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Original Article

Ganoderma Lucidum Extract Regulates Gut Morphology and Microbial Community in Lipopolysaccharide-Challenged Broilers

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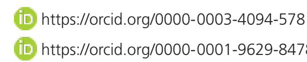
Chuang KB[I]  https://orcid.org/0000-0003-4094-5781
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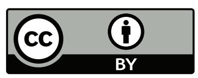
■Keywords

Broiler, Ganoderma lucidum,gut, lipopolysaccharide, microbiota.



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ABSTRACT

This study was conducted to investigate the effect of *Ganoderma lucidum* extract (GLE) on the gut morphology and cecal microbial community of broilers challenged with lipopolysaccharide (LPS). 144 one-day-old unsexed broiler chicks were randomly distributed into four treatments: non-challenged broilers fed a basal diet; LPS-challenged broilers fed a basal diet; LPS challenged broilers fed a basal diet supplemented with 1 mL/L of GLE in the drinking water; and LPS challenged broilers fed a basal diet supplemented with 1.33 mL/L of GLE in the drinking water. Results showed that supplementationwith1.33 mL/L of GLE alleviated intestinal inflammatory gene expression in LPS-challenged broilers ($p \leq 0.05$). Supplementation of GLE (1 and 1.33 mL/L) increased the villus height in the jejunum and ileum of LPS-challenged broilers ($p \leq 0.001$). Weighted principal coordinate analysis, heat map of species abundance, and microbial function pathway revealed distinct separation between the groups treated with LPS only and LPS in combination with GLE supplementation (1 and 1.33 mL/L). The abundance of the genus *Faecalibacterium* was increased in the cecal digesta of LPS-challenged broilers receiving GLE(1 and 1.33 mL/L) compared with the LPS challenge-only group ($p \leq 0.001$). The growth performance parameter of broilers was positively associated with the abundance of the genus *Faecalibacterium* in the cecal digesta. In conclusion, GLE supplementation could modulate gut morphology and cecal microbiota composition of broilers under inflammatory challenge.

INTRODUCTION

Broilers that are raised under the conditions of intensive farming and high stocking densities are exposed to immunological stress, leading to poorer feed conversion ratio and impaired growth (Zulkiflid *et al*., 2009; Wasti *et al*., 2020). Broilers under immunological stress are susceptible to pathogen infection due to an imbalance in immune response and cecal microbiota (Yang *et al*., 2011). In the past, antibiotics used as growth promoters were commonly administered to animals for the prevention of infectious diseases, thereby alleviating inflammatory response in broilers (Khan *et al*., 2021a). However, the European Union has banned the use of antibiotics as growth promoters in animal feed since 2006. Therefore, developing antibiotic-free solutions for reducing immunological stress and preventing pathogen infection in broilers is urgent.

It has been demonstrated that regulation of immune response through feed additives exerts beneficial effects on poultry health and growth (Kiczorowska *et al*., 2017; Alhotan *et al*., 2021; Hafeez *et al*., 2021; Khan *et al*., 2021b). *Ganoderma lucidum*, a medicinal fungus, is a potent immune modulator and exhibits several pharmacological

functions, including antiatherosclerotic, antioxidant, antiviral, and antitumor properties (Boh *et al*., 2007; Sanodiya *et al*., 2009). The polysaccharides purified from *G. lucidum* are able to regulate immune cell proliferation and cytokine production (Mao *et al*., 1999; Chen *et al*., 2004). Dietary supplementation of *G. lucidum* extract (GLE) in the diet or drinking water enhances the immunity of broilers (AL-Zuhariy & Hassan, 2017; Chen & Yu, 2020). GLE supplementation can ameliorate growth performance in broilers (Ogbe *et al*., 2008; Ogbe *et al*., 2009; Sofyan *et al*., 2012; Liu *et al*., 2016).

Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, has been shown to induce inflammatory responses in broilers (De Boever *et al*., 2008; De Boever *et al*., 2009; Chen & Yu, 2021). Systemic inflammation induced by LPS can reallocate nutrient utilization and disturb the cecal microbial composition, resulting in impaired growth of broilers (Yang *et al*., 2011; Liu *et al*., 2014; Chen & Yu, 2021).

The intestinal microbiota regulates several physiological responses in poultry, such as nutrient utilization, gut morphology, and immune response (Diaz Carrasco *et al*., 2019). Disruption of intestinal microbiota reduces nutrient metabolism and disrupts the immune system, leading to growth retardation in broilers (Dibner & Richards, 2005). LPS-induced inflammation not only cause damage to the gut morphology, they also disrupt the intestinal microbial composition (Metzler-Zebeli *et al*.,2020). Our previous study demonstrated that GLE supplementation in drinking water regulates the immune system and gut microbiota in broilers (Chen & Yu, 2020). However, it remains unclear whether GLE has the ability to normalize the gut microbiota imbalance induced by LPS in broilers.

Therefore, the objective of the current research was to determine the effect of GLE on the gut morphology and cecal microbial community in broilers challenged with LPS. These findings could provide a theoretical basis for the amelioration of inflammation-induced gut microbiota disturbance and new insight for the application of GLE as a potential alternative to antibiotics to improve poultry production efficiency.

MATERIALS AND METHODS

G. lucidum extract

The GLE from powdered fruiting bodies (53 mg/mL, Life Rainbow Biotech, Yilan, Taiwan) was prepared using a hot water extraction method and polysaccharide concentration in GLE was verified using phenol-sulfuric acid. The polysaccharide quantity in GLE was 3 mg/mL (Chen & Yu, 2020).

Animal experiment

The experimental protocols were in accordance with guidelines set by National Ilan University Institutional Animal Care and Use Committee (IACUC, protocol number 109-9). A total of 144 one-day-old healthy unsexed Ross 308 broiler chicks (with average body weight of 47.4 ± 0.17 g)were obtained from a commercial hatchery. The chicks were allocated to four treatments and six replicates each containing six birds (36 broilers per group), in a completely randomized design. Broilers were reared in stainless-steel and temperature-controlled cages (89 cm × 56.5 cm × 60 cm).The experimental diets were (1) non-challenged broilers fed a basal diet (C); (2) LPS-challenged broilers fed a basal diet (L); (3) LPS challenged broilers fed a basal diet supplemented with 1 mL/L of GLE (LL)in the drinking water; and (4) LPS challenged broilers fed a basal diet supplemented with 1.33 mL/L of GLE (HL) in the drinking water. GLE was supplied in the chickens' drinking water during the entire period. At 14, 16, 18, 20 d of age, broilers were intraperitoneally injected with LPS (serotype 0111:B4, Sigma-Aldrich, St. Louis, MO, USA) at a dosage of 5 mg/kg of body weight (L, LL, and HL group) or equivalent volume of 0.9% sterile saline solution (C group). The experimental diets were formulated to meet or exceed the requirements of birds according to the National Research Council recommendations (Table 1). No antibiotics or coccidiostats were included in the diets. Feed and water were provided *ad libitum* during the 21 day duration of the experiment. The feeding program consisted of 2 phases: days 1 through 14 and days 15 through 21. Room temperature was maintained between 32 and 34 °C for the initial 3 d, and then gradually decreased by 2 to 3°C a week until the final temperature reached 26 °C. The birds received continuous light for the first three days and were then maintained under a 20 h light/4 h darkness regime for the remainder of the study. Body weight and feed intake on a cage (replicate) basis was recorded to calculate average daily gain, average daily feed intake, and feed conversion ratio. The mortality of broilers was recorded daily.

Intestinal gene expression and morphology analysis

Two broilers per replicate were chosen based on their cage's average body weight and euthanized

Table 1 – Composition of basal diets.

Item	Day 1 to 14	Day 15 to 21
Ingredient, g kg^{-1}, as fed basis		
Corn, yellow	554.2	607.3
Soybean meal	355.2	315.3
Vegetable oil	35.2	30.2
Fish meal	39.9	36.3
Limestone	15.2	12.7
Monocalcium phosphate	9.2	7.8
Salt	3.0	3.0
L-lysine	1.0	0.6
DL-methionine	2.0	2.0
Choline chloride	0.5	0.5
Vitamin premix[1]	2.0	2.0
Mineral premix[2]	2.0	2.0
Calculated composition, g kg^{-1}		
Dry matter	88.9	88.7
Crude protein	221.6	206.3
Lysine	11.2	9.5
Methionine+Cystine	8.5	7.6
Analyzed calcium	10.2	8.7
Analyzed total phosphorus	6.9	6.3
ME, kcal/kg	3081.1	3057.2

[1]Vitamin premix provided per kg of diet: 10 mg of nicotine amid, 0.02 mg of cholecalciferol, 0.3 mg of folic acid, 2 mg of pyridoxine HCl, 1.8 mg of all-trans-retinyl acetate, 8 mg of cyanocobalamin, 2.2 mg of menadione, 8.3 mg of alpha-tocopheryl acetate, 160 mg of choline chloride, and 20 mg of D-biotin.

[2]Mineral premix provided per kg of diet: 60 µg of Se, 200 µg of Co ($CoSO_4$), 800 µg of I (KI), 2 mg of Cu ($CuSO_4 \cdot 5H_2O$), 24 mg of Zn (ZnO), 16 mg of Fe ($FeSO_4 \cdot 7H_2O$), and 32 mg of Mn ($MnSO_4 \cdot H_2O$).

using carbon dioxide inhalation at the end of the experiment. Four replicates per group were used for gene expression analysis (n = 4). Small intestine was collected at 3 locations: duodenum (2 cm after the gizzard), jejunum (1 cm proximal to Meckel's diverticulum), and ileum (1 cm proximal to the ileocecal junction). Total RNA was extracted from the small intestine using the TRIzol reagent extraction method (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The expression of inflammatory genes (*cox2* and *inos*) was measured on the Miniopticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA). The *18S* rRNA expression was used for normalization control. The specific oligonucleotide primers were as follows:*18S* rRNA forward: 5'-ATA ACG AAC GAG ACT CTG GCA-3', and reverse: 5'-CGG ACA TCT AAG GGC ATC ACA-3'; cyclooxygenase 2 (*cox2*) forward: 5'-AAC ACA ATA GAG TCT GTG ACG TCT T-3', and reverse: 5'-TAT TGA ATT CAG CTG CGA TTC GG-3'; inducible nitric oxide synthase (*inos*) forward: 5'-AGG CCA AAC ATC CTG GAG GTC-3', and reverse: 5'-TCA TAG AGA CGC TGC TGC CAG-3'.Threshold cycle (Ct) values were obtained and the relative gene expression was calculated using the formula $2^{-\Delta\Delta Ct}$.Small intestines were fixed in 10% neutral-buffered formalin solution (Sigma, St. Louis, MO, USA) at 4°C, sectioned at 5 µm thickness (3 cross-sections from each sample), and then stained with hematoxylin and eosin. The villus length and crypt depth were measured randomly on thirty villi by Olympus CKX41 microscope (Olympus Corporation, Tokyo, Japan). The images were analyzed using stereological image software, Cast Image System (Version 2.3.1.3, Visiopharm Albertslund, Hørsholm, Denmark).

16S rRNA sequencing and analysis

Birds chosen for microbiota analysis were identical to those for intestinal gene expression and morphology analysis. Cecal digesta was freshly collected and DNA from cecal digesta was purified using Zymo BIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) and quantified on the Quibt 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA). The V3 and V4 hypervariable region of the 16S rRNA gene was amplified by 341F-805R primers. PCR products were purified using QIAquick Gel Extraction kit (QIAGEN, Germantown, MD, USA). The library construction, sequencing, operational taxonomic unit (OTU) alignment and analysis has been described previously (Chen & Yu, 2020). Naïve Bayesian classification method and QIIME 2 software (version 2017.4) were used forphylogenetic assignment and alpha diversity (richness and evenness), respectively. UniFrac distances coupled with standard multivariate statistics (QIIME 2 software) was used for principal component analysis (PCA) and principal coordinate analysis (PCoA). Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories were performed to predict cecal microbial function (PICRUSt software, version 1.1.4).Visualization of Pearson correlation analysis was created using R package corrplot (version 0.84).

Statistical analysis

Statistical analysis was performed using SAS software (version 9.4, 2012; SAS Institute, Cary, NC, USA). Replicates were used as the experimental unit. One-way ANOVA was performed and Tukey's honestly significant difference test was used for multiple comparisons. $p \leq 0.05$ indicated significant difference.

RESULTS

Effect of *G. lucidum* extract on the growth performance and inflammatory gene expression of broilers challenged with lipopolysaccharide

The effect of dietary GLE supplementation on the growth performance of broilers challenged with LPS is described in Table 2. No dead birds were observed over the experimental period. LPS challenges (L, LL, and HL) decreased body weight at 21 days of age ($p \leq 0.01$). The average daily gain of broilers at 15 to 21 days of age ($p=0.02$) and the whole trial period ($p \leq 0.01$) was reduced in the LPS challenge groups (L, LL, and HL groups) compared with the C group. LPS challenges (L, LL, and HL) also decreased the average daily feed intake at 15 to 21 days of age compared with the C group ($p \leq 0.01$). No significant differences in feed conversion ratio were observed between groups. The effect of dietary GLE supplementation on inflammatory gene expression in the broiler´s small intestine under the LPS challenge is described in Table 3. GLE supplementation at 1.33 mL/L reduced the *cox2* mRNA expression in the duodenum of broilers compared with LPS challenge only group ($p=0.01$). Similarly, the *cox2* mRNA expression was decreased in the jejunum of broilers challenged with LPS in combination with 1.33 mL/L GLE compared with the LPS challenge only group ($p=0.03$). LPS challenge only increased the *cox2* mRNA expression in the ileum of broilers compared with the C group, whereas 1.33 mL/L GLE supplementation decreased the *cox2* mRNA expression ($p=0.02$). The effect of dietary GLE supplementation on the gut morphology of broilers challenged with LPS is described in Table 4. LPS challenge-only increased the crypt depth in the duodenum compared with the C group ($p \leq 0.05$), whereas LPS challenge in combination with 1.33 mL/L of GLE supplementation reduced the crypt depth compared with the CL group ($p \leq 0.05$). LPS challenge-only reduced the ratio of villus length to crypt depth

Table 2 – Effect of *Ganoderma lucidum* extract on the growth performance parameter of broilers under lipopolysaccharide challenge.

	C[1]	L	LL	HL	SEM	pvalue
Body weight (g/bird)						
1 d	47.4	47.3	47.5	47.5	0.04	0.288
14 d	409.9	405.6	403.1	410.2	1.69	0.573
21 d	780.1[a]	720.2[b]	715.3[b]	713.1[b]	8.42	0.003
Average daily gain (g/d/bird)						
1-14 d	25.9	25.6	25.4	25.9	0.26	0.564
15-21d	52.9[a]	44.9[b]	44.6[b]	43.3[b]	1.08	0.018
1-21d	34.9[a]	32.0[b]	31.8[b]	31.7[b]	0.40	0.003
Average daily feed intake (g/d/bird)						
1-14 d	32.9	31.0	29.3	30.5	0.63	0.527
15-21d	89.8[a]	75.8[b]	79.1[b]	74.3[b]	1.62	0.006
1-21d	52.6	45.2	44.5	42.9	1.11	0.083
Feed conversion ratio						
1-14 d	1.3	1.2	1.2	1.2	0.02	0.630
15-21d	1.7	1.7	1.8	1.7	0.03	0.773
1-21d	1.5	1.4	1.4	1.4	0.03	0.787

[1] C= No LPS challenge; L = LPS challenge-only; LL = LPS challenge plus 1 mL/L of GLE; HL = LPS challenge plus 1.33 mL /L of GLE

[a-b]Means in a row without a common superscript letter differ ($p \leq 0.05$)

Table 3 – Effect of *Ganoderma lucidum* extract on the intestinal inflammatory gene expression of broilers under lipopolysaccharide challenge.

	C[1]	L	LL	HL	SEM	*p* value
Duodenum						
cox2	1.4[ab]	2.2[a]	1.8[ab]	0.9[b]	0.18	0.011
inos	1.3	1.3	1.2	0.9	0.07	0.269
Jejunum						
cox2	1.3[ab]	2.0[a]	1.2[ab]	0.7[b]	0.17	0.026
inos	1.1	1.4	0.9	0.9	0.08	0.060
Ileum						
cox2	0.9[b]	2.7[a]	2.0[ab]	0.9[b]	0.26	0.015
inos	1.1	1.7	2.3	1.6	0.19	0.102

[1] C= No LPS challenge; L = LPS challenge-only; LL = LPS challenge plus 1 mL/L of GLE; HL = LPS challenge plus 1.33 mL /L of GLE.

[a-b]Means in a row without a common superscript letter differ ($p \leq 0.05$).

Table 4 – Effect of *Ganoderma lucidum* extract on the gut morpholgy of broilers under lipopolysaccharide challenge.

		C[1]	CL	LL	HL	SEM	*p* value
Duodenum	Villus length (μm)	1369.6	1303.7	1412.74	1308.4	26.60	0.432
	Crypt depth (μm)	137.5[c]	177.8[a]	166.4[ab]	149.8[bc]	4.49	0.002
	Villus length: Crypt depth	10.0[a]	7.4[b]	8.5[ab]	8.8[ab]	0.26	0.002
Jejunum	Villus length (μm)	636.7[c]	656.6[c]	773.2[b]	919.2[a]	26.39	≤ 0.001
	Crypt depth (μm)	89.1[b]	109.5[ab]	122.8[a]	131.8[a]	4.74	0.003
	Villus length: Crypt depth	7.2	6.1	6.3	7.2	0.21	0.165
Ileum	Villus length (μm)	562.3[b]	488.3[b]	690.7[a]	708.3[a]	23.4	≤ 0.001
	Crypt depth (μm)	107.1[b]	102.4[b]	156.4[a]	136.5[a]	5.69	≤ 0.001
	Villus length: Crypt depth	5.3	4.8	4.4	5.3	0.13	0.058

[1] C = No LPS challenge; L = LPS challenge-only; LL = LPS challenge plus 1 mL/L of GLE; HL = LPS challenge plus 1.33 mL /L of GLE.

[a-c] Means in a row without a common superscript letter differ ($p \leq 0.05$).

in the duodenum ($p \leq 0.05$), whereas supplementation of GLE (1 and 1.33 mL/L) partially improved the ratio of villus length to crypt depth. Supplementation of GLE (1 and 1.33 mL/L) increased the villus height in the jejunum and ileum compared with the C and CL group ($p \leq 0.001$). GLE supplementation at 1.33 mL/L increased the crypt depth in the jejunum and ileum compared with the C group ($p \leq 0.05$).

Effect of *G. lucidum* extract on the microbial composition in the cecal digesta of broilers challenged with lipopolysaccharide

No significant difference in bacterial species richness (Chao1 and Fisher alpha estimator) in the cecal digesta among the groups was observed (Table 5). The bacterial species evenness (Shannon estimator) in the cecal digesta of LPS-challenged broilers receiving 1.33 mL/L of GLE was decreased compared with the L and LL groups ($p \leq 0.001$) (Table 5). LPS challenge-only and LPS challenge in combination with 1 mL/L of GLE supplementation increased the bacterial species evenness (Enspie estimator) compared with the C group, whereas the bacterial species evenness (Enspie estimator) was decreased in LPS-challenged broilers receiving 1.33 mL/L of GLE ($p \leq 0.001$) (Table 5). PCA and unweighted UniFrac distances of PCoA (qualitative traits) revealed that the cecal microbial composition was not well-separated among the groups (Fig. 1A and 1B). In contrast, weighted UniFrac distances of PCoA (quantitative traits) indicated significant discrimination among the groups (Fig. 1C).

Table 5 – Microbial diversity in the cecal digesta of broilers under lipopolysaccharide challenge.

	C[1]	L	LL	HL	SEM	*p* value
Chao1	102.8	98.5	105.8	100.0	1.20	0.253
Fisher alpha	12.1	11.0	12.4	11.1	0.22	0.076
Shannon	4.2[ab]	4.3[a]	4.3[a]	4.1[b]	0.03	≤ 0.001
Enspie	10.2[b]	11.0[a]	11.4[a]	9.5[c]	0.20	≤ 0.001

[1] C = No LPS challenge; L = LPS challenge-only; LL = LPS challenge plus 1 mL/L of GLE; HL = LPS challenge plus 1.33 mL /L of GLE.

[a-c]Means in a row without a common superscript letter differ ($p \leq 0.05$).

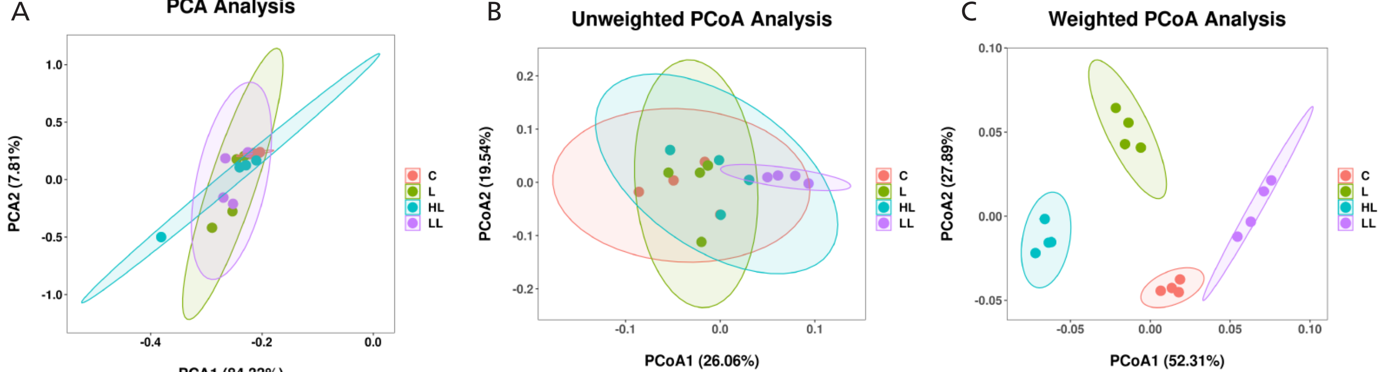

Figure 1 – Advanced analysis of the microbial community. (A) PCA of the cecal microbiota of non-challenged broilers fed a basal diet (C), LPS-challenged broilers fed a basal diet (L), LPS challenged broilers fed a basal diet supplemented with 1 mL/L of GLE (LL), and LPS challenged broilers fed a basal diet supplemented with 1.33 mL/L of GLE (HL) ($n = 4$). (B) Unweighted PCoA and (C) weighted PCoA of the cecal microbiota from C, L, LL, and HL ($n = 4$).

Effects of *G. lucidum* extract on the bacterial taxonomic composition in the cecal digesta of broilers challenged with lipopolysaccharide

The results of bacterial taxonomic distribution and abundance in the cecal digesta of broilers challenged with LPS are shown in Table 6. The abundance of the phylum Firmicutes was decreased in the HL group compared with the L group ($p=0.01$). LPS challenge only decreased the abundance of the phylum Bacteroidetes compared with the other groups ($p \leq 0.01$). Supplementation with GLE (1.33 mL/L) in combination with LPS challenge increased the abundance of the phylum Bacteroidetes compared with the other groups ($p \leq 0.01$). At the genus level, 1.33 mL/L of GLE supplementation increased the abundance of the genera *Barnesiella* and *Lactobacillus* in the cecal digesta of LPS-challenged broilers compared with the other groups ($p \leq 0.001$). GLE supplementation (1 and 1.33 mL/L) in combination with LPS challenge decreased the abundance of the genus *Lachnospiraceae_unclassified* compared with the C group ($p=0.04$). The abundance of the genera *Ruminococcus torques group* and *Ruminiclostridium_9* was increased in the L and LL groups compared with the other group ($p \leq 0.001$). LPS challenges (L, LL, and HL) decreased the abundance of the genus *Faecalibacterium* compared with the C group ($p \leq 0.001$).1 mL/L of GLE supplementation increased the abundance of the genera *Alistipes*, *Erysipelatoclostridium*, and *Blautia* in the cecal digesta of LPS-challenged broilers compared

Table 6 – Bacterial taxonomy within the cecal digesta of broilers under lipopolysaccharide challenge.

	Relative abundance (%)					
	C[1]	L	LL	HL	SEM	*p* value
Phylum						
Firmicutes	72.0^{ab}	73.9^{a}	72.2^{ab}	70.3^{b}	0.38	0.010
Bacteroidetes	27.2^{b}	24.9^{c}	26.9^{b}	29.1^{a}	0.42	0.004
Genus						
Barnesiella	18.6^{b}	16.6^{c}	16.6^{c}	20.7^{a}	0.46	≤ 0.001
Lachnospiraceae_unclassified	19.0^{a}	17.1^{ab}	17.0^{b}	16.7^{b}	0.30	0.043
Lactobacillus	10.0^{c}	14.3^{b}	7.0^{d}	17.5^{a}	1.05	≤ 0.001
Ruminococcus torques group	8.9^{b}	12.8^{a}	12.0^{a}	9.5^{b}	0.44	≤ 0.001
Faecalibacterium	9.4^{a}	2.6^{d}	6.8^{b}	5.6^{c}	0.63	≤ 0.001
Alistipes	6.0^{b}	4.7^{c}	8.6^{a}	4.3^{c}	0.44	≤ 0.001
Ruminiclostridium_9	3.0^{c}	4.2^{b}	4.6^{a}	2.5^{d}	0.23	≤ 0.001
Bacteroides	2.5^{b}	3.5^{a}	1.6^{c}	4.0^{a}	0.24	≤ 0.001
Erysipelatoclostridium	2.2^{c}	2.6^{b}	3.7^{a}	2.2^{c}	0.15	≤ 0.001
Blautia	2.6^{b}	1.7^{c}	4.1^{a}	2.0^{c}	0.24	≤ 0.001
Ruminococcaceae_UCG_014	2.3^{a}	1.8^{b}	2.3^{a}	1.4^{c}	0.10	≤ 0.001
Anaerostipes	1.3^{c}	2.8^{a}	1.2^{c}	1.9^{b}	0.17	≤ 0.001
Butyricicoccus	1.5^{a}	1.5^{a}	1.2^{b}	0.9^{c}	0.07	≤ 0.001
Eubacterium_hallii_group	0.9^{c}	0.6^{d}	1.1^{b}	1.3^{a}	0.07	≤ 0.001
Ruminiclostridium_5	1.0^{a}	1.0^{a}	0.8^{b}	0.8^{b}	0.03	≤ 0.001

[1] C = No LPS challenge; L = LPS challenge-only; LL = LPS challenge plus 1 mL/L of GLE; HL = LPS challenge plus 1.33 mL /L of GLE.

[a-d]Means in a row without a common superscript letter differ ($p \leq 0.05$).

with the other group ($p \leq 0.001$). The abundance of the genus *Bacteroides* was increased in the L and HL groups compared with the other group ($p \leq 0.001$). Supplementation with GLE (1.33 mL/L) in combination with LPS challenge decreased the abundance of the genus *Ruminococcaceae_UCG_014* compared with the other group ($p \leq 0.001$). The abundance of the genus *Anaerostipes* was increased in the L group compared with the other group ($p \leq 0.001$). The abundance of the genera *Butyricicoccus* and *Ruminiclostridium_5* was decreased and the abundance of the genus *Eubacterium_hallii_group* was increased in LPS-challenged broilers receiving of GLE (1 and 1.33 mL/L) compared with the other group ($p \leq 0.001$). An overview of the species abundance heat map of the dominant 35 genera in the cecal digesta is shown in Fig. 2A. The results show that some microbial community clusters were specifically increased in the L group, such as genera *Bacillaceae_unclassified*, *Anaerostipes*, *Erysipelotrichaceae_unclassified*, and *Gastranaerophilales_unclassified*. Similar microbial community clusters were observed between the L and LL groups, such as genera *Akkermansia*, *Ruminococcus_torques_group*, and *Ruminiclostridium_9*. GLE supplementation at 1 mL/L in combination with LPS challenge resulted in unique bacterial community clusters compared with other groups, such as the genera *Oscillibacter*, *Erysipelatoclostridium*, *Sellimonas*, *Christensenellaceae_R_7_group*, *Blautia*, and *Ruminococcaceae_UCG_004*. Some bacterial community clusters were specifically decreased in the HL group, such as the genera*GCA_900066575*, *Ruminococcaceae_UCG_014*, *Escherichia_Shigella*, *Butyricicoccus*, and *Negativibacillus*. An overview of the microbial function heat map within the cecal digesta of broilers is presented in Fig. 2B. The results indicated that the microbial function was well-separated among the groups. Some microbial functions were specifically decreased in the L group, such as nucleotide metabolism, amino acid metabolism, and metabolism of cofactors and vitamins. The membrane transport, immune diseases, and infectious diseases: parasitics were increased compared with the other groups. The energy metabolism and nervous system functions were specifically decreased in the HL group compared with the other groups. Some microbial functions were increased in the C and LL group, such as endocrine and metabolic diseases.

Correlation between cecal microbiota and growth performance

Correlation among the dominant 10 genera in the cecal digesta of broilers is presented in Fig. 3A. The abundance of the genera *Barnesiella*, *Bacteroides*, and *Lactobacillus* was positively associated with each other. The abundance of the genus *Faecalibacterium* was positively associated with the abundance of the genus *Lachnospiraceae_unclassified*. The abundance of the

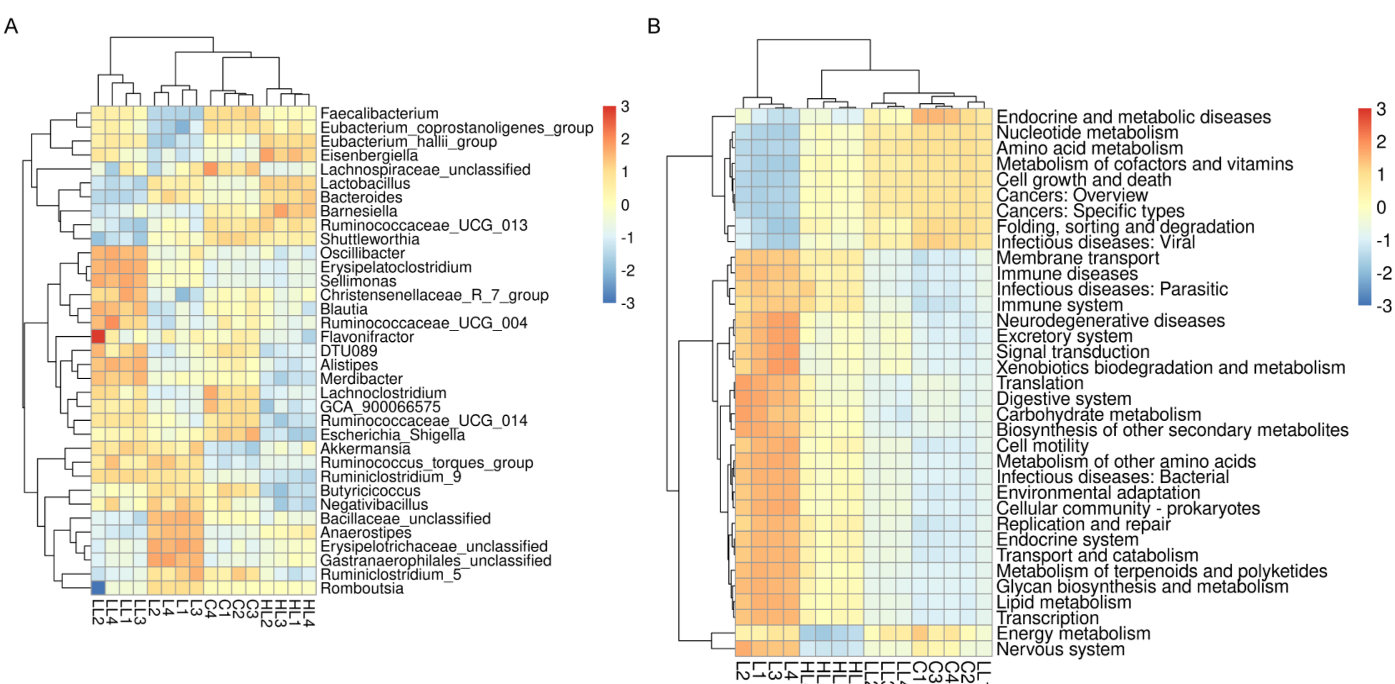

Figure 2 – Heat map of bacterial abundance distribution and microbial functions. (A)Species abundance distribution of the dominant 35 genera of cecal microbiota of broilers challenged with LPS. Samples from non-challenged broilers fed a basal diet (C), LPS-challenged broilers fed a basal diet (L), LPS challenged broilers fed a basal diet supplemented with 1 mL/L of GLE (LL), and LPS challenged broilers fed a basal diet supplemented with 1.33 mL/L of GLE (HL) ($n = 4$) is plotted on the X-axis ($n = 4$), and the Y-axis represents the genus. (B) Cecal microbial functions based on KEGG functional categories in broilers challenged with LPS.

genus *Faecalibacterium* was negatively associated with the abundance of the genera *Bacteroides*, *Lactobacillus*, *Ruminococcus_torques_group*, and *Ruminiclostridium_9*. The abundance of the genera *Ruminococcus_torques_group*, *Erysipelatoclostridium*, and *Ruminiclostridium_9* was positively associated with each other. The abundance of the genera *Faecalibacterium* and *Lachnospiraceae_unclassified* was positively associated with growth performance (BW, ADG, ADFI, and FCR) (Fig. 3B). In contrast, the abundance of the genera *Bacteroides*, *Lactobacillus*, *Ruminococcus_torques_group*, *Erysipelatoclostridium*,

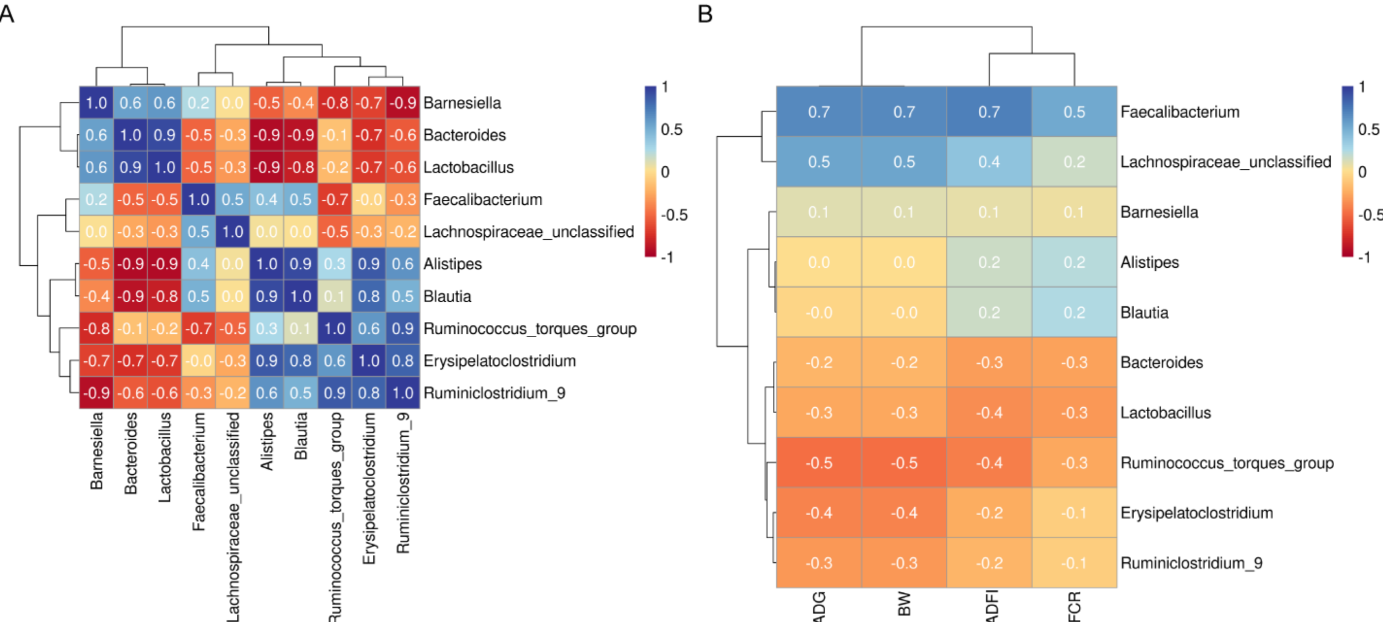

Figure 3 – Correlation between cecal microbiota and growth performance of broilers. (A) Correlation analysis between the abundance of dominant genera in broilers of different groups. (B)Correlation analysis between growth performance and abundant genera in broilers of different groups. The average body weight (BW) at 21 days of age, average daily gain (ADG) at 1 to 21 days of age, average daily feed intake (ADFI) at 1 to 21 days of age, and feed conversion ratio (FCR) at 1 to 21 days of age were used for Pearson correlation analysis.

and *Ruminiclostridium_9* was negatively associated with growth performance (Fig. 3B).

DISCUSSION

Gut microbiota is strongly shaped by host environments and developing a healthy intestinal microbial community can prevent inflammatory response and ameliorate the growth of broilers (Pourabedin & Zhao, 2015).Intestinal inflammation disturbs microbial communities, which results in intestinal microbiota dysbiosis (Lupp *et al*., 2007; Lobionda *et al*., 2019).It has been demonstrated that LPS-induced inflammation disrupts the cecal microbial composition, thereby impairing the health and growth of broilers (Yang *et al*., 2011; Liu *et al*., 2014; Chen & Yu, 2021). In this study, growth performance was impaired and intestinal inflammatory response was induced in the LPS challenge-only group, which is in agreement with the previous study (Chen & Yu, 2021). LPS challenge did not affect the richness of bacterial species in the cecal digesta of broilers in the present study. This observation is in agreement with the results of Metzler-Zebeli *et al*. (2020), who also observed the richness of bacterial species in the gut is not altered by LPS treatment in broilers. However, LPS challenge-only increased the evenness of bacterial species in the cecal digesta of broilers, indicating that LPS may mainly regulate bacteria proportion in the gut. Our previous study demonstrated that GLE supplementation decreases the richness and evenness of fecal microbiota in broilers (Chen & Yu, 2020). GLE supplementation at 1.33 mL/L decreased the evenness of bacterial species in the cecal digesta of LPS challenged broilers compared with the LPS challenge-only group. Thus, these results indicate that the LPS challenge induces intestinal inflammation and disturbs cecal bacterial composition in broilers. GLE supplementation can alleviate the intestinal inflammatory response and modulate gut microbial diversity in broilers under LPS challenge.

A previous study revealed that the phylum Bacteroidetes is essential in developing a stable and healthy gut microbiota (Jandhyala *et al*., 2015). The phylum Bacteroidetes species are able to synthesize short-chain fatty acids in the gut through the production of polysaccharide-degrading enzymes and can be considered as beneficial microbes (Beckmann *et al*., 2006; Wall *et al*., 2012). The short-chain fatty acids have been reported to have antibacterial properties in broilers (Ricke, 2003).In this study, the abundance of the phylum Bacteroidetes was decreased in the cecal digesta of LPS challenge-only group, whereas 1.33 mL/L of GLE supplementation increased the abundance of the phylum Bacteroidetes in the cecal digesta of LPS-treated broilers. At genus level, the abundance of the genera *Barnesiella*, *Faecalibacterium*, and *Alistipes* was reduced in the cecal digesta of LPS challenge-only group. It has been demonstrated that the genus *Barnesiella* members are able to synthesize short-chain fatty acids in the gut and can be classified as beneficial bacteria (Wei *et al*., 2018). It has been found that the genus *Faecalibacterium* members maintain epithelial health by increasing the ratio of villus to crypt and butyrate production (Miquel *et al*., 2013; Gangadoo *et al*., 2018). The abundance of the genus *Alistipes* in the gut is positively associated with the growth of broilers (Torok *et al*., 2011). Supplementation with GLE at 1 mL/L increased the abundance of the genera *Barnesiella* and *Faecalibacterium* in the cecal digesta of LPS-treated broilers. The *Eubacterium_hallii_group* genus members are involved in intestinal metabolic balance due to their ability to utilize glucose and its intermediates to form short-chain fatty acids (Engels *et al*.,2016). In this study, the genus *Eubacterium_hallii_group* abundance was specifically increased in the cecal digesta of LPS-treated broilers in response to GLE supplementation. Furthermore, correlation analysis results demonstrated that the abundance of the genus *Faecalibacterium* was positively correlated with the growth performance in the present study. The abundance of the genus *Faecalibacterium* was negatively correlated with the abundance of the genus *Ruminococcus_torques_group*. The abundance of the genus *Ruminococcus_torques_group* was increased in the LPS challenge-only group. The genus *Ruminococcustorques* members is correlated with gastrointestinal diseases and has the ability to degrade mucin in the gastrointestinal tract (Malinen *et al*., 2010; De Cesare *et al*., 2017). These results imply that the genus *Faecalibacterium* may promote growth of broilers challenged with LPS by decreasing the abundance of the genus *Ruminococcus_torques_group*. Taken together, these findings indicate that GLE supplementation can modulate cecal microbiota in broilers under LPS challenge by increasing the abundance of short-chain fatty acid-producing bacteria. Whether short-chain fatty acid levels in the cecum are elevated in GLE-treated broilers under LPS challenge remains to be investigated.

Gut microbial composition contributes to the development of intestinal structure in the growing period of broilers (Liao *et al*., 2020). In this study,

supplementation of GLE (1 and 1.33 mL/L) could increase the villus height in the jejunum and ileum of LPS-challenged broilers. Furthermore, cecal microbiota was also modulated in LPS-challenged broilers in response to GLE supplementation. This observation is in agreement with the results of Chen & Yu (2020), who also observed the gut morphology and microbiota is improved by GLE treatment in broilers. However, the growth performance was not improved in GLE-treated broilers under LPS challenge at the end of the experimental period (21 days) in the present study. But the LPS-induced inflammatory gene expression in the small intestine of GLE-treated broilers was inhibited. The heat map of bacterial cluster results also demonstrated that the GLE supplementationre-shaped the cecal microbial community in broilers under LPS challenge. Some bacterial cluster abundance returned to normal levels when GLE was supplied in the diet, such as *Faecalibacterium*, *Eubacterium_coprostanoligenes_group*, *Erysipelotrichaceae_unclassified*, and *Gastranaerophilales_unclassified*. Furthermore, the heat map of KEGG metabolic pathway results confirmed that GLE supplementation normalized several metabolic pathways in broilers under LPS challenge, such as nucleotide metabolism, amino acid metabolism, metabolism of cofactors and vitamins, and cell growth and death. These results imply that GLE supplementation may correct the LPS-induced gut dysbiosis in broilers. Thus, we speculate that the beneficial effect of GLE on the growth performance of broilers under inflammatory challenge may be observed after a prolonged feeding period (market age of 35 days).

CONCLUSION

The result of this study showed, for the first time, that GLE supplementation could improve gut morphology and normalize the cecal microbial community of broilers under inflammatory challenge through elevating beneficial bacteria and reducing harmful bacteria. Whether altered gut microbiota caused by GLE has a direct impact on health and growth remains to be confirmed in the future.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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