16S rRNA gene sequencing reveals effects of photoperiod on cecal microbiota of broiler roosters

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Photoperiod is an important factor in stimulating broiler performance in commercial poultry practice. However, the mechanism by which photoperiod affects the performance of broiler chickens has not been adequately explored. The current study evaluated the effects of 3 different photoperiod regimes (short day (LD) = 8 h light, control (CTR) = 12.5 h light, and long day (SD) = 16 h light) on the cecal microbiota of broiler roosters by sequencing bacterial 16S rRNA genes. At the phylum level, the dominant bacteria were Firmicutes (CTR: 68%, SD: 69%, LD: 67%) and Bacteroidetes (CTR: 25%, SD: 26%, and LD: 28 %). There was a greater abundance of Proteobacteria (p<0.01) and Cyanobacteria (p<0.05) in chickens in the LD group than in those in the CTR group. A significantly greater abundance of Actinobacteria was observed in CTR chickens than in SD and LD chickens (p<0.01). The abundance of Deferribacteres was significantly higher in LD chickens than in SD chickens (p<0.01). Fusobacteria and Proteobacteria were more abundant in SD chickens than in CTR and LD chickens. The predicted functional properties indicate that cellular processes may be influenced by photoperiod. Conversely, carbohydrate metabolism was enhanced in CTR chickens as compared to that in SD and LD chickens. The current results indicate that different photoperiod regimes may influence the abundance of specific bacterial populations and then contribute to differences in the functional properties of gut microbiota of broiler roosters.
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ABSTRACT
Photoperiod is an important factor in stimulating broiler performance in commercial poultry practice. However, the mechanism by which photoperiod affects the performance of broiler chickens has not been adequately explored. The current study evaluated the effects of 3 different photoperiod regimes (short day (LD) = 8 h light, control (CTR) = 12.5 h light, and long day (SD) = 16 h light) on the cecal microbiota of broiler roosters by sequencing bacterial 16S rRNA genes. At the phylum level, the dominant bacteria were Firmicutes (CTR: 68%, SD: 69%, LD: 67%) and Bacteroidetes (CTR: 25%, SD: 26%, and LD: 28%). There was a greater abundance of Proteobacteria ($p<0.01$) and Cyanobacteria ($p<0.05$) in chickens in the LD group than in those in the CTR group. A significantly greater abundance of Actinobacteria was observed in CTR chickens than in SD and LD chickens ($p<0.01$). The abundance of Deferribacteres was significantly higher in LD chickens than in SD chickens ($p<0.01$). Fusobacteria and Proteobacteria were more abundant in SD chickens than in CTR and LD chickens. The predicted functional properties indicate that cellular processes may be influenced by photoperiod. Conversely, carbohydrate metabolism was enhanced in CTR chickens as compared to that in SD and LD chickens. The current results indicate that different photoperiod regimes may influence the abundance of specific bacterial populations and then contribute to differences in the functional properties of gut microbiota of broiler roosters.

INTRODUCTION
Photoperiod is defined as the relative amount of light per day to which an organism is exposed (Lee et al. 2017). This period of exposure to light can influence different aspects of physiology in avian as well as mammalian species, such as reproduction, behavior, and immune functions, to different magnitudes (Pittendrigh & Daan 1976; Walton et al. 2011). Following photoperiod, animals tend to undergo a suite of adaptive responses by altering their physiology and reproductive state for survival (Walton et al. 2011). An increased photoperiod length has been reported to result in lower incidence of skeletal diseases and increase in weight gain with slower growth in broiler chickens (Classen et al. 1991). Decreasing or increasing photoperiod can also
be used to reduce the early growth rate of broilers but allow them to compensate as they approach market age (Downs et al. 2006). Photoperiod was also found to affect the physiology of chickens; birds exposed to short days had a higher expression of gonadotropin-inhibitory hormone expression compared to birds exposed to long days (Dixit et al. 2017). Furthermore, it was reported that long photoperiods promote the development of the gonads in poultry (Kang & Kuenzel 2015), although the exact mechanism underlying this effect is still unclear. Given the many roles that photoperiod plays in various aspects of the physiology of avian and mammalian species, it is of scientific interest to evaluate its role in other inadequately explored aspects, such as the bacterial structure and functional properties of the gut microbiota.

Gut bacteria, which form part of the gut microbiota, have been shown to play important roles in digestion, metabolism, and health in avian species (Waite & Taylor 2014; Waite & Taylor 2015). Gut microbiota have been widely reported to be affected by factors such as diet and age (Waite & Taylor 2014; Zhao et al. 2017; Zhu et al. 2017). However, other factors that may affect the structure and functional properties of chicken gut microbiota, such as photoperiod, have yet to be evaluated. Photoperiod may play a significant role in determining most physiological functions by altering the gut microbiota. At present, there is a gap in the knowledge on the role of photoperiod in gut microbiota structure and function. The present study evaluated the effect of photoperiod on the abundance, diversity, and predicted functional properties of cecal microbiota in broiler roosters by sequencing the 16S rRNA gene.

**MATERIALS AND METHODS**

Ethical approval for the present study was obtained from the Ethical Committee of the Jilin Agricultural University, China.

**Photoperiod treatments**

One hundred and twenty AA+ Broilers (20 weeks of age, average weight: 2806 g) were randomly divided into three groups (n=40) and subjected to different photoperiodic regimes for 5 weeks. Group I was designated the Control group (CTR; 12.5 h Light:11.5 h Dark, i.e., lights on at 08:00 a.m. and lights off at 08:30 p.m.), Group II the Long-day photoperiod group (SD; 16 h Light:8 h Dark, i.e., lights on at 04:00 p.m. and lights off at 08:00 a.m.), and Group III the Short-
day photoperiod group (LD; 8 h Light:16 h Dark, i.e., lights on at 08:00 a.m. and lights off at
04:00 p.m.). A 60 W incandescent lamp with an illuminating intensity of 30 lux was used as the
source for artificial illumination and was positioned at the height of the head of standing birds.
All the broiler roosters were maintained in cages of equal size. Each rooster was fed 115 g of
commercial broiler diet per day for 20 weeks via restricted feeding before the experiment, and
then the amount of feed was increased by 5 g every week. In order to ensure that each rooster
was fed the same amount of diet, each rooster was kept in an individual cage. Water was
provided ad libitum during the whole experimental period.

2.2 Sample collection

All the roosters were slaughtered at about 25 weeks of age. Luminal cecum contents were
collected from 7 randomly selected broilers from each group. All samples were harvested within
30 min after slaughter and immediately frozen in liquid nitrogen. The frozen luminal samples
were stored in a freezer at −80 °C until further use.

DNA Extraction and 16S rRNA Amplification

Samples were allowed to thaw at room temperature before DNA extraction. Total genomic DNA
was extracted using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA),
following the manufacturer’s instructions. DNA concentration was evaluated by measuring
optical density using Nano-Drop 2000 (Thermo Electron Corporation, USA) at wavelengths of
260 and 280 nm. The integrity of the DNA extracts was assessed by electrophoresis on 1.0%
agarose gels. The V4–V5 regions of the bacterial 16S rRNA gene were amplified from the total
microbial genomic DNA via PCR using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 907R (5′-
CCGTCATATTGTTAGTTT-3′). The amplification was carried out in 25μL reactions
containing 5 μL of Q5 reaction buffer (5×), 5 μL of Q5 High-Fidelity GC buffer (5×), 0.25 μL of
Q5 High-Fidelity DNA Polymerase (5 U/μL), 2 μL of dNTPs (2.5 mM), 1 μL each of the
forward and reverse primer (10 uM), 2 μL of DNA template, and 8.75 μL of ddH2O. PCR
conditions were as follows: initial denaturation at 98 °C for 2 min; followed by 25 cycles of
denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and
then a final extension at 72 °C for 5 min. The PCR products were separated on 2% agarose gels
...and subsequently extracted from the gels. Samples with a bright band with a size between 200–
450 bp were chosen for downstream experiments. PCR products were purified using a GeneJET
Gel Extraction Kit (Thermo Scientific, Waltham, USA). Products were quantified using a
PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After quantification, the
amplicons were pooled in equal amounts, and pair-end 2×300-bp sequencing was performed
using the Illlumina MiSeq platform and a MiSeq Reagent Kit v3 at Shanghai Biotechnology Co.,
Ltd (Shanghai, China).

Bioinformatics and Statistical Analysis

The quality control and analysis of the sequences were performed using the software
Quantitative Insights into Microbial Ecology (QIIME, v1.8.0) (Caporaso et al. 2010). The
paired-end reads from the DNA fragments were merged using FLASH (Magoc & Salzberg 2011).
The UCLUST (Edgar 2010) clustering method was used to cluster operational taxonomic units
(OTUs) with ≥97% sequence identity. OTU classification was conducted by running a BLAST
search against the Greengenes Database (DeSantis et al. 2006) using the representative sequence
set as a query (Altschul et al. 1997). To minimize the difference in sequencing depth across
samples, an averaged, rounded, and rarefied OTU table was generated by averaging 100 evenly
re-sampled OTU subsets under the 90% of the minimum sequencing depth. These were then
used for further analysis.

Bioinformatics and statistical analyses were performed using the QIIME and R packages (v3.2.0).
The alpha-diversity indices (Chao1, ACE metric, Shannon diversity index, and Simpson index)
were calculated using the QIIME software to establish the abundance and diversity of the
sequences. Beta-diversity was determined using unweighted UniFrac distance metrics to evaluate
the structure and distribution of the microbial genetic communities among the samples
(Lozupone & Knight 2005; Lozupone et al. 2007). Differences in the Unifrac distances for
pairwise comparisons among groups were calculated using Student’s t-test and the Monte Carlo
permutation test with 1000 permutations. Significance was assigned when p<0.05 and p<0.01.
The differences and similarities between the compared groups were evaluated using ANOSIM
(analysis of similarities) in the R package “vegan.” A Venn diagram was generated using the R
package “VennDiagram” to visualize the shared and unique OTUs among samples or groups.
Functional genes were predicted using PICRUSt (phylogenetic investigation of communities by
reconstruction of unobserved states) using high-quality sequences as the input (Langille et al. 2013).

**RESULTS**

**Sequencing overview**

A total of 21 samples were obtained from three groups (n=7 per group) of broiler roosters and subsequently sequenced to generate V4–V5 16S rRNA gene profiles. A total of 398445, 328235, and 375402 sequences were obtained for the CTR, SD, and LD groups, respectively. There was an average of 56920, 46890, and 53628 reads per sample in the CTR, SD, and LD groups, respectively.

**Validation and structure determination of the sequences**

The variation in data distribution between the groups was analyzed using ANOSIM, which indicated a significant difference ($p<0.01$) between the three groups under unweighted Unifrac. The alpha-diversity indices (chao1, Simpson and Shannon index) are reported in Table 1, there was a significant difference between CTR and SD groups when comparing a chao1 indices mean. The results of the beta diversity analysis and the PLS-discriminant analysis are shown in Fig. 1 and 2, respectively. Samples from LD and SD indicated to be clustered similar where the CTR samples were different in NMDS analysis. The PLS-discriminant analysis indicated that the two groups are different with the exception of one sample from LD which was clustered with SD.

**Abundance and Significant difference between the three groups at the Phylum level**

The most abundant bacteria at the phylum level were *Firmicutes*, with abundances of 68%, 69%, and 67% in the CTR, SD, and LD groups, respectively, followed by *Bacteroidetes* with abundances at 25%, 26%, and 28% in the CTR, SD, and LD groups, respectively (Fig. 3). The other bacterial phyla had abundances lower than 3% in all groups at varying magnitudes. As shown in Fig. 2, *Proteobacteria* ($p<0.01$) and *Cyanobacteria* ($p<0.05$) were more abundant in LD chickens than in CTR chickens, while *Actinobacteria* was more abundant in chicken from the CTR group than in those from the LD group ($p<0.01$). Between the CTR and SD groups, there was a significant difference in the abundance of *Actinobacteria*, which was more abundant in the CTR group than in the SD group ($p<0.01$). *Deferribacteres* was more abundant in LD roosters than in SD roosters ($p<0.05$). *Fusobacteria* and *Proteobacteria* were significantly more
abundant \((p<0.01)\) in chickens from the SD group than in those from the CTR and LD groups, as indicated in Fig. 4.

**Abundance and Significance difference between the three groups at the Genus level**

The most abundant bacteria at the genus level were *Bacteroides*, with 15%, 13%, and 15% abundances in the CTR, SD, and LD groups, respectively. This was followed by unclassified *Ruminococcaceae* at 13%, 14%, and 14% abundances in the CTR, SD, and LD groups, respectively (Fig. 5). Other abundant genera included *Ruminococcus* (CTR: 14%, SD: 9%, LD: 10%), unclassified *Clostridiales* (CTR: 9%, SD: 11%, LD: 12%), and *Faecalibacterium* (CTR: 8%, SD: 10%, LD: 8%). Ten genera were significantly \((p<0.01)\) different in abundance between the CTR and SD groups, 7 between the CTR and LD groups, and 5 between the SD and LD groups. Also importantly the genus *Aeriscardovia* was significantly more abundant \((p<0.01)\) in the LD than in the SD and CTR groups (Fig. 6). Interestingly, *Megamonas*, *Ochrobactrum*, and *Selenomonas* were significantly more abundant \((p<0.01)\) in the CTR group than in the other two groups. *Aeriscardovia*, *Delftia*, and *Rikenella* were significantly more abundant \((p<0.01)\) in the LD group than in the CTR and SD groups. *Lactococcus* and *Fusobacterium* were significantly more abundant \((p<0.01)\) in the SD group than in the other two groups (Fig. 6). A heat map indicating significantly expressed genera is shown in Fig. 7.

**Differences in Predicted Functional properties between the three groups**

The differences in the effect of photoperiod on the functional properties across the three groups were further evaluated. Moderate differences were observed in cellular processes, particularly, in cell motility (Fig. 8). Cell motility was relatively low in samples from the CTR group compared to the motility in samples from the SD and LD groups. However, there were no notable differences in other functions such as transport and catabolism, cell growth and death, and cell communication across the groups. Analysis of the metabolism of the samples showed that carbohydrate metabolism was enhanced in CTR samples as compared to those in the SD and LD samples. Other functions did not exhibit any differences across the three tested groups (Fig. 9). Similar results were observed in other functional processes, such as genetic information processes and environmental information processes (results not shown).

**DISCUSSION**
Photoperiodism provides animals with the ability to change many physiological aspects and, consequently, adapting their body to the environment depending on the duration of light exposure (Bailey et al. 2010). The current study evaluated the structure and functional properties of the cecal microbiota of roosters that were subjected to three different photoperiodic regimes. Our data indicates that the length of time of light exposure may affect the abundance of specific bacteria in the cecum, leading to possible changes in functional properties. These changes may range across a variety of aspects the underlying mechanism of which has not been sufficiently explored.

To the best of our knowledge, there are few reports on the effects of photoperiod on gut microbiota in chickens and even in other species. Recently, a study demonstrated the role of photoperiod in changing gut microbiota. It indicated that different photoperiodic regimes (8 h dark/16 h light, 12 h dark/12 h light, and 16 h dark/8 h light cycles) could shape the gut microbiota of mice and thereby affect host radio sensitivity (Cui et al. 2016). The results of the present study are in general agreement with the findings of previous studies. Previously, it was reported that *Firmicutes* and *Bacteroidetes* dominate the broiler gut microbiota (Cui et al. 2017; Oh et al. 2017a; Oh et al. 2017b; Zhou et al. 2017), although effects of photoperiod on their abundances were not demonstrated. It was noted that *Megamonas* was significantly more abundant in CTR group as compared to LD and SD groups. It is of interest to note that *Megamonas* has been previously reported to play a significant role in fermenting glucose into acetate and propionate, which is pivotal for health benefits in a few species such as humans and ducks (Chevrot et al. 2008; Sakon et al. 2008; Zhang et al. 2013). These findings may indicate a new platform for manipulating acetate and propionate in broiler roosters by varying photoperiod regimes. Short term (LD) exposure to light has also been indicated to significantly increase the abundance of the novel genus *Aeriscardovia* (Simpson et al. 2004) and of the gram-negative bacteria *Delftia*, which has been reported to be associated with infectious diseases (Bilgin et al. 2015; Calzada et al. 2015). Long term (SD) exposure to light significantly increases the abundance of gram-positive *Lactococcus*, which has been reported to have potential for use in preventing infectious diseases (Hanniffy et al. 2007). The gram-negative genus *Fusobacterium*, which has been reported to be associated with infections in humans (Kostic et al. 2013), was also significantly more abundant under an SD regime. Our results seem to indicate that short term (LD) photoperiod (8 h light) could increase the abundance of bacterial genera associated with
infectious diseases in the rooster gut, while long term photoperiod (16 h light) could increase the
abundance of bacterial genera associated with preventing infectious diseases. However, this
deduction needs to be verified by more extensive scientific investigation. These results are of
importance to prompt more studies on the role that photoperiod may play with regards to
physiology in animals. It is of note that different results may arise due to differences in time of
exposure, light intensity, animal and growth stages, and other factors employed in the study.

The effect on several functional properties of the roosters may be attributed to increases in the
abundance of specific bacteria caused by light exposure duration. Analysis of predicted
functional properties in the present study indicated that metabolism may be influenced by
photoperiod. Carbohydrate metabolism was enhanced in the CTR group, as compared to the SD
and LD groups. Previous studies have demonstrated that gut microbiota plays an important role
in the life activities of chickens (Waite & Taylor 2014). The present study is limited by the fact
that the change in gut microbiota was not correlated with performance (e.g., testis development
or body weight). Further studies are suggested to investigate the effects of photoperiod on gut
microbiota and their relationship with growth or reproduction performance.

CONCLUSIONS

Our results indicate that photoperiod may affect the abundance of specific bacteria in the gut and
thereby contribute to differences in the functional properties of the gut microbiota in broiler
roosters.

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The average alpha-diversity indexes (choa1, Simpson and Shannon index) of the data distribution

Numbers with asterisks are significantly different (p value < 0.05).
| Group | Chao1 | Simpson | Shannon |
|-------|-------|---------|---------|
|       | Mean  | STD     | Mean    | STD     | Mean   | STD    |
| CTR   | 1799.593* | 256.3406 | 0.972857 | 0.00488 | 7.411429 | 0.281569 |
| SD    | 1461.779* | 310.5823 | 0.977143 | 0.00488 | 7.525714 | 0.227146 |
| LD    | 1729.097  | 224.2392 | 0.975714 | 0.007868 | 7.607143 | 0.223958 |
Figure 1

The beta diversity results of NMDS indicating the data distribution between the groups. LD refers to the 8 h light group samples, CTR refers to the 12.5 h light and SD refers to the 16 h light group samples respectively.
Figure 2

The PLS-discriminant analysis

LD refers to the 8 h light group samples, CTR refers to the 12.5 h light and SD refers to the 16 h light group samples respectively.
Figure 3

Taxonomic profiles of the microbial communities at the phylum level

LD refers to the 8 h light group, CTR refers to the 12.5 h light and SD refers to the 16 h light group. Samples are presented along with the horizontal axis and relative abundance at the vertical axis.
Figure 4

Taxonomic profiles of the notable significant different bacterium at the phylum level

Samples/groups are as previously explained. Different uppercase and lowercase letters indicate significance of difference at $p<0.01$ and $p<0.05$, respectively. Same letters indicate no significant difference.
Figure 5

Taxonomic profiles of the microbial communities at the genus level

Samples/groups are as previously explained.
Figure 6

Taxonomic profiles of the notable significant different bacterium at the genus level

Samples/groups are as previously explained. Different uppercase and lowercase letters indicate significance of difference at $p < 0.01$ and $p < 0.05$, respectively. Same letters indicate no significant difference.
Figure 7

Heatmap showing the genera with significant differences of relative abundances amongst the 3 groups

Heatmap is color-coded based on the scale of -4 to 4.
Figure 8

Representing the functional differences at the cellular processes

Samples/groups are as previously explained.
Figure 9

Representing the functional differences at the metabolism level between the 3 groups.

Samples/groups are as previously explained.