Analysis of Colon Microbial Diversity of African Ostriches at Different Ages

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Research

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Abstract

Background: The colon is the unique digestive organ of the African ostrich. It has a large microbial population, which plays an important role in the digestive process of the African ostriches.

Methods: In order to understand the diversity of colon microbes in African ostriches, this study used metagenomics sequencing technology to sequence and analyze the colonic microbes of African ostriches at the age of 7, 30, 60 and 180 days.

Results: The results showed that with the increase of age, the microbial richness index first increased and then decreased, the highest at 60 days of age (ACE 271.5, Chao1 274.097), while the microbial diversity gradually reduced with the increase of age, the highest at 7 days (Shannon 3.203, Simpson 0.104). At the phylum level, the dominant bacteria phylum at 7 days of age is Firmicutes, while at 30 days of age, the phyla Firmicutes and Tenericutes are dominant phyla, and the abundance of Proteobacteria is highest at 60 days of age and 180 days of age. At the genus level, the dominant bacteria in the colon at 7 and 30 days are Anaeroplasma and Bacteroides. Acinetobacter is the dominant genus at 60 days, and the dominant genus at 180 days is Pseudomonas. PCA analysis showed that the microbial composition in the 180-day-old colon was very different from other days. The microbial composition in the anterior and middle part of the colon at 7-day-old was similar to that of 30-day-old, while the microbial composition in the latter part was similar to that of 60-day-old. Results of LEfSe analysis showed that there were 34, 37, and 36 differential flora in the colon at 7, 30, and 60 days, respectively, while there was only one differential flora at 180 days of age.

Conclusion: The results of the study showed that the composition of the microbial community in the colon of African ostriches of different ages is different and there are different flora, but the microbes in the colon are still mainly concentrated in the phylum Firmicutes, Tenericutes, and Proteobacteria, which gives the intestinal tract and function of the African ostrich further research provides a theoretical basis.

Background

The intestinal microflora is the general term for all microorganisms inhabiting the gastrointestinal tract of an organism [1], including archaeabacteria, bacteria, and eukaryotes. These microorganisms are mostly enriched in the large intestine, among which Bacteroides and Firmicutes account for more than 95% of the total number of bacteria, and most of them are enriched in the colon [2]. The colon has the functions of absorption, bacterial digestion, and assisting defecation, and because the chyme moves slowly in the colon, it is more conducive to the reproduction and growth of bacteria [3]. Studies have shown that colonic microorganisms can ferment and break down undigested carbohydrates, food protein residues, and enzymes secreted by the small intestine to produce short-chain fatty acids (SCFA) and other metabolites. These substances can regulate the development of immune cells, provide energy, and inhibit inflammation [4, 5]. This shows that the microorganisms in the colon are of great significance for maintaining the health of the body. Therefore, more and more scholars have launched research on
colonic microorganisms. Lu et al. found that Firmicutes is the dominant phylum in piglet colon [6]. Guo et al. found that the colonic microbial diversity of breeding Jinfen white pigs was significantly higher than that of local Mashen pigs [7]. Hu et al. found that the microbiota in the colon has a certain connection with the occurrence of cardiovascular disease [8]. Ji et al. found that short-term feeding of high-nutrient diets can change the microbial content in the colon of some Huan Jiang Xiang pigs [9].

The African ostrich is the largest herbivorous bird in the world. Unlike other poultry, it has a well-developed colon, which is about 2.5 times the length of the small intestine, accounting for 84.2% of the large intestine [10], and its colon is the site with the highest degree of microbial accumulation. At present, research on colonic microorganisms has mainly focused on mammals such as pigs and mice. There is no report about the related research of African ostrich. Understanding the composition and succession of the colonic microorganisms of the African ostrich is of great significance to promote the growth and development of the African ostrich and improve its production performance. Therefore, this experiment uses metagenomics methods to sequence and analyze the diversity of microorganisms in the colon of African ostriches of different ages.

Methods

Sample Collection

Test animals selected 4 healthy African ostriches, which were 7 days, 30 days, 60 days, and 180 days. Adjust the diet ratio according to the dietary structure of experimental animals of different ages and meet the requirements of the National Research Council (1994). Use 20% Urethane (1 g/kg) for anesthesia. After general anesthesia, open the abdominal cavity and quickly remove the colon. Extract the contents from the front, middle and back positions of the colon into a 2 mL EP tube, collect 10 samples in total, and label each sample. The specific group numbers are shown in Table 1.

| Intestine | Location | 7-day-old | 30-day-old | 60-day-old | 180-day-old |
|----------|----------|-----------|------------|------------|-------------|
| Anterior | E11      | E21       | E31        | E41        |
| colon    | Middle   | E12       | E22        | E32        |
| Posterior| E13      | E23       | E33        |

Entrust Annoroad Gene Technology (Beijing) Co., Ltd. to conduct microbial mutagenesis sequencing and biological information analysis of the resulting sequence, and the specific process is as follows:

Test procedure

After extracting the total DNA of the sample, primers are designed according to the conserved regions, and sequencing adapters are added to the ends of the primers to perform PCR amplification, and the
products are purified, quantified, and homogenized to form a sequencing library. The constructed library is first subjected to library quality inspection, and the qualified library is sequenced with Illumina HiSeq 2500.

**Information analysis process**

According to the overlap relationship between PE reads, the paired-end sequence data obtained by HiSeq sequencing is merged into a sequence Tag. At the same time, the quality of Reads and the effect of Merge is filtered to obtain Effective Tags.

**Drawing Rarefaction curve**

The rarefaction curve is to randomly select a certain number of sequences from a sample, count the number of species represented by these sequences, and construct a curve based on the number of sequences and species to verify whether the amount of sequencing data is sufficient to reflect the sample species diversity. The rarefaction curve reflects the rate of emergence of new OTUs (new species) under continuous sampling: within a certain range, as the number of sequencing items increases, if the curve shows a sharp rise, it means that a large number of species have been discovered in the community; The curve tends to be flat, indicating that the species in this environment will not increase significantly with the increase in the number of sequencing.

The Shannon diversity index curve is drawn using Mothur software and R language tools according to the Shannon index of each sample at different sequencing depths. The larger the Shannon index, the more OTU species and the richer the species, indicating that most of the microbial species information is covered in the sample. When the curve tends to be flat, it indicates that the amount of sequencing data is large enough, and the OTU types will not increase with the increase in the amount of sequencing; if the curve does not tend to be flat, it indicates that it is not saturated, and increasing the amount of data can reveal more OTU.

**Alpha diversity analysis**

Alpha diversity can reflect the species diversity within a single sample, and it has multiple metrics. Chao1 and Ace indexes are used to measure the abundance of the community. The Shannon index can reflect the diversity of the community, and the Simpson reflects the concentration of dominant populations in the community. Therefore, the larger the Chao1, Ace, and Shannon index values, and the smaller the Simpson, the higher the species diversity of the sample.

**Analysis of species composition and abundance**

Comparing the representative sequence of OTU with the microbial reference database, the species classification information corresponding to each OTU can be obtained, and then the composition of each sample community at each level (phyla, family, class, family, genus, species) is counted. First, use QIIME software to generate species abundance tables at different taxonomic levels, and then use R language tools to draw community structure diagrams at each taxonomic level of the sample.
Principal component analysis

Principal component analysis (PCA) is a technique used to analyze and simplify data sets. By decomposing the composition of different sample OTUs, the differences between multiple sets of data will be reflected on the two-dimensional coordinate graph, and the coordinate axis can best reflect the two characteristic values of the variance. The closer the distance between the two samples, the more similar the composition of the two samples.

LEfSe analysis of samples between groups

Linear discriminant analysis (LDA) is used to find statistically different flora in the colon of African ostriches of different ages. Use the LDA value distribution histogram to display species with LDA scores greater than the set value (the default setting is 2.0). The length of the histogram indicates the influence of different species (LDA score). The longer the length of the histogram, the greater the influence of species on the differences between groups.

Results

Rarefaction curves

In this experiment, 10 samples obtained were sequenced and analyzed. A total of 873,733 optimized sequences and 771,878 valid sequences were obtained, and a total of 2,280 OTUs were defined. The rarefaction curve began to flatten at around 10,000, indicating that the number of sequencing is gradually reasonable, and the increase in the number of sequencing has little contribution to the discovery of new OTUs. (Fig. 1) In addition, the Shannon Index is used to indicate the diversity of microorganisms in the sample. The curve of each sample tends to be flat, indicating that the sample covers most of the microbial information. Microbial diversity in the sample can be fully displayed, and the reliability of subsequent analysis can be guaranteed. (Fig. 2)

Alpha diversity analyses

The Alpha diversity index of the African ostrich colon is shown in Table 2, and the statistical histogram is shown in Fig. 3. Combining Table 2 and Fig. 3, the abundance indexes ACE and Chao1 both increased first and then decreased with increasing age, reaching the highest at 60 days of age (ACE 271.5, Chao1 274.097), and compared with other ages, 60 days old has the highest microbial richness. ACE and Chao1 indexes were the lowest at 180 days of age, 209.465, and 210, respectively, and the microbial abundance was the lowest. The diversity index Shannon index gradually decreases with age, while the Simpson index shows the opposite trend, indicating that the microbial diversity in the African ostrich colon decreases with age, and the diversity is highest at 7 days of age, the lowest diversity at 180 days of age.
**Table 2**
The colonic microbial diversity index of African ostriches at different ages

| Sample ID      | ACE               | Chao1            | Shannon          | Simpson          |
|---------------|-------------------|------------------|------------------|------------------|
| E1 (7-day-old)| 241.790 ± 5.973   | 251.303 ± 6.831  | 3.203 ± 0.229    | 0.104 ± 0.034    |
| E2 (30-day-old)| 259.887 ± 21.542  | 267.139 ± 22.808 | 3.173 ± 0.099    | 0.130 ± 0.016    |
| E3 (60-day-old)| 271.500 ± 19.444  | 274.097 ± 21.476 | 2.768 ± 0.041    | 0.173 ± 0.009    |
| E4 (180-day-old)| 209.465          | 210.000          | 1.388            | 0.374            |

**The composition of the flora in the colon of African ostriches of different ages**

Analyzed at the phylum level, there are differences in the composition and abundance of flora in the colon of African ostriches of different ages. When the African ostrich is 7 days old, there are differences in the composition and abundance of the flora in different intestinal segments of the colon. The phyla with higher abundance in the anterior and middle segment of the colon are *Firmicutes, Tenericutes, Bacteroidetes*, and *Verrucomicrobia*, and *Firmicutes* have the highest relative abundance, 52.8%, and 51.2%, respectively. The relative abundance of *Proteobacteria* in the front and middle segment is extremely low, 1.1% and 2.1%, respectively. In the 7-day-old posterior segment of the colon, *Proteobacteria* is the most important phylum, with a relative abundance of 39.7%, which is significantly higher than the anterior and middle segments. *Firmicutes* is the second dominant species, accounting for 37.8%, significantly lower than the anterior and middle segment. In addition, the relative abundance of *Bacteroidetes* and *Verrucomicrobia* in the posterior segment is also significantly lower than the relative abundance of the anterior and middle segments. At the age of 30 days, the composition and abundance of the intestinal segments of the colon are not much different, and they are mainly composed of *Firmicutes, Tenericutes*, and *Bacteroidetes*. In addition, each segment has a certain abundance of *Proteobacteria*, and the anterior segment is significantly higher than the middle and posterior segments. At the age of 60 and 180 days, the Proteobacteria was the most dominant phylum in the colon, with an average relative abundance of 51.7% and 56.5%, respectively, followed by *Firmicutes*, accounting for 25.9% and 42.9%. It can be seen that the abundance of *Firmicutes* at 180 days is significantly higher than that at 60 days. At 60 days of age, in the colon, besides the Proteus and *Firmicutes*, there are also the phylum *Tenericutes, Bacteroidetes* and *Verrucomicrobia*, which have high abundance, while in the colon at 180 days of age, the *Proteobacteria* and *Firmicutes* the relative abundance have exceeded 98%, and the abundance of other bacteria phyla are extremely low. (Fig. 4)

At the genus level, at 7 days of age, the colon is mainly composed of *Aneaploasma, Bacteroides, Akkermania, Escherichia-Shigella*, and in its posterior colon, the relative abundance of the *Acinetobacter* is 35.8%, which is significantly higher than the former and middle segment. The microorganisms in the colon at the age of 30 days are mainly classified into *Aneaploasma, Bacteroides, Christensenellaceae-R-7-group, Acinetobacter*, and *Escherichia-Shigella*, among which *Aneaploasma* is the most dominant
genus in each segment, with an average relative abundance of 15.9%. There was no significant difference in the composition and relative abundance of the bacterial communities of each segment of the colon at the age of 60 days, mainly composed of Acinetobacter, Anaeroplasma, Bacteroides, Christensenellaceae-R-7-group, and Akkermania. Acinetobacter is the most dominant genes, with an average relative abundance of 50.9%. At 180 days of age, the composition of the colon flora is concentrated in Pseudomonas, and its relative abundance reached 56.1%. (Fig. 5)

**PCA analyses**

Perform principal component analysis based on the OTU types and abundance obtained from all samples, and draw PCA diagrams. As showed in Fig. 6, except for the larger points of dispersion in the 7-day-old group, the dispersion in the 30-day and 60-day-old groups is smaller. It shows that the composition of the colon flora is quite different at the age of 7 days. The composition of the colonic colon at 30 days and 60 days is similar. Among the different ages, the 7-day and 30-day age graphs are closer, and both are farther away from the 60-day and 180-day age. It shows that the composition of colonic flora at 7-day and 30-day-old is relatively similar and is quite different from that of 60-day and 180-day-old. The degree of dispersion between 60-day-old and 180-day-old is relatively large, showing a large difference in floral composition.

**LEfSe analyses of the differential flora in the colon contents of ostriches of different ages**

The results showed that there were different flora in the colon of the African ostrich of different ages. At the age of 180 days, the Planococcaceae have a higher abundance. At 60 days of age, there are 36 bacterial groups, including Ruminococcaceae-UCG-011, Christensenellaceae-R-7_group, Christensenellaceae, Gordonibacter, Synergistia, Synergistetes, Aerococcus, Ruminococcaceae-NK4A214_group, Lachnocostrum, Family-XIII, Family-XIII-AD3011-group, Exiguobacterium, Clostridium-sensu-stricto-6, Erysipelotrichaceae-UCG-004, Eubacterium-coprostanoligenes-group, etc. have a high abundance. At 30 days of age, there are 37 bacterial groups with high abundance, including Anaeroplasma, Anaeroplasmataceae, Eubacteriaceae, Mollicutes, Lachnospiraceae, Clostridiales-vadinBB60-group, Coprococcus-3, Ruminiclostridium-5, Ruminiclostridium-9, Lachnospiraceae-ND3007-group, Angelakisella, Acetotomaculum, Muribaculaceae, Lachnospiraceae-NK4A136-group, Lachnospiraceae-FCS020-group, Enterococcus, Cyanobacteria, etc. At 7 days of age, there are 34 bacterial groups with high abundance, including Erysipelotrichaceae, Lachnocostrum-10, Mollicutes-RF39, Subdoligranulum, Lachnospiraceae-NC2004-group, Ruminococcaceae-UCG-013, T2WK15B57, Odoribacter, Marinilaceae, Coprococcus-1, Ruminococcus-gauvreauii-group, Barnesiellaceae, Ruminococcus-2, etc. (Fig. 7)

**Discussion**
The colonization and development of the microbial community in the animal intestines are not static but dynamic, and a diversified microecosystem can be formed in the first few weeks after the animal is born [11]. Studies have shown that the composition of the intestinal microbiota of young animals is highly fluctuating. But as age increases, they gradually stabilize in the end. In this process, the intestinal tract at different developmental stages will form a dominant flora that adapts to the developmental needs of this stage and plays a corresponding role [12]. The taxonomic annotation results of this study show that *Firmicutes*, *Tenericutes*, and *Bacteroidetes* are the dominant phyla in the colon of African ostriches at the age of 7 and 30 days. *Proteobacteria* and *Firmicutes* in the colon were the dominant phyla at 60 and 80 days of age. Studies have shown that *Bacteroidetes* are directly related to the body's protein metabolism and lipid metabolism [13]. The high abundance of *Bacteroidetes* may be adapted to the digestion of high-protein and high-nutrient diets of African ostriches at the juvenile stage. *Verrucomicrobia* with higher abundance appeared at 7 and 60 days of ages. Studies have shown that the increase in the abundance of *Verrucobacterium* may be related to the lack of peptidoglycan in the body [14]. Peptidoglycan has a variety of biological effects such as anti-infection, anti-tumor, and immune regulation. The lack of peptidoglycan in the body will lead to reducing immune function and cause the invasion of pathogenic bacteria [15]. This suggests that the changes in the microbial community in the intestine can reflect the health of the body at this time and the hidden risk of pathogenicity. In addition, at the genus level, *Anaeroplasma* is the dominant genus at 7 and 30 days old. *Acinetobacter* is the dominant genus at 60 days of age, and *Pseudomonas* is the dominant genus at 180 days of age. The results of LEfSe analysis show that there are different bacterial groups in the colon of each age, indicating that the abundance and composition of microorganisms in the intestine will change significantly at different stages of growth and development. This is consistent with the research of Yatsunenko et al.[12].

The results of the Alpha diversity analysis of this study showed that the abundance of microorganisms in the colon first increased and then decreased with age, while the diversity gradually decreased with age. It showed the highest microbial diversity at 7 days of age and the lowest at 180 days of age. PCA analysis shows that the 180-day-old colonic microbial composition is very different from other days. The microbiological composition of the front part of the 7-day-old colon is similar to that of the 30-day-old colon, and the latter part is similar to the 60-day-old colon. This shows that the colonic microbial population of African ostriches has stabilized in the adult stage, and with age, the gut microbes are also changing.

## Conclusion

The results of the study showed that the composition of the microbial community in the colon of African ostriches of different ages is different and there are different flora, but the microbes in the colon are still mainly concentrated in the phylum Firmicutes, Tenericutes, and Proteobacteria, which gives the intestinal tract and function of the African ostrich further research provides a theoretical basis.

## Abbreviations
OTU
Operational Taxonomic Units
PCA
Principal Component Analysis
LEfSe
Line Discriminant Analysis (LDA) Effect Size

Declarations

Acknowledgments

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Authors’ contributions

Yan Zhang and Jie Yang performed animal trial, laboratory experiments, and statistical analysis, designed the study and drafted the manuscript; Xiaoting Zhang and Lixun Ye assisted in study design, feed formulation, data evaluation and reviewed manuscript; Baitao Li assisted in laboratory work, collection and analysis of data; Qingping Luo contributed to study design and reviewed the manuscript; Jiaxiang Wang and Peng Li coordinated and designed the study, contributed to data collection and statistical analyses, and critically reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article can be obtained in the Figshare database and the attachment of this article.

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https://figshare.com/s/202e691bdefb77807e7d

The research on live animals in this article complies with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) and was approved by the Animal Ethics Committee of Yangtze University.

Consent for publication
Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

Sample rarefaction curve Note: E11: 7-day-old anterior colon; E12: 7-day-old middle colon; E13: 7-day-old posterior colon; E21: 30-day-old anterior colon; E22: 30 Day-old middle colon; E23: 30-day-old posterior colon; E31: 60-day-old anterior colon; E32: 60-day-old middle colon; E33: 60-day-old posterior colon; E41: 180-day-old anterior colon
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Figure 2

Shannon index curve Note: E11: 7-day-old anterior colon; E12: 7-day-old middle colon; E13: 7-day-old posterior colon; E21: 30-day-old anterior colon; E22: 30 Day-old middle colon; E23: 30-day-old posterior colon; E31: 60-day-old anterior colon; E32: 60-day-old middle colon; E33: 60-day-old posterior colon; E41: 180-day-old anterior colon
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Figure 3

Statistical histogram of Alpha diversity index (including ACE Index, Chao 1 Index, Shannon Index, Simpson Index) Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old
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Figure 4

Histogram of phylum horizontal species distribution Note: The histogram only shows the top ten species in abundance level, and other species are merged into Others and shown in the figure. E11: 7-day-old anterior colon; E12: 7-day-old middle colon; E13: 7-day-old posterior colon; E21: 30-day-old anterior colon; E22: 30 Day-old middle colon; E23: 30-day-old posterior colon; E31: 60-day-old anterior colon; E32: 60-day-old middle colon; E33: 60-day-old posterior colon; E41: 180-day-old anterior colon
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Figure 5

Histogram of genus horizontal species distribution Note: The histogram only shows the top ten species in abundance level, and other species are merged into Others and shown in the figure. E11: 7-day-old anterior colon; E12: 7-day-old middle colon; E13: 7-day-old posterior colon; E21: 30-day-old anterior colon; E22: 30 Day-old middle colon; E23: 30-day-old posterior colon; E31: 60-day-old anterior colon; E32: 60-day-old middle colon; E33: 60-day-old posterior colon ; E41: 180-day-old anterior colon
Figure 5

Histogram of genus horizontal species distribution Note: The histogram only shows the top ten species in abundance level, and other species are merged into Others and shown in the figure. E11: 7-day-old anterior colon; E12: 7-day-old middle colon; E13: 7-day-old posterior colon; E21: 30-day-old anterior colon; E22: 30 Day-old middle colon; E23: 30-day-old posterior colon; E31: 60-day-old anterior colon; E32: 60-day-old middle colon; E33: 60-day-old posterior colon; E41: 180-day-old anterior colon.
Figure 5

Histogram of genus horizontal species distribution Note: The histogram only shows the top ten species in abundance level, and other species are merged into Others and shown in the figure. E11: 7-day-old anterior colon; E12: 7-day-old middle colon; E13: 7-day-old posterior colon; E21: 30-day-old anterior colon; E22: 30 Day-old middle colon; E23: 30-day-old posterior colon; E31: 60-day-old anterior colon; E32: 60-day-old middle colon; E33: 60-day-old posterior colon ; E41: 180-day-old anterior colon
Figure 6

PCA analysis chart Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old
Figure 6

PCA analysis chart Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old
Figure 6

PCA analysis chart Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old
Figure 7

Histogram of LDA value distribution Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old
Figure 7

Histogram of LDA value distribution Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old
Figure 7

Histogram of LDA value distribution Note: E1: 7-day-old; E2: 30-day-old; E3: 60-day-old; E4: 180-day-old