RESEARCH ARTICLE Indian Journal of Animal Research, Volume 58 Issue 5: 753-758 (May 2024)

Analysis of the Difference of Intestinal Microbes in Muchuan Black-bone Chickens with Two Skin Colors

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ABSTRACT

Background: Skin color is an important economic trait in black-bone chicken production and it has been identified that the gut microbiota is an important factor in animal health and physiology. However, it is unknown about whether the gut microbiota in black-bone chickens is connected to the coloration of chicken skin.

Methods: To investigate the relationship between the gut microbiota and skin coloration, comparing the differentiation of the gut microbiota structure and abundances in black- (B group) and white-skinned (W group) Muchuan black-bone chicken. Here, we characterized the microbiota in the jejunal contents of both the B and W groups using the Illumina MiSeq platform, targeting the genomic V4 region of the 16S rRNA gene.

Result: In terms of community richness, the ACE community richness index of the B group was significantly higher than that of the W group (p<0.05) and the proportion of unique OTUs was higher in group B than in group W. According to the species annotation and abundance information of all samples at the genus level, *Parabacteroides*, *Faecalicoccus* and *Alistipes* were enriched in group B, whereas *Actinomyces*, *Elstera* and *Nosocomiicoccus* were enriched in group W. In the evolutionary branch diagram, the relative abundances of Enterobacteriaceae (Gammaproteobacteria, Enterobacteriales) were significantly higher in group B than in group W (LDA Score>4). Our data provide a basis for the hypothesis that the discriminating bacterial taxa in gut is associated with the feature variants for skin coloration of *Muchuan* black-bone chickens, which were fed in the same feed and stocking niches.

Key words: 16S rRNA, Gut microbiota, Muchuan Black-bone chicken, Skin coloration.

INTRODUCTION

The black-bone chicken is a very nutrient-rich food with high medicinal value (Sehrawat *et al*., 2021). The Muchuan black-bone chicken is an important economic breed originating from Muchuan County, Sichuan Province, China. It is characterized by an all-black body, including its feathers, skin, muscle, cockscomb and claws -(Yu *et al*., 2018). Skin color is a very important economic trait for black-bone chickens; consumers prefer to purchase black-bone chickens with darker skin (Wang *et al*., 2018). However, individuals with lighter or even white skin are produced during the breeding process of the Muchuan black-bone chicken, causing serious economic losses.

The phenotypic pigmentation variations in black-bone chickens result from complex interactions between genetic and physiological factors (Bourgeois *et al*., 2016) and these variations are primarily influenced by factors such as genetics, nutrition and environmental conditions (Yu *et al*., 2017). The gut microbiota can affect the host's energy metabolism and lipid metabolism and plays important roles in animal health (Zeng *et al*., 2015). Previous studies have shown that the gut microbiota is closely related to obesity, metabolic diseases and gut immune maturation (Jiao *et al*., 2019). However, studies on whether the gut microbiota can affect the skin color of black-bone chicken have not been reported.

16S rRNA is highly conserved and specific and the gene sequence is long enough (containing about 50 functional domains). With the development of PCR and nucleic acid technology, 16S rRNA gene detection has become a powerful tool for bacterial detection and identification. With the continuous improvement of the database, the technology can be used to classify and detect the pathogenic bacteria rapidly, microscopically, accurately and simply. Here, we analyzed the differentiation in gut microbiota between white-skinned and black-skinned chickens based on 16S rRNA gene sequencing to explore the relationship between gut microbial diversity and skin color in black-bone chickens.

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How to cite this article: Liao, J., Shen, X., Long, W., Yu, S., Wang, G., Du, C. and Liang, Z. (2024). Analysis of the Difference of Intestinal Microbes in Muchuan Black-bone Chickens with Two Skin Colors. Indian Journal of Animal Research. 58(5): 753-758. doi: 10.18805/IJAR.BF-1752.

Submitted: 03-01-2024 **Accepted:** 02-03-2024 **Online:** 14-03-2024

MATERIALS AND METHODS

Sample collection and preparation

A total of 92 Muchuan black-bone chickens were tested and the dorsal skin of each chicken was measured using a portable Colorimeter (NR10QC; 3nh, Shenzhen, China). Ten female Muchuan black-bone chickens, including five of the blackest (group B; lightness (L*) ≤45) and five of the whitest (group W; L* ≥60) black-bone chickens, were selected (Fig 1). All chickens were fed the same feeding and management routines at the Experimental Chicken Farm of Leshan Normal University, Leshan, China. All chickens were executed by exsanguinations to obtain jejunal content samples. The samples were immediately stored in liquid nitrogen and transported to the laboratory for storage at 80°C.

DNA extraction and amplicon generation

DNA extraction and sequencing were performed at Novogene Bioinformatics Technology Co., Beijing, China. Total bacterial DNA was extracted from the samples using the sodium-dodecyl sulfate method. DNA concentration and purity were determined using 1% agarose gel electrophoresis and DNA samples were diluted to 1 ng/μL with sterile water. The DNA was amplified using the barcoded primer set 515f/806r, which targets the V4 region of the bacterial 16S rRNA gene.

All PCR reactions were performed using Phusion High-Fidelity PCR Master Mix (New England Biolabs, Beijing, China). The PCR products were mixed with an equal volume of 1× loading buffer (containing SYB green), detected via electrophoresis in 1.2% (w/v) agarose gels and purified using a Gene JETTM Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA). Sequencing libraries were generated using an Ion Plus Fragment Library Kit (48 reactions; Thermo Scientific) and the quality of the library was assessed using a Qubit 2.0 Fluorometer (Thermo Scientific). Finally, the library was sequenced using an Ion S5 XL platform (Thermo Scientific).



Fig 1: Phenotypic characterization. Skin color of two types of Muchuan black-boned chickens: Black skin (B) and White skin (W).

Sequence data processing

To obtain high-quality clean reads, quality filtering of raw reads was performed using Cut adapt (v1.9.1; http://cutadapt.readthedocs.io/en/stable/) (Martin, 2011) and chimera filtering was performed using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar, 2011).

Operational taxonomic unit (OTU) clustering and species annotation

To examine species composition and diversity in the samples, sequence analyses were performed using Uparse software (v7.0.1001; http://drive5.com/uparse/) (Edgar, 2013). Sequences sharing ≥97% identity were assigned to the same OTU. Representative sequences for each OTU were screened and used to assign taxonomy according to the Silva database (v132) (Quast *et al*., 2013). The taxon abundances in each sample were determined at several taxonomic ranks (phylum, class, order, family and genus).

Analysis of alpha and beta diversity

To estimate alpha diversity we calculated observed species, Chao1, Shannon, Simpson, ACE and Good's coverage indices. Beta diversity was calculated using both weighted and unweighted Unifrac distances. All analyses were performed using QIIME (v1.7.0) and displayed using the R software (v2.15.3).

RESULTS AND DISCUSSION

Metadata and sequencing

A total of 10 jejunal content samples from five black-skinned (B) and five white-skinned (W) Muchuan black-bone chickens were collected and sequenced using the Ion S5 XL platform. After quality and chimera filtering, a total of 667, 456 clean reads (sample minimum: 47,678; maximum: 86,260) with an average length of 253 bp were obtained. A total of 3,154 OTUs were assigned to 560 taxa at the genus level. The dilution curves tended to be flat, indicating that the 16S rRNA gene sequencing depth was sufficient to reflect the microbial diversity of the samples.

OTU analysis

The relative abundances of bacteria in groups B and W at the phylum and genus levels are presented in Fig 2. Firmicutes was the most abundant bacterial phylum, with an average relative abundance of >50% (54.31% in group B, 64.04% in group W). Bacteroidetes and Proteobacteria had higher relative abundances compared to other phyla. At the genus level, *Lactobacillus* was the most abundant (13.1% in group B, 16.57% in group W).

We analyzed the shared and unique OTUs between groups B and W at the phylum and genus levels. At the phylum level, a total of 34 OTUs were shared between the

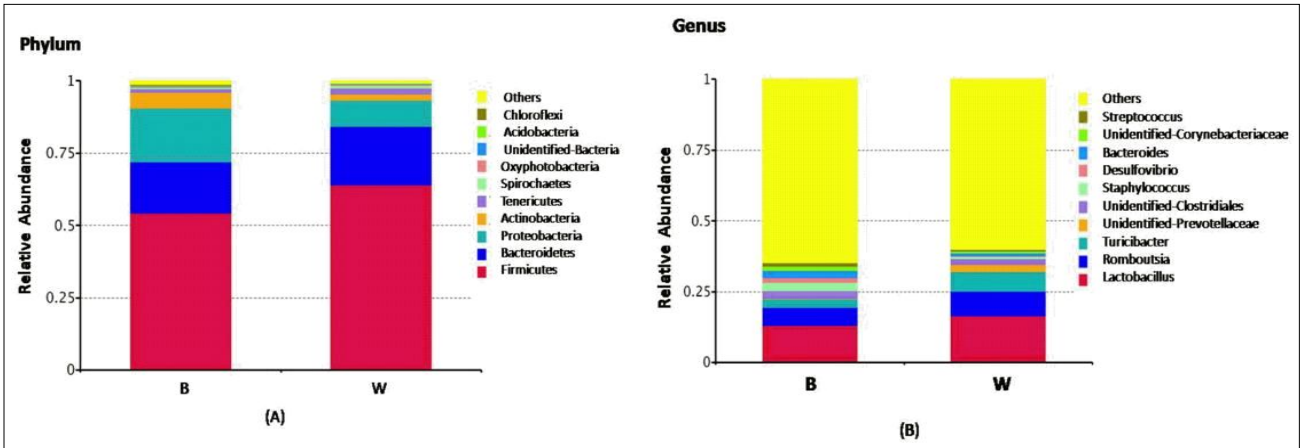


Fig 2: Relative abundance of the dominant bacterial in group B and group W at phylum and genus level.

two groups, representing 85% of the OTUs in both groups. At the genus level, groups B and W had 147 and 36 unique OTUs, respectively, representing 23.56% and 8.72% of the total OTUs in groups B and W, respectively; the proportion of unique OTUs was higher in group B than in group W.

Differences in bacterial communities between groups B and W

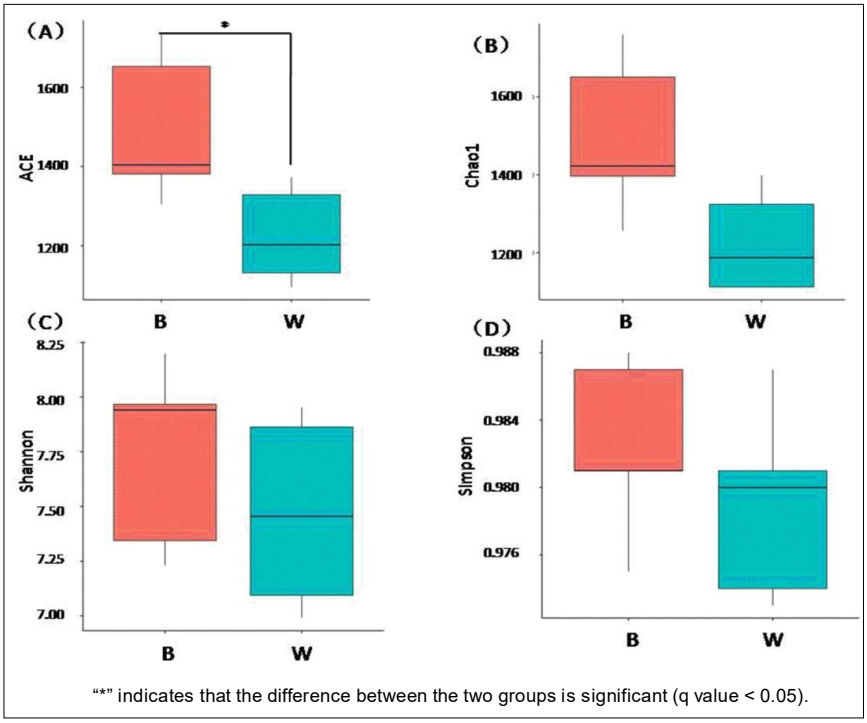
Alpha diversity was used to analyze the complexity of sample microbial diversity. Six alpha diversity indices, including the Shannon, Simpson, Chao1, ACE, observed species and Good's coverage indices, were calculated using the QIIME toolkit and displayed using R software. We found no significant differences in the observed species or Good's coverage index between groups B and W. In terms of community richness, the ACE index of group B was significantly higher than that of group W (P<0.05, Fig 3). No significant differences in the Shannon or Simpson diversity index were observed.

According to the species annotation and abundance information of all samples (the 35 most abundant taxa are depicted), at the phylum level, Calditrichaeota, Armatimonadetes and Chlamydiae were enriched in group B, whereas Thermotogae, Cloacimonetes and Firmicutes were enriched in group W. At the genus level, *Parabacteroides*, *Faecalicoccus* and *Alistipes* were enriched in group B, whereas *Actinomyces*, *Elstera* and *Nosocomiicoccus* were enriched in group W.

To identify the bacterial taxa that differed significantly between groups B and W, linear discriminant analysis (LDA) effect size analyses was performed. From the LDA value distribution histogram, species with an LDA score greater than the set value (the default setting is 4) can be identified. Bacterial taxa that differed significantly between the two groups are shown in Fig 4. In the evolutionary branch diagram, the relative abundances of Enterobacteriaceae (Gammaproteobacteria, Enterobacteriales) were significantly higher in group B than in group W (Fig 4).

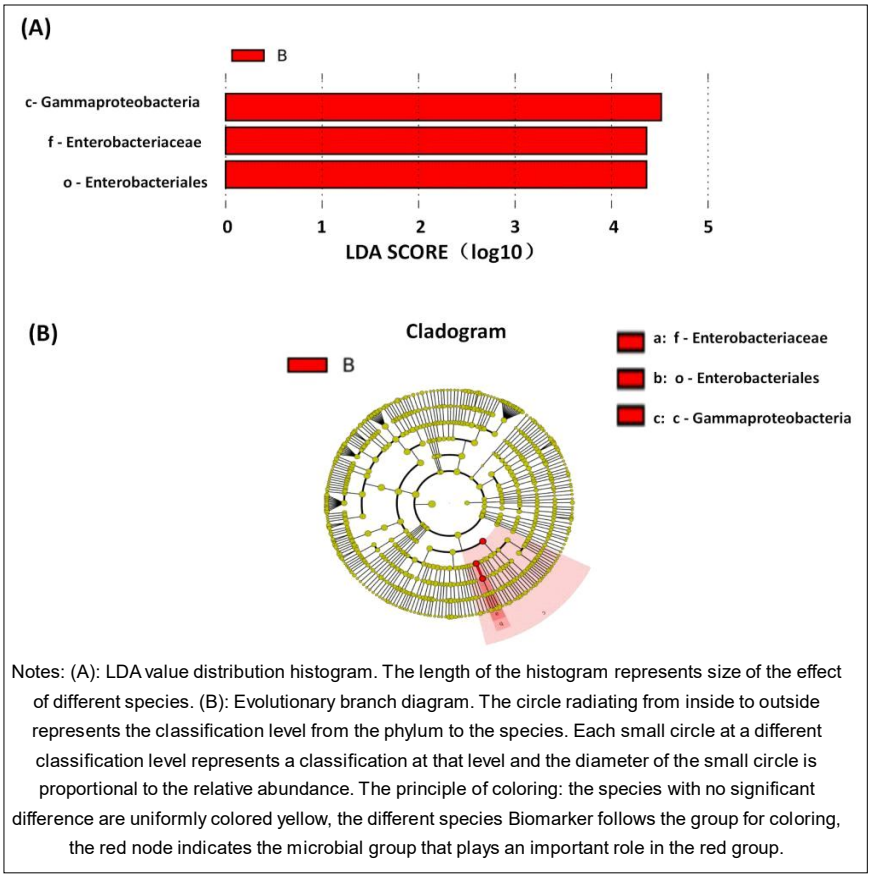
The gut microbiota plays a fundamental role in host nutrition, development, immunity and metabolism (Pandit *et al*., 2018). The gastrointestinal compartments of chickens are densely populated with a variety of microorganisms and the microorganisms at each location have specific characteristics. In this study, we characterized the correlation between the gut microbiota in Muchuan black-bone chickens and their skin color. In terms of community richness, the ACE index of group B was significantly higher than that of group W (P <0.05) and the proportion of unique OTUs was higher in group B than in group W. The composition and diversity of the gut microbiota are closely associated with the host species and several host attributes such as diet, habitat and hormones (Hanning *et al*., 2015). Changes in the gut microbiota may affect the production and regulation of host hormones .

The gut microbiota consumes, stores and redistributes energy, as well as mediates important physiological and chemical processes (Adak *et al*., 2019). It can modify caloric intake by using carbohydrates (such as cellulose) that are indigestible to the host. The gut microbiota is essential to the development and maturation of the intestinal immune system during the early stages of life and to maintain the intestinal epithelial barrier (Rodrguez *et al*., 2015). Recent studies have reported that the gut microbiota can communicate with the brain *via* the brain-gut axis to influence brain development and behavior (Silva *et al*., 2020). Even more interestingly, it is well documented that variation of the gut microbiota influences host gene expression. For example, altering the gut microbiota in mice can regulate intestinal epithelial gene expression by suppressing the transcription factor Hepatocyte nuclear factor 4 alpha (Davison *et al*., 2017). Studies have shown that the expression of genes involved in epithelial-to-mesenchymal transition and the (re) organization of the extracellular matrix is closely related to specific bacterial genera (*e.g*., *Bifidobacterium* spp.) in the gut (van der Lug *et al*., 2018).



"*" indicates that the difference between the two groups is significant (q value < 0.05).

Fig 3: Differences in bacterial community diversity and richness between B group and W group.



Notes: (A): LDA value distribution histogram. The length of the histogram represents size of the effect of different species. (B): Evolutionary branch diagram. The circle radiating from inside to outside represents the classification level from the phylum to the species. Each small circle at a different classification level represents a classification at that level and the diameter of the small circle is proportional to the relative abundance. The principle of coloring: the species with no significant difference are uniformly colored yellow, the different species Biomarker follows the group for coloring, the red node indicates the microbial group that plays an important role in the red group.

Fig 4: Bacterial taxa significantly differentiated between the B group and W group identified by LEfSe with LDA values of 4.0.

Therefore, symbiotic interactions between the host and gut microbiota are fundamental to host health (Shang *et al.*, 2018).

In this study, the bacterial taxa of Enterobacteriaceae (Gammaproteobacteria, Enterobacteriales) were overrepresented in group B. Gammaproteobacteria are a common member of the gut community and a grain-based diet may result in a higher relative abundance of Gammaproteobacteria (Bergmann *et al.*, 2017). Enterobacteriales is a diverse order of Gammaproteobacteria. A higher abundance of Enterobacteriales may increase intestinal permeability (Pedersen *et al.*, 2018) and the increased diversity of the Enterobacteriales may be beneficial to piglets during the first week after weaning to combat *Escherichia coli*-induced diarrhea (Starke *et al.*, 2014). Enterobacteriaceae are Gram-negative, non-spore-forming, rod-shaped, facultative anaerobes that can obtain energy by oxidizing a variety of simple organic compounds, fermented sugars, organic acids, or polyols (Ye *et al.*, 2018). The family Enterobacteriaceae includes non-pathogenic, autochthonous (commensal) microbes as well as pathogens (Mellen *et al.*, 2014). In human studies, Enterobacteriaceae are common pathogens that can cause a variety of infections, such as hospital-acquired pneumonia, community-acquired pneumonia, complicated urinary tract infections and complicated intra-abdominal infections (Sheu *et al.*, 2019). However, Parmentier *et al.* (2016) reported that a large uptake of Enterobacteriaceae by bumblebees when they had just moved to a new environment did not necessarily indicate a state of dysbiosis, but that this uptake possibly contributed to host nutritional function. Consistent with these studies, our study found that members of the Enterobacteriaceae were significantly enriched (P<0.05) in black-skinned Muchuan black-bone chickens, suggesting that members of Enterobacteriaceae might contribute to the digestion and absorption of nutrients and thereby affect the formation of melanin. However, the pigmentary phenotype is mainly affected by genetic factors and previous research has shown that gene and protein expression in the chicken caecum is dependent on and influenced by the composition of the microbiota (Volf *et al.*, 2017; Volf *et al.*, 2016). Studies have shown that the microbiota can modulate host microRNA expression, which could in turn regulate host gene expression (Richards *et al.*, 2017). The roles of the Enterobacteriaceae in the expression of melanogenesis-related genes require further study.

CONCLUSION

In summary, we characterized the gut microbiota of black-skinned and white-skinned Muchuan black-bone chickens and the bacterial taxa of Enterobacteriaceae (gamma-proteobacteria, Enterobacteria), were overrepresented in black-skinned chickens. These bacterial taxa can serve as immediate targets for future studies to examine their roles in the formation of melanin in the Muchuan black-bone chicken.

ACKNOWLEDGEMENT

This study was supported by discipline construction and cultivation project of Leshan Normal College (No. 2022SSDJS013) and Scientific Research Funding Project of Leshan Normal University (No. DGZZ202003), Leshan, China.

Ethics statement

The experiment was conducted at Leshan Normal University from March to September 2022. The animal experiment was approved by the Leshan Normal University Animal Care and Use Committee (approval number:23-2018). The methods were carried out in strict accordance with the approved guidelines and regulations of the Animal Ethics Committee.

Conflict of interest

The author declares that they have no conflicts of interest in the research presented in this manuscript.

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