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Original Article

Gut Microbiome Revealed the Dominant Microbes Affecting the Feed Efficiency of Wannan Yellow Chicken

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■Keywords

Feed conversion ratio, gut microbiota, ileum, Lactobacillus, microbial community.

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ABSTRACT

Intestinal digestibility can be affected by the interaction between host and gut microbiota, which could influence feed efficiency in poultry. In this study, the performance of 1480 10-week-old Wannan Yellow chickens was recorded for 6 weeks, and the feed conversion ratio (FCR) was calculated.

Thirteen 16-week-old chickens (7 in the high and 6 in the low FCR group) were selected for carcass performance, ileum structure, and microbiota composition analysis. The results showed that ileum weight and length were significantly higher (p<0.05) in the high feed conversion ratio (HFCR) group. However, compared with the low feed conversion ratio (LFCR) group, villus length was significantly lower (p<0.05) in the HFCR group.

The microbial community between the HFCR and LFCR groups showed no differences, but the dominant microbiota varied between them. The proportions of Acidobacteria were significantly higher in the LFCR group compared with the HFCR group. At the species level, the content of most microbiota in the LFCR group was higher than in the HFCR group and most of these microbiotas are Lactobacillus. According to the results of RDA analysis, Lactobacillus was positively correlated with FCR, ileum length, ileum weight, and ileal villi length.

These results suggested that a prominent effect of ileum microbiota on chicken feed efficiency, and that *Lactobacillus* could be used as a biomarker for improving the growth performance of Wannan yellow chickens.

INTRODUCTION

Feed cost in broilers is as high as 70% of production costs (Aggrey *et al*., 2010). Moreover, with the extension of feeding time, feed costs have also increased (Zhang *et al*., 2003). Therefore, it has been a worldwide concern to improve feed efficiency. In poultry research, feed conversion ratio (FCR) is the most commonly used indicator to evaluate feed efficiency, which is expressed as the ratio of feed intake to body weight gain or egg weight in a certain period (Reyer *et al*., 2015). Thus, chickens with low FCR are considered to have high feed efficiency. FCR is affected by many factors, such as genetics, feed composition, feeding cycle and rearing environment. Under modern intensive breeding conditions, the growth rate of broilers in the same house is basically not affected by the environment and nutrition. However, within the same group, broilers might exhibit quite different FCR between individuals (Van Eerden *et al*., 2004). Recent evidence proved that gut microbiota plays a significant role in the growth and health performance of broilers (Wu *et al*., 2019). Probiotic bacteria (such as Lactobacillus) were beneficial, decreasing the susceptibility to Salmonella and enhancing immune systems so as to

decrease the pathogen carriage in mucous layers (Seo *et al*., 2000, Maki *et al*., 2019). Previous studies have confirmed that gut microbes regulate gut peptides and hormones via the brain-gut axis, thereby influencing feed intake and growth performance in chickens (Fetissov *et al*., 2017, Fulling *et al*., 2019). At present, the relationship between gut microbes and FCR has been widely studied in pigs (Tan *et al*., 2018), cattle (Zhang *et al*., 2017), and Cobb 500FF broiler chickens (Siegerstetter *et al*., 2017). However, different growth rates in broiler species resulted in different microbiotas (Videnska *et al*., 2014), and given the low growth rate of indigenous species, Wannan yellow chickens might exhibit different microbiota as compared to commercial broiler chickens.

In animals, each segment of the small intestine has different a function of digestion and absorption. Along the proximal to distal end of the small intestine, the number of villi and villus height decreases, and the thickness of the intestinal wall becomes thinner (Ito, 1981). Therefore, the duodenum is the main place for digestion, while the absorption of digestive products mostly occurs in the jejunum and ileum in poultry (Rodriguez-Sanchez *et al*., 2019).

The aim of this study was to investigate the relationship between FCR and slaughtering performance, ileal villus structure, and the composition of gut microbiota in the ileum of Chinese indigenous Wannan yellow chicken by high-throughput sequencing technique, to explore the function of gut microbiota in regulating FCR in indigenous broilers.

MATERIALS AND METHODS HEADINGS

Experimental Animals and Sample Collection

A total of 1480 Wannan yellow male chickens at 10-week-old were provided by Anhui Huadong Mountain Fresh Agricultural Development Co.; Ltd. The basic diet was also provided by Anhui Huadong Mountain Fresh Agricultural Development Co.; Ltd., the components and nutritional concentrations of which are shown in Table 1. Birds with similar body weights (0.93±0.10kg) were selected and raised in three-layer cascade cages with one bird per cage and one cage for separation to avoid interference between individuals. The daily photoperiod was 12L: 12D. Feed in powder was sufficiently provided once a day in individual boxes, and daily feed intake was calculated as feed supplied minus residue in the box. Water was provided *ad libitum*. Body weight was recorded in each week.

Table 1 – Feed composition (g/kg) and nutritional concentration.

Ingredients	g/kg	indicators	Nutrient concentration
Corn	630	Metabolic energy (MJ/kg)	11.24
Soybean meal	200	Crude Protein (%)	15.14
Wheat bran	105	Lysine (%)	0.76
Stone powder	15	Methionine (%)	0.26
Premix	50	Ca (%)	1.63
-	-	P (%)	0.69

The feed conversion ratio (FCR) was calculated as the ratio between feed intake and weight gained, which means that lower FCR values reflect higher feed efficiencies. FCR values at the two extreme points that were exhibited more than three times were set as low and high FCR. A total of 13 16-week-old birds (7 in high and 6 in low FCR group) were selected and euthanized by carbon dioxide anesthesia after 12 hours of fasting for performance determination and intestinal contents collection.

Carcass performance determination

Body weight was measured after 12 hours of fasting, and then the carcass weight, eviscerated weight, breast muscle weight, leg muscle weight, abdominal fat weight, ileum weight, ileum length, ileal villi length, and ileal crypt depth were recorded.

Sample collection and ileal slice preparation

About 2g of contents from the middle part of the ileum were collected and frozen in liquid nitrogen rapidly, then stored at –80 °C for DNA extraction and microbiota analysis. After carefully cleaning with normal saline, the middle part of the fresh ileum was collected and placed in 4 % paraformaldehyde fixation fluid for HE staining, which was based on the description reported by Feldman and Wolfe (Feldman *et al*., 2014). After gradient dewaxing of the paraffin sections, hematoxylin was added for 0.5-1 minutes, and then rinsed with tap water. The following steps were followed: differentiation of 1% hydrochloric acid alcohol for a few seconds; rinsing with tap water; addition of 1% ammonia aqueous solution back to blue for 1 minute; rinsing with running water for a few seconds; addition of eosin dyeing solution for a few seconds; rinsing with running water. After rinsing, 75% ethanol was added for 2 min, 85% ethanol for another 2 min, then anhydrous ethanol for 5min, anhydrous ethanol for 5 minutes, xylene for 5min until the slices are transparent (light can penetrate), and finally the slices were removed from xylene and sealed with neutral gum. The sections were observed under an Olympus IX73 microscope (Olympus, Japan).

Genomic DNA extraction

Total genomic DNA was extracted with a QIAamp Fast DNA Extraction Kit (QIAGEN, Germany, 51604). About 100 µl lysis buffer were added to 100 µg ileum content, which was subjected to incubation at 70 °C for 10 minutes. All the operations were performed according to the manufacturer's instructions. Quality and quantity of DNA were detected by agarose gel electrophoresis and concentration determination (Nanodrop2000, Thermofisher, USA). Samples that met the sequencing requirements (DNA concentration > 50 ng/µL, with clear bands) were stored in a -80 °C freezer for future use.

PCR and high-throughput 16s rRNA sequencing

Primers 343F: (TACGGRAGGCAGCAG) and 798R (AGGGTATCTAATCCT) were used to amplify the V3-V4 region of the bacterial 16 S rRNA. The diluted DNA was used as the template for PCR. PCR amplification included the following steps: initial denaturation at 94 °C for 2 min, and then 30 cycles with 94 °C for 30 sec, 57 °C for 30 seconds, 72 °C for 30 sec, and final extension at 72° C for 5min after the cycles. The PCR product was detected by electrophoresis, purified by magnetic beads, and then purified by magnetic beads using Qubit 7 for quantification and sequencing through the Illumina MiSeq PE300 high-throughput platform with paired-end sequencing. The original data were submitted to the NCBI database and the BioProject number is PRJNA784012.

Bioinformatic Analysis

The double-terminal sequence of the raw data was removed using the Trimmomatic software (version 0.35). The sliding window method was used to check the average base mass. When the mass was less than 20, the previous high-quality sequence was truncated. After impurity removal, the double-terminal sequence was assembled using the FLASH software (version 1.2.11) to obtain a complete paired-end sequence. The splicing parameters were as follows: the minimum overlap was 10 bp, the maximum over-lap was 200 bp, and the maximum mismatch rate was 20%. The chimera sequence was detected and removed using the UCHIME software. After the sequencing data were pre-processed to generate high-quality sequences, Vsearch software (version 2.4.2) was used to analyze the valid tags obtained from quality control, and subsequently for OTU classification according to 97% similarity. The QIIME software (version 1.8.0) was used to select the most abundant sequence in each OTU as the representative sequence of the OTU. The RDP classifier Naive Bayesian classification algorithm was used to compare and annotate the representative sequence with the Greengenes database to obtain the OTU annotation information.

Alpha diversity was analyzed using the Chao_1 index and Shannon diversity index. The PICRUSt function prediction analysis was based on the 16s sequencing data annotated by the Greengenes database. The redundancy analysis (RDA) was made using Canoco for Windows (version 4.5), and visualization with an R package.

Statistical Analysis

The significance of flora diversity was analyzed by Wilcoxon algorithm between high and low FCR groups. The slaughter performance and flora abundance between high and low FCR groups were analyzed by the independent sample t test of the SPSS 20.0 software. Redundancy analysis (RDA) was used to analyze the relationship between the relative content of the top 15 bacterial genera and the carcass performance with significant differences. The results were expressed as mean ± SEM, and the difference was deemed significant when $p<0.05$.

RESULTS

Weight gain of Wannan yellow male chicken

According to the weight gain of the 1480 male chickens during the six weeks feeding, an inflection point of weight gain appeared at 14 weeks of age, indicating that the rapid growth of the Wannan yellow male chickens mainly occurred before 14 weeks of age (Figure 1). Therefore, the 14-week-old FCR was used as indicator for chicken selection with low and high FCR to further analyze the compositions of gut microbiota.

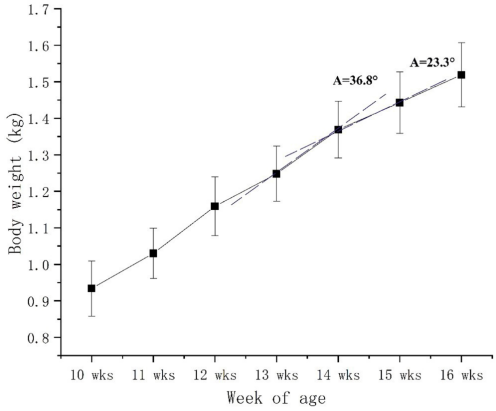

Figure 1 – Weight record of Wannan yellow male chicken. A is the angle between the connecting line of two adjacent points and the horizontal line. The larger the angle, the greater the weight gain.

Carcass performance and ileal histochemical analysis of Wannan yellow chicken

FCR in the high FCR group was 12.83, which was significantly higher ($p<0.05$) than that of the low FCR group (Figure 2A). The levels of body weight, carcass weight, eviscerated weight, breast muscle weight, leg muscle weight and abdominal fat weight showed no significant difference ($p>0.05$) between high and low FCR groups (Figure 2B). Simultaneously, the levels of ileum weight and ileum length in the HFCR group were significantly higher ($p<0.05$) than that of the LFCR group, while ileal villi lengths in the HFCR group were significantly lower ($p<0.05$) than that of the LFCR group. However, ileal crypt depth showed no significant difference ($p>0.05$) between the two groups (Figure 2C). Figures 2D and 2E present the ileum structure of the HFCR and LFCR groups, the numbers on the figure indicate ileum villi length and ileum crypt depth.

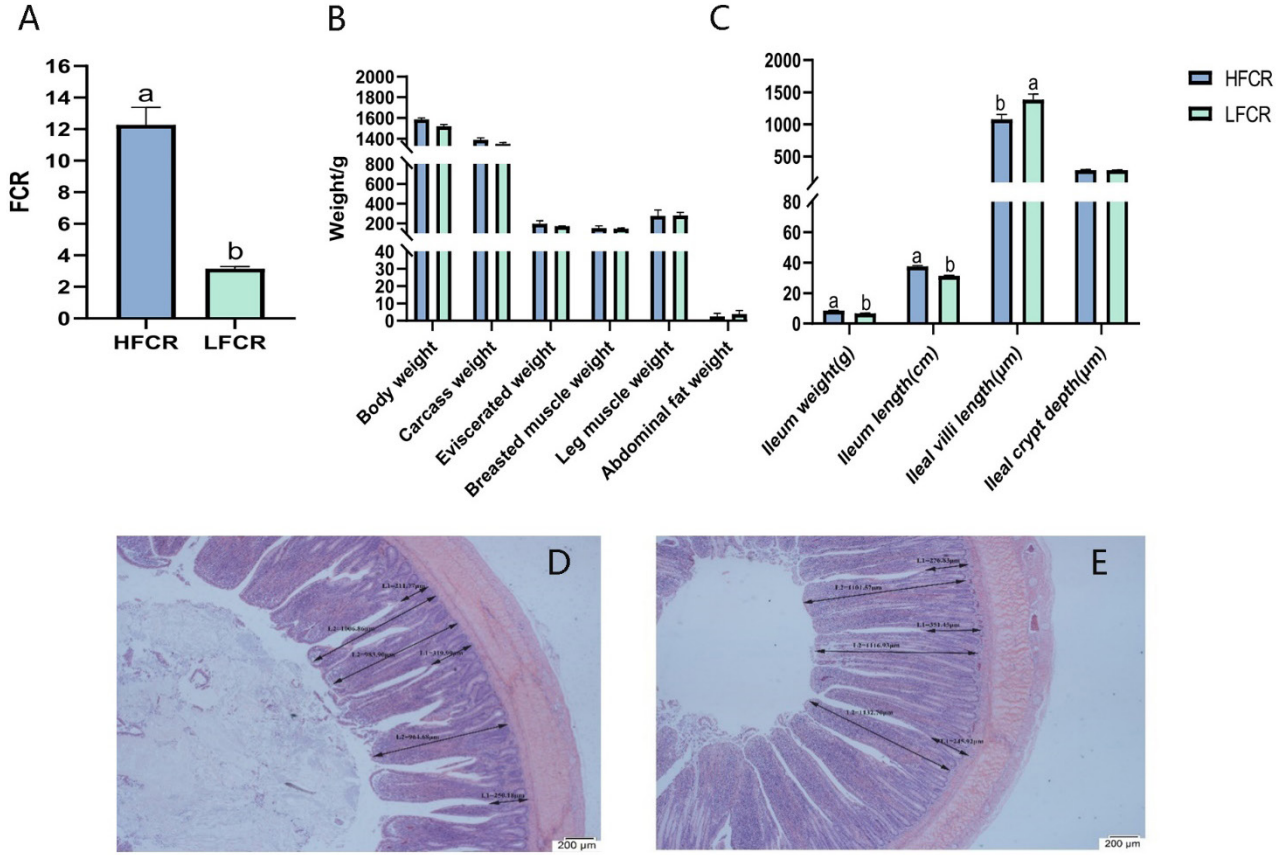

Figure 2 – Carcass performance and ileal histochemical analysis of Wannan yellow chicken. (A) The level of FCR in the HFCR and LFCR groups; (B) The carcass performance of Wannan yellow chickens (such as body weight, carcass weight, eviscerated weight, breast muscle weight, leg muscle weight and abdominal fat weight); (C) The intestinal performance of Wannan yellow chickens (such as ileum weight, ileum weight, ileal villi length and ileal crypt depth). Different letters (a, b,) in the same column indicate significant differences (P< 0.05); (D) Ileum HE stained sections of the high FCR group; (E) Ileum HE stained sections of the low FCR group. L1 is the depth of ileal crypts, L2 is the length of ileal villi.

The analysis of the gut microbiota structure and relative abundance between high and low FCR

The composition of microbiota of the ileum was analyzed by 16S rRNA high-throughput sequencing. In total, 4,201,828 bacterial 16S rRNA raw reads were found in 13 samples, with 131,307 reads per sample, and 816 distinct operational taxonomic units (OTUs) (Figure 3A). The α diversity dilution curve was drawn according to the Shannon index. When the curve tended to be flat, it could be considered that the sequencing depth had basically covered all species in the sample. As shown in Figure 3B, the results showed that the sequencing results could reflect the real microbial structure situation of Wannan yellow chickens. PCA analysis showed good reproducibility in both groups (Figure 3C). The boxplot was plotted according to the Simpson index calculated at the same sequencing depth, and the results showed that there were no significant differences in the diversity of intestinal microorganisms between the high and low feed conversion ratio groups (Figure 3D).

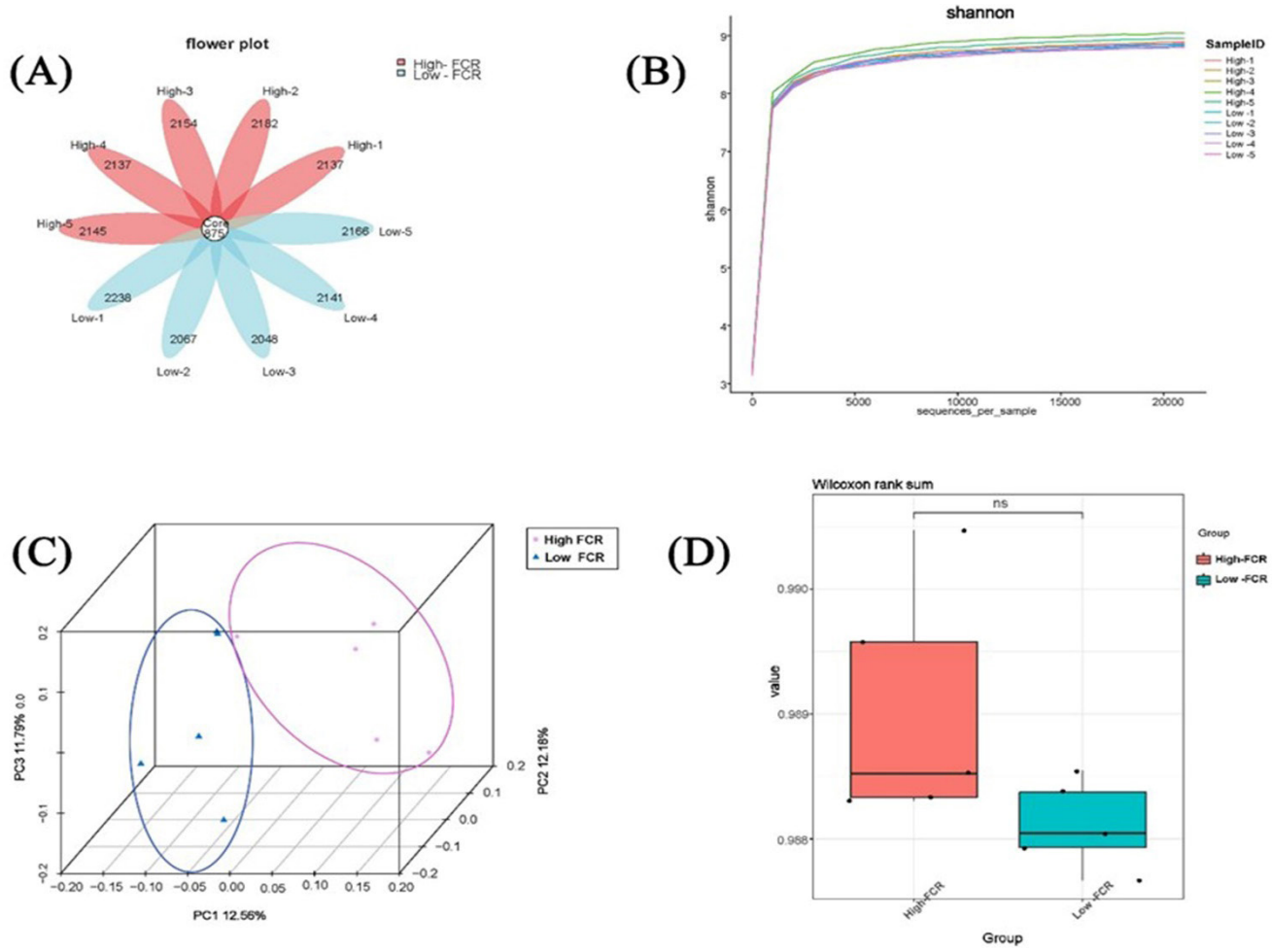

Figure 3 – The OUT number of each sample (A), α diversity dilution curve (B), PCA analysis (C) and diversity difference analysis (D) of Wannan Yellow chickens in the high and low feed conversion ratio groups in ileum.

As shown in Figure 4A-C, the microbial structure of high and low FCR groups was similar. At phylum level, *Bacteroidetes* and *Firmicutes* accounted for the largest proportion, and the relative abundance of *Acidobacteria* and *Gemmatimonadetes* in the LFCR group was significantly higher ($p<0.05$) than that of the HFCR group (Figure 4D). At the genus level, there was no significant difference in the relative abundance of ileum microbiota between the HFCR and LFCR groups (Figure 4E). At species level, Lactobacillus were the most diverse species, and the relative abundance of the LFCR group was higher than that of the HFCR group.

PICRUSt analysis of high and low FCR group

In order to explore whether the intestinal microbial differences in the ileum could affect FCR, the PICRUSt software was used to predict the function of known microbiota. Combined with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, the different functions of differential microbiota between the two groups were predicted. The LFCR groups had significantly higher function in metabolism, organismal system, genetic information processing, and human disease ($p<0.05$) when compared with the HFCR group (Table 2).

Table 2 – Differences in the abundance of KEGG analysis between the LFCR and HFCR groups.

OTU	LFCR	HFCR	*p* Value
Metabolism	6820159[a]	6480049[b]	0.026
Organismal Systems	106592[a]	100882[b]	0.026
Genetic Information Processing	2684524[a]	2568534[b]	0.029
Human Diseases	122492[a]	116466[b]	0.029
Unclassified	1940333[a]	1855049[b]	0.049

Redundancy analysis of microbial flora in the ileum and carcass performance

In order to determine the bacteria that caused the difference in carcass performance between the two groups, a redundancy analysis (RDA) on the intestinal flora with FCR, ileum length, ileum weight and ileal villi length was perform and the top 15 genera were listed. According to the results (Figure 5), ileum weight, ileum

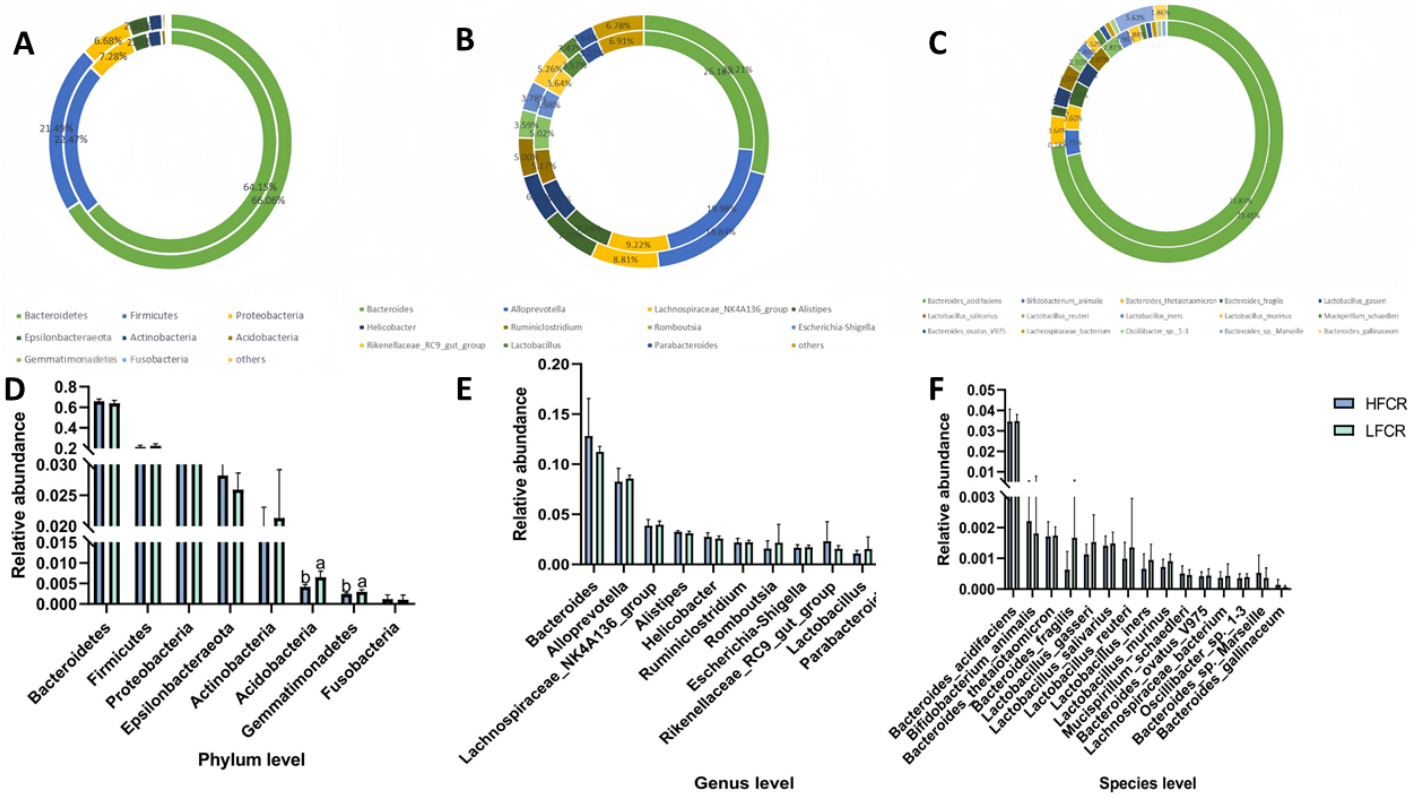

Figure 4 – Ileal microbial structure and relative abundance. A-C, Bacterial community structure of ileal at phylum, genus and species levels in the HFCR and LFCR groups. The inner and outer rings represent the HFCR and LFCR groups, respectively. D-F, Relative abundance of gut bacteria at phylum, genus and species levels in the HFCR and LFCR groups.

length and ileal villi length were positively correlated with FCR. *Lactobacillus* was positively correlated with FCR, ileum length, ileum weight and ileal villi length.

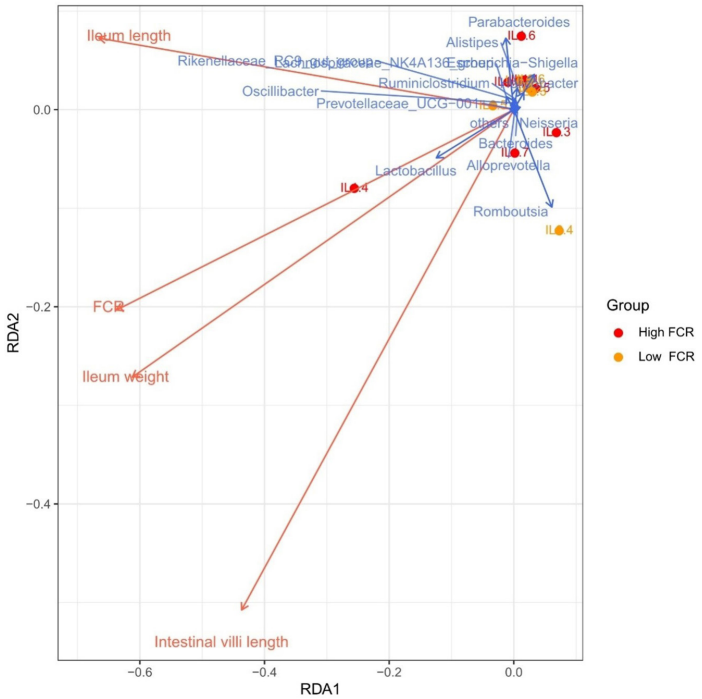

Figure 5 – Redundancy analysis (RDA) between FCR, carcass, and ileum microbes (genus level). The red arrows in the figure indicate different performance factors, and the blue arrows indicate different microorganisms. The red dots and yellow dots represent the samples of the high and low groups, respectively.

Correlation between differential Microbial Genera in HFE and LFE Groups

In order to explore the relationship among different microorganisms, correlation analysis was conducted for the top 30 microbial genera. As shown in Figure 6, *Lachnospiraceae* had strong positive correlation with most of microbiota, such as *Alloprevotella*, *Ruminiclostridium* and *Oscillibacter*.

DISCUSSION

Indigenous chickens are usually raised up to marketing conditions in 12 weeks of age. Wannan yellow chicken is an indigenous Chinese chicken breed, and the males are usually raised to 12-16 weeks of age for marketing. Therefore, the late period (after 10 weeks of age) of FCR before marketing is highly important for economic reasons. In this experiment, 10-week-old Wannan yellow male chickens were thus used for the FCR measurement from 10 to 16 weeks of age.

The small intestine is an important digestive organ, since its developmental status is closely related to poultry growth performance. Research showed that feed utilization efficiency was closely related to the development of digestive organs (Huang *et al*., 2022).

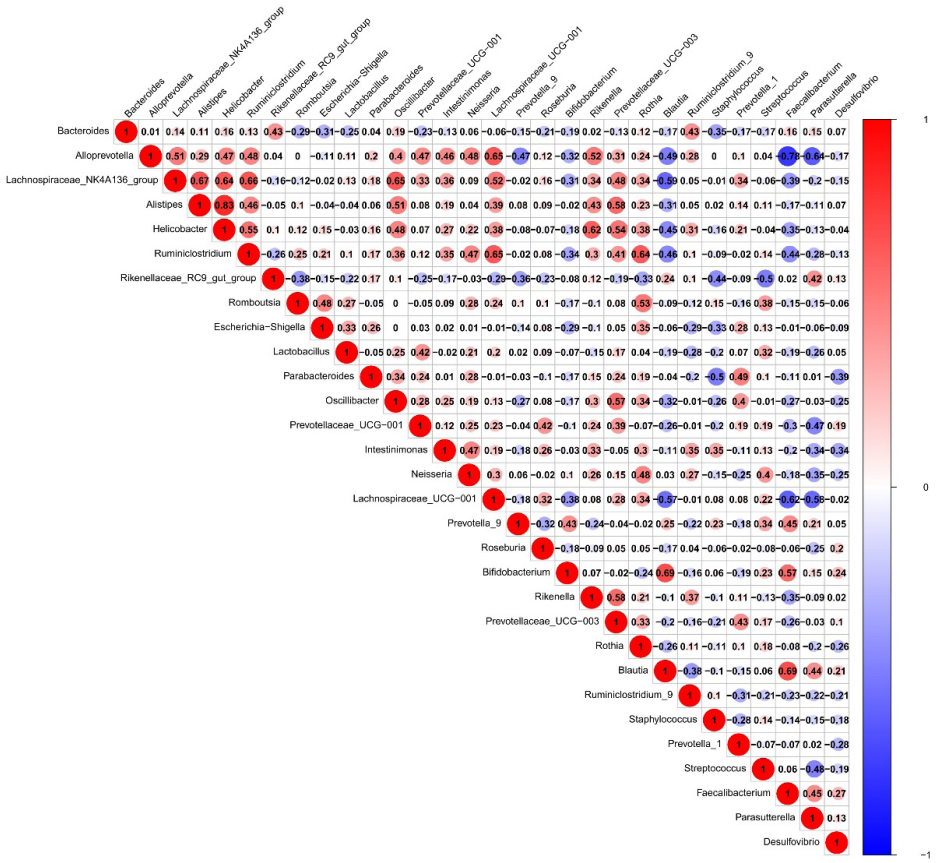

Figure 6 – Correlation plot of the top 30 most abundant genera. Red dots represent positive correlation while blue dots represent negative correlation, and larger dot areas represent stronger correlations.

This experiment also found that the weight and length of ileum was increased and villi length was decreased in chickens with high FCR. Similar to the results of this experiment, de Verdal *et al*. (Verdal *et al*., 2011) also suggested that the ileum weight and ileum length in the high FCR group were significantly higher than those in the low FCR group. This might be due to the thicker epithelium and muscle layer of small intestine in the high FCR group as compared to those in the low FCR group.

The growth performance is positively related to the diversity and abundance of the intestinal flora (Yang *et al*., 2015), and the higher the richness of the microbial community, the higher the utilization of nutrients in the gut (Lozupone *et al*., 2012). Therefore, the abundance of ileum microflora was analyzed in the high and low FCR groups. According to our result, there were no significant differences in alpha diversity between high FCR and low FCR chickens, which is similar to previous studies on fecal microflora. We inferred that as chickens mature, gut microbiome also becomes stable, so the composition of microbiota may have no significant effect on host feed efficiency while the dominant microbiota may play the main role.

Firmicutes, *Bacteroides* and *Proteobacteria* are dominant flora in the intestinal tract of chickens. The ratio of *Firmicutes* to *Bacteroides* can be used as a marker of obesity-related metabolic diseases (Magne *et al*., 2020). According to the experiment of Huang *et al* (2021), the changes in the relative abundance of *Firmicutes* and *Bacteroidetes* may be linked to feed efficiency. In our study, the ileum of the low FCR group had the higher ratio of *Firmicutes* to *Bacteroides,* which may cause the difference of feed efficiency between the two groups. The effect of intestinal microbiota on host metabolism may be the result of bacteria and host interaction: host genes influence microbial composition and microbial metabolic activity has a feedback effect on the host, which together influence the host phenotype (Wei *et al*., 2017). *Lactobacillus* is considered a type of gut probiotic bacteria. Studies have reported that *Lactobacillus* participate in the metabolic process of bile, phenols and phenyl derivatives in the body, which could accelerate fat metabolism (Yoon *et al*., 2020). Previous studies suggested that *Lactobacillus* are associated with the weight of human and animal infants (Crovesy *et al*., 2017). However, the body weight of the LFCR group was not significantly higher than that of the HFCR group, results which were consistent with Wei *et al* (Wei *et al*., 2017). According to Altaher (Altaher *et al*., 2015), supplementation of *Lactobacillus* did not affect body weight gain, but feed efficiency improved due to a reduction in feed intake. Moreover, when *Lactobacillus* is added as feed additive, it can increase nutrient digestibility and intestinal villus length of the jejunum of broilers, and the longer length of intestinal villus leads to a an improvement in the capacity of digestion and absorption (Wu *et al*., 2021). In this study, the relative content of ileum *Lactobacillus* in the LFCR group was higher than that of the HFCR group, and according to the result of RDA, *Lactobacillus* has a positive correlation with ileum length, ileum weight and ileal villi length. Therefore, we inferred that *Lactobacillus* could affect host ileum phenotypic traits by promoting the length of villi of the ileum, thereby

increasing feed efficiency without affecting body weight.

Moreover, according the result of the PICRUSt analysis, the microbiota of the LFCR group had higher abundance in metabolism function than that of the HFCR group, which may reflect in a more active metabolic activity in the intestinal tract of the LFCR group and improve host feed efficiency. The gut microflora represents a systematically balanced organ that affects poultry growth performance (Abd El-Hack *et al*., 2020), and could perform many physiological functions, including immunization and growth. Research by Mazmanian *et al*. (2008) showed that the balance between various bacterial species in the intestine is an important factor in maintaining health. *Lachnospiraceae* are among the main producers of short-chain fatty acids (SCFAs). *Lactobacillaceae* and *Ruminiclostridium* hydrolyze starch and other sugars to produce butyrate and other SCFAs (Mazmanian *et al*., 2008). Acetate is produced by *Alloprevotella* and *Ruminiclostridium* (Vogt *et al*., 2015). According to the result of the correlation analysis, *Lachnospiraceae* have strong positive correlation with *Alloprevotella* and *Ruminiclostridium* and have weak positive correlation with *Lactobacillaceae*, which reflects the these microbiota affect metabolic efficiency and energy utilization by co-participating in host intestinal fatty acid metabolism.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Xingyong Chen contributed to conception and design of the study. Zhaoyu Geng organized the database. Kaiqin He performed the statistical analysis. Qianyun Ji wrote the first draft of the manuscript. Qianyun Ji, Jinzhou Peng and Penghui Chang wrote sections of the manuscript. Xiaoxiu Zhu offered the experiment site. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

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DATA AVAILABILITY STATEMENT

The gut microbiome sequencing data have been deposited in the NCBI (https://www.ncbi.nlm.nih.gov/sra/PRJNA784012).

ETHICAL STATEMENTS

The animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Anhui Agricultural University (no. SYDW–P20201225211). The experiments were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and the Standards for the Administration of Experimental Practices.

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