Effects of Compound Probiotic Preparation on Apparent Nutrient Utilization, Serum Biochemical Indices, and Intestinal Mucosal Morphology in Broiler Chickens (Postprint)

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Abstract

This study aimed to investigate the effects of compound probiotic preparations with different ratios on apparent nutrient utilization, serum biochemical parameters, and intestinal mucosal morphology in broiler chickens. A total of 320 healthy 1-day-old male Arbor Acres (AA) broiler chickens were selected and randomly allocated into 4 groups, with 4 replicates per group and 20 birds per replicate. Group served as the control group and was fed a basal diet; Groups , , and were experimental groups and were fed the basal diet supplemented with 1,000 mg/kg compound probiotic preparation, in which the ratios of Bacillus subtilis: Saccharomyces cerevisiae: Lactobacillus acidophilus: Bifidobacterium lactis were 2:1:1:1, 1:2:1:1, and 1:1:1.5:1.5, respectively. The experimental period was 42 d. The results showed: 1) The apparent utilization rates of dry matter, crude protein, crude fat, and calcium in Groups , , and were significantly higher than those in the control group (P<0.05); 2) At 42 days of age, the serum total protein content in Groups , , and was significantly higher than that in the control group (P<0.05); 3) The villus height, crypt depth, and villus height to crypt depth ratio in the duodenum, jejunum, and ileum of Groups , , and were superior to those of the control group (P>0.05). It can be concluded that supplementation of the basal diet with compound probiotic preparations at different ratios can improve apparent nutrient utilization, serum biochemical parameters, and intestinal mucosal morphology in broiler chickens.

Full Text

Effects of Compound Probiotics on Nutrient Apparent Availability, Serum Biochemical Indexes and Intestinal Mucosal Morphological Structure of Broilers

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Abstract

This experiment was conducted to investigate the effects of different proportions of compound probiotics on nutrient apparent availability, serum biochemical indexes and intestinal mucosal morphology in broilers. A total of 320 healthy 1-day-old male Arbor Acres (AA) broilers were randomly divided into 4 groups with 4 replicates per group and 20 broilers per replicate. Group I served as the control group and was fed a basal diet. Groups II, III and IV were experimental groups fed the basal diet supplemented with 1,000 mg/kg compound probiotics, with *Bacillus subtilis*:*Saccharomyces cerevisiae*:*Lactobacillus acidophilus*:*Bifidobacterium lactis* ratios of 2:1:1:1, 1:2:1:1 and 1:1:1.5:1.5, respectively. The experimental period lasted 42 days. The results showed: 1) The apparent availability of dry matter, crude protein, ether extract and calcium in groups II, III and IV was significantly higher than that in the control group ($P<0.05$). 2) At 42 days of age, serum total protein content in groups II, III and IV was significantly higher than that in the control group ($P<0.05$). 3) The villus height, crypt depth and villus height/crypt depth ratio in the duodenum, jejunum and ileum of groups II, III and IV were superior to those of the control group ($P>0.05$). These findings indicate that supplementation with different proportions of compound probiotics in the basal diet can improve nutrient apparent availability, serum biochemical indexes and intestinal mucosal morphology in broilers.

Key words: compound probiotics; nutrient apparent availability; serum biochemical indexes; intestinal mucosal morphology; broilers

Introduction

Compound probiotics are composite biological preparations made from individual beneficial microorganisms for animal organisms. In livestock and poultry production, compound probiotics can not only promote animal growth and development but also improve intestinal flora balance, enhance feed utilization efficiency and boost animal immunity. The main microbial strains in compound probiotics include *Bacillus*, *Lactobacillus*, *Enterococcus* and yeast. Among them, *Bacillus subtilis* serves as a novel expression vector that can express various proteins such as enzymes and growth factors [1], making it a commonly used genetically engineered bacterium in molecular microbiology research. Sun Xifeng

[2] reported that supplementation with different concentrations of compound probiotics (mainly composed of marine red yeast BSH, *Bacillus subtilis* and lactic acid bacteria) in basal diets significantly increased average daily gain and daily feed intake of broilers, improved apparent availability of energy, dry matter, crude protein and ether extract, enhanced small intestinal morphological structure, and significantly elevated the villus height/crypt depth ratio (V/C) in all intestinal segments. Zhao Jianwen et al. [3] found that adding compound bacterial preparations (mainly composed of *Lactobacillus* and *Bacillus*) to basal diets could reduce ammonia concentration in chicken houses to some extent, increase total protein and globulin content in blood, and enhance humoral immunity, though the effect on cellular immunity was not significant. Luan Chao [4] utilized small ubiquitin-related modifier (SUMO) fusion technology to express the antimicrobial peptide Cathelicidin-BF (CBF) in *Bacillus subtilis*. Subsequent studies demonstrated that antimicrobial peptide CBF possesses efficient broad-spectrum antibacterial activity and immunomodulatory functions, with good safety profile and low risk of resistance development, making it an excellent novel antibacterial substance. Our laboratory employed the antimicrobial peptide gene fragment from housefly larvae and fused it into *Bacillus subtilis* engineering bacteria using overlapping extension PCR to express *Bacillus subtilis* genetically engineered bacteria containing antimicrobial peptides [5]. Currently, numerous studies have investigated the individual use of probiotics or antimicrobial peptides in livestock and poultry, but no reports have documented the combined application of probiotics and antimicrobial peptide-containing genetically engineered bacteria in broiler production. Therefore, this experiment utilized compound probiotics composed of antimicrobial peptide-containing *Bacillus subtilis* genetically engineered bacteria, *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* as test materials. Three different proportions of compound probiotics determined through previous artificial simulated gastrointestinal digestive fluid tolerance tests were added to broiler basal diets to observe their effects on nutrient apparent availability, serum biochemical indexes and intestinal mucosal morphology, aiming to identify the optimal compound probiotics ratio and provide data reference for practical application and promotion of compound probiotics in the broiler industry chain.

Materials and Methods

1.1 Experimental Materials

The probiotic preparations used in this experiment were all solid powders, with viable *Bacillus subtilis* count of 9.00×10 CFU/g, viable *Saccharomyces cerevisiae* count of 4.00×10 CFU/g, viable *Lactobacillus acidophilus* count of 1.00×10^1 CFU/g, and viable *Bifidobacterium lactis* count of 1.00×10^1 CFU/g. *Bifidobacterium lactis* was provided by Shaanxi Sciphar Biotechnology Co., Ltd., while *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* were prepared in our laboratory. The *Bacillus subtilis* used was a genetically engineered strain containing antimicrobial peptides. Based on the principle of overlapping extension PCR, two pairs of four primers were designed to synthesize the antimicrobial peptide gene, with each antimicrobial peptide gene completed through two-step overlapping extension. The final PCR product was recovered and ligated with pMD18-T Vector to successfully construct the cloning vector pMD18-T/CC34. The expression vector pHT43 was selected to construct the recombinant expression vector, which was then transformed into *Bacillus subtilis* WB800N to obtain the genetically engineered bacterium pHT43/CC34/WB800N. In LB medium, heterologous antimicrobial peptide CC34 was successfully expressed upon induction with isopropyl -D-1-thiogalactopyranoside (IPTG). The expressed product was purified using high-performance liquid chromatography (HPLC), and the purified protein was identified as the target protein by mass spectrometry (MS) with a molecular mass of 3.7 kDa and expression level of 31.98 mg/L. Additionally, the antibacterial effect of the antimicrobial peptide against pathogenic bacteria was detected using a combination of the inhibition zone method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [5].

1.2 Experimental Animals and Basal Diet

Experimental animals were 1-day-old healthy male Arbor Acres (AA) broilers purchased from a hatchery in Dehui City, Jilin Province. The basal diet used in this experiment was formulated according to the NRC (1994) broiler feeding standards and combined with China' s "Feeding Standard of Chicken" (NY/T 33-2004). The composition and nutrient levels are shown in Table 1 . Feed ingredients were purchased from Daqing Hefeng Bayi Agricultural University Animal Science and Technology Co., Ltd., and the diet was in powder form, ground and mixed in our laboratory.

1.3 Experimental Design

This experiment employed a single-factor completely randomized design. A total of 320 healthy 1-day-old male Arbor Acres (AA) broilers were randomly divided into 4 groups with 4 replicates per group and 20 broilers per replicate, with no significant difference in body weight among groups (P>0.05). Group I served as the control group and was fed the basal diet. Groups II, III and IV were experimental groups fed the basal diet supplemented with 1,000 mg/kg compound probiotics, with *Bacillus subtilis*:*Saccharomyces cerevisiae*:*Lactobacillus acidophilus*:*Bifidobacterium lactis* ratios of 2:1:1:1, 1:2:1:1 and 1:1:1.5:1.5, respectively. The experimental period lasted 42 days.

1.4 Feeding Management

Before chick arrival, the chicken house was thoroughly cleaned and disinfected without dead angles. As the experiment was conducted in spring, preheating began 2 days before chick arrival. Within 2 hours of the experimental chicks' arrival, they were provided with a low-concentration (5%) glucose solution at moderate temperature. Broilers were raised in cages at a stocking density of 20 birds/m² during days 1-21 and 10 birds/m² during days 22-42, with free access to water and feed. Temperature, humidity and lighting duration in the house were strictly controlled according to conventional feeding management requirements, with good ventilation ensured. Water troughs were cleaned and disinfected daily before water replacement, and cooled boiled water was provided throughout the experimental period. Chicks were immunized against Newcastle disease and infectious bursal disease according to conventional immunization programs.

1.5 Determination Methods

1.5.1 Nutrient Apparent Availability On day 35 of the feeding trial, one healthy broiler with similar body weight was randomly selected from each replicate of each group and housed individually in cages for a total fecal collection metabolism trial. At 20:00 on day 37, the metabolism broilers began fasting to eliminate the influence of intestinal contents on the metabolism trial, with free access to water during fasting while other feeding conditions remained unchanged. At 08:00 on day 39, a 3-day total fecal collection metabolism trial commenced, during which feed intake was accurately recorded and all excreta were collected daily at fixed times (with careful removal of feathers and debris). After collection, 10 mL of 10% hydrochloric acid was added per 100 g of excreta, and samples were immediately stored at -20 °C. On the final night of the feeding trial, metabolism broilers underwent fasting metabolism. After collection, excreta were thawed, mixed uniformly, dried to constant weight at 65 °C, equilibrated at room temperature for 24 hours, weighed, ground to pass through a 40-mesh sieve, and sealed in bags for subsequent analysis.

Nutrient contents in diets and excreta were determined using conventional methods: dry matter content by oven drying at 103-105 °C (GB/T 6435-2014), crude ash content by high-temperature incineration (GB/T 6438-2007), crude protein content by semi-micro Kjeldahl method (GB/T 6432-1994), ether extract content by Soxhlet extraction (GB/T 6433-2006), crude fiber content by filtration method (GB/T 6434-2006), calcium content by potassium permanganate titration (GB/T 6436-2002), and phosphorus content by spectrophotometry (GB/T 6437-2002) [6]. Nutrient apparent availability was calculated using the following formula: Nutrient apparent availability (%) = 100 × (nutrient intake - nutrient content in excreta) / nutrient intake.

1.5.2 Serum Biochemical Indexes At 21 and 42 days of age, 3 broilers were randomly selected from each replicate of each group, and 5 mL of blood was collected from the heart. After centrifugation at 3,000 r/min for 10 minutes, serum was separated into 1.5 mL EP tubes and stored at -20 °C for later analysis. Serum total protein (TP), albumin (ALB) content, urea nitrogen (UN) concentration and alanine aminotransferase (ALT) activity were measured using assay kits according to the manufacturer' s instructions. All kits were purchased from Nanjing Jiancheng Bioengineering Institute.

1.5.3 Intestinal Mucosal Morphology On day 42 of the feeding trial, 3 healthy broilers with similar body weight were randomly selected from each replicate of each group. After slaughter, the entire intestine was immediately removed, dissected and separated. Segments of 2-3 cm from the middle of the duodenum, jejunum and ileum were taken, gently rinsed with 0.9% saline solution to remove intestinal chyme, and fixed in 10% neutral formalin solution for 24 hours. Conventional hematoxylin-eosin (HE) staining was performed on small intestinal tissues to prepare paraffin sections. Using an ML-50 microscopic image acquisition and analysis system under low magnification (40×) of an optical microscope, multiple non-consecutive fields were randomly selected on tissue sections from the duodenum, jejunum and ileum for observation. Typical fields were photographed, and small intestinal villus height and crypt depth were measured. The average values were taken as fixed data, and the villus height/crypt depth ratio was calculated.

1.6 Data Statistics and Processing

In this experiment, nutrient apparent availability was analyzed statistically using the replicate as the unit, with 4 mean values per group. Serum biochemical indexes and intestinal mucosal morphology were analyzed using 12 samples per group. Data were initially processed and organized using Excel 2007, and one-way ANOVA was performed using SAS 8.0 software. Duncan' s method was used for significance analysis, and results were expressed as "mean ± standard deviation." $P<0.05$ was considered statistically significant.

Results

2.1 Effects of Compound Probiotics on Nutrient Apparent Availability in Broilers

As shown in Table 2 , there was no significant difference in apparent availability of crude fiber and phosphorus among groups ($P>0.05$). Compared with the control group, the apparent availability of dry matter, crude protein, ether extract and calcium in groups II, III and IV was significantly increased ($P<0.05$). Specifically, dry matter apparent availability increased by 1.83%, 1.95% and 1.43%; crude protein apparent availability increased by 6.75%, 8.60% and 5.25%; ether extract apparent availability increased by 9.64%, 13.89% and 10.15%; and calcium apparent availability increased by 14.70%, 15.19% and 14.24%, respectively.

2.2 Effects of Compound Probiotics on Serum Biochemical Indexes in Broilers

As shown in Table 3 , at 21 days of age, there were no significant differences among groups in serum total protein, albumin content, urea nitrogen concentration or alanine aminotransferase activity ($P>0.05$). At 42 days of age, serum albumin content, urea nitrogen concentration and alanine aminotransferase activity showed no significant differences among groups (P>0.05). However, compared with the control group, serum total protein content in groups II, III and IV was significantly increased (P<0.05) by 13.84%, 14.79% and 14.04%, respectively.

2.3 Effects of Compound Probiotics on Intestinal Mucosal Morphology in Broilers

As shown in Table 4 , the villus height, crypt depth and villus height/crypt depth ratio in the duodenum, jejunum and ileum of groups II, III and IV were superior to those of the control group, though the differences were not significant (P>0.05).

Figure 1 [Figure 1: see original paper] shows the intestinal mucosal morphological structure of broilers in each group. The figure reveals that some intestinal villus tip epithelial cells were shed in the control group, while groups II, III and IV exhibited longer villi with better integrity. A, B, C and D represent duodenum sections from groups I, II, III and IV, respectively; E, F, G and H represent jejunum sections from groups I, II, III and IV, respectively; I, J, K and L represent ileum sections from groups I, II, III and IV, respectively.

Discussion

3.1 Effects of Compound Probiotics on Nutrient Apparent Availability in Broilers

Nutrient apparent availability is one of the most important indicators for measuring whether livestock and poultry can fully utilize dietary nutritional value and represents the most intuitive experimental result for evaluating broiler health. It is influenced by factors such as dietary nutrient levels, feed processing technology, animal growth stage and intestinal health status [7]. After compound probiotics enter the intestinal tract of livestock and poultry, synergistic and symbiotic effects with complementary advantages can occur among different microbial strains, between microbial preparations and intestinal microorganisms, and between microbial preparations and the host [8]. Sun Xifeng et al. [9] found that compared with the control group, supplementation with different concentrations of compound probiotics in broiler diets improved apparent availability of energy, crude protein, dry matter and ether extract to varying degrees during both early and late growth stages. Mountzouris et al. [10] demonstrated that adding compound probiotics to broiler basal diets could improve nutrient digestibility. The results of this experiment indicate that after supplementation with different proportions of compound probiotics, nutrient apparent availability in groups II, III and IV increased to varying degrees compared with the control group. This improvement can be attributed to the fact that compound probiotics, antimicrobial peptide-containing genetically engineered bacteria and their various metabolites and antimicrobial peptides can improve the intestinal microecological environment and facilitate digestion and utilization of dietary nutrients. *Bacillus subtilis* regulates key rate-limiting enzymes in nitrogen metabolism such as xanthine oxidase and glutamine synthetase, thereby modulating nitrogen metabolism [11]. It also possesses strong oxygen-scavenging capacity [12], creating a favorable environment for beneficial intestinal bacteria and the co-administered *Lactobacillus*, *Bifidobacterium* and yeast, resulting in synergistic and complementary effects. Additionally, the *Bacillus subtilis* genetically engineered bacterium used in this experiment can secrete antimicrobial peptides that inhibit and reduce proliferation of harmful bacteria in the intestine. The protoplast of *Saccharomyces cerevisiae* cells is rich in nucleotides and essential amino acids (especially lysine) and contains abundant digestive enzymes that promote intestinal digestion and absorption of macromolecular nutrients. The polysaccharides in yeast cell walls are mainly composed of -1,3-glucan and mannan oligosaccharides (MOS) [13]. These two polysaccharides can be decomposed by enzymes produced by *Bifidobacterium* in the small intestine to become carbon sources for *Bifidobacterium*, promoting its proliferation, acidifying the intestinal environment, reducing intestinal redox potential and inhibiting growth of harmful bacteria. *Lactobacillus* and *Bifidobacterium* are the main bacterial groups maintaining microbial flora balance in the animal intestine. They can also metabolize and produce organic acids, promote intestinal peristalsis and digestive juice secretion, and facilitate nutrient digestion and absorption. Moreover, this acidic environment is conducive to releasing bound or chelated mineral elements in free form, thereby improving utilization of calcium, phosphorus and other minerals. Group III showed the highest nutrient apparent availability, possibly because the powder feed used in this experiment was thoroughly mixed with the compound probiotics dominated by *Saccharomyces cerevisiae*. After broiler consumption, yeast exerted its fermentation characteristics in the gastrointestinal tract, decomposing, transforming and synthesizing nutrients in the diet to produce enzymes, bacterial bodies and various metabolites required by broilers, thereby improving nutrient utilization.

3.2 Effects of Compound Probiotics on Serum Biochemical Indexes in Broilers

Blood is an important component of the internal environment and the primary site for material exchange and metabolism. Its index changes are influenced by factors such as broiler growth and development status, nutritional level and endocrine secretion [14]. Only when biochemical indexes in blood remain relatively stable can normal metabolism occur and satisfactory production performance be achieved. Therefore, blood or serum biochemical indexes can serve as important indicators reflecting physiological status and health condition. Serum protein, urea nitrogen content and alanine aminotransferase activity are commonly used indexes reflecting protein metabolism level in broilers. Serum total protein and albumin content can reflect protein digestion status and immunity. Serum proteins are important blood components that not only reflect nutritional status but also maintain colloidal osmotic pressure and perform functions such as ion transport, immunity and tissue repair [15]. Good nutritional status can maintain serum total protein and albumin at relatively high levels, and elevated levels of both indicate vigorous metabolic activity [16]. Urea nitrogen is an important indicator reflecting poultry nitrogen metabolism as it is a product of protein metabolism. Its concentration can accurately reflect protein metabolism and amino acid balance in animals [17], with lower urea nitrogen concentration indicating better protein metabolism [18]. Tang Zhigang et al. [19] found that probiotics significantly reduced urea nitrogen concentration in broilers during the early stage and increased serum total protein, albumin and globulin content to some extent, indicating that probiotics can regulate protein metabolism. Liu Xiaolong et al. [20] reported that individual or combined supplementation of antimicrobial peptides or synbiotics (composed of *Lactobacillus plantarum*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and chitosan oligosaccharide) in AA broiler diets could significantly increase serum total protein and albumin content and reduce alanine aminotransferase activity.

The improvement in nutrient apparent availability indicates enhanced broiler capacity to absorb various nutrients (protein, fat and carbohydrates) from the diet, thereby increasing serum total protein and albumin content and reducing urea nitrogen concentration and alanine aminotransferase activity to some extent. The results of this experiment showed that different proportions of compound probiotics significantly increased serum total protein content in 42-day-old broilers. Although serum albumin content, alanine aminotransferase activity and urea nitrogen concentration did not differ significantly from the control group, they were numerically higher. This suggests that compound probiotics can regulate protein metabolism. The increase in serum total protein and albumin content and decrease in alanine aminotransferase activity and serum urea nitrogen concentration may be attributed to the ability of compound probiotics to balance intestinal microbiota, reduce intestinal pH, produce microbial protein and secrete proteases. The antimicrobial peptides secreted by *Bacillus subtilis* genetically engineered bacteria can also improve gastrointestinal digestive enzyme activity in broilers, enhance protein and amino acid utilization and strengthen protein anabolism [21]. These findings are consistent with Chen Jing et al. [22], who reported that compound microecological preparations significantly increased serum total protein content and reduced serum urea nitrogen concentration in broilers.

3.3 Effects of Compound Probiotics on Intestinal Mucosal Morphology in Broilers

The intestine is an important organ for digestion and absorption of nutrients in broilers. Changes in small intestinal villus height, crypt depth and villus height/crypt depth ratio are important indicators reflecting digestive and absorptive capacity of the digestive tract. Therefore, small intestinal mucosal morphology significantly influences nutrient apparent availability. Villus height results from cell proliferation, and higher villus height facilitates nutrient absorption in the small intestine. Crypts, also known as intestinal glands, reflect cell generation rate, with shallower crypts indicating more mature cell development [23]. The villus height/crypt depth ratio comprehensively reflects small intestinal function status. A decreased ratio indicates weakened intestinal absorption capacity, while an increased ratio suggests improved mucosal structure and enhanced nutrient digestion and absorption capacity [24]. Qi Fenghua et al. [25] found that supplementation with *Bacillus subtilis* and *Lactobacillus acidophilus* preparations in basal diets significantly reduced crypt depth and increased villus height/crypt depth ratio in the duodenum, jejunum and ileum. Zhang Caifeng et al. [26] reported that adding 1,000 mg/kg compound bacterial preparation composed of *Lactobacillus* and yeast to basal diets significantly increased jejunal villus height and villus height/crypt depth ratio, improved intestinal mucosal morphology and helped maintain healthy intestinal mucosal structure. The results of this experiment showed that although differences in villus height, crypt depth and villus height/crypt depth ratio in the duodenum, jejunum and ileum among groups II, III and IV were not significant, they were all superior to those of the control group. The possible reason is that *Bacillus subtilis* is an aerobic bacterium that can proliferate and secrete antimicrobial peptides in the anterior gastrointestinal tract where oxygen is abundant. As *Bacillus subtilis* moves posteriorly along the small intestine where oxygen becomes scarce, proliferation ceases, antimicrobial peptide secretion decreases and its effects gradually weaken, resulting in greater effects on the duodenum than on the jejunum and ileum [27]. After compound probiotics enter the digestive tract with the diet, they not only help the body decompose and utilize nutrients but also continuously stimulate small intestinal mucosa, promoting its growth and development and increasing villus height. Furthermore, compound probiotics can improve intestinal mucus secretion, enhance tight junctions of intestinal epithelial cells, regulate proliferation and differentiation of intestinal epithelial cells, repair damaged intestines, reduce crypt depth and thereby increase villus height/crypt depth ratio [28]. Finally, after entering the body, compound probiotics inhibit proliferation of harmful bacteria in the gastrointestinal tract through oxygen consumption in the intestinal lumen, competitive inhibition and production of metabolites, thereby preventing damage to intestinal mucosa and maintaining good intestinal mucosal morphology and barrier function [29]. Lam et al. [30] demonstrated that probiotics can promote recovery of damaged intestines by inducing phosphorylation of epidermal growth factor receptors and can stimulate proliferation and differentiation of intestinal epithelial cells by regulating secretion of polyamines such as putrescine and spermidine and expression of ornithine decarboxylase genes. In this experiment, supplementation with different proportions of compound probiotics in diets improved small intestinal mucosal morphology in broilers, though the specific mechanisms require further investigation.

Conclusion

Supplementation with different proportions of compound probiotics in diets can significantly improve nutrient apparent availability of dry matter, crude protein, ether extract and calcium in broilers, with group III showing the highest nutrient apparent availability. Different proportions of compound probiotics in diets can significantly increase serum total protein content in 42-day-old broilers. Different proportions of compound probiotics in diets can improve villus length, crypt depth and villus height/crypt depth ratio in the duodenum, jejunum and ileum of broilers to varying degrees.

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