28 wk had the significant increased ($P < 0.05$) T-SOD activities in the liver and jejunum (Table 5). The 1,000 mg/kg GCM group had the lowest H_2O_2 content in the liver and MDA content in the jejunum ($P < 0.05$). Furthermore, GCM supplementation significantly increased ($P < 0.05$) the jejunal GSH-Px activities in laying hens.

Table 4. Effects of GCM supplementation on the concentration of serum immunoglobulins of laying hens at the age of 20, 24, and 28 wk.[1]

Items	Control	GCM[2], mg/kg			SEM	*P*-value
		250	500	1,000		
20 wk						
IgA[2], mg/L	32.67^{b}	42.38^{ab}	46.89^{a}	42.81^{a}	1.60	0.005
IgG[2], mg/L	147.60	174.98	171.26	170.78	4.28	0.083
IgM[2], mg/L	58.48	72.49	57.30	60.33	2.67	0.158
24 wk						
IgA, mg/L	32.53^{b}	40.23^{b}	51.97^{a}	39.14^{b}	1.89	0.000
IgG, mg/L	154.97	182.89	169.71	170.88	5.78	0.424
IgM, mg/L	68.49^{b}	105.58^{ab}	128.95^{a}	111.11^{ab}	7.46	0.013
28 wk						
IgA, mg/L	32.35	38.50	37.89	35.01	1.35	0.124
IgG, mg/L	859.24	840.40	955.46	836.01	33.90	0.608
IgM, mg/L	146.20^{b}	210.20^{ab}	237.54^{ab}	258.03^{a}	14.69	0.025

[1]Data are means and SEM (n = 10). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ($P < 0.05$).
[2]GCM: the mixture of glycerol monolaurate and cinnamaldehyde; IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M.

Intestinal Microbiota

For α diversity, Chao1 index and Shannon index of 1,000 mg/kg GCM group were significantly higher than those of control group and 250 mg/kg GCM group (Figure 2A, B). Simpson index showed that species uniformity was highest in 1,000 mg/kg GCM group, reaching a maximum of 0.99 (Figure 2C). Principal coordinate analysis (**PCoA**) results showed that there were significant differences between the microflora of the GCM addition groups and the control group. The number of OTUs increased gradually with the increase of

Table 5. Effects of GCM supplementation on antioxidant status in liver and jejunum of laying hens at the age of 28 wk.[1]

Items	Control	GCM[2], mg/kg			SEM	*P*-value
		250	500	1,000		
Liver						
T-SOD[2], U mg/prot	1,418.27^{b}	1,642.26^{ab}	1,770.81^{a}	1,390.72^{b}	43.10	0.001
GSH-Px[2], U mg/prot	30.50	31.88	33.28	34.50	0.89	0.434
CAT[2], U mg/prot	4.59	4.54	4.64	4.64	0.15	0.995
MDA[2], nmol mg/prot	1.34	0.94	1.18	0.72	0.08	0.129
H_2O_2[2], nmol mg/prot	35.88^{a}	34.62^{a}	30.64^{ab}	23.09^{b}	1.49	0.006
Jejunum						
T-SOD, U mg/prot	549.13^{b}	571.01^{ab}	655.83^{a}	556.72^{b}	14.02	0.019
GSH-Px, U mg/prot	25.70^{c}	37.87^{b}	36.86^{b}	129.38^{a}	7.63	0.000
CAT, U mg/prot	3.64	4.34	4.38	4.19	0.11	0.055
MDA, nmol mg/prot	1.18^{a}	1.01^{ab}	1.09^{a}	0.69^{b}	0.05	0.002
H_2O_2, nmol mg/prot	31.34	28.11	32.66	29.86	1.55	0.767

[1]Data are means and SEM (n = 10). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ($P < 0.05$).

[2]GCM: the mixture of glycerol monolaurate and cinnamaldehyde; T-SOD: total superoxide dismutase; GSH-Px: glutathione peroxidase; CAT: catalase; MDA: malondialdehyde; H_2O_2: hydrogen peroxide.

GCM addition concentration, and the number of OTUs reached the highest in 1,000 mg/kg GCM group (Figure 2D).

The GCM supplementation changed the composition of cecal microbiota of laying hens. The predominant bacteria from each group were *Bacteroidota* and *Firmicutes*

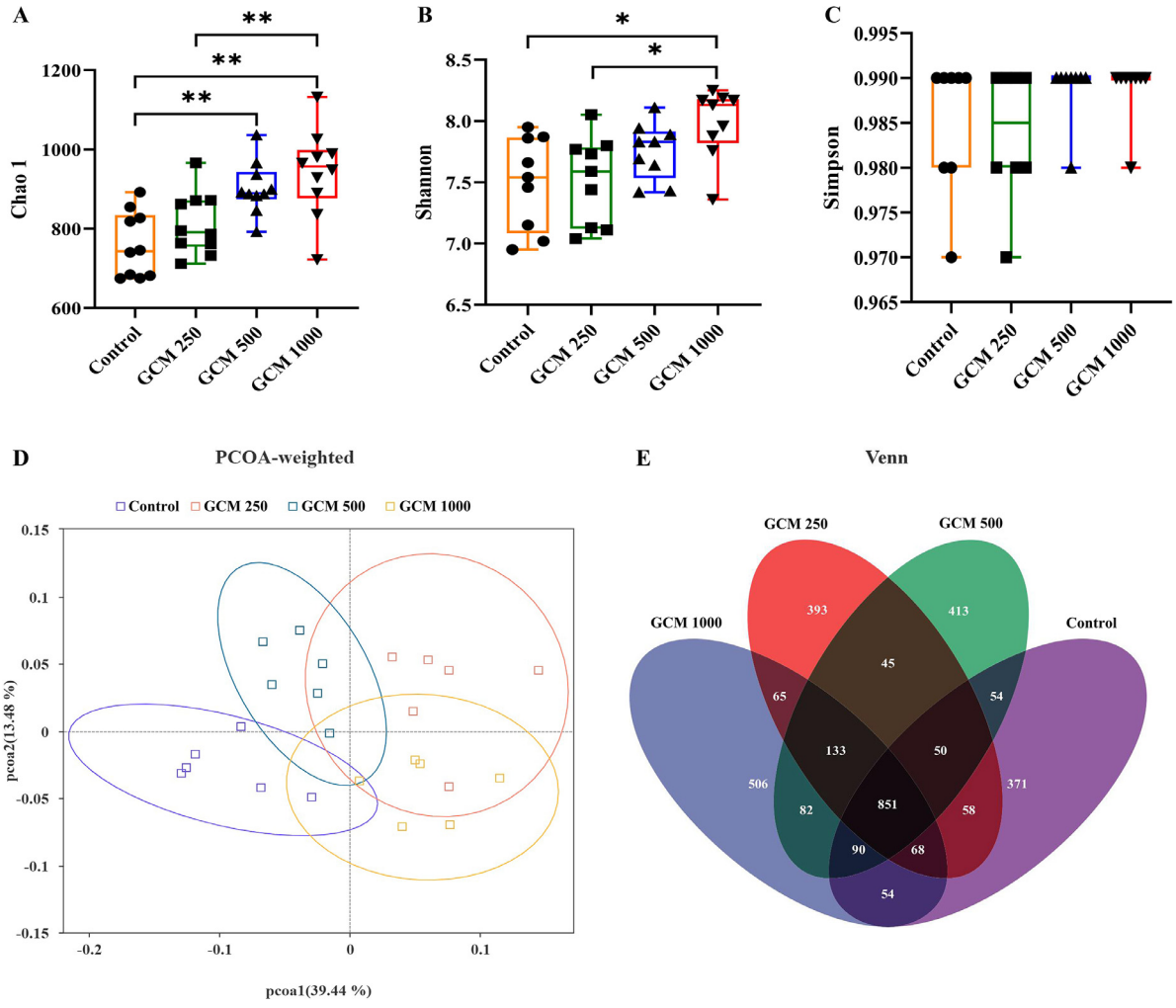

Figure 2. Effects of the mixture of glycerol monolaurate and cinnamaldehyde (GCM) on cecum microbiota diversity and composition in layers. (A) Chao 1 diversity index boxplot; (B) Shannon diversity index boxplot; (C) Simpson diversity index plot; (D) The principal coordinate analysis (PCoA) of the cecum microbiota based on unweighted UniFrac metric; (E) Venn diagrams of amplicon sequence variant (ASV) distributions in layers fed with basal diets supplemented with 0 (control), 250, 500, and 1,000 mg/kg GCM (n = 10 each group). GCM, the mixture of glycerol monolaurate and cinnamaldehyde.

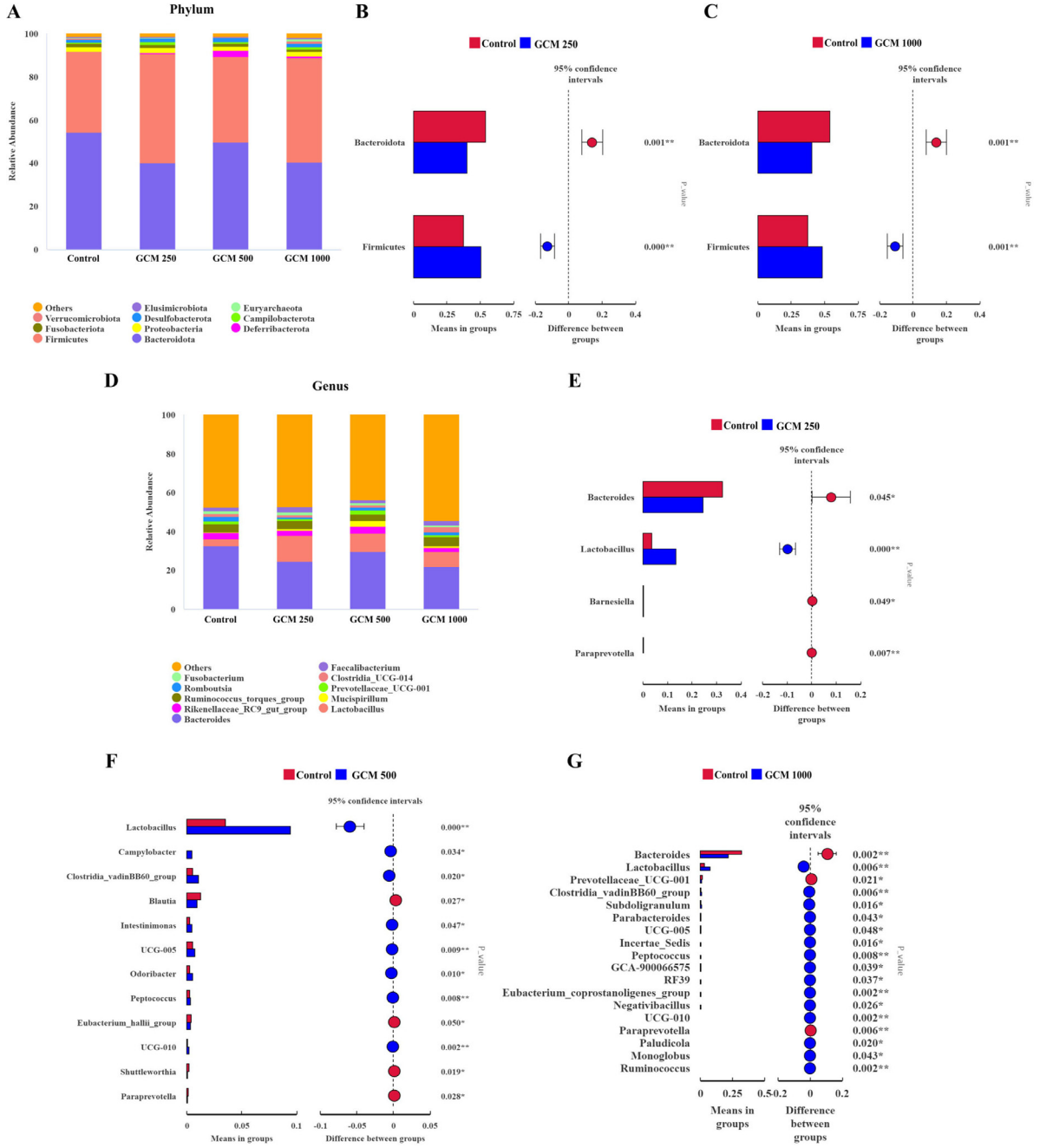

Figure 3. (A) Barplot difference analysis of cecal microbiota in phylum level of laying hens fed with diet supplemented with 250 mg/kg GCM; (B) Barplot difference analysis of cecal microbiota in phylum level of laying hens fed with diet supplemented with 1,000 mg/kg GCM; (C) The relative abundance of cecal microbiota at phylum level in laying hens; (D) Barplot difference analysis of cecal microbiota in genus level of laying hens fed with diet supplemented with 250 mg/kg GCM; (E) Barplot difference analysis of cecal microbiota in genus level of laying hens fed with diet supplemented with 500 mg/kg GCM; (F) Barplot difference analysis of cecal microbiota in genus level of laying hens fed with diet supplemented with 1,000 mg/kg GCM; (G) The relative abundance of cecal microbiota at genus level in laying hens. n=10 hens per group. GCM, the mixture of glycerol monolaurate and cinnamaldehyde.

at the phylum level and *Bacteroides* and *Lactobacillus* were the dominant genera (Figures 3A and 3D). It was found that the relative abundance of *Firmicutes* significantly increased and the relative abundance of *Bateroidota* decreased in the 250 mg/kg and 1,000 mg/kg GCM groups (Figures 3B and 2C). Moreover, the relative abundance of *Lactobacillus* increased significantly and the relative abundance of the *Bacteroldes, Barnesiella* and *Pareprevotella* decreased significantly in the 250 mg/kg GCM group (Figure 3E). The 500 mg/kg GCM group had the increased relative abundance of the *Lactobacillus, Campylobacter, Clostridia_vadinBB60_group, Intestinimonas, UCG-005, Odoribacter, Peptococcus* and *UCG-010* and the decreased relative abundance of the *Blautia, Eubacterium_hallii_group, Shuttleworthia* and *Parapervotella* Compared with the control group (Figure 3F). It was noticeable that most microorganisms in the 1,000 mg/kg GCM group significantly increased including *Lactobacillus, Closridia_vadinBB60_group, Subdoligranulum, Parabacteroides, UCG-005, Incertae_Sedis, Peptococcus, GCA-900066575, RF39, Eubacterium_coprostanoli-genes_*

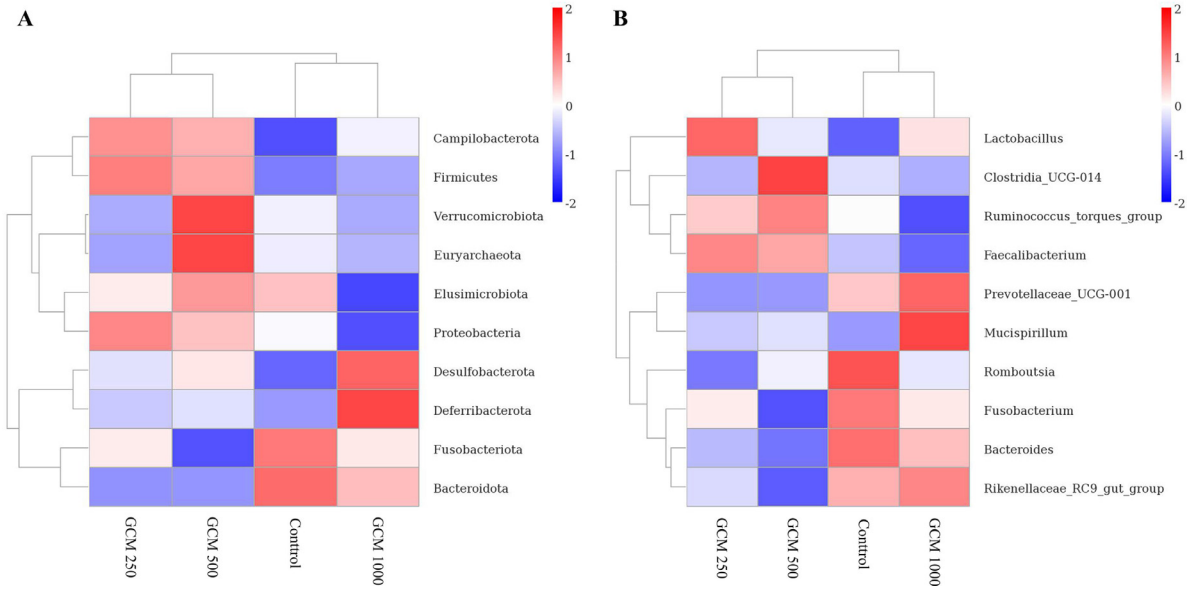

Figure 4. Clustering heat map of cecal microbiota at phylum level (A) and genus level (B) of laying hens between groups. The horizontal axis represents the group, and the vertical axis represents one genus per row. The heatmap clusters high and low abundance species into blocks based on color variation and similarity, demonstrating similarity and variation between groups. n = 10 hens per group. GCM, the mixture of glycerol monolaurate and cinnamaldehyde.

group, Negativibacillus, UCG-010, Paludicola, Monoglobus and *Ruminococcus,* and significantly decreased including *Bacteroides, Prevotellaceae_UCG-001* and *Paraprevotella* (Figure 3G). At the phylum level, 500 mg/kg GCM group increased the relative abundance of *Campilobacterota, Firmicutes, Verrucomicrobiota, Euryarchaeota* and *Proteobacteria*, and reduced *Fusobacteriota* and *Bacteroidota* compared with the control group (Figure 4A). At the genus level, 500 mg/kg GCM group increased the relative abundance of *Clostridia_UCG-014, Ruminococcus_torques_group* and *Fecalibacterium*, and reduced *Prevotellaceae_UCG-001, Romboutsia, Fusobacterium, Bacteroides* and *Rikenellaceae_RC9_gut_group* compared with the control group (Figure 4B).

Difference in cecal microbiota in layers fed with basal diets supplemented with 0 (control), 250, 500, and 1,000 mg/kg GCM using Linear discriminant analysis (**LDA**) effect size (**LEfSe**) (LDA score > 4) (Figure 5). The results showed that *Bacteroidota* (from phylum to genus) *s_Bacteroides_salanitronis*, and *Prevotellaceae* were significantly enriched in the control group. In the 250 mg/kg GCM group, *Firmicutes, Bacilli, Lactobacillale* (from order to genus), *Oscillospirales*, and *Ruminococcaceae* were detected to be significantly enriched. In the 500 mg/kg GCM group, *Deferribacterales* (from phylum to family), *Mucispirillum*, and *s_Bacteroides_caecicola* were significantly enriched. In the 1,000 mg/kg GCM group, *Clostridia* and *Lachnospirales* (from order to family) were significantly enriched.

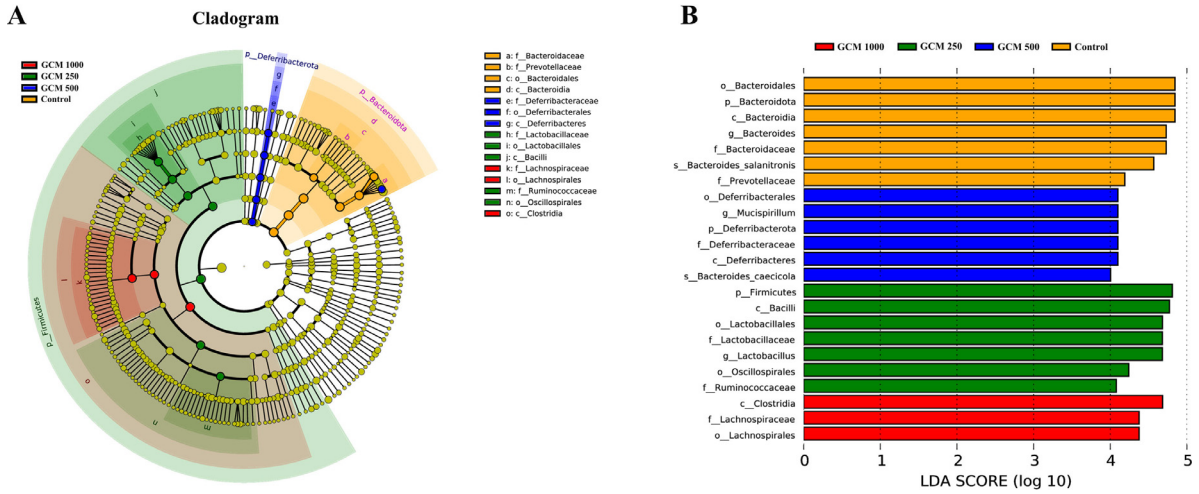

Figure 5. Differential cecal microbiota in layers fed with basal diets supplemented with 0 (control), 250, 500 and 1,000 mg/kg GCM (n = 10 each group) were determined by linear discriminant analysis Effect Size (LEfSe) analysis. GCM, the mixture of glycerol monolaurate and cinnamaldehyde.

DISCUSSION

Nutrient absorption of poultry is closely related to intestinal villus morphology. When the intestinal VH is higher, the CD is smaller and the VH/CD is larger, indicating the ability to absorb nutrients is stronger (Zhang et al., 2005). An increase in the intestinal VH can increase the absorption area, increase the number of mucosal glands and enzymes production resulting in better digestion and absorption of nutrients (Mohan et al., 1996). Kong et al. (2023) reported that GML had a protective effect on intestinal morphology by improving the VH of the small intestine in challenged chicken embryos. Shao et al. (2023) reported that the essential oil containing 13.8% cinnamaldehyde can significantly increase the VH and the VH/CD in the ileum of piglets, thus promoting the development of intestinal epithelium. In the current study, it was observed that the combination of GML and cinnamaldehyde in the diet of laying hens significantly increased the VH, the VH/CD of small intestine, and the duodenal villus area, decreased the duodenal and jejunal CD, and thus improved the digestibility and absorption rate of feed.

Liver and spleen are the main immune organs of the body, so the immune status of the body can be reflected by measuring the relative weight of the immune organs, and greater weights of immune organs usually represent stronger immune functions to some extents (Ravis et al., 1988). In the current study, GCM addition had no significant effect on the organ (liver, spleen and abdominal fat) index of laying hens. Due to the important role of immunoglobulins in immune function, it has been widely accepted that immunoglobulins can be used to assess immune status (Liao et al., 2015). The results of this experiment indicated that dietary GCM supplementation can significantly increase the contents of IgA and IgM in serum. The contents of IgA in the early stage of the experiment and IgM in the later stage of the experiment are mainly increased. But the improvement of GCM on serum immunoglobulin level was not corresponding to no difference in immune organ index of laying hens. Therefore, the results of GCM on immune in laying hens need to be further studied.

It is well known that MDA is a secondary lipid oxidation product and is considered to be the main product in evaluating lipid peroxidation (Botsoglou et al., 2012). The activities of CAT, T-SOD and GPH-Px reflects the antioxidant status of the body (Wang et al., 2023). In the current study, GCM supplemented with 500 mg/kg or 1,000 mg/kg showed a significant increment in the activities of hepatic and jejunal T-SOD and jejunal GSH-Px and a decrease in content of hepatic H_2O_2 and jejunal MDA, which was consistent with the results of Zhao et al. (2019) and Li et al. (2022b). Interestingly, there is evidence that changes in intestine microbes regulate the metabolism of major intracellular antioxidants (GSH) in the host body (Mardinoglu et al., 2015). Therefore, it is necessary to investigate the changes of microbial composition through sequencing analysis of cecum microorganisms.

As one of the 3 intestinal barriers, intestinal flora can not only block the invasion of pathogenic bacteria, but also participate in food digestion, absorption and metabolism, body growth and development, immune suppression and activation (Xue et al., 2018; Gao et al., 2022). In this study, the diversity, richness and structure of intestinal flora were significantly different between the GCM addition groups and the control group, which may be related to the regulation of intestinal homeostasis by the addition of GCM. In addition, the relative abundance of *Firmicutes* and *Lactobacillus* in the all GCM supplementation groups were significantly increased compared with the control group. Colonized *Bacillus, Clostridium*, and *Lactobacillus* are often used as probiotics in the poultry industry (Liu et al., 2023). A study has shown that lactic acid bacteria, as a beneficial bacterium, can improve the feed efficiency of chickens (Yan et al., 2017), which is consistent with the results of our previous study that 1,000 mg/kg GCM supplementation can significantly improve the laying performance, egg quality and serum antioxidant status of laying hens (Li et al., 2024). It was found in this experiment that *Clostridia* was significantly enriched in 1,000 mg/kg GCM group using LEfSe. Short-chain fatty acids (**SCFA**) are known to reduce inflammation, eliminate drug-resistant pathogens, regulate key gene expression in the gut (Serino Matteo, 2019), and are important metabolites of C*lostridia* and other microbiota (Yao et al., 2020). *Clostridium* contains both beneficial bacteria (such as *Clostridium butyricum*) (Wang et al., 2020) and pathogenic bacteria (such as *Clostridium perfringens*) (Zhang et al., 2018). From its beneficial effect, studies have shown that the high relative abundance of C*lostridia* improves the absorption and utilization of nutrients in the gut (He et al., 2019), and *Bacillus* also improves nutrient utilization and intestinal morphology (Souza et al., 2021). These suggest that GCM supplementation may maintain the integrity of the intestinal barrier and stimulate the antioxidant status of the gut and the immune system by optimizing the composition of the gut microbiome and increasing the abundance of beneficial bacteria (*Lactobacillus* and *Clostridium*).

CONCLUSIONS

In conclusion, dietary GCM (500−1,000 mg/kg diet) effectively improved intestinal morphology, immune function, intestinal and liver antioxidant status and intestinal flora of laying hens, thereby improving intestinal digestion and absorption capacity. In addition, the mechanism of GML combined with cinnamaldehyde to improve intestinal digestion and absorption function in laying hens remains to be further studied.

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Ethical Approval: All experimental procedures were conducted with approval from the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (WPU202204007) (Wuhan, China).

DISCLOSURES

The authors declare that there are no conflicts of interest.

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