Effects of Dietary Folic Acid and Vitamin B12 Supplementation on Cecal Microbiota Structure in Goslings (Postprint)

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Abstract

This experiment aimed to investigate the effects of different supplemental levels of folic acid and vitamin B12 in diets on the cecal microbiota structure of goslings. A total of 360 healthy 1-day-old Wulong geese were selected and randomly divided into 6 groups, with 6 replicates per group and 10 geese per replicate (half male and half female). The experiment adopted a 2$×$3 (folic acid × vitamin B12) two-factor cross-equal-replicate factorial design. The supplemental levels of folic acid in the diets were 0.55 and 2.50 mg/kg, and the supplemental levels of vitamin B12 were 0.009, 0.018, and 0.036 mg/kg. Groups I–VI had folic acid and vitamin B12 supplemental levels in diets of 0.55 and 0.009 mg/kg, 2.50 and 0.018 mg/kg, 0.55 and 0.036 mg/kg, 2.50 and 0.009 mg/kg, 0.55 and 0.018 mg/kg, and 2.50 and 0.036 mg/kg, respectively. The experimental period lasted for 4 weeks. After the experiment, 16S rRNA high-throughput sequencing technology was used to determine the cecal microbiota composition of goslings. Based on the Illumina HiSeq sequencing platform, paired-end sequencing was performed, a short-insert library was constructed for sequencing, and α-diversity and differential significant species analyses were conducted. The results showed: 1) Different supplemental levels of folic acid and vitamin B12 in diets had significant effects on final body weight and average daily gain of goslings ($P<0.05$), but no significant effect on feed-to-gain ratio ($P>0.05$). Group IV had significantly higher final body weight and average daily gain than the other 5 groups ($P<0.05$). 2) Group IV had the highest operational taxonomic unit (OTU) number, ACE index, and Chao1 index, indicating that the cecal microbial species richness was higher than in the other 5 groups. Cluster analysis showed that groups I and VI had the highest similarity in cecal microbiota, while groups I and II had the lowest similarity. 3) Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla in the cecum

of goslings. Dietary supplementation with different levels of folic acid and vitamin B12 altered the phylum-level abundance of cecal microbiota in goslings, with obvious changes in Firmicutes, Bacteroidetes, and Proteobacteria. A total of 91 genera were detected in cecal samples of goslings, with groups I–VI detecting 86, 84, 83, 87, 87, and 83 genera, respectively. The five genera with relatively high abundance were Desulfovibrio, Bacillus, Bacteroides, Alistipes, and Barnesiella. The relative abundance of dominant genera differed substantially among groups. At the species level, Bacillus held an absolutely dominant position in the cecal microbiota of all groups, with relative abundances of 93.5%, 93.7%, 87.8%, 95.2%, 93.4%, and 87.9% in groups I–VI, respectively, with no significant difference among groups (P>0.05). It was concluded that dietary supplementation with 2.50 mg/kg folic acid and 0.009 mg/kg vitamin B12 can optimize the cecal microbiota structure of goslings, increase the abundance of beneficial bacteria, and thereby improve growth performance.

Full Text

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Abstract

This experiment aimed to investigate the effects of different dietary levels of folic acid and vitamin B12 on the cecal microflora structure of goslings. A total of 360 healthy 1-day-old Wulong geese were randomly divided into 6 groups, with 6 replicates per group and 10 geese per replicate (half male and half female). A 2$×$3 (folic acid × vitamin B12) two-factor crossed equal-replication factorial design was employed. The dietary folic acid supplemental levels were 0.55 and 2.50 mg/kg, and vitamin B12 supplemental levels were 0.009, 0.018, and 0.036 mg/kg. Groups I to VI received folic acid and vitamin B12 at levels of 0.55 and 0.009 mg/kg, 2.50 and 0.018 mg/kg, 0.55 and 0.036 mg/kg, 2.50 and 0.009 mg/kg, 0.55 and 0.018 mg/kg, and 2.50 and 0.036 mg/kg, respectively. The experimental period lasted 4 weeks.

After the experiment, 16S rRNA high-throughput sequencing technology was used to determine the cecal microflora composition of goslings. Based on the Illumina HiSeq sequencing platform, paired-end sequencing was performed, a small-fragment library was constructed, and α-diversity and significantly different species analyses were conducted. The results showed: (1) Dietary supplementation with different levels of folic acid and vitamin B12 had significant effects on final body weight and average daily gain of goslings (P<0.05), but

no significant effect on feed-to-gain ratio (P>0.05). Group IV had significantly higher final body weight and average daily gain than the other five groups (P<0.05). (2) Group IV had the highest operational taxonomic unit (OTU) number, ACE index, and Chao1 index, indicating that the species richness of cecal microorganisms was higher than in the other five groups. Cluster analysis showed that groups I and VI had the highest similarity in cecal microflora, while groups I and II had the lowest similarity. (3) Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla in the gosling cecum. Dietary supplementation with different levels of folic acid and vitamin B12 altered the abundance of cecal microflora at the phylum level, with obvious changes in Firmicutes, Bacteroidetes, and Proteobacteria. A total of 91 genera were detected in the cecal samples, with groups I to VI detecting 86, 84, 83, 87, 87, and 83 genera, respectively. The five genera with relatively high abundance were Desulfovibrio, Bacterium, Bacteroides, Alistipes, and Barnesiella. The relative abundance of dominant genera differed substantially among groups. At the species level, Bacillus held an absolute advantage in the cecal microflora of all groups, with relative abundances of 93.5%, 93.7%, 87.8%, 95.2%, 93.4%, and 87.9% in groups I to VI, respectively, with no significant difference among groups (P>0.05).

It is concluded that dietary supplementation with 2.50 mg/kg folic acid and 0.009 mg/kg vitamin B12 can optimize the cecal microflora structure, increase the abundance of beneficial bacteria, and thereby improve growth performance.

Keywords: folic acid; vitamin B12; gosling; growth performance; cecal microflora structure

Introduction

The gut microbiota is closely related to animal nutrition metabolism, obesity, fat deposition, and diabetes. Gut microorganisms primarily act on carbohydrate metabolism, protein and amino acid metabolism, lipid metabolism, vitamin metabolism, and mineral metabolism in terms of nutritional metabolism [1]. Research indicates that gut microbiota is also closely related to host functions, capable of maintaining host health, improving production performance, reducing environmental pollution, and enhancing animal product safety [2-4]. The large number of bacterial flora present in the animal intestine plays a significant role in improving nutrient utilization and maintaining animal health. Therefore, further research on the relationship between gut microflora structure and nutrient utilization and production performance is important for guiding animal diet formulation.

Studies have shown that folic acid is an important B vitamin that plays a vital role in maintaining animal health, normal physiological functions, and production performance, and is an essential substance for gut microorganisms, which competitively synthesize and utilize a certain amount of folic acid [5]. Vitamin

B12, as a coenzyme for one-carbon metabolism, can improve folic acid utilization and promote various DNA syntheses [6]. Folic acid and vitamin B12 deficiency can not only reduce white blood cell count but also impair lymphocyte function, affect humoral immunity, and cause decreased neutrophil bactericidal capacity [7]. High-throughput sequencing technology has long been used to study poultry gut microbial diversity, with research results indicating that Firmicutes, Bacteroidetes, and Proteobacteria are the main dominant phyla in chicken ceca [8-9]. Studies on the effects of dietary folic acid and vitamin B12 supplementation on poultry production performance have been reported [10-12], but research on the effects of different combinations of folic acid and vitamin B12 levels on gosling gut microflora is still blank. This experiment added different levels of folic acid and vitamin B12 to diets, and based on studying their effects on gosling growth performance, used 16S rRNA high-throughput sequencing technology to analyze the cecal microflora of goslings fed diets with different folic acid and vitamin B12 levels, compared the composition and structure of gosling cecal microflora, and further studied the effects of folic acid and vitamin B12 on gosling gut microflora structure, aiming to determine the relationship between folic acid and vitamin B12 supplementation levels and gosling cecal microflora structure, explore ways to improve poultry growth performance, and provide a theoretical basis for establishing goose nutrient requirement standards.

Materials and Methods

1.1 Test Materials and Diet Composition

Folic acid: feed grade, with a folic acid content of 96%, product of Ningxia Jinwei Pharmaceutical Co., Ltd. Vitamin B12: with a vitamin B12 content of 1%, product of Ningxia Jinwei Pharmaceutical Co., Ltd.

The basal diet was formulated based primarily on NRC (1994) [13], and its composition and nutrient levels are shown in Table 1 . The basal diet was measured to contain 0.42 mg/kg folic acid and 0.00 mg/kg vitamin B12 using high-performance liquid chromatography.

1.2 Experimental Design

The experimental geese were provided by Gaomi Yinhe Runyan Goose Industry Co., Ltd., a breeding base of the China Waterfowl Industry Technology System. A total of 360 1-day-old Wulong geese with no significant difference in initial body weight ($P>0.05$) were randomly divided into 6 groups, with 6 replicates per group and 10 geese per replicate (half male and half female). A 2$×$3 (folic acid × vitamin B12) two-factor crossed equal-replication factorial design was employed, with dietary folic acid supplemental levels of 0.55 and 2.50 mg/kg, and vitamin B12 supplemental levels of 0.009, 0.018, and 0.036 mg/kg. The experimental grouping is shown in Table 2 . The experimental period lasted 4 weeks (1-28 days of age).

1.3 Management

Before the experiment, the goose house was thoroughly disinfected to prevent disease transmission. Full-period house feeding was adopted with floor rearing. Experimental geese had free access to water and feed, with frequent small feedings. Environmental sanitation in the goose house was maintained, the floor was kept clean and dry, the health status of geese was monitored, and disease prevention and control measures were implemented.

1.4 Measurements

1.4.1 Growth Performance After the feeding experiment, feed was withheld for 6 hours, then each goose was weighed individually on an empty stomach. The body weight and weight gain of each group were recorded to calculate final body weight (FBW), average daily gain (ADG), and feed-to-gain ratio (F/G).

1.4.2 Cecal Microflora Structure Sample Collection: After empty weighting, 2 geese were randomly selected from each replicate (half male and half female), euthanized by jugular vein bleeding, the abdominal cavity was quickly opened, the cecum was aseptically removed, rapidly collected in cryovials, stored in liquid nitrogen, and then transferred to a -80°C freezer for later analysis.

Total DNA Extraction: Each sample was analyzed individually. Total bacterial DNA from cecal contents was extracted using a genomic DNA kit from Tiangen Biotech Co., Ltd.

DNA Quantification and Purity Assessment: Total DNA content was measured using a DNA quantifier, DNA purity was expressed as OD260/OD280, and DNA fragment size was detected by 0.8% agarose gel electrophoresis.

PCR Amplification and 16S rRNA Sequencing Analysis: Primers were synthesized according to the bacterial 16S rRNA (V3+V4) region: forward primer, 5' -ACTCCTACGGGAGGCAGCA-3' ; reverse primer, 5' -GGACTACHVGGGTWTCTAAT-3' . The PCR pre-experiment program for sample detection was as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 40 s, for a total of 25 cycles.

1.5 Statistical Analysis

SPSS 17.0 software was used to analyze main effects and interactions using the General Linear Model (GLM). Data were then analyzed for significant differences using ANOVA and LSD methods. Optimized sequences were clustered to define operational taxonomic units (OTUs). Based on OTU analysis results, taxonomic analysis was performed at various classification levels to obtain microflora structure diagrams and species abundance clustering heatmaps at phylum, genus, and species levels for each sample.

Results

2.1 Effects of Dietary Folic Acid and Vitamin B12 on Growth Performance of Goslings

The effects of dietary folic acid and vitamin B12 levels on final body weight, average daily gain, and feed-to-gain ratio of goslings are shown in Table 3 . Dietary supplementation with different folic acid levels had no significant effects on final body weight, average daily gain, or feed-to-gain ratio of goslings ($P>0.05$). Dietary supplementation with different vitamin B12 levels had significant effects on final body weight and average daily gain ($P<0.05$), but no significant effect on feed-to-gain ratio ($P>0.05$). The interaction between folic acid and vitamin B12 had significant effects on final body weight and average daily gain ($P<0.05$), but no significant effect on feed-to-gain ratio ($P>0.05$). Group IV had significantly higher final body weight and average daily gain than the other five groups ($P<0.05$), indicating that dietary supplementation with 2.50 mg/kg folic acid and 0.09 mg/kg vitamin B12 resulted in the best growth performance.

2.2.1 Alpha Diversity Analysis Results

As shown in Table 4 , different dietary combinations of folic acid and vitamin B12 altered the abundance and diversity of cecal microorganisms in goslings. Group IV had the highest OTU number, ACE index, and Chao1 index, indicating that the species richness of cecal microorganisms was higher than in the other five groups. Group V had the highest Shannon index and lowest Simpson index, indicating that group V had the highest community diversity and most uniform individual distribution. Additionally, coverage index, which reflects OTU sequencing depth, was calculated. The coverage values of all six groups were greater than 0.999, indicating a high probability of species detection in the samples.

The Shannon curve was constructed using microbial diversity indices at different sequencing depths to reflect microbial diversity in each sample at different sequencing quantities. As shown in Figure 1 [Figure 1: see original paper], the curve initially rose sharply, then slowly increased until plateauing, with all six groups showing a trend of rapid initial increase followed by stabilization, indicating that the sequencing quantity was sufficient to cover the vast majority of microorganisms in each sample.

As shown in Figure 2 [Figure 2: see original paper], the red box curve composed of samples in this experiment first increased slowly and then plateaued, indicating that species did not increase significantly with sample size. The green box curve first decreased and then plateaued, indicating that the common species in the sampled samples tended toward saturation. Both red and green box curve results demonstrate that the sample size in this experiment was sufficient to reflect all current microbial species.

2.3 Microflora Similarity Analysis Results

Analyzing the OTU composition of different groups can reflect sample differences and distances. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used for sample clustering analysis. As shown by the sample UPGMA clustering tree (Figure 3 [Figure 3: see original paper]), groups I and VI had the smallest distance, indicating the highest similarity in cecal microflora composition between groups I and VI. Groups I and II had the largest distance, indicating the lowest similarity in cecal microflora composition between groups I and II. This demonstrates that dietary supplementation with different folic acid and vitamin B12 levels had certain effects on gosling cecal microflora structure. The inter-group Principal Coordinates Analysis (PCoA) results shown in Figure 4 [Figure 4: see original paper] were similar to these findings.

Figure 5 [Figure 5: see original paper] shows the species abundance clustering heatmap at the genus level of gosling cecal microflora. As shown, groups II and IV had the greatest color difference and farthest branch distance, indicating the lowest similarity in microflora at the genus level between groups II and IV. Groups I and III, and groups I and VI had smaller color differences and closer distances, indicating higher similarity in microflora at the genus level. Groups II and V had the smallest color difference and closest distance, indicating the highest similarity in microflora at the genus level. Groups III and IV had the smallest color difference and closest distance, indicating the highest similarity in microflora at the genus level. These results demonstrate that dietary supplementation with different folic acid and vitamin B12 levels altered gosling cecal microflora structure.

2.4 Dominant Microflora Analysis

Figure 6 [Figure 6: see original paper] shows the effects of dietary supplementation with different folic acid and vitamin B12 levels on cecal microflora abundance at the phylum level in goslings. At the 97% similarity phylum level, 10 phyla were detected in all cecal samples, with groups VI, V, and IV each detecting 9 phyla, and groups III and II detecting 8 and 7 phyla, respectively. Calculating the relative abundance of detected phyla in cecal samples revealed that Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla. Figure 7 [Figure 7: see original paper] shows the relative abundance of dominant phyla. Group II had the highest relative abundance of Firmicutes at 61.5%, significantly higher than other groups ($P<0.05$). Group VI had the highest relative abundance of Bacteroidetes at 38.4%, with no significant difference from groups III and V ($P>0.05$). Group I had the highest relative abundance of Proteobacteria at 37.7%, significantly higher than groups II and V ($P<0.05$). Additionally, Cyanobacteria had the highest relative abundance in group IV at 1.89%, Verrucomicrobia had the highest relative abundance in group VI at 3.1%, and Tenericutes had the highest relative abundance in group V at 2.7%. Other phyla such as Actinobacteria and Deferribacteres had relatively low abundance (<0.1%) and were even undetectable in some samples. Dietary supplementation

with different folic acid and vitamin B12 levels altered cecal microflora abundance at the phylum level, with obvious changes in Firmicutes, Bacteroidetes, and Proteobacteria, indicating that dietary supplementation with different folic acid and vitamin B12 levels changed gosling cecal microflora structure.

Figure 8 [Figure 8: see original paper] shows the effects of dietary supplementation with different folic acid and vitamin B12 levels on cecal microflora abundance at the genus level in goslings. A total of 91 genera were detected in all cecal samples, with groups I to VI detecting 86, 84, 83, 87, 87, and 83 genera, respectively. As shown in Figure 9, the five genera with relatively high abundance were Desulfovibrio, Bacterium, Bacteroides, Alistipes, and Barnesiella. As shown in Figure 8, the relative abundance of the top five dominant genera differed substantially among groups. Desulfovibrio had relatively high abundance in groups I, II, and IV, significantly different from the other three groups ($P<0.05$). Group IV had the highest relative abundance of Bacterium, significantly higher than the other five groups ($P<0.05$). Group V had the highest relative abundance of Bacteroides, significantly higher than groups I, III, and IV ($P<0.05$). Group VI had the highest relative abundance of Alistipes, significantly higher than all groups except III ($P<0.05$). Group III had the highest relative abundance of Barnesiella, significantly higher than the other five groups ($P<0.05$).

Figure 10 [Figure 10: see original paper] shows the effects of dietary supplementation with different folic acid and vitamin B12 levels on cecal microflora abundance at the species level in goslings. As shown in Figure 11, at the species level, Bacillus had absolute dominance in all six groups, with relative abundances of 93.5%, 93.7%, 87.8%, 95.2%, 93.4%, and 87.9% in groups I to VI, respectively. The effects of different dietary folic acid and vitamin B12 combinations on the relative abundance of other bacterial species in gosling cecum did not reach significant levels ($P>0.05$).

Discussion

3.1 Function and Relationship of Folic Acid and Vitamin B12

Gut microorganisms affect not only the digestion, absorption, and energy supply of nutrients but also regulate normal physiological functions and disease occurrence and development in the host. The gut microecosystem has a crucial impact on the normal functioning of the organism [14]. Currently, high-throughput sequencing has been reported for determining poultry gut microflora. Research indicates that gut microorganisms and their metabolism have important effects and regulatory roles on nutrition, health, and disease in broiler chickens, and nutritional intervention in host gut microbial health has become a hotspot in animal nutrition research [10].

Studies have shown that adding folic acid to corn-soybean broiler diets can increase feed intake and body weight, with the most significant effect in the 3.0 mg/kg folic acid group [11]. Xue et al. [12] found that adding folic acid to 8-

week-old broiler diets resulted in significant differences in daily feed intake and daily gain between the 1.64 mg/kg folic acid group and other groups; compared with the control group, daily gain and daily feed intake showed an increasing trend in folic acid-supplemented groups. Yu Y G [15] demonstrated that folic acid maintains normal immune system function in animals, and its deficiency increases animal susceptibility to bacteria, hinders normal lymphocyte function, and impedes antibody synthesis.

Vitamin B12 participates in one-carbon unit synthesis as a coenzyme and plays a very important role in DNA methylation. Vitamin B12 affects the metabolic efficiency of folic acid and participates in purine and nucleotide synthesis while maintaining DNA synthesis and repair and ensuring chromosome stability. Vitamin B12 is mainly absorbed and transported in the body through two substances: intrinsic factor (IF) and transcobalamin (TC). Therefore, vitamin B12 is actually closely related to nucleic acid and protein synthesis [16]. Vitamin B12-dependent methionine synthase can catalyze the transfer of a methyl group from methyltetrahydrofolate to homocysteine (Hcy) to form methionine, ultimately forming S-adenosylmethionine (SAM). Vitamin B12 deficiency will reduce the available amount of SAM for DNA methylation, thereby affecting gene expression [17].

Studies on the combined application of folic acid and vitamin B12 are commonly reported in medical literature but are basically blank in poultry nutrition research. This experiment is the first to use high-throughput sequencing technology to study the effects of combined supplementation with different folic acid and vitamin B12 levels on gosling cecal microflora structure, to explore the intervention effects of different folic acid and vitamin B12 combination levels on cecal microflora structure. In this experiment, group IV (2.50 mg/kg folic acid × 0.009 mg/kg vitamin B12) had the highest OTU, ACE index, and Chao1 index, indicating that group IV had higher cecal microbial species richness than the other five groups. Dietary supplementation with different folic acid and vitamin B12 levels can affect the composition and quantity of gosling cecal microflora.

3.2 Effects of Dietary Folic Acid and Vitamin B12 on Gosling Cecal Microflora Structure

Research demonstrates that Firmicutes is closely related to intestinal nutrient absorption. Obese individuals generally maintain a higher proportion of Firmicutes with poorer bacterial diversity, and increased proportions of Firmicutes species may contribute to the pathophysiology of obesity, with obesity severity often positively correlated with Firmicutes proportion [18]. The main function of Firmicutes is to hydrolyze carbohydrates and proteins, while Bacteroidetes mainly acts on steroid, polysaccharide, and bile acid metabolism, helping the host absorb polysaccharides and synthesize proteins [19]. Hildebrandt et al. [20] found that when RELII gene knockout mice and wild-type mice switched from normal to high-fat diets, Bacteroidetes numbers decreased while Firmicutes and Proteobacteria numbers increased. The authors speculated that this change was

caused by high-fat diet rather than obesity itself.

This experiment detected 10 phyla in all gosling cecal samples, with Firmicutes, Bacteroidetes, and Proteobacteria as the dominant phyla, consistent with previous reports [21]. The distribution of microflora in cecal samples differed among groups at both phylum and genus levels. At the phylum level, group II had the highest relative abundance of Firmicutes, group VI had the highest relative abundance of Bacteroidetes, and group I had the highest relative abundance of Proteobacteria. At the genus level, the five genera with highest relative abundance were Desulfovibrio, Bacterium, Bacteroides, Alistipes, and Barnesiella, with substantial differences in relative abundance of dominant genera among groups. At the species level, Bacillus had absolute dominance in cecal samples from all six groups, but differences among groups were not significant. This indicates that dietary supplementation with different folic acid and vitamin B12 levels affected gosling cecal microflora structure. Combined with growth performance results, the group supplemented with 2.50 mg/kg folic acid and 0.009 mg/kg vitamin B12 had the highest final body weight and average daily gain, further indicating that the combination of folic acid and vitamin B12 in the diet affects growth performance by regulating gosling cecal microflora structure.

Conclusion

1. The group supplemented with 2.50 mg/kg folic acid and 0.009 mg/kg vitamin B12 had the highest final body weight and average daily gain in goslings.
2. The group supplemented with 2.50 mg/kg folic acid and 0.009 mg/kg vitamin B12 had the highest cecal microflora OTU, ACE index, and Chao1 index, indicating the highest species richness.
3. The relative abundance of dominant phyla and genera in the cecum differed substantially among groups, but Bacillus held an absolute advantage in all groups.
4. It is concluded that dietary supplementation with 2.50 mg/kg folic acid and 0.009 mg/kg vitamin B12 can optimize gosling cecal microflora structure, increase the abundance of beneficial bacteria, and thereby improve growth performance.

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