Effects of Acidifiers and Antibiotics on Ileal Mucosal Microbiota in Yellow-Feathered Broiler Chickens: Postprint

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Abstract

This study aimed to investigate the effects of long-term dietary supplementation of acidifiers and sub-dose neomycin sulfate on the dynamic changes and composition of the ileal mucosal microbiota in yellow-feathered broilers. A total of 800 healthy 1-day-old Lingnan yellow-feathered broilers were randomly allocated into 4 groups, with 5 replicates per group and 40 birds per replicate. The control group (Group A) received a basal diet, while Groups B, C, and D received the basal diet supplemented with 0.2% acidifier I, 0.3% acidifier II, and 50 mg/kg neomycin sulfate, respectively. The experimental period lasted 42 days. At 1, 7, 14, 21, 28, 35, and 42 days of age, ileal mucosa was collected for bacterial DNA extraction. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was employed to analyze the ileal mucosal microbiota structure, and 16S rDNA gene sequencing was used to construct a random clone library of bacterial 16S rDNA from the ileal mucosa of 42-day-old broilers. Dynamic change trend analysis revealed that the four groups exhibited different degrees of structural changes in the ileal mucosal microbiota, with dynamic change values of 9%–33%, 8%–90%, 8%–56%, and 17%–45% during days 1–28, respectively. Analysis of the random clone library of ileal mucosal bacteria showed that Group A' s library contained 94 sequences, generating 16 operational taxonomic units (OTUs); Group B had 87 sequences, generating 7 OTUs; Group C had 98 sequences, generating 5 OTUs; and Group D had 94 sequences, generating 9 OTUs. The proportions of Lactobacillus in total clones for Groups A and D were 39.36% and 50.00%, respectively, which were extremely significantly higher than those for Group B (6.90%) and Group C (10.20%) ($P<0.01$). The types and compositional proportions of other bacterial genera in Group A differed from those in the other groups, and no sequences related to Escherichia were detected in any group. These results indicate that long-term dietary supplementation of acidifiers and sub-dose neomycin sulfate

caused dramatic alterations in the ileal mucosal microbiota structure of Lingnan yellow-feathered broilers during days 1–28, reduced the diversity of the ileal mucosal microbiota at 42 days of age, and modified the composition of Lactobacillus. These changes and differences may be attributed to the continuous pressure exerted by acidifiers and antibiotics.

Full Text

Effects of Acidifiers and Antibiotics on Ileal Mucosa Microbiota of Yellow-Feathered Broilers

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Abstract

This experiment investigated the effects of long-term dietary supplementation with acidifiers and subclinical doses of neomycin sulfate on the dynamic changes and composition of ileal mucosa microbiota in yellow-feathered broilers. Eight hundred 1-day-old healthy Lingnan yellow-feathered broilers were randomly allocated into four groups with five replicates per group and 40 broilers per replicate. Broilers in the control group (Group A) received a basal diet, while those in Groups B, C, and D received the basal diet supplemented with 0.2% acidifier I, 0.3% acidifier II, and 50 mg/kg neomycin sulfate, respectively. The experimental period lasted 42 days. Ileal mucosa samples were collected, and bacterial DNA was extracted at 1, 7, 14, 21, 28, 35, and 42 days of age. The microbiota structure was analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and a random clone library of 16S rDNA from ileal mucosa bacteria was constructed for 42-day-old broilers using 16S rDNA gene sequencing technology. Dynamic change trend analysis revealed that the four groups exhibited different degrees of alteration in ileal mucosa microbiota structure, with dynamic change values ranging from 9%–33%, 8%–90%, 8%–56%, and 17%–45% during days 1–28, respectively. Analysis of the random clone library showed that Group A contained 94 sequences forming 16 operational taxonomic units (OTUs), Group B had 87 sequences forming 7 OTUs, Group C had 98 sequences forming 5 OTUs, and Group D had 94 sequences forming 9 OTUs. The proportion of *Lactobacillus* in Groups A and D was 39.36% and 50.00% of total clones, respectively, which was significantly higher than in

Groups B (6.90%) and C (10.20%) ($P < 0.01$). Differences were observed between Group A and other groups in the types and compositional proportions of other bacterial genera, and no sequences related to *Escherichia* were detected in any group. These findings indicate that long-term dietary supplementation with acidifiers and subclinical doses of neomycin sulfate caused dramatic changes in the ileal mucosa microbiota structure of Lingnan yellow-feathered broilers during days 1–28, reduced microbiota diversity at 42 days of age, and altered the composition of *Lactobacillus*, likely due to continuous pressure from acidifiers and antibiotics.

Keywords: acidifier; neomycin sulfate; Lingnan yellow-feathered broilers; intestinal mucosa microbiota; PCR-DGGE; 16S rDNA clone library

Introduction

The animal gastrointestinal tract is an open and complex ecosystem containing 10^{11}–10^{14} microorganisms per gram of intestinal content, with rich species diversity. These microorganisms significantly influence host nutrition, physiology, and immunity [1-2]. Consequently, the impact of gastrointestinal microbiota on host health has attracted considerable research attention. The irrational and non-standardized use of feed antibiotics has led to decreased animal immunity, reduced feed intake, and intestinal microbiota dysbiosis. In response, functional feed additives such as acidifiers, enzymes, and probiotics have been extensively studied and applied as antibiotic alternatives over the past two decades. Research indicates that acidifiers can improve livestock and poultry performance and intestinal microbiota by enhancing nutrient digestibility, improving the intestinal microenvironment, optimizing small intestinal histomorphology, promoting beneficial bacterial proliferation, and reducing pathogenic bacteria and bacterial toxins [3-8]. Neomycin sulfate, an aminoglycoside broad-spectrum antibiotic, is primarily used clinically to treat bacterial gastrointestinal infections in livestock and poultry. Oral administration rarely causes toxic reactions, with most of the drug excreted unchanged in feces, resulting in no drug residues in animal tissues. Due to its high safety profile for livestock and poultry and low potential for developing drug resistance and cross-resistance, neomycin sulfate has become one of the most commonly used veterinary drugs internationally. However, most studies have focused on the effects of acidifiers and antibiotic growth promoters on intestinal content microbiota, while research on the effects of neomycin sulfate as a prophylactic agent and acidifiers on gastrointestinal mucosa microbiota remains unreported. Therefore, this study utilized Lingnan yellow-feathered broilers as experimental subjects and employed PCR-DGGE technology and 16S rDNA gene sequencing to analyze the effects of acidifiers and subclinical doses of neomycin sulfate on the composition and diversity of ileal mucosa microbiota, providing a basis for future in-depth research on the application of feed antibiotics and acidifiers.

Materials and Methods

Experimental Design The experiment was conducted from October 29 to December 9, 2014, at the experimental facility of the Institute of Animal Science, Guangdong Academy of Agricultural Sciences, with a duration of 42 days. Eight hundred 1-day-old healthy Lingnan yellow-feathered broilers were randomly divided into four groups with five replicates per group and 40 broilers per replicate. Initial body weight showed no significant differences among groups ($P > 0.05$). The control group (Group A) received a basal diet, while Groups B, C, and D received the basal diet supplemented with 0.2% acidifier I, 0.3% acidifier II, and 50 mg/kg neomycin sulfate, respectively. Acidifier I [(2009) Wai Si Zhun Zi 198] was an imported product composed of phosphoric acid, formic acid, lactic acid, malic acid, citric acid, and tartaric acid. Acidifier II [Yue Si Tian Zi (2007) 186007] was a domestic product composed of fumaric acid, lactic acid, citric acid, propionic acid, and formic acid. Both acidifiers were used at the dosages recommended by their manufacturers. Neomycin sulfate (potency: 675 U/mg) was purchased from Yichang Three Gorges Pharmaceutical Co., Ltd., with dosage based on the *Import Veterinary Drug Quality Standards* (1999).

Management Practices Experimental broilers were raised on floor pens with wood shavings in a closed broiler house under 24-hour lighting, with free access to feed and water. A corn-soybean meal basal diet was formulated in two phases: days 1–21 and days 22–42, according to NRC (1994) *Nutrient Requirements of Chickens*, *Feeding Standard of Chickens* (NY/T 33–2004), and the Chinese Feed Composition and Nutritional Value Table (2005). The phase 1 diet contained 21% crude protein and 12.12 MJ/kg metabolizable energy, while the phase 2 diet contained 19% crude protein and 12.54 MJ/kg metabolizable energy. Conventional vaccination programs were implemented. The average house temperature was approximately 22°C, with relative humidity maintained at 60%–65%. No diseases occurred during the experiment, and routine disinfection was performed.

Performance Measurements At days 1, 21, and 42, broilers were fasted for 12 hours (water provided) and weighed at 08:00 the following day by replicate. Average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F/G) were calculated for each replicate.

Sample Collection, Pretreatment, and DNA Extraction At days 1, 7, 14, 21, 28, 35, and 42, two healthy broilers were randomly selected from each replicate (10 per group) before morning feeding, euthanized, and ileal mucosa samples (middle 2 cm segment) were collected. One centimeter was immediately snap-frozen in liquid nitrogen and stored at –80°C. Pretreatment followed methods described in references [9-10]: samples were thawed, the 1 cm segment was longitudinally opened, and large content particles were rinsed away with sterile physiological saline (repeated twice) to separate loosely attached digesta and bacteria from the intestinal wall. The rinsed segment was placed in 1 mL

sterile physiological saline (containing 0.1% Tween 80), vortexed for 30 s to detach mucosa-associated bacteria (repeated once), and the bacterial suspension was centrifuged at 13,000 r/min for 30 min at 4°C to collect bacterial cells. The remaining 1 cm ileal mucosa samples from the 10 broilers per group were pooled. Bacterial pellets were resuspended in 1.5 mL TE buffer [10 mmol/L Tris-HCl (pH = 8.0), 1 mmol/L ethylenediaminetetraacetic acid (EDTA)], and 500 µL was used for DNA extraction; the remaining 1 mL was snap-frozen in liquid nitrogen and stored at -80°C. DNA was extracted using the EZgene™ Bacterial gDNA Kit (Biomiga, USA) according to the manufacturer' s instructions.

PCR-DGGE Analysis

PCR Amplification Based on reference [11], primers for the V3 region of 16S rDNA were designed and synthesized by Shanghai Bioengineering Co., Ltd.: 341F-GC (5 -CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCA 3) and 518R (5 -ATTACCGCGGCTGCTGG-3). The PCR reaction mixture (50.0 µL) contained: 5 µL 10×buffer (with $MgCl_2$), 4 µL dNTPs (10 mmol/L), 0.5 µL each of forward and reverse primers (10 µmol/L), 5 µL template DNA (20 ng/µL), 0.25 µL Taq DNA polymerase (5 U/µL), and ddH_2O to 50 µL. A negative control without template was included. PCR conditions were: 94°C for 3 min; 30 cycles of 94°C for 30 s, 57.5°C for 40 s, and 72°C for 50 s; and final extension at 72°C for 7 min. Five microliters of PCR product was verified by electrophoresis on 1.2% agarose gel in 1×TAE buffer. PCR products were purified using a kit (Shanghai Bioengineering Co., Ltd.) according to the manufacturer' s instructions.

DGGE Electrophoresis PCR-DGGE was performed using the Bio-Rad Dcode system with a 40%-60% denaturing gradient parallel to the electrophoresis direction. The 100% denaturant solution contained 7 mol/L urea and 40% deionized formamide. Electrophoresis was conducted at 70 V and 60°C for 14 h in 1×TAE buffer, with 20 µL of PCR product loaded per well. Silver nitrate staining was used, and stained gels were photographed on a white light transilluminator.

16S rDNA Random Clone Library Construction

PCR Amplification of Bacterial 16S rDNA V3 Region Using genomic DNA from ileal mucosa bacteria of 42-day-old broilers as template, primers for the V3 region of 16S rDNA (without GC clamp) were designed based on reference [11] and synthesized by Shanghai Bioengineering Co., Ltd.: 341F (5 -CCTACGGGAGGCAGCAG-3) and 518R (5 -ATTACCGCGGCTGCTGG-3). PCR reaction mixture, amplification conditions, electrophoresis detection, and product purification were as described in Section 1.5.1.

Clone Library Construction and Data Analysis Purified PCR products were ligated into pMD18-T vector (purchased from TaKaRa Bio, Dalian, China) overnight at 4°C, then transformed into DH5α competent cells (TaKaRa Bio). Transformants were cultured in LB medium at 37°C and 160 r/min for 1 h, then plated on LB agar containing 50 μg/mL ampicillin (Amp), 20% isopropyl β-D-1-thiogalactopyranoside (IPTG), and 2.5% 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). Plates were incubated at 37°C for 12–14 h, then held at 4°C overnight. One hundred white colonies were randomly selected from each plate, inoculated into LB broth (with 50 μg/mL Amp), and cultured at 170 r/min and 37°C for 12–14 h. Colony PCR was performed using BcaBEST Primer M13-47 (TaKaRa Bio) to amplify the insert from pMD18-T vector for positive clone identification. Positive clones were sequenced (BGI) to establish the clone libraries.

Data Processing and Analysis Broiler performance data were analyzed using SPSS 13.0 software by one-way ANOVA followed by Duncan' s multiple comparison test. Results were expressed as "mean ± standard error." The 16S rDNA V3 region sequences obtained were analyzed using DNASTAR to identify target fragments, and similar sequences (similarity $\geq 95\%$) were identified in the RDP database. The proportions of clones in the ileal random clone libraries were analyzed for significant differences using the u-test.

Results

Performance of Lingnan Yellow-Feathered Broilers As shown in , no significant differences were observed among groups in final body weight, average daily gain, average daily feed intake, or feed-to-gain ratio at any stage ($P > 0.05$). These results indicate that dietary supplementation with acidifiers and neomycin sulfate had no significant effect on the performance of Lingnan yellow-feathered broilers at different growth stages.

DNA Extraction and 16S rDNA V3 Region PCR Amplification from Ileal Mucosa Bacteria Electrophoresis of ileal mucosa bacterial DNA revealed fragments of approximately 23 kb, which were suitable as templates for PCR amplification of the 16S rDNA V3 region as determined by nucleic acid-protein analyzer measurement. The 16S rDNA V3 region PCR products were approximately 240 bp, consistent with the expected size (figure omitted). Clone PCR identification results ([Figure 1: see original paper]) showed that products amplified with BcaBEST Primer M13-47 were approximately 330 bp. Clones with single bands and no primer dimers were identified as positive. One hundred positive clones were randomly selected from LB agar plates for each group, yielding 94 suitable clones for sequencing in Group A, 87 in Group B, 98 in Group C, and 94 in Group D.

Similarity and Dynamic Change Analysis of Ileal Mucosa Microbiota Structure Phoretix 1D Pro software analysis of PCR-DGGE profiles revealed

low similarity (36%–75%, 33%–75%, and 40%–71%) between Group A and Groups B, C, and D, respectively, throughout days 1–42. Cluster analysis ([Figure 2: see original paper]) showed that day 1 samples from Groups A and B clustered together, while other ages from Group A formed a separate cluster that did not group with any ages from other groups. Dynamic change trend analysis ([Figure 3: see original paper]) demonstrated varying degrees of structural change among the four groups: during days 1–28, dynamic change values were 9%–33% for Group A, 8%–90% for Group B, 8%–56% for Group C, and 17%–45% for Group D; during days 35–42, values were 11%–17% for Group A, 5%–67% for Group B, 12%–20% for Group C, and 29%–33% for Group D. These results indicate that long-term dietary supplementation with acidifiers and subclinical neomycin sulfate caused dramatic changes in ileal mucosa microbiota structure in Group B throughout days 1–42 and in Groups C and D during days 1–28.

Diversity Analysis of Ileal Mucosa Microbiota As shown in , Group A exhibited lower coverage but higher OTUs, Shannon index, evenness, richness, and Simpson index compared to Groups B and C. Group A' s Shannon index and richness were higher than Group D, while evenness and Simpson index were comparable. These findings indicate that the diversity of ileal mucosa microbiota in 42-day-old broilers was higher in Group A than in Groups B, C, and D, with abundant diversity information and high species richness.

Analysis of Random Clone Libraries from Ileal Mucosa Bacteria As shown in , the 16S rDNA random clone library from Group A ileal mucosa bacteria contained 94 sequences representing 6 genera, 4 families, and 2 phyla. Group B contained 87 sequences representing 3 genera, 3 families, and 1 phylum. Group C contained 98 sequences representing 2 genera, 2 families, and 1 phylum. Group D contained 94 sequences representing 3 genera, 3 families, and 2 phyla. Unknown sequences accounted for 55.32%, 86.21%, 88.78%, and 47.87% of total clones in Groups A, B, C, and D, respectively. These bacteria showed 100.0% similarity to sequence FJ471462.1 in GenBank. Firmicutes was the second most abundant phylum, representing 43.62%, 13.79%, 11.22%, and 51.06% of total clones, respectively, with Lactobacillaceae being the predominant family (39.36%, 6.90%, 10.20%, and 50.00% of total clones). The proportion of *Lactobacillus* in Groups A and D (39.36% and 50.00%) was significantly higher than in Groups B (6.90%) and C (10.20%) ($P < 0.01$). Differences in types and compositional proportions of other bacterial genera were observed between Group A and other groups, and no *Escherichia*-related sequences were detected in any group. These results demonstrate that long-term dietary supplementation with acidifiers and subclinical neomycin sulfate affected bacterial species and compositional proportions in the ileal mucosa of 42-day-old broilers, particularly the proportion of the dominant *Lactobacillus* genus.

Discussion

Broilers have rapid growth rates and are susceptible to intestinal pH imbalances and inflammation due to physiological and environmental factors. Acidifiers can regulate appropriate acidic conditions in the intestine, and both acidifiers and antibiotics can inhibit harmful bacteria, promote beneficial bacterial proliferation, and eliminate intestinal inflammation. However, long-term use of acidifiers and antibiotics can disrupt the balance of host intestinal microbiota. PCR-DGGE profile analysis in this study showed low similarity between Group A and Groups B, C, and D throughout days 1–42, with dramatic changes in ileal mucosa microbiota structure in Groups B, C, and D during days 1–28, and continued dramatic changes in Group B during days 35–42. Throughout the experiment, broilers in all groups exhibited normal feeding, drinking, and excretory behaviors, with no significant differences in performance, indicating that continuous pressure from acidifiers and antibiotics altered the intestinal environment, forcing reselection and colonization of mucosa-associated bacteria and resulting in relatively reduced microbiota diversity. Differences were observed among groups in the types and compositional proportions of ileal mucosal bacterial genera, particularly *Lactobacillus*. The maturation and stability of host intestinal microbiota are influenced by age, dietary composition, antibiotics, genetics, and other factors, which subsequently affect host physiological activities [14-15].

Research on acidifier application in poultry production lags behind that in piglets. Studies have shown that dietary acidifiers significantly reduce *Escherichia coli* populations in duodenum [16], ileum [16-18], jejunum [19], and cecum [17,19-22] contents of chickens, but reports on their effects on *Lactobacillus* populations remain controversial. The primary functions of antibiotics are disease prevention and growth promotion, with few studies investigating their effects on intestinal microecology, most focusing on common antibiotic growth promoters such as avermectin, bacitracin methylene disalicylate, enramycin, virginiamycin, and salinomycin [14,23-27]. However, antibiotic growth promoters have been banned or restricted in livestock feed worldwide, resulting in minimal recent research on feed antibiotics' effects on animal intestinal microecology. This study investigated the effects of acidifiers and neomycin sulfate (approved by the Ministry of Agriculture) on broiler ileal mucosa microbiota. The significantly higher proportion of *Lactobacillus* in Groups A and D compared to Groups B and C contradicts most reports on acidifiers' effects on ileal *Lactobacillus* content, possibly due to differences in acidifier composition and processing technology. Uncoated acidifiers rapidly acidify the gastrointestinal tract upon ingestion, lowering intestinal pH and inhibiting harmful bacteria like *E. coli* without significantly affecting beneficial bacteria. Coated acidifiers resist neutralization by dietary components, providing sustained release to regulate intestinal pH and significantly enhance beneficial bacteria such as *Lactobacillus* [20,28]. No *Escherichia*-related sequences were detected in any group' s ileal mucosa bacterial 16S rDNA random clone library, possibly due to: (1) different

broiler breeds selecting for different intestinal microbiota—*Escherichia*-related sequences have been detected in ileal mucosa of 5–6-week-old Ross broilers [29-30], contrary to our findings; and (2) different optimal growth environments for bacteria—most harmful bacteria thrive in neutral to alkaline conditions (e.g., *E. coli* pH 6.0–8.0), while beneficial bacteria prefer acidic conditions (pH 5.4–6.4). In this study, intestinal contents from crop to cecum in 42-day-old broilers were acidic (pH 3.2–6.7) (data not shown), favoring beneficial bacterial growth. Additionally, long-term dietary supplementation with subclinical neomycin sulfate increased the proportion of the dominant *Lactobacillus* genus in ileal mucosa, suggesting that subclinical antibiotic use as a prophylactic agent represents a new research direction in livestock production—a positive signal not yet reported in literature and warranting further investigation. The interactions among acidifiers or antibiotics, luminal microbiota, and mucosal microbiota remain unclear, and further research is needed on the effects of feed antibiotics on intestinal mucosa microbiota.

Conclusion

Long-term dietary supplementation with acidifiers and subclinical doses of neomycin sulfate caused dramatic changes in the ileal mucosa microbiota structure of Lingnan yellow-feathered broilers during days 1–28, reduced microbiota diversity at 42 days of age, and altered the composition of *Lactobacillus*.

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