Improvement in ovarian function following fecal microbiota transplantation from high-laying rate breeders

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ABSTRACT The underlying mechanism between the gut microbiota and reproductive function is not yet well-known. This study was conducted to investigate the effect of the administration of fecal microbiota transplantation (**FMT**) from highly laying rate donors on the cecal microbiota, intestinal health and ovarian function in broiler breeders. A total of 60 broiler breeders (53 wk of age) were selected by their laying rate [high (**HP**, 90.67 ± 0.69%; $n = 10$) and low (**LP**, 70.23 ± 0.87%; $n = 20$)]. The LP breeders were then be transplanted with fecal microbiota from HP hens (**FMTHP**; $n = 10$) or the same dosage of PBS (**FMTCON**; $n = 10$) for 28 d. The results revealed that FMT from HP donors increased egg-laying rate and serum hormone levels [17β-estradiol (**E2**), anti-Müller hormone], also decreased proinflammatory cytokine levels (interleukin-6, interleukin-8, tumor necrosis factor-α) of LP breeders ($P < 0.05$). The FMTHP group breeders had higher villus height, villus height/crypt depth ratio, and upregulated mRNA expression of jejunum barrier-related gene (***ZO-2*** and *mucin-2*) and estrogen, follicle-stimulating hormone (**FSH**) and anti-Müller hormone (**AMH**) receptor genes (***ESR1***, ***ESR2***, ***FSHR***, ***AMHR***) ($P < 0.05$) than FMTCON group. FMT from HP donors led to higher mRNA expression of *Bcl2* and sirtuin1 (***SIRT1***), while it downregulated the proapoptotic genes (*Bax, caspase-3, caspase-8*, and *caspase-9*) mRNA expressions in ovary compared with the FMTCON breeders ($P < 0.05$), and this pattern was also observed in HP donors. Also, HP breeder had higher observed_species and alpha-diversity indexes (Chao1 and ACE) than FMTCON group, while FMTHP can increase observed_species and alpha-diversity indexes (Chao1 and ACE) than FMTCON group ($P < 0.05$). The bacteria enrichment of Firmicutes (phylum), Bacteroidetes (phylum), *Lactobacillus* (genus), *Enterococcus* (genus), and *Bacteroides* (genus) were increased by FMTHP treatment. The genera *Butyricicoccus, Enterococcus*, and *Lactobacillus* were positively correlated with egg-laying rate. Therefore, cecal microbiomes of breeders with high egg-laying performance have more diverse activities, which may be related to the metabolism and health of the host; and FMT from high-yield donors can increase the hormone secretion, intestinal health, and ovarian function to improve egg-laying performance and the **SIRT1**-related apoptosis and cytokine signaling pathway were involved in this process.

Key words: egg-laying rate, cytokine signaling pathway, ovarian function, cecal microbiota, fecal microbiota transplantation

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INTRODUCTION

The gut microbes perform several key roles in maintaining host health and aging by participation in host metabolism, immunity, and endocrine system (Wu and Wu, 2012; Chang and Kao, 2019; Hussain et al., 2021; Qi et al., 2021). It is generally accepted that the gut microbes can break down different food components and nutrients and synthase a range of metabolites to interact with host (Rastelli et al., 2018; Rowland et al., 2018;

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Rong et al., 2019; Shock et al., 2021; Ricke et al., 2022). Several studies have shown that the status of the intestinal microbiota of hens is closely associated with their health and productivity (Zhao et al., 2019; Wang et al., 2020; Yang et al., 2020; Wang et al., 2021a). The egg-laying rates show great differences despite the same genetic background, age, feed nutrition, and living environment in breeders and laying hens (Pedroso et al., 2006; Wang et al., 2020; Yang et al., 2020). It has been proposed that dysbiosis of gut microbiota may be a potential pathogenetic factor in the development of ovarian dysfunction and decreasing in fertility (Yurtdaş and Akdevelioğlu, 2020; Qi et al., 2021; Wang et al., 2021b,c). Also, the gut microbiota was shown difference between high- and low-yield layers and breeders in previous studies (Wang et al., 2020; Yang et al., 2020; Wang et al., 2021a), and transplanted with the fecal microbiota from high-yield layers can improve the egg-laying rate of low-yield layers (Wang et al., 2020), which indicate that the microbiota also plays an important role reproduction function. Sex steroid hormones, such as estradiol, progesterone, and testosterone play an important physiological role in reproduction, differentiation, cell proliferation, apoptosis, inflammation, metabolism, homeostasis, and brain function (Edwards, 2005; Qi et al., 2021). Bacteria can produce and secrete hormones, and the crosstalk between microbes and hormones can affect host metabolism, immunity, and behavior (Markle et al., 2013; Neuman et al., 2015). The microbiota also reported to participate the reproductive endocrine system by producing and interacting with estrogen, androgens, insulin, and other hormones (insulin like growth factor 1, leptin, serotonin, melatonin, etc.) in humans and animal models (Neuman et al., 2015; Yan and Charles, 2018; Jenson et al., 2020; Qi et al., 2021). Imbalance of the gut microbiota composition can lead to several reproduction dysfunction, such as polycystic ovary syndrome (**PCOS**), endometriosis, and ovarian cancer in mammal (Baker et al., 2017; Insenser et al., 2018; Giudice et al., 2021; Qi et al., 2021). However, the mechanism how the microbiota is involved in the regulation of ovarian function is still not well-known.

Hence, the objective of this study was to determine: 1) the characteristics of the cecal microbiota of broiler breeders with high egg laying, 2) the relationship between the structure of the intestinal microbiota and ovarian function by fecal microbiota transplantation (**FMT**) in poultry model.

MATERIALS AND METHODS

Animals, Diets, and Design

The experimental protocol used in the study was approved by the Animal Care and Use Committee of the Sichuan Agricultural University (SYXK2020-067). A parent breeders of the same age (47 wk of age) were individually housed with free access to water under the same feed nutrition, management and environmental condition. Egg-laying rate were recorded for 56 consecutive days to select the high- (HP) and low- (LP) laying rate breeders. Based on the egg-laying level, 20 breeders were assigned to the HP (laying rates = 90.67% ± 1.31) group, and 40 breeders were assigned to the LP (laying rates = 70.23% ± 1.31) group.

Fecal Microbiota Transplantation Procedure

The total experimental period was 5 wk and was divided into 2 stages: preadministration (7 d, microbiota depletion by antibiotics: streptomycin and penicillin administration) and FMT (4 wk). Forty microbiota-depleted breeders (55 wk of age) were chosen for this study. Then, the microbiota-depleted breeder that were originally fed the control diet received FMT from donor layer (**FMTHP**, $n = 10$) that had higher egg-laying rate or received same dosage of PBS (**FMTCON**, $n = 10$). Before the fecal transfer, recipient breeder was treated with streptomycin and penicillin to deplete endogenous gut microbiota. Penicillin (1 g/L) and streptomycin (1 g/L) were dissolved in sterile water and 100 μL were fed into breeder by oral gavage once a day for 7 consecutive days as previously described (Duan et al., 2019; Wang et al., 2020). Depletion of microbiota was validated by PCR analysis using 16S rRNA gene. For FMT, approximately 500 g of fresh fecal excreta were extracted via donor breeder followed by resuspension in 2.5 L of sterile PBS (0.1 M, pH 7.2) under anaerobic conditions. Then, the supernatant was collected and FMT was performed by a single oral administration of 2 mL of suspension ($1.4 \text{ CFU} \times 10^{12}$/mL) according to the method described by Ma et al. (2018) and Wang et al. (2020). Broiler breeders were fed a complete feeding mixture in a mash form. There were 10 replicates with 2 birds per replicate; room environment was controlled at 22°C by a daily lighting schedule of 16 h light and 8 h dark. Birds were allowed free access to water and restricted access to feed (154 g/d/breeder).

Productive Performance and Sample Collection

Egg-laying rate was recorded every day and the body weight of the layer was recorded on the onset and end day of the experiment ($n = 10$). On d 24 of the animal trial, blood samples were collected ($n = 10$) from the wing after 12 h of fasting and the serum were separated by incubation at 4°C for 30 min and subsequent centrifugation at 1,500 × g for 20 min.

Serum Reproductive Hormones and Inflammatory Cytokines Analysis

Egg-laying rate was recorded every day and the body weight of the layer was recorded on the onset and end day of the experiment ($n = 10$). On d 28 of the animal trial, blood samples were collected ($n = 10$) 12 h before

the expected times for ovulation (based on prior observation) from the wing after 12 h of fasting and the serum were separated by incubation at 4°C for 30 min and subsequent centrifugation at 1,500 × *g* for 20 min. Serum concentration of 17*β*-estradiol (**E2**, CHEB0528), follicle-stimulating hormone (**FSH**, CHFI00020), anti-Müller hormone (**AMH**, abx585111), luteinizing hormone (**LH**, CHF100113), progesterone (**PROG**, CHF100061), interleukin-1*β* (**IL-1*β***, CHDL00034), interleukin-6 (**IL-6**, CHDL00038), interleukin-6 (IL-8, CHDL00039), interleukin-10 (**IL-10**, CHDL00028), and tumor necrosis factor-*α* (**TNF-*α***, CHFI00014) were examined by commercial enzyme-linked immunosorbent assay (**ELISA**) kits according to the manufacturer's instructions (ELISAGenie, Dublin, Ireland).

Ovarian TUNEL Assay

The ovary ($n = 10$) was quickly removed and placed immediately into methylaldehyde, then were histochemical stained using TUNEL technique by an in situ apoptosis detection kit (in situ cell death detection kit POD, Roche Group, Basel, Switzerland). Using BA200Digital (Mike Audi Industrial Group Co., Ltd., Chengdu, China) to image acquisition. Apoptotic color is light yellow or brown yellow, and negative expression is blue with white back ground. Overall, 100 images were taken to measure cell apoptosis, and apoptosis rate was defined as the percentage of apoptotic cells in 100 cells counted as described in our previous studies (Wang et al., 2021a,b).

Real-Time PCR for Jejunum Barrier, Short Chain Fatty Acids Receptors, and Ovarian Function-Related mRNA Expression

The total RNA and real-time RT-PCR were carried out as described previously (Wang et al., 2021a). In brief, total RNA were extracted with Trizol followed by DNase1 treatment to remove genomic DNA. Gene expression of jejunum barrier (***ZO-1***, ***ZO-2***, *Occludin, Mucin-2*), SCFAs receptors [free fatty acids receptor 2 (***FFAR2***), free fatty acids receptor 3 (***FFAR3***)] and ovarian function related [sirtuin1 (***SIRT1***), nuclear factor-kappaB (**NF-*κ*B**), poly ADP-ribose polymerase (***PARP***), ***estrogen receptor 1*** (*ESR1*), ***ESR2***, ***FSHR*** (*FSH receptor*), ***AMHR*** (*AMH receptor*), ***Bcl-2*** (B-cell lymphoma-2), *Bax, caspase-3, caspase-8, caspase-9*] was determined by quantitative real-time PCR in the ovary of broiler breeders by ABI 7900 Real-Time PCR system (ABI Biotechnology, Eldersburg, MD). The primer information for all the genes is listed in Supplementary Table S2. Each sample was assayed in triplicate and *β*−actin was used as the house-keeper genes. The $2^{-\Delta\Delta CT}$ method was used to calculate target gene expression, and mRNA expression in HP was used as baseline relative to treatment groups (i.e., fold-change).

Gut Microbiota Analysis and Short Chain Fatty Acids Quantification in Cecum

Microbial profile in the cecum digesta ($n = 10$) was evaluated by the sequencing and clustering of 16S rRNA gene with high-throughput pyrosequencing, the sequencing and bioinformatics analysis were performed by Novogene Bioinformatics185-187, Technology Co. (Tianjin, China), and the method was used as recently described by Yang et al. (2020). SCFA ($n = 10$) including acetate, propionate, and butyrate in the cecum content was also analyzed using Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) following previous protocols (Wang et al., 2021b).

Statistical Analysis

Data were analyzed by ANOVA using GLM procedure of SAS 9.2 (SAS Institute, Cary, NC) and GraphPad Prism (GraphPad Inc., La Jolla, CA). For the microbiota data, alpha indexes were analyzed by Wilcox rank sum test, differences among treatments were considered significant at $P < 0.05$. Beta diversity based on the weighted UniFrac distance matrices were calculated with QIIME (Version 1.7.0) and cluster analysis was preceded by principal coordinate analysis (**PCoA**). Differentially represented bacterial taxa between different samples were analyzed using the linear discriminant analysis effect size (**LEfSe**). The results are presented as mean and SEM.

RESULTS

Production Performance and Plasma Hormone and Cytokine Levels

As shown in Table 1, LP breeders were observed to have lower egg-laying rate compared with the HP breeders at the initial and 21-day post-FMT ($P < 0.05$), while the FMT from the HP donors increased egg-laying rate when compared to the FMTCON breeders. Sex hormones (E2 and AMH) and anti-inflammatory cytokine IL-10 concentration in plasma were lower in FMTCON breeders than those in HP breeders (Tables 2 and 3; $P < 0.05$). The FMPHP breeders enhanced E2 and AMH concentration, also decreased proinflammatory cytokine levels (IL-6, IL-8, TNF-*α*) of LP breeders ($P < 0.05$). The serum levels of FSH, PROG, LH, and IL-1*β* in serum were not affected among 3 treatments ($P > 0.05$).

Intestine Morphology and Tight Junction Protein mRNA Expression

As shown in Tables 4 and 5, the FMTCON group breeders had lower villus height and V:C ratio than the HP group, while it also had lower jejunum barrier-related mRNA expression of *ZO-2* and *Mucin 2* in

Table 1. Effect of FMT on egg-laying rate of broiler breeders.

Item[1]	Initial	After antibiotics	FMT period			
			D 1−7	D 8−14	D 15−21	D 22−28
HP	90.67[a]	88.79[a]	86.22[a]	85.79[a]	84.29[a]	86.67[a]
FMTCON	70.23[b]	47.32[b]	53.41[b]	58.67[b]	62.33[c]	59.71[c]
FMTHP	70.23[b]	46.79[b]	55.27[b]	61.29[b]	72.22[b]	76.67[b]
SEM	1.31	1.43	2.69	2.44	2.37	2.34
P value	0.010	0.012	0.021	0.032	0.035	0.024

[a,b,c]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 2 breeders/replicate. Abbreviations: FMT, fecal microbiota transplantation; FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from HP donor breeder; HP, high-laying rate.

Table 2. Effect of FMT on serum hormone levels of broiler breeders.

Item[1]	E2, pmol/L	FSH, U/L	AMH, pg/mL	LH, ng/L	PROG, pmol/L
HP	86.53[a]	8.30	267.79[a]	392.80	2409.43
FMTCON	67.33[b]	8.73	151.53[c]	396.29	2452.09
FMTHP	82.26[a]	7.16	207.20[b]	365.51	2265.28
SEM	4.29	0.42	14.39	18.74	108.06
P value	0.011	0.121	0.008	0.776	0.466

[a,b,c]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: AMH, anti-Müller hormone; E2, 17β-estradiol; FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from HP donor breeder; FSH, follicle-stimulating hormone; HP, high-laying rate; LH, luteinizing hormone; PROG, progesterone.

Table 3. Effect of FMT on egg-laying rate of broiler breeders.

Item[1]	IL-1β, ng/L	IL-6, ng/L	IL-8, ng/L	IL-10, ng/L	TNF-α, n/L
HP	28.02	5.41[b]	26.81[c]	9.45[a]	931.02[c]
FMTCON	27.62	6.59[a]	41.82[a]	7.84[b]	1399.34[a]
FMTHP	28.57	5.33[b]	33.32[b]	9.13[b]	1018.93[b]
SEM	2.26	0.56	2.48	0.86	96.56
P value	0.951	0.008	0.009	0.012	0.017

[a,b,c]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from HP donor breeder; HP, high-laying rate.

jejunum mucosa ($P < 0.05$). The FMTHP group breeder had higher villus height, V:C ratio, and upregulated mRNA expression *ZO-2* and *mucin-2* than FMTCON group ($P < 0.05$). The crypt depth and mRNA expression of *occludin* and *ZO-1* were not affected by experimental treatments at present study.

Table 4. Effect of FMT on duodenum morphology of broiler breeders.

Item[1]	Villus height, mm	Crypt depth, mm	V:C ratio
HP	1307.52[a]	204.80	6.38[a]
FMTCON	1044.92[b]	264.57	3.95[b]
FMTHP	1322.55[a]	239.49	5.52[a]
SEM	76.05	24.21	0.44
P value	0.009	0.334	0.031

[a,b]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from HP donor breeder; HP, high-laying rate; V:C, villus height to crypt depth ratio.

Cecum SCFA and Its Receptors-Related Gene Expression

The LP breeders had lower cecum content concentration of the main short chain fatty acids (propionate, butyrate) and lower total SCFAs than those observed in

Table 5. Effect of FMT on jejunum barrier protein-related gene expression of broiler breeders.

Item[1]	Occludin	ZO-2	ZO-1	Mucin-2
HP	1.00	1.00[a]	1.00	1.00[a]
FMTCON	0.97	0.53[b]	0.90	0.62[b]
FMTHP	1.02	0.97[a]	0.96	1.15[a]
SEM	0.24	0.19	0.16	0.24
P value	0.967	0.028	0.570	0.010

[a,b]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); HP, high-laying rate; ZO-1, zonula occudens-1; ZO-2, zonula occudens-2.

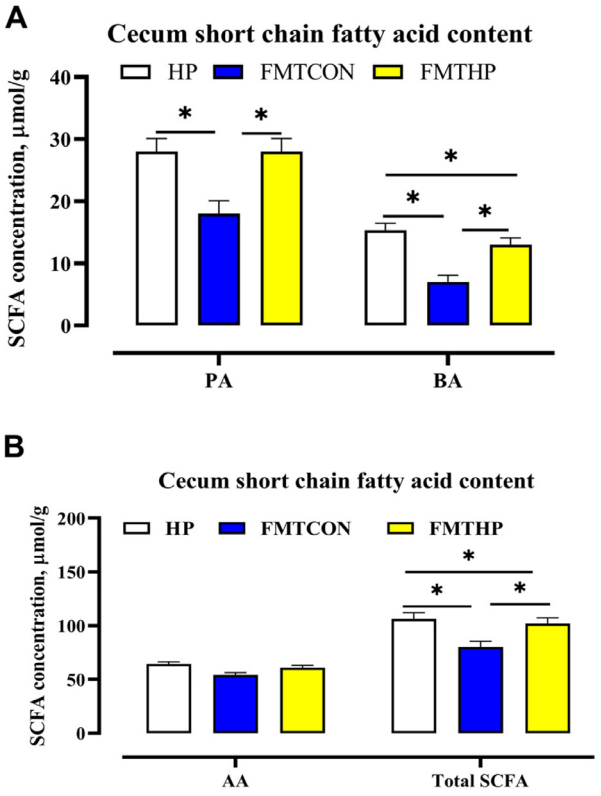

Figure 1. The effect of FMT on hormone receptors-related gene expression. Each mean represents 10 replicates, with 1 breeder/replicate. (A, B) Short chain fatty acid concentration in cecal digestion. Abbreviations: AA, acetate; BA, butyrate; EHP, high egg-laying rate; FMTCON, lower laying rate breeder received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); PA, propionate; SCFA, short chain fatty acid. Statistical significance was evaluated by *t* test, $^{*}P < 0.05$.

the HP groups (Figure 1A and B; $P < 0.05$), while the FMTHP breeders had higher propionic and butyric acids and total SCFAs concentration when compared with the FMTCON breeders ($P < 0.05$). The mRNA expression of SCFAs receptors (*FFAR2* and *FFAR3*) was also lower in FMTCON breeders and upregulated by FMTHP when compared with the FMTCON breeders (Figure 2A and B, $P < 0.05$).

The Cell Apoptosis Rate and Relative mRNA Expression of Ovary Function-Related Genes

The number of the ovary cell apoptosis rate were lower in the HP group than in the FMTCON ones (Figure 3A and B; $P < 0.05$). The HP breeders had higher mRNA expression of hormone receptors gene (*ESR1, ESR2, FSHR, AMHR*) and antiapoptotic gene (*SIRT1, Bcl2, PARP*) compared to FMTCON breeders (Figures 2C, D and 3C−F; $P < 0.05$). Transplanted microbiota from HP donors to LP breeders led to lower cell apoptosis rate and higher mRNA expression of *SIRT1, PARP*, and *Bcl2* ($P < 0.05$, while it downregulated the cytokine pathway-related gene *NF-κB* and proapoptotic genes (*Bax, caspase-3, caspase-8*, and *caspase-9*) mRNA expressions in ovary compared with the FMTCON breeders ($P < 0.05$).

Cecum Microbiota Composition

Relative microbial abundances of the cecum at phylum level indicated that Firmicutes was the dominant phylum in all dietary treatments (HP, 52.11%; FMTCON, 60.86%; FMTHP, 53.98%). Firmicutes, Bacteroidetes, and Proteobacteria comprised of 93.44, 94.97, and 91.93% microflora in the HP, FMTCON, and FMTHP groups, respectively (Table 7). The HP and FMTHP group had higher abundance of Firmicutes and Bacteroidetes, and lower enrichment of Proteobacteria and Firmicutes/Bacteroidetes ratio value at the phylum level ($P < 0.05$). At the genus level, we observed that abundance of *Lactobacillus* and *Enterococcus* was increased in HP and FMTHP group ($P < 0.05$). The shared OUT among 3 groups were presented in Figure 4A. These data showed that while the FMTCON and FMTHP led to microbial variation but did not change the dominant species at phylum level in the breeder cecum.

Alpha Diversity of Cecum Microbiota

Alpha diversity refers to the diversity in a region or ecosystem. Diversity indices, including Chao1, ACE, Simpson, and Shannon index, were used to reflect the richness and uniformity of the samples. As shown in Table 6, HP breeder had higher observed_species and alpha-diversity indexes (Chao1 and ACE) than FMTCON group ($P < 0.05$), while FMTHP can increase observed_species and alpha-diversity indexes (Chao1 and ACE) than FMTCON group ($P < 0.05$). These results indicate that the diversity of the cecum microbiota in the HP and FMTHP group was higher than that in LP group.

Beta Diversity of Cecum Microbiota

The PCA of the 3 groups of related bacterial communities showed differences in clustering in the high-yield group and low-yield group (Figure 4B; HP vs. FMTCON). As shown in Tables 7 and 8, Figures 4C, D and 5A, B (LEfSe), the bacteria enrichment of Firmicutes (phylum), Acinobacteria (phylum), *Streptococcaceae* (Family), *Lactobacillus* (genus), *Enterococcus* (genus), and *Lactobacilus_gasseri* (Species) were increased by FMTHP treatment, while Bacteroidetes (phylum) were lower and *Weisssela_cibaria* (species) in FMTCON breeders ($P < 0.05$).

Correlations Between Cecum Microbiota and Laying Rate of Breeders

A Spearman correlation analysis was performed to evaluate the potential link between alterations in gut microbiota composition and the egg-laying rate in

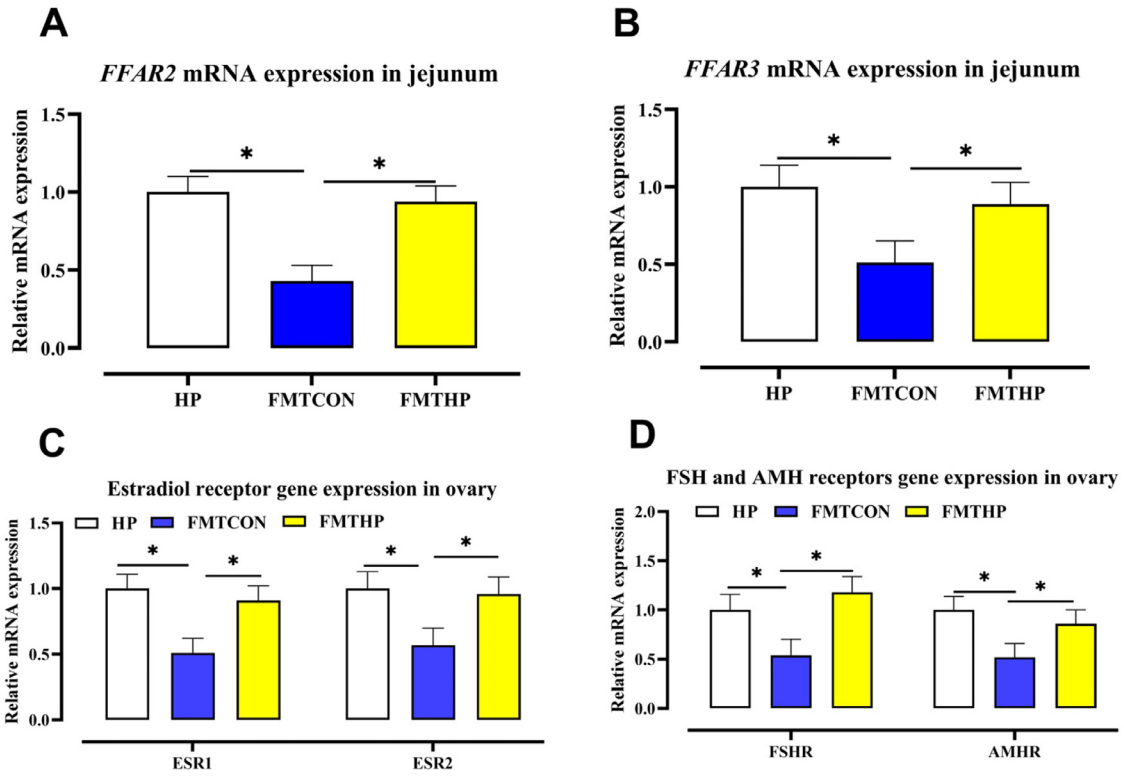

Figure 2. The effect of FMT on SCFAs and SCFAs receptors gene expression. Each mean represents 10 replicates, with 1 breeder/replicate. (A) FFAR2 mRNA expression in jejunum; (B) FFAR3 mRNA expression in jejunum; (C) estradiol receptors genes expression in ovary; (D) FSH and AMH receptors genes expression in ovary. Abbreviations: AMHR, anti-Müllerian hormone receptor; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; FFAR2, free fatty acid receptor 2; FFAR3, free fatty acid receptor 3; FMTCON, lower laying rate breeder received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); FSHR, follicle-stimulating hormone receptors. Statistical significance was evaluated by *t* test, $^*P < 0.05$.

breeders (Figure 6). The genera *Butyricicoccus, Enterococcus*, and *Lactobacillus* were positively correlated with egg-laying rate ($r = 0.85, 0.59, 0.78$; $P < 0.05$), but genera *Streptococcus* (-0.67; $P < 0.05$) was negatively correlated with egg-laying rate ($P < 0.05$).

DISCUSSION

There are many factors other than genomic background can affect the reproductive performance of breeders, including feed nutrition, management, environmental stressors, and diseases (Rozenboim et al., 2007; Du et al., 2020). The endocrine factors, such as gonadotropin-releasing hormone, estradiol, FSH, and LH, can modulate egg-laying rate through regulating follicle development (Palmer and Bahr, 1992; Du et al., 2019). It has been observed that the HP breeders had higher reproductive hormone levels (E2, AMH, and leptin), and higher gene expression of hormone receptor genes (ESR1, ESR2, AMHR, and FSHR) in ovary at present study. Estrogen receptors are key receptors to maintain ovarian granulosa cell differentiation, follicle and oocyte growth and development, and ovulation function. The abnormal functions of estrogen, its receptors, and estradiol synthesis-related enzymes (aromatase) are closely related to ovarian aging and infertility (Isola et al., 2020). Also, the number of antral follicles selected for dominance and ovulation is largely dependent on the regulatory action and the density of FSHR and LHR on the granulosa cell surface (Cai et al., 2007; Regan et al., 2021). These results are in agreement with our previous study, which is also found that higher egg-laying rate breeders had higher serum AMH and leptin levels, together with higher gene expression of FSHR and AMHR in ovary (Wang et al., 2021a,b). However, the estradiol level was not affected between 2 different egg-laying rate (about 80 vs. 70%) breeders in above studies. This may due to the egg-laying rate in current study (about 90 vs. 70%) is higher than the previous studies.

It is well-accepted that productive and reproductive performances are influenced by absorption and utilization of nutrients in poultry. Intestinal morphology determines the nutrient absorption capacity and villus height is generally recognized as a good indicator for intestinal health. Also, studies have suggested that stressors and aging-induced can induce inflammation (indicated by higher mRNA expression of proinflammatory cytokines and its upstreaming nuclear factor NF-κB), decreased the villus height and antioxidant capacities of small intestine (Ding et al., 2020; Kogut et al., 2018). The LP breeder had higher proinflammatory cytokine levels (IL-6 and TNF-α) and lower mRNA expression of intestinal barrier-related protein in the jejunum. Similarly, Mao et al. (2021) also reported that IL-6 and TNF-α mRNA expression were higher in the jejunal mucosa of low-laying breeders. As also suggested in our study, the

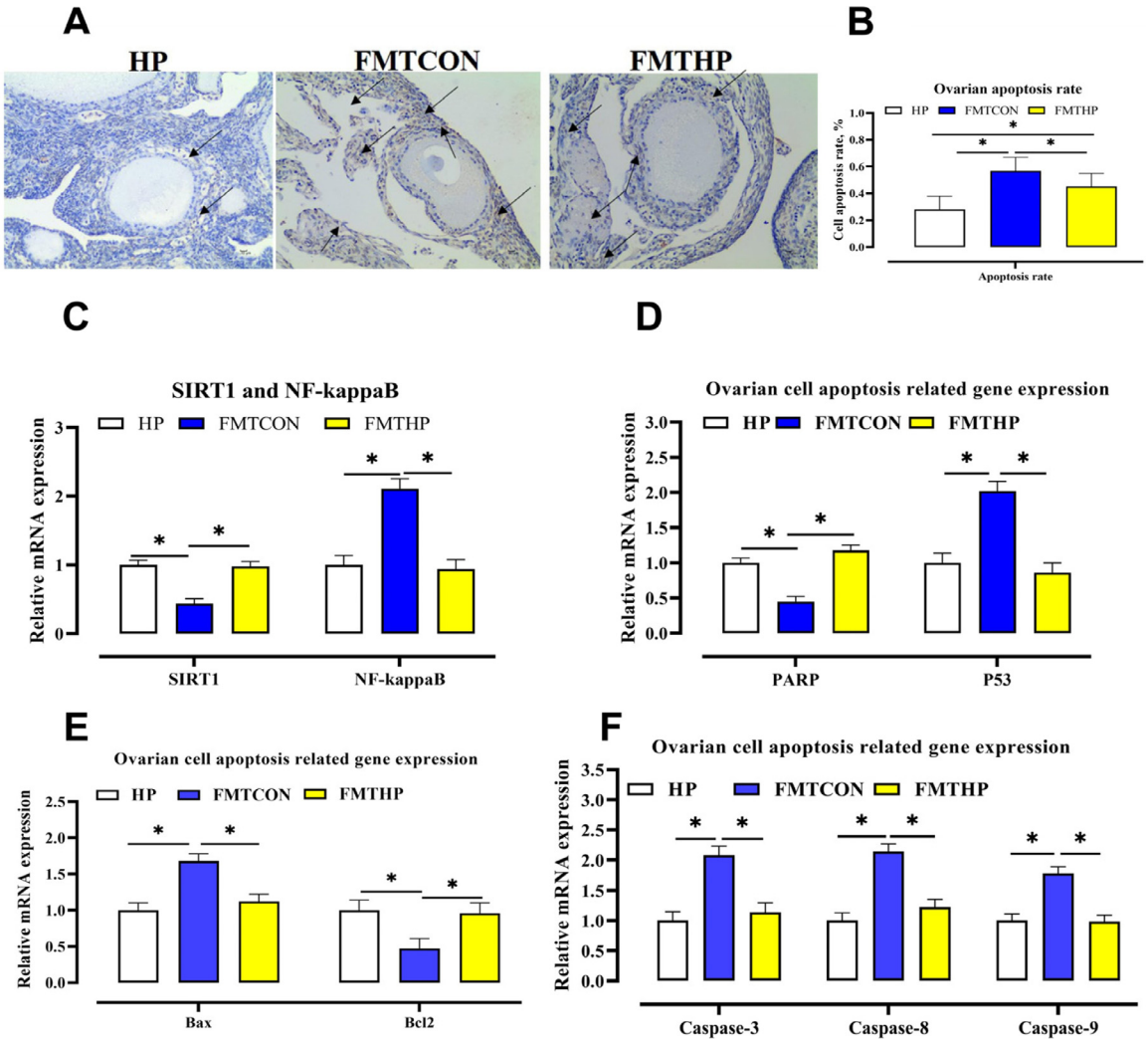

Figure 3. The effect of FMT on ovarian apoptosis rate and ovarian function-related gene expression. Each mean represents 10 replicates, with 1 breeder/replicate. (A, B) TUNEL analysis for cell apoptosis in ovary with the brown color presents the positive cells; (C) RT-PCR analysis for SIRT1 and NF-kappaB; (D−F) mRNA expression level related to ovarian follicular apoptosis-related gene expression. Abbreviations: Bcl-2, B-cell lymphoma-2; FMTCON, lower laying rate breeder received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); PARP, poly ADP-ribose polymerase; SIRT1, sirtuin 1. Statistical significance was evaluated by t test, $^*P < 0.05$.

FMTHP group breeder had higher villus height, V:C ratio, and upregulated mRNA expression ZO-2 and mucin-2 than FMTCON group.

Also, the intestinal microbiota was reported to participate in reproductive function of humans and animals (Wang et al., 2020; Qi et al., 2021), but the mechanism is still not clear. Microbiota balance is important for nutrient digestion, absorption, and utilization in poultry. Torok et al. (2011) also showed that the intestinal microbiota structure was significantly different between broilers with feed efficiency and this may cause differences in nutrient digestion and utilization. In current study, we selected broiler breeders with high- and low-laying rate to study the role of microbiota by FMT. We found that the egg-laying rate of LP breeders was increased after FMT from HP. Similarly, Wang et al. (2020) reported that the egg-laying rates were significantly increased after transplantation with fecal microbiota from high egg-laying rate hens. These may indicate that the microbiota does have positive influence on egg-laying performance in laying hens and breeders. Previous studies have implicated that intestinal microbiome of chickens with high egg-laying performance have more diversities than those of chickens with low egg-laying performance, which may be related to the metabolism and health of the host and egg production variation (Elokil et al., 2020; Yang et al., 2020; Wang et al., 2020). In current study, the HP breeders had higher observed species and greater Chao1 and ACE indexes than LP breeders, while the FMT from the HP led to an increase in Chao1 and ACE indexes of LP breeders. Similarly, Wang et al. (2020, 2021a) also found that observed_species, Chao1, ACE, and Shannon indices were higher in cecum of laying hens and breeders with high-laying rate. Furthermore, there are significant differences in microbial composition between the high-yield and low-yield breeders. Taxonomic analysis at the phyla level showed that the most abundant phyla identified in the HP and LP (FMTCON) groups were Firmicutes, Bacteroidetes, Proteobacteria, and

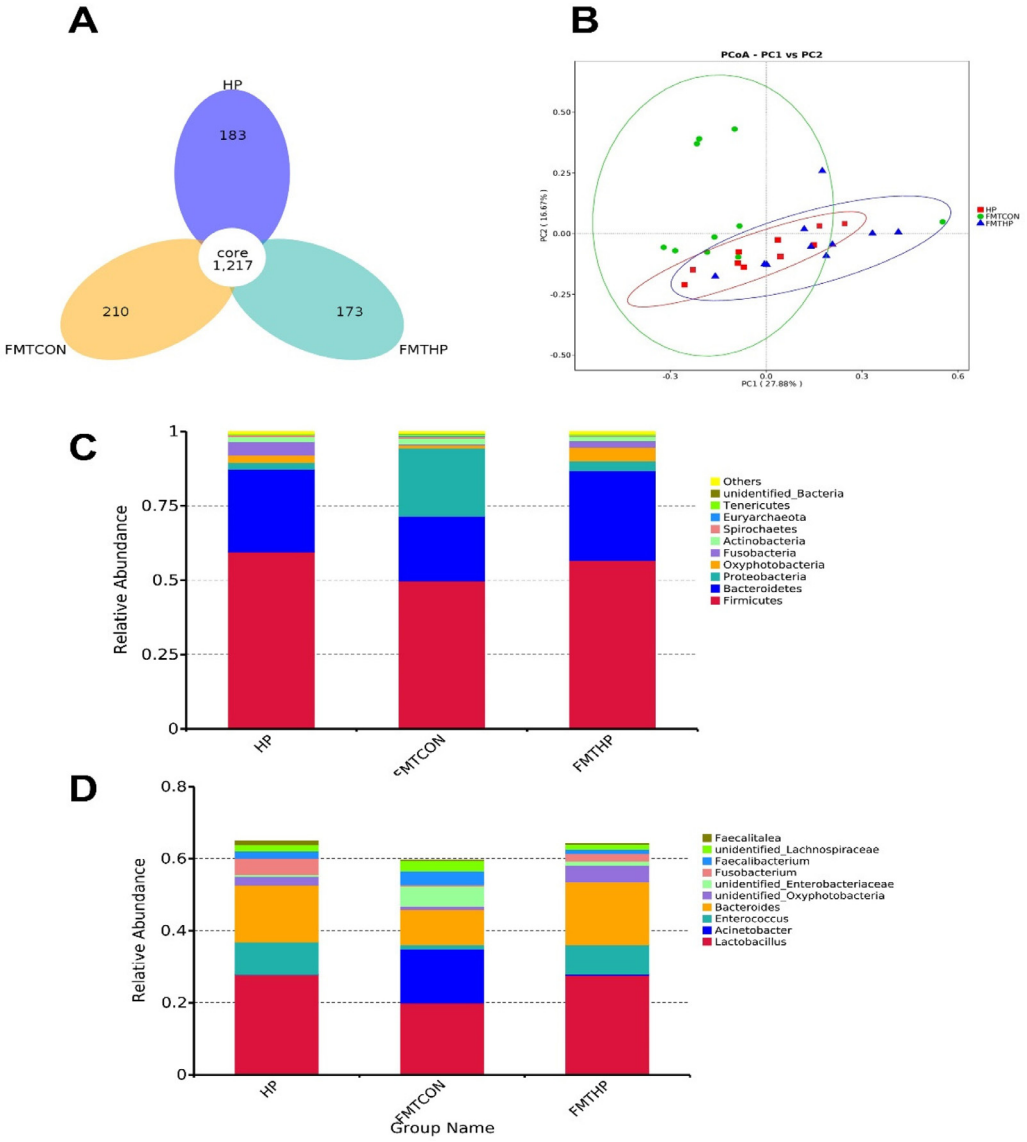

Figure 4. The effect of FMT on reproductive performance-related gene expression. (A) Principal coordinate analysis plot of the cecum microbiota based on the unweighted UniFrac metric. (C) Flower diagram illustrated in cecum microbiota among the samples. (B, D) The relative abundance of the top 10 phylum (B) and genus (D) from samples. (E) Taxonomic cladogram obtained from LEfSe analysis of 16SrRNA sequencing. Biomarker taxa are heighted by colored circles and shaded areas. Each circle's diameter is relative to abundance of taxa in the community. (F) Only taxa meeting an LDA significant threshold >4 are show. (Red) AP enriched taxa; (Green) LP enriched taxa; (Blue) LPE enriched taxa. Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, lower laying rate breeder received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP).

Actinobacteria (Tables 7 and 8; Figure 6), which agree with previous studies (Yang et al., 2020; Wang et al., 2020, 2021a,b; Mao et al., 2021). Firmicutes participate in the metabolism of energy substances and play an important role in the digestion of food (Krajmalnik-Brown et al., 2012; Ma et al., 2018; Rowland et al., 2018). In the current study, the HP and FMTHP group had higher abundance of Firmicutes and Bacteroidetes, while resulted in lower enrichment of Proteobacteria and Firmicutes/Bacteroidetes ratio. Previous studies

Table 6. Effect of FMT on bacteria enrichment in the top 5 phylum of broiler breeders.

Item[1]	Observed_species	Shannon	Simpson	Chao1	ACE
HP	953.60[a]	6.19	0.93	1174.71[a]	1171.98[a]
FMTCON	747.60[c]	5.88	0.93	868.00[c]	878.93[c]
FMTHP	868.40[b]	5.98	0.94	933.77[b]	925.8[b]
SEM	28.59	0.44	0.02	25.29	25.2
P value	0.007	0.956	0.910	0.009	0.006

[a,b,c]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); HP, high-laying rate.

Table 7. Effect of FMT on bacteria enrichment in the top 5 phylum of broiler breeders.

Item[1]	Firmicutes	Bacteroidetes	Proteobacteria	Oxyphotobacteria	Actinobacteria	Firmicutes/Bacteroidetes
HP	59.56[a]	27.75[a]	2.32[b]	2.40	1.66[b]	2.15[b]
FMTCON	49.77[b]	21.76[b]	22.97[a]	0.90	2.04[a]	2.28[a]
FMTHP	56.70[a]	30.17[a]	3.26[b]	4.60	1.51[b]	1.87[c]
SEM	2.81	2.28	0.76	0.37	0.28	0.05
P value	0.090	0.142	0.398	0.190	0.069	0.045

[a,b,c]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, microbiota-depleted lower laying rate breeder (LP) received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); HP, high-laying rate.

Table 8. Effect of FMT on cecum bacteria enrichment in the top 5 genus of broiler breeders.

Item[1]	Lactobacillus	Acinetobacter	Enterococcus	Bacteroides	Alloprevotella
HP	27.82[a]	0.03[b]	9.05[a]	15.78[a]	1.20
FMTCON	19.95[b]	15.00[a]	1.20[b]	9.79[b]	2.79
FMTHP	27.64[a]	0.34[b]	8.11[a]	17.43[a]	2.71
SEM	1.37	1.50	1.08	0.89	0.80
P value	0.030	0.798	0.232	0.655	0.483

[a,b]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, lower laying rate breeder received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP); HP, high-laying rate.

have also demonstrated that the gut microbiota of obese animals and humans exhibits lower abundance of Firmicutes and higher abundance of Bacteroidetes than their lean counterpart (Levy et al., 2016; Ma et al., 2018; Zhang et al., 2019). Additionally, both the Firmicutes and Bacteroidetes phylum comprised most of the intestinal chicken microbiota, and these organisms have been associated with short-chain fatty acid metabolism, with Firmicutes contributing to the synthesis propionate and butyrate and Bacteroidetes primarily contributing to the biosynthesis of propionate (Fei and Zhao, 2013; Morrison and Preston, 2016; Louis and Flint, 2017; Bielka et al., 2022). SCFAs, consisting predominantly of acetate, propionate, and butyrate, regulate the proper function, motility, and integrity of the gastrointestinal tract. Decreased SCFA production is associated with metabolic diseases (Qin et al., 2012). The HP and FMTHP group increased the concentration of total SCFAs, propionate and butyrate at present study, which can be also associated with the increased abundance of Firmicutes and Bacteroidetes in the cecum of breeders. Surpportedly, butyrate, as a main product of gut microbial fermentation, has regulatory effects on host energy homeostasis by regulating glucose and lipid metabolism (Zhang et al., 2021). Butyrate can serve as signaling molecules via the pathway of specific G protein-coupled receptors GPR41 (FFAR3), GPR43 (FFAR2), and GPR109a, which can result in further signaling cascades including AMP-activated protein kinase (AMPK), nuclear factor-κB (NF-κB), and other pathways (Xiong et al., 2004; Zhang et al., 2021). As shown in current study, the FFAR2 (GPR42) and FFAR3 (GPR43) were upregulated by FMT from HP donors. Evidence suggested that butyrate directly stimulated adipocyte leptin production via the activation of the GPR41 and GPR43 signaling pathways (Xiong et al., 2004). SCFAs have been shown to repair intestinal mucosa and alleviate intestinal inflammation by activating GPRs, inhibiting histone deacetylases (**HDACs**), and downregulating the expression of proinflammatory factor genes (Säemann et al., 2000; Liu et al., 2021). Butyrate plays an important role in inflammation regulation, especially in the intestines (Smith et al., 2013; Chang et al., 2014), which also can be used to explain the down regulation of proinflammatory cytokine levels (IL-6, IL-8, TNF-α) in FMTHP group in current study too. On the other hand, high abundances of Proteobacteria, which was also shown to decrease in FMTHP in current study, have been associated with dysbiosis in hosts with metabolic or inflammatory disorders in human and other animal species (Shin et al., 2015).

As demonstrated in previous studies, sex hormones, such as progesterone, estradiol, and testosterone, also participate in communication between microorganisms and their hosts and play several important physiological roles in reproduction, differentiation, cell proliferation, apoptosis, inflammation, metabolism, homeostasis, and brain function (Qi et al., 2021). Disruption in this mechanism may lead to reproductive disorders. Commensal bacteria can produce and secrete hormones, and the crosstalk between microbes and hormones can affect host metabolism, immunity, and behavior (Markel et al., 2013; Kaliannan et al., 2018). It is indicated that dysbiosis of gut microbiota, characterized by lower microbial diversity resulted in a reduction of circulating estrogens (Karavolos et al., 2013; Baker et al., 2017). As shown in current study, FMT from HP donors led to higher serum reproduction function-related hormone levels (E2 and AMH) than in LP breeders transplanted with PBS. Bacterial phyla, which are localized in the gut, allow for the metabolization of steroid hormones through the stimulation of different enzymes. Reproductive hormones such as progesterone, estrogen, and testosterone play a pivotal role in the successful completion

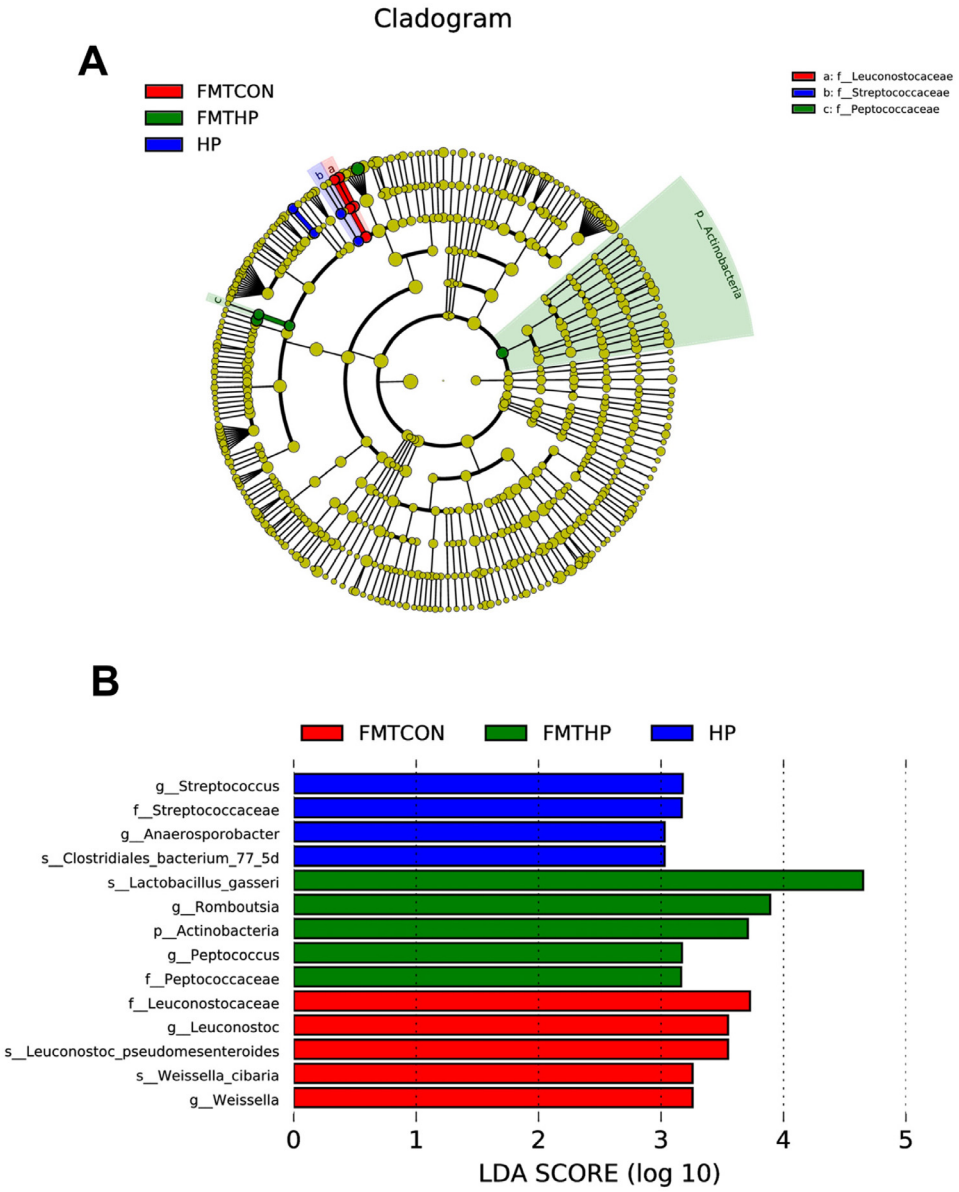

Figure 5. The effect of FMT on reproductive performance-related gene expression. (A) Principal coordinate analysis plot of the cecum microbiota based on the unweighted UniFrac metric. (C) Flower diagram illustrated in cecum microbiota among the samples. (B, D) The relative abundance of the top 10 phylum (B) and genus (D) from samples. (E) Taxonomic cladogram obtained from LEfSe analysis of 16SrRNA sequencing. Biomarker taxa are heighted by colored circles and shaded areas. Each circle's diameter is relative to abundance of taxa in the community. (F) Only taxa meeting an LDA significant threshold >4 are show. (Red) AP enriched taxa; (Green) LP enriched taxa; (Blue) LPE enriched taxa. Each mean represents 10 replicates, with 1 breeder/replicate. Abbreviations: FMTCON, lower laying rate breeder received the same dosage of PBS; FMTHP, microbiota-depleted lower laying rate breeder were received microbiota transplantations from donor layer with high-laying rate (HP).

of reproductive events. Disruption in this mechanism may lead to reproductive disorders. Lactobacillus was the dominant genus in the small intestines while Bacteroides was the dominant genus in the cecum. In our study we observed that *Butyricicoccus, Enterococcus*, and *Lactobacillus* (genus) abundances in cecum were positively related to the reproductive performance (higher ELR), while genera *Streptococcus* was negatively correlated with egg-laying rate. The Firmicutes/Bacteroidetes ratio is considered a biomarker of gastrointestinal functionality and can be indicative of eubiosis conditions in the gastrointestinal tract (Magne et al., 2020; Wang et al., 2021b). It has been indicated that Firmicutes such as *Lactobacillus* and *Lactococcus* spp. have biotechnological value in fermentation and bacteriocin production, which may benefit the host and increase the reproductive performance. In this study, FMT from HP donors also upregulated hormone receptors expression (*ESR1, ESR2, FSHR*, and *AMHR*), decreased ovarian cell apoptosis and its proapoptotic-related mRNA expression (*Bax, caspase-3, caspase-8, caspase-9*), and increased SIRT1 and antiapoptotic-related gene expression (*Bcl2, PARP*). It has been suggested that follicle atresia contributed to the main factor led to the inferior total laying performance and the early culling in practice (Chen et al., 2021; Wang et al., 2021b). Increasing evidence suggested that follicle granulosa cells apoptosis may be one of the main reasons that led to follicle atresia

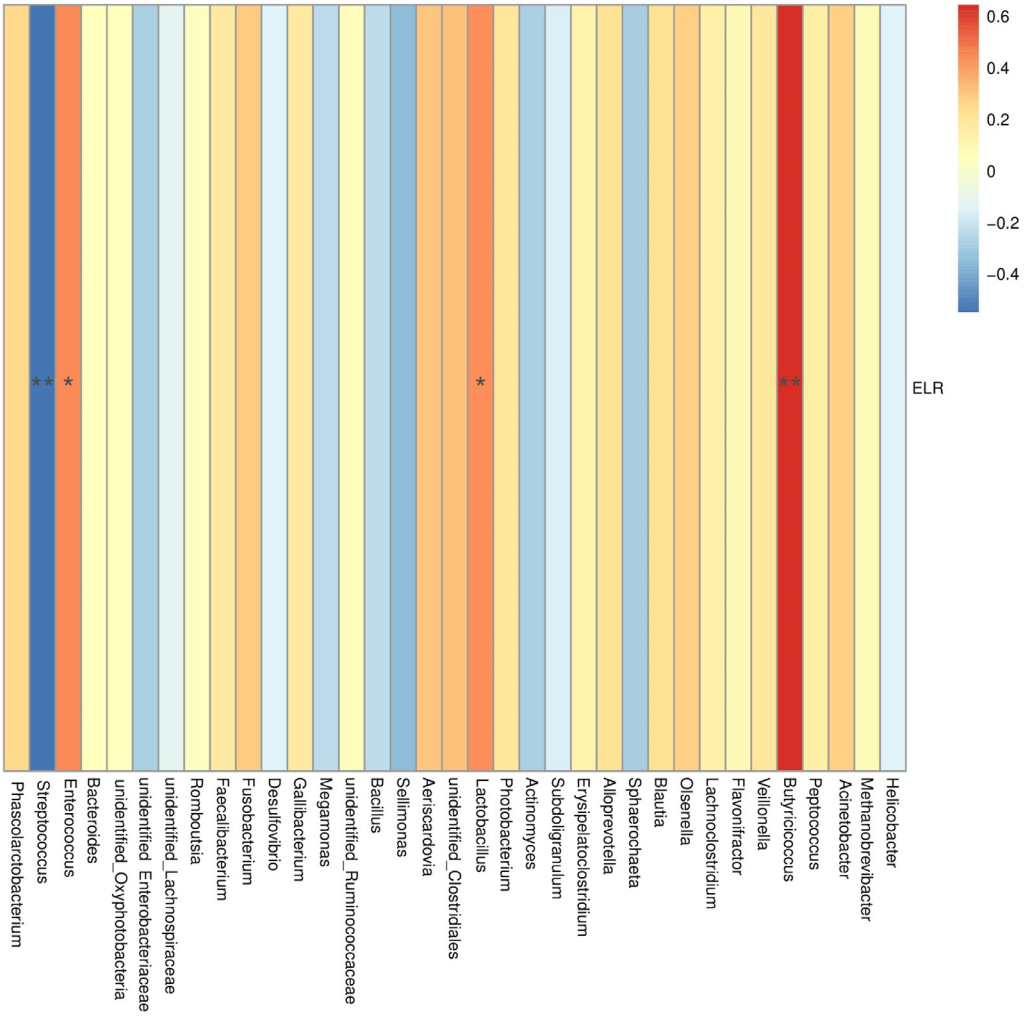

Figure 6. Heatmap of spearman r correlations between the gut microbiota significantly modified by different reproductive performance at genus level (Top 35). Red indicates positive correlation and blue indicates negative correlation; while the color is darker, the correlation is higher. $^{*}P < 0.05$ and $^{**}P < 0.01$. Each mean represents 10 replicates, with a breeder/replicate. Abbreviation: ELR, egg-laying rate.

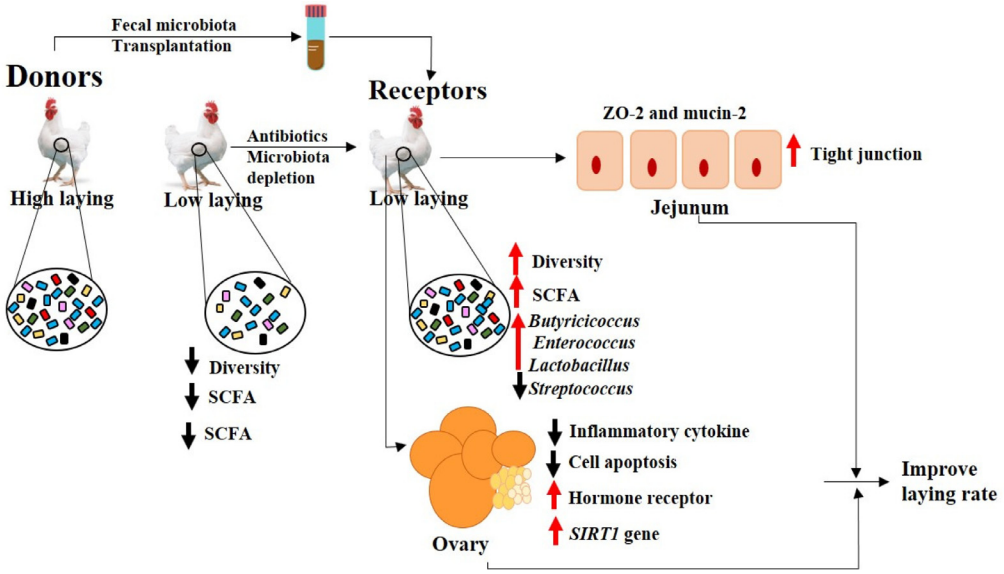

Figure 7. Graphic abstract of this study. Cecal microbiomes of breeders with high egg-laying performance have more diverse activities, which may be related to the metabolism and health of the host; and FMT from high-yield donors can increase the microbiota diversity and enrichment of *Butyricicoccus, Enterococcus, Lactobacillus*, jejunum health, and ovarian function to improve egg-laying performance and the SIRT1-related apoptosis and cytokine signaling pathway were involved in this process.

and ovary atrophy (Hussein, 2005; Krysko et al., 2008; Regan et al., 2016). Proapoptotic caspases (caspase 2, 3, 6, 7, 8, 9, 10) and Bax are known to be mainly involved in mediating cell death signaling transduction, whereas Bcl-2 and PRAP play a key role in reducing cell apoptosis (Krysko et al., 2008; Shi and Kehrl, 2019). Studies in pigs have reported an intensive expression of proapoptotic genes (Bax, Bim, caspase-8, caspase 9) in the granulosa cells of early atretic and progressed atretic follicles but not in the granulosa cells of healthy follicles (Edlich, 2018). Similarly, the LP breeders were found to exhibited higher gene expression of caspase-9 and Bax and lover gene expression of Bcl-2 in previous studies (Wang et al., 2021a,d). Sirtuin 1 or silent information regulator 1 (SIRT1), is an NAD-dependent nuclear class III HDAC and were been involved in the regulation of follicular development, ovarian aging, and stress-related infertility (Salminen et al., 2013; Alam et al., 2021; Wang et al., 2021b). Therefore, our results also suggest that FMT from HP donors can upregulate the SIRT1-related apoptosis and cytokine signaling pathway to improve ovarian function and egg-laying rate in current study; which is also indicate that the microbiota plays a vital role in this process.

CONCLUSIONS

Overall, our findings suggest that cecal microbiomes of breeders with high egg-laying performance have more diverse activities, which may be related to the metabolism and health of the host; and FMT can increase the hormone secretion, intestinal health, and ovarian function to improve egg-laying performance of breeders with lower egg-laying rate. The SIRT1-related apoptosis and cytokine signaling pathway to improve ovarian function and egg-laying rate in current study (Figure 7).

DISCLOSURES

The authors declare no conflict of interest.

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my coauthors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have been approved the manuscript that is enclosed.

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Author contributions: S.C., D.G., and J.W. conceived and designed the experiments; J.W., S.Z., and D.G. performed the experiments; S.Z., D.G., and J.W. analyzed the data; S.C. and D.G. wrote the paper; S.Z., S.B., Z.Q., X.M., J.L., Y.Z., and J.L. helped revise this manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102467.

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