The effects of dietary supplementation with lotus leaf extract on the immune response and intestinal microbiota composition of broiler chickens

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ABSTRACT This study aimed to assess the effect of lotus leaf extract (**LLE**) on the immune response and intestinal microbiota composition of broiler chickens. One-day-old birds were assigned to 7 treatments. Two maize-based control diets were each given with or without 50 mg/kg chlortetracycline (antibiotics and blank control groups, respectively). Five experimental diets were each given with 1.0, 2.5, 5.0, 7.5, or 10.0 g/kg LLE. Average daily weight gain (**ADG**) was assessed, and the immune organ index was calculated. Serum cytokine and immunoglobulin levels were determined, and intestinal microbiota composition was analyzed via high-throughput sequencing of the 16S rRNA gene. Results showed that in the LLE5 group, ADG was higher than that of the antibiotics and blank control groups ($P < 0.05$) from d 7 to 21, the thymus index at d14, spleen index at d 21, and bursa index at d 14 and 21 were increased markedly ($P < 0.05$). In the LLE5 and LLE7.5 groups, serum total IgG and sIgA concentrations were higher than those of the antibiotics and blank control groups ($P < 0.05$) at d 7 and higher than those of the antibiotics group ($P < 0.05$) at d 14. No significant effect was observed for interferon-gamma concentrations between the antibiotics and LLE5 or LLE7.5 groups; compared with the antibiotics group, IL2 concentrations were increased in the LLE5 group at d 7 and in the LLE7.5 group at d 21 ($P < 0.05$). 16s rRNA sequencing analysis revealed that there were 1,704, 232, and 4,814 operational taxonomic unit in the blank control group, antibiotics group, and LLE groups, respectively. The intestinal microbiota consisted mainly of Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes ($>95\%$) at the phylum level; at the family level, the abundance of Clostridiaceae and Bacteroidales S24-7 was increased, whereas that of Peptostreptococcaceae was reduced in LLE5 group ($P < 0.05$). These findings suggest that LLE may be a good source of prebiotics, helping to modulate the immune response and boost the levels of beneficial bacteria.

Key words: lotus leaf extract, immune stimulation, intestinal microbiota

INTRODUCTION

Antibiotic-mediated growth enhancement was first reported by Moore et al. (1946). Following the discovery that aureomycin could significantly stimulate the growth of chickens, cattle, and pigs (Jukes, 1972), the use of antibiotic growth promoters (**AGP**) became widespread in the agricultural industry. The use of in-feed prophylactic antibiotics as growth promoters has contributed to large financial profits for producers and low costs for consumer over the past 50 y; however, the world is now facing an increasing crisis concerning the increased use of antibiotics in animal agriculture and the emergence of multidrug-resistant superbugs that threaten disease management in both animals and humans (Cowieson and Kluenter, 2019; Woo and Lillehoj, 2019). Consequently, there is a growing global trend for the removal of in-feed prophylactic antibiotics as growth promoters from the diets of animals that enter the human food chain (Yadav and Jha, 2019). This poses immense challenges for the sustainability of the poultry production industry, necessitating the development of alternative strategies to achieve optimum growth performance and combat the many infectious diseases of poultry. This is especially true when the prophylactic use

of enteric conditioning antibiotics is limited owing to the risk of antibiotic resistance (Jazi et al., 2018a,b; Mohebodini et al., 2019; Shirani et al., 2019).

The commonly used alternatives to antibiotics in the animal industry include prebiotics, probiotics, phytonutrients (herbs and botanical products), hyperimmune antibodies, bacteriophages, antimicrobial peptides, and minerals (Cao et al., 2013; Gadde et al., 2017; Jazi et al., 2018a,b; Patra et al., 2018). Phytogenic feed additives are promising alternatives to antibiotics as natural growth promoters in a global strategy to reduce the use of drugs in animal production (Lillehoj et al., 2018). For example, a commercial blend of phytonutrients (containing carvacrol, cinnamaldehyde, and capsicum oleoresin) was approved in the European Union as the first botanical feed additive for improving broiler performance (Bravo et al., 2014). Supplementation of phytogenic feed additives to broiler diets is based on their bioactive constituents, which have been shown to improve growth performance, dietary available energy, nutrient digestibility, immune function, and antioxidant status, as well as to stabilize intestinal microbiomes (Pirgozliev et al., 2015; Bravo et al., 2014; Patra et al., 2018).

Lotus (Nelumbo nucifera Gaertn.), a perennial aquatic herb widely cultivated in Eastern Asia, and especially in China, is increasingly used owing to its varied beneficial effects (Xiong et al., 2016). The extracts of lotus rhizomes, seeds, flowers, and leaves are reported to possess varied therapeutic potential. The leaves are widely used in traditional medicine (Mukherjee et al., 2009) owing to their substantial alkaloid and flavonoid contents and have been applied for the treatment of hematemesis, epistaxis, hemoptysis, hematuria, metrorrhagia, and hyperlipidemia (Huang et al., 2011; Ding et al., 2017). Lotus leaves also exhibit antioxidant, antiobesity, antimalarial, antifungal, and potential anti-HIV activities and can inhibit the proliferation of breast cancer cells (Ahn et al., 2013; Zhao and Deng, 2013; Zhu et al., 2019). The potential biological effects of lotus leaf extract (**LLE**) have been shown in animals, including humans, rats, and fish (Lee et al., 2015; Munglue, 2015; Su et al., 2015; Zhang et al., 2015; Ding et al., 2017). Most of the relevant studies to date have focused on its pharmacological functions in mammals as a traditional Chinese medicine (Mukherjee et al., 2009), and knowledge of the effects of dietary LLE supplementation on poultry is currently limited to its growth-promoting influence (Munglue, 2015).

In the present study, a growth trial with 7 groups of 1-day-old male broiler chickens fed with different levels of dietary LLE was conducted to explore the effects of dietary LLE supplementation on the growth performance, immunostimulation, and intestinal microbiota composition of broiler chickens.

MATERIALS AND METHODS

Lotus Leaf Extract Preparation

The lotus leaf extract was prepared as previously described (Huang et al., 2010), with minor modifications.

In brief, dried lotus leaves were ground into a fine powder through a 420-µm mesh. The powder was decocted in 80% ethanol for 1 h at 80°C, and then filtered. The filtrate was condensed using an EYELA SB-1100 rotatory evaporator (Eyela Rikakikai Co., Ltd., Tokyo, Japan). The residues were vacuum-dried, and the resulting dry powder was stored at 4°C.

The concentrations of the main components of the LLE, nuciferine and total flavonoids (nuciferine: 0.71 mg/g; total flavonoids: 2.49 mg/g), were calculated using external standard high-performance liquid chromatography and the colorimetric method, respectively (Zhu et al., 2019; Chinese Pharmacopoeia Commission, 2020).

Animals, Experimental Design, and Management

All procedures used in this study were in strict accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals approved by the Animal Ethics Committee of Yangtze University. A total of two hundred ten, 1-day-old broiler chicks (Ross 308) were randomly assigned to 7 treatment groups with 5 replicate cages (6 birds per cage) in a completely randomized design. Seven dietary treatments were administered as follows: (i) a basal diet (blank control); (ii) the basal diet supplemented with 50 mg/kg chlortetracycline (antibiotics control); (iii) the basal diet supplemented with 1.0 g/kg LLE (LLE1); (iv) the basal diet supplemented with 2.5 g/kg LLE (LLE2.5); (v) the basal diet supplemented with 5.0 g/kg LLE (LLE5); (vi) the basal diet supplemented with 7.5 g/kg LLE (LLE7.5); and (vii) the basal diet supplemented with 10.0 g/kg LLE (LLE10). The basal diet was formulated to meet or exceed the requirements recommended by the Feeding Standard of Chicken (Ministry of Agricultural of the People's Republic of China, 2004) for broilers (Table 1). The LLE replaced the same amount of maize in the experimental diets. All the birds were housed in wire-floored cages (110 × 80 × 45 cm) in a room with a controlled environment and under constant light (5 lx). Feed and water were provided ad libitum. All the chicks were vaccinated against Marek's disease (subcutaneous injection) at the hatchery, Newcastle disease (intranasal) on d 7, and infectious bronchitis and infectious bursa disease (water) on d 14 according to the vaccination procedure guide for Ross broilers (www.aviagen.com). The test period lasted until the age of 21 d.

Average Daily Weight Gain

On d 7, 14, and 21, the BW was measured for each replicate cage. The average daily weight gain (ADG) was calculated between d 7 and 14, d 14 and 21, and d 7 to 21. On d 21, one chicken per replicate cage was randomly selected and euthanized by cervical dislocation. After exsanguination, the thymus, spleen, and bursa of each bird were removed, drained with blotting paper, and weighed. Immune organ development was expressed as relative weight respect to live BW (g/kg).

Blood Sample, Intestinal Tissue, and Content Collection

On d 7, 14, and 21, one chicken was randomly selected from each replicate cage of all the groups (a total of 6 chickens per group) and euthanized for the evaluation of intestinal morphology and gut microbiota, as well as for blood sample collection for immune index analysis. Blood samples were obtained as quickly as possible from the wing vein, immediately transferred to the laboratory, and allowed to clot at $37°C$ for 2 h. Serum separated by centrifugation at $4,000 \times g$ for 15 min at $4°C$. Serum samples were stored at $-20°C$ for further analysis. The chickens were then euthanized by cervical dislocation. The viscera were removed and separated, and the small intestine was isolated. The contents of the small intestine were immediately collected and placed in microtubes in an icebox with ice packs. All the samples were immediately brought back to the laboratory and stored at $-80°C$ for further microbial assay or histological analysis.

Determination of Serum Cytokine and Immunoglobulin Levels by ELISA

The serum levels of sIgA, IgG, IL-2, and interferon-gamma (**IFNG**) were measured using ready-to-use sandwich ELISA kits according to the manufacturer's instructions (Biosamite Biotechnology Co., Ltd., Shanghai, China).

Intestinal Microbiome Analysis

The composition of the microbial community in the contents of the small intestine of the chickens was analyzed via high-throughput sequencing of the 16S rRNA gene (Shanghai Personal Biotechnology Co., Ltd., China). The total DNA of the intestinal bacteria was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and purity were assessed on 1% agarose gels. The DNA was diluted to $1 \, \text{ng}/\mu\text{L}$ using sterile water. PCR amplification of the V3–V4 region of the 16S rRNA gene was performed using the forward primer 5′- ACTCCTACGGGAGGCAGCA-3′ and the reverse primer 5′-GGACTACHVGGGTWTCTAAT-3′. Barcodes were incorporated into the primers for multiplex sequencing. Each PCR reaction contained $5 \, \mu\text{L}$ of Q5 reaction buffer ($5 \times$), $5 \, \mu\text{L}$ of Q5 GC buffer ($5 \times$), $0.25 \, \mu\text{L}$ of Q5 DNA polymerase ($5 \, \text{U}/\mu\text{L}$), $2 \, \mu\text{L}$ of dNTPs (2.5 mmol), $1 \, \mu\text{L}$ (10 μmol) of each forward and reverse primer, $2 \, \mu\text{L}$ of DNA template, and $8.75 \, \mu\text{L}$ of ddH$_2$O. Thermal cycling consisted of an initial denaturation at $98°C$ for 2 min, followed by 25 cycles of denaturation at $98°C$ for 15 s, annealing at $55°C$ for 30 s, and extension at $72°C$ for 30 s, with a final extension of 5 min at $72°C$. The PCR amplicons were purified using Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). After individual quantification, the amplicons were pooled in equal amounts, and paired-end, $2 \times 300 \, \text{bp}$ sequencing was performed using the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd., China. The sequencing data were processed using the QIIME v.1.8.0 pipeline. Briefly, raw sequencing reads with exact matches to the barcodes were assigned to the respective samples and identified as valid sequences. Low-quality sequences were filtered using the following criteria: sequences that had a length of <150 bp, sequences that had average Phred scores <20, sequences that contained ambiguous bases, and sequences that contained mononucleotide repeats >8 bp. Paired-end reads were assembled using FLASH. After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (**OTU**) at 97% identity using UPARSE. Taxonomies were assigned to each OTU using UCLUST. Alpha and beta diversity analyses were performed for each library using QIIME. Moreover, the community structure was analyzed at different classification levels.

Statistical Analysis

All data were presented as means ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test to identify significant differences among groups. All analyses were performed using IBM SPSS 23 and Origin 8.5. The statistical significance level was set at $P < 0.05$.

RESULTS

ADG of the Chickens

After 21 d of experimental diet administration, the chickens of the LLE groups had a markedly greater ADG than the antibiotics and blank control groups. From d 7 to d 14, the ADG of all the LLE groups was greater than that of the antibiotics control group ($P < 0.05$). Notably, the ADG of the LLE5, LLE7.5, and LLE10 groups was considerably greater than that of the blank control group ($P < 0.05$). From d 14 to d 21, the ADG of the LLE5 and LLE7.5 groups was greater than that of the blank control group ($P < 0.05$); importantly, the ADG of the LLE5 group was greater than that of the antibiotics control group ($P < 0.05$). From d 7 to d 21, the ADG of the LLE2.5, LLE5, and LLE7.5 groups was greater than that of the antibiotics and blank control groups ($P < 0.05$) (Figure 1).

The Viscera Index

The thymus index is presented in Table 2. From d 7 to d 21, the thymus index of the LLE5 and LLE7.5 groups was higher when compared with that of the antibiotics and blank control groups, especially on d 14, when the difference was significant ($P < 0.05$).

On d 21, the spleen index (Table 2) was markedly higher in the LLE2.5, LLE5, and LLE7.5 groups than in the antibiotics control group ($P < 0.05$); notably, the spleen

Table 1. Dietary composition and nutrient levels of the basal diets.

Ingredient	Content (%)	Nutrient levels[3]	Content
Corn	55.22	Metabolizable energy MJ/kg	12.34
Soybean oil	3.50	Crude protein %	21.00
Soybean meal (43% CP)	36.92	Lysine %	1.2
Lysine hydrochloride (98%)	0.22	Methionine + Cystine %	0.85
DL- methionine	0.30	Threonine	0.66
Ground limestone	1.12	Tryptophan %	0.22
Dicalcium phosphate	2.10	Calcium %	1.00
Salt	0.30	Total phosphorus %	0.67
Choline chloride (70%)	0.09	Nonphytate phosphorus %	0.45
Poultry vit mix[1]	0.03		
Poultry mineral mix[2]	0.20		
Total	100.00		

[1]Poultry vit mix provided the following per kilogram of diets: VA 8,000 IU, VD$_3$ 1,000 IU, VE 20 IU, VK$_3$ 0.5 mg, VB$_1$ 2 mg, VB$_2$ 9.60 mg, VB$_6$ 3.5 mg, VB$_{12}$ 10 μg, niacin 35 mg, calcium pantothenic acid 10 mg, folic acid 0.55 mg, biotin 0.18 mg.

[2]Poultry mineral mix provided the following per kilogram of diets: Fe 80 mg, Cu 8 mg, Mn 100 mg, Zn 80 mg, I 0.7 mg, Se 0.3 mg.

[3]ME was calculated value, while the others were measured values.

index in the LLE5 group was also substantially higher than that of the blank control group ($P < 0.05$).

The bursa index (Table 2) of LLE-treated chickens was greatly increased compared with those of the antibiotics and blank control groups. On d 14, the bursa index of the LLE1, LLE2.5, LLE5, LLE7.5, and LLE10 groups was higher than that of the antibiotics control group ($P < 0.05$); in particular, the bursa index of the LLE5 group was also markedly higher than that of the blank control group ($P < 0.05$). On d 21, the bursa index of the LLE5, LLE7.5, and LLE10 groups was higher compared with those of the antibiotics and blank control groups ($P < 0.05$).

Serum Antibody Levels

Serum antibody concentrations (total IgG and sIgA) in the chickens were determined at 7, 14, and 21 d of age. On d 7, the serum total IgG concentration in the LLE2.5, LLE5, and LLE7.5 groups was higher than that of the antibiotics control group ($P < 0.05$) and was also higher in the LLE5 and LLE7.5 groups than

in the blank control group ($P < 0.05$). On d 14, except for the LLE2.5 group, the serum total IgG concentration was markedly higher in the LLE groups than in the antibiotics control group ($P < 0.05$), with the LLE5 group showing the highest concentration. Furthermore, compared with the antibiotics control group, the total IgG concentration increased on d 14 and 21 ($P < 0.05$) in the LLE1 group (Figure 2A).

The serum concentrations of sIgA are presented in Figure 2B. On d 14, the serum concentration of sIgA in the LLE5 and LLE7.5 groups was higher than that in the antibiotics and blank control groups ($P < 0.05$). On d 21, the serum concentration of sIgA was higher in the LLE7.5 group than in the blank control group ($P < 0.05$); however, no significant differences were detected among the other groups.

Changes in Serum Cytokine Levels

The serum concentrations of IFNG are presented in Figure 3A. On d 7, except for the LLE10 group, the serum concentration of IFNG was higher in the LLE
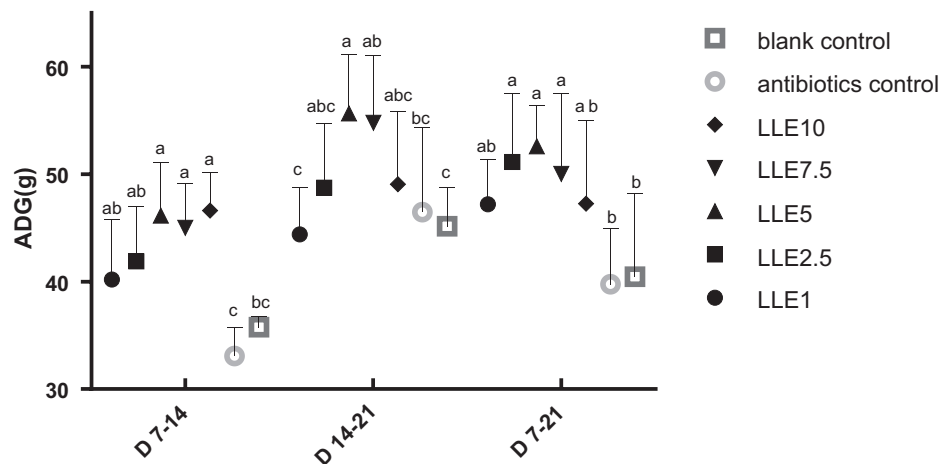

Figure 1. Effect of supplement LLE diet on ADG of chickens from d 7 to 21 (g). The abscissa is the age of collecting samples. [a-c]Bars in the histogram with different letters were significantly different ($P < 0.05$), n = 6. Abbreviations: LLE, lotus leaf extract; ADG, average daily weight gain.

Table 2. Effect of supplement LLE diet on viscera index (g/kg BW).

Organs	D	LLE1	LLE2.5	LLE5	LLE7.5	LLE10	Antibiotics control	Blank control
Thymus index	D 7	$3.41 \pm 0.52^{a,b}$	$3.72 \pm 0.25a$	$3.31 \pm 0.47^{a,b}$	2.78 ± 0.22^{b}	$3.36 \pm 0.43^{a,b}$	$3.09 \pm 0.57^{a,b}$	$3.03 \pm 0.12^{a,b}$
	D 14	2.65 ± 0.24^{a}	$2.47 \pm 0.34^{a,b}$	2.63 ± 0.13^{a}	2.68 ± 0.25^{a}	$2.38 \pm 0.25^{a,b}$	1.63 ± 0.21^{c}	2.12 ± 0.26^{b}
	D 21	3.18 ± 0.24^{b}	3.05 ± 0.14^{b}	$3.56 \pm 0.18^{a,b}$	$3.52 \pm 0.08^{a,b}$	3.97 ± 0.34^{a}	3.12 ± 0.22^{b}	3.24 ± 0.69^{b}
Spleen index	D 7	$1.02 \pm 0.14^{a,b}$	1.39 ± 0.05^{a}	0.92 ± 0.12^{b}	0.74 ± 0.16^{b}	0.73 ± 0.12^{b}	$1.12 \pm 0.37^{a,b}$	$1.01 \pm 0.04^{a,b}$
	D 14	1.18 ± 0.20^{a}	0.58 ± 0.12^{b}	0.79 ± 0.18^{b}	0.70 ± 0.15^{b}	0.68 ± 0.13^{b}	0.62 ± 0.02^{b}	0.80 ± 0.01^{b}
	D 21	0.63 ± 0.15^{c}	$1.07 \pm 0.03^{a,b}$	1.33 ± 0.42^{a}	$1.04 \pm 0.20^{a,b}$	$0.89 \pm 0.19^{b,c}$	0.64 ± 0.07^{c}	$0.89 \pm 0.18^{b,c}$
Bursa index	D 7	$2.35 \pm 0.32^{a,b}$	2.08 ± 0.34^{b}	2.00 ± 0.11^{b}	2.67 ± 0.26^{a}	2.17 ± 0.05^{b}	1.99 ± 0.01^{b}	1.89 ± 0.02^{b}
	D 14	$2.17 \pm 0.16^{a,b}$	$2.01 \pm 0.06^{a,b}$	2.27 ± 0.13^{a}	$2.22 \pm 0.28^{a,b}$	$2.20 \pm 0.30^{a,b}$	1.50 ± 0.42^{c}	$1.80 \pm 0.06^{b,c}$
	D 21	2.69 ± 0.30^{a}	$2.41 \pm 0.32^{a,b}$	2.72 ± 0.24^{a}	2.81 ± 0.42^{a}	2.84 ± 0.21^{a}	2.26 ± 0.17^{b}	2.16 ± 0.10^{b}

a, b within the same line, mean values with different letters are significantly different at $P < 0.05$, n = 6.
Abbreviation: LLE, lotus leaf extract.

groups than in the blank control group ($P < 0.05$), but did not differ markedly from the levels in the antibiotics control group ($P > 0.05$). On d 14, except for the LLE10 group, the serum IFNG concentration in the LLE groups was similar to that of the antibiotics and blank control groups ($P > 0.05$). On d 21, the serum IFNG concentration in the LLE5, LLE7.5, and LLE10 groups was higher than that in the blank control group ($P < 0.05$), but no differences were found when compared with the antibiotics control group ($P > 0.05$).

On d 7, the serum concentration of IL2 (Figure 3B) in the LLE2.5 and LLE5 groups was higher compared with both the antibiotics and blank control groups ($P < 0.05$). On d 14, the serum IL2 concentration was considerably lower in the LLE1 and LLE10 groups, but not in the LLE2.5, LLE5, and LLE7.5 groups, when
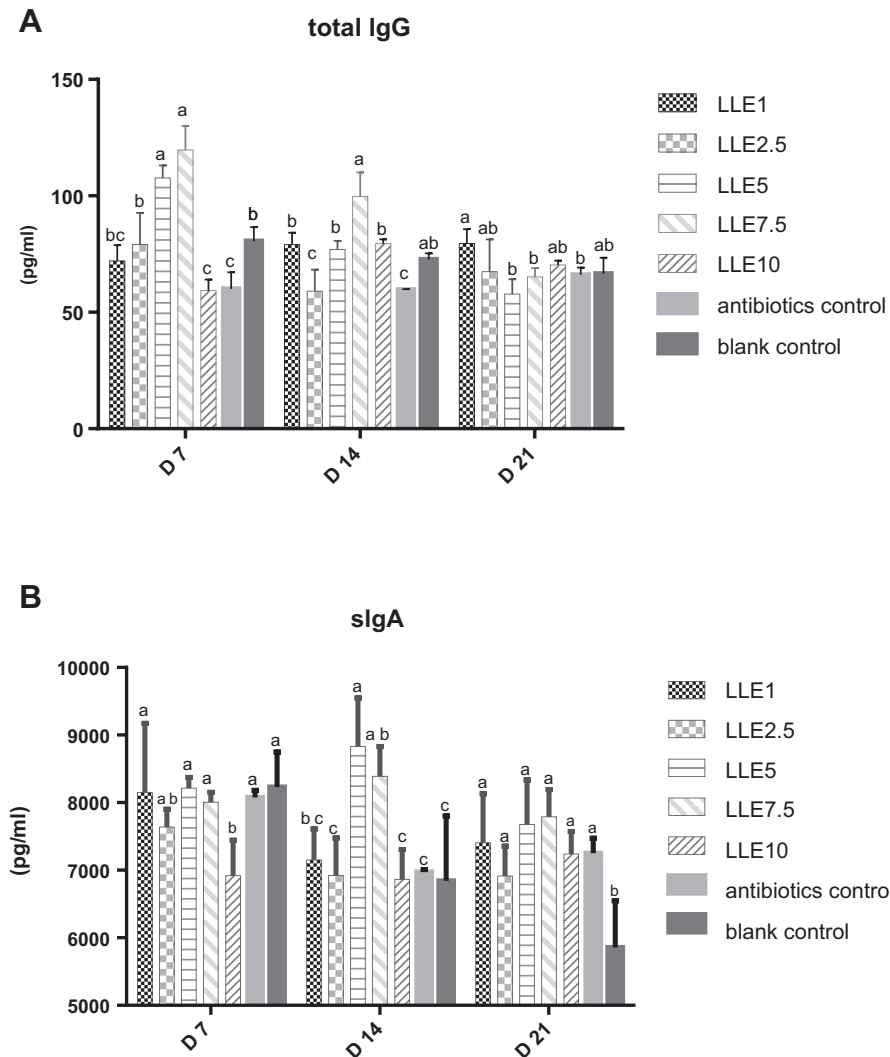

Figure 2. Concentrations of antibody levels in serum. The abscissa is the age of collecting samples. [a-c]Bars in the histogram with different letters were significantly different ($P < 0.05$), n = 6. Abbreviation: LLE, lotus leaf extract.
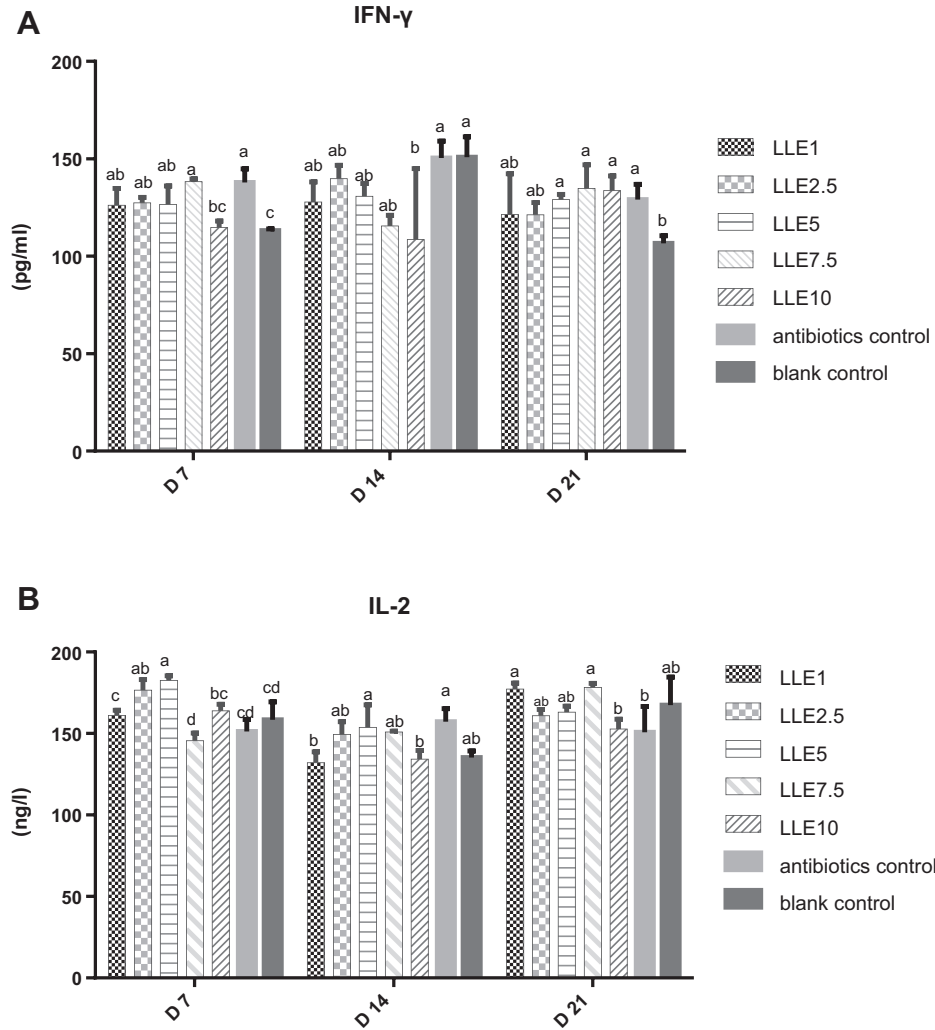
Figure 3. Concentrations of cytokines levels in serum. The abscissa is the age of collecting samples. [a-d]Bars in the histogram with different letters were significantly different ($P < 0.05$), n = 6. Abbreviation: LLE, lotus leaf extract.

compared with the antibiotics and blank control groups ($P > 0.05$). On d 21, the serum concentration of IL2 was higher in the LLE7.5 and LLE1 groups than in the antibiotics control group ($P < 0.05$); however, no differences were found when compared with the blank control group ($P > 0.05$).

The Effects of LLE on the Microbiota Composition of the Small Intestine

High-throughput sequencing analysis of the V3–V4 region of the bacterial 16S rRNA gene was conducted on samples of the content of the small intestine. After filtering the low-quality reads, trimming the longer homopolymer runs, adapters, barcodes, primers, removing all cyanobacteria/chloroplast sequences, and rarefying the datasets, the analysis revealed a highly diverse microbiota with a total of 1,121,483 16S rRNA high-quality sequences, and an average of 35,046 sequences per sample. Species accumulation curves showed that the number of observed species per sample was sufficient (Figure 4). A species accumulation curve was drawn (using R software) for the total number of OTU

corresponding to each sample in the OTU abundance matrix. The result showed that the curve tended to flatten with increasing sample size, indicating that the sample size of this experiment was enough to reflect the richness of the community.
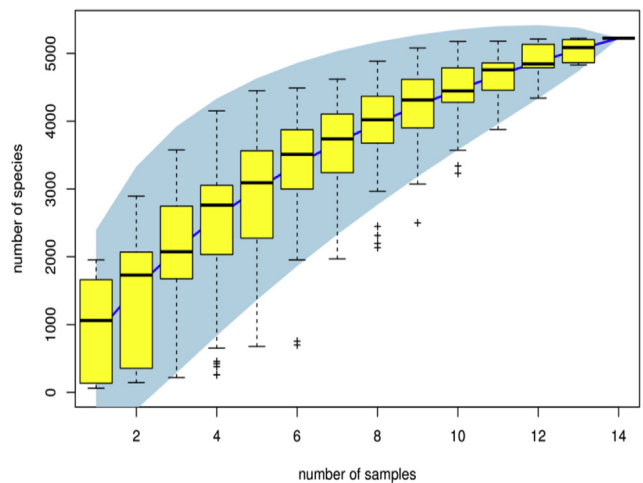

Figure 4. Species accumulation curve of samples.

Table 3. Intestinal microbial diversity indices of chickens after dietary LLE supplementation.

Group	Simpson	Chao1	ACE	Shannon
LLE1	$0.63 \pm 0.09^{b,c,d}$	953.53 ± 129.76^{b}	942.43 ± 106.27^{b}	5.13 ± 1.12^{b}
LLE2.5	0.99 ± 0.01^{a}	$1,725.08 \pm 240.20^{a}$	$1,764.76 \pm 255.99^{a}$	8.84 ± 0.28^{a}
LLE5	$0.89 \pm 0.14^{a,b}$	$1,343.73 \pm 392.67^{a,b}$	$1,391.93 \pm 348.87^{a,b}$	$7.04 \pm 0.41^{a,b}$
LLE7.5	$0.83 \pm 0.22^{a,b,c}$	$1,124.71 \pm 143.32^{b}$	$1,171.88 \pm 111.89^{b}$	5.93 ± 1.39^{b}
LLE10	$0.56 \pm 0.16^{c,d}$	$14,040 \pm 3.68^{c}$	142.12 ± 2.67^{c}	2.51 ± 1.05^{c}
Antibiotics control	0.45 ± 0.04^{d}	148.69 ± 0.27^{c}	159.55 ± 5.63^{c}	1.67 ± 0.83^{c}
Blank control	$0.67 \pm 0.45^{b,c,d}$	905.41 ± 189.10^{b}	923.26 ± 199.03^{b}	4.92 ± 1.26^{b}

[a–d]within the same column, mean values with different letters are significantly different at $P < 0.05$, n = 6.
Abbreviation: LLE, lotus leaf extract.

Operational Taxonomic Unit Clusters and Alpha Diversity of the Microbiota in the Intestinal Contents

To explore the diversity of the intestinal microbiota of chickens after dietary LLE supplementation, the composition and species distribution of the intestinal microbiota were investigated by 16s rRNA gene sequencing. Intestinal samples were analyzed by assigning OTU at the 97% identity level. Our results showed that alpha diversity, including species richness (ACE and Chao 1 indices) and diversity (Shannon and Simpson indices), differed markedly between the intestines of chickens in the LLE groups and the antibiotics control groups ($P < 0.05$; Table 3). The abundance distribution of microbiota components between the LLE groups and the antibiotics control groups were evaluated using the Metastats algorithm (Table 4, Figure 5 and Figure 6). Venn diagram analysis showed that 102 OTU were shared among the 3 treatment types. There were 1,704 OTU in the blank control group, 232 in the antibiotics control group, and 4,814 in the LLE supplementation groups (Figure 7). These results demonstrated that LLE could modulate the gut microbiota of the chickens, promoting a high diversity of the intestinal microbiota.

Beta Diversity of the Microbiota in the Intestinal Contents

Beta diversity was compared between the dietary LLE supplementation groups and control groups. For this, we performed principal components analysis and nonmetric multidimensional scaling of weighted and unweighted UniFrac distances to compare the diversity of each intestinal microbiome sample. The first and second principal coordinates accounted for 31.77% and 25.69% of the variance (Figure 8A). There was considerable overlap

Table 4. Comparisons of Metastats between groups were performed in pairs.

Group	Phylum	Genus
Antibiotics control–blank control	1	0
Antibiotics control–LLE	0	7
Blank control–LLE	0	0

Abbreviation: LLE, lotus leaf extract.

between the LLE groups and the antibiotics control group. Nonmetric multidimensional scaling results also showed that there were marked differences between the microbial community structure of the LLE groups and that of the antibiotics control group (Figure 8B). This suggested that the intestinal microbial composition and species distribution of the chickens were altered following dietary LLE supplementation.

Changes in Intestinal Microbial Structure at Different Taxonomic Levels

The intestinal microbial composition and species distribution of the chickens were altered after dietary LLE supplementation at both the phylum and family levels. The dominant populations in the intestinal microbiota of the chickens at the phylum level were *Firmicutes, Proteobacteria, Actinobacteria,* and *Bacteroidetes.* The other phylum with a relative abundance >1% was *Tenericutes* (Figure 9A). Additionally, we found marked differences in the levels of *Firmicutes, Proteobacteria, Bacteroidetes,* and *Actinobacteria* between the intestinal samples of the LLE2.5, LLE5, and LLE7.5 groups and those of the antibiotics control group ($P < 0.05$) (Figure 9B).

Comparisons of the top 20 genera in the gut microbiota are shown in Figure 10A. At the family level, the dominant populations (relative abundances >5%) in the intestinal microbiota of the chickens were *Clostridiaceae, Peptostreptococcaceae, Enterobacteriaceae, Enterococcus*, and *Lactobacillus*. Moreover, numerous genera were affected by dietary LLE supplementation. Members of Clostridiaceae in LLE1 and LLE5 groups and Bacteroidales S24-7 in LLE1, LLE2.5, LLE5, and LLE7.5 groups were increased, whereas members of Peptostreptococcaceae in LLE1, LLE2.5, LLE5, and LLE7.5 groups were reduced ($P < 0.05$) (Figure 10B). There were also marked changes in the other groups compared with both the antibiotics and blank control groups; however, these changes were not significant ($P > 0.05$).

DISCUSSION

It is well-known that AGP have also been linked to the increased incidence of antimicrobial-resistant bacterial infections, and extensive restrictions have been imposed on their use. The discovery and development of effective
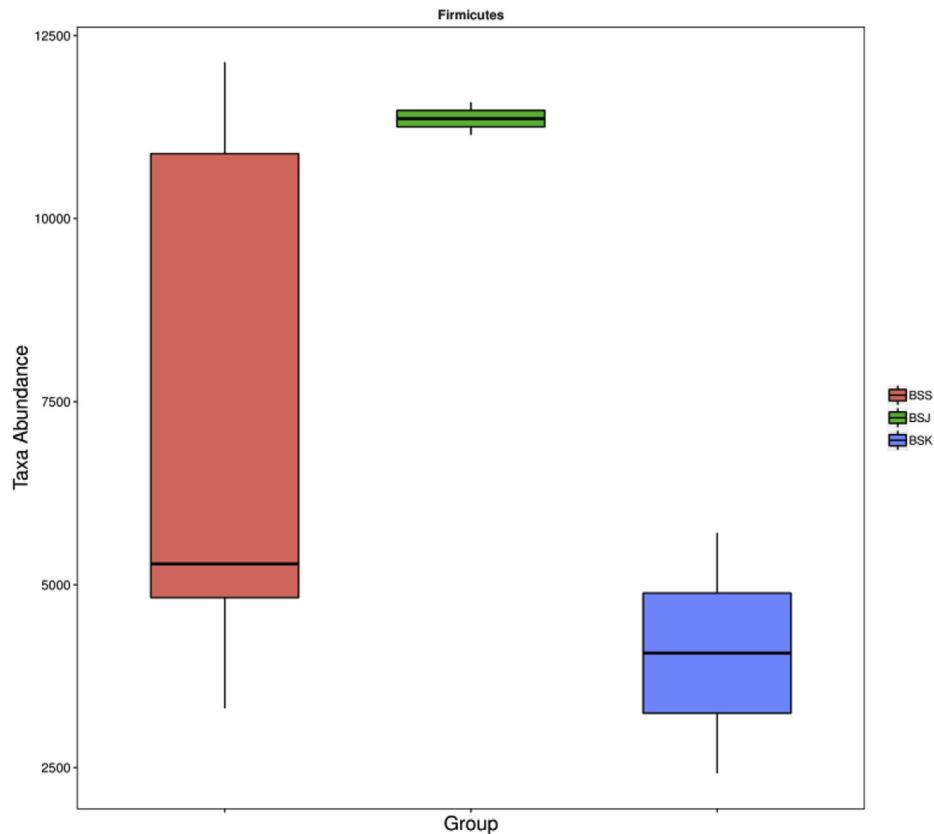
Figure 5. Metastats test samples in pairs between antibiotics control and blank control groups at the phylum level. Abbreviations: BSS, lotus leaf extract groups; BSJ, antibiotics control group; BSK,blank control group.

alternatives to AGP are, therefore, of great economic significance (Milad et al., 2019). In this study, we investigated the effects of LLE on growth performance, immunostimulation, and intestinal microbiota composition in broiler chickens.

The greater ADG at 1 to 21 d and 14 to 21 d for broilers fed a diet supplemented with 0.5% LLE could be attributed to the growth-promoting properties of LLE. In addition, the ADG was significantly greater in the LLE7.5 group than in the antibiotics and blank control groups at 7 to 14, 1 to 21, and 14 to 21 d, with the other LLE supplementation groups also showing an increasing trend. The effect of LLE on growth performance may be dose dependent. Previous studies have indicated that diets containing LLE can improve the growth performance of tilapia and catfish in a dose-dependent manner (Zhu et al., 2019). Similarly, the weight of yellow broilers fed a diet containing LLE was reported to be slightly greater than that of control animals (Chen et al., 2010).

Lymphoid organ weights can be easily measured and reflect the body's ability to provide lymphoid cells during an immune response (Heckert et al., 2002). The thymus weight is related to the production of developing T cells, the spleen weight is related to the proliferation of immune cells within the secondary lymphoid tissue during periods of infection, and the bursa helps to fight several types of bacteria through different immune response mechanisms (Oso et al., 2017; Ahiwe et al.,

2019). The morphometric measures of the immune organs were determined to assess the immune status. Of all the parameters assessed, the bursa to BW ratio of all the LLE groups at 14 d and the LLE5, LLE7.5, and LLE10 groups at 21 d gave the most consistent and reliable indication of the immunological status; 0.5% and 0.75% LLE supplementation elicited a better effect on the thymus to BW ratio and spleen to BW ratio. The results indicated that these LLE dietary inclusion levels may have been optimal for immune organs development. Furthermore, these results suggested that LLE dietary supplementation was beneficial for both the adaptive and innate immune responses.

Among the blood serum immunoglobins, sIgA and IgG have more vital roles in the protection of vascular compartments than in the protection against pathogenic microorganisms (Mohebodini et al., 2019). Meanwhile, the maturation and secretion of sIgA are highly associated with the development and composition of the indigenous microflora (Long et al., 2018). In the present study, the concentrations of IgG and sIgA tended to increase with LLE supplementation, especially at the 0.5% and 0.75% LLE levels. Similar results were also reported by Zhu et al. (2019), who demonstrated that an alcohol extract of lotus leaf could be used as an additive to boost the immunocompetence of grass carp.

Cytokines play a key role in both adaptive and innate immunity by aiding cell-to-cell communication during immune responses (Pirgozliev et al., 2019). In the
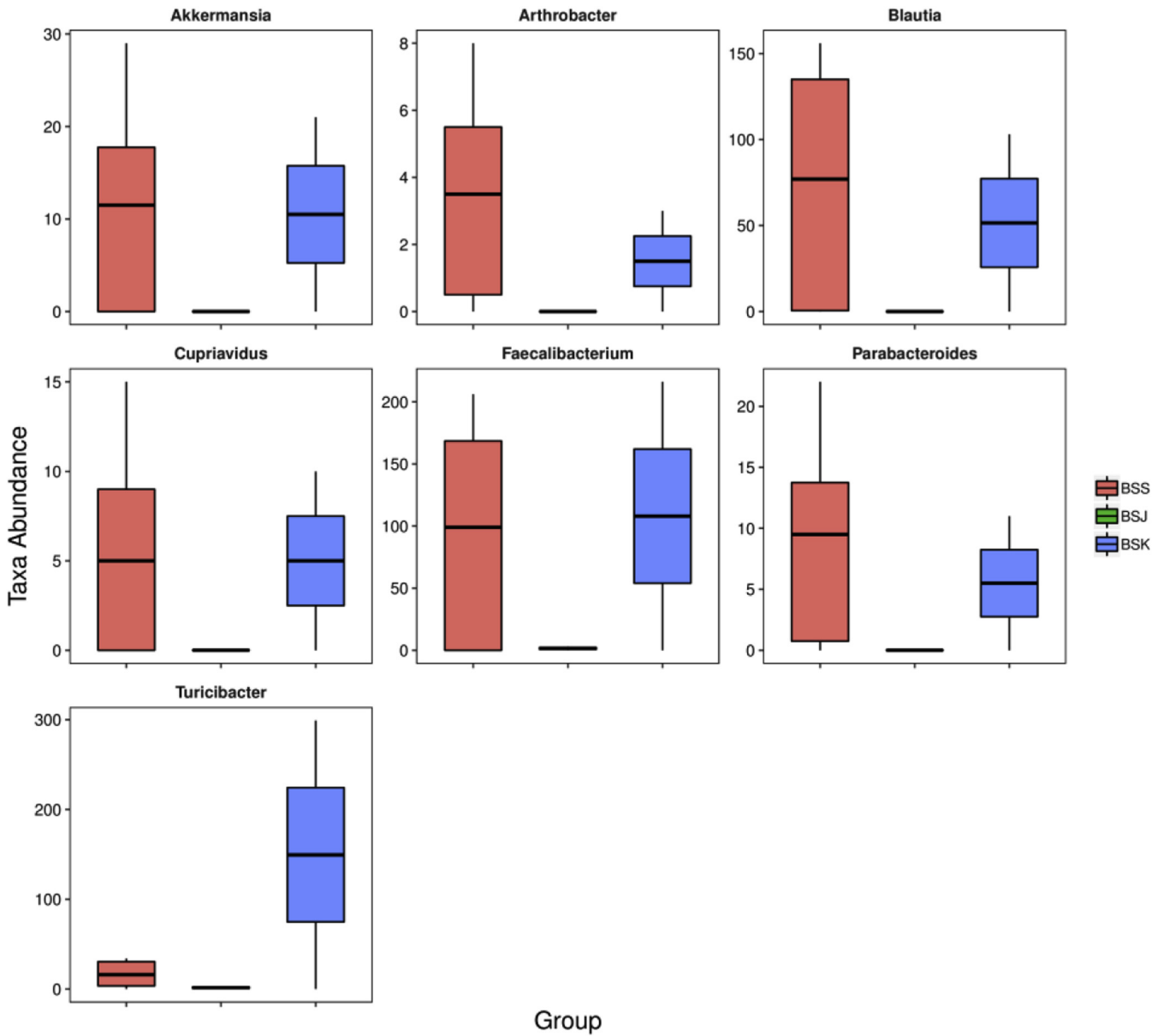
Figure 6. Metastats test samples in pairs between antibiotics control and LLE groups at the genus level. Samples were the first 20 taxa with the most significant differences. Abbreviations: BSS, lotus leaf extract groups; BSJ, antibiotics control group; BSK,blank control group; LLE, lotus leaf extract.

present study, the LLE5 and LLE7.5 groups showed significantly higher serum IFNG values when compared with the blank control group, but not the antibiotics control group. Meanwhile, serum IL2 concentrations only showed statistical increase in the LLE2.5 and LLE5 groups at 7 d and the LLE7.5 group at 21 d. Interferon-gamma contributes to the activation of antigen-presenting cells, and IL2 has a stimulatory effect on the body's defense mechanisms (Rochman et al., 2009; Khaled et al., 2020). The LLE-mediated activity of these cytokines in poultry would stimulate the recognition of LLE as an antigen, as well as the cytotoxicity of Tc lymphocytes, thereby promoting cellular immune responses. These results suggested that the addition of LLE as a feed additive at the 0.5% and 0.75% levels may be the most beneficial for the long-term maintenance of homeostasis in poultry.

The gastrointestinal microbiome plays an important role in maintaining gut and nutritional health in poultry production. Phytogenics, probiotics, prebiotics, and exogenous enzymes are commonly used to modulate the gut microbiome (Herrero-Encinas et al., 2020). Accordingly, bioactive plant-derived compounds are good candidates to modulate the composition of gut microbiota and enhance gut integrity. Bioactive phytochemicals patented worldwide include isoflavones, saponins, alkaloids, and lignocellulose, all of which are reported to effectively modulate the gut microflora, improve immune function, and promote both the absorption and digestion of nutrients by both livestock and poultry (Peng et al., 2014). Many traditional Chinese medicines are food and medicine homologs, possessing prebiotic properties that can alter the bacterial diversity and composition of fecal flora (Zhu et al., 2020). In this study, the bacterial
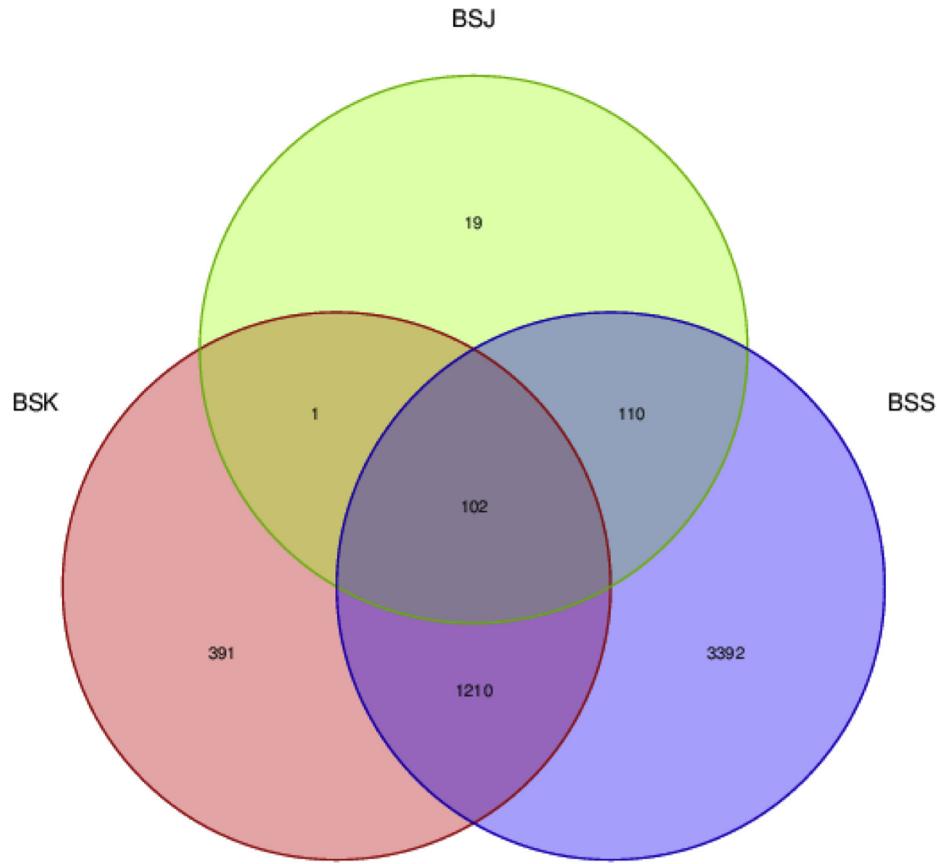
Figure 7. Venn diagram analysis of proportion of the intestinal microbes in different treatment. Abbreviations: BSS, lotus leaf extract groups; BSJ, antibiotics control group; BSK,blank control group.

diversity of broiler chicken intestines increased with LLE supplementation, suggesting that LLE may modulate the health of the intestine. In agreement with most studies, our data also showed that the broiler intestinal microbiota was mainly composed of Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (>95%), with

Firmicutes being the predominant phylum (61.1%) (Islam et al., 2019). A moderate shift in the proportion of Bacteroidetes to Firmicutes was evident in the control and LLE2.5, LLE5, and LLE7.5 groups throughout the trial. Wei et al. (2013) previously reported temporal shifts in the Bacteroidetes-to-Firmicutes ratio in the
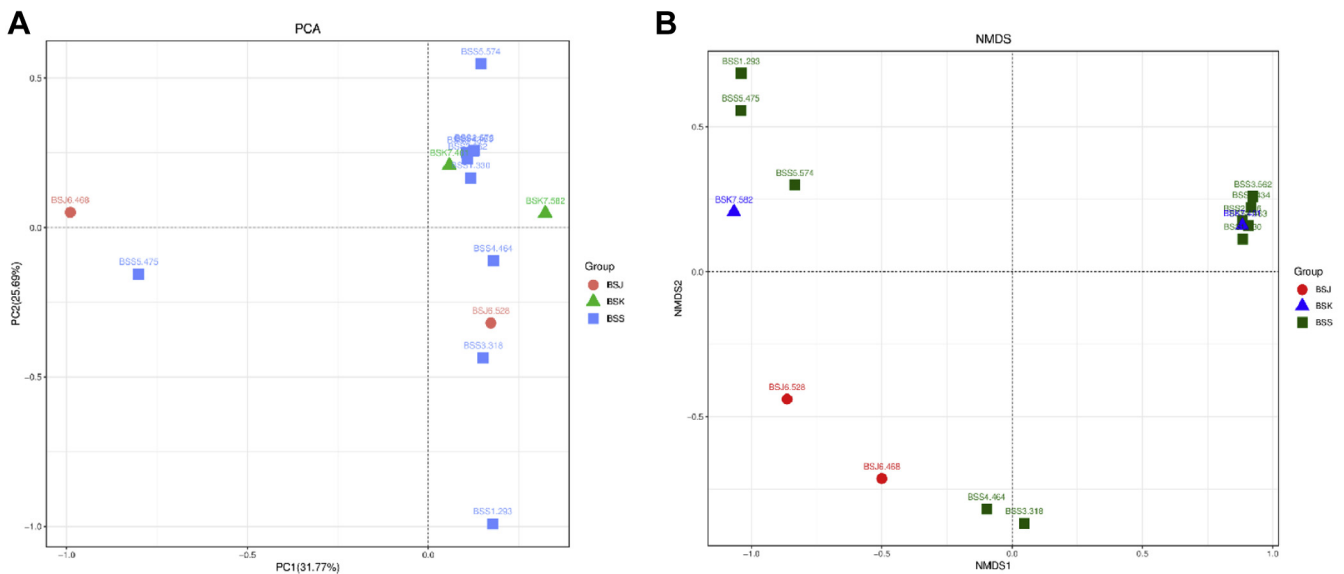

Figure 8. Results of β diversity analysis showed as principal coordinates analysis (PCoA) plots based on weighted Unifrac metrics. Abbreviations: BSS, lotus leaf extract groups; BSJ, antibiotics control group; BSK,blank control group.
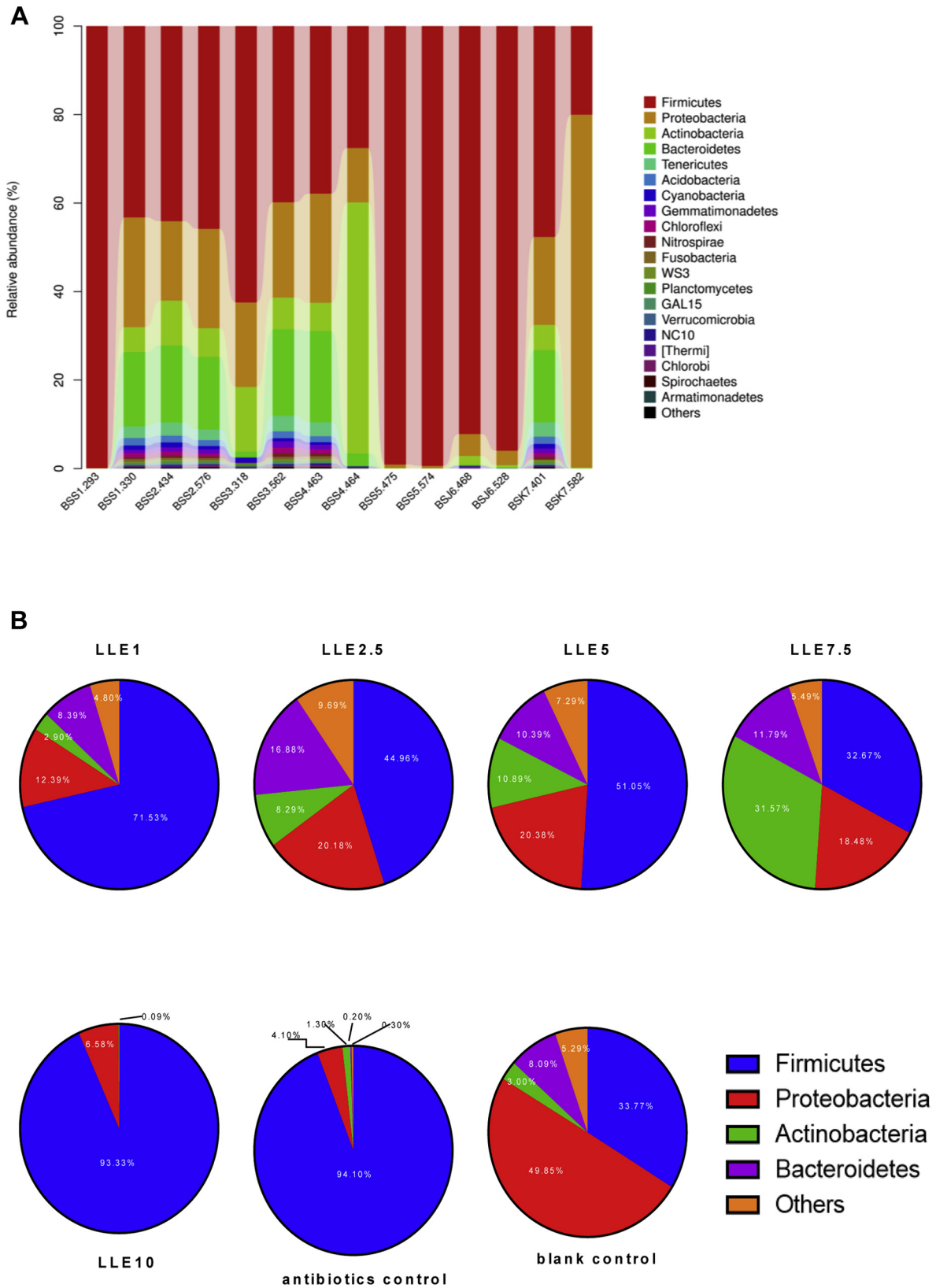
Figure 9. The intestinal microbial composition and species diversity of chickens fed diets with different concentration of LLE at the phylum level. LLE, lotus leaf extract. The numbers after BSS, BSJ and BSK is the chicken wing number in different groups. Abbreviations: BSS, lotus leaf extract groups; BSJ, antibiotics control group; BSK,blank control group.

chicken gut microbiota. Bacteroidetes and Firmicutes contribute to the fermentation of indigestible carbohydrates to short-chain fatty acids; members of the former phylum mainly produce butyrate, whereas members of the latter phylum produce acetate and propionate as primary metabolic end products (Islam et al., 2019;
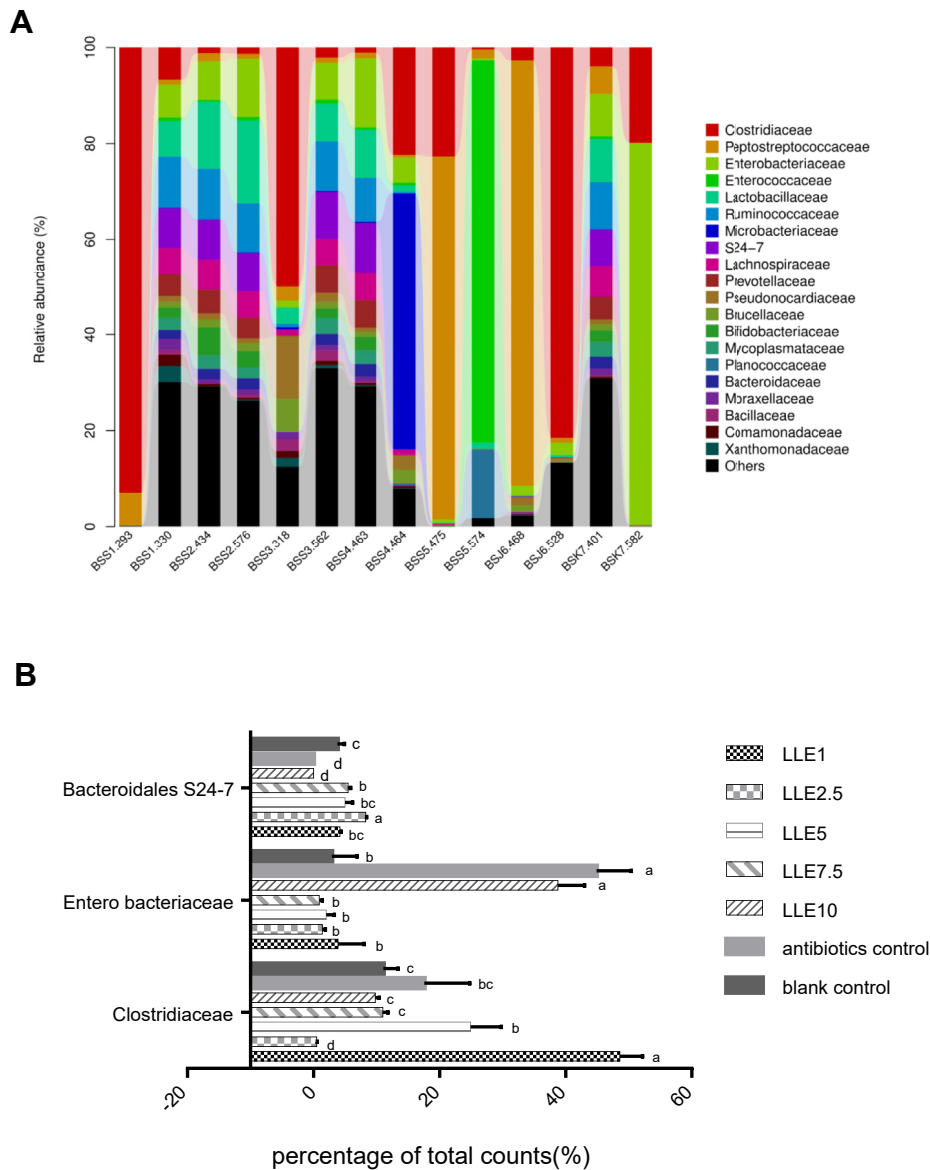
A

B

Figure 10. The intestinal microbial composition and species diversity of chickens fed diets with different concentration of LLE at the family level. LLE, lotus leaf extract. The numbers after BSS, BSJ and BSK is the chicken wing number in different groups. [a-d]Bars in the histogram with different letters were significantly different ($P < 0.05$), n = 2. Abbreviations: BSS, lotus leaf extract groups; BSJ, antibiotics control group; BSK, blank control group

Macfarlane and Macfarlane, 2003). Throughout the trial, the ADG was significantly greater in the LLE2.5, LLE5, and LLE7.5 groups than in the antibiotics and blank control groups, suggesting that the composition of the gut microbiota differed markedly between LLE-treated and LLE-untreated broilers that were genetically similar, of the same flock, and reared under identical conditions. At the family level, the abundance of Peptostreptococcaceae decreased, whereas that of Clostridiaceae and Bacteroidales increased. This is in agreement with results obtained by Mohd Shaufi et al. (2015), who observed a large abundance of *Clostridium* (47 to 70%) and *Bacteroides* in the ileal microbiome of broilers fed a commercial diet. Members of the genus *Bacteroides* are among the most effective degraders of indigestible carbohydrates and are characterized as short-chain fatty acid producers (Islam et al., 2019). The predominance of *Bacteroides* in

LLE-fed birds could prove advantageous for the digestion of otherwise indigestible carbohydrates in the host. Together, these results revealed that feeding an LEE-supplemented diet in poultry increased the bacterial diversity and abundance of the intestinal microbial community, which would be related to the function and stability in small intestine.

In conclusion, the present study provided experimental support for the prebiotic effects of LLE, with the results showing that LLE can enhance innate immunity and modulate the intestinal microbial communities in broiler chickens. These results help in the understanding of how feed LLE supplementation affects chickens and provide an interesting insight into the intricacy of chicken gut bacterial communities with phytogenics. Additional studies are needed to better understand the mechanisms underlying how LLE interacts with the

gut microbiota and the host to further enhance overall broiler health.

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DISCLOSURES

The authors in this manuscript have declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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