

Effects of different duck rearing systems on egg flavor and quality and microbial diversity

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ABSTRACT The fishy odor of duck eggs has restricted their consumption and industrial development, a problem that producers need to address. We estimated the effects of cage, floor, and pond rearing systems on duck egg flavor, egg quality, and microbial diversity by evaluating yolk trimethylamine (TMA) content, egg quality, and the differences between duck cecum (cage cecum, **CC**; floor cecum, **FC**; pond cecum, **PC**) and the environment (cage environment, **CE**; floor environment, **FE**; pond environment, **PE**). The results show that the yolk TMA content of the floor-rearing and pond-rearing systems was significantly higher than that of the cage-rearing system ($P < 0.001$), with no difference between the floor and pond-rearing systems. No significant differences were detected in egg quality among the rearing systems. Firmicutes, Actinobacteria, and Bacteroidetes were the dominant phyla in the

cecum, and in the rearing environment, Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria were the dominant phyla. The results of α and β diversity analyses show that changes in the rearing system affected the composition and diversity of duck cecal microbes. In addition, we screened several genera that may be related to the production of TMA in duck cecum under different rearing systems using LEfSe analysis; for example, *Subdoligranulum* in the CC group; *Romboutsia* in the FC group; and *Lactobacillus*, *Clostridium*, and *Streptococcus* in the PC group. In conclusion, the rearing system affects the cecal microbes of ducks, which in turn affect the deposition of TMA in duck eggs but have no adverse effect on egg quality. This study provides a basis for the development of rearing strategies to reduce the fishy odor of egg yolk in the duck industry.

Key words: duck, rearing system, egg flavor, egg quality, microorganisms

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INTRODUCTION

Duck eggs are common among poultry eggs and are mainly distributed in Asia, with China accounting for the largest proportion. Duck eggs are the second most widely consumed poultry eggs worldwide. Duck eggs are an inexpensive and high-quality source of nutrients, particularly proteins, iron, vitamins, and phosphorus (Thammarat et al., 2008). In addition, compared to laying hens, laying ducks have the advantages of stronger disease resistance and lower mortality during feeding (Smith et al., 2015). According to the Food and Agriculture Organization of the United Nations (FAO), the total egg production (excluding chicken eggs) has been

rising steadily worldwide since the early 20th century (Windhorst, 2006). Compared with chicken eggs, duck eggs are richer in Omega-3 fatty acids and have high potential economic value (Sinanoglou et al., 2011; Yang et al., 2020). However, in most cases, duck eggs have a stronger fishy odor than other poultry eggs, and are not preferred by consumers (Li et al., 2017). Therefore, the fishy odor of duck eggs is the main limiting factor for their consumption and commercial development.

Factors such as feed, genetics, and intestinal microbes can all contribute to the fishy odor of poultry eggs. The fishy odor may result from feeding genetically deficient laying hens high levels of choline and n-3 polyunsaturated fatty acids (Fraeye et al., 2012; Goldberg et al., 2016). TMA precursors, such as lecithin, betaine, and carnitine, as well as choline-rich food, are degraded into TMA by microorganisms in the intestines of animals (Zeisel et al., 1989; Zhang et al., 1999). TMA is primarily oxidized to trimethylamine oxide (TMAO) in the liver

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after absorption (Acara et al., 1977). However, studies have shown that once the metabolic pathway is blocked, TMA is deposited in the eggs, resulting in a fishy odor (Butler and Fenwick, 1984). Li et al. (2019) showed that the high content of TMA in egg yolk is the main factor leading to fishy odor in duck eggs.

The microbial flora of the poultry farm environment plays an important role in the microbial colonization and development of the poultry gut (Wang et al., 2016; Kers et al., 2018). The gut microbiota of poultry develops during the early stages of life (Yadav and Jha, 2019). In commercial production, chicks do not develop microbiota through contact with adult hens after hatching (Rychlik, 2020). Young chicks are transported from the hatchery to the chicken house as soon as they are born, and their gut microbiota is therefore very simple and contains a very small number of bacteria belonging to a few species (Cox et al., 2012). Chicks are exposed to several bacterial sources that can enter the immature intestine. These exogenous bacterial sources include feed, water, and ambient air in the chicken house (Locatelli et al., 2017; Zuzana et al., 2020). As chicks grow, their gut microbiota becomes increasingly diverse and complex (Wei et al., 2013). Different rearing environments affect the diversity, composition, and structure of the gut microbiota in poultry (Cui et al., 2017).

To improve the production efficiency of duck eggs, rearing of laying ducks has gradually changed from traditional free-rearing to cage rearing to achieve large-scale intensive production. Most previous studies have focused on the effects of different rearing systems on the feed conversion rate and production performance of laying ducks (Zhang et al., 2018; Bai et al., 2022). However, few studies have explored the effects of different rearing systems on the differences in egg flavor and gut microbiota of laying ducks. The overall aim of this study was to compare the diversity, composition, and structure of the microbiota of ducks in cages, ponds, and floor duck rearing systems using 16S rRNA amplicon sequencing techniques. In addition, we determined the TMA content in the yolks of duck eggs and egg quality in the 3 rearing systems. This study also sought to investigate the microbial taxa associated with TMA levels in egg yolks. The results of this study would be useful in improving the flavor of duck eggs and promoting duck production by changing the rearing system.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures were conducted following the Guidelines for Experimental Animals established by the Animal Care and Use Committee of China Agricultural University (permit number: AW52501202-1-1). Animal studies were reported in compliance with the ARRIVE guidelines (Percie et al., 2020).

Sample Collection

All experimental samples were collected from Hubei Shendan duck farms (Hubei, China) with cage, floor, and pond-rearing systems in August 2021. The cage-rearing system for laying ducks adopted three-tiered stepped cages. The duck cage was 400 mm deep and 400 mm high, and each cage housed 2 ducks. The duck house was equipped with feeding, nipple water, manure removal, ventilation, and lighting systems. The floor-rearing duck house was semi-open, with sunshade, using the online floor-rearing method, laying bran shells as bedding, and equipped with a nipple water supply system. The duck density of the floor-rearing system is 0.20 m²/duck. The pond-rearing system adopts an open culture mode, equipped with the feeding trough. The duck density of the pond-rearing system is 0.08 m²/duck. The duck breed, age (weeks), and diets of the 3 rearing systems were the same. The composition and nutritional level of the basic diet are shown in Table 1. We collected 80 duck eggs from each rearing system, and the 240 duck eggs collected were used to determine egg quality and TMA content in yolk. We randomly selected seven healthy ducks (Shaoxing, 50 wk, 1.40 ± 0.25 kg) from each rearing system and collected their cecal feces. In addition, 9 environmental samples were collected from the different rearing systems. We used sterile cotton swabs to wipe the ground at 3 different positions in each rearing environment. Following collection, cecal samples and environmental samples were immediately snap-frozen in liquid nitrogen and stored at −80°C.

Yolk TMA Content Determination

Thirty duck eggs from each rearing system were used to determine the TMA content in the egg yolk, following those previously described for quail (Mo et al., 2013). The yellow TMA-N-picric acid complex obtained was measured at 410 nm using an ultraviolet spectrophotometer (Infinite 200 Pro, Beijing, China). A standard curve

Table 1. Composition and nutrient levels of basal diet (% air-dry basis).

Items	Dietary energy	Items	Dietary energy
Ingredients		Nutrient levels	
Corn	47.40	Metabolic energy (Mcal/kg)	2.60
Soybean meal	12.00	Crude protein	17.06
Rice bran	5.00	Crude fat	2.61
Corn gluten meal	1.60	Ca	4.35
Sunflower seed meal	11.00	Lysine	0.89
Sesame meal	3.00	Methionine	0.26
wheat shorts	10.00	Methionine + Cystine	0.53
Limestone	5.80	Available phosphorus	0.45
Fine stone	2.00		
Vitamin premix ¹	1.40		
Calcium hydrogen phosphate	0.80		

¹Provided per kg of feed: Vitamin A, 10,000 IU; Vitamin D, 32,500 IU; Vitamin E, 30 mg; Vitamin B1, 1 mg; Vitamin B2, 4 mg; Vitamin B6, 3 mg; Vitamin B12, 15 mg; Pantothenic acid, 8 mg; Niacin 30 mg; Folic acid, 0.5 mg; Biotin 25 μg; Fe, 25 mg; Cu, 5mg; Mn, 100 mg; Zn, 60 mg; Se, 0.2 mg; I, 0.5 mg; Co, 0.1 mg.

(TMA concentration = $0.0308 \times A + 0.2315$; A = absorbance at 410 nm, $R^2 = 0.9965$) was produced from 10 TMA standard solutions (0, 0.5, 1, 2, 4, 5, 6, 8, and 10 $\mu\text{g}/\text{mL}$ of TMA-N) to calculate the TMA-N concentration in the egg yolk. The TMA content was then presented as TMA-N in the egg yolk.

Egg Quality Determination

Fifty duck eggs were collected from each rearing system to determine egg quality, including egg weight (EW), egg white height (AH), Haugh units (HU), yolk color (YC), egg shape index (ESI), eggshell strength (ESS), and eggshell thickness (EST). The EW, AH, HU, and YC of duck eggs were measured using a multifunctional egg tester (Robotmation EMT-5200; Tokyo, Japan). The ESS were measured using a Model-II eggshell strength tester (Robotmation). The ESI was measured using an egg-shaped index tester. EST was measured at 3 locations: the lower, middle, and upper ends, using a micrometer screw gauge.

Microbiota DNA Extraction and 16S rRNA Amplicon Sequencing

Microbial genomic DNA was extracted from cecal and environmental samples using a Stool DNA kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's instructions. The DNA extract was analyzed on a 1% agarose gel, and DNA concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, NC). The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCC-TACGGGAGGCAGCAG-3') and 806R (5'-GGAC-TACHVGGGTWCTAAT-3') using an ABI GeneAmp 9700 PCR thermocycler (ABI, CA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and finally at 4°C. The PCR mixtures contained 4 μL 5 \times TransStart FastPfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL forward primer (5 μM), 0.8 μL reverse primer (5 μM), 0.4 μL TransStart FastPfu DNA Polymerase, 10 ng template DNA, and ddH₂O to 20 μL . PCR was performed in triplicate. The PCR product was extracted from a 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) according to the manufacturer's instructions, and quantified using a Quantus Fluorometer (Promega, Beijing, China).

Bioinformatic Analysis

The purified amplicons were sequenced on an Illumina HiSeq 2000 according to the protocols of Majorbio BioPharm Technology (Shanghai, China) and Wekemo Tech Group (Shenzhen, China). Quality control and

operational taxonomic unit (OTU) binning were performed using QIIME and UPARSE, and the sequences were clustered with 97% similarity. Sequence files were analyzed using QIIME2 software (Bolyen et al., 2019). Denoising, chimera removal, and amplicon sequence variant (OTU) table generation were performed using DADA2 (Callahan et al., 2016). Alpha diversity was calculated using the observed OTUs. Chao1, ACE, Shannon–Wiener diversity (H), and inverse Simpson indices were computed using the phyloseq v1.26.1 package in R v1.2.1335 (McMurdie and Holmes, 2013). Beta diversity patterns were explored by performing principal coordinate analysis (PCoA) with phylogeny-based (UniFrac) weighted distances between samples. The differences in abundance were tested using LEfSe. All statistical analyses were performed at $\alpha = 0.05$.

Statistics Analysis

Egg quality data were analyzed using Statistical software (SPSS 25.0; Chicago, IL) for analysis of variance and Duncan's tests. Statistical significance was set at $P < 0.05$. The basic descriptive statistical results are presented as the mean and standard deviation (mean \pm SD). Differences between multiple groups were compared using analysis of variance (ANOVA). Data plot analysis was performed using the ggplot2 package in R software (version 4.0.2).

RESULTS

Effects of Different Rearing Systems on TMA Content in Egg Yolk

The TMA content in duck egg yolks from the 3 rearing systems is shown in Figure 1. The contents of TMA in egg yolks from cage, floor, and pond-rearing systems were $3.17 \pm 0.36 \mu\text{g}/\text{g}$, $4.56 \pm 0.56 \mu\text{g}/\text{g}$, and $4.79 \pm 0.57 \mu\text{g}/\text{g}$, respectively. There was no significant difference in TMA in the egg yolks between the floor and pond groups. The TMA content in the egg yolk in the cage group was significantly lower than that in the floor and pond groups ($P < 0.001$).

Effects of Different Rearing Systems on Egg Quality

The egg quality results for the 3 rearing systems are shown in Table 2. There were no significant differences among the groups in the ESS, AH, or HU indices. There was no difference in EW between the floor and cage groups or between the floor and pond groups, but there was a significant difference between the cage and pond groups ($P < 0.05$). There were significant differences in ESI between the cage, floor, and pond groups. There was no significant difference in the YC between the cage and pond groups, but significantly higher in these than the floor group that in the floor group ($P < 0.05$). The EST of the floor group was significantly higher than

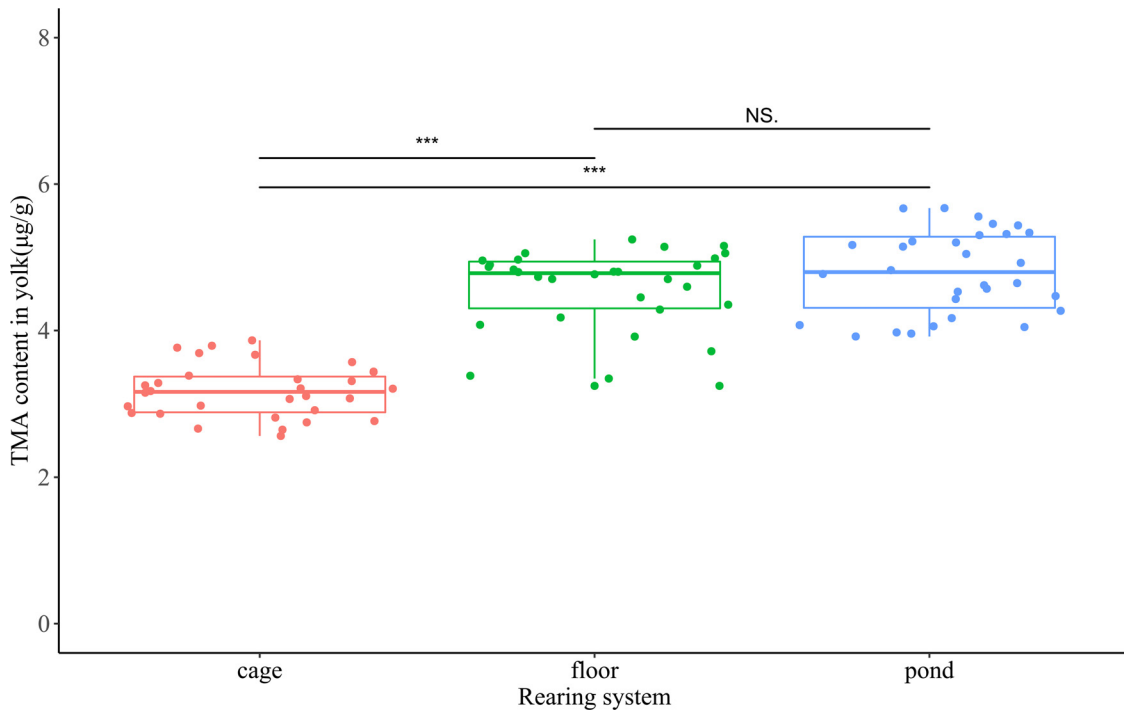


Figure 1. Effect of different rearing systems on the TMA content in duck egg yolk (** $P < 0.001$). Abbreviation: TMA, trimethylamine.

that of the cage and pond groups ($P < 0.05$); however, there was no difference between the cage and pond groups.

Microbial Composition of Cage, Floor, and Pond-Rearing Systems Based on 16S rRNA Amplicon Sequencing

A total of 1,497,671 pairs of reads were obtained from sequencing all cecal samples and environmental samples, and 979,758 clean reads were produced after quality control and splicing of double-ended reads. Following amplicon denoising and filtering, 698 OTU were identified, 307 of which were shared by cecal and environmental samples. The microbial community structures of the cecal and environmental samples are shown in Figure 2.

Figures 2A and 2C show the relative abundance of the microbial composition at the phylum level. The cecal samples in the cage, floor, and pond-rearing systems were dominated by 4 bacterial phyla: *Firmicutes* (51.51, 42.19, and 51.47%, respectively), *Bacteroidetes* (39.87, 23.51, and 11.61%, respectively), *Actinobacteria* (3.22, 5.83, and 5.56%, respectively), and *Deferribacteres*

(1.49, 4.29, and 0.41%, respectively; Figure 2A). Among them, the relative abundance of *Firmicutes* in the CC and PC groups was higher than that in the FC group ($P < 0.05$), the relative abundance of *Actinobacteria* in the CC group was lower than that in the FC and PC groups ($P < 0.05$), the relative abundance of *Bacteroidetes* in the 3 different rearing systems were all different from one another ($P < 0.05$), and the relative abundance of *Deferribacteres* species in both CC and PC groups was significantly lower than that in the floor group ($P < 0.05$). Thus, at the bacterial phylum level, changes in the rearing system had an impact on the cecal microbial composition of ducks. The environmental samples in the cage, floor, and pond-rearing systems mainly consisted of 4 bacterial phyla: *Firmicutes* (29.03, 42.21, and 7.42%, respectively), *Actinobacteria* (59.43, 57.31, and 29.62%, respectively), *Bacteroidetes* (7.64, 0.06, and 8.14%, respectively), and *Proteobacteria* (3.86, 0.33, and 38.96%, respectively; Figure 2C). Among them, the relative abundance of *Firmicutes* and *Proteobacteria* showed differences in the CE, FE, and PE groups ($P < 0.05$); the relative abundance of *Actinobacteria* in the CE and FE groups was higher than that in the PE group ($P < 0.05$), and the relative abundance of *Bacteroidetes*

Table 2. Effect of different rearing systems on the egg quality.

Groups	Items						
	EW (g)	ESI	ESS (kg/cm ²)	AH (mm)	HU	YC	EST (µm)
Cage	69.77 ± 5.42 ^a	1.31 ± 0.04 ^c	3.61 ± 0.75	6.63 ± 1.39	78.20 ± 9.35	13.14 ± 0.45 ^a	323.66 ± 27.80 ^b
Floor	67.901 ± 4.86 ^{ab}	1.34 ± 0.05 ^b	3.72 ± 0.71	6.43 ± 1.34	75.89 ± 11.15	11.77 ± 0.60 ^b	339.63 ± 21.28 ^a
Pond	66.31 ± 4.10 ^b	1.36 ± 0.05 ^a	3.52 ± 0.66	6.49 ± 1.25	77.08 ± 9.82	13.29 ± 0.43 ^a	324.31 ± 28.70 ^b
<i>P</i> -value	0.002	<0.001	0.364	0.753	0.526	<0.001	0.004

Abbreviations: AH, egg white height; EW, egg weight; ESI, egg shape index; ESS, eggshell strength; EST, eggshell thickness HU, Haugh units; YC, yolk color.

^{a-c}In the same column, means with different superscripts indicate significant differences ($P < 0.05$). Data are expressed as mean ± SD.

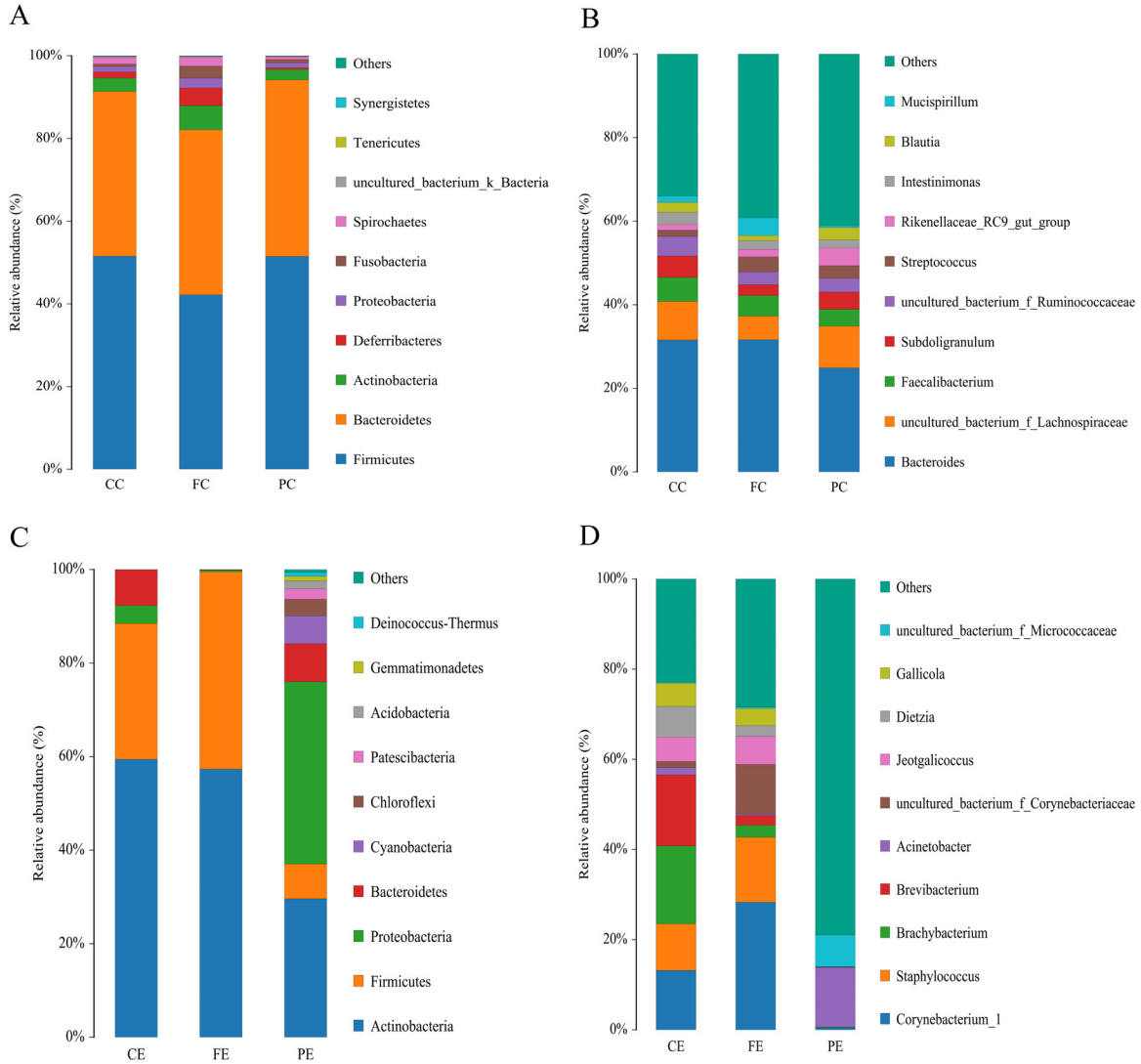


Figure 2. The relative abundance taxa in CC, CE, FC, FE, PC and PE groups. (A) and (C) Relative abundance of taxa by phylum. (B) and (D) Relative abundance of taxa by genus. Relative abundances in the top ten taxa were shown, and other taxon were combined, which was labeled “Others”. Abbreviations: CC, cage cecum; CE, cage environment; FC, floor environment; floor cecum; FE, pond environment; PC, pond cecum; and PE, pond environment.

in the CE and PE groups was higher than that in the floor group ($P < 0.05$). There were differences in environmental microbial composition among the 3 rearing systems. Other phyla present in the top 10 taxa included *Cyanobacteria*, *Fusobacteria*, *Chloroflexi*, and *Patescibacteria*.

Figures 2B and 2D show the relative abundance of the microbial composition at the genus level. The cecal samples from the cage, floor, and pond-rearing systems were dominated by 4 bacterial genera: *Bacteroides* (31.56, 31.64, 24.90%, respectively), *Faecalibacterium* (5.76%, 4.93%, and 4.04%, respectively), *uncultured_bacterium_f_Lachnospiraceae* (9.21, 5.64, and 9.96%, respectively), and *Subdoligranulum* (5.16, 2.58, 4.13%, respectively; Figure 2B). Among them, the relative abundance of *Bacteroides* in the CC and FC groups was higher than that in the PC group ($P < 0.05$), the relative abundance of *Faecalibacterium* and *Subdoligranulum* was significantly different among the CC, FC, and PC groups ($P < 0.05$), and the relative abundance of

uncultured_bacterium_f_Lachnospiraceae in the FC group was lower than that in the CC and PC groups ($P < 0.05$). The relative abundance of other genera of the top 10 taxa appearing in the three different rearing systems in the cecal samples showed variability. Changes in rearing system affected the relative abundance of duck cecal microorganisms at the genus level. The taxonomic groups with the most abundant environmental samples in the cage, floor, and pond-rearing systems were *Corynebacterium* (13.21, 28.32, and 0.43%, respectively), *Staphylococcus* (10.30, 14.38, and <0.001%, respectively), *Brachybacterium* (17.28, 2.64, and 0.18%, respectively), and *Brevibacterium* (15.76, 2.16, and <0.001%, respectively; Figure 2D). These main bacterial genera showed differences in the environments of different rearing systems ($P < 0.05$). In addition to these 4 main bacterial genera, the remaining 6 bacterial genera showed differences in their relative abundances. The composition of microorganisms in the environmental samples also showed differences at the genus level.

Alpha and Beta Diversity of Samples From Different Rearing Systems

To determine the alpha diversity of the samples, the ACE, Chao1, Simpson, and Shannon indices were measured (Figure 3C). The Shannon curves and the species accumulation curve of sequenced cecal content samples and environmental samples indicated that the sequencing depth was sufficient for all samples by their saturated trend (Figures 3A and 3B). Chao1 and ACE indices measure species abundance, that is, the number of species. Shannon and Simpson indices were used to measure species diversity, which is affected by species abundance and community evenness. No considerable variability was observed when comparing the cecal samples from different rearing systems using these 4 alpha diversity indices. The microbial richness and evenness of the floor group in the cecal samples were higher than

those of the cage and pond groups, but there was no difference among them. However, considerable variability was observed when comparing the environmental samples from different rearing systems. In the environmental samples, the microbial richness and evenness of the floor group were significantly higher than those of the cage and pond groups ($P < 0.05$), whereas no difference was detected between the cage and pond groups. Overall, microbial richness and evenness were higher in cecal samples than in environmental samples from the different rearing systems. In addition, the differences in microbial richness and evenness between cecal samples from different rearing systems were smaller than those in the environmental samples.

Beta diversity, a measure of variance in taxa composition between sampling sites, was visualized by plotting the distances between samples on a PCoA biplot (Figure 4). The first and second plot coordinates

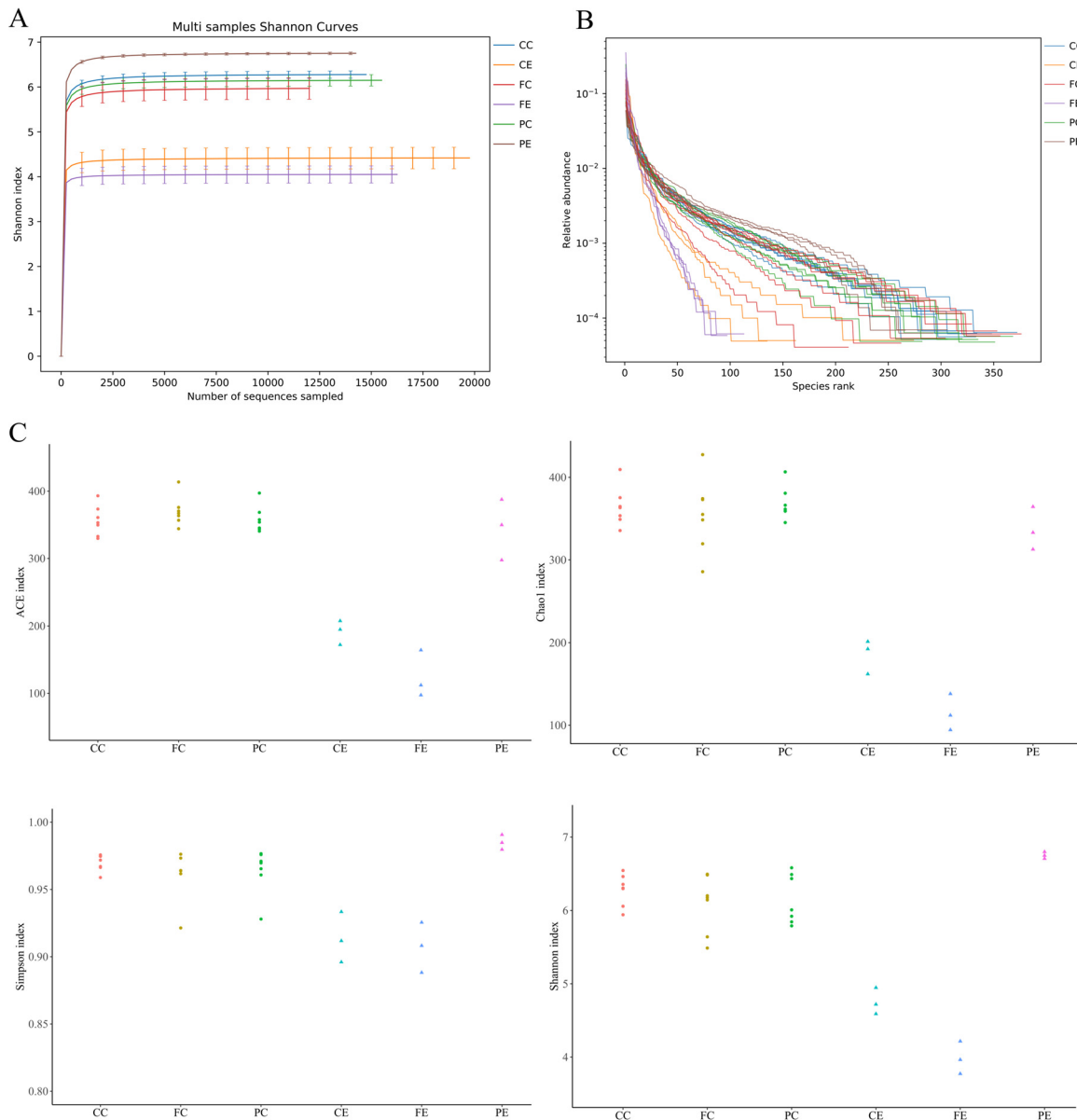


Figure 3. (A) The alpha diversity Shannon curves of 16S rRNA gene sequence to estimate the rationality of sequencing depth (at 97% similarity). (B) Species accumulation curve is used to estimate the rationality of sequencing sample quantity. (C) Alpha diversity index ACE, Chao1, Shannon, and Simpson by sampling site.

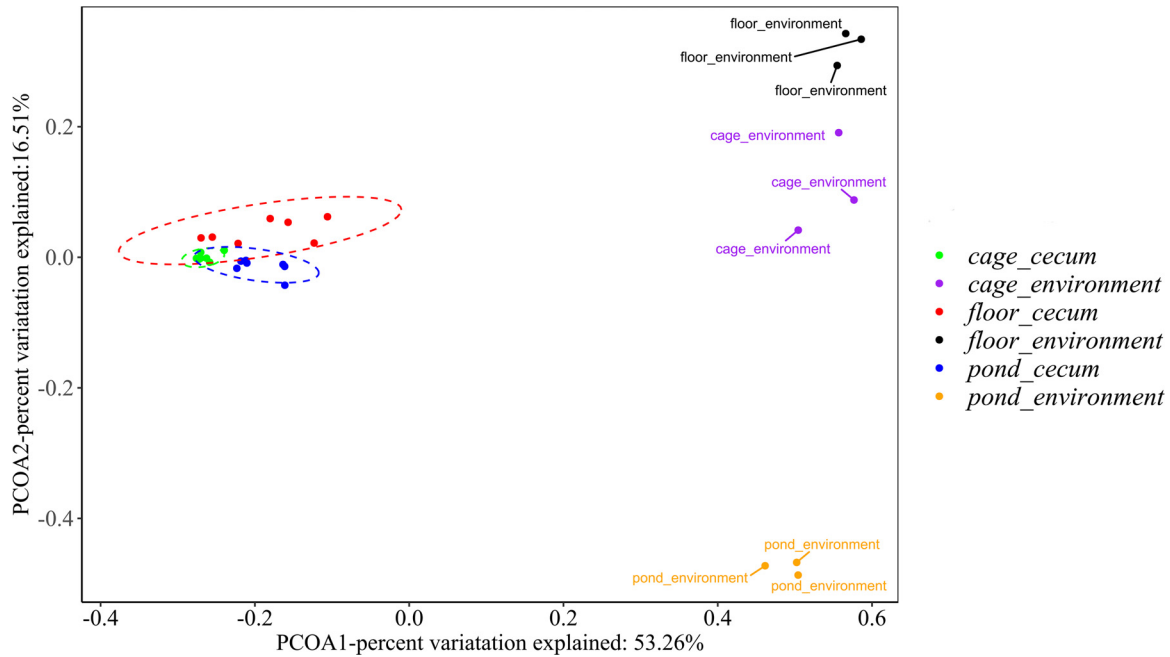


Figure 4. Compositional biplot of bacterial community composition of cecal samples and environmental samples in different rearing systems. The proportion of variation is stated in the axis titles.

represented 16.51 and 53.26% of the variation in beta diversity, respectively. It is clear from the biplot that the cecal samples were clustered separately from the environmental samples. The environmental samples from the cage, floor, and pond groups were significantly separated and clustered. The cecal samples from the cage, floor, and pond groups were also significantly separated and clustered. However, the cecal samples did not vary considerably among the cage, floor, and pond groups. This variation can be explained by the inclusion of fewer OTUs and the average lower taxonomic evenness observed in these samples.

Significant Differences Between Groups in Microbial Diversity

To determine the classified bacterial taxa with significant differences in abundance among cecal or environmental samples in different rearing systems, we performed a biomarker analysis using the linear discriminant analysis (LDA) effect size (LEfSe) method. LEfSe analysis of cecal samples is shown in Figure 5A. The PC group was characterized by a higher abundance of *Lachnospirales*, *Lachnospiraceae*, *Bacilli*, and *Lactobacillales*, whereas *Peptostreptococcales tissierellales*, *Peptostreptococcaceae*, *Romboutsia ilealis*, and *Romboutsia* were predominant in the FC group. However, the CC group was characterized by a higher abundance of *Ruminococcaceae*, *Subdoligranulum*, *Subdoligranulum variable*, and *Ruminococcaceae* (from genus to species). LEfSe analysis of environmental samples (Figure 5B) shows that *Proteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Pseudomonadales* were significantly enriched in the PE group; *Corynebacteriales*, *Corynebacterium*, *Corynebacteriaceae*, and

Corynebacterium freneyi were significantly enriched in the FE group; and in the CE group, the abundance of *Brachybacterium paraconglomeratum*, *Brachybacterium*, *Dermabacteraceae*, and *Brevibacterium* increased significantly. In addition, we identified 10 microorganisms that differed among the three groups at the genus level. Among them, the 5 microorganisms related to TMA metabolism in ducks were *Subdoligranulum* in the CC group, *Romboutsia* in the FC group, and *Lactobacillus*, *Clostridium*, and *Streptococcus* in the PC group. The relative abundances of the 5 bacteria in the cecum and the environment are shown in Figure 5C.

DISCUSSION

Fresh duck eggs usually have an unpleasant odor, and Li et al. (2017) showed that TMA is the main reason. Therefore, TMA, a quantitative indicator of this odor, can be targeted for selective breeding of ducks to produce duck eggs with low fishy odor levels. No effective method to improve the fishy odor of duck eggs has been described. Some methods have been proposed for the treatment of trimethylaminuria (TMAU) in humans. TMAU is a genetic defect that causes patients to overproduce TMA, which in turn produces a strong fishy odor through sweat, urine, and breath (Zschocke et al., 1999). As TMA production is related to dietary choline, the first step in treating TMAU is to reduce dietary choline intake. Girdwichei et al. (2015) reported that the fishy odor in the urine and sweat of TMAU patients is reduced after dietary choline restriction. However, choline deficiency can lead to liver cell damage, neurological diseases, and cancer (Wallace et al., 2018). Another method is to use oral drugs, such as neomycin and metronidazole, to relieve the symptoms of TMAU patients

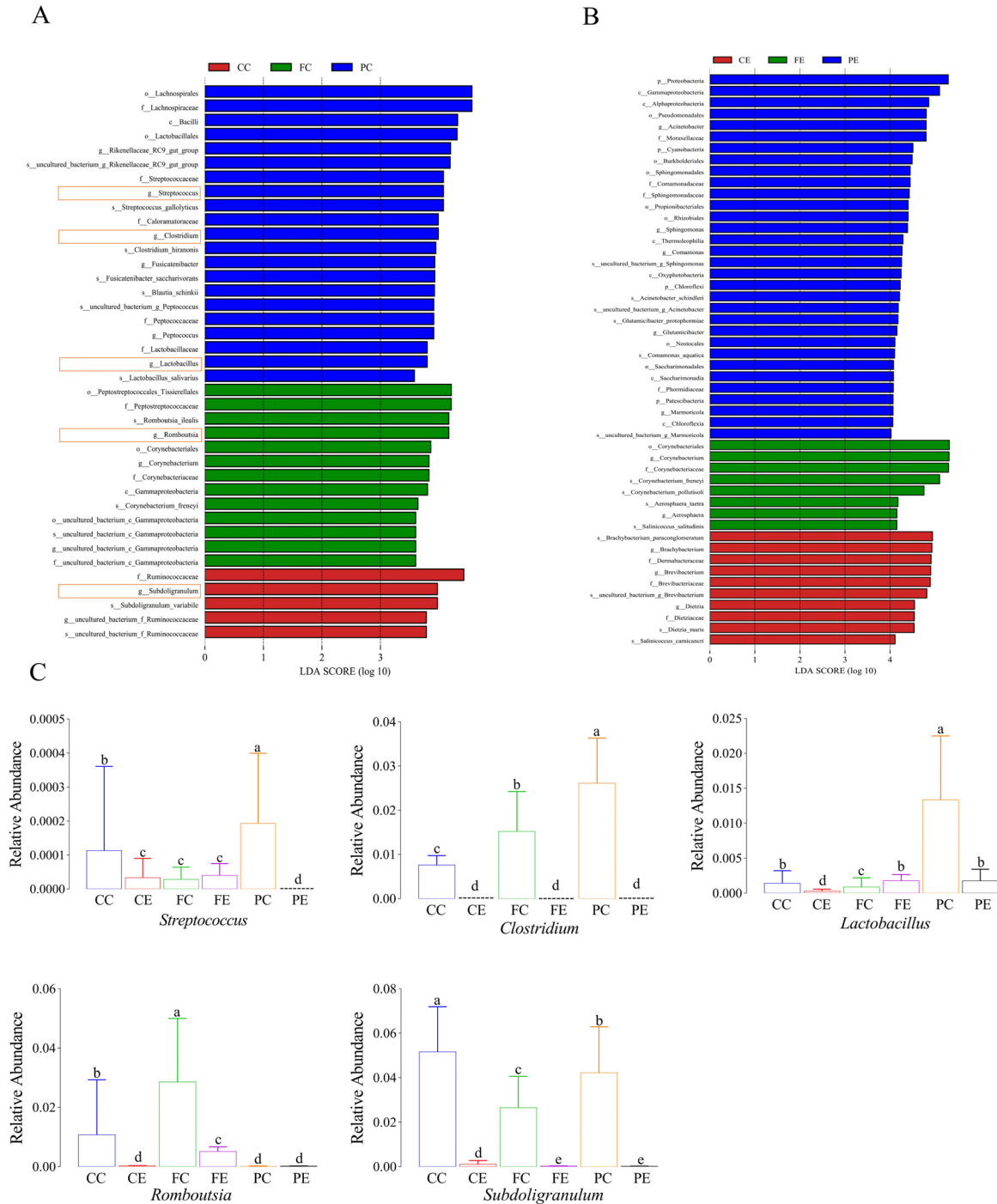


Figure 5. Cecal and environmental microbial markers in different rearing system. (A) Linear discriminant analysis (LDA) scores identified the size of cecal bacterial differentiation among the cage, floor and pond groups with a threshold of 3.5. (B) Linear discriminant analysis (LDA) scores identified the size of environmental bacterial differentiation among the cage, floor and pond groups with a threshold of 4.0. (C) At the genus level, the relative abundances of microbial communities associated with TMA production were compared using analysis of variance. Different letters indicate significant differences ($P < 0.05$). Abbreviations: p, phylum; c, class; o, order; f, family; g, genus; s, species.

(Schmidt and Leroux, 2020). The principle is that drugs inhibit some intestinal microorganisms that are related to the production of TMA, but long-term use can cause side effects (Schmidt and Leroux, 2020). Additionally, it has been found that fecal transplantation can also reduce fishy smell symptoms, because fecal transplantation can change the composition of the gut microbiota (Lahtinen et al., 2017). However, regardless of the method, the high cost and complicated operation are not suitable for actual production of livestock and

poultry products. Existing research shows that we cannot find a simple method for improving the fishy odor of duck eggs using TMAU treatment methods. In this study, we attempted to improve the fishy odor of duck eggs by changing the rearing environment of ducks. Surprisingly, the TMA content in egg yolks showed significant differences between different rearing systems. The TMA content in the cage group was significantly lower than that in the pond and floor groups ($P < 0.001$). These results suggest that the fishy odor of duck eggs can

be improved by changing the rearing system of ducks, thereby promoting their commercial consumption.

As a new rearing system for laying ducks cage rearing not only fundamentally solves the pollution problem of duck production and improves biosafety and product quality, but also exhibits the benefits of implementing standardized production compared with floor rearing (Zhang et al., 2019). In the present study, we found that changes in the rearing system did not adversely affect the quality of duck eggs. The EW and ESI of the cage-rearing group were better than those of the floor- and pond-rearing groups. The color intensity of the yolk was higher in the cage-rearing group. Dong et al. (2017) found that Xianju chickens from cage systems have advantages in terms of production parameters, whereas hens from the floor rearing system and free-range system exhibit lower serum concentrations of lipids and glucose, and the effect of changes in the rearing system on egg quality was negligible, which is consistent with the results of the present study. Zhu et al. (2020) found that the final body weight of Gaoyou ducks is higher in the cage group than in the floor group. Wang et al. (2021) showed that cage-reared broilers exhibit better production performance compared to floor-raised broilers. Similarly, Bai et al. (2022) showed that the cage-rearing system improves the production performance and some important meat quality traits of ducks, such as brightness, pH, shear force, WLR, moisture content, and IMF content, thereby enhancing the nutritional and economic value of duck meat. In addition, when collecting duck eggs, we found that the eggshell surface of duck eggs in the cage group was cleaner than that in the floor and pond groups. Owing to the rearing environment, there were many stains on the surface of the duck eggs in the pond group. From a biosecurity perspective, duck eggs are generally considered to carry greater microbiological contamination than chicken eggs, including *Salmonella*-type bacteria (Kokoszynski et al., 2007).

It is well known that microbial localization, diversity, and composition vary throughout the gastrointestinal tract and are regulated by drivers such as pH and oxygen concentrations. Here, we performed a comparative structural analysis of the gut microbiota of duck cecal contents and rearing environmental samples under different rearing conditions. Previous studies have shown that the gut microbiota is a dynamic entity that is affected by environmental and nutritional inputs (Paoli et al., 2019). The rearing system affects the gastrointestinal microbiota, which, in turn, affects the production performance and health of animals. Intriguingly, our taxonomic classification showed that Firmicutes, Bacteroidetes, Actinobacteria, and Deferribacteres were the most abundant bacteria in the cecum, which is in agreement with previous studies on chickens and ducks (Onrust et al., 2015; Xiao et al., 2017; Wang et al., 2021; Chen et al., 2022). Results of 16S rRNA sequencing show that human and mouse cecal bacteria were mainly composed of Bacteroidetes and Firmicutes, while Bacteroidetes and Firmicutes in the pig cecal digestive tract were the main phyla from farrow to finish (Brücher and

Jamall, 2019; Yue et al., 2019; Quan et al., 2020). In addition, among the top 10 dominant bacterial phyla in the duck cecum, Fusobacteria was found (CC, 0.60%; FC, 2.93%; PC, 0.81%). Zhu et al. (2020) reported for the first time that the relative abundance of Fusobacteria in the duck cecum is >1%. Sun et al. (2018) detected Fusobacteria in the cecum of chickens under free-range breeding conditions but not in chickens raised in cages. Elokil et al. (2019) indicated that the abundance of Fusobacteria in the cecum of laying hens housed in individual cages is 0.01 to 0.06%. Based on these previous studies and the results of the present study, it is suggested that Fusobacteria may be a normal part of the gut microbiota, although some studies have not detected the same relative abundance. At the phylum level, Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria comprised more than 90% of all bacteria in environmental samples from cage, floor, and pond-rearing systems. To the best of our knowledge, no studies have reported differences in environmental microbial community diversity in reared ducks, although some studies have been conducted on chickens. Wilson et al. (2021) compared the microbial composition of the environment in cages and free-range egg production, and found that the free-range samples consist primarily of 3 bacterial phyla: Firmicutes, Proteobacteria, and Actinobacteria, which is consistent with the present study.

At the genus level, both cecal content and environmental samples showed differences in microbial composition and abundance in different rearing systems. Interestingly, in addition to the common Bacteroides, we found several bacterial genera in the cecum that are associated with host health, including *Faecalibacterium*, *Subdoligranulum*, *Intestinimonas*, and *Blautia*. The relative abundances of these bacterial genera were greater than 1% and were related to lipid metabolism in the body. *Faecalibacterium* is an important producer of butyric acid, which has anti-inflammatory properties, maintains the activity of bacterial enzymes, and protects the digestive system from intestinal pathogens (Lopez-Siles et al., 2017). Roy et al. (2022) found that *Subdoligranulum*, a newly isolated human commensal bacterium that prevents diet-induced obesity and metabolic disorders in mice, has a function similar to *Akkermansia muciniphila*. Furthermore, *Faecalibacterium* and *Intestinimonas* in the gastrointestinal tract are significantly reduced with obesity (Thingholm et al., 2019). *Blautia* probably helps reduce inflammation associated with obesity-related complications (Liu et al., 2021). In terms of relative abundance, these bacterial genera differed among different rearing systems. Whether different rearing systems affect lipid metabolism in ducks requires further research. *Corynebacterium*, *Staphylococcus*, *Brachybacterium*, and *Acinetobacter* were central in the 3 rearing systems and were present in high proportions. *Staphylococcus* is the most common genus on farms. Syed et al. (2019) pointed out that *Staphylococcus* is highly abundant in chicken houses.

As there are vast differences in the environments of the 3 rearing systems, with considerable variation in

microbial composition and diversity. In fact, analysis of alpha and beta diversity indicators shows that there were not many differences in the bacterial composition and diversity of duck cecum contents in cage, floor, and pond-rearing systems, but there were significant differences in bacterial composition and diversity in the rearing system environment. Alpha diversity within the microbiota is correlated with health status. Our study shows that the Shannon index of duck cecum ranged from 6 to 8, which is consistent with previous studies on ducks (Zhu et al., 2020). Previously, it was found that the Shannon index of chickens ranged from 4 to 5, which was lower than that of ducks (Wen et al., 2019). In addition, the ACE and Chao1 index ranges are consistent with those of previous studies (Zhao et al., 2019). We speculate that, although the microbial composition and diversity of the duck cecum can be affected by factors such as diet, environment, and health, it has a certain range for the collective species. The range of alpha diversity in the environmental samples of the cage and floor groups is consistent with previous studies, although it was higher in the pond group, which may be related to the easier colonization and growth of microorganisms in the hydroponic environment (Wilson et al., 2021). The gut microbes of newly hatched chicks are only gradually propagated from environmental sources (Rychlik, 2020). Furthermore, the alpha diversity of the duck cecum varied with the rearing environment, suggesting that different rearing environments have an impact on the composition of the cecal biota. Previous reports have demonstrated significant changes in the gastrointestinal microbiota in response to housing conditions in mice and other animals, further confirming the importance of the living environment in shaping gastrointestinal microbiota (Ericsson et al., 2018; Kers et al., 2018; Patil et al., 2020). The results of the β -diversity analysis show that there were differences in microbial composition between the CC, FC, and PC groups, as well as between the CE, FE, and PE groups.

Previous studies have confirmed that factors such as diet, genetics, and gut microbes contribute to the fishy odor of eggs (Li et al., 2017, 2019). In the present study, the same batch of ducks was fed the same diet, and only the rearing environment was varied. Therefore, we can rule out the effects of diet and genetic factors on TMA content in the egg yolk. As we all know, the cecum is a major functional part of the distal gut that plays an important role in preventing pathogen colonization, detoxifying harmful substances, and absorbing additional nutrients (Sergeant et al., 2014). Therefore, we inferred that gut microbes affect TMA content in egg yolk.

Using LEfSe analysis, we identified several bacteria associated with TMA deposition in the different flora of the CC, FC, and PC groups. Hsu et al. (2020) showed that plasma TMA concentrations increase when the relative abundances of *Subdoligranulum*, *Streptococcus*, and *Akkermansia* decrease. Studies have shown that *Akkermansia* affects plasma TMA concentrations by regulating the production or metabolism of intestinal

TMA (Chen et al., 2016). Ji et al. (2021) administered rhubarb enema to rats in the treatment group, and compared with the control group, serum TMA and TMAO concentrations increase. *Romboutsia* was positively correlated with TMA concentrations, whereas *Lactobacillus* was negatively correlated. Chen et al. (2020) showed that *Lactobacillus* is negatively correlated with TMA concentrations after probiotic supplementation in young men. Xu et al. (2017) showed that, compared with healthy patients, the levels of *Clostridium* and TMAO in patients with chronic kidney disease were higher. In our study, although 3 bacteria related to TMA production were found in the duck cecum of the PC group, the relative abundance of *Clostridium* was much higher than that of *Lactobacillus* and *Streptococcus* (Figure 5C), and thus the main differential bacteria were *Clostridium*. In conclusion, previous studies suggest that *Romboutsia* and *Clostridium* are positively associated with TMA production in the gut, whereas *Subdoligranulum* is negatively associated with TMA. Interestingly, the TMA content in the egg yolks of the FC and PC groups was higher than that of the CC group in the 3 rearing systems, which is consistent with the results of the intestinal flora. Based on the α and β diversity analyses, we speculate that changes in the rearing environment affect the composition of duck cecal microflora, which in turn affects the deposition of TMA in egg yolk. However, the specific bacteria that affect TMA deposition in egg yolk require further investigation.

Therefore, our study shows that the cage rearing of laying ducks is expected to be further developed. Compared with other methods, it is a relatively simple and low-cost way to reduce the fishy odor of duck eggs. Moreover, the flavor of duck eggs has been improved under caged conditions, making them more acceptable to consumers, which could support increases consumption of duck eggs and the development of the industry.

CONCLUSIONS

In conclusion, we demonstrate that changes in the rearing system affect TMA deposition in the yolk, egg quality, and the composition and diversity of duck cecal microbes. Furthermore, the analysis indicates that the rearing system may affect the deposition of TMA in yolk by affecting cecal microbiota diversity and composition. The cage rearing system it is potentially a low-cost way to reduce the fishy odor of duck eggs, promoting consumer acceptance, and therefore, consumption and development if the industry.

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DISCLOSURES

The authors declared that no conflict of interest exists in the submission of this manuscript.

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