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Full-Length Article

LAYING RATE WAS CORRELATED WITH MICROBIAL Fecal microbiota transplantation improves the laying performance by changing the gut microbiota composition in late laying period

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ABSTRACT

This research investigated the differences and succession patterns of microbes in different ages, the performance of laying hens, and the effect of Fecal Microbiota Transplantation (FMT) on aged laying hens. First, based on the different laying rates and age, we divided the laying hens into four groups: 75-week-old high-yield (OH, laying rate (LR) > 90%), 75-week-old low-yield (OL, LR < 60%), 75-week-old non-laying hens (OZ, LR = 0%) and 35-week-old high-yield (YH, LR > 90%) with 5 replicates in each group and 6 chickens in each replicate. The microbial metabolic patterns between different ages and laying rates were determined using 16S rDNA technology. Then, to verify the results of microbiome research, we utilized FMT technology to transplant the gut microbiota from OH to OZ ($OZ_{FMT\text{-}OH}$), thereby revealing the connection between gut microbes and production performance. The results showed that high-yielding hens (YH and OH groups) had higher levels of Superoxide dismutase (SOD) and Immunoglobulin A (IgA) compared to OL and OZ groups. The Villus height to Crypt depth ratio(V/C) was significantly higher in the YH group than in 75-week-old hens ($P < 0.05$). Alpha diversity indicated higher microbial diversity in the YH group compared to older hens ($P < 0.05$), with YH hens harboring more *Megamonas*, OH hens more *Bacteroides*, and OL and OZ groups showing higher levels of harmful bacteria. The villus height, V/C, mucosal layer thickness, cup cell number acetic acid level, and LR in the $OZ_{FMT\text{-}OH}$ group were significantly higher than those in the OZ group ($P < 0.05$), while the IL-2 level, crypt depth and cecal intestinal wall thickness were significantly lower than those in OZ group ($P < 0.05$). FMT also changed the morphological structure of grade follicles and small yellow follicles, improved the microbe composition of cecum and increased *Bacteroides* abundance. In the late laying period, if the intestinal flora cannot maintain the dynamic balance and carry out timely replacement, the production performance may be decreased, and the increase of *Bacteroides* abundance in the intestinal tract can improve the intestinal health and production performance of laying hens in the late laying period.

Introduction

The intestinal microbiota of chickens constitutes a dynamic ecosystem whose continuous turnover plays a pivotal role in maintaining intestinal homeostasis (Lu et al., 2003). Accumulating evidence indicates that host age serves as a primary determinant of microbial succession in poultry intestines (Ballou et al., 2016; Shi et al., 2019; Elokil et al., 2020). The gut microbiota of laying hens undergoes distinct compositional shifts across developmental stages (Li et al., 2018; Dai et al., 2022). During the brooding phase, Proteobacteria and Firmicutes dominate the gut microbial profile of newly hatched chicks. This initial colonization pattern transitions within two weeks, marked by a decline in Proteobacteria and concurrent expansion of Firmicutes and Bacteroidetes (Ballou et al., 2016; Dai et al., 2020). Such transient Proteobacterial dominance may facilitate intestinal immune system maturation through microbial-epithelial crosstalk (Ekino et al., 1980;

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Oakley et al., 2014).

By the peak laying period, Firmicutes emerges as the predominant phylum in the cecal microbiota, followed by Bacteroidetes surpassing Proteobacteria in relative abundance (Joat et al., 2021). Intriguingly, this hierarchy undergoes inversion during late laying stages, with Bacteroidetes ultimately displacing Firmicutes as the dominant taxon while Proteobacteria abundance diminishes further (Dai et al., 2022). These stage-specific microbial signatures underscore the dynamic co-evolution between gut microbiota and host physiology. Nevertheless, critical knowledge gaps regarding the functional consequences of delayed microbial succession persist. For instance, whether senile non-laying hens could regain productivity through microbiota remodeling resembling high-performing counterparts remains unexplored.

Age-related intestinal dysbiosis in aging layers correlates with declining performance metrics and heightened disease susceptibility (Guo et al., 2017; Wang et al., 2018; Feng et al., 2020). The gut microbiota exerts multifaceted influences on layer physiology, including nutrient metabolism optimization (Dai et al., 2020; Wang et al., 2020), intestinal barrier reinforcement (Khan et al., 2020; Miao et al., 2020), and systemic regulation of egg production through gut-organ axes (Agus et al., 2021; Wang et al., 2023). The sustained high productivity observed in subsets of aged layers is particularly noteworthy, suggesting potential microbiota-mediated mechanisms buffering age-related decline. This observation necessitates comparative investigations into microbial community structures between contemporaneous high and low-performing layers, which may reveal actionable targets for precision microbiota modulation to enhance productivity.

Fecal microbiota transplantation (FMT), the therapeutic transfer of functional microbial consortia between donors and recipients, has emerged as a transformative approach for modulating gut ecosystem dynamics and enhancing host phenotypes. Emerging evidence demonstrates its multifaceted applications in poultry production systems. Beyond restoring microbial diversity (Glendinning et al., 2022), FMT exerts multifaceted functional benefits through coordinated biological mechanisms. Its immunomodulatory effects manifest in suppressing intestinal inflammation (Fu et al., 2022; Zhang et al., 2022) and improving the structure of intestinal flora during aging (Liu et al., 2025). Concurrently, FMT drives metabolic reprogramming by transferring feed efficiency-associated microbiota to enhance nutrient utilization (Siegerstetter et al., 2018; Elokil et al., 2022), with cecal transplants from high-fat donors further modifying lipid metabolism pathways to regulate fat deposition (Song et al., 2023). Neuroendocrine regulation constitutes another critical pathway, where FMT enhances growth performance through Th17/Treg balance modulation (Ma et al., 2023) while alleviating stress responses via gut-brain axis interactions (Yan et al., 2021). In laying hen-specific applications, FMT from high-performance donors exerts systemic effects, improving ovarian steroidogenesis, intestinal barrier integrity, and reproductive tract function (Cao et al., 2023). Strategic microbiota transfer enhances disease resilience by competitively excluding pathogens, such as transplantation-induced *Bacteroides* enrichment reduces *Salmonella* enteritidis colonization while strengthening mucosal immunity (Wang et al., 2022). These findings collectively position FMT as an effective means of improving the gut microbiota in poultry.

Based on the above findings, in this study, FMT technology was used to reconstruct the gut microbiota of non-laying hens at the late laying stage, and 16S rDNA gene sequencing technology was used to investigate the specific effects of delayed replacement of gut microbiota on performance of laying hens. This is of great significance in improving the overall laying rate of laying hens in the late laying period and increasing farm income.

Table 1
Composition and nutrient levels of the basal diet.

Items	Content %
Ingredients	
Corn	62.60
Soybean meal	23.90
Wheat bran	1.80
Soybean oil	0.90
Limestone	8.00
$CaHPO_4$	1.50
NaCl	0.30
Premix(1)	1.00
Total	100.00
Nutrient levels(2)	
ME/(MJ/kg)	11.24
CP	16.70
Ca	3.50
AP	0.44
Met	0.34
Lys	0.81

(1) The premix provided the following per kg of the diet: VA 10000 IU, VD_3 2000 IU, VE 15 IU, VK_3 2.0 mg, VB_1 1.2 mg, VB_2 7mg, VB_6 6 mg, VB_{12} 0.08 mg, niacin 40 mg, biotin 0.15 mg, folic acid 1.0 mg, pantothenic acid 15 mg, choline 420 mg, Cu 10 mg, Fe 60 mg, Zn 80mg, Mn 80 mg, I 1mg, Se 0.3 mg. (2) CP was a measured value, while the others were calculated values.

Materials and methods

Experimental design and animal management

Forty Hy-line grey laying hens aged 28 weeks and 250 Hy-line grey laying hens aged 68 weeks were used in this research. The laying hens used in this experiment were all from the same farm, and the environmental conditions, such as temperature, humidity, and ventilation, were consistent with good repeatability. The egg-laying rate was observed before selection and was observed again for 7 weeks after being transported to the test site. Before the test began, the chicken coop was thoroughly cleaned and disinfected to ensure a healthy and stable test environment.

Based on the laying performance, they were divided into 75-week-old high-yield (OH, laying rate (LR) $>$ 90%, 76 in total), 75-week-old low-yield (OL, 30%$<$ LR $<$ 60%, 104 in total), 75-week-old non-laying hens (OZ, LR $=$ 0%, 62 in total) and 35-week-old high-yield (YH, LR $>$ 90%, 37 in total). The egg-laying rate is calculated as eggs per chicken per week /7 $\times$ 100%. In the 8th week, 15 chickens from the OH group were selected as donors, and FMT was performed. Fecal bacteria from laying hens in the 75-week-old OH group were transplanted into laying hens in the 75-week-old OZ group ($OZ_{FMT\text{-}OH}$ group, 30 in total), and the control group was the OZ group (30 in total), with 5 replicates in each group and 6 chickens in each replicate. Each hen was kept in a single cage, fed and watered freely, and given 16 h of light and 8 h of darkness a day. The composition and nutrient levels of the basal diet are shown in Table 1.

The management procedures and use of animals for research adhered to institutional and national guidelines and were endorsed by Hebei Agriculture University's Animal Care and Use Committee.

FMT treatment

FMT operations refer to the Ma (2023) method (Ma et al., 2023); the specific operation is Collect fresh feces (white urate removed) from OH laying hens in a sterile manner, then immediately transport them to the laboratory for treatment. Weigh 20 g of feces, add 120 mL of normal saline, thoroughly mix, filter the sterile gauze twice, take filtrate, centrifuge 1000r/ min for 10 min, and take supernatant for irrigation, 3

mL/ day/ hen. The control group was continuously given the same volume of normal saline, FMT, for 4 weeks, and then the laying rate during the FMT colonization period was observed for 2 weeks.

Sample collection

At the end of the treatment period, one chicken was randomly selected for each replicate and blood samples were collected in 5 mL tubes from the wing vein. The blood samples were then centrifuged at 4 °C for 15 min at 3000 rpm, followed by storage of the serum at -80 °C.

Ovarian follicle tissue was retrieved and counted, and the middle sections (2 cm) of the jejunum and cecum, after flushing with physiological saline, were placed in a 4% paraformaldehyde solution for fixation. These samples were used for subsequent morphological analysis.

The cecum contents were meticulously gathered into sterile tubes, promptly plunged into liquid nitrogen for freezing, and then carefully preserved at -80 °C to ensure optimal conditions for subsequent 16S rDNA sequencing.

Egg quality

After the egg-laying rate record was completed at week seven, twelve eggs in the YH group, OH group and OL group were randomly selected for egg quality determination, while the egg-laying rate in the OZ group was 0%, so no relevant determination was performed. The eggs quality was rigorously assessed. The sophisticated multi-functional egg analyzer measured the egg weight, yolk color, and the Haugh unit (Bulader Co., Ltd., Beijing, China). The egg shape was determined using an egg-shape determinator (FHK, Fujihira Industry Co., Ltd., Tokyo, Japan). Yolk weight was gauged using a precise digital scale (Huachaoice Electrical Appliances, Shanghai, China). The Egg Force Reader (ESTG-01, ORKA, Israel) was used to examine eggshell strength rigorously.

Blood parameter

Commercial ELISA kits (Jianglai Biotechnology Co., Ltd., Shanghai, China) were used to measure the serum levels of superoxide dismutase (SOD), malondialdehyde (MDA), interferon-gamma (IFN-γ) interleukin-2 (IL-2), immunoglobulin A (IgA), and immunoglobulin M (IgM).

Morphological measurements

After fixation in a 4% paraformaldehyde solution for 48 h, the hierarchical follicles (F follicles, >10 mm), small yellow follicles (SYF, 6-10 mm), large white follicles (LWF, 2–6 mm), and the jejunum and cecum, were dehydrated, embedded, and sectioned. The sections were stained with hematoxylin and eosin (HE) and evaluated under a fluorescence microscope (DP80; Olympus, Japan). Periodic Acid-Schiff (PAS) staining was performed on jejunum and goblet cell count was recorded. The magnification of the images of intestinal tissue morphology was 40 times, and the magnification of the images of follicle tissue morphology and jejunum goblet cells was 400 times.

16S rDNA gene amplicon sequencing

Microbial genomic DNA was extracted from fecal samples using a specialized Stool DNA kit (Tiangen, Beijing, China). The purity and integrity of the genomic DNA were validated through 1.0% agarose gel electrophoresis. The V3-V4 regions of the bacterial 16S rDNA gene, spanning nucleotides 341 to 806, were amplified from the DNA using specifically designed barcoded primers: 341F (5′-CCTACGGGNGGCWGCAG −3′) and 806 R (5′ GGACTACNVGGGTATC-TAAT-3′). PCR was carried out using a high-precision Biometra TOne 96 G PCR thermocycler (Germany). The 50 μL reaction system incorporated 1.5 μL of each primer, 100 ng of template DNA, 5μL of 10 × KOD Buffer, 5 μL of 2.5 mM dNTPs, and 1 μL of KOD polymerase. The purified amplicons were combined in equimolar proportions and sequenced using paired-end sequencing on the Illumina Hiseq PE250 platform, following standard protocols by Genedenovo Inc. (Guangzhou, China). In microbiota analysis, the α-diversity of the bacterial community was computed using Mothur software, version 1.30.1. β-diversity was assessed using principal coordinate analysis (PcoA). Disparities in taxonomic composition among samples were discerned using linear discriminant analysis (LDA) effect size (LefSe) analysis and the Kruskal–Wallis test.

Table 2
Influence of different age groups and egg production rates on egg quality.

Item/Group	YH	OH	OL	*P*-value
Egg length, mm	56.13 ± 1.60^{b}	57.27 ± 1.61^{b}	60.68 ± 1.55^{a}	<0.01
Egg width, mm	42.76 ± 0.92^{b}	43.18 ± 1.07^{b}	44.80 ± 0.25^{a}	<0.01
Egg shape index	1.31 ± 0.05	1.33 ± 0.05	1.35 ± 0.04	0.26
Egg weight, g	58.36 ± 2.51^{c}	60.97 ± 2.97^{b}	66.84 ± 1.67^{a}	<0.01
Yolk color	8.71 ± 0.41	8.72 ± 0.27	9.80 ± 0.41	0.26
Egg yolk weight, g	16.02 ± 0.97	17.05 ± 1.30	17.36 ± 1.16	0.08
Haugh unit	94.02 ± 7.27^{a}	84.30 ± 7.87^{ab}	79.34 ± 5.48^{b}	0.04
Eggshell strength, N	48.96 ± 2.45^{a}	36.81 ± 1.49^{b}	34.62 ± 2.37^{b}	<0.01

All data are expressed as mean ± standard deviation. For the same line of data, different lowercase superscripts indicate significant differences ($P < 0.05$), and no letters or the same letters indicate no significant differences ($P > 0.05$). Abbreviations: YH, 35-week-old high-yield; OH, 75-week-old high-yield; OL, 75-week-old low-yield.

Metabolomics analysis of blood sample

After thawing, a sufficient amount of serum was immersed in a methanol/acetonitrile/water solution (2:2:1). The mixture was then extracted and introduced into 100 μL of aqueous acetonitrile solution for effective re-solubilization. The mixture was vigorously vortexed for 30 s to ensure thorough mixing. The resulting solution was then prepared for comprehensive mass spectrometric evaluation. UHPLC-MS (Vanquish LC, Thermo) analysis was conducted with a HILIC column (Waters, ACQUITY, 2.1 mm × 10 cm × 1.7 μm). The instrument settings were as follows: Gas1 at 60 kPa, Gas2 at 60 kPa, Ion source temperature at 600 °C, CUR at 30 psi, and Spray voltage (ISVF) at ± 5500 V. For metabolomics analysis, a mixed-mode approach was used, scrutinizing the biological implications of potential biomarkers with the HMDB and the Kyoto Encyclopedia of Genes and Genomes (KEGG). In-depth pathway and enrichment analyses were conducted using the MetaboAnalyst 4.0 platform.

Determination of the concentration of short-chain fatty acids

The concentration of short-chain fatty acids (SCFAs) in the cecum chyme was measured using the internal standard method of high-Performance gas chromatography (Agilent Technologies Inc, USA). Concentration (mmol/L) = (peak area of certain acid of sample × peak area of crotonic acid in standard solution × mol concentration of certain acid)/ (peak area of crotonic acid in sample × peak area of certain acid in standard solution).

Data analysis

Results are presented as mean ± standard deviation (mean ± SD). Data analysis and graphical representation were performed using SPSS 27 and GraphPad Prism 9. Turkey's method was used in the YH group, OH group, OL group, and OZ group analysis for multiple comparisons,

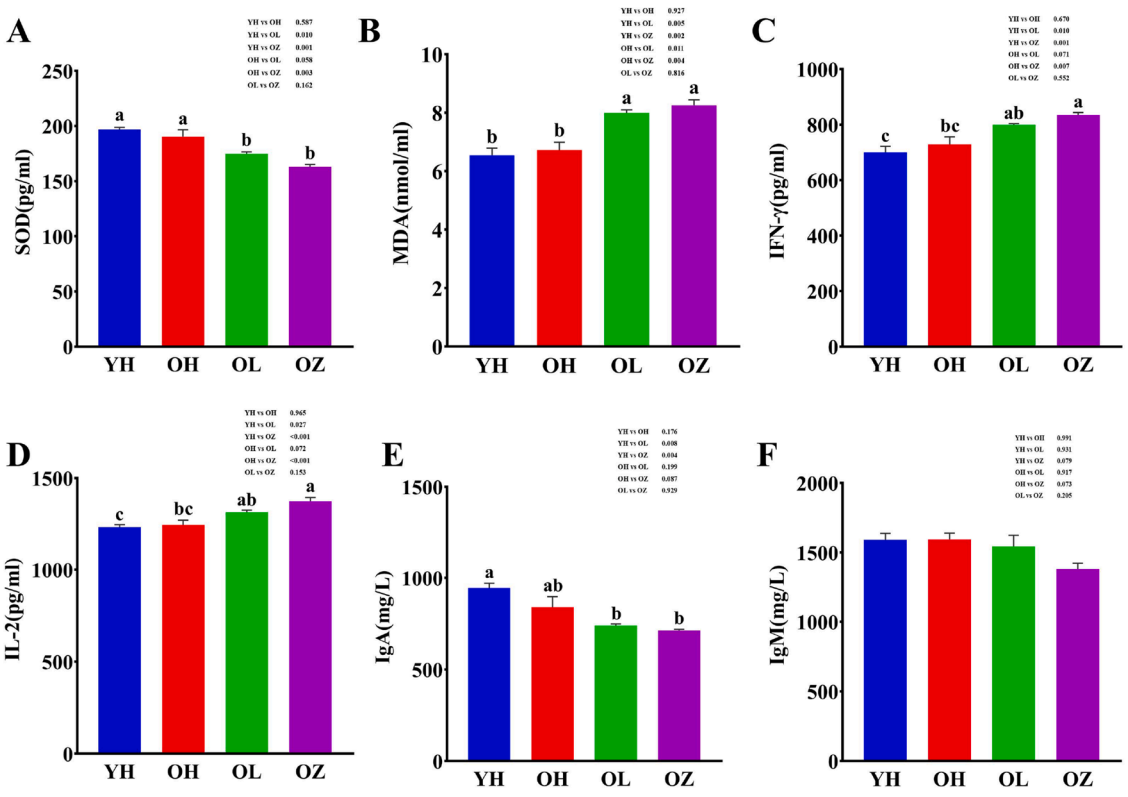


Fig. 1. The effect of age and egg production rate on blood parameters of laying hens. YH, 35-week-old high-yield; OH, 75-week-old high-yield; OL, 75-week-old low-yield. OZ, 75-week-old non-laying hens. Serum levels of (A) superoxide dismutase (SOD), (B) malondialdehyde (MDA), (C) Interferon (IFN-γ), (D) gammainterleukin-2 (IL-2), (E) immunoglobulin A (IgA), and (F) immunoglobulin M (IgM). Data are the mean ± SD. Different superscript letters in the bar chart indicate significant differences ($P < 0.05$).

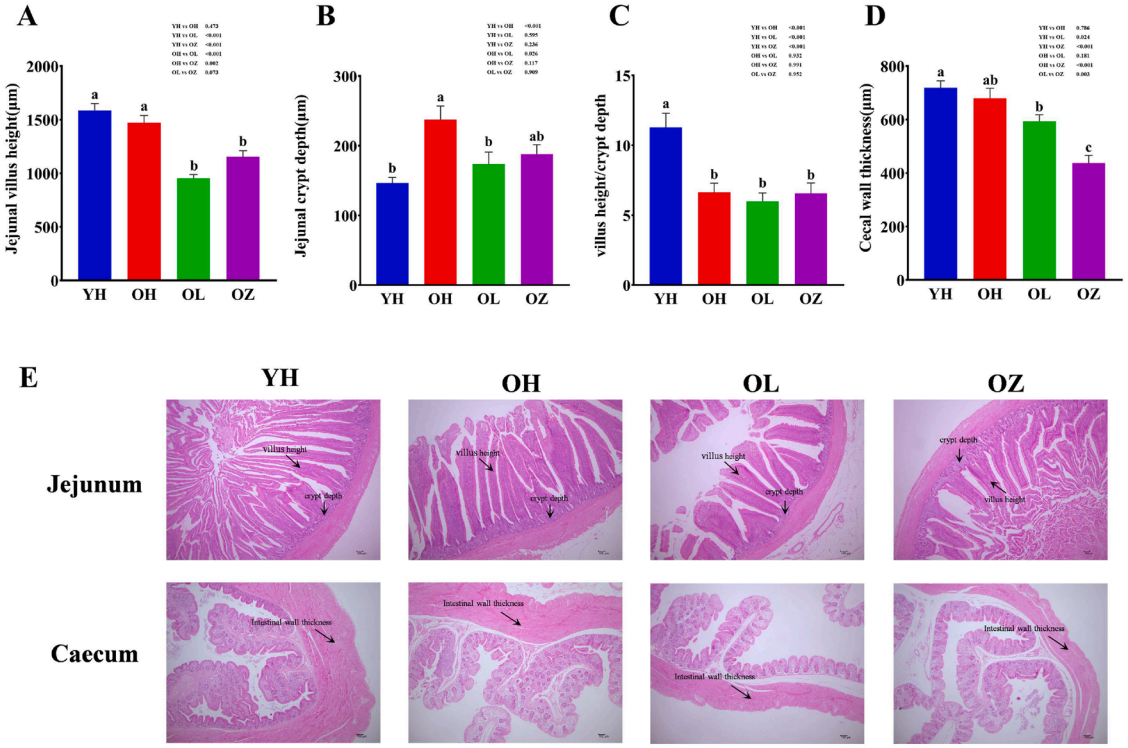


Fig. 2. The effect of age and egg production rates on the intestinal morphology of laying hens. YH, 35-week-old high-yield; OH, 75-week-old high-yield; OL, 75-week-old low-yield; OZ, 75-week-old non-laying hens. (A) Jejunal villus height; (B) Jejunal crypt depth; (C) Villus height/crypt depth. (D) Cecal wall thickness; (E) Intestinal histomorphology. Data are mean ± SD. Different superscript letters in the bar chart indicate significant differences ($P < 0.05$).

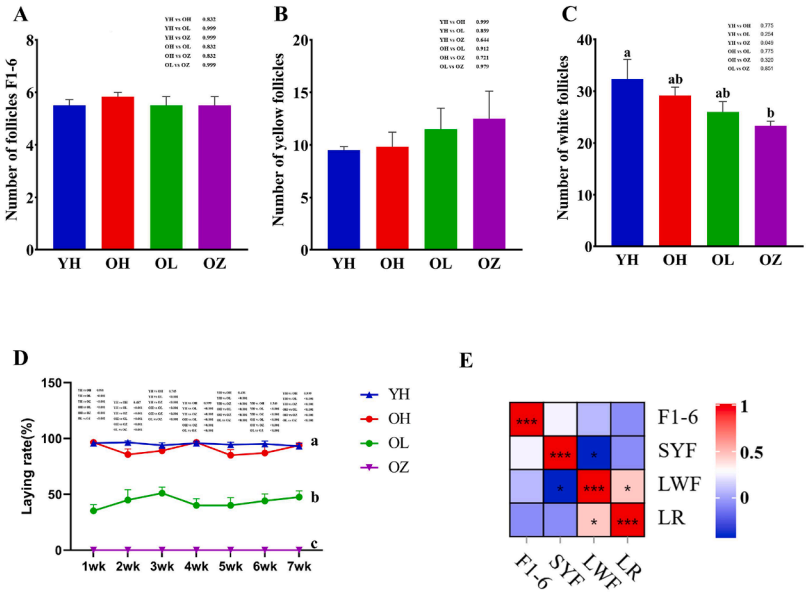


Fig. 3. The effect of age and laying rate on the number of follicles. YH, 35-week-old high-yield; OH, 75-week-old high-yield; OL, 75-week-old low-yield; OZ, 75-week-old non-laying hens. (A) F1-6 number of follicles (B) Number of small yellow follicles (C) Number of larger white follicles (D) Laying rate (E) Heat map of correlation between follicle and egg production rate. Data are mean ± SD. Different superscript letters in the bar chart and line chart indicate significant differences ($P < 0.05$). Red and blue are positively and negatively correlated, respectively. *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$p \leq 0.001$.

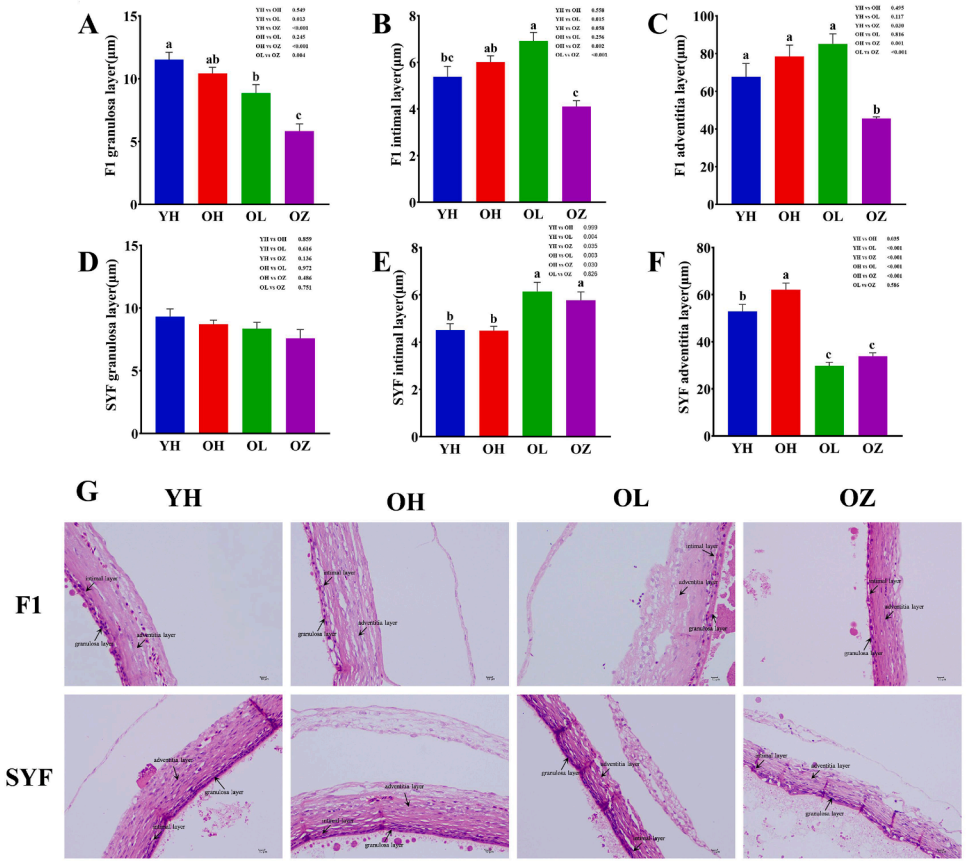


Fig. 4. The effects of hen age and egg production rates on the morphology of follicles. YH, 35-week-old high-yield; OH, 75-week-old high-yield; OL, 75-week-old low-yield; OZ, 75-week-old non-laying hens. (A) F1 follicular granular layer; (B) F1 follicular inner layer; (C) F1 follicular outer membrane; (D) SYF follicular granular layer; (E) SYF follicular inner layer; (F) SYF follicular outer membrane; and (G) Follicular histomorphology. Data are mean ± SD. Different superscript letters in the bar chart indicate significant differences ($P < 0.05$).

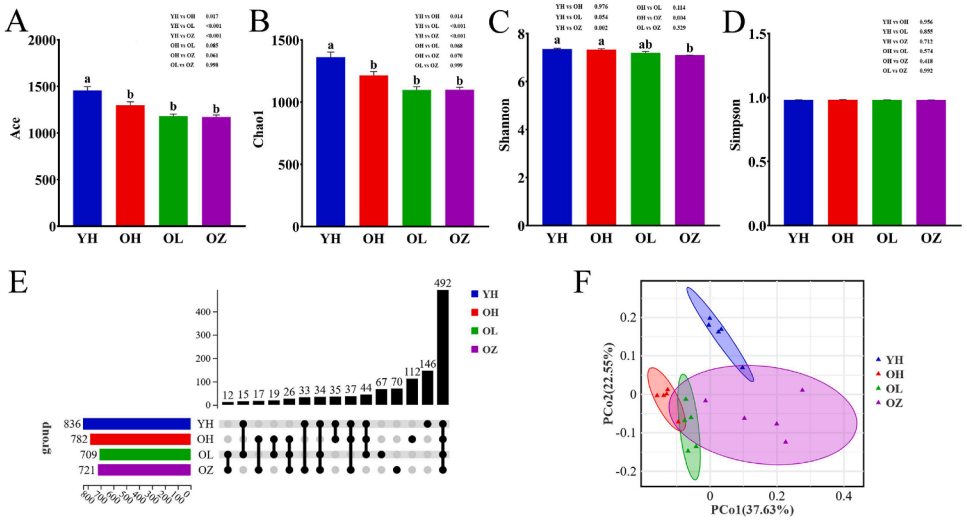


Fig. 5. Analysis of intestinal flora at different ages and laying rates. (A–D) Alpha diversity among groups (Ace, Chao1, Shannon, and Simpson index). (E) The Upset diagram illustrates the overlap of operational taxonomic unit (OTU) in all tested chickens. (F) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity.

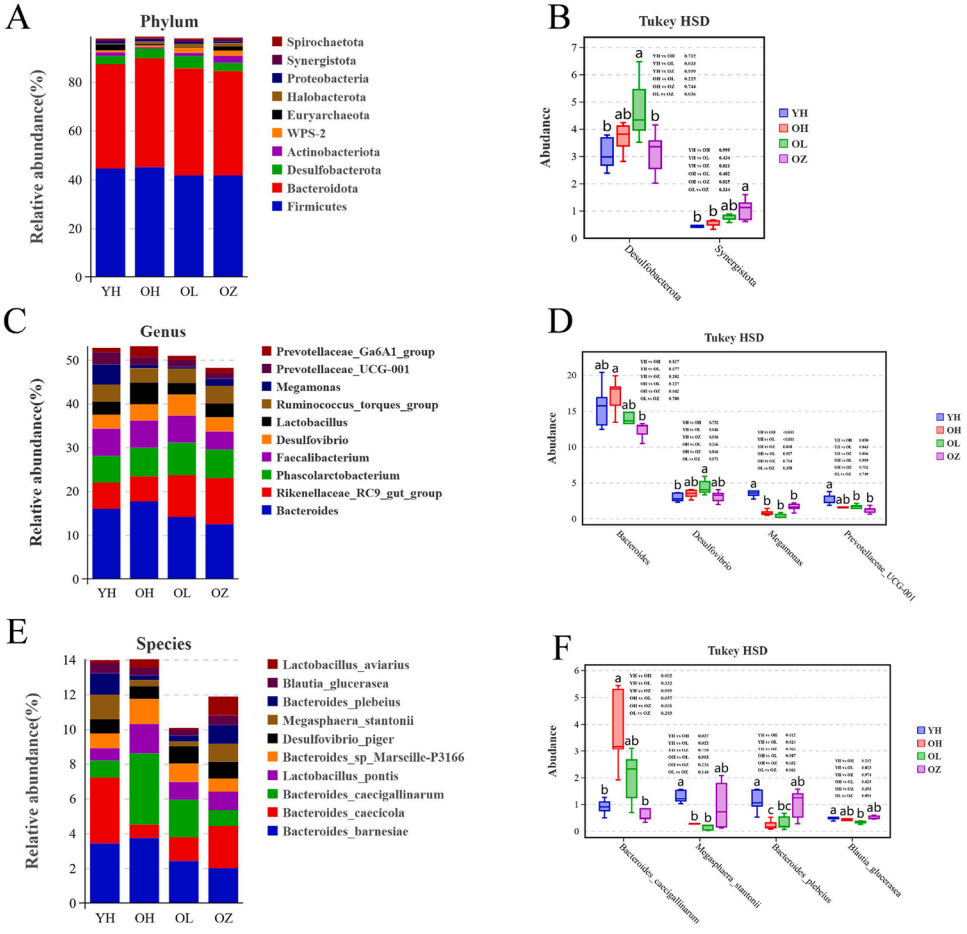


Fig. 6. (A) The relatively abundant cecal microbiota at the phylum level in laying hens; Differential analysis of cecal microflora at the (B) phylum level across various ages and laying rates and (C) at genus level; (D) Differential analysis of microflora at cecal genus level in laying hens with different age and laying rate; (E) The relative abundance of cecal microbiota at the species level in laying hens; (F) Differential analysis of microflora at cecal species level in laying hens with different ages and laying rates.

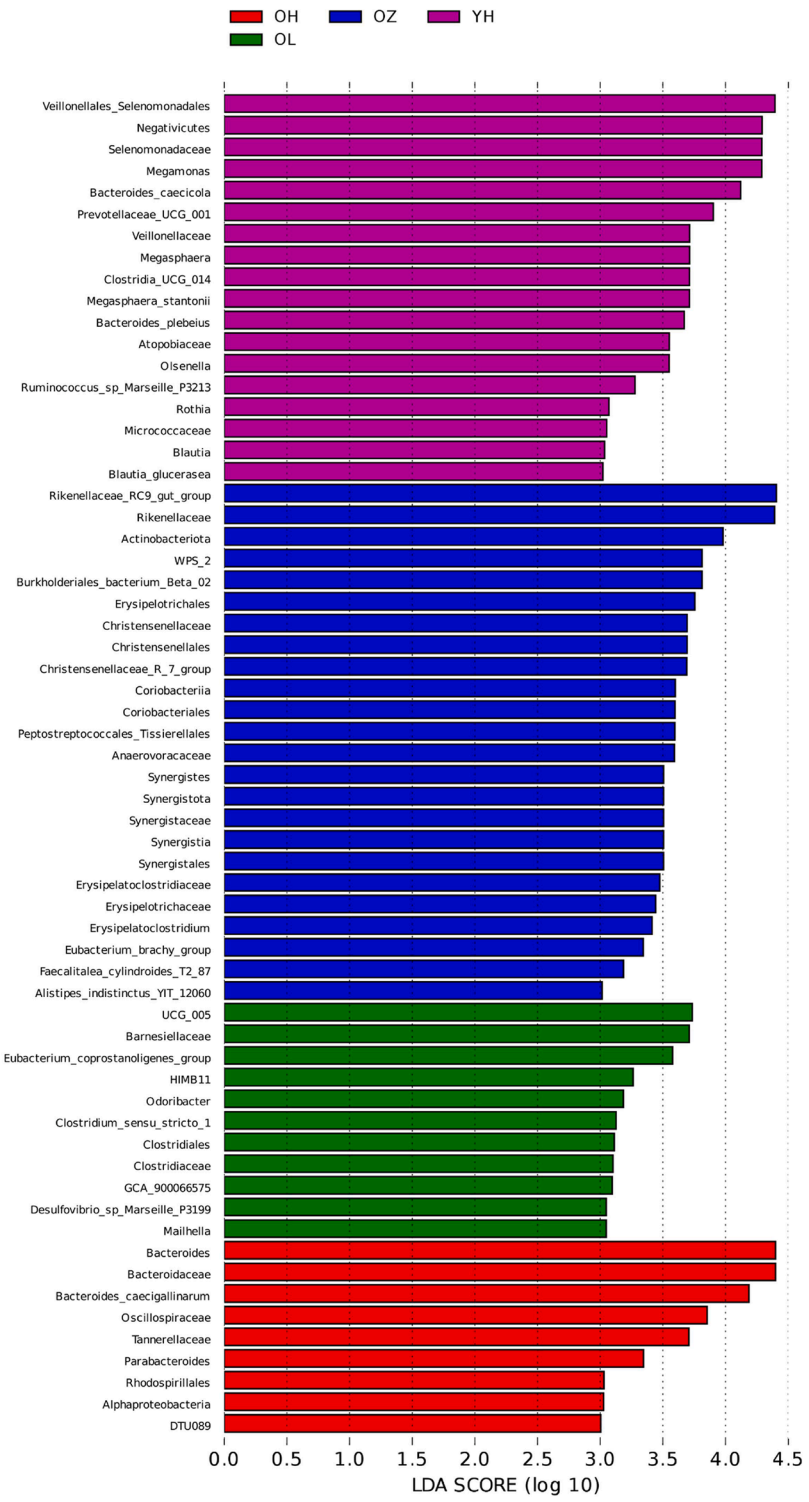


Fig. 7. Linear Discriminant Effect Size (LEfSe) analysis to identify distinct cecal microbiota profiles across varying ages and laying rates.

the egg quality was compared in YH group, OH group and OL group, and the other data was compared in four groups. Student t-test was used to compare the $OZ_{FMT\text{-}OH}$ group and OZ group. Statistical significance was determined at a *P*-value less than 0.05. Spearman correlation analysis was used to identify bivariate relationships, and heatmaps were generated using an online platform.

Results

Effect of microbial flora succession on egg quality

The egg quality data of YH group, OH group and OL group were analyzed. As shown in Table 2, the lengths, widths, and weights of eggs in the OL group surpassed those in the other two groups, while the Haugh unit was lower than that in the YH group ($P < 0.05$). The eggshell strength in the YH group was greater than in the other two

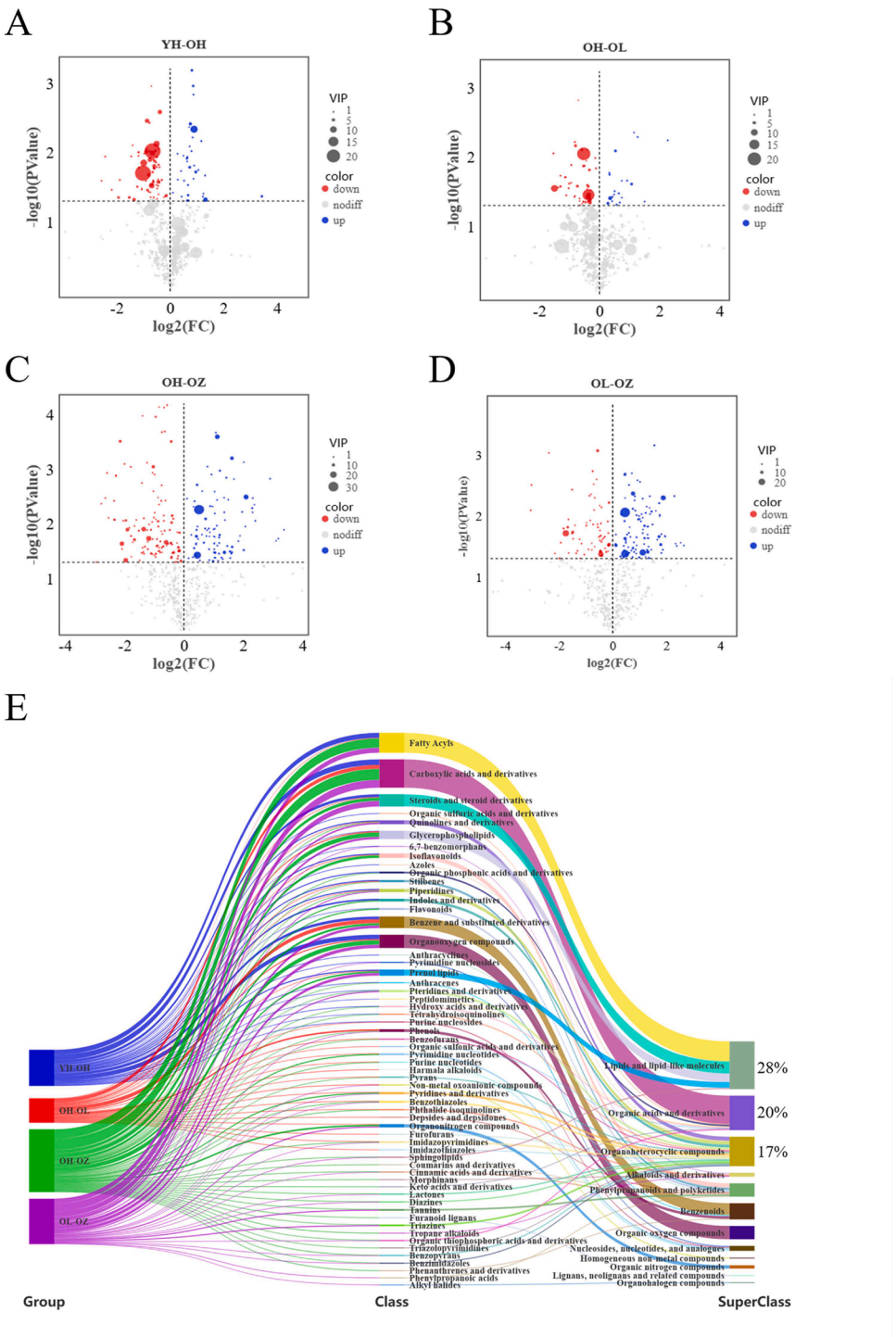


Fig. 8. Volcano plot of differential metabolites. Comparison between the (A) YH and OH groups, (B) OH and OL groups, (C) OH and OZ groups, (D) OL and OZ groups. The horizontal axis represents the fold change of each substance in the respective group (logarithm base 2), and the vertical axis represents the P value (logarithm base 10 is negative). The magnitude of the scatter points signifies the VIP value, with a direct correlation: the larger the scatter points, the higher the VIP value. Significantly upregulated metabolites are shown in blue, downregulated are in red, and insignificantly varied metabolites are marked in the gray realm. (E) Alluvial plot, the first column represents the different groups, the second column is the metabolite classification of the substance matched by the secondary spectra in the different groups, and the third column is Super Class.

groups, showing a statistically significant difference ($P < 0.05$).

Effect of microbial flora succession on blood parameter

The blood parameter data of YH group, OH group, OL group and OZ group were analyzed. As shown in Fig. 1, the serum SOD levels in the high-yield laying hens (YH group and OH group) were higher than in the OL group and OZ group ($P < 0.05$, Fig. 1A, E). The levels of MDA, IFN-γ, and IL-2 were lower compared to those in the OL group and OZ group ($P < 0.05$, Fig. 1B–D). The level of IgA in YH group was significantly higher than that in OL and OZ groups.

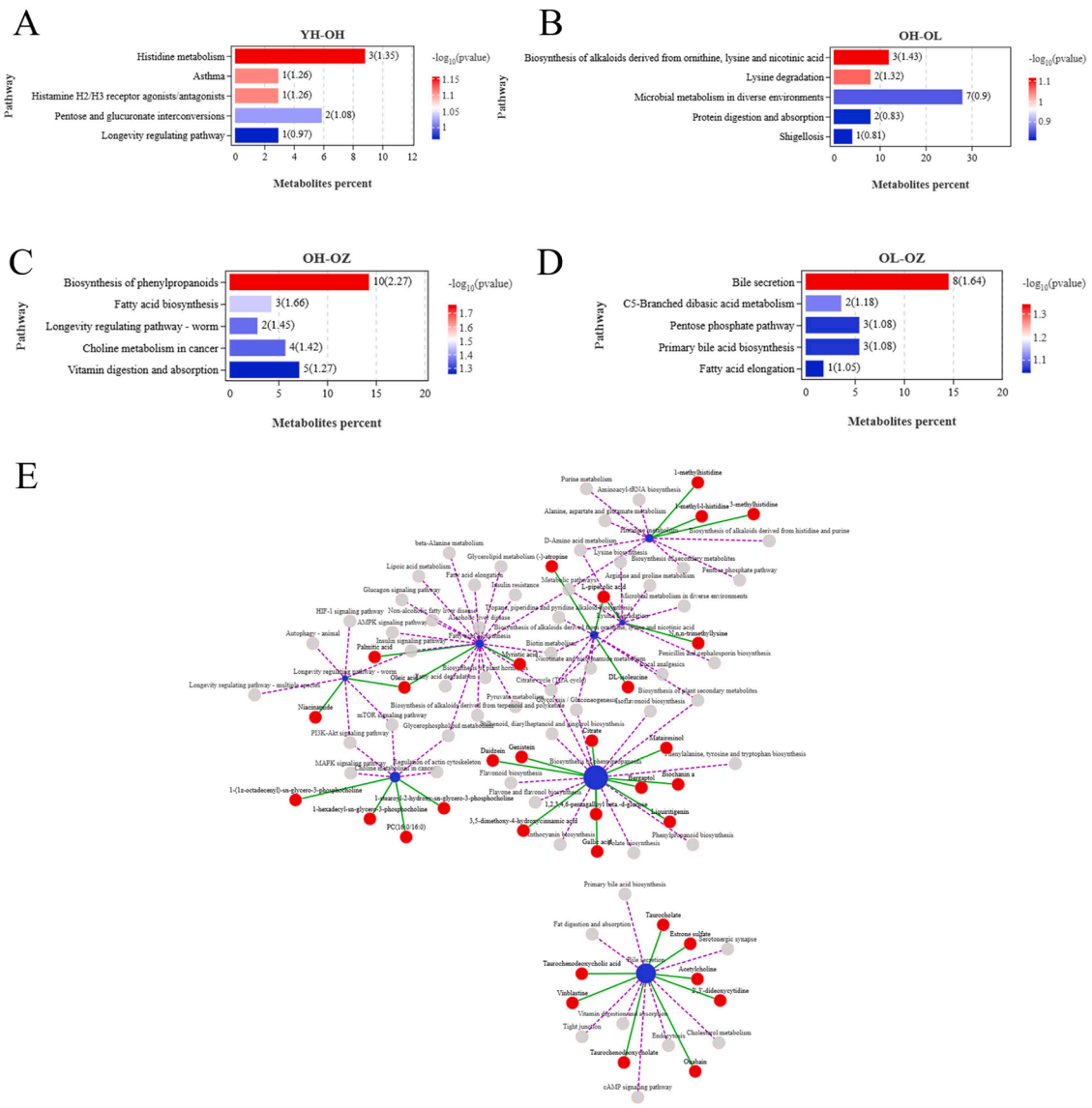


Fig. 9. (A–D) KEGG enrichment bar chart. The abscissa signifies the percentage of differential metabolites annotated under a specific pathway concerning the total number of annotated differential metabolites, while the ordinate denotes the name of the enriched KEGG metabolic pathway. (E) Within the regulatory framework about differential metabolites, the presence of red dots serves as an indication of differential metabolites. Blue dots indicate enriched pathways and gray dots indicate adjacent pathways.

Effect of microbial flora succession on intestinal morphology

The intestinal morphology data of YH group, OH group, OL group and OZ group were analyzed. The jejunal villus height in the YH and OH groups were higher than in the OL and OZ groups ($P < 0.05$, Fig. 2A). The jejunal crypt depth in the OH group was deeper than in the YH and OL groups ($P < 0.05$, Fig. 2B). The jejunal V/C ratio in the YH group was higher than in the other three groups ($P < 0.05$, Fig. 2C). In terms of cecal wall thickness, the YH group had a thicker wall compared to the OL and OZ groups ($P < 0.05$); no significant difference was noted between the YH and OH groups ($P > 0.05$, Fig. 2D).

Effect of microbial flora succession on laying rate and number of follicles

The laying rate and number of follicles data of YH group, OH group, OL group and OZ group were analyzed. The quantity of LWF follicles was notably greater in the YH group compared to the OZ group ($P < 0.05$, Fig. 3C), and the egg-laying rate of the YH and OH group was significantly higher than that of the OL and OZ groups ($P < 0.05$, Fig. 3D). A prominent positive correlation was noted between the egg-laying rate and LWF ($P < 0.05$, Fig. 3E).

Effect of microbial flora succession on morphology of follicles

The morphology of follicles data of YH group, OH group, OL group and OZ group were analyzed. In F1 follicles, the YH group had significantly thicker granulosa layers (Fig. 4A). The OL group exhibited significantly thicker inner layers (Fig. 4B). The OZ group had thinner follicular structures. In SYF, the OL and OZ groups had thicker endometrium layers (Fig. 4E), and the OH group had the thickest outer membrane layer (Fig. 4F).

Effect of microbial flora succession on microbe composition and difference of cecum

The microbe composition and difference of cecum data of YH group, OH group, OL group and OZ group were analyzed. The cecal contents from the YH, OH, OL, and OZ groups were analyzed to characterize the

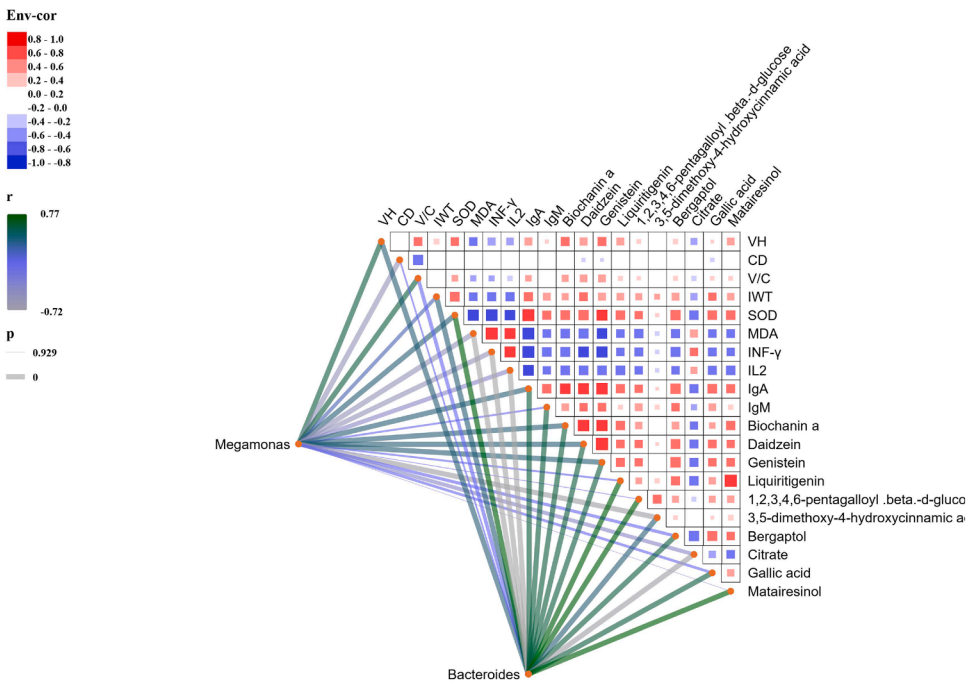


Fig. 10. Correlation analysis between remarkable genus, metabolites, intestinal morphology, and blood indexes; red, green and blue, gray indicate positive and negative correlations, respectively.

microbial structure and community. Community richness was assessed using the Abundance-based Coverage Estimator (ACE) and Chao1 indices, while community diversity was evaluated using the Shannon and Simpson indices. As depicted in Fig. 5A–D, the YH and OH groups, high-yield laying hens, showed elevated diversity and richness levels. The YH group significantly surpassed the OH group in both metrics ($P < 0.05$). Fig. 5E shows that 146, 112, 67, and 70 distinct OTUs were identified in the YH, OH, OL, and OZ groups, respectively. The β-diversity indices are illustrated through PCoA, based on Bray-Curtis distances and the results showed that there was an obvious separation between the four groups (Fig. 5F).

At the phylum level, the dominant phyla were Firmicutes and Bacteroidota, accounting for approximately 85% (Fig. 6A). In the OL group, the abundance of Desulfobacterota was notably higher than in both the YH and OZ groups. The abundance of Synergistota in the OZ group was notably higher compared to that in the YH and OH groups ($P < 0.05$, Fig. 6B). At the genus level, the dominant bacterial genera included *Bacteroides, Rikenellaceae_RC9_gut_group*, and *Phascolarctobacterium* (Fig. 6C). Among the 10 most abundant genera, the OH group showed a notably greater abundance of *Bacteroides* compared to the OZ group ($P < 0.05$). The abundance of *Desulfovibrio* in the OL group was markedly higher than in the YH group ($P < 0.05$). The abundance of *Megamonas* in the YH group was notably greater than in the other three groups ($P < 0.05$), and the preponderance of *Prevotellaceae_UCG-001* in the YH group was markedly greater than in both the OL and OZ groups ($P < 0.05$, Fig. 6D). At the species level, the dominant bacterial species included *Bacteroides barnesiae, Bacteroides caecicola*, and *Bacteroides caecigallinarum* (Fig. 6E). Among the top 10 most abundant species, the abundance of *Bacteroides caecigallinarum* in the OH group was notably greater than in the YH and OZ groups ($P < 0.05$). The abundances of *Megasphaera stantonii* and *Bacteroides plebeius* in the YH group were notably and substantially elevated compared to those in the OH and OL groups ($P < 0.05$, Fig. 6E). Both the YH and OZ groups exhibited similarity in the abundance of Desulfobacterota, *Bacteroides*, and *Megasphaera stantonii* ($P > 0.05$).

Through LEfSe (LDA score > 3) analysis, we pinpointed specific bacterial taxa associated with various treatments. Our findings uncovered a diverse array of 62 distinct bacterial taxa among the three treatment regimens (Fig. 7). Among these bacterial taxa, 18 characterized the YH group distinctly, 9 were unique to the OH group, 11 stood out as typical of the OL group, and 24 were emblematic of the OZ group. The differentially abundant bacteria in the YH group were primarily enriched in the Firmicutes, Actinobacteria, and Bacteroidota phyla. In the OH group, the differentially abundant bacteria were mainly enriched in the Bacteroidota and Firmicutes phyla. The OL group showed enrichment in the Firmicutes, Bacteroidota, Desulfobacterota, and Proteobacteria phyla. The OZ group exhibited enrichment in the Firmicutes and Synergistota phyla.

Effect of microbial flora succession on differential metabolites and KEGG pathway

The differential metabolites and KEGG pathway data of YH group, OH group, OL group and OZ group were analyzed. In this study, the metabolic profile in serum samples was examined using a cutting-edge LC-QTOF/MS system. The identification of differential metabolites was conducted by combining the VIP value and p-value thresholds. Metabolites with a p-value less than 0.05, a VIP value above 1, and a $\log_2$FC value greater than 0 were designated as upregulated. Conversely, metabolites with a $\log_2$FC value less than 0 were designated as downregulated, illustrating a nuanced portrait of metabolic alterations. There were 100 differential metabolites in the YH-OH group, with 66 downregulated and 34 upregulated (Fig. 8A, Table S1). In the OH-OL group, 69 differential metabolites were identified: 49 downregulated and 20 upregulated (Fig. 8B, Table S1). Within the OH-OZ group, 204 differential metabolites were identified: 114 downregulated and 90 upregulated (Fig. 8C, Table S1). In the OL-OZ group, 159 differential metabolites were found: 67 downregulated and 92 upregulated (Fig. 8D, Table S1). These metabolites were predominantly lipids and lipid-like molecules, organic acids and their derivatives, and various organoheterocyclic compounds (Fig. 8E).

Based on the KEGG database, the metabolic pathways were analyzed to identify the distinct influence of metabolites from this research. In the YH-OH group, three metabolites significantly affected the Histidine

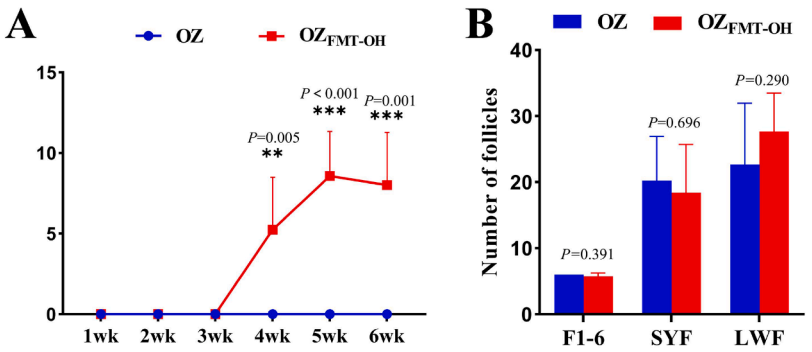


Fig. 11. Effect of FMT on laying rate and follicle number in aged non-laying hens. *0.01 < P < 0.05, **0.001< P < 0.01, ***$p \leq 0.001$.

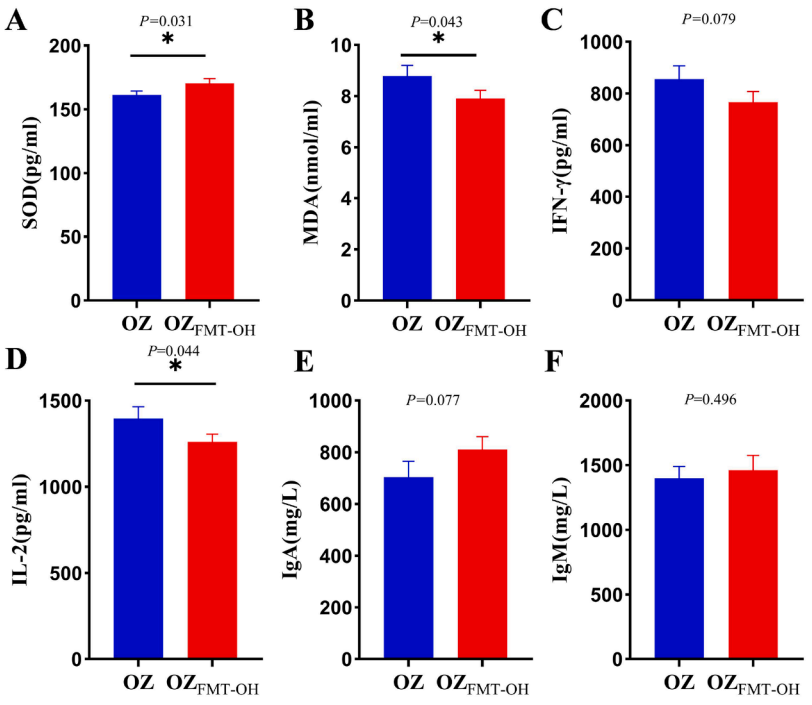


Fig. 12. Effect of FMT on blood indexes of aged non-laying hens. *0.01 < P < 0.05, **0.001< P < 0.01, ***$p \leq 0.001$.

metabolism pathway (P < 0.05, Fig. 9A, Table S1). In the OH-OL group, three metabolites affected the Biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid (P < 0.05), while two other metabolites affected the Lysine degradation pathway (P < 0.05, Fig. 9B, Table S1). The metabolites in the OH-OZ group significantly affected four pathways: ten metabolites affected the biosynthesis of phenylpropanoids. Three metabolites affected the fatty acid biosynthesis. Two metabolites affected the longevity-regulating pathway - worm. Four metabolites affected the choline metabolism in cancer pathway (P < 0.05, Fig. 9C, Table S1). In the OL-OZ group, eight metabolites significantly affected the bile secretion pathway (P < 0.05, Fig. 9D, Table S1).

Relevance analysis

We selected the differential genera (*Bacteroides*) of Bacteroidota, the differential genera of Firmicutes (*Megamonas*), and biosynthesis of phenylpropanoids (The highest enrichment of differentiated metabolites) differential metabolites in the pathway, intestinal morphological indexes and blood parameters for correlation analysis (Fig. 10).

Bacteroides are significantly negatively correlated with MDA, INF, and IL2, and significantly positively correlated with VH, IWT, SOD, IgA, and IgM. *Bacteroides* are significantly positively correlated with other metabolites such as Biochanin A, except for Citrate. *Megamonas* is significantly positively correlated with VH, V/C, SOD, IgA, Biochanin a, Daidzein, Genistein, and significantly negatively correlated with CD, MDA, 3,5-dimethoxy-4-hydroxycinnamic acid. These key differentiating microorganisms and metabolites may influence intestinal health, antioxidant function, and immune homeostasis in laying hens.

Effects of FMT on laying rate and follicle numbers in aged non-laying hens

The laying rate and follicle numbers data of OZ_{FMT-OH} group and OZ group were analyzed. The laying rate of OZ_{FMT-OH} group was significantly higher than that of OZ group at the fourth week of FMT and two weeks of planting period (P < 0.05, Fig. 11A), there was no significant difference in follicle numbers (P > 0.05, Fig. 11B).

Effect of FMT on blood parameters in aged non-laying hens

The blood parameters data of OZ_{FMT-OH} group and OZ group were analyzed. SOD level of OZ_{FMT-OH} group was significantly higher than that of OZ group, MDA and IL-2 levels were significantly lower than that of OZ group (P < 0.05, Fig. 12D), and other indexes had no significant changes. It indicates that FMT significantly enhances the antioxidant

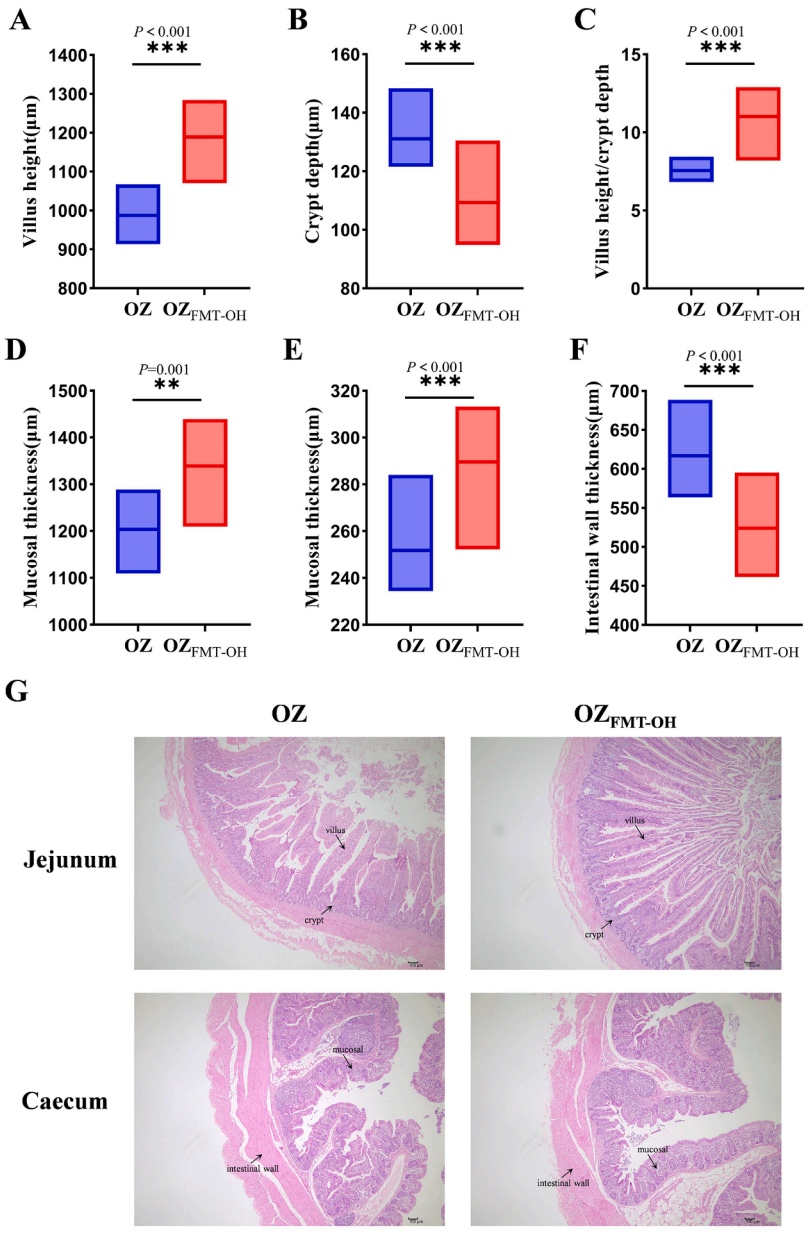


Fig. 13. Effect of FMT on intestinal morphology of aged non-laying hens. *0.01 < P < 0.05, **0.001< P < 0.01, ***$p \leq 0.001$.

performance of the aged non-laying hens.

Effect of FMT on intestinal morphology in aged non-laying hens

The intestinal morphology data of $OZ_{FMT\text{-}OH}$ group and OZ group were analyzed. The jejunum villus height, V/C, mucosal thickness and cecal mucosal thickness in $OZ_{FMT\text{-}OH}$ group were significantly higher than those in OZ group ($P < 0.05$, Fig. 13A C–E), and the jejunum crypt depth and cecal intestinal wall thickness in $OZ_{FMT\text{-}OH}$ group were significantly lower than those in OZ group ($P < 0.05$, Fig. 13B and F). The number of goblet cells in jejunum in $OZ_{FMT\text{-}OH}$ group was significantly higher than that in OZ group ($P < 0.05$, Fig. 14). The results indicate that FMT significantly improved the intestinal morphology in aged non-laying hens.

Effect of FMT on follicles morphology in aged non-laying hens

The follicles morphology data of $OZ_{FMT\text{-}OH}$ group and OZ group were analyzed. The F1 granule layer and intima layer, and the SYF intima layer and outer membrane layer in $OZ_{FMT\text{-}OH}$ group were significantly lower than those in OZ group ($P < 0.05$, Fig. 15A B E and F), and the particle layer of SYF was significantly higher than that in OZ group ($P < 0.05$, Fig. 15 D). The results indicate that FMT significantly improved the follicular morphology in aged non-laying hens.

Effect of FMT on cecal microbial composition in aged non-laying hens

The cecal microbial composition of $OZ_{FMT\text{-}OH}$ group and OZ group were analyzed. There was no significant difference in α diversity between the two groups ($P > 0.05$, Fig. 16 A–D), OTUs in the $OZ_{FMT\text{-}OH}$ group was higher than that in the OZ group, and β diversity showed A significant separation between the two groups (Fig. 16 F). After FMT, cecal microbial composition in the $OZ_{FMT\text{-}OH}$ group was changed, with Firmicutes decreasing and Bacteroidota increasing (Fig. 16 G–L). These results indicate that FMT significantly improved the microbial community structure in aged non-laying hens.

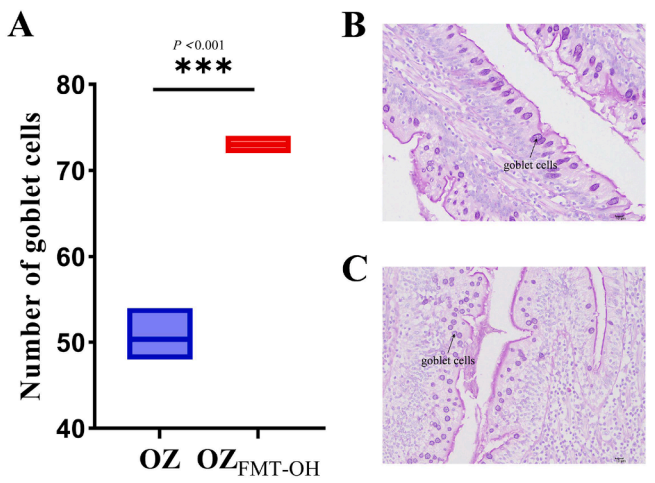


Fig. 14. Effect of FMT on jejunum goblet cells of aged non-laying hens. *0.01 < P < 0.05, **0.001< P < 0.01, ***$p \leq 0.001$.

Effect of FMT on short-chain fatty acids in aged non-laying hens

The short-chain fatty acids data of OZ_{FMT-OH} group and OZ group were analyzed. Acetic acid in OZ_{FMT-OH} group was significantly higher than that in OZ group ($P < 0.05$, Fig. 17), while propionic acid and butyric acid were not significantly different between the two groups.

Discussion

Effects of different weeks of age on egg quality

Egg quality serves as a pivotal determinant in poultry production efficiency. Our study revealed distinct differences between the OL (older laying hens) and YH (younger hens) groups, eggs from OL hens exhibited significantly greater length, short diameter, and weight compared to YH hens, these were accompanied by compromised quality parameters including reduced Haugh units and diminished eggshell strength. This paradoxical pattern aligns with established observations in aging layers - although egg production rates decline markedly during late laying cycles, individual egg mass tends to increase concomitantly (Wistedt et al., 2019; Sharma et al., 2022). This may be due to age-related intestinal degeneration impairs nutrient absorption efficiency, paradoxically triggering compensatory mechanisms that redirect metabolic resources toward fewer but larger eggs rather than maintaining production quantity (Wistedt et al., 2019). The observed reduction in Haugh units, a key indicator of albumen integrity, may correlate with diminished phospholipid content in aging hen eggs, this biochemical alteration likely reflects age-dependent changes in oviductal protein secretion patterns (Tumová and Gous, 2012; Silversides and Budgell, 2004). The compromised eggshell strength and thickness in OL hens arise from dual deficiencies in calcium homeostasis, specifically age-related declines in intestinal calcium absorption efficiency and uterine calcium carbonate deposition capacity. This disrupts the precisely coordinated ion transport system within the shell gland (uterus), ultimately impairing the structural organization of the mammillary layer and palisade matrix (Gu et al., 2021). Notably, these age-related changes appear synergistic, where reduced nutrient absorption exacerbates calcium metabolism deficiencies.

Effects of different weeks of age and egg-laying rate on blood parameters

Our hematological analyses revealed significant immunological improvements following fecal microbiota transplantation (FMT). Compared to aged non-laying hens (OZ group), young (YH) and treated aged hens exhibited enhanced antioxidant profiles (elevated SOD activity and reduced MDA levels) alongside modulated immune responses (increased IgA levels with decreased IFN-γ and IL-2 concentrations). The inverse correlation between serum SOD and MDA levels (Szczubiał et al., 2004) indicates FMT-induced restoration of redox homeostasis. Reduced lipid peroxidation (as evidenced by lower MDA) coupled with elevated SOD activity suggests improved scavenging of reactive oxygen species in metabolically active laying hens. Elevated serum IgA and IgM levels in productive hens reflect robust antibody-mediated protection. As primary effectors of mucosal immunity (Xu et al., 2024), these immunoglobulins neutralize pathogens through antigen binding (Ohashi et al., 2006), with subsequent phagocytic clearance by macrophages, this aligns with the delayed immunoglobulin class switching observed during primary immune responses (Rezaei et al., 2015). The suppressed IFN-γ and IL-2 levels in high-yield hens demonstrate controlled cellular immunity. While IL-2 facilitates T lymphocyte proliferation post-antigen challenge (Jeon and Oh, 2017), its downregulation here suggests attenuated inflammatory signaling (Hotamisligil, 2017). Notably, the IFN-γ/IL-12/IL-18 axis role in macrophage antimicrobial responses (Lalsiamthara and Lee, 2017) implies that reduced pro-inflammatory cytokine production may reflect optimized immune resource allocation in productive hens. Collectively, these hematological parameters demonstrate that peak laying performance correlates with enhanced antioxidant capacity and balanced immune homeostasis, where effective microbial modulation through FMT can mitigate age-related immune senescence.

Effects of different weeks of age and egg-laying rate on intestinal morphology and follicle morphology

Intestinal villus architecture serves as the structural foundation for nutrient assimilation in poultry, VH and CD collectively determine absorptive efficiency through their ratio (VH/CD). Our findings demonstrate that FMT administration induced significant intestinal remodeling, both YH (young hens) and OZ (treated aged hens) groups exhibited enhanced villus architecture, with YH showing superior VH/CD ratios. Notably, aged non-laying hens receiving FMT displayed restored intestinal morphology through increased VH/CD ratios and goblet cell proliferation. Elevated goblet cell numbers, as observed in plant extract-supplemented diets (Alip et al., 2021), enhance mucin secretion to protect against luminal pathogens while maintaining epithelial integrity (Lv et al., 2022). This mucosal restoration likely synergizes with FMT-induced microbial modulation to protect gut health. The VH/CD ratio's positive correlation with nutrient uptake capacity (Chuang et al., 2021) explains the improved performance in YH and OH groups, increased VH expands the absorptive surface area (Jing et al., 2014), improvement of the microbial structure can stabilize this morphology through microbial metabolic crosstalk (Kong et al., 2023; Liu et al., 2024). The age-dependent ROS accumulation (Jing et al., 2014) induces intestinal degeneration through oxidative damage (Tang et al., 2019). FMT counteracts this by microbial-mediated redox homeostasis, as evidenced in murine models (Yan et al., 2021; Ma et al., 2023). Restored villus architecture in aged hens suggests microbial metabolites may attenuate ROS-induced epithelial apoptosis. The synergistic interaction of barrier enhancement, structural optimization, and oxidative balance mechanisms collectively establishes FMT as a multifactorial intervention. FMT appears to reverse age-related intestinal decline through ecological succession in the gut microbiome.

Follicular development constitutes the biological cornerstone of egg production, where both follicular quantity and structural integrity determine laying performance. The YH (young high-yield) group exhibited significantly higher large white follicle (LWF) counts compared to aged counterparts (OZ group), aligning with nutritional studies demonstrating LWF proliferation through dietary optimization (Yao et al., 2022; Lu et al., 2023). While total follicle numbers remained comparable across groups, FMT-treated aged hens showed restored follicular ultrastructure specifically, granulosa layer thickening and

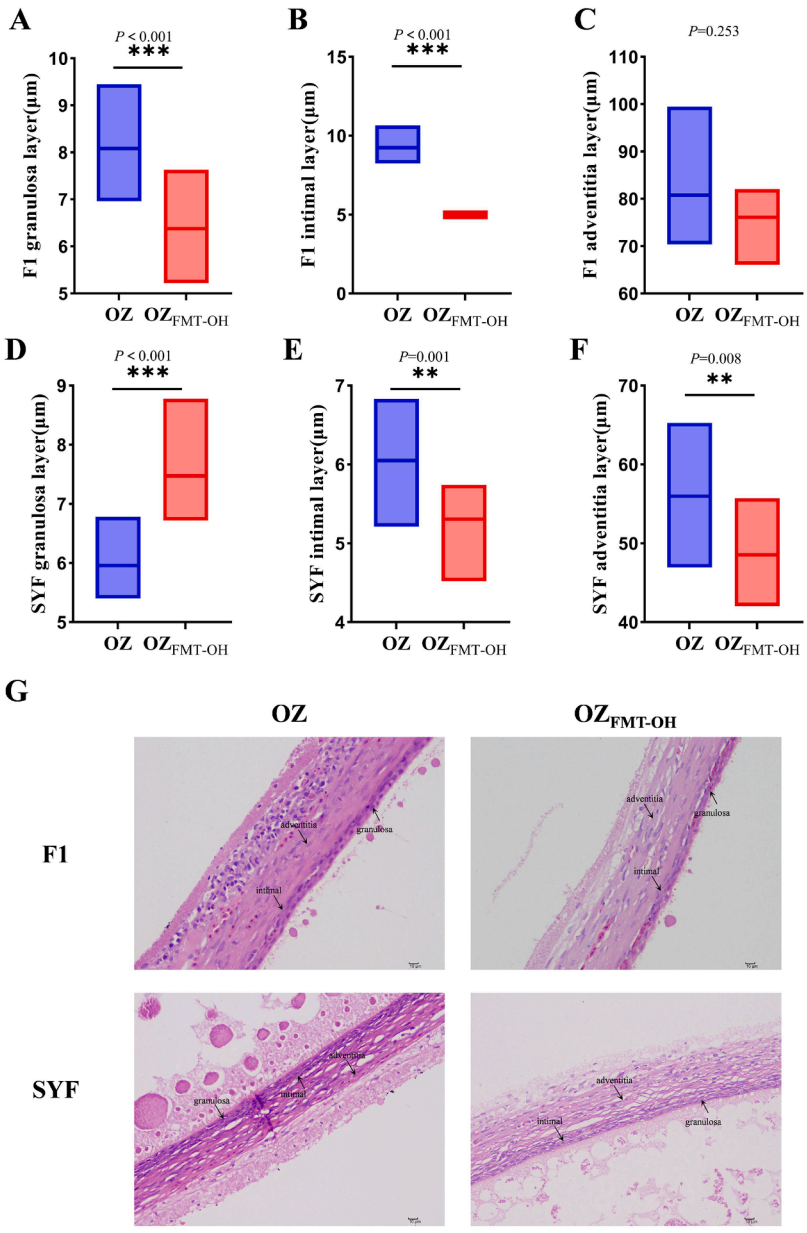


Fig. 15. Effect of FMT on Follicular morphology of aged non-laying hens. *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$p \leq 0.001$.

optimized theca interna and externa. These morphological parameters, crucial for oocyte protection and steroidogenesis (Tajima et al., 2006; Ma et al., 2024), correlate with functional recovery in treated hens. Age-related ovulatory decline may stem from microbial dysbiosis-induced endocrine disruption. Gut microbiota modulates estrogen synthesis via enterohepatic recirculation (Baker et al., 2017), while FMT from high-yielding donors can upregulate SIRT1-related apoptosis and cytokine signaling pathways, thereby improving ovarian function and oviposition rate, accompanied by an enrichment of *Bacteroides* and an increase in SCFAs. This also suggests that the microbiota plays a crucial role in this process (Qi et al., 2021; Cao et al., 2023). We hypothesize that age-associated oviductal inflammation during ovulation-potentially mediated by microbial metabolites impair oocyte release efficiency. This premise, supported by microbial links to reproductive senescence (Bendikov-Bar et al., 2021), warrants targeted investigation through oviductal cytokine profiling and microbial metabolite tracing. Collectively, these findings position FMT as a systems-level intervention, simultaneously addressing follicular quality, microbial-endocrine signaling, and potential inflammatory barriers to optimize late-cycle laying performance.

Effects of different weeks of age and egg-laying rate on blood metabolome

Microbial-derived metabolites serve as pivotal mediators in host-microbe interactions, with our metabolomic analysis revealing significant suppression of phenylpropanoid biosynthesis pathways in aged low-yield hens (OZ group) compared to OH group. This metabolic attenuation carries profound biological implications, given phenylpropanoids well-documented roles as multifunctional bioactive compounds. These secondary metabolites exhibit potent antioxidant activity through free radical scavenging, modulate inflammatory responses via NF-κB pathway regulation, and demonstrate antimicrobial efficacy against enteric pathogens, pharmacological properties which have been extensively harnessed in therapeutic development (Korkina et al., 2011; Berköz et al., 2020). Nutritional interventions using phenylpropanoid-rich supplements, such as 3% ethanol-purified flavonoids (EPF), have shown promise in poultry production by restoring metabolic flux through this pathway, thereby enhancing oxidative

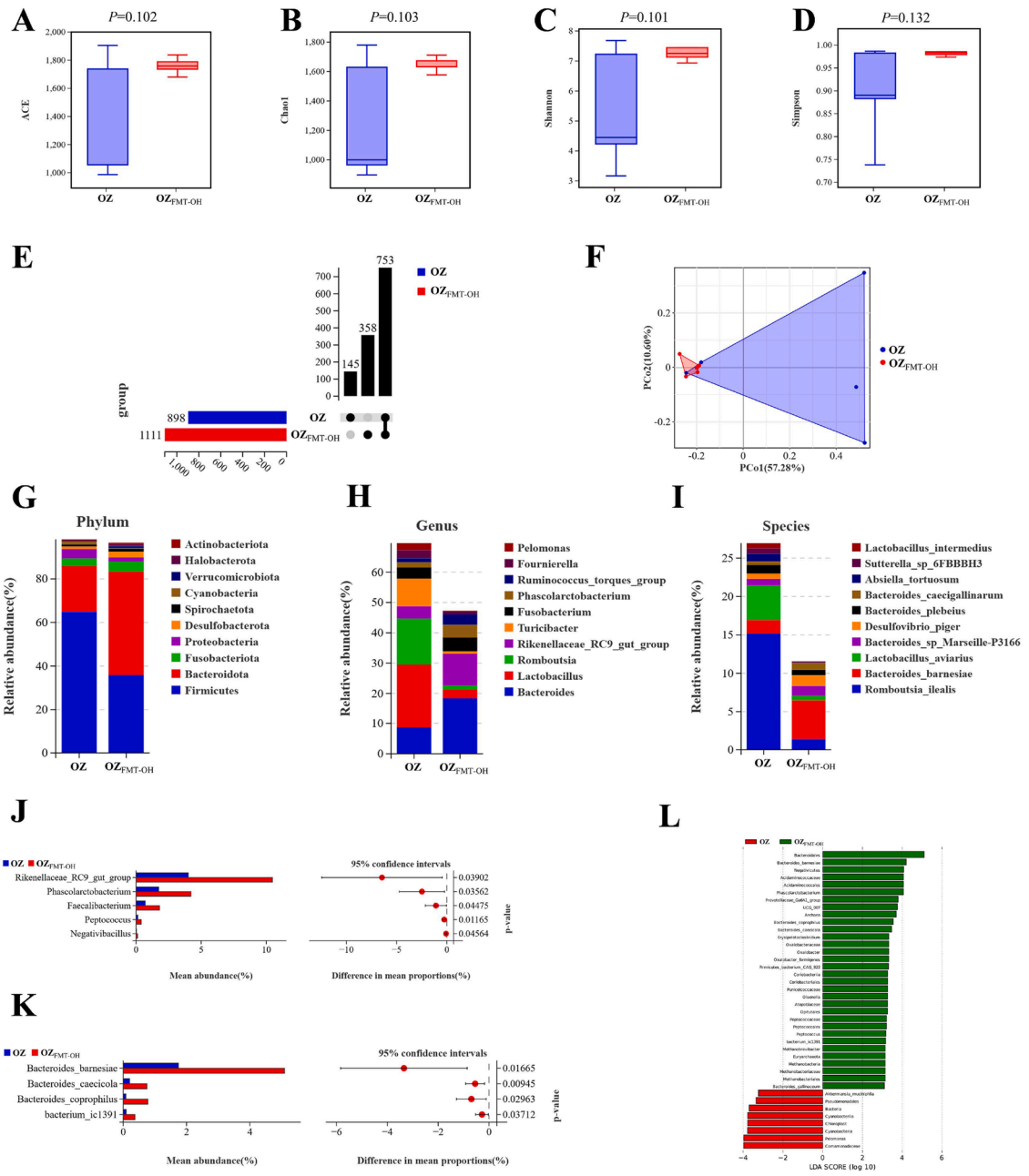
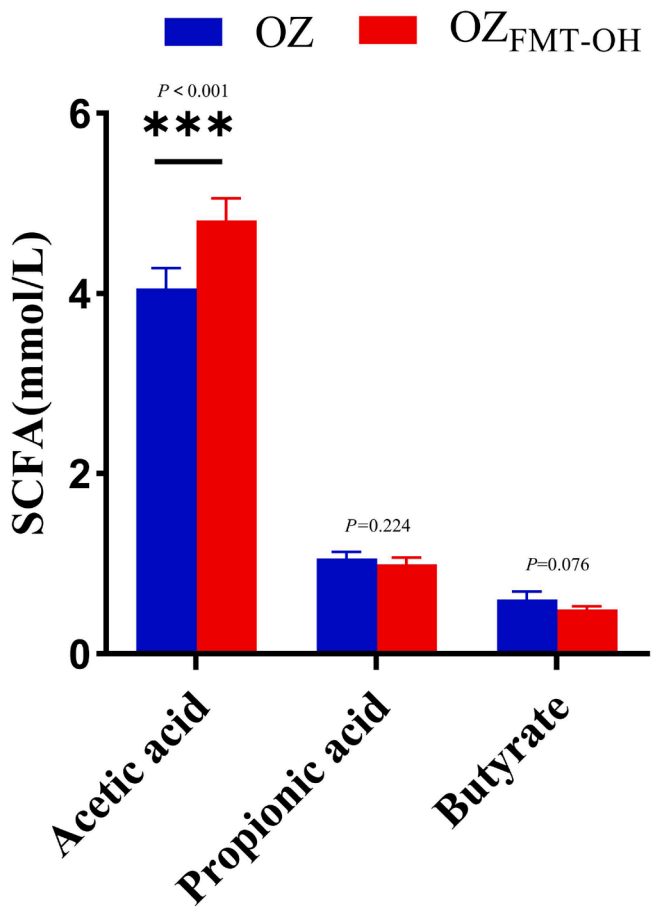


Fig. 16. Effect of FMT on Cecal microbial of aged non-laying hens. (A–D) Alpha diversity among groups (Ace, Chao1, Shannon, and Simpson index). (E) The Upset diagram illustrates the overlap of operational taxonomic unit (OTU) in all tested chickens. (F) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. (G-I) Phylum, genus, species composition. (J-K) Genus and species differences. (L) Linear Discriminant Effect Size (LEfSe) analysis.

homeostasis and intestinal barrier integrity (Hu et al., 2021; Ju et al., 2023). Notably, structural parallels between microbial phenylpropanoids and legume-derived isoflavones, as demonstrated by chickpea biochanin A-suggest evolutionary conservation in their bioactive mechanisms. These phytochemicals demonstrate osteoprotective effects through estrogen receptor-mediated calcium regulation, cardioprotective properties via LDL oxidation inhibition, and neuroprotective capacity through amyloid-β fibril disruption, establishing cross-species therapeutic potential (Liang et al., 2019; Yu et al., 2020; Zhang et al., 2020). The metabolic defects observed in OZ hens highlight the opportunity for their performance to be transformation. By leveraging the molecular mimicry between microbial and metabolites, strategic modulation of this pathway through combined pharmabiotic formulations and precision nutrition could address both poultry production challenges and human health issues, particularly menopause-associated disorders (Jalaludeen et al., 2016).

Effects of different weeks of age and laying rate on gut microbial composition of laying hens

The gut microbial landscape across all groups exhibited characteristic dominance of Firmicutes and Bacteroidetes, aligning with established avian gut microbiota profiles (Zhang et al., 2021). Notably, age-related dysbiosis showed significant differences between groups. Phylum-level dynamics revealed elevated Desulfobacterota in OL hens, whose lipopolysaccharide production may drive intestinal inflammation and epithelial dysfunction (Huang et al., 2021). Concurrently, OZ hens showed marked Synergistota enrichment-a taxa implicated in enteric pathogenesis through mucosal barrier disruption (Adhikari et al., 2020; Sun et al., 2022; Tang et al., 2024). This proliferation of



Fig, 17. Effect of FMT on Short-chain fatty acid of aged non-laying hens. *0.01 $< P <$ 0.05, **0.001$< P <$ 0.01, ***$p \leq$ 0.001.

pro-inflammatory taxa (Desulfobacterota and Synergistota) likely contributes to the observed villus atrophy (reduced V/C ratios) in aged layers via chronic mucosal injury. Genus-level stratification demonstrated production-performance correlations, *Megamonas* (Firmicutes) dominated YH hens, potentially enhancing carbohydrate metabolism through polysaccharide fermentation (Nuli et al., 2019), while *Bacteroides* prevailed in OH hens. The vitamin E-responsive *Megamonas* (Wang et al., 2024) displayed age-dependent decline patterns, suggesting microbial biomarkers of senescence (Wang et al., 2015). Contrastingly, *Bacteroides* immunomodulatory capacity mediated through TLR4/5 signaling via flagellin (Spase et al., 2020) correlated with enhanced gut barrier function via tight junction upregulation (Feng et al., 2024). These microbial signatures aligned with physiological metrics: superior antioxidant (elevated SOD, reduced MDA) and anti-inflammatory (high IgA/IgM, low IFN-γ/IL-2) profiles in YH/OH groups versus OL/OZ hens. Species-specific patterns identified *Bacteroides caecigallinarum* and *Bacteroides plebeius* as conserved cecal symbionts (Saputra et al., 2015; Borrelli et al., 2017) with group-dependent abundance shifts. Intriguingly, YH and OZ groups shared comparable abundances of these species alongside *Megasphaera stantonii*, a paradoxical similarity extending to phylum/genus-level profiles. This suggests that aging layers failed to undergo appropriate microbial succession, resulting in Firmicutes and Bacteroidetes abundance inversion may retain "pseudo-juvenile" microbiota configurations ill-adapted to aged physiology, ultimately compromising production performance (Stanley et al., 2013; Adhikari et al., 2020; Ricke et al., 2022). Collectively, these multi-level microbial signatures delineate how dysregulated community succession-marked by pro-inflammatory taxa persistence and symbiont ratio distortion-undermines intestinal homeostasis in aging layers, providing mechanistic insights for microbiota-targeted productivity preservation strategies.

Effect of FMT on gut microbiota composition in aged non-laying hens

The strategic modulation of gut microbiota, particularly through fecal microbiota transplantation (FMT), demonstrates profound impacts on late-cycle laying performance. Our findings reveal that transplanting microbiota from high-yield hens to aged non-laying significantly enriched *Bacteroides* populations, at the same time, the production performance is improved. *Bacteroides-mediated* polysaccharide fermentation enhances nutrient bioavailability while generating immunomodulatory metabolites. This genus promotes IL-10-dependent Treg cell differentiation (Tan et al., 2019), critical for maintaining intestinal immune homeostasis during prolonged laying cycles (Dagdeviren et al., 2017), and can produce short-chain fatty acid (SCFA), reinforces gut barrier integrity through tight junction protein upregulation (Fan et al., 2023), while suppressing pro-inflammatory LPS signaling (Serino, 2019). The *Bacteroides* can dynamically regulates energy partitioning. Enriched *Bacteroides* reduce fat signaling through PPARγ inhibition (Xiang et al., 2021), thereby redirecting lipid resources toward egg production rather than visceral fat deposition, a metabolic shift empirically linked to sustained laying capacity (Wu et al., 2022). This aligns with FMT-induced improvements in glucose homeostasis (Sung et al., 2017) and hepatic vitellogenin synthesis efficiency. FMT from high-yield laying hens could up-regulate hormone receptor expression (ESR1, ESR2, FSHR and AMHR) and reduce ovarian cell apoptosis and the expression of pro-apoptotic mRNA (Bax, caspase-3, caspase-8, caspase-9). Moreover, it can increase the expression of anti-apoptosis related genes (Bcl2, PARP), and finally improve ovarian function and ovulation rate by up-regulating SIRT1 related apoptosis and cytokine signaling (Cao et al., 2023). Notably, our findings resonate with diverse microbiota-modulation strategies, phytogenic interventions (e.g., *Ligustrum* supplementation), fermented feeds, and herbal formulations all converge on a common target, abundance of *Bacteroides* (Chen et al., 2020; Guo et al., 2022; Li et al., 2024). The resultant microbial ecology fosters IL-10-producing Treg cell expansion (Tan et al., 2019), establishing an anti-inflammatory milieu that extends laying cycles by preserving ovarian function (Dagdeviren et al., 2017; Liao et al., 2022). This evidence positions *Bacteroides* as a keystone taxon in poultry microbiomes, where its enrichment through FMT or nutritional strategies can reverse age-related dysbiosis. By simultaneously enhancing nutrient assimilation, calcium transport, and metabolic efficiency while curbing inflammation, *Bacteroides*-centric microbial management emerges as a holistic approach to sustain late-cycle productivity in aging flocks.

Conclusion

The intestinal flora of aged high-yield laying hens shows differences compared to those of low-yield and non-laying hens, Aged high-yield laying hens have a higher abundance of *Bacteroides*, while the flora structure of aged non-laying hens is more similar to that of young hens. FMT can change the intestinal flora structure, increase *Bacteroides* abundance, improve intestinal and follicular morphology, improve anti-inflammatory ability and increase laying rate in aged non-laying hens.

Disclosures

The authors declare no conflicts of interest.

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105064.

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