Effects of Ligustrum lucidum on egg production, egg quality, and caecal microbiota of hens during the late laying period

Xiaochen Chen*, Yaowhen Zhang*, Wenfeng Ma and Zhanbin Wang\footnote{These authors contributed equally to this work.}

Henan Provincal Academician Workstation of Feed Resource Development and Healthy Livestock, Department of Animal Science and Technology, Henan University of Science and Technology, Luoyang, China

ABSTRACT
This study was conducted to determine the effects of diet supplementation of laying hens with Ligustrum lucidum (LL) on egg production, egg quality and caecal microbiota. A total of 360 72-week-old Hy-Line Brown laying hens were divided into three groups with four replicates of 30 birds each. The laying hens were fed with basal diet (control group), basal diet + 1% LL (group I) and basal diet + 2% LL (group II). The experiment lasted for 45 d. Eggs were collected daily and caecal samples were collected at the end of the experiment. Results showed that dietary supplementation with LL did not affect the average daily egg weight, the average daily feed intake, the cracked egg rate, the mortality and the egg quality (\(p > 0.05\)). However, groups I and II showed significantly increased laying rate and decreased feed/egg ratio (\(p < 0.05\)). The differences in caecal microbiota between group II and the control group were significant. The relative abundance of Bacteroidetes, Firmicutes, Saccharibacteria and Verrucomicrobia at the phylum level; Ruminococcaceae, Rikenellaceae, Acidaminococcaceae, unclassified_Bacteroidales, S24-7 group and ODP1230B8.23 at the family level; RC9 gut group, Phascolarctobacterium, unclassified_Bacteroidales, Butyricicoccaceae, norank_f__Ruminococcaceae, Ruminococcaceae_UCG-014, coprostanoligenes group and Ruminococcaceae_UCG-005 at the genus level in group II changed significantly compared with that in the control group (\(p < 0.05\)). Dietary supplementation with 1% and 2% LL could improve the laying performance and affect the caecal microbial community structure of laying hens during the late laying period.

HIGHLIGHTS
- Dietary supplementations with Ligustrum lucidum significantly increased laying performance of hens during the late laying period.
- Dietary supplementations with Ligustrum lucidum not affected the egg quality of laying hens.
- Dietary supplementations with Ligustrum lucidum affected the microbial structure of caecum of laying hens.

Introduction
The trend in the use of phytobiotics in animal feed has been increased during last two decades. Phytobiotics includes a wide range of plant-derived products such as essential oils, herbs and oleoresins (Mohammadi Gheisar and Kim 2018). Ligustrum lucidum (LL) is a crucial Chinese herb rich in oleanolic acid, ursolic acid, triterpenoids, iridoids, flavones and phenolic glucosides (Huang and Wang 2011). Oleanolic and ursolic acids are well known for their anti-oxidant and anti-inflammatory activities (Liu 2005). Oleanolic acid also has higher antibacterial activity than ursolic acid (Ayeleso et al. 2017). In China, LL is traditionally used as a tonic agent to nourish the liver and kidney, strengthen the bone and muscle and prevent some diseases, such as diabetes (Gao et al. 2009; Li et al. 2015). Modern medical studies have demonstrated that LL possesses the functions of immune regulation, antitumor and anti-inflammatory; it can also protect cells from lipid peroxidation damage stimulated by oxidative stress (Huang et al. 2010; Hui et al. 2014; Liu et al. 2014). Recent studies on LL supplementations illustrated that LL could increase the laying rate; decrease the mortality and cracked-egg...
rate of laying hens; improve the biochemical blood markers, immune function, and antioxidant status and reduce the harmful effects of heat stress on hens (Ma et al. 2005; Ma et al. 2007; Li et al. 2017). They could also improve the growth performance of broilers and growing-finishing pigs (Chen et al. 2009; Zhang et al. 2012).

Gut microbial communities play a vital role in the health and function of the host. Animal gut microbiota are a complicated and diverse system easily affected by many factors, such as the environment, age, diet, feed additive, and hygiene level. Diet-related differences are the main cause of the total variations, indicating that diet components could influence the composition and diversity of gut microbiota (Zhang et al. 2010).

Hen egg production and egg quality generally decrease with age, thereby reducing the forming profits of poultry farmers. Therefore, keeping the egg production at a high rate is important. Few reports on the effects of LL as a feed additive on poultry diets are available, particularly on the intestinal microbiota in late laying period. Thus, the present study was conducted to determine the effects of dietary supplementation with LL on the egg production, egg quality, and caecal microbiota of laying hens during the late laying period to provide valuable references for the application of LL as a feed additive.

Materials and methods

The animal experimentation procedures were approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology, Luoyang, China.

**LL Preparation**

LL fruits were purchased from a Chinese herbal market in Luoyang, Henan Province, China. The main active constituents of fresh LL fruits were investigated using HPLC-photodiode array detection previously (Li et al. 2017) in our institute: oleanolic acid (7.49 mg/g), ursolic acid (1.607 mg/g) and total flavonoids (78.142 mg/g). After the LL was dried at 60 °C, it was grounded into fine powder in a knife mill and strained through a 40-mesh sieve.

**Diets, experimental design and laying hens**

A total of 360 72-week-old Hy-Line Brown laying hens were assigned to three groups, with four replicates of 30 hens in each group. The hens were housed in 120 cages with a size of 64 cm × 35 cm × 35 cm (three hens each). The hens had free access to feed and water during the experiment and exposed to a 16:8 light–dark cycle. The average room temperature was 20 °C ± 3 °C. The differences in egg production rate among the three groups before the start of the experiment were insignificant.

The laying hens were fed with basal diet (control group), basal diet + 1% LL (group I) and basal diet + 2% LL (group II). The diets were formulated to be isocaloric and isonitrogenous and to meet the nutrient requirements of laying hens according to the Management Guide of National Research Council (NRC, 1994). Table 1 shows the composition and nutrient levels of the basal diet. Crude protein (method 976.06), available phosphorus (method 993.31), calcium (method 927.02) and amino acid composition (method 994.12) were analysed in accordance with the method of AOAC (1999). The experiment lasted for 45 d.

### Data collection

**Egg production**

Daily records of egg production, cracked-eggs, egg weight, hen mortality and feed consumption were recorded. The feed was supplied ad libitum and the daily feed consumption was recorded. The egg production rate was calculated as the proportion of the eggs produced to the total number of hens. The egg weight was calculated as the ratio of the total weight of the eggs produced to the number of eggs produced.

**Table 1.** Ingredient composition and analysed nutrient contents of the basal diet [g/kg] (data is based on feed).

| Items                  | Content |
|------------------------|---------|
| Ingredients             |         |
| Corn grain              | 645.00  |
| Soybean meal            | 130.00  |
| Rapeseed meal           | 40.00   |
| Limestone               | 80.00   |
| Premix<sup>a</sup>      | 50.00   |
| Wheat bran              | 50.00   |
| Soybean oil             | 5.00    |
| Metabolisable energy<sup>b</sup>[MJ/kg] | 10.71 |
| CP                     | 14.13   |
| Calcium                 | 3.88    |
| Total phosphorus        | 0.63    |
| Non-phytate phosphorus  | 0.42    |
| Digestible lysine       | 0.68    |
| Digestible methionine   | 0.33    |
| Digestible methionine + cysteine | 0.57 |
| Digestible isoleucine   | 0.53    |
| Digestible threonine    | 0.48    |
| Digestible serine       | 0.17    |
| Digestible valine       | 0.62    |
| Analysed nutrient composition |     |
| Dry matter              | 91.25   |
| CP                     | 15.42   |
| Calcium                 | 3.95    |
| Total phosphorus        | 0.57    |

<sup>a</sup>Provide per kg of diet: retinol A, 1.32 mg; Cholecalciferol, 0.03 mg; tocopherol, 6 mg; thiamine, 1.4 mg; Riboflavin, 3 mg; pyridoxine, 1.0 mg; Cyanocobalamin, 0.01 mg; pantothenic acid, 7.5 mg; choline chloride, 500 mg; thiamine, 0.15 mg; Ca,7500 mg; P, 3000 mg; Mn, 72 mg; Zn, 56 mg; Fe, 60 mg; Cu, 25 mg; I, 0.50 mg; Se, 0.10 mg.

<sup>b</sup>Metabolizable energy was calculated from data provided by Feed Database in China 2009.
maintained during the experimental period. Laying rate (including cracked eggs), average daily egg weight, average daily feed intake, cracked-egg rate and feed/egg ratio were analysed.

**Egg quality**
Six saleable eggs (i.e., no shell defects or cracks) were randomly selected from each replication on day 45 and utilised to determine the egg quality. Egg albumen height and Haugh units were determined using a digital egg tester (TSS, England, UK), and eggshell breaking strength was determined using In-Spec 2200 (Instron Corporation, Canton, Massachusetts, USA). Egg shape index was assessed using the following formula: vertical diameter/transect diameter. Eggshell thickness was assessed using a propeller micrometre and a spiral rangefinder.

**Caecal microbiome**
Four hens were randomly selected from the control group and group II (one for each replication) and sacrificed on the last day of the experiment. Caecum samples were aseptically collected from each bird, immediately stored at $-80^\circ C$ and promptly processed.

**DNA extraction and PCR amplification**
Microbial DNA was extracted from the caecum samples by using the EZNA Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) in accordance with the manufacturer’s protocols. Final DNA concentration and purification were checked via a NanoDrop 2000 UV vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked via 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 341 F (5’-CCTAYGGGRBGCASCAG-3’) and 806 R (5’-GGACTACNNGGGTATCTAAT-3’) on a thermocycler PCR system (GeneAmp 9700, ABI, USA). PCRs were conducted as follows: denaturation at $95^\circ C$ for 5 min, followed by 27 cycles at $95^\circ C$ for 30 s, $55^\circ C$ for 30 s, and $72^\circ C$ for 45 s and a final extension at $72^\circ C$ for 10 min. They were performed in triplicate with $20 \mu L$ of mixture containing 4 µL of 5 × FastPfu buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase and 10 ng of template DNA. The PCR products were electrophoresed using 2% agarose gel, further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST (Promega, USA) in accordance with the manufacturer’s protocol.

**Illumina MiSeq sequencing**
The purified amplicons were pooled in equimolar and paired-end sequenced ($2 	imes 300$) on an Illumina MiSeq platform (Illumina, San Diego, USA) in accordance with the standard protocols of Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive database (accession no.: SRP158075).

**Sequencing data processing**
Raw fastq files were demultiplexed, quality filtered using Trimmomatic and merged using FLASH with the following criteria: (i) the reads were truncated at any site receiving an average quality score < 20 over a 50-base pair (bp) sliding window; (ii) the primers were exactly matched, thereby allowing two-nucleotide mismatching, and the reads containing ambiguous bases were removed; and (iii) sequences with overlaps longer than 10 bp were merged on the basis of their overlap sequences.

Operational taxonomic units (OTUs) were clustered with 97% similarity cut-off using UPARSE (version 7.1, http://drive5.com/uparse/). Chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analysed using the RDP classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database with a confidence threshold of 70%.

**Statistical analysis**
Data were analysed using one-way ANOVA on SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The significance of the mean differences among the groups was identified using the Tukey test. Data significance was determined at $p < .05$. Alpha diversity and rarefaction curve analyses consisting of community diversity (Simpson and Shannon indices) and richness indices (Sobs, Chao and ACE) were performed using mothur that is based on summary single command. Beta diversity analysis was performed to investigate the diversity among the samples. Principal component analysis (PCA) was calculated using mothur to describe the distances among the samples, and permutational multivariate ANOVA (PERMANOVA) was used to conduct statistically significant analysis on the OTU level. Welch’s t-test was used to determine the significant differences in terms of bacterial relative abundance at the phylum, family and genus levels between the LL and the control group.
Results

Egg production

Table 2 shows the laying performance of hens. Compared with the control group, groups I and II exhibited significantly increased laying rate and decreased feed/egg ratio (p < 0.05), respectively. However, the differences in average daily egg weight, daily feed intake, broken egg rate and mortality among the three groups were insignificant (p > 0.05).

Egg quality

Table 3 shows the egg quality of hens. The differences in egg albumen height, Haugh units, egg shape index, eggshell breaking strength and eggshell thickness among the three groups were insignificant.

Caecal microbiota

A total of 383,098 DNA sequence reads with an average length of 416 bp were generated from all samples. The average number of sequence reads per sample was 47,887 (minimum of 37,571 and maximum of 57,048). The sequences were further clustered into 781 OTUs by using a 97% similarity cut off. The rarefaction curves generated from the OTU suggested that a high sampling coverage (~99%) was achieved in all samples (Supplementary Figure S1). Supplementary Table S1 shows the Alpha diversity indices of the caecal microbiota. The average values of microbial diversity indices in the LL group were lower than those in the control group.

PCA on the OTU level showed the changes in the community structure (Figure 1). The microbial community of the LL group was clearly separated from that of the control group. The results revealed a significant difference in bacterial structure in the caecum between LL and control groups (PERMANOVA, p = .031).

Figure 2 and Supplementary Table S2 demonstrate the phylum distributions of the microbial composition. The predominant phyla in the LL and control groups were Firmicutes, Bacteroidetes and Proteobacteria. The relative abundance of Bacteroidetes and Saccharibacteria was significantly increased and that of Firmicutes and Verrucomicrobia was significantly decreased in the LL group compared with those in the control group (p < .05).

The family distribution results of the microbial composition are shown in Figure 3 and Supplementary Table S3. The main families of the LL and control groups were Ruminococcaceae, Rikenellaceae, Bacteroidaceae and Lachnospiraceae, with Ruminococcaceae as the most abundant one. The average proportion of Ruminococcaceae was significantly decreased and that of Rikenellaceae was significantly increased in the LL group compared with those in the control group (p < .05). In addition, several families with small relative abundance (Acidaminococcaceae, unclassified_Bacteroidales, S24-7_group and ODP123088.23) in the LL group changed significantly compared with those in the control group (p < .05).

The microbial composition was also comparable at the genus level (Figure 4 and Supplementary Table S4). The main genera of LL and the control groups were RC9 gut group and Bacteroides. Between these genera, the relative abundance of the RC9 gut group was significantly increased in the LL group compared with those in the control group (p < .05). Dietary with LL also significantly effected the average proportion of some low relative abundance genus (Phascolarctobacterium, unclassified_Bacteroidales, Butyriviricoccus, norank_f__Ruminococcaceae, Ruminococcaceae_UGC-014, coprostanogenes_group and Ruminococcaceae_UGC-005) compared with the control group (p < .05).
Figure 1. Principal Component Analysis (PCA) of the community membership using Bray-Curtis distance. CON, control group; LL, group II. The abscissa and ordinate represent the two selected principal components, and the percentage represents the contribution of the principal component to the difference in sample composition. Points of different colours and shapes represent samples of different groups, and the closer the two sample points are, the more similar the composition of the two samples species is.

Figure 2. Welch’s t-test bar plot on Phylum level. CON, control group; LL, group II. The ordinate (left) represents the phyla name and the ordinate (right) represents the \( p \)-Value.
Discussion

Effects of LL on egg production

Herbs possess beneficial effects on growth performance, antioxidation as well as nutrient and energy utilisation for animals (Wenk 2003). The dietary supplement of LL can significantly increase the egg production rate (Ma et al. 2005), and decrease the mortality rate of laying hens (Li et al. 2017). Similar to previous research, our results demonstrated that the dietary inclusion of 1% and 2% LL significantly increased the egg production rate and decreased the feed/egg ratio of hens in late laying period. These changes may be related to the positive effects in the biological activity of bioactive
components, such as oleanolic acid, ursolic acid and flavonoids present in LL, which can improve the efficiency and utilisation of nutrients, thereby enhance the productive performance (Gao et al. 2009).

**Effects of LL on egg quality**

Eggs are an excellent source of many essential nutrients. Egg quality is still a topic of concern in the poultry industry and for consumers worldwide. In the present study, the differences of egg quality among the three groups were insignificant.

**Effects of LL on caecal microbiota**

Chicken’s intestinal tract microbiota is important for their health, nutrient absorption and immune system. Some authors suggested that almost 10% of energy needs is recovered from a well-functioning caecum (Hegde et al. 1982; Józefiak et al. 2004).

The alpha diversity of microbial communities was measured using the indices of Sobs, Shannon, Simpson, Ace and Chao. The Shannon and Simpson indices represent how much of the differences are among the abundance of different taxa (diversity), whereas the Sobs, Chao and Ace indices reflect the number of the different taxa present in the sample (richness). Our study showed significant differences in the caecal microbiota diversity between the LL and the control groups. Combined with the result of the microbiota composition, the differences of the microbial structure may be due to the increase relative abundance of Bacteroidetes and the concurrent decrease of Firmicutes at the phylum level in the LL group. The results revealed the significantly lower average values of microbial diversity and richness in the LL group compared with those in the control group. These results indicated that dietary supplementation with LL seemed to reduce the bacterial species in the chicken caecal gut. The specific effect mechanism is still unclear.

Consistent with previous studies (Pan and Yu 2014; Zheng et al. 2019), our results also demonstrated that the caecal microbial communicates were dominated by Firmicutes, Bacteroidetes and Proteobacteria in chickens, which made up > 90% of the total sequences. Meanwhile, the higher relative abundance of Bacteroidetes and the lower Firmicutes than that of the control group were observed in the LL group. Bacteroides could digest complex carbohydrates and maintain an intestinal micro-ecological balance (Hooper 2004; Spence et al. 2006). Bacteroides could promote the development of the immune system (Sears 2005). Bacteroidetes is related to the development of interleukin-17-producing T-helper cells (Mazmanian et al. 2005).

Firmicutes comprises a lot of genera of outstanding relevance in health care such as Staphylococcus, Listeria, and lactic acid bacteria (Lanza et al. 2015) and can obtain more energy from fibrous feed to satisfy the requirement of animal growth (Brulc et al. 2009). In the present study, we also observed significant differences in the relative abundance of Verrucomicrobia and Saccharibacteria between the LL and the control group, but the function of these phyla in the hen's caecum was unclear and needed further investigations.

At the family level, the relative abundance of Rikenellaceae significantly increased in the LL group which may have partially contributed to the increased Bacteroidetes relative abundance in the LL group. Rikenellaceae is a relatively new taxonomic classification that belongs to the order Bacteroidales, with only three genera currently described belonging to this family (Graf 2014). Some bacteria in Rikenellaceae can produce acetate (Su et al. 2014), thereby suggesting a high level of functional redundancy for this metabolite in the animal gut microbiome. The high abundance of Rikenellaceae in the LL group may be due to the increase in the proportion of the RC9 gut group which belongs to Rikenellaceae in the LL group at the genus level. Ruminococcaceae family is a major population of phylum Firmicutes, the lower relative abundance of Ruminococcaceae may cause a decrease of Firmicutes in the LL group. Ruminococcaceae can produce short-chain fatty acids and degrade diverse polysaccharides and fibres in the human gut (Hooda et al. 2012). Except of the unclassified bacteria, the relative abundance of Acidaminococcaceae, Bacteroidiales_S24-7_group and ODP12308.23 also changed significantly in the LL group compared with that in the control group at family level. However, further studies are needed to investigate the function of these families in the chicken’s gut.

Similarly, as reflected in the phylum and family, our results showed considerable differences in genus composition. The main genera of the LL and the control groups were RC9 gut group and Bacteroides. A large proportion of Firmicutes are composed of the genus Clostridium in chicken’s caecum (Pan and Yu 2014). However, in our study, Firmicutes was predominated by sequences belonging to Faecalibacterium and Phascolarctobacterium genus. Many factors can contribute to these differences. These factors include different chicken types, ages, diets and environmental factors. For example, the abundance of Clostridium increases and the proportion of lactobacilli decreases as the chicken aged (Shang et al. 2018). Different diet ingredients can also affect the relative abundance of Firmicutes and Bacteroidetes in chickens (Kumar et al. 2019).
Cage type can also influence the composition of ileal and caecal microbiota of laying hens (Nordentoft et al. 2011). Genotype and gender can affect some microbiome species in the chicken gut (Zhao et al. 2013). These complexities make comparison with other studies difficult.

Sequences related to the genera Butyricicoccus were enriched in the caecum of hens in the LL group. The species of Butyricicoccus genera include Butyricicoccus pullicaecorum which is a main butyrate-producing strain in the intestine (Eckhaut et al. 2008). Butyrate mediates beneficial effects on the growth performance and intestinal integrity of chickens (Mountzouris et al. 2010; Ahmed et al. 2014). Butyrate can also induce the synthesis of a subset of host defense peptides (cathelicidins) in jejunal and caecal explants from the chicken origin, with a natural broad antimicrobial spectrum that is important as the first line of defense (Sunkara et al. 2011). The relative abundance of Phascolarctobacterium, Ruminococcaceae_UCG-014 and Ruminococcaceae_UCG-005 were all significantly decreased in the LL group. The decrease of Ruminococcaceae_UCG-014 and Ruminococcaceae_UCG-005 might explain the decreased average proportion of Ruminococcaceae family. These results might be detrimental for hens because Phascolarctobacterium is an acetate/propanoate-producer (Wu et al. 2017), and Ruminococcaceae_UCG-014 is positively related to butyrate concentrations (Han et al. 2018). However, information about the RC9 gut group, norank_f_Ruminococcaceae and coprostanoligenes group is lacking. Therefore, evaluating the possible effect of the changes in these genera on the chicken’s caecum microbial community is difficult. To the best of our knowledge, the effects of dietary supplementation with LL on the hen’s caecal microbiota have not been reported yet. Therefore, the cause and effect relationships driving these changes in the present study still need to be determined. However, since herbs can influence selectively the microorganisms by an anti-microbial activity or by a favourable stimulation of the aerobiosis of the microflora (Wenk 2003), LL may have also acted in this way in the present research. The exact reason for these results needs to be further studied.

**Conclusions**

The results indicated that dietary supplementation with 1% and 2% LL could improve egg production rate and significantly decrease feed/egg ratio compared with unsupplemented hens, respectively. Dietary supplementation with 2% LL can significantly alter the caecal microbiota composition with the changes in the phylum, family and genus levels. Therefore, 2% LL was the more appropriate level in this experiment. These findings provide an insight into hen’s egg production and gut microbiota modulations in response to different LL dosages and emphasise the requirement for additional research to determine the effects of LL on the chicken gut microbiota to predict the response of the microbiota to these agents.

**Ethical approval**

This research did not involve the introduction of any intervention on hens. The data collection was obtained with humanly handled, which according of animal care and welfare standard of The People’s Republic of China. Animal experimentation procedures were approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology, Luoyang, China.

**Disclosure statement**

No potential conflict of interests was reported by the authors.

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**ORCID**

Zhanbin Wang @ http://orcid.org/0000-0001-7466-7754

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