

Effects of Cordyceps Militaris Polysaccharide on Egg Production, Egg Quality and Caecal Microbiota of Layer Hens

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ABSTRACT

The present study was conducted to determine the effects of the diet supplementation of laying hens with Cordyceps Militaris Polysaccharide (CMP) on egg production and quality, and also caecal microbiota. A total of 360 Hy-Line Brown laying hens with 72-week-old were divided into three groups with four replicates of 30 birds each. The laying hens were fed with basal diet (control group), basal diet +100 mg CMP/kg (group 1) and basal diet + 200 mg CMP/kg (group 2). The experiment lasted 45 days. Eggs were collected daily and caecal samples were collected at the end of the experiment. Results showed that dietary supplementation with CMP did not affect albumen height, shape index, Haugh units, eggshell breaking strength and eggshell thickness. The laying rate significantly increased and the feed-egg ratio decreased in groups 1 and 2, the average daily egg weight significantly increased in group 2 compared with those in the control group. However, insignificant differences were found in broken egg rate and mortality among the three groups. The differences in caecal microbiota between group one and the control group were significant. The relative abundance of Firmicutes, Bacteroidetes and Proteobacteria at the phylum level, Christensenellaceae and Veillonellaceae at the family level, and the no rank_ Ruminococcaceae, Phascolarctobacterium and no rank Christensenellaceae at the genus level changed significantly in group one compared with those in the control group. In conclusion, dietary supplementation with 100 and 200 mg CMP/kg could improve product performance and affect the caecal microbial community structure of laying hens during the late laying period.

Key words: Cordyceps militaris polysaccharide, Egg production, Egg quality, Gut microbiome, Hens

INTRODUCTION

Antibiotics, a feed additive to improve animal production performance and disease resistance, are widely used in animal production. However, abuse of antibiotics during feeding had led to drug resistance of pathogens, which led to an imbalance in the intestinal flora of livestock and poultry (Chang et al., 2015). Antibiotics residues in livestock products and caused environmental pollution (Martinez, 2009). The researchers were therefore trying to find better antibiotic alternatives for laying hens.

Cordyceps Militaris Polysaccharide (CMP), one of the most important active substances from *C. militaris*, was composed of mannose, cordycepin, adenosine, arabinose, and fucose. CMPs extracted from *C. militaris* and *C. sinensis* had the same effect, and *C. sinensis* was rare, so Cordyceps polysaccharide was often extracted from *C. militaris* (Das et al., 2010). Studies had shown that

Cordyceps polysaccharides had some positive effects on improving immunity, reducing blood lipids and protecting the liver and the kidney (Cheung, et al., 2009; Lee, et al., 2010a; Shin, et al., 2010). Cordyceps polysaccharides, as a feed additive had received wide attention in Asia due to their diverse functions in the livestock industry. For example, Cordyceps polysaccharides could promote the early growth of chickens, increase the feed conversion ratio and enhance the daily growth gain of broilers (Han et al., 2015). *C. militaris* waster medium increased the egg production rate and decreased the feed- egg ratio (F/G) (Wang et al., 2015). Hot-water extract from the mycelia of *C. sinensis* maintained the better microbial flora from the intestines of chickens and improved the growth performance and health index of chickens (Koh et al., 2003).

Microbial communities in the intestine played a

crucial role in the health and function of hosts. Animal intestinal flora was a complex and diverse system and could easily be influenced by many factors, such as environment, age, nutrition, feed additive and hygiene level. Nutritional differences were the main cause of the overall variations, suggesting that food components could influence the composition and diversity of the intestinal flora (Zhang et al., 2010). Chicken egg production and quality generally decreased with age, reducing the agricultural profits of poultry farmers. It was therefore important to keep the egg production at a high level. There had been few reports on the effects of CMP as a feed additive on poultry nutrition, particularly on the intestinal flora in the late laying period. Hence, this study was conducted to determine the effects of CMP supplementation on egg production and quality, and also caecal microbiota during the late laying period of chickens and to provide valuable information on the use of CMP as feed additives.

MATERIALS AND METHODS

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.

Preparation of *Cordyceps militaris* polysaccharide

Freeze-dried *C. militaris* polysaccharide was purchased from Yangshao Biology and Chemical Engineering Company, Mianchi City, Henan Province, China.

Laying hens, diets and experimental design

A total of 360 Hy-Line Brown laying hens at 72-week of age were assigned to three groups with four replicates of 30 hens in each group. The hens were housed in 120 cages sized 64 cm × 35 cm × 35 cm (three hens each) During the experiment, the hens were free to obtain feed and water and were exposed to a light dark cycle of 16 hours: 8 hours. The average room temperature was 20 ± 3°C. Before the experiment started, there was no differences in the egg production rate between the three groups. The laying hens were fed basal diet (control group), and basal diet + 100 mg/kg CMP (group 1) and basal diet + 200 mg/kg CMP (group 2). The entire process

included a 7-day pre-experiment and a 45-day formal experiment. The diets consisted of corn-based basal rations formulated according to the Management Guide of National Research Council (NRC, 1994) to meet the nutritional requirements of laying hens. Composition of basal feed and nutrients content in proximate analysis were showed in Table 1.

Table 1. Composition and nutrients content of basal feed

Feedstuffs ingredients	Percentage (%)
Corn grain	64.50
Soybean meal	13.00
Rapeseed meal	4.00
Limestone	8.00
Premix ¹	5.00
Wheat bran	5.00
Soybean oil	0.50
Total	100
Analyzed nutrient composition	Content
Dry matter (%)	88.89
Ash (%)	13.87
Crude Fiber (%)	4.17
Crude Protein (%)	19.90
Crude Fat (%)	4.59
Gross Energy (MJ/ kg)	16.58
Metabolizable Energy ² (MJ/ kg)	11.61

¹ = Provide per kg of diet: vitamin A, 4000 IU; vitamin D3, 1200 IU; vitamin E, 6 mg; vitamin B1, 1.4 mg; vitamin B2, 3 mg; vitamin B6, 1.0 mg; vitamin B12, 0.01 mg; pantothenic acid, 7.5 mg; choline chloride, 500 mg; biotin, 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. ² = Metabolizable energy was calculated from data provided by Feed Database in China 2009. MJ/ kg = Mega Joule per kilogram

Egg production

During the experimental period, daily records of egg production, egg cracking, egg weight and mortality of hens and records of feed consumption were maintained. The Lay rate (including cracked eggs), average daily egg weight, cracked egg rate, and feed to egg ratio were analyzed.

Egg quality

Six saleable eggs (no shell defects or cracks) were randomly selected from each replication on day 45 and used to determine egg quality, egg albumen height and Haugh units were determined with a digital egg tester (TSS, England, UK), eggshell breaking strength was identified with a strength instruction (In-spec 2200, Instron Corporation, Canton, Massachusetts, USA), egg shape index was assessed using the following formula including vertical diameter/transverse diameter, egg diameter

was assessed with a vernier caliper and eggshell thickness was assessed with a Spiral range finder.

Caecal microbiome

Four hens were randomly selected from the control group and group 2 (one for each replication) and sacrificed on the 45th day. The caecum samples were collected aseptically from each bird, immediately stored at -80°C and processed promptly.

DNA extraction and PCR amplification

According to the manufacturer's protocol, an E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA) was used to extract microbial DNA from cecum samples. Final DNA concentration and purification were determined using a Nano Drop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked through 1% agarose gel electrophoresis. The hypervariable regions (variable 3-variable 4) of the bacterial 16S rRNA gene were amplified with the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3') by using a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR was conducted by denaturing at 95°C for 5 minutes (min.), followed by 27 cycles at 95°C for 30 second (s), 55°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. PCR was performed in triplicate with a 20 μL mixture containing 4 μL of $5\times$ 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. PCR products were electrophoresed using a 2% agarose gel, purified using 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

Purified amplicates were separated pooled in equimolar and paired-end sequenced (2 \times 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw data was stored into the NCBI Sequence Read Archive database (accession nos. SRP154946 and PRJNA555247).

Sequencing data processing

Trimmomatic were used to demultiplex and do quality-filter for the raw fastq files, then merged them by FLASH according to the following criteria. First, reads

were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window; then, primers were exactly matched, allowing two nucleotide mismatching, and the reads containing ambiguous bases were removed; eventually, sequences with overlaps longer than 10 bp were merged on the basis of their overlap sequence.

Using UPARSE version 7.1 the operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off, the UCHIME were used to identify and remove chimeric sequences. RDP Classifier algorithm were used to analyse the taxonomy of each 16S rRNA gene sequence, which against the Silva (SSU123) 16S rRNA database with a confidence threshold of 70%.

Data analysis

Data were analyzed by one-way ANOVA using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The significance of the mean differences between the groups was identified by a Tukey test. Data significance was determined at $p < 0.05$. Alpha diversity and dilution curve analyses consisted of community diversity (simpson and shannon), richness (sobs, chao and ace) were performed using mothur (a 16S rRNA clustering tool) based on a single summary command. Beta diversity analysis was carried out to investigate the diversity between the samples. Principal component analysis (PCA) was conducted with mothur to describe the distances between the samples, and a permutative multivariate analysis of variance (PERMANOVA) was performed to analyze the significance of the OTU levels. Wilcoxon rank sum test was used to determine the significant differences in the relative abundance of bacteria at phylum and genus level between the CMP and the control group.

RESULTS

Egg production

Table 2 shows the laying performance of hens. The egg production increased significantly and the feed-egg ratio decreased ($p < 0.01$) in groups 1 and 2 compared to those in the control group. The average daily egg weight increased significantly in group 2 ($p < 0.05$). However, insignificant differences in the cracked egg rate and mortality were found among the three groups ($p > 0.05$). The egg production in group 2 increased significantly compared to group 1 ($p < 0.01$).

Egg quality

Table 3 shows the egg quality of hens. Insignificant differences were observed in the egg albumen height, the egg shape index, the Haugh units, eggshell breaking strength and eggshell thickness between three groups ($p > 0.05$).

Caecal microbiota

A total of 380,227 DNA sequence read with an average length of 415 bp were generated from all of samples. The average number of sequence readings per sample was 47,528, with a minimum number of 35,454 and a maximum number of 58,207. The sequences were further clustered into 749 OTUs by using a 97% similarity cut off. The sparse curve generated by the OTU shows that all samples achieved a high sampling coverage (~ 99%) (Figure 1). Table 4 shows the alpha diversity indices of caecal microbiota. The average values of ace, chao and sobs in the CMP group were significantly lower than those in the control group.

The PCA on the OTU level showed the changes in the community structure (Figure 2). The microbial community of the CMP group was clearly separated from that of the control group. The results revealed a significant difference in the bacterial structure in the caecum between the CMP and control groups (PERMANOVA, $p = 0.026$).

Figure 3 and table 5 demonstrate the phylum distributions of the microbial composition. In the CMP group and the control group, the main flora were

Firmicutes, Bacteroidetes and Proteobacteria. Firmicutes significantly decreased in the CMP group compared with that in the control group ($p < 0.05$). In contrast, the proportions of Bacteroidetes in the CMP group increased compared to those in the control group ($p < 0.05$). The two phyla with a low relative abundance (Synergistetes and Elusimicrobia) decreased significantly in the CMP group compared to those in the control group ($p < 0.05$).

The family distribution results of the microbial composition are shown in figure 4 and table 6. The main families of the CMP and control groups were Ruminococcaceae, Bacteroidales and Lachnospiraceae. Among them, Ruminococcaceae was the most abundant. The two families with a low relative abundance (Veillonellaceae and Christensenellaceae) decreased significantly in the CMP group compared to those in the control group ($p < 0.05$).

The microbial composition was also comparable at the genus level (Figure 5 and table 7). The main genera of the CMP and control groups were norank Bacteroidales, norank Ruminococcaceae, Bacteroides Bacteroidaceae and Oscillospira. Among these genera, the relative abundance of norank Ruminococcaceae decreased significantly in the CMP group compared to those in the control group ($p < 0.05$). The two genera with a low relative abundance (Phascolarctobacterium and norank_Christensenellaceae) decreased significantly in the CMP group compared to those in the control group ($p < 0.05$).

Table 2. Effects of *Cordyceps militaris* polysaccharide on laying performance in 72-week-old laying hen at Louyang, China.

Items ¹	Treatment Groups			SEM ²	P value
	Control	Group 1	Group 2		
Egg production (%)	79.95 ^c	83.26 ^b	85.55 ^a	0.508	<0.01
Cracked egg rate (%)	0.26	0.39	0.22	0.084	0.45
Average daily egg weight (g)	64.14 ^b	64.97 ^{ab}	65.62 ^a	0.221	0.02
Feed to egg ratio	2.17 ^a	1.96 ^b	1.98 ^b	0.023	<0.01
Mortality (%)	4.17	3.33	4.17	0.804	0.71

¹Control, Group 1, Group 2 with 0, 100, 200 mg CMP/kg, respectively; ²SEM= pooled standard error of mean. ^{ab}= Means not sharing the same superscripts in a row differ significantly ($p < 0.05$).

Table 3. Effects of *Cordyceps* Polysaccharide on egg quality in 72-week-old laying hen at Louyang, China.

Items ¹	Treatment Groups			SEM ²	P value
	Control	Group 1	Group 2		
Egg shape index	1.25	1.35	1.30	0.024	0.21
Eggshell thickness (mm)	0.35	0.34	0.31	0.012	0.28
Eggshell breaking strength (kg/cm ²) ³	2.48	3.37	3.61	0.361	0.46
Albumen height (mm)	5.41	5.43	5.34	0.282	0.49
Haugh unit	70.24	70.43	71.56	2.016	0.86

¹Control, Group 1, Group 2 with 0, 100, 200mgCMP/kg, respectively; ²SEM= pooled standard error of mean. ³kg/cm²= Kilogram per square centimetre

Table 4. Alpha Indices data of caecum samples in 72-week-old laying hen at Louyang, China.

Estimators	CON-Mean ¹	CON-Sd ²	CMP-Mean	CMP-Sd	P Value
Ace	710.73	15.594	629.72	42.39	0.012
Chao	712.82	12.958	638.82	45.648	0.021
Coverage	0.99834	0.0007961	0.9979	0.00093558	0.502
Shannon	5.1584	0.080538	4.9022	0.25779	0.107
Simpson	0.011827	0.0018573	0.017143	0.0077245	0.230
Sobs	664.75	27.244	577.5	49.89	0.022

¹Mean= the mean number; ²Sd = the standard deviation; CON = control group; CMP = Cordyceps Militaris Polysaccharide

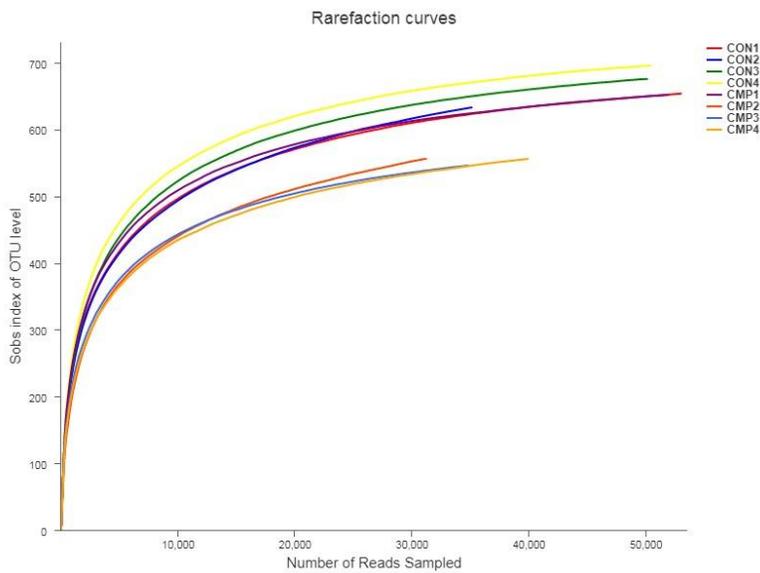


Figure 1. Rarefaction curves of samples (n=8) clustered at 97% sequences identity. OTU= Operational Taxonomic Unit; CON= control group; CMP= Cordyceps Militaris Polysaccharide.

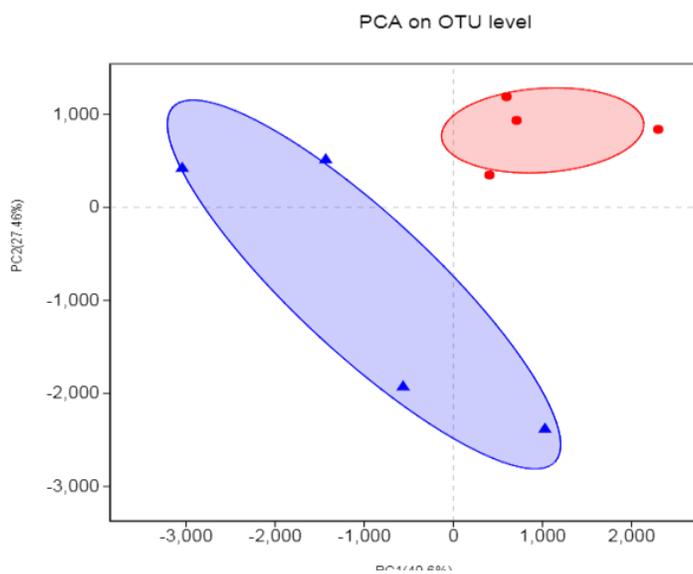


Figure 2. Principal Component Analysis (PCA) of the community membership using Bray-Curtis distance. CON = control group; CMP= Cordyceps Militaris Polysaccharide. The abscissa and ordinate represent the two selected principal components, and the percentage represents the contribution of the principal component to the difference in sample composition. Points of

different colors 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.

Wilcoxon rank-sum test bar plot on Phylum level

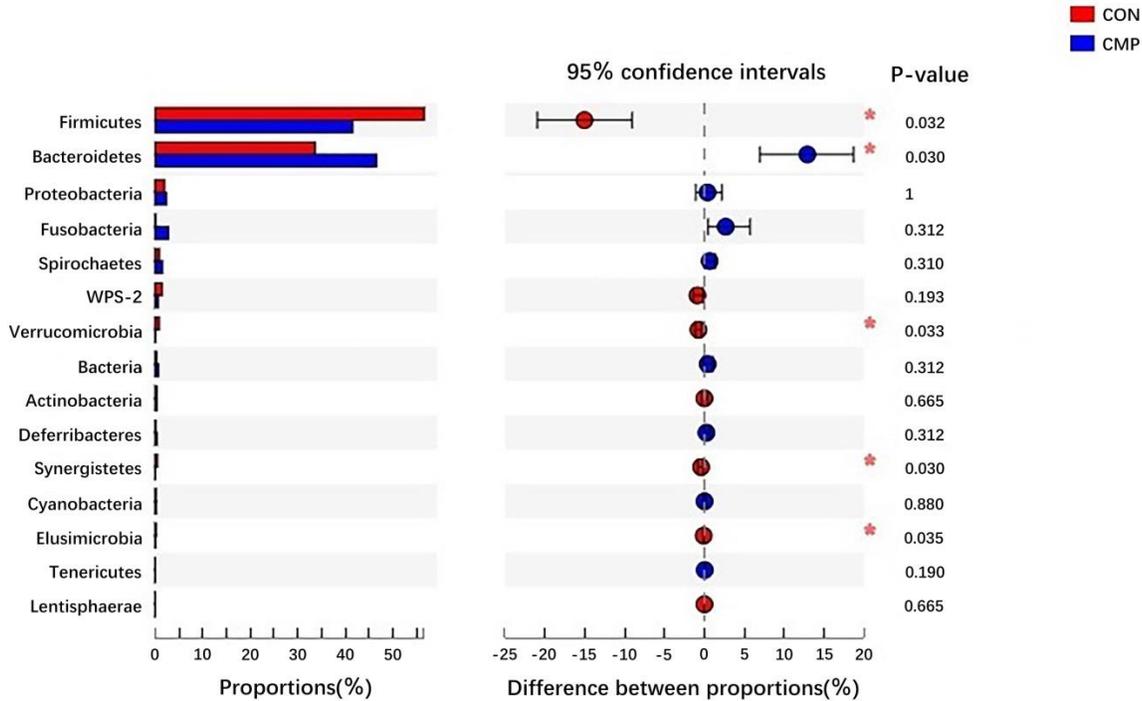


Figure 3. Welch’s t-test bar plot on Phylum level. CON = control group; CMP = Cordyceps Militaris Polysaccharide. The ordinate (left) represents the phyla name and the ordinate (right) represents the *P*-Value.

Wilcoxon rank-sum test bar plot on Family level

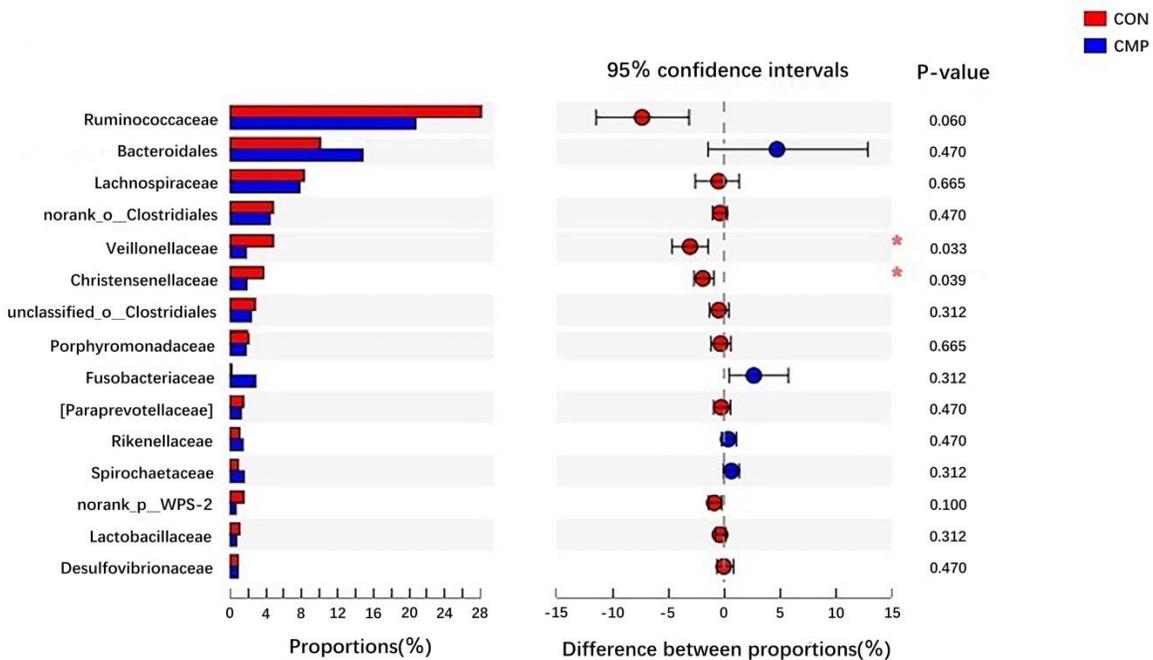


Figure 4. Welch’s t-test bar plot on Family level. CON= control group; CMP = Cordyceps Militaris Polysaccharide. The ordinate (left) represents the family name and the ordinate (right) represents the p-Value.

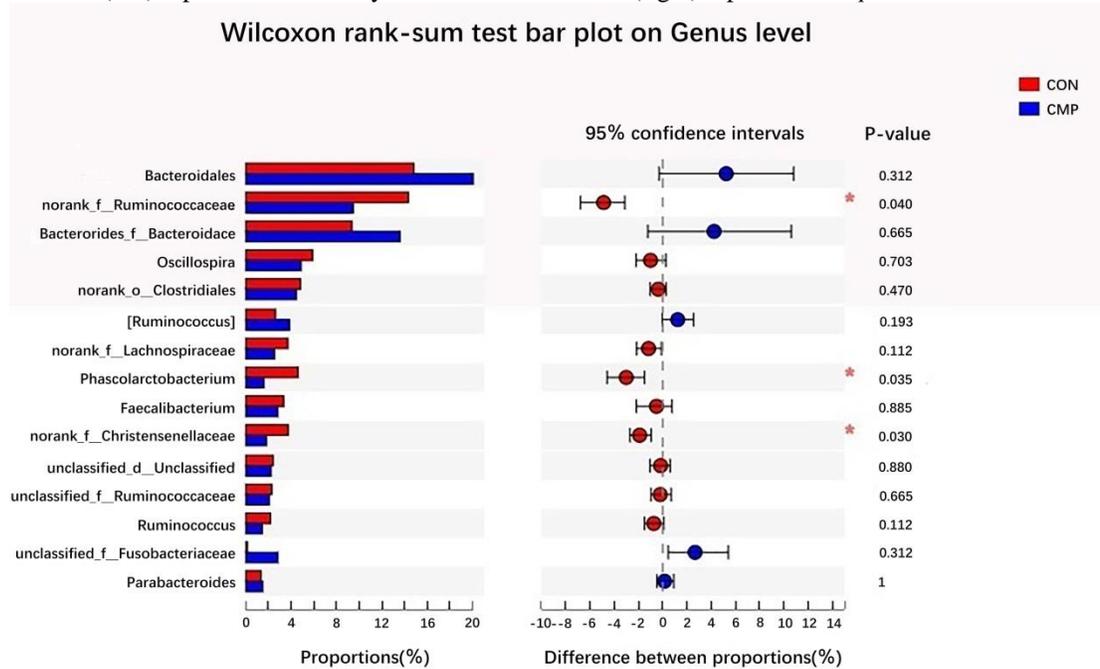


Figure 5. Welch’s t-test bar plot on Genus level. CON= control group; CMP = Cordyceps Militaris Polysaccharide. The ordinate (left) represents the genus name and the ordinate (right) represents the p-Value.

Table 5. Wilcoxon rank sum test data on Phylum level (cut off at 1%).

Species name	CMP-Mean ¹ (%)	CMP-Sd ² (%)	CON-Mean (%)	CON-Sd (%)	P value
Firmicutes	41.61	6.022	56.65	4.489	0.032
Bacteroidetes	46.57	6.96	33.66	1.768	0.030
Proteobacteria	2.339	1.855	1.952	0.7248	1.000
Fusobacteria	2.827	3.139	0.1601	0.06761	0.312
Spirochaetes	1.559	0.7397	0.9031	0.3315	0.312

¹Mean = the percentage of the mean number of Phylum; ²Sd = the percentage of standard deviation of Phylum number; CON = control group; CMP = Cordyceps Militaris Polysaccharide.

Table 6. Wilcoxon rank sum test data on Family level (cutoff at 1%).

Species name	CMP-Mean ¹ (%)	CMP-Sd ² (%)	CON-Mean (%)	CON-Sd (%)	P value
Ruminococcaceae	20.74	4.057	28.08	3.225	0.060
Bacteroidales	14.81	8.024	10.07	0.7542	0.470
Lachnospiraceae	7.763	1.558	8.253	1.746	0.665
norank_o__Clostridiales	4.45	0.5395	4.818	0.5201	0.470
Veillonellaceae	1.784	0.9881	4.831	1.653	0.030
Christensenellaceae	1.832	0.9219	3.735	0.4204	0.039
unclassified_o__Clostridiales	2.329	0.9786	2.805	0.3763	0.312
Porphyromonadaceae	1.769	1.058	2.084	0.2	0.665
Fusobacteriaceae	2.827	3.139	0.1601	0.0676	0.312
Paraprevotellaceae	1.233	0.8325	1.478	0.4542	0.470
Rikenellaceae	1.433	0.7874	1.072	0.1486	0.470
Spirochaetaceae	1.559	0.7397	0.9031	0.3315	0.312
norank_p__WPS-2	0.6253	0.6725	1.501	0.2236	0.193

Lactobacillaceae	0.7092	0.5013	1.071	0.2273	0.312
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¹Mean = the percentage of the mean number of Family; ²Sd = the percentage of standard deviation of Family number; CON = control group; CMP = Cordyceps Militaris Polysaccharide.

Table7. Wilcoxon rank sum test data on Genus level (cutoff at 1%).

Species name	CMP-Mean ¹ (%)	CMP-Sd ² (%)	CON-Mean (%)	CON-Sd (%)	P value
norank_o__Bacteroidales	20.05	6.837	14.82	0.3941	0.312
norank_f__Ruminococcaceae	9.476	1.981	14.35	0.838	0.040
Bacteroides_f__Bacteroidaceae	13.59	7.388	9.351	0.7964	0.665
Oscillospira	4.879	1.219	5.884	0.8032	0.470
norank_o__Clostridiales	4.45	0.5395	4.818	0.5201	0.470
Ruminococcus	3.85	1.248	2.612	0.8816	0.193
norank_f__Lachnospiraceae	2.534	0.9579	3.701	0.8048	0.112
Phascolarctobacterium	1.611	0.8582	4.618	1.682	0.035
Faecalibacterium	2.824	1.488	3.339	0.7728	0.885
norank_f__Christensenellaceae	1.832	0.9219	3.735	0.4204	0.030
unclassified_d__Unclassified	2.233	0.2937	2.405	0.9561	0.885
unclassified_f__Ruminococcaceae	2.087	0.7055	2.294	0.6626	0.665
Ruminococcus	1.447	0.7955	2.184	0.6644	0.112
unclassified_f__Fusobacteriaceae	2.827	3.139	0.1601	0.0676	0.312
Parabacteroides	1.501	0.8043	1.349	0.1146	1.000
norank_f__Rikenellaceae	1.432	0.7874	1.071	0.1485	0.470
Treponema	1.559	0.7397	0.9031	0.3315	0.312
norank_f__Paraprevotellaceae	1.122	0.7804	1.197	0.3533	0.470
unclassified_f__Lachnospiraceae	0.8786	0.1701	1.352	0.2803	0.061
norank_p__WPS-2	0.6253	0.6725	1.501	0.2236	0.194
unclassified_f__Bacteroidaceae					
_o__Bacteroidales	1.219	0.7175	0.7158	0.132	0.312
Lactobacillus	0.7086	0.5018	1.07	0.2276	0.312

¹Mean = the percentage of the mean number of Genus; ²Sd = the percentage of standard deviation of Genus number; CON = control group; CMP = Cordyceps Militaris Polysaccharide.

DISCUSSION

Effects of CMP on egg production and egg quality

It has been clinically confirmed that *C. militaries* had several functions, such as antitumor, anti-inflammatory and antioxidant effects (Chen, et al., 2010; Lee, et al., 2010b; Li, et al., 2015). *Cordyceps* polysaccharide isolated from *C. militaries* could suppress a cyclophosphamide-induced immune response to improve lymphocyte and macrophage activities, increased superoxide dismutase, catalase and glutathione peroxidase levels and reduced malondialdehyde content in the blood in mice (Wang, et al., 2012). However, information on the impact of CMP supplementation on laying hens is limited. Researchers emphasized that feeding laying hens with *C. militaries* waster medium decreased significantly feed conversion ratio (FCR) and increased egg production. They also showed that ascorbic acid and *C. militaries* waster medium have approximately the same reducing power (Wang et al., 2015). Ciftici et al. indicated that ascorbic acid can partially inhibit adverse oxidative protein denaturation and improve nutrient digestibility and FCR (Ciftici, et al., 2005). As such, the feed-egg ratio of the hens

receiving the added CMP in the present study was likely to decrease and egg production to increase.

Eggshell thickness, eggshell strength, Haugh unit and egg shape index were important indicators for egg quality measurement, and these parameters were related to the shelling rate and freshness of eggs. In the present study, the differences in the egg quality between the three groups were insignificant. Similarly, scholars observed that supplementing a diet with *C. militaries* waster medium did not improve eggshell thickness and egg shell strength (Wang et al., 2015).

Effects of CMP on ceecal microbiota

Chicken intestinal microflora had a positive effect on food intake and the immune system (Shang et al., 2018b). Studies had shown that a well-functioning caecum could cover 10% of the body's energy needs (Hegde, et al., 1982; Józefiak, et al., 2004).

Our study determined the alpha diversity of microbial communities by measuring Sobs, Shannon's, Simpson's, Ace and Chao indices. Shannon's and Simpson's indices represent the degree of differences in the frequencies of different taxa (diversity), while Sobs, Chao and Ace indices reflect the number of different taxa present in the

sample (richness). The present study showed the significant differences in the caecal microbial density between the CMP and control groups. Consistent with previous findings (Bederska-ojewaska et al., 2017), our results revealed significantly lower averages for microbial diversity and richness in the CMP group compared to those in the control group. Scientists found that the addition of cordycepin had an antimicrobial function similar to tetracycline and chloramphenicol (Ahn et al., 2000). These results indicated that CMP supplementation appeared to reduce the bacterial species in the caecal intestine of chicken. Further studies were needed to investigate the specific mode of action of CMP.

At the phylum level, differences in microbial structures could mostly be attributed to changes in the microbiome in Firmicutes, Bacteroidetes, Synergistetes and Elusimicrobia in the CMP group. In accordance with a previous research (Pan and Yu, 2014), the present study also found that Firmicutes, Bacteroidetes and Proteobacteria accounted for more than 90% in the chicken caecal microflora. The relative abundance of Bacteroidetes in the CMP group was higher than in the control group, while the Firmicutes content decreased significantly. Bacteroides could digest complex carbohydrates and maintain an intestinal micro-ecological balance (Józefiak et al., 2004; Sears 2005). Bacteroides could promote the development of the immune system (Spence et al., 2006). Bacteroidetes was related to the development of interleukin-17-producing T-helper cells (Mazmanian et al., 2005). Some studies have shown that with the increase of body mass index (BMI), the content of A increases gradually, while the content of B decreases (Koliada et al., 2017). Some studies had found differences between infants, adults and the elderly in relation to the Firmicutes to Bacteroidetes ratio. Among them, adults had the highest value (Mariat et al., 2009). Therefore, our results might had a positive effect on the health and fat accumulation of hens during the late laying period. In the present study, we also observed significant differences in the relative abundance of Synergist and Elusimicrobia between the CMP and control groups, but the functions of these phyla were unclear and should be investigated further.

At the family level, the relative abundance of Veillonellaceae and Christensenellaceae decreased significantly in the CMP group, but was low in our experimental hens. Christensenellaceae belongs to the order of the Clostridiales within the Clostridia class of Firmicutes (Morotomi et al., 2012) and had emerged as the intestinal flora component, which the abundance was most

influenced by host genetics (Goodrich et al., 2014). Christensenellaceae might be a symbol of the ecosystem of long-lived people (Biagi et al., 2016), Goodrich et al reported the relationship between Christensenellaceae and the weight of humans and mice, that reduced the degree of receptor weight gain (Goodrich et al., 2014). Similarly, in the addition of a Christensella strain in a mouse model, reduced the degree of receptor weight gain (Fischbach and Segre, 2016). The colonisation of *Campylobacter jejuni*, a foodborne zoonotic pathogen, could increase the relative abundance of Christensenellaceae in a chicken intestine (Alexandre et al., 2015). However, the role of this new family in chicken intestine health was unknown. Veillonellaceae had an excellent ability to ferment lactic acid; it was known as a short-chain fatty acid acetate and propionate producer (Lecomte et al., 2015). Other studies had shown that the number of families defined as the chicken's core microbiome might increase or decrease, but families such as Veillonellaceae had been reported common members of the chicken (Qu et al., 2008; Callaway et al., 2009; Filip, et al., 2011). Veillonellaceae had a similar effect on Campylobacteraceae, so Veillonellaceae could reduce the colonisation of Campylobacteraceae in the chicken intestines (Videnska et al., 2014). However, further studies were needed to investigate the function of Veillonellaceae in the chicken intestine.

At the genus level, the main genera of the CMP and the control groups were norank Bacteroidales, norank Ruminococcaceae, Bacteroides, Bacteroidaceae and Oscillospira. Scientists reported that the caecum of chickens consisted mainly of Clostridia, followed by Lactobacillus and Ruminococcus (Gong et al., 2010). This change might be due to the addition of CMP in the diet. Other factors could also cause these changes. These factors include different types of chicken, age and environmental factors. For example, the abundance of *Clostridium* increased and the proportion of lactobacilli decreased as the chicken aged (Shang et al., 2018a). Cage type could affect the composition of caecal microflora in laying hens (Filip et al., 2011). Genotype and gender might also affect changes in the intestinal microflora of chicken (Zhao et al., 2013). Norank_Ruminococcaceae and norank_Christensenellaceae were unknown bacterial residents, and their relative abundance decreased significantly in the CMP group. Scientists reported that the caecum was rich in unknown and unclassified bacterial residents (Stanley et al., 2013). The relative abundance of *Phascolarctobacterium* also decreased significantly in the CMP group compared to

those in the control group. The *Phascolarctobacterium* within the *Sporomusa* branch of the *Clostridium/Bacillus* subphylum of Gram-positive bacteria and is a neighbor of *Acidaminococcus fermentans* (Del Dot et al., 1993). *Phascolarctobacterium* complements the lack of carbohydrate metabolism by increasing lipid metabolism with methylmalonyl-CoA mutase, and methylmalonyl-CoA carboxyltransferase was the most abundant among proteins involved in lipid metabolism (Polansky et al., 2015). However, evaluating the possible effect of the changes in these genera on the chicken's caecal microbial community was difficult because of the lack of reports about *Phascolarctobacterium* in the health of chicken intestine.

CONCLUSION

Dietary supplementation with 100 and 200 mg CMP/kg can improve the laying rat and decrease feed to egg ratio significantly compared to those none-supplemented hens, respectively. Dietary supplementation with CMP could alter significantly the caecal microbiota composition with the changes in the phylum, family and genus levels. These findings provided insights into egg production of chickens and intestinal microbiota modulations in response to different CMP dosages and emphasise the requirement for further research to determine the effects of CMP on the chicken intestine microbiota and predict the response of the microbiota to these agents.

DECLARATIONS

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Consent to publish

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author's contribution

Xiaochen Chen carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Yaowen Zhang, and Wenfeng Ma carried out the immunoassays. Xi Wu participated in the sequence alignment. Yanzi Zhu participated in the design of the

study and performed the statistical analysis. Zhanbin Wang conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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