

# Exploring the effect of the microbiota on the production of duck striped eggs

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**ABSTRACT** The microbiota has received plenty of attention in recent years due to its influence on host health and productivity. The striped eggs have reduced hatching performance and resulted in economic loss. The reasons are still unknown. Microbiota is one of the potentially important factors contributing to striped egg formation. This study investigates the relationship between the microbiota and striped eggs. The litter samples, feed samples, and cloacal swab samples of female ducks that produce striped eggs and normal eggs were performed for microbial diversity and composition using 16S rRNA sequencing. The results showed that there was no significant difference between feed microbiota and cloacal swab microbiota by alpha diversity, whereas, the number of microorganisms in the litter

samples of female ducks that produced striped eggs was less than those of female ducks with normal eggs. There were compositional differences in litter microbiota of female ducks between the striped egg and the normal eggs. Among them, the abundance of *Staphylococcus*, *Corynebacterium*, and *Brevibacterium* in the litter of female ducks that produced striped eggs was significantly higher than that produced normal eggs. And these differential bacteria maybe affect the health of female ducks and cause abnormalities in the formation process of duck eggs. Therefore, the reduction of harmful bacteria may protect the reproductive health of female ducks and decrease the proportion of striped eggs. It provides an important reference to explore why female ducks produce striped eggs.

**Key words:** duck, striped eggs, microbiota, litter microbiota

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## INTRODUCTION

Poultry eggs are rich in protein, lipids, and other nutrients, making them a relatively inexpensive source of high-quality protein (Gao et al., 2021). With the increasing population of China, the demand for duck eggs is also increasing (Hou and Liu, 2022). The importance of good quality eggs is self-evident. In recent years, it has been found that there is a phenomenon of duck striped eggs, that is, oblique striped strips of different depths can be seen on the surface of duck eggs (Sang et al., 2022). Striped eggs had lower eggshell weight, thick albumen weight, and thin albumen crude protein than normal eggs, which caused lower hatchability (Yuan et al., 2013). So, it brings great trouble to the breeding process and causes considerable economic

losses. Until now, there are few studies on the specific causes of striped eggs.

Egg quality is affected by many factors such as feed, heredity, age, and environment (Nasri et al., 2020). There are many kinds of microorganisms anywhere, and the microbes in different environments have different functions (Tropini et al., 2017). It has been reported that intestinal microbes and their metabolites act as signaling molecules linking the gut, liver, brain, and reproductive tract (Nicholson et al., 2012), which in turn has a direct or indirect impact on poultry health and egg quality (Zhan et al., 2019; Feng et al., 2021). And in rearing, it is also important for the influence of microbiota in the environment on animal production. Wang et al. showed that fresh and reused litter affects the chicken GI microbiota, which may impact the host's nutritional status and intestinal health (Wang et al., 2016). When laying hens are infected with pathogenic bacteria like *Salmonella*, intestinal *Salmonella* will spread to the oviduct through the cloaca (Gantois et al., 2009). Furthermore, *Salmonella* alters the expression of toll-like receptors, NOD-like receptors,  $\beta$ -defensins, and cytokine family genes in the oviduct, resulting in the decline of egg quality (Zhang et al., 2019). Studies have

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shown that duck excrement contains a large number of different pathogenic bacteria (Jeong et al., 2021; Wang et al., 2021), which will affect the composition of litter microorganisms in the duck house. It has a certain impact on the health of ducks and egg quality.

Previous studies on striped eggs mainly focused on the analysis of the differences between striped and normal laying ducks, but there are few studies about the effects of environmental microbiota on striped eggs. This study explored the influence of cloacal swabs, litter, and feed microbiota on striped eggs through 16S rRNA sequencing, and provided a research basis for studying the causes of striped egg production.

## MATERIALS AND METHODS

### *Animals and Sample Collection*

A total of 260 female Pekin ducks from a purebred line were used in the present study. All the ducks were reared individually in each cage from the beginning and managed according to the Pekin duck rearing standards. The cage is 1 meters long, 0.8 meters wide, and 1 meters high. The duck house is semienclosed with 17 h of light a day. The duck house were auto ventilated by monitoring ammonia, and temperature ranged from 15°C to 25°C during experiment. The ducks were provided ad libitum commercial diets: a diet containing 21% crude protein (CP), 2,800 MJ/kg dietary metabolizable energy (ME). When the female ducks were 66 wk old, the eggs were collected for 2 or 3 consecutive days. The striped eggs could be classified into 1 to 4 categories (SANG et al., 2022). Based on the level of laying striped eggs, 15 female ducks that produced striped eggs were assigned to the striped egg group, and 15 female ducks that produced normal eggs were assigned to the normal egg group. All samples were collected when the female ducks had mostly finished feeding for the day. The litter sample of the cage where the female duck was located was taken, and the sterile cotton swab was used to sample the litter at 5 points.

A total of 90 samples were collected, 45 from each of the striped egg and normal egg groups, comprised of the cloacal swabs, litter, and feed. Each duck was collected the cloacal swabs, the litter, and the feed. Sterile cotton swabs were taken from inside the cloaca of the female duck and placed in 2 mL cryopreserved tubes. And the samples were stored in liquid nitrogen. Subsequently, the leftover feed of one day's feed trough of the female duck was taken and put in a sterile sealed bag. The samples were stored at -80°C. Meanwhile, the litter samples of the cage where the female ducks were housed were taken. Sterile cotton swabs were used to sample the litter at five points. The litter was stored in liquid nitrogen.

### *DNA Extraction and Sequencing*

Total bacterial genomic DNA samples were extracted using the Fast DNA Spin Kits for soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The completeness of the DNA sample was

then assessed by 1% agarose gel electrophoresis. The V3 to V4 hypervariable region of the bacterial 16S rRNA gene was amplified with the primers 338F (5'-ACTCC-TACGGGAGGCAGCAG-3') and 806R (5'-GGAC-TACNNGGGTATCTAAT-3') (Munyaka et al., 2015). The PCR was carried out on a Master cycler Gradient (Eppendorf, Germany) using 25  $\mu$ L reaction volumes, containing 12.5  $\mu$ L 2  $\times$  Taq PCR MasterMix, 3  $\mu$ L BSA (2 ng/ $\mu$ L), 1  $\mu$ L Forward Primer (5  $\mu$ M), 1  $\mu$ L Reverse Primer (5  $\mu$ M), 2  $\mu$ L template DNA, and 5.5  $\mu$ L ddH<sub>2</sub>O. Cycling parameters were 95°C for 5 min, followed by 28 cycles of 95°C for 45 s, 55°C for 50 s, and 72°C for 45 s with a final extension at 72°C for 10 min. The raw paired-end (PE) reads data was obtained through the Illumina Miseq platform.

### *Bioinformatics Analysis*

After sample splitting of PE reads obtained by MiSeq sequencing, quality control and filtering of double-ended reads were performed based on sequencing quality. In brief, use FASTP and FLASH to optimize and filter the data. The bases of the tail quality value of reads below 20, the reads below 50 bp, and the reads containing N bases were filtered. The sequence information for each sample is presented in Supplementary Material Table S1.

Qualified tags were denoised into amplicon sequence variants (ASVs) by DADA2 (Callahan et al., 2016, p. 2) or Deblur (Amir et al., 2017). The BLAST tool (Altschul et al., 1990) was used to classify all sequences into different taxonomic groups against the Silva138 database (Quast et al., 2013). The Alpha diversity analysis of microbial communities reflects the species richness and the diversity of microorganisms in the samples including the Chao index, Sobs index, Shannon index, and Simpson index. QIIME (v1.8.0) was used to calculate Alpha diversity based on the ASV information (Caporaso et al., 2010). To examine the similarity between different samples, each sample analyzed clustering and PCoA by R (v3.6.0) (Wang et al., 2012).

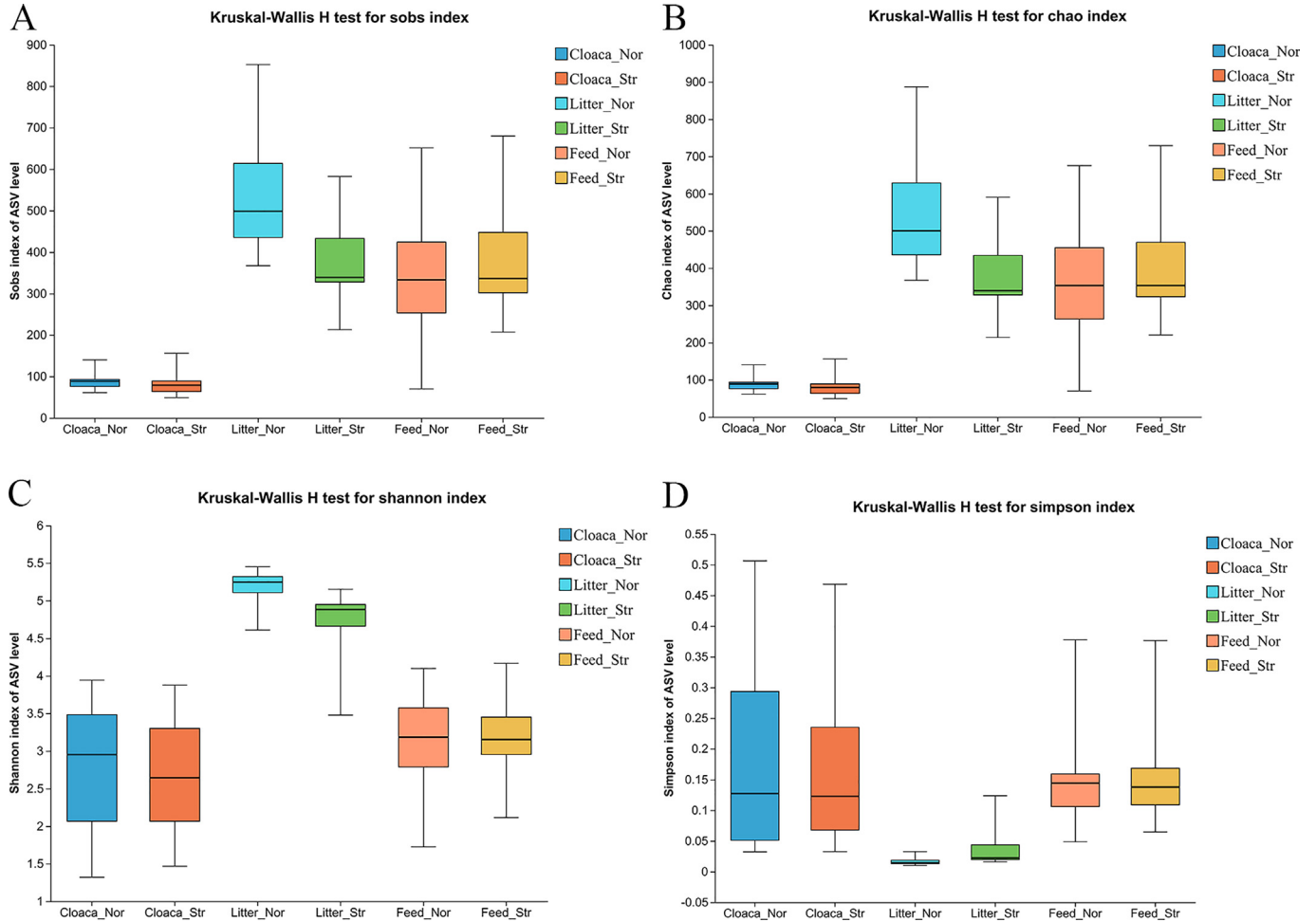
### *Statistical Analysis*

The community structure data of microbial flora was presented in the form of an average. The Chao index, Sobs index, Shannon index, and Simpson index were analyzed by one-way ANOVA, and Duncan's multiple comparisons were performed for those with significant differences. Kruskal-Wallis rank sum test was used for the differences in microbial community structure. R (v3.6.0) software was used for basic statistical analysis.

## RESULTS

### *Sequencing Output and Alpha Diversity of the Samples*

The 16S rRNA gene sequencing analysis produced a total of 3,929,490 quality-filtered sequences from 90



**Figure 1.** Comparison of the microbiome Alpha diversity in the cloacal swabs, the litter and the feed of female ducks that produced striped eggs and normal eggs, respectively. (A) Sobs index, (B) the Chao index, (C) Shannon index (D) Simpson index of Alpha diversity at the ASV level. Boxes denote the interquartile (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the line inside denotes the median. Whiskers denote the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively. Anal\_Nor, Litter\_Nor, and Feed\_Nor are cloacal swab samples, litter samples, and feed samples of female ducks that produced normal eggs, respectively. Anal\_Str, Litter\_Str, and Feed\_Str are cloacal swab samples, litter samples, and feed samples of female ducks that produced striped eggs.

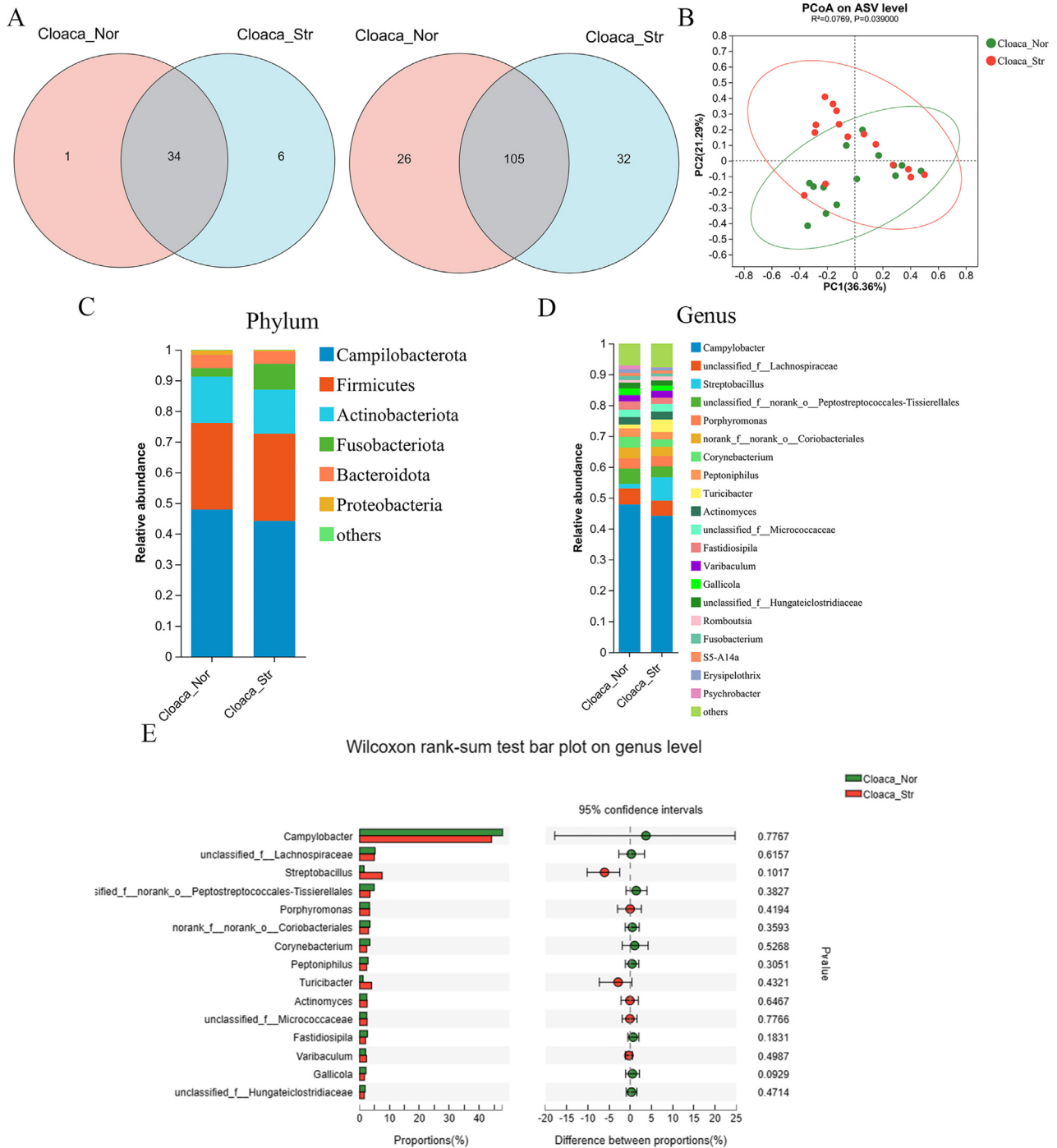
samples with an average length of 416 bp. Denoizing was performed on the optimized data, and the statistical sequence information was shown in Table S2. Comparing ASVs numbers between groups, the number of ASVs in cloacal swabs of female ducks that produce striped eggs and normal eggs was significantly different, the number of ASVs in the litter of female ducks was similar, and the number of ASVs in the feed was little difference (Table S3). We subsequently classified the ASVs into 1,666 species, 983 genera, 395 families, 207 orders, 85 classes, and 31 phyla. The Alpha diversity indices showed a significant difference in ASVs richness between striped duck eggs and normal duck eggs (Figure 1, Table S4). Noticeably, there were no significant differences in cloacal swabs and feed, while there were significant differences in the litter between the Sobs index and the Chao index ( $P < 0.05$ ).

### Differences in the Diversity and Composition Feature of Cloacal Microbiota

A Venn diagram was created to show the number of orders and genera shared or unique to cloacal swabs of

female ducks that produced striped eggs and normal eggs (Figure 2A). At the order level, the microbiota of the Cloaca\_Nor and Cloaca\_Str groups shared 34 orders, whereas 1 and 6 orders were uniquely identified in the Cloaca\_Nor and Cloaca\_Str. At the genus level, the cloacal microbiota of the two groups shared 105 genera, with 26 and 32 genera uniquely identified in the Cloaca\_Nor and Cloaca\_Str group, respectively. The principal coordinates analysis (PCoA) of ASVs indicated that the microbiota is an insignificant difference between the striped egg ducks and normal egg ducks (Figure 2B).

Campylobacter, Firmicutes, and Actinobacteriota were the dominant phyla in cloacal swabs (Figure 2C). At the genus level, *Campylobacter* (47.87% in the Cloaca\_Nor group and 44.17% in the Cloaca\_Str group), *Lachnospiraceae* (5.20% in the Cloaca\_Nor group and 4.95% in the Cloaca\_Str group), and *Streptobacillus* (1.51% in the Cloaca\_Nor group and 7.57% in the Cloaca\_Str group) were enriched in cloacal swabs (Figure 2D). Then the difference analysis found that there was no significant difference between Cloaca\_Nor and Cloaca\_Str among the top 15 genera (Figure 2E).



**Figure 2.** Compositions of the cloacal microbiota of female ducks that produced the striped eggs and normal eggs. (A) The number of orders and genera shared by the two groups are shown in Venn diagrams. (B) The principal coordinates analysis (PCoA) plot at the ASV level. (C) Relative abundances in the dominant phyla across the cloacal microbiota. (D) Relative abundances of dominant genera across the cloacal microbiota. Only the genera with an abundance of >1.0% in any segment were plotted. (E) The genera represented at significantly different levels in the microbiota of the two groups were shown in an extended error bar plot. The differences in the compositions were tested using Wilcoxon rank-sum test, and  $P < 0.05$  was marked with “\*”.

### Differences in the Diversity and Composition Feature of Litter Microbiota

At the order level, the microbiota of the Litter\_Nor and Litter\_Str groups shared 113 orders, 32 orders were unique to Litter\_Nor and 21 orders were unique to Litter\_Str (Figure 3A). At the genus level, the microbiota of the Litter\_Nor and Litter\_Str groups shared 441 genera, whereas 189 and 85 genera

were uniquely identified in the Litter\_Nor group and the Litter\_Str group. The PCoA of ASVs indicated that the litter microbiota was different in female ducks that produced striped eggs and normal eggs (Figure 3B).

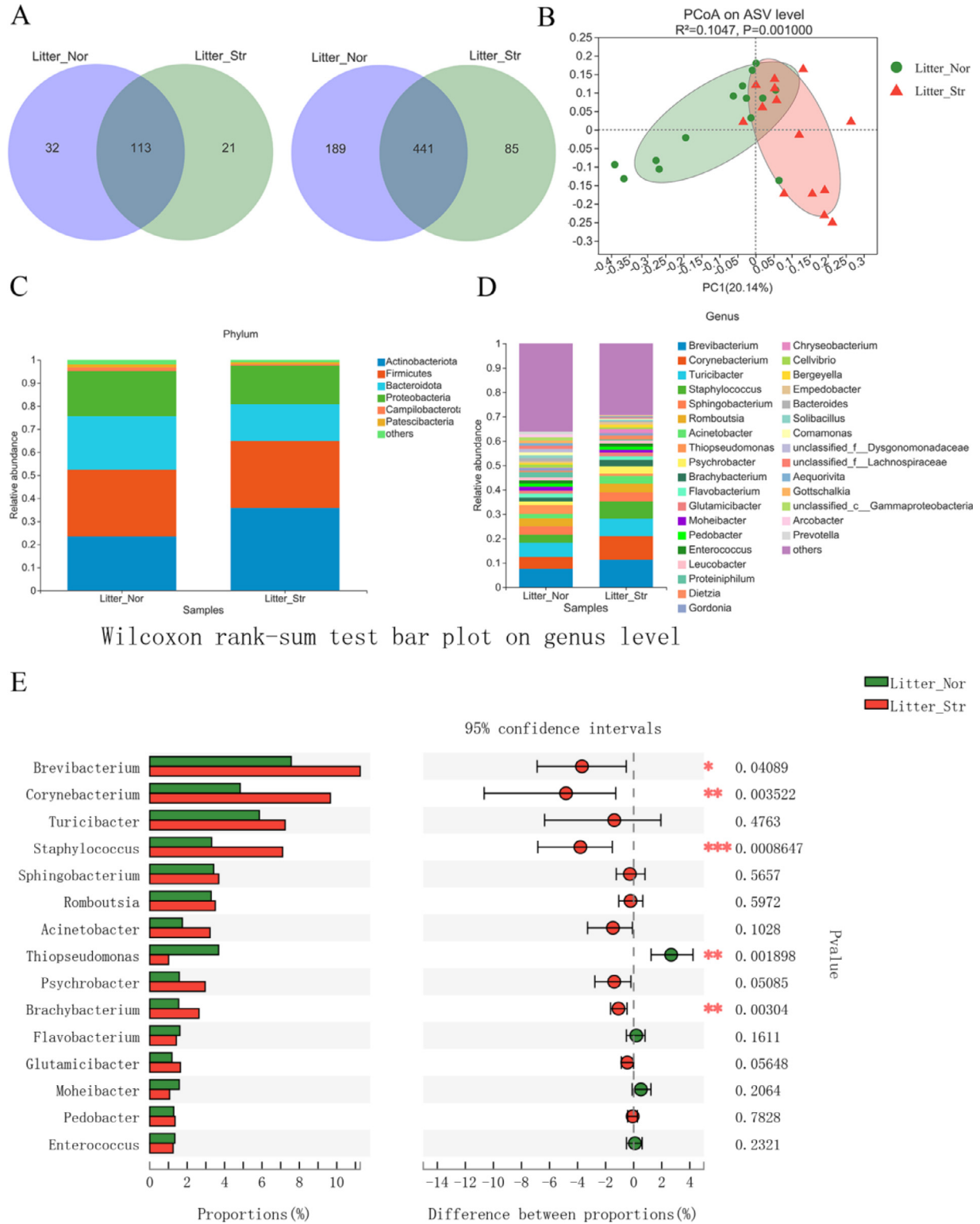
Firmicutes, Actinobacteria, and Bacteroidota were the main components of the microbial community, and the total proportion of the three was more than 70% (Figure 3C). At the genus level, the 4 dominant genera



detected in both groups were *Brevibacterium* (7.56% in the Litter\_Nor group and 11.25% in the Litter\_Str group), *Corynebacterium* (4.84% in the Litter\_Nor group and 9.66% in the Litter\_Str group), *Turicibacter* (5.86% in the Litter\_Nor group and 7.24% in the

Litter\_Str group) and *Staphylococcus* (3.31% in the Litter\_Nor group and 7.10% in the Litter\_Str group) (Figure 3D).

Then, we compared the relative abundance of microbiota between the two groups and found significantly



**Figure 3.** Compositions of the litter microbiota of female ducks that produced striped eggs and normal eggs. (A) The number of orders and genera shared by the two groups are shown in Venn diagrams. (B) The principal coordinates analysis (PCoA) plot at the ASV level. (C) Relative abundances in the dominant phyla of the litter microbiota. (D) Relative abundances of dominant genera of the litter microbiota. Only the genera with an abundance of > 1.0% in the litter were plotted. (E) The genera represented at significantly different levels in the microbiota of the two groups were shown in an extended error bar plot. The differences in the compositions were tested using Wilcoxon rank-sum test, and  $P < 0.05$  was marked with “\*”.

different phyla and genera ( $P < 0.05$ ). Between Litter\_Nor and Litter\_Str, Actinobacteria, Bacteroidota, Campylobacter, Patensibacteria, Desulfobacterota, and Fibrobacterota were significantly different (Figure S1). At the genus level, the abundance of *Corynebacterium*, *Brevibacterium*, *staphylococcus*, *Thiopseudomonas*, and *Brachybacterium* in the litter of female ducks that produced striped eggs was significantly higher than that produced normal eggs (Figure 3E).

### **Differences in the Diversity and Composition Feature of Feed Microbiota**

At the order level, the microbiota of the Feed\_Nor and Feed\_Str groups shared 117 orders, whereas 27 orders were unique to Feed\_Nor and 30 orders were unique to Feed\_Str (Figure 4A). At the genus level, there were shared 431 genera in the feed microbiota, whereas 154 and 158 genera were uniquely identified in the Feed\_Nor group and the Feed\_Str group (Figure 4A). PCoA was used to compare the total microbial composition of the Feed\_Nor and Feed\_Str groups at the ASV, but the microbial communities were not separated (Figure 4B).

Firmicutes, Cyanobacteria, and Actinobacteria were the main components of the microbial community, and the total proportion of the three was more than 92% (Figure 4C). At the genus level, The dominant genera detected in both groups were *Staphylococcus* (61.65% in the Feed\_Nor group and 51.53% in the Feed\_Str group), *Brevibacterium* (2.11% in the Feed\_Nor group and 1.68% in the Feed\_Str group), *Corynebacterium* (2.53% in the Feed\_Nor group and 2.92% in the Feed\_Str group), and *Chloroplast* (13.64% in the Feed\_Nor group and 21.68% in the Feed\_Str group) (Figure 4D). Then, we found some significantly different phyla and genera between the 2 groups ( $P < 0.05$ ). Between Feed\_Nor and Feed\_Str, Myxococcota was significantly different at the phylum level ( $P < 0.05$ , Figure S2). At the genus level, the abundance of *Dietzia* in the Feed\_Str was significantly higher than those in the Feed\_Nor group (Figure 4E).

## **DISCUSSION**

The hatching rate and quality of breeding eggs are important to control indexes in the current breeding process, so abnormal eggs should be decreased as much as possible. Striped eggs showed significantly lower fertility and hatchability compared with normal eggs (Yuan et al., 2013). We need to understand why female ducks produced striped eggs. The microbiota of the poultry farm environment, including pathogenic bacteria, play an important role in the microbial colonization and development of the hen's gastrointestinal tract during production (Cressman et al., 2010; Wang et al., 2016). In addition to direct effects on the egg quality and safety via vertical transmission route in the intestine-oviduct-egg, intestinal microbiota and its metabolites such as

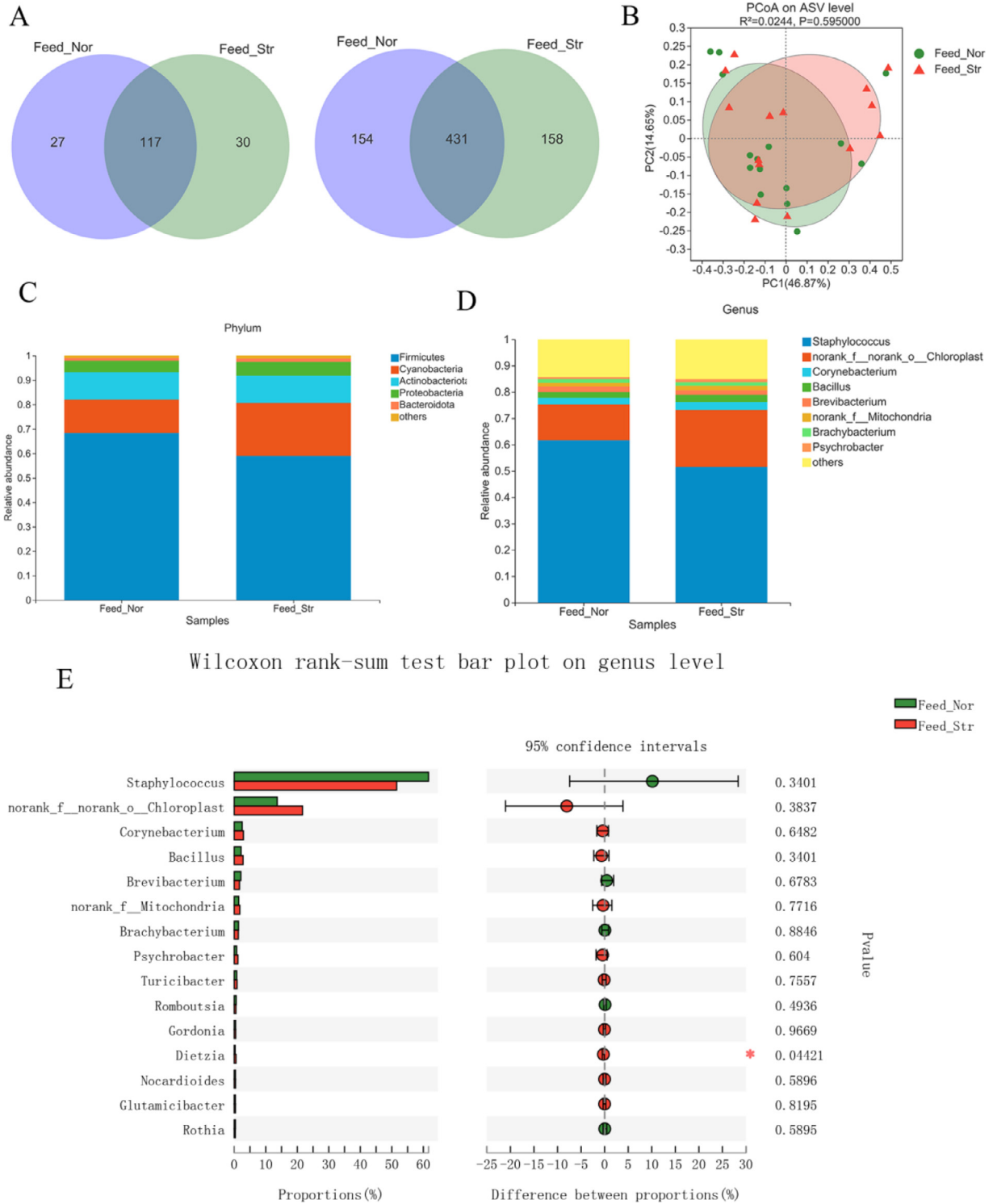
SCFAs, BA, and tryptophan derivatives are indirectly involved in regulating egg quality through the microbiota-gut-liver/brain-reproductive tract axis (Dai et al., 2022).

Alpha diversity showed that the number of microorganisms in the litter of female ducks that produced striped eggs was less than those that produced normal eggs. And there was no significant difference in the feed and cloacal swab samples. This phenomenon may be due to the maturation of some environmental microbial communities, which leads to the reduction of microbial communities through the competition of microbial communities and the filtering effect of the environment (Bearson et al., 2013; Rivera-Chávez and Bäumlner, 2015; Redweik et al., 2019; Rogers et al., 2021). Lower microbial diversity indicates a reduced ability of the microbiota to maintain metabolic and cellular functionality (Lee and Hase, 2014; Polansky et al., 2015). The results of PCoA showed that there was no significant difference in the microbial community between the control group and the experimental group of the cloacal swabs and feed, while there was a significant difference between the control group and the experimental group of the litter. It may indicate that the litter microbiota is not only affected by fecal microbiota, but also by other factors such as the environment.

We identified specific microbes in the feed, litter, and cloacal swabs at the phyla and genus levels of taxa to provide novel information regarding the diversity and composition of microbiota about striped eggs. In this study, Firmicutes and Actinobacteria are common dominant phyla. Firmicutes participate in the metabolism of energy substances and play an important role in the digestion of food (Mariat et al., 2009; Ma et al., 2018). Actinobacteria has a vast pharmacological potential that remains unopposed among other microbial groups (Puttaswamygowda et al., 2019).

At the genus level, *Campylobacter*, *Lachnospiraceae*, and *Streptobacillus* were enriched in cloacal swabs. *Brevibacterium*, *Corynebacterium*, and *Turicibacter* were enriched in the litter. The feed riched in *Staphylococcus*, *Brevibacterium*, *Corynebacterium*, and *Chloroplast*. The dominant genera in the litter and feed samples were similar. It indicates that litter microbiota and feed microbiota may interact with each other (Radon et al., 2002; Weidhaas et al., 2010; O'Brien et al., 2016). No differential bacterial genera were found in the cloacal swab microbiota, suggesting that cloacal swab microbes are not the main factor responsible for striped eggs. The differences in the litter and feed microbiota between groups may be related to the striped eggs. *Corynebacterium*, *Brevibacterium*, *staphylococcus*, *Thiopseudomonas*, and *Brachybacterium* were significantly different between striped eggs and normal eggs.

Previous studies have shown that *Corynebacterium* can cause severe respiratory disease (Trost et al., 2012; Sangal and Hoskisson, 2016; David and Daum, 2017; Timms et al., 2018). Meanwhile, high levels of *Corynebacterium* in the air of duck houses can significantly affect duck performance, leading to increased immune



**Figure 4.** Compositions of the feed microbiota of female ducks with striped eggs and normal eggs. (A) The number of orders and genera shared by the two groups were shown in Venn diagrams. (B) The principal coordinates analysis (PCoA) plot at the ASV level. (C) Relative abundances in the dominant phyla across the feed microbiota. (D) Relative abundances of dominant genera across the feed microbiota. Only the genera with an abundance of >1.0% in any segment were plotted. (E) The genera represented at significantly different levels in the microbiota of the two groups were shown in an extended error bar plot. The differences in the compositions were tested using Wilcoxon rank-sum test, and  $P < 0.05$  was marked with “\*”.

suppression and disease susceptibility (Martin et al., 2010; Wu et al., 2019; Zhu et al., 2022). *Staphylococci* were widely distributed and were found in many parts of healthy birds, including skin, mucous membranes, and intestines (Szafraniec et al., 2020). Previous studies

have found that *Staphylococcus aureus* can not only cause skin and soft tissue infection but also cause genital tract abscess and microbiota disorder, thereby causing the occurrence of endometritis (David and Daum, 2017). The uterus and reproductive tract of female ducks

producing striped eggs also showed lesions and inflammation, and *Staphylococcus* may be one of the factors. In addition, previous studies have found the presence of *Brevibacterium* in poultry feces (Weidhaas et al., 2010; Ryu et al., 2014). *Brevibacterium* in the litter may come from duck feces. At present, there are many diseases caused by *Brevibacterium*, such as osteomyelitis and sepsis (Funke et al., 1997; Riegel, 1998; Peel et al., 2022). We hypothesize that different microbiota in the feed and litter may contribute to the production of striped eggs by influencing host health.

## CONCLUSION

The 16S rRNA gene sequencing results showed that the microbial community structure was different in the litter and feed of female ducks that produced striped eggs and normal eggs. The abundance of *Staphylococcus*, *Corynebacterium*, and *Brevibacterium* in the litter of female ducks that produced striped eggs was significantly higher than those of normal eggs. Therefore, the increase of these different genera may affect the production function of female ducks and lead to striped egg production. These observations provide insights into the causes of the production of striped eggs, and more studies should be conducted in the future.

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Authors' contributions: LZ wrote the paper and performed data analysis; SQQ performed research and analyzed data; YX and LY performed research; ZCH designed this study and revised the manuscript. All authors edited the manuscript and agreed with its final form. All authors read and approved the final manuscript

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Ethics approval and consent to participate: The animal care and experimental procedures were approved by the Animal Care and Use Committee of China Agricultural University and adhered to the university's guidelines for animal research.

## DISCLOSURES

The authors declare that they have no competing interests, and the manuscript is approved by all authors for publication. This manuscript is original and has not been published in whole or in part previously.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.psj.2022.102436](https://doi.org/10.1016/j.psj.2022.102436).

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