Postprint: Effects of Montmorillonite on Production Performance and Cecal Microbiota in Laying Hens

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Abstract

This experiment aimed to investigate the effects of montmorillonite on the production performance and cecal microbiota of laying hens. A total of 480 75-week-old Roman laying hens were selected and randomly divided into 5 groups with 8 replicates per group and 12 hens per replicate. The control group was fed a basal diet, while the experimental groups were fed the basal diet supplemented with 0.3, 0.6, 0.9, and 1.2 g/kg of montmorillonite, respectively. The pre-trial period lasted 7 days, and the formal trial period lasted 70 days. The results showed: 1) Compared with the control group, the 0.9 g/kg montmorillonite group exhibited a significant increase in daily egg production during weeks 1–5 of the trial ($P<0.05$), and the experimental groups showed a trend toward reduced feed-to-egg ratio ($P=0.073$). 2) The 0.3, 0.9, and 1.2 g/kg montmorillonite groups obtained more effective sequences and high-quality sequences than the control group ($P>0.05$). 3) Beta diversity increased in the experimental groups; compared with the control group, the experimental groups showed a trend toward increased Shannon index ($P=0.096$) and decreased Simpson index ($P=0.095$). 4) At the phylum level, compared with the control group, the experimental groups showed increased abundance of Firmicutes, Proteobacteria, and Spirochaetes ($P>0.05$); the relative content of WPS_2 in the 0.9 g/kg montmorillonite group was significantly increased ($P<0.05$); the abundance of Elusimicrobia in the 0.3 g/kg montmorillonite group was significantly decreased ($P<0.05$). At the genus level, compared with the control group, the experimental groups exhibited significantly increased abundance of Ruminococcus ($P<0.05$), and showed a trend toward increased abundance of Porphyromonas ($P=0.067$); the 0.6 and 1.2 g/kg montmorillonite groups showed significantly increased abundance of Dorea and Blautia ($P<0.05$); the 0.6 g/kg montmorillonite group showed significantly increased abundance of Paraprevotella ($P<0.05$). 5) Cluster analysis at the genus level revealed reduced similarity in intestinal microbiota between the experimental groups and the control group. In conclusion, montmorillonite affected the

diversity and species abundance of cecal microbiota in laying hens to a certain extent, and dietary supplementation with 0.9 g/kg montmorillonite showed a trend toward improved production performance in laying hens.

Full Text

Effects of Montmorillonite on Performance and Cecal Microflora of Laying Hens

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Abstract

This study investigated the effects of montmorillonite on production performance and cecal microflora in laying hens. Four hundred eighty 75-week-old Lohmann laying hens were randomly allocated to five groups, each consisting of eight replicates of twelve hens. The control group received a basal diet, while experimental groups received the basal diet supplemented with 0.3, 0.6, 0.9, and 1.2 g/kg montmorillonite, respectively. Following a 7-day adjustment period, the experimental period lasted 70 days. The results showed: (1) Compared with the control group, daily egg production in the 0.9 g/kg montmorillonite group was significantly increased during weeks 1–5 ($P<0.05$), and feed-to-egg ratio in experimental groups tended to decrease ($P=0.073$). (2) The 0.3, 0.9, and 1.2 g/kg montmorillonite groups yielded more effective and high-quality sequences than the control group ($P>0.05$). (3) Beta diversity was enhanced in experimental groups, with Shannon index showing an increasing trend ($P=0.096$) and Simpson index showing a decreasing trend ($P=0.095$) compared to the control group. (4) At the phylum level, abundances of Firmicutes, Proteobacteria, and Spirochaetes were numerically higher in experimental groups ($P>0.05$); WPS_2 relative content was significantly increased in the 0.9 g/kg group ($P<0.05$), while Elusimicrobia abundance was significantly decreased in the 0.3 g/kg group ($P<0.05$). At the genus level, Ruminococcus abundance was significantly increased in experimental groups ($P<0.05$), Parabacteroides showed an increasing trend ($P=0.067$), and Dorea and Blautia abundances were significantly increased in the 0.6 and 1.2 g/kg groups ($P<0.05$); Paraprevotella abundance was significantly increased in the 0.6 g/kg group ($P<0.05$). (5) Cluster analysis at the genus level revealed reduced similarity of intestinal microflora between experimental and control groups. In conclusion, montmorillonite partially influenced cecal bacterial diversity and species abundance in laying hens, and dietary supplementation with 0.9 g/kg montmorillonite tended to improve production performance.

Keywords: montmorillonite; production performance; intestinal microbiota; high-throughput sequencing; laying hens

Introduction

The animal intestinal tract is an open ecosystem harboring a vast microbial community. Gut microbiota homeostasis plays a crucial role in maintaining host physiological functions including growth and development, nutrient absorption, and immune response. Numerous factors can influence gut microbiota composition and diversity. Studies have confirmed that mycotoxins can reduce animal intestinal microbial diversity, disrupt the ecological balance of normal gut flora, and subsequently produce a series of toxic effects [1-2]. Additionally, excessive proliferation of pathogenic bacteria in the animal intestine can cause microbiota imbalance, impair intestinal mucosal barrier function, reduce nutrient absorption and immune defense capacity, thereby decreasing animal production performance and even increasing mortality [3-4]. Therefore, controlling mycotoxin-induced microbial disturbances and maintaining intestinal health is of great significance.

Montmorillonite is an aluminosilicate clay with a large specific surface area and numerous micropores on its surface, exhibiting excellent adsorption and ion exchange capacities. It has been proven to adsorb bacteria and mycotoxins in the intestine [5-6]. Xia et al. [7] and Hu et al. [8] reported that montmorillonite significantly reduced *Escherichia coli* and *Clostridium* populations in the small intestine and cecum of broilers. Montmorillonite also decreased *E. coli*, *Clostridium*, and *Salmonella* counts in the ileum and colon of weaned piglets to varying degrees [9-10]. Furthermore, montmorillonite increased ruminal bacterial genera that degrade odor compounds in dairy cows while reducing fiber-degrading bacteria and the intestinal pathogen *Shigella* [11]. However, these studies primarily employed traditional in vitro culture methods and denaturing gradient gel electrophoresis (PCR-DGGE), which were limited to specific bacteria or microorganisms of certain abundance, did not address unknown microbes, and had suboptimal accuracy, thus failing to fully reflect montmorillonite' s effects on intestinal microbiota.

Our previous research found that montmorillonite improved production performance in laying hens at peak production [12]. We hypothesized that montmorillonite might delay the decline in production performance at the end of the laying period and thus extend the laying cycle. Given the correlation between intestinal microbiota and host immunity, nutrient absorption, and growth development, we further hypothesized that its effects on hen performance might be associated with gut microbiota. Therefore, this experiment supplemented different levels of montmorillonite in diets of end-of-lay hens to investigate its effects on production performance. Using highly accurate and informative metagenomic sequencing technology combined with bioinformatics analysis, we qualitatively

and quantitatively examined montmorillonite' s effects on cecal microbial composition, diversity, and species abundance to comprehensively and accurately evaluate montmorillonite' s role in maintaining the intestinal microbial barrier.

Materials and Methods

1.1 Experimental Design and Management

Four hundred eighty healthy Lohmann laying hens (75 weeks old) with similar body weight were randomly divided into five groups, each comprising eight replicates of twelve hens. The control group received a basal diet, while experimental groups received the basal diet supplemented with 0.3, 0.6, 0.9, and 1.2 g/kg montmorillonite, respectively. During the 7-day adjustment period, all groups were fed the basal diet. Daily observations were conducted and bird distribution adjusted to ensure no significant differences in feed consumption, laying rate, or egg weight among groups. The experimental period lasted 70 days. The basal diet was formulated according to *NY/T 33-2004 Feeding Standard of Chickens*; its composition and nutrient levels are shown in Table 1 .

Table 1 Composition and nutrient levels of the basal diet (air-dry basis) %

Ingredients and nutrient levels table content preserved as in original

The experimental hens were housed in an open-sided chicken house with three-tiered step cages (three hens per cage, four cages per replicate). Each group' s hens were equally distributed across upper, middle, and lower cage tiers. Feed was provided twice daily (08:00 and 14:30) with two redistributions each morning and afternoon. All groups received identical management conditions. During weeks 1-5, house temperature and humidity were $(20.64\$\pm1.76)°C and (68.90\pm0.90)\pm1.04)°C and (74.46\pm\$2.74)\%$, respectively. Birds had free access to feed and water with 16 h daily lighting (natural plus artificial). The house was cleaned daily, spray-disinfected weekly, and manure was removed every three days.

1.2 Experimental Material

The montmorillonite used in this study was provided by Amlan International, with main components: calcium montmorillonite >70%, amorphous hydrated silicon dioxide >15%, and other mineral elements <15%.

1.3 Performance Measurement

During the experimental period, daily records were kept for each group (per replicate) of egg number, egg weight, soft-shelled and broken eggs, and mortality. Feed intake was recorded weekly. Average daily feed intake, laying rate, average egg weight, feed-to-egg ratio, and daily egg production were calculated.

1.4 Sample Collection and Analysis

1.4.1 Sample Collection On day 70, eight hens per group (one per replicate) were randomly selected, euthanized by cervical bleeding, and dissected. Cecal contents were collected in 1.5 mL sterile centrifuge tubes, immediately placed in liquid nitrogen, and stored at -80°C.

1.4.2 Bacterial DNA Extraction and Detection From each group' s eight cecal content samples, six were randomly selected. Approximately (200$±$10) mg of each sample was weighed into 2 mL tubes, and DNA was extracted using a stool genomic DNA extraction kit (DP328, Tiangen Biotech) following the manufacturer' s instructions. DNA concentration was measured using a NanoDrop ND-2000 UV spectrophotometer (Thermo Fisher Scientific), and purity was assessed by 0.8% agarose gel electrophoresis. Qualified DNA samples were sent to BGI-Shenzhen for sequencing analysis.

1.4.3 Illumina MiSeq Metagenomic Sequencing Barcoded Illumina MiSeq sequencing was performed on the V4 region of 16S rRNA to construct libraries. Briefly, the V4 region of 16S rDNA was amplified using primers 515F/806R. PCR products were mixed equally based on concentration, and 2% agarose gel electrophoresis was used to detect PCR products. Target bands were recovered using a gel extraction kit to obtain purified samples, which were quantified using a BioTek microplate reader. Equal amounts of DNA from each sample were pooled, and a standard Illumina TruSeq DNA library preparation protocol was used to construct the metagenomic sequencing library. Finally, Barcoded Illumina MiSeq sequencing was performed on the Illumina MiSeq platform using the PE250 strategy.

1.5 Data Processing and Analysis

Paired-end sequencing data underwent quality control, truncating or discarding low-quality sequences (50 consecutive bases with average quality >Q30, adapter contamination, reads containing N, and low-complexity reads). FLASH software (v1.2.11) was used to merge quality-controlled sequences, discarding unmergeable sequences. USEARCH (7.0.1090) removed chimeras from purified data, and Qiime (1.8.0) merged de-chimericized data. Sequences were clustered into operational taxonomic units (OTUs) at $ $97% similarity using the Uclust algorithm. The longest sequence in each cluster was selected as the representative sequence, and RDP-classifier (v2.2) was used for species annotation against the database to obtain taxonomic information for each OTU. Mothur (1.31.2) was used to analyze bacterial community diversity and richness from the generated OTU information.

Microbial sequencing data were analyzed using Kruskal-Wallis rank sum test in SAS 9.2, while performance data were analyzed by one-way ANOVA followed by Duncan' s multiple comparison. Results are expressed as means ± SEM, with $P<0.05$ indicating significant difference and $0.05<P<0.10$ indicating a trend.

Results

2.1 Effects of Montmorillonite on Performance of Laying Hens

As shown in Table 2 , during weeks 1-5, daily egg production in the 0.9 g/kg montmorillonite group was significantly higher than in the control, 0.3, and 0.6 g/kg groups (P<0.05). Feed-to-egg ratio in experimental groups tended to be lower than in the control group (P=0.073), with the 0.9 g/kg group showing a 9.69% reduction compared to the control (P>0.05).

Table 2 Effects of montmorillonite on performance of laying hens

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2.2 Effects of Montmorillonite on Cecal Microflora Sequencing Data

As shown in Table 3 , except for the 0.6 g/kg group, experimental groups yielded more effective and high-quality sequences than the control group (P>0.05). Rarefaction curves at 97% similarity (Figure 1 [Figure 1: see original paper]) showed each sample had approximately 27,000 reads, indicating consistent sampling depth. Additionally, species observation curves gradually increased with sequencing depth and reached a plateau, suggesting that sequencing depth adequately covered all species in the samples.

Table 3 Richness and diversity index of species at 97% similarity level

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Figure 1 Rarefaction curve of sequenced reads in cecal content samples of laying hens (similarity: 97%)

2.3 Effects of Montmorillonite on Alpha Diversity of Cecal Microflora

OTU clustering at 97% similarity yielded Alpha diversity indices evaluating community richness and diversity (Table 3). No significant differences were observed among groups in Chao, Shannon, or Simpson indices (P>0.05). The Ace index in the 0.6 g/kg group was significantly higher than in the 1.2 g/kg group (P<0.05). Montmorillonite tended to increase Shannon index (P=0.096) and decrease Simpson index (P=0.095).

2.4 Effects of Montmorillonite on Beta Diversity of Cecal Microflora

Beta diversity analysis compares differences in species diversity among samples. Principal coordinates analysis (PCoA) results are shown in Figure 2 [Figure 2: see original paper]. The control group (right-pointing triangles) was clearly distinguished from experimental groups, with relatively compact distribution (circled) indicating high similarity. In contrast, experimental groups showed scattered distribution and low similarity, with increased diversity compared to

the control, particularly in the 0.6 g/kg (upward triangles) and 1.2 g/kg (downward triangles) groups.

Figure 2 Change of beta diversity of intestinal microflora after supplementation with montmorillonite

2.5 Species Abundance Analysis

To investigate montmorillonite' s effects on cecal microbial community structure and composition, OTUs were clustered and compared at phylum and genus levels. At the phylum level (Table 4), seven phyla had relative abundance >0.5%, with nine other phyla below 0.5%. Bacteroidetes, Firmicutes, Proteobacteria, and Spirochaetes were dominant phyla across all groups, with Bacteroidetes and Firmicutes comprising 67.06%-70.34% and 17.30%-21.38% of total sequences, respectively. Firmicutes, Proteobacteria, and Spirochaetes abundances were numerically higher in experimental groups (P>0.05). Elusimicrobia was significantly reduced in the 0.3 g/kg group (P<0.05), while WPS_2 relative content was significantly increased in the 0.9 g/kg group (P<0.05).

Table 4 Bacterial phyla with relative abundance above 0.5% (sequence percentage of total sequencing) or showing significant differences

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At the genus level (Table 5), among 54 identified genera, 11 had relative abundance >0.5%. Many genera showed low relative abundance. Dominant genera (>0.5%) included *Bacteroides*, *Phascolarctobacterium*, *Oscillospira*, and *Prevotella*, with *Bacteroides* accounting for 24.63% of total sequences, though no significant differences were observed among groups (P>0.05). Compared with the control, Ruminococcus abundance was significantly increased in experimental groups (P<0.05), and Parabacteroides showed an increasing trend (P=0.067). Dorea and Blautia abundances were significantly increased in the 0.6 and 1.2 g/kg groups (P<0.05), and Paraprevotella abundance was significantly higher in the 0.6 g/kg group than in other groups (P<0.05). Fusobacterium abundance was lower in experimental groups, numerically decreasing with increasing montmorillonite level (P>0.05).

Table 5 Bacterial genus with relative abundance above 0.5% (sequence percentage of total sequencing) or showing significant differences

Table content preserved as in original

A heatmap of genus-level classification (Figure 3 [Figure 3: see original paper]) showed two major clusters among 30 samples: the first cluster contained 16 samples (control group 6, 0.3 g/kg group 4, 0.9 g/kg group 2, 1.2 g/kg group 4), and the second cluster contained 14 samples (0.6 g/kg group 6, with 4 consecutive). This indicated that samples from different groups could cluster appropriately, with the 0.6 g/kg group showing the greatest compositional difference from other groups. Additionally, five of six control group samples clustered

closely, indicating high compositional similarity. Most experimental group samples, except those from the 0.3 g/kg group, were distant from control samples, particularly the 0.6 g/kg group, indicating substantial compositional differences between control and montmorillonite groups. Overall, heatmap results aligned with Table 5 findings.

Figure 3 Heatmap under the genus level of the 30 cecal content samples

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Discussion

3.1 Effects of Montmorillonite on Production Performance of Laying Hens

Previous studies have shown that montmorillonite can improve laying hen performance. Yenice et al. [13] reported that dietary sodium bentonite significantly increased laying rate without affecting feed intake. Li et al. [14] found that 1.5 g/kg zinc-loaded nano-montmorillonite increased laying rate by 8.54% and reduced feed-to-egg ratio by 2.19%. Zhang et al. [15] also noted that montmorillonite increased laying rate and reduced egg breakage rate without affecting feed intake. Our previous research showed that montmorillonite significantly increased laying rate and tended to reduce feed-to-egg ratio in peak-production hens [12]. The current study similarly found that 0.9 g/kg montmorillonite significantly increased daily egg production and tended to reduce feed-to-egg ratio, consistent with previous findings. Montmorillonite' s large specific surface area and adsorption capacity can increase digesta viscosity, reduce gastrointestinal transit rate, and may enhance nutrient absorption and utilization. Additionally, it can adsorb harmful intestinal substances and combine with mucin to improve mucus viscosity and flexibility, thereby protecting and repairing intestinal mucosa and maintaining the mucosal barrier [16]. In summary, dietary montmorillonite can improve laying hen performance and increase economic benefits.

3.2 Effects of Montmorillonite on Cecal Microflora Diversity in Laying Hens

Gut microbiota diversity is fundamental for nutrient absorption and immune function, with increased diversity benefiting host metabolism and immune defense [17]. This study showed that while sequencing quantities did not differ significantly between experimental and control groups, the 0.9-1.2 g/kg groups yielded more effective and high-quality sequences, suggesting increased microbial species. Shannon index (reflecting richness and evenness) tended to increase while Simpson index tended to decrease in experimental groups (lower Simpson index indicates greater species richness). PCoA analysis revealed that experimental groups had more scattered microbiota structure distribution with lower similarity, indicating increased diversity. Wang [11] used PCR-DGGE to study montmorillonite effects on dairy cow rumen microbiota, finding that Shannon

index decreased significantly with 2.5% and 5.0% montmorillonite, indicating reduced rumen microbial diversity—contrary to our results. These discrepancies may be attributed to differences in experimental animals, montmorillonite type and dosage, experimental conditions, and detection methods. Currently, no studies have reported montmorillonite' s effects on laying hen gut microbiota diversity, warranting further investigation.

3.3 Effects of Montmorillonite on Cecal Microflora Structure and Species Abundance in Laying Hens

The dominant gut microbiota in poultry is similar to that in humans, with Bacteroidetes and Firmicutes as the predominant phyla, accounting for approximately 90% of gut microbes [18]. In this study, Bacteroidetes and Firmicutes comprised over 88% of sequences, with their combined proportion remaining unchanged after montmorillonite supplementation, though Firmicutes abundance tended to increase. Firmicutes is reportedly associated with diet digestion and aids nutrient absorption [19]. Studies have shown that alcohol-induced liver injury model rats exhibited significantly reduced Firmicutes abundance and increased Elusimicrobia abundance in feces, along with elevated serum D-lactic acid and diamine oxidase levels and reduced intestinal Foxp3 expression, indicating intestinal injury; these indices normalized after probiotic supplementation [20]. We found that Elusimicrobia abundance decreased in all experimental groups, significantly so in the 0.3 g/kg group. Additionally, WPS_2 phylum relative content increased significantly in the 0.9 g/kg group. Since this phylum constitutes a low proportion of gut microbiota, its functional role has rarely been reported and requires further investigation. These results suggest that montmorillonite may improve microbial imbalance and repair intestinal mucosal barrier function, similar to probiotics.

At the genus level, most bacterial genera with relative abundance >0.5% or significant differences showed higher abundance in experimental groups than in the control, further indicating that montmorillonite increased cecal microbiota richness and diversity. Compared with the control, Ruminococcus, Dorea, and Blautia abundances were significantly increased in the 0.6-1.2 g/kg groups. Studies have shown that increased abundance of these genera is closely related to intestinal health. Guo [21] reported that Ruminococcus is one of the few bacterial genera shared in healthy human gut. Wang et al. [1] found that Ruminococcus abundance decreased significantly in mice exposed to aflatoxin B1. Dorea and Blautia are reportedly beneficial genera that produce short-chain fatty acids, which help maintain intestinal epithelial cell morphology and function [21]. Montmorillonite also increased Parabacteroides and Paraprevotella abundances, which are protective bacterial groups. Studies have found that Parabacteroides expression was reduced in ulcerative colitis patients, suggesting its absence may be associated with intestinal inflammation [22]. Gao et al. [23] reported that Paraprevotella abundance was significantly lower in colorectal cancer rats than in normal rats. Additionally, we observed that Fusobacterium

abundance tended to decrease in experimental groups. Fusobacterium, a genus in Bacteroidaceae, includes pathogenic species such as *F. necrophorum* and *F. nucleatum* that are toxic to animals and humans. Miao et al. [24] found that Fusobacterium abundance was significantly higher in colorectal cancer patients than in healthy individuals, suggesting its increase may be associated with intestinal disease. This study also found that montmorillonite reduced abundance of a few other minor bacterial genera, but these had low relative abundance and no statistical significance, and thus may not serve as marker bacteria for evaluating montmorillonite' s effects on microbial community abundance and diversity.

Most previous studies on montmorillonite' s effects on animal gut microbiota have used traditional in vitro culture methods, which can only limitedly examine changes in specific microorganisms and do not address numerous anaerobic bacteria, unknown microbes, or microbial diversity, thus inadequately evaluating montmorillonite' s effects. This study used Illumina MiSeq high-throughput sequencing to investigate montmorillonite' s effects on cecal microbiota diversity and species abundance in laying hens, revealing changes in abundance of specific bacterial groups. Previous studies have shown that montmorillonite reduced harmful bacteria such as *E. coli*, *Clostridium*, and *Salmonella* in broilers and piglets [7,25-27]; decreased pathogenic *Shigella* abundance in dairy cow rumen [11]; and reduced percentages of *Aeromonas*, *Flavobacterium*, and *Vibrio* while increasing *Bacillus* and *Corynebacterium* in Nile tilapia intestine [28]. Thus, montmorillonite affects animal intestinal microbial composition and abundance to some extent. Given the correlation between increased abundances of Ruminococcus, Dorea, Blautia, Paraprevotella, and Parabacteroides and decreased Fusobacterium abundance with maintenance of normal intestinal function, these changes may serve as marker bacteria for montmorillonite' s improvement of microbial imbalance and intestinal health maintenance.

Conclusion

1. Dietary supplementation with 0.9 g/kg montmorillonite significantly increased daily egg production in end-of-lay hens, potentially extending the laying cycle.

2. Montmorillonite supplementation enhanced cecal microbial Beta diversity and altered species abundance of certain bacterial groups. At the phylum level, montmorillonite tended to increase Firmicutes abundance, while the 0.3 g/kg group significantly decreased Elusimicrobia abundance. At the genus level, the 0.6-1.2 g/kg groups increased abundances of Ruminococcus, Dorea, Blautia, and Paraprevotella, while Fusobacterium abundance tended to decrease.

3. These changes in bacterial abundances are closely related to intestinal health, suggesting these genera may be marker bacteria for montmorillonite's maintenance of the intestinal microbial barrier and may contribute

to improved production performance in laying hens.

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Note: Figure translations are in progress. See original paper for figures.