Effects of Different Relative Humidity on Cecal Microbiota Diversity in Broiler Chickens under Intermittent 26 °C Conditions: Postprint

Authors: Peng Qianqian, Zhou Ying, Wang Xuemin, Feng Jinghai, Zhen Long, Zhang Shaoshuai, Chang Yu, Shi Yuxiang, Zhang Minhong

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Abstract

This study aimed to investigate the effects of different relative humidity (RH) levels on cecal microbial diversity in broiler chickens under intermittent 26 °C conditions. A total of 180 Arbor Acres (AA) broiler chickens at 29 days of age were transferred to environmental control chambers and randomly assigned to three groups (RH at 30%, 60%, and 85%), with six replicates per group and ten chickens per replicate (five males and five females). Starting from day 29, the temperature was maintained at 26 °C with RH at 30%, 60%, and 85% from 10:00 to 16:00 (6 h) daily, while the remaining time was at 21 °C with 60% RH. The experiment lasted for 14 days. The 16S rDNA denaturing gradient gel electrophoresis (DGGE) technique, combined with excision and DNA recovery of specific and common bands for cloning and sequencing, was employed to analyze the effects of RH on cecal content microbial structure and diversity on days 7 and 14 of intermittent 26 °C heat treatment. The results showed: 1) On day 7, the number of DGGE bands (microbial richness) in the cecum of broilers in the 30% RH group was higher than that in the 60% RH group, while the 85% RH group was lower than the 60% RH group; on day 14, the number of cecal DGGE bands in both the 30% and 85% RH groups was higher than that in the 60% RH group. 2) Cluster analysis revealed that on day 7, 85% RH had a significant effect on the cecal microbiota; on day 14, 30% RH had a significant effect on the cecal microbiota. However, as the treatment progressed, the effect of 30% RH on the cecal microbiota of broilers became greater. 3) The common microbiota in the cecum of broilers under different RH groups in the intermittent 26 °C environment was Faecalibacterium prausnitzii; on day 7 of the experiment, the specific microbiota in the cecum of broilers in the 30% and 85% RH groups was Stomatobaculum longum. These results suggest that under intermittent 26 °C conditions, both low humidity (30% RH) and high humidity

(85% RH) affect the structure and diversity of the cecal microbiota in broilers, and the effects of RH differ with different treatment durations.

Full Text

Effects of Different Relative Humidity on Cecal Microflora Diversity of Broilers under Intermittent 26 ℃ Environment

PENG Qianqian[1,2], **ZHOU Ying**[1], ***WANG Xuemin***[2], ***FENG Jinghai***[1], ***ZHEN Long***[1,2], ***ZHANG Shaoshuai***[1], ***CHANG Yu***[1], ***SHI Yuxiang***[2], ***ZHANG Minhong***[1]

[1]State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[2]College of Agriculture, Hebei University of Engineering, Handan 056021, China

Abstract

This study investigated the effects of different relative humidity (RH) levels on cecal microflora diversity in broilers exposed to an intermittent 26 ℃ environment. One hundred eighty 29-day-old Arbor Acres (AA) broilers were transferred to environmental control chambers and randomly divided into three groups (RH levels of 30%, 60%, and 85%), with six replicates per group and ten birds per replicate (five males and five females). Beginning at 29 days of age, the temperature was maintained at 26 ℃ with RH levels of 30%, 60%, and 85% daily from 10:00 to 16:00 (6 h). During the remaining time, the temperature was 21 ℃ with 60% RH. The experiment lasted for 14 days. Using 16S rDNA-based denaturing gradient gel electrophoresis (DGGE) combined with cloning and sequencing of specific and common bands, we analyzed the effects of RH on cecal microbial community structure and diversity on days 7 and 14 of the intermittent 26 ℃ treatment. The results showed that: (1) On day 7, the number of DGGE bands in the 30% RH group (indicating microbial richness) was higher than that in the 60% RH group, while the 85% RH group showed lower band numbers than the 60% RH group. On day 14, both the 30% and 85% RH groups exhibited higher band numbers than the 60% RH group. (2) Cluster analysis revealed that on day 7, 85% RH had a pronounced effect on the cecal microflora, whereas on day 14, 30% RH showed a more significant impact. Moreover, the influence of 30% RH on cecal microflora increased with treatment duration. (3) The common microflora across different RH groups under the intermittent 26 ℃ environment was *Faecalibacterium prausnitzii*, while the specific microflora in the 30% and 85% RH groups on day 7 was *Stomatobaculum longum*. These findings indicate that both low (30% RH) and high (85% RH) humidity levels affect the structure and diversity of cecal microflora in broilers under intermittent 26 ℃ conditions, with differential effects observed at different treatment time points.

Keywords: relative humidity; intermittent heat; broilers; cecal microbiota;

denaturing gradient gel electrophoresis

Introduction

The intestinal microflora of poultry plays a crucial role in host nutrient absorption and intestinal development, thereby influencing growth performance and health status. Environmental factors, dietary composition, and age can all affect intestinal microflora composition. Previous studies have reported that under heat stress, significant changes occur in the cecal microflora of broilers, with marked reductions in *Lactobacillus* and *Bifidobacterium* populations and significant increases in *Escherichia coli* and *Clostridium perfringens*. Dietary factors can also substantially influence gastrointestinal microflora composition and metabolic activity.

Traditionally, research on poultry digestive tract microflora has relied on conventional pure culture methods to quantify bacterial populations, but these results are often inaccurate. Since the vast majority of gastrointestinal microflora are anaerobic and current cultivation techniques remain inadequate, traditional culture-based methods have significant limitations for analyzing intestinal microflora. It has been reported that more than 50% of normal microflora in the chicken intestine cannot be detected using conventional culture techniques.

With the development of modern technologies, molecular biology has provided scientific and convenient methods for studying intestinal microflora. Our laboratory previously used DGGE technology to demonstrate that continuous mild heat treatment at 26 ℃ reduced cecal microflora diversity in broilers. Additionally, we found that intermittent mild heat environments (26 ℃ and 31 ℃) combined with RH-induced stress significantly affected broiler production performance, body temperature, and acid-base balance. To date, DGGE-based studies on factors influencing broiler intestinal microflora have examined dietary components, age, and heat stress, but no reports have investigated the effects of RH on broiler intestinal microflora under intermittent 26 ℃ conditions. Therefore, this study analyzed DGGE profiles of 16S rDNA genes from broiler cecal microflora to investigate the effects of different RH levels on cecal microflora diversity under intermittent 26 ℃ conditions.

Materials and Methods

Experimental Animals and Management

One hundred eighty healthy 29-day-old Arbor Acres (AA) broilers with a body weight of (1,210 ± 13) g were randomly divided into three groups, with six replicates per group and ten birds per replicate (five males and five females). The experiment was conducted in environmental control chambers at the State Key

Laboratory of Animal Nutrition, with automatic control of temperature and RH (precision $±$1 ℃ and $±$7%), no air movement, and 24 h lighting. Broilers were housed in single-tier cages developed by our laboratory and provided with free access to feed and water. The dietary composition was consistent with that described in previous studies.

Environmental Conditions

From 22 days of age, broilers were acclimated for one week at 21 ℃ and 60% RH. At 29 days of age, they were transferred to three environmental chambers with RH levels of 30%, 60%, and 85%. Temperature was maintained at 26 ℃ from 10:00 to 16:00 (6 h) daily, while the remaining time was kept at 21 ℃ with 60% RH until the end of the 14-day experimental period.

Sample Collection and Processing

On days 7 and 14 of the experiment, six birds per group (three males and three females, one per replicate) were randomly selected, fasted for 12 h, and then euthanized. After sterilization by immersion in 5% benzalkonium bromide for 3 min, the abdominal cavity was opened, both ends of the cecum were ligated, and the cecum was excised and transferred to a laminar flow hood. The cecal wall was opened with sterile scissors, and six samples from the same group were rapidly mixed, placed in sterile 2 mL centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80 ℃ until analysis.

DNA Extraction and PCR Amplification

Bacterial Total DNA Extraction Genomic DNA was extracted from samples using the cetyltrimethylammonium bromide (CTAB) method as described in reference [23] (completed by Beijing Yiming Fuxing Biotechnology Co., Ltd.). Extracted genomic DNA was stored at -20 ℃.

16S rDNA V3 Region Amplification Primers for the 16S rDNA V3 region were designed based on reference [24] and synthesized by Beijing Yiming Fuxing Biotechnology Co., Ltd. (Table 1).

Table 1 Primer sequences

Primers	Sequence (5’ $→$3’)
338F	CCTACGGGAGGCAGCAG
518R	ATTACCGCGGCTGCTGG
GC357F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG

The PCR amplification system (50 L) consisted of: 10×PCR buffer 5 L, dNTPs (2.5 mmol/ L) 3.2 L, Taq DNA polymerase (5 U/ L) 0.4 L, GC-338F (20

mol/ L) 1 L, 518R (20 mol/ L) 1 L, template DNA 50 ng, and ddH_2O to 50 L. The PCR program was: 94 ℃ pre-denaturation for 5 min, 30 cycles of 94 ℃ denaturation for 1 min, 55 ℃ annealing for 0.5 s, and 72 ℃ extension for 1 min, followed by final extension at 72 ℃ for 10 min. PCR products were purified using the OMEGA DNA Gel Extraction Kit. The PCR instrument was a T-gradient from Biometra.

DGGE Analysis of 16S rDNA V3 Amplicons Ten microliters of PCR product were analyzed by gel electrophoresis using the Bio-Rad Gel-Doc2000 system. Electrophoresis was performed on 7% polyacrylamide gels with a 30%-60% denaturing gradient at 150 V and 60 ℃ in 1×TAE buffer for 5-8 h. After DGGE, gels were silver-stained according to reference [25] and scanned using the Bio-Rad Gel-Doc2000 system.

Excision, Recovery, Cloning, and Sequencing of Bands Specific and common bands were excised with a sterile scalpel, and DNA was recovered using the OMEGA Poly-Gel DNA Extraction Kit. The recovered products were re-amplified using the method described in section 1.4.2, and the amplified DNA fragments were excised, purified, ligated into Pmd18-T vectors, and transformed into DH5α competent cells. Positive clones were sequenced by Beijing Yiming Fuxing Biotechnology Co., Ltd., and sequences were analyzed using BLAST in GenBank.

Data Analysis

DGGE profiles were quantitatively analyzed for band numbers using Quantity One software, and cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Bacterial diversity indices were calculated based on band numbers and intensities in DGGE profiles. Diversity was expressed using the Shannon-Wiener index (H), evenness (E), and richness (S) as follows:

$$H = -\sum_{i=1}^{S} P_i \ln P_i$$

where P_i is the proportion of intensity of a single band relative to the total intensity of all bands in a sample.

$$E = \frac{H}{H_{\max}} = \frac{H}{\ln S}$$

where S is the total number of bands in a sample.

Results

Cecal Microflora Structure Analysis

All samples represented pooled cecal contents from six birds. The PCR-DGGE profiles showed that intermittent mild heat treatment affected broiler cecal microflora (Figure 1 [Figure 1: see original paper]), with distinct differences observed among RH treatments at the same time point. Digital analysis of the 16S rDNA V3 region PCR-DGGE profiles using Quantity One software revealed that on day 7, the 30% RH group had two more bands than the 60% RH group, while the 85% RH group had one fewer band. On day 14, the 30% and 85% RH groups had eight and five more bands, respectively, compared to the 60% RH group. These results indicate that on day 7, 30% RH increased cecal microflora diversity whereas 85% RH decreased it, but on day 14, both 30% and 85% RH increased diversity. Differences were also observed between time points within the same RH treatment. The 30% and 85% RH groups showed five additional bands on day 14 compared to day 7, while the 60% RH group showed one fewer band, suggesting that treatment duration had a greater impact on microflora diversity in the 30% and 85% RH groups than in the 60% RH group.

Cluster analysis results (Figure 2 [Figure 2: see original paper]) showed that on day 7, the similarity coefficients between the 30% RH group and 60% RH group, and between the 85% RH group and 60% RH group, were 71.2% and 64.4%, respectively. On day 14, these similarity coefficients were 52.9% and 65.2%, respectively. The similarity coefficient for the 30% RH group decreased more markedly from day 7 to day 14 compared to the 85% RH group. These findings indicate that 85% RH had a more pronounced effect on cecal microflora on day 7, while 30% RH showed a more significant impact on day 14, with the effect of 30% RH becoming more pronounced over time.

Cecal Microflora Diversity Analysis

Bacterial diversity indices were analyzed based on band numbers and intensities in PCR-DGGE profiles. As shown in Table 2 , diversity indices differed among RH treatments. On day 7, the Shannon-Wiener index and richness in the 30% RH group were 2.81 and 20, respectively, while those in the 85% RH group were 2.65 and 17. On day 14, the corresponding values were 3.03 and 25 for the 30% RH group, and 2.96 and 22 for the 85% RH group. Throughout the experimental period, the Shannon-Wiener index and richness were highest in the 30% RH group. On day 7, the 85% RH group showed lower Shannon-Wiener index and richness than the 60% RH group, but on day 14, both the 30% and 85% RH groups exhibited higher values than the 60% RH group. Evenness exceeded 92% in all groups during the entire experimental period. These results demonstrate that 30% RH increased cecal microflora diversity indices and richness, while 85% RH decreased these parameters on day 7 but increased them on day 14.

Analysis of Specific and Common Cecal Microflora

Two common bands and four specific bands were excised from the 16S rDNA V3 region PCR-DGGE profiles (indicated by arrows in Figure 1 [Figure 1: see original paper]). After PCR amplification, cloning into Pmd18-T vectors, and sequencing, the results were compared against the GenBank database (Table 3). Bands 2 and 6 (*Faecalibacterium prausnitzii*) were detected in all groups on both days 7 and 14. However, distinct differences emerged among RH treatments. On day 7, band 1 (*Stomatobaculum longum*) appeared in both the 30% and 85% RH groups but was absent in the 60% RH group. On day 14, bands 1 and 5 (*Stomatobaculum longum* and *Faecalibacterium prausnitzii*) were not detected in the 30% RH group but were present in both the 60% and 85% RH groups. Band 4 (*Clostridium thermosuccinogenes*) was present in the 30% and 60% RH groups but absent in the 85% RH group.

These findings indicate that on day 7, 30% and 85% RH promoted the growth of *Stomatobaculum longum*, while on day 14, 30% RH inhibited colonization by *Faecalibacterium prausnitzii* and 85% RH inhibited colonization by *Clostridium thermosuccinogenes*.

Among the six sequenced bands, sequences were distributed in the phyla Firmicutes and Bacteroidetes. Most showed >95% homology with bacteria in the GenBank database, except for band 4, which showed only 84% homology with the most closely related identified strain, suggesting that this sequence may represent a novel unculturable bacterium.

Discussion

Effects of Intermittent Mild Heat Environment on Cecal Microflora Diversity

Zhou et al. [26] demonstrated that appropriate sample sizes are essential for PCR-DGGE analysis of poultry intestinal microflora, with five birds identified as the optimal sample size. Gong et al. [27] used the same analytical technique to study bacterial composition from crop to cecal mucosa in broilers, also using five birds per sample. In our 2015 study on the effects of continuous mild heat on broiler cecal microflora diversity using DGGE technology, we employed six birds per sample [11]. Therefore, the current study used pooled cecal contents from six broilers for DGGE analysis of RH effects under intermittent 26 ℃ conditions.

The cecum has a substantial impact on microflora diversity, and its rich microbial community—containing 10^{11} CFU per gram of feces—makes it a focal point for research. Numerous studies have reported that the cecum harbors the most abundant microflora in the broiler intestine [29]. Li [19] found that the growth period has the greatest influence on cecal microflora, with microbial richness

increasing with age and stabilizing by 28 days [27]. Based on these findings, we selected the cecum for investigating intestinal microflora diversity in this study.

Our results showed that on day 7, 85% RH reduced total cecal bacterial numbers and decreased microflora diversity, whereas on day 14, both 30% and 85% RH increased total bacterial numbers and altered microflora diversity. The 30% and 85% RH groups exhibited five additional bands on day 14 compared to day 7, while the 60% RH group showed one fewer band, indicating that treatment duration had a greater impact on cecal microflora diversity in the 30% and 85% RH groups than in the 60% RH group.

Previous research has shown that intermittent mild heat (26 ℃ and 31 ℃) combined with RH significantly affects broiler growth performance, body temperature, and acid-base balance. Specifically, 85% RH significantly reduced average daily feed intake and average daily gain, elevated body temperature, and caused acid-base imbalance [12]. Adams et al. [30] reported that high humidity (80% vs. 40%) reduced growth rate in 4- to 8-week-old broilers at 29 ℃. Our study suggests that 85% RH initially decreased but subsequently increased cecal microflora diversity, possibly by altering intestinal acid-base balance and disrupting microflora homeostasis, thereby reducing the host' s capacity to cope with environmental stressors.

Effects of Intermittent Mild Heat Environment on Cecal Microflora Structure

The broiler intestine is dominated by Firmicutes [27]. Our results indicate that under intermittent 26 ℃ conditions, 30% RH inhibited the growth of *Faecalibacterium prausnitzii* and *Stomatobaculum longum*, while 85% RH inhibited colonization by *Clostridium thermosuccinogenes*.

Faecalibacterium prausnitzii metabolizes unabsorbed carbohydrate products to produce large amounts of butyrate and is a major butyrate-producing bacterium in the intestine. It can also secrete unidentified substances that, together with butyrate, exert anti-inflammatory effects and correct intestinal dysbiosis [31-32]. *Stomatobaculum longum* primarily produces butyrate, lactate, isovalerate, and acetate as end products in anaerobic culture [33]. *Clostridium thermosuccinogenes* ferments various carbohydrates, mainly producing succinate, acetate, and formate [33]. All three bacteria belong to unculturable Firmicutes. Most bacteria in Firmicutes and Bacteroidetes produce enzymes that degrade plant cell walls, participating in plant cell wall degradation and thus contributing to intestinal digestive function. Butyrate plays an important role in host intestinal health [34-37].

On day 14, the 30% and 85% RH groups showed five additional bands compared to day 7, while the 60% RH group showed only one fewer band, demonstrating that 30% and 85% RH had greater effects on cecal microflora diversity than 60% RH. However, whether the changes in microflora quantity and species induced by high and low humidity stress have positive or negative effects on nutrient

digestion and absorption requires further comprehensive investigation.

Conclusions

1. Under intermittent 26 ℃ conditions, both 30% and 85% RH altered cecal microflora diversity in broilers.
2. Under intermittent 26 ℃ conditions, the specific bacteria in the 30% RH treatment were *Stomatobaculum longum* and *Faecalibacterium prausnitzii*, while 85% RH inhibited colonization by *Stomatobaculum longum* and *Clostridium thermosuccinogenes*.

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