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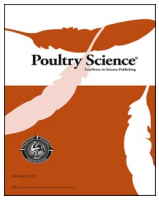

Dietary *Saccharomyces cerevisiae* fermentation product improved egg quality by modulating intestinal health, ovarian function, and cecal microbiota in post-peak laying hens

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ABSTRACT

Saccharomyces cerevisiae fermentation product (**SCFP**), a postbiotic feed additive, has potential to improve animal growth and productivity. However, its effects on post-peak laying hens have not been thoroughly investigated. Therefore, this study aimed to explore the effects of SCFP on production, egg quality, intestinal health, ovarian function, and cecal microbiota in post-peak laying hens. A total of 600 45-week-old Lohmann pink laying hens were randomly assigned into three treatments, with ten replicates and twenty hens per replicate. The hens were fed either a basal diet (**CON**) or basal diet supplemented with SCFP at 750 mg/kg (**SCFP1**) and 1250 mg/kg (**SCFP2**) for 16 weeks. The results showed no significant effects on the laying performance ($P > 0.05$). SCFP supplementation increased Haugh unit, yolk color, albumen height, and eggshell ratio compared to the CON diet ($P < 0.05$). Hens received SCFP diets exhibited a higher intestinal villus height-to-crypt depth ratio ($P < 0.05$) and up-regulated the expression of jejunal occludin, zonula occluden-1 (***ZO-1***), and mucin 2 (***MUC-2***) ($P < 0.05$). Additionally, SCFP supplementation increased the concentration of jejunal secretory immunoglobulin A (**SIgA**) ($P < 0.05$), elevated serum levels of immunoglobulin A (**IgA**), IgG, interleukin-10 (**IL-10**), and interferon-γ (**IFN-γ**) ($P < 0.05$). Furthermore, dietary SCFP tended to decrease ovarian cell apoptosis and enhanced antioxidant capacity in laying hens ($P < 0.05$). Compared to CON group, the SCFP1 and SCFP2 groups had lower total bacteria and *Escherichia coli*, higher *Lactobacillus* ($P < 0.05$), and a greater abundance of *Streptococcus, Pedosphaerales, Christensenellales*, and *Prevotellaceae* in cecum. Significant correlations were observed between egg quality, intestinal health, ovarian function, and cecal microbiota. In addition, cecal microbial functional prediction indicated that SCFP altered various nutritional metabolism pathways. Dietary SCFP supplementation effectively improved egg quality in post-peak laying hens by modulating intestinal health, ovarian function, and cecal microbiota. Collectively, SCFP could be used as a valuable feed additive for post-peak laying hens, with 1250 mg/kg SCFP showing the better effects.

Introduction

With the development of the economy, people's living standards have greatly improved, leading to higher demands for livestock products such as meat, eggs, and milk. The global egg industry has proposed that producing up to 500 eggs in a laying production cycle of 100 weeks, with a focus on long-term growth (Arulnathan et al., 2024). As the laying cycle lengthens, laying performance and egg quality steadily decline. Post-peak laying hens usually experience intestinal and ovarian degeneration, resulting in metabolism disorder, oxidative stress, reproductive dysfunction, and inflammatory response, which ultimately affect egg quantity and quality (Bain et al., 2016; Hou et al., 2024). Although antibiotics were previously used as growth promoters and to protect health, they pose serious threat to food safety, human health, and the environment (Pandey et al., 2024). Therefore, it is necessary to explore safe and effective feed additives to promote or maintain the production and egg quality of post-peak laying hens.

Previous studies have shown that acidifiers, probiotics and plant

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extracts effectively improve the production performance and health of laying hens (Gong et al., 2021; Zhang et al., 2023; Xu et al., 2023, 2024). Increasing studies have confirmed that postbiotics derived from probiotics such as *Lactobacillus, Streptococcus, Bifidobacterium*, and *Bacillus* have positive effects on the production and egg quality of laying hens (Mazanko et al., 2018; Guo et al., 2021; Saeed et al., 2023). *Saccharomyces cerevisiae*, among numerous probiotics, is considered the most valuable species for various industrial processes, including wine, food, ethanol production, and feed additives (Parapouli et al., 2020). SCFP is obtained through anaerobic fermentation of *Saccharomyces cerevisiae,* accompanied by the secretion and release of cellular products, cell wall compounds, stabilized bacteria, and metabolic by-product (Soren et al., 2024). As a result, it contains various nutrients, including amino acids, vitamin, organic acids, and polysaccharides (Heitmann et al., 2018). The *Saccharomyces cerevisiae* cell wall is rich in active polysaccharides such as β-glucans, mannan, and chitin (Klis et al., 2006), which are associated with the immunological, antioxidant, anti-inflammatory, and anti-bacterial effects of SCFP (Sivinski et al., 2022; Branco et al., 2023). Recent reports indicated that SCFP is extensively used in ruminant diets and has beneficial effects on growth and milk production performance (Alugongo et al., 2017; Olagaray et al., 2019; Lei et al., 2024). In practice, SCFP has considerable potential for application in monogastric animals. Adding SCFP to the gestation and lactation sow diets can increase milk production, promote litter body weight gain, and improve sow health (Shen et al., 2011). Similarly, SCFP acting as an alternative to antibiotic growth promoters, improves the feed conversion ratio in broilers aged 1 to 42 days (Soren et al., 2024). Moreover, *Saccharomyces cerevisiae* principally colonizes the intestines of animals, where it can agglutinate pathobionts and enhance the immune system (Chou et al., 2017). SCFP supplementation in poultry diet can resist *Escherichia coli* (Kiarie et al., 2011), *Salmonella* (Rubinelli et al., 2016), *Campylobacter* (Feye et al., 2020), *Mycoplasma gallisepticum* (Elliott et al., 2020), and other bacterial challenges, improving disease resistance and reducing stress susceptibility (Price et al., 2018). Only a few studies have reported that SCFP supplementation improves yolk quality and alleviates intestinal damage in peak laying hens infected with microbial pathogens (Lensing et al., 2012; Elliott et al., 2020). However, few studies are available regarding the effects of SCFP on laying performance, egg quality, and related mechanisms in post-peak laying hens.

This study hypothesizes that dietary supplementation with SCFP improves egg quality in post-peak laying hens by modulating intestinal health, ovarian function, and cecal microbiota communities. To verify our hypothesis, we designed a feeding experiment to explore the effect of SCFP on laying performance and egg quality in hens. Additionally, we evaluated intestinal health, ovarian function and cecal microbiota. The findings provide a theoretical and practical basis for using SCFP as a functional feed additive in post-peak laying hens and offer valuable insights for its widespread application.

Materials and methods

Animal ethics statement

All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Sichuan Agricultural University. The study was reviewed and approved by the Animal Ethics Committee of Sichuan Agricultural University (Chengdu, China; No. 20221116004)

Experimental animals and design

A total of 600 healthy 45-week-old Lohmann pink laying hens were randomly assigned to 3 treatment groups, each with 10 replicates containing 20 birds per replicate (4 layers/cage, 5 adjacent cages/replicate): (1) CON group: basal diet; (2) SCFP1 group: basal diet + 750 mg/kg SCFP; (3) SCFP2 group: basal diet + 1250 mg/kg SCFP. The *Saccharomyces cerevisiae* fermentation product (Original^{TM} XPC) is a fully fermented yeast culture containing *Saccharomyces cerevisiae* cells, produced by Diamond V Mills (Cedar Rapids, IA). The basal diet (Table S1) was formulated according to the China National Feeding Standard for laying hens (NY/T 33-2004). All experimental birds had ad libitum access to feed and water and were housed in an environmental control room. The controlled environment maintained a temperature of 22 ± 1°C, relative humidity of 40 ± 10 %, and 16-hour light:8-hour dark photoperiodic cycle. Prior to the trial, all birds were pre-fed the corn-soybean meal basal diet for 2 weeks to adapt to the experimental environment. Egg production rates were adjusted to ensure no differences between groups. The experimental period lasted 16 weeks.

Date and sample collection

In the whole experiment, egg production and egg weight were recorded daily, and feed intake was recorded weekly by replicate. Laying rate, average daily feed intake (ADFI), feed conversation ratio and egg mass were calculated based on the 16-week average. In this study, the feed conversation ratio was determined as the ratio of total feed intake (g) to total egg weight (g), and egg mass was calculated as the laying rate (%) multiplied by the average weight of eggs (g).

At the end of the 16-week trial, 3 eggs from each replicate were collected to assess egg quality. On the last day of the experiment, one laying hen was randomly selected from each replicate for blood sampling from the jugular vein. The samples were centrifuged at 3000 × g for 15 min to isolate serum, which was then stored at −20°C. After slaughter by cervical dislocation, a 1 cm^2 segment of the mid-jejunum was collected for histomorphology analysis. The jejunal mucosa was scraped for immunity and barrier function analysis, and cecal contents were collected for bacterial quantitative analysis and microbiome sequencing. Ovaries were obtained for antioxidant capacity, and the number follicles (primordial, primary, pre-hierarchial, atretic and total) and ovarian cells apoptosis were analyzed.

Egg quality

The Haugh unit, albumen height, and yolk color were measured using an Egg Analyzer (EMT-7300, Robotmation Co., Ltd.). Eggshell strength was assessed using an Egg Force Gauge (model II, Robotmation Co., Ltd., Tokyo, Japan), and eggshell thickness was determined with an Eggshell Thickness Gauge (Robotmation Co., Ltd.). The egg and yolk index were calculated as [height (cm)/ diameter (cm)] × 100 %). The yolk and albumen ratio were calculated as [weight (g)/egg weight (g)] × 100 %.

Jejunum histological analysis

Jejunal histological analysis was performed according to established methods (Wang et al., 2021b). Briefly, a jejunal segment (1 bird/-replicate) was isolated, fixed in 10 % buffered formaldehyde, dehydrated and embedded in paraffin. It was then sliced (5-μm thickness) and stained with hematoxylin-eosin (H&E). The jejunal villus height and crypt depth were measured using an optical microscope (NIKON Eclipse ci, Nikon Precision (Shanghai) Co., Ltd, China), and the ratio of villus height to crypt depth (V/C) was calculated.

Serum, jejunal immunity and inflammatory status

Serum immunoglobulins (IgA, IgG, and IgM) were quantified using commercial chicken-specific enzyme-linked-immunosorbent assay (ELISA) kits (Meimian, Jiangsu, China) with diluted serum samples. The concentrations of serum inflammatory factors, including IL-10, IFN-γ were quantified using chicken -specific ELISA kits (Meimian, Jiangsu, China). The jejunal mucosa was homogenized in a saline solution (1:9), centrifuged at 3000 × g for 10 min, and the supernatant was collected

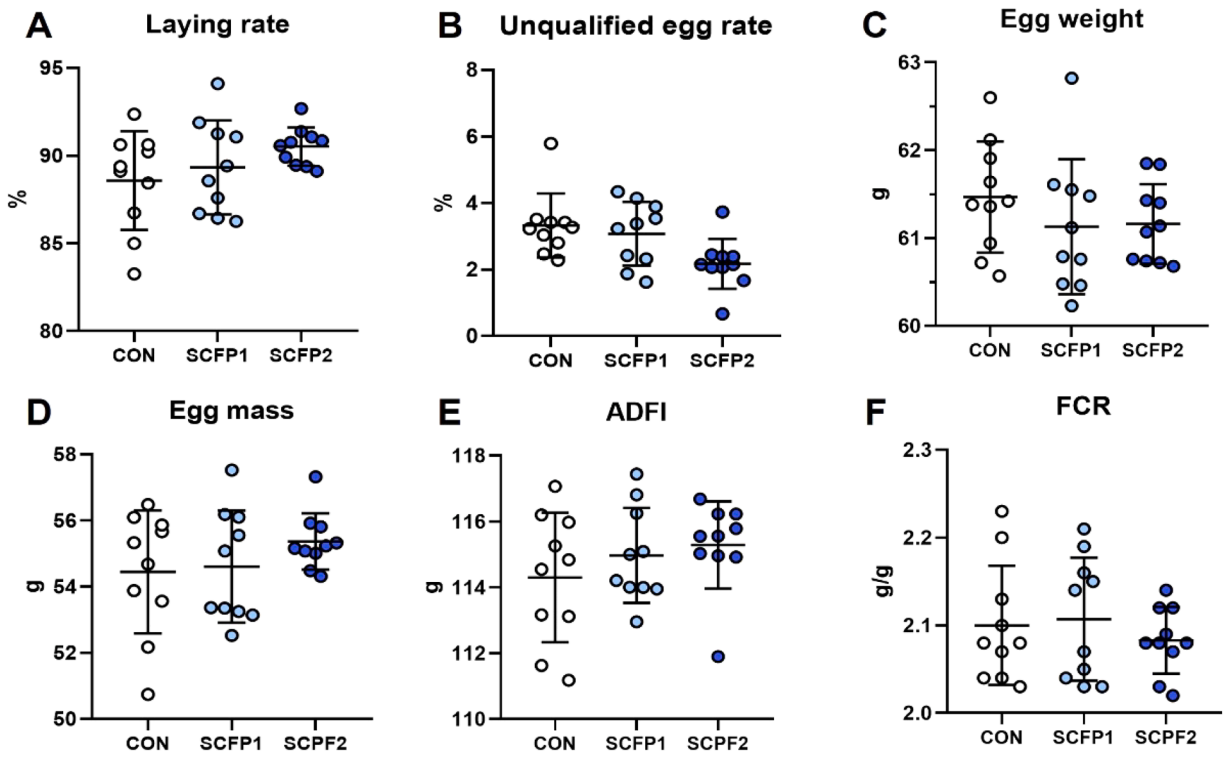

Fig. 1. Effects of dietary supplementation with different levels of *Saccharomyces cerevisiae* fermentation product (SCFP) on laying performance of 62-week-old laying hens. (A) Laying rate. (B) Unqualified egg rate, including broken eggs, dirty eggs, misshapen eggs, pimpled eggs. (C) Egg weight. (D) Egg mass was calculated laying rate multiplied by egg weight. (E) Average daily feed intake (ADFI). (F) Feed conversion ratio (FCR)was determined as the ratio of feed intake to the egg weight. Data are expressed as mean and standard deviation ($n = 10$), the point represents the mean of each replicate. $^{*}P < 0.05$.

for determining immune factors, including SIgA and IFN-γ, using the commercial chicken-specific ELISA kits (Meimian, Jiangsu, China).

Ovarian antioxidant capacity

The activities of ovarian antioxidant enzymes, including glutathione (GSH), glutathione peroxidase (GSH-PX), glutathione s-transferase (GST), superoxide dismutase (SOD), and catalase (CAT), as well as the contents of ovarian oxidized products, including malondialdehyde (MDA) and protein carbonyl (PC), and ovarian total antioxidant capacity (T-AOC) were determined by colorimetric kits. All kits were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

TUNEL assay

Ovarian tissues were isolated and fixed in 10 % buffered polyformaldehyde. The TUNEL assay was performed using the In Situ Cell Apoptosis Detection Kit I (Roche Group, Switzerland) according to the manufacturer's protocol. Ovarian tissues were counterstained with 4′,6 diamidino-2-phenylindole (DAPI) for 5 min, generating an insoluble brown signal. All images were observed using ortho-fluorescence microscopy (Olympus, Co., Japan). The TUNEL-positive cells (apoptosis cells) appeared bright green, while TUNEL-negative cells showed blue-green to greenish-tan. The apoptotic rate was calculated as the percentage of bright green cells relative to the total number of ovarian cells. If the apoptotic cells in a follicle exceeded one-third, the follicle was defined as an atretic follicle.

Gene expression of jejunal barrier function by real time PCR

Total RNA was extracted from the jejunal mucosa using TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instructions. The quality and concentration of RNA were assessed by nucleic acid quantification using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Kusatsu, Japan). Quantitative real-time PCR was performed using the Prism 7000 detection system and SYBR Green qPCR master mix (Takara, Dalian, China). Specific primers of target genes (Table S2), including *occludin, Claudin-1, zonula occluden-1* (*ZO-1*), and *mucin 2* (*MUC2)* were designed online (https://bioinfo.ut.ee/primer3-0.4.0/) and purchased from Takara Co., Ltd (Dalian, China). The $2^{-\triangle\triangle CT}$ method was used for the quantification with *β-actin* as housekeeping gene, and relative abundance was normalized to the control group.

Cecal microbiota

The quantitative determination of cecal bacteria via RT-PCR was performed as described in a previous study (Lund et al., 2010). Briefly, cecal contents were immediately frozen in liquid nitrogen after collection and stored at −80°C. Total DNA was extracted from cecal contents using a Fecal Genomic Extraction Kit (Tiangen Biotech, Beijing, China). Specific primers were designed based on the bacterial 16S rRNA sequence (Table S3), and RT-PCR was performed using the SuperReal PreMix kit (Tiangen Biotech, Beijing, China) on a CFX96 RT-qPCR instrument (Bio-Rad, California, American). Results were expressed as the logarithm of bacterial copies per milligram of cecal contents [log (copies/mg)]. The structure and composition of the cecal microbiota were assessed using high-throughput pyrosequencing, as previously described (Wang et al., 2021b). Total genomic DNA extracted from cecal contents using a Stool DNA kit (Qiagen, United States). PCR amplification of the V4 region of the 16S rRNA gene was amplified using specific primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). After generating amplicon sequence library, sequencing was performed on the Illumina HiSeq platform (Novogene Biotech Co., Ltd., Beijing).

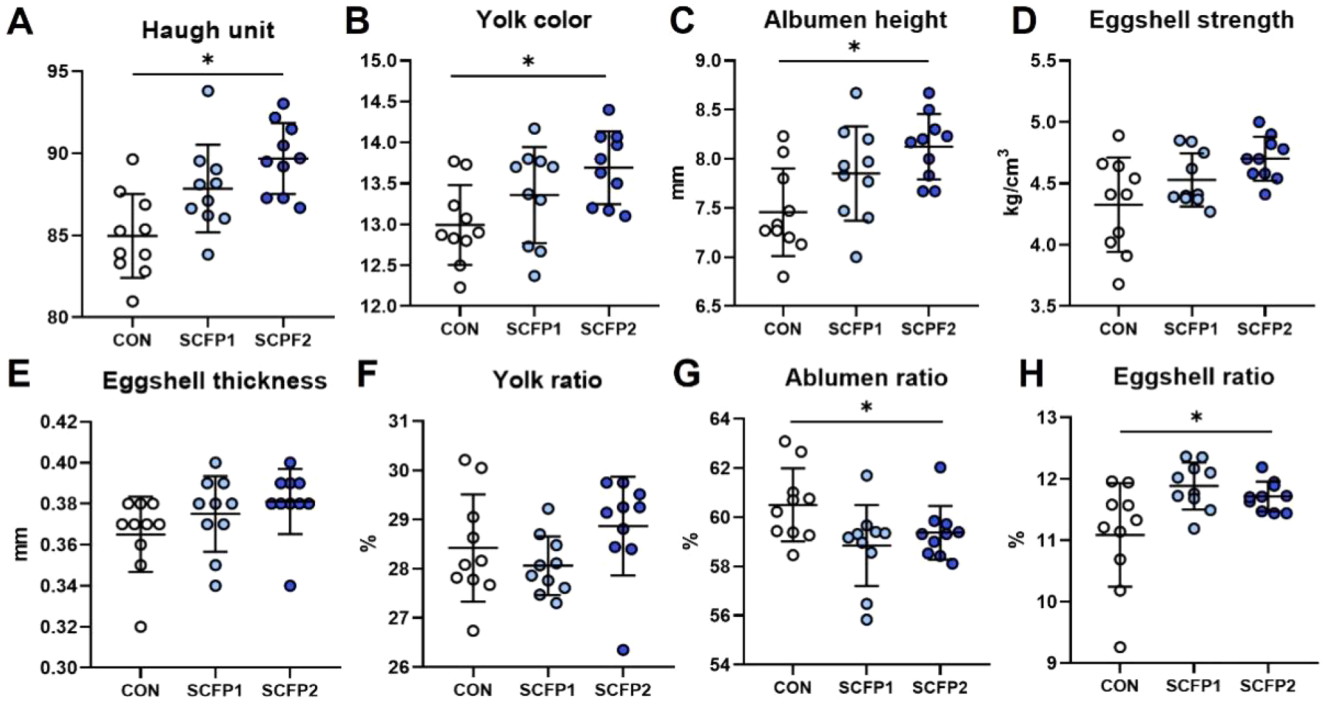

Fig. 2. Effects of dietary supplementation with different levels of *Saccharomyces cerevisiae* fermentation product (SCFP) on egg quality of 62-week-old laying hens. (A) The Haugh unit. (B) yolk color. (C) Albumen height, (D) eggshell strength, (E) eggshell thickness, (F) yolk ratio, (G) albumen ratio, and (H) eggshell ratio. Data are expressed as mean and standard deviation ($n = 10$), the point represents the mean of each replicate. *$P < 0.05$.

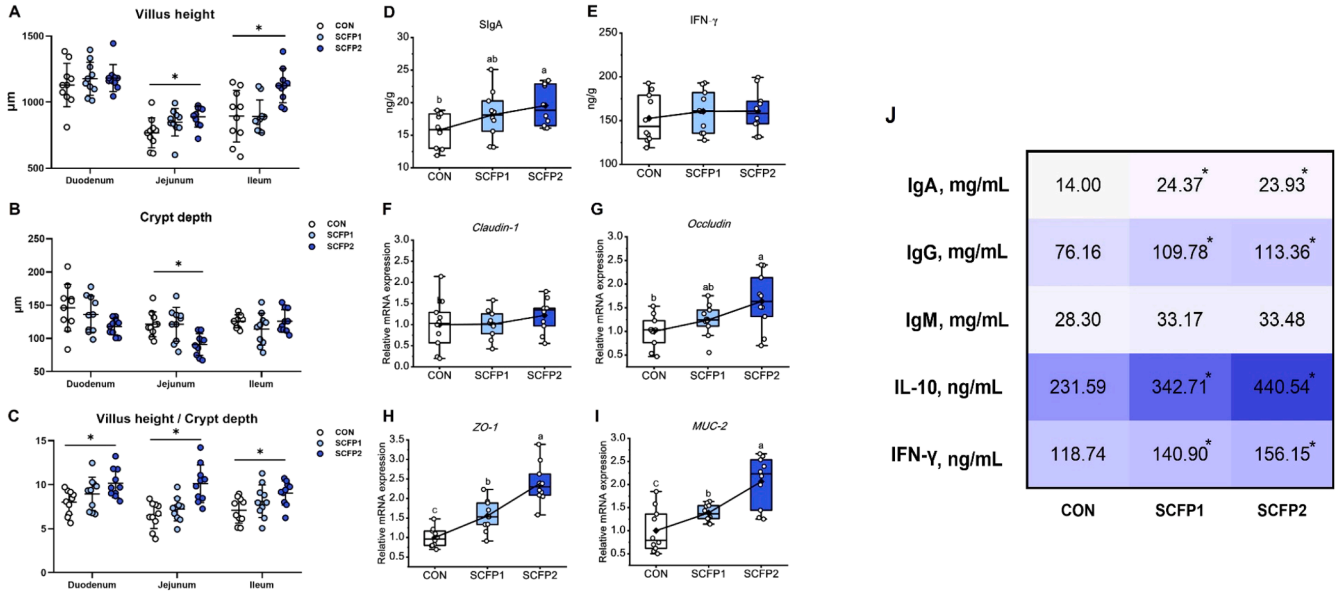

Fig. 3. Effects of dietary supplementation with different levels of *Saccharomyces cerevisiae* fermentation product (SCFP) on the intestinal morphology, immune response and function, and the inflammatory status of serum of 62-week-old laying hens. (A) The jejunal villus height, (B) crypt depth, and (C) villus height to crypt depth ratio were obtained from the section (magnification, 100 ×). The content of immune response factors (D) Secretory Immunoglobulin A (SIgA) and (E) Interferon-γ (IFN-γ) in jejunal mucosa. The mRNA expression of tight junction protein (F) *claudin-1*, (G) *occludin*, (H) *zonula occludens-1* (*ZO-1*), and (I) *mucin-2* (*MUC-2*) in jejunal mucosa. Data are expressed as mean and standard deviation ($n = 10$), the point represents the mean of each replicate. Heatmap shown the concentration of the immunoglobulins (Ig) A, IgG, and IgM and pro-inflammatory factors interleukin (IL)−10 and IFN-γ. Values are shown as mean ($n = 10$). *$P < 0.05$.

Statistical analysis

The data were subjected to the Shapiro-Wilk and Levene's test for normal distribution and homogeneity of variances, respectively. If normal distribution was confirmed, data were analyzed using one-way analysis of variance (ANOVA) with the GLM procedure of SAS 9.2 (SAS Institute, Cart, NC, USA) and GraphPad Prism (GraphPad Inc., La Jolla, CA, USA). Differences between two treatments were performed using the Student's t-test and Tukey's test. Statistical significance was set at $P < 0.05$, and the data are presented as mean ± standard deviation (SD).

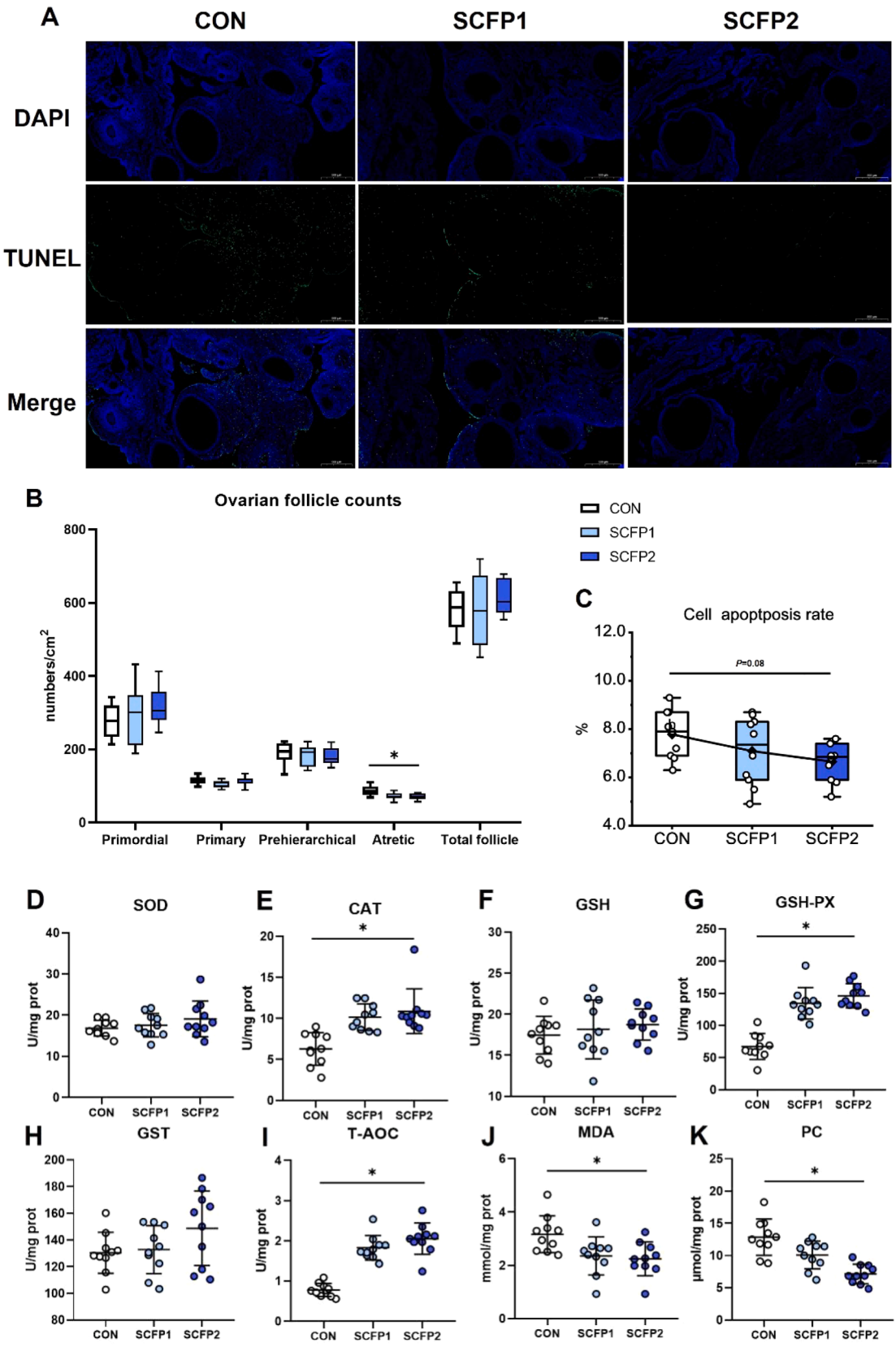

Fig. 4. Effects of dietary supplementation with different levels of *Saccharomyces cerevisiae* fermentation product (SCFP) on the ovarian function of 62-week-old laying hens. (A) TUNEL analysis for cell apoptosis in ovary (5 ×, 200μm) the positive cells have green nuclei. (B) The number of various follicles. (C) The ovarian cell apoptosis rate was calculated as the ratio of TUNEL positive cells to total cells. (D-K) The ovarian antioxidative capacity analysis with antioxidant enzyme activities and oxidation product. Data are expressed as mean and standard deviation ($n = 10$). *$P < 0.05$.

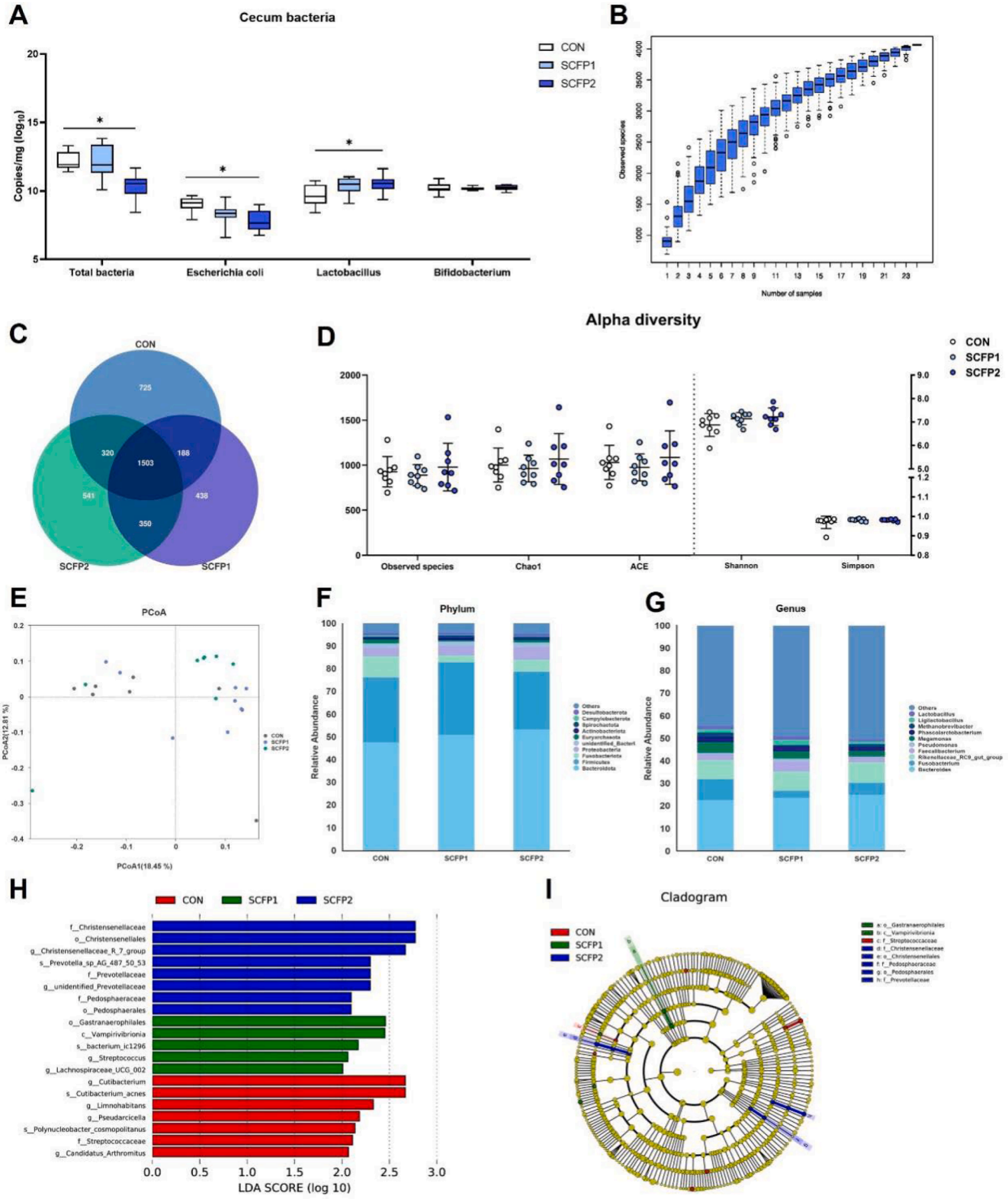

Fig. 5. Effects of dietary supplementation with different levels of *Saccharomyces cerevisiae* fermentation product (SCFP) on cecal microbiota structure and composition of 62-week-old laying hens. (A) The absolute abundance of bacteria includes total bacteria, *Escherichia coli, Lactobacillus* and *Bifidobacterium*. (B) The species accumulation boxplot. (C) Venn diagram for bacterial OUT. (D) Comparison of α-diversity indices includes observed species, Chao1, ACE, Shannon, and Simpson. (E) Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities were used to evaluate microbial β-diversity. (F-G) Relative abundances of the top 10 microbiota at phylum and genus levels. (H-I) Linear discriminant analysis effect size (LEfSe) based on order to genus level (LDA > 2), and biomarker taxa are heighted by colored circles and shaded areas. Data are expressed as mean and standard deviation ($n = 8$). *$P < 0.05$.

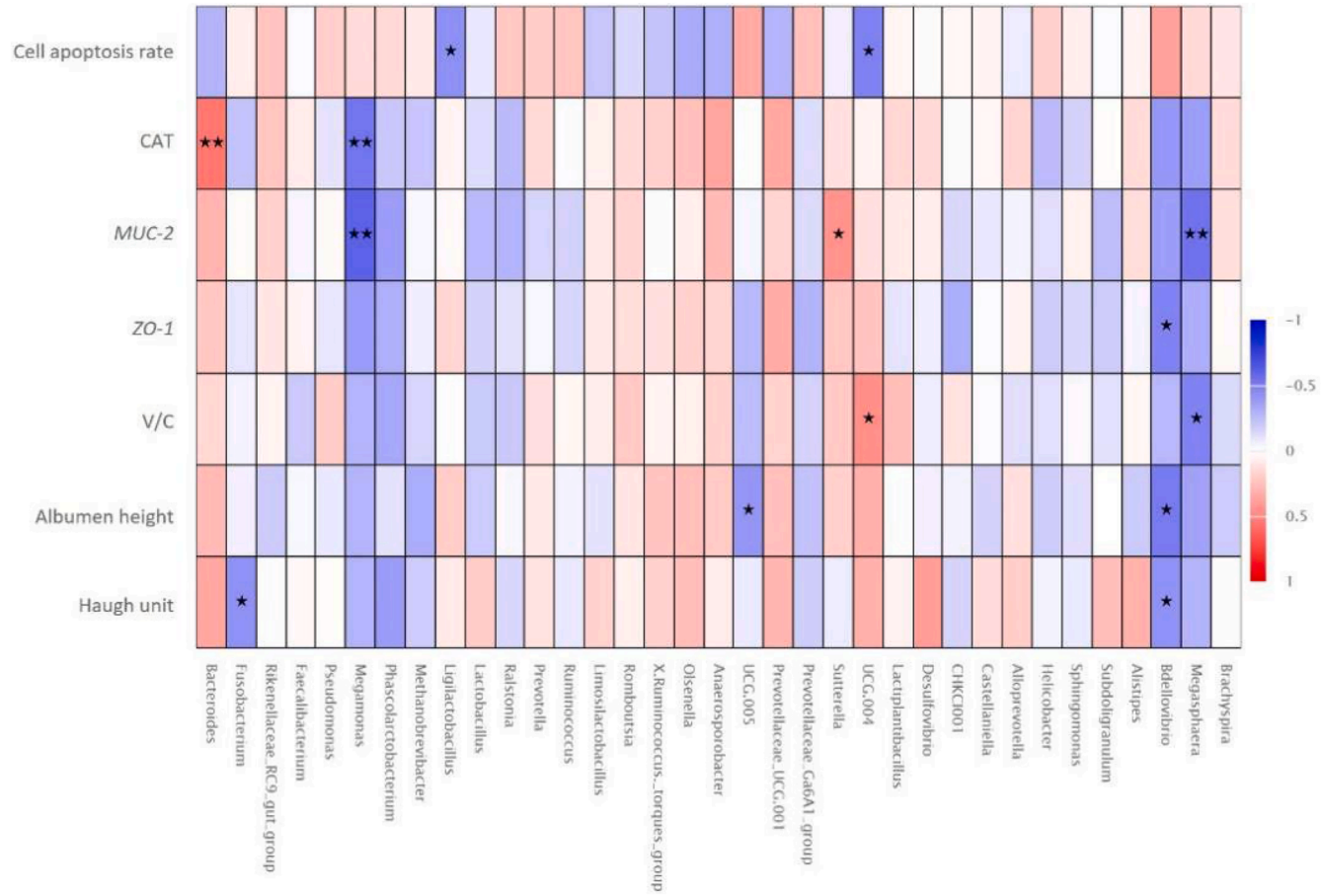

Fig. 6. Heatmap of Spearman correlations among egg quality, intestinal health, ovarian function, and differential cecal microbiota at genus level (Top 35). Color in each block represents the correlation coefficient, red indicates positive correlation, and blue indicates negative correlation ($n = 8$). *$P < 0.05$, **$P < 0.01$.

Results

Laying performance

Dietary supplementation with 750 or 1250 mg/kg SCFP had no detectable influence on the laying rate, unqualified egg rate, egg weight, egg mass, ADFI, and feed conversion ration from 45 to 60 weeks in comparison to the CON group (Fig. 1A-F).

Egg quality

At 60 weeks of age, hens fed SCFP diets had higher Haugh unit, albumen height, yolk color, and eggshell ratio ($P < 0.05$, Fig. 2A-C, H), along with lower albumen ration ($P < 0.05$, Fig. 2G) compared to the CON group. However, dietary SCFP had not significant effect eggshell strength, eggshell thickness, and yolk ratio (Fig. 2D-F).

Intestinal morphology and barrier function

The effect of SCFP on intestinal morphology is shown in Fig. 3. Dietary 750 or 1250 mg SCFP supplementation markedly increased jejunal villus height and crypt depth, and ileal villus height ($P < 0.05$, Fig. 3A and B). Of note, the villus height to crypt depth ratio in the duodenum, jejunum, and ileum of hens was greater in the SCFP1 and SCFP2 groups than CON group ($P < 0.05$, Fig. 3C). Dietary addition to SCFP showed the most significant improvement in jejunal morphology, therefore the jejunal barrier function was evaluated further. As shown in Fig. 3F-I, hens fed SCFP diets displayed significantly upregulated mRNA levels of *Occludin, ZO-1, MUC-2* than the CON group ($P < 0.05$).

Immune and inflammatory status

The effects of SCFP on jejunal and serum immune and inflammatory response are presented in Fig. 3. Dietary supplementation with SCFP significantly increased SIgA level in the jejunum ($P < 0.05$, Fig. 3D and E). Furthermore, the significantly increased IgA, IgG, IL-10, and IFN-γ were observed in hens fed SCFP diets versus the CON diet ($P < 0.05$, Fig. 3J).

Ovarian follicular counts, cells apoptosis, and antioxidant capacity

Dietary supplementation with SCFP did not significantly affect the number of primordial, primary, prehierarchical, and total follicles in ovaries of 60-week laying hens, whereas hens fed with SCFP diets had fewer atretic follicles ($P < 0.05$, Fig. 4B). TUNEL analysis showed that ovarian cell apoptosis rate was lower in the SCFP1 and SCFP2 groups than the CON group ($P = 0.08$, Fig. 4A and C). Regarding ovarian antioxidant capacity, the levels of antioxidant enzymes, including CAT, GSH-PX, and T-AOC were significantly increased, while the concentration of oxidant products MDA and PC were lower in the hens fed SCFP diets ($P < 0.05$, Fig. 4D-K).

Cecal microbial structure and composition

To investigate the effect of SCFP on gut microbiota in hens, cecal bacteria were acquired for RT-PCR quantitative profiling. As shown in Fig. 5A, total bacteria and *Escherichia coli* were markedly lower, while *Lactobacillus* was higher in hens fed with SCFP diets compared to CON diet ($P < 0.05$). 16S rRNA sequencing of the cecal microbial community

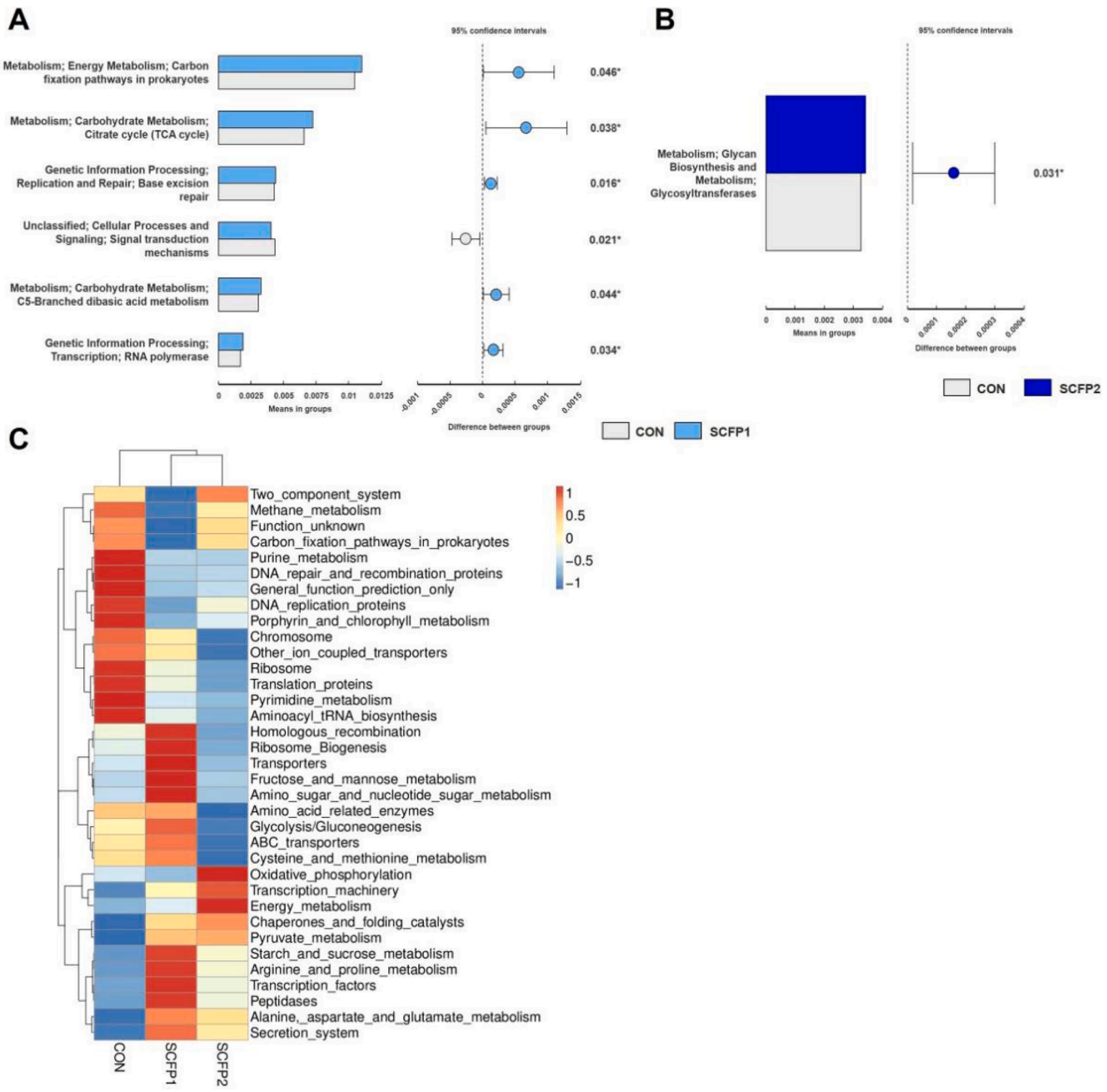

Fig. 7. Functional metagenomics prediction analysis of cecal microbiota by using PICRUSt. (A). Differences in microbial function prediction between CON and SCFP1 groups by t-test. (B). Differences in microbial functional prediction between CON and SCFP2 groups by t-test. (C) Heatmap of microbial functional prediction at level 3 among all groups($n = 8$). *$P < 0.05$.

was further analyzed. The species accumulation curve gradually flattened as the number of samples increased (Fig. 5B), indicating that the sequencing results were reliable and met the requirements for subsequent analysis. Fig. 5C showed that 725, 438, and 541 unique OUTs were identified in the CON, SCFP1 and SCFP2 groups, respectively. Dietary SCFP did not affect the α-diversity indices (Fig. 5D) PCoA analysis showed that PCoA1 and PCoA2 explained 18.45 % and 12.81 % of the variation in microbial β-diversity, respectively (Fig. 5E). The results revealed that SCFP did not markedly altered microbial communities β-diversity.

The relative abundances of the top 10 phyla and genera were displayed in Fig. 5F and G. At the phylum level, the predominant bacteria were *Bacteroidota, Firmicutes, Fusobacteriota, Proteobacteria*, and others. At the genus level, *Bacteroides, Fusobacterium, Faecalibacterium, Pseudomonas*, and others were predominant in the cecal microbiota of laying hens. Compared to the CON group, the SCFP diet did not change the top 10 dominant bacteria.

LEfSe analysis revealed significant changes in the relative abundance of bacteria in the cecal microbiota across the three treatments (Fig. 5H and I). Specific bacterial taxa were selected as biomarker taxa (LDA>2), with 7, 5 and 8 taxa characteristic of the CON, SCFP1, and SCFP2 groups, respectively. Particularly, dietary SCFP significantly enriched *Vampirivibrionia (class), Gastranaerophilales (order), Pedosphaerales (order), Christensenellales (order), Prevotellaceae (family)*, and *Streptococcus (genus)* in the cecal microbiota communities.

The correlations among egg quality, intestinal health, ovarian function and differential cecal microbiota

Spearman correlation analysis was performed to better understand the function of gut microbiota in regulating egg quality, intestinal health, and ovarian health (Fig. 6). Haugh unit and albumen height were negatively correlated with *Bdellovibrio, Fusobacterium*, and *UCG.005* ($P < 0.05$). Jejunal morphology was positively correlated with *UCG.005* but negatively correlated with *Megasphaera* ($P < 0.05$). Jejunal barrier function-related gene expression (*ZO-1* and *MUC-2*) was positively correlated with *Sutterella* ($P < 0.05$), but was negatively correlated with *Bdellovibrio, Megamonas*, and *Megasphaera* ($P < 0.05$). Ovarian CAT level was positively correlated with *Bacteroides* ($P < 0.01$), but negatively correlated with *Megamonas*. In addition, the abundance of *Ligilactobacillus* had a negative correlation with ovarian cell apoptosis rate ($P < 0.05$).

Functional prediction of cecal microbiota

Phylogenetic investigation of communities by reconstruction of observed states (PICRUST) was used to analyze the abundance data from the original 16S rRNA sequences and obtain the predicted functions of the cecal microbiota. Statistical analysis using t-test at level 1 revealed that functions involved nutrition metabolism, gene information process, and cellular process were dominant in the SCFP1 and SCFP2 groups. Compared to the CON group, the SCFP1 group significantly enhanced energy metabolism, while carbohydrate metabolism and glycan metabolism were upregulated in the SCFP2 group ($P < 0.05$, Fig. 7A and B). Moreover, the heatmap of predicted functions at level 3 showed that the positive results for SCFP was associated with amino acid metabolism, peptidases metabolism, pyruvate metabolism, starch and sucrose metabolism (Fig. 7C). In addition, the pathways associated with antioxidant and energy metabolism were more active in the SCFP2 group.

Discussion

Nowadays, the global egg industry aims to extend the laying cycle until 100 weeks and ensure that each hen eventually produces 500 eggs or more (Arulnathan et al., 2024). However, sustained high-intensity production negatively affect laying performance, egg quality, and bone health, leading to intestinal dysbiosis and ovarian dysfunction, which may result in economic losses that outweigh the benefits (Bain et al., 2016; Alfonso-Carrillo et al., 2021). Owing to the restrictions on antibiotics in animal feed, exploring effective alternatives to enhance laying performance and egg quality is crucial for extending the laying period of modern laying hens. SCFP, derived from *Saccharomyces cerevisiae*, is considered a promising probiotic candidate due to its variety of bioactive substances (Alugongo et al., 2017). It is reported that feed SCFP diets can improve growth and production performance by resisting intestinal pathogen colonization and activating the immune system in poultry (Gao et al., 2009; Elliott et al., 2020; Soren et al., 2024). However, there are no reports on the effect of SCFP supplementation on post-peak laying hens. In this study, the dietary SCFP (750 mg/kg or 1250 mg/kg) was found no noticeable effect on production performance in post-peak laying hens. Similar results were reported, suggesting that the addition of 2.0 g/kg yeast culture to diet did not affect egg production, feed intake, and FCR in laying hens at 55-63 weeks (Liu et al., 2021). Conversely, other studies demonstrated that dietary 3.0 g/kg yeast culture enhanced laying performance and reduced feed/egg ratio in 67-week-old laying hens (Zhang et al., 2020). Additionally, supplementation with 2.0 g/kg yeast culture (from *Kluyveromyces marxianus*) improved egg production performance parameters in 40-week-old laying hens (Qiu et al., 2024). It is speculated that yeast cultures, which exhibited different strain sources, varied fermentation process and differing dosages in feed, hereby causing the discrepancy between the outcomes were observed on the laying performance of post-peak laying hens, and further study is needed to illustrate this discrepancy.

As is known to us all, yolk, albumen, and eggshell are the three primary components of the egg that are separated from each other by membranes. The Haugh unit is determined by the egg weight and the albumen height. It is noted that Haugh unit and albumen height are considered to be pivotal parameters for evaluating egg freshness (Ledvinka et al., 2012). Our results showed that SCFP at 750 or 1250 mg/kg markedly enhanced Haugh unit and albumen height compared to the CON. These findings are consistent with previous studies showing that yeast culture positively affects egg quality (Özsoy et al., 2018; Liu et al., 2021; Qiu et al., 2024). Moreover, we also observed that SCFP greatly increase yolk color, an essential quality trait strongly correlated with consumer preference (Altmann et al., 2023). The *Saccharomyces cerevisiae* fermentation product is rich in bioactive substances, including β-glucan, which promotes the secretion of gastrointestinal enzymes and enhances nutrient digestion, absorption, and utilization of nutrients, thereby benefiting egg quality and extending the shelf-life (Zhen et al., 2020). In consideration of the improvements of SCFP on egg quality of post-peak laying hens, it is suggested that greater intestinal integrity and barrier function may be the potential mechanism. The small intestine of poultry is responsible for digesting and absorbing nutrients, and villus height and crypt depth are directly correlated with intestinal function (Metzler-Zebeli et al., 2018). Similar to the positive role of yeast culture in intestinal health (Zhang et al., 2020, 2023; Qiu et al., 2024), the diet supplemented with 750 mg/kg or 1250 mg/kg SCFP facilitated intestinal morphology, increasing villus height to crypt depth ratio of duodenum, jejunum, and ileum in post-peak laying hens. Additionally, the intestinal mucosal barrier is mainly supported by epithelial cell tight junctions, which defend against pathogen invasion and control inflammation (Dong et al., 2022). This study found that dietary SCFP supplementation (750, 1250 mg/kg) increased the expression levels of tight junction proteins, including *ZO-1, Occludin*, and *MUC-2*. Furthermore, the concentrations of jejunal SIgA, serum IgA, IgG, IL-10 and IFN-γ were also elevated. Intestinal integrity and permeability may have improved due to bioactive molecules from SCFP, which continue to support intestinal epithelial cell proliferation and inhibit apoptosis (Perricone et al., 2022; Aquino et al., 2024). Moreover, the higher intestinal villus and shallower crypts serve the purpose of enhancing the nutrients absorption, indirectly demonstrating that intestinal health is beneficial to improve egg quality of laying hens (Zhang et al., 2022).

Laying performance and egg quality are generally acknowledged to be influenced by multiple factors, including genetics, breeding, nutrition, environment and age of laying hens. The ovary is the primary organ responsible for maintaining reproductive and endocrine functions (Wang et al., 2021a). Previous studies have proved that the female reproductive system is the first to exhibit signs of senescence in animals, with functional decline occurring before other organs (Lu et al., 2022). Ovarian follicle development is associated with ovarian function, and the number of primordial follicles determines the follicular reserve and reproductive ability (Johnson, 2015). Ovarian aging is mainly characterized by progressive decline in the quality and quantity of oocytes, negatively affecting egg quality, particularly in the later stage when the primordial follicle reserve pool is being depleted (Xu et al., 2023). The present study investigated the effects of SCFP on ovarian follicle count in post-peak laying hens and found that SCFP groups had fewer atretic follicles. This effect may be attributed to reduced ovarian cells apoptosis, a mechanism previously demonstrated in studies (Yeung et al., 2017). Our results suggested that dietary SCFP supplementation tended to reduce ovarian cell apoptosis rate. Moreover, oxidative stress, which is regarded as an additional crucial inducement for follicular atresia, occurs when reactive oxygen species production overwhelms the intrinsic antioxidant defenses (Burton and Jauniaux, 2011; Wang et al., 2021a). An increasing number of studies suggested that elevated laying frequency and prolonged laying cycles would accelerate reactive oxygen species accumulation in the ovaries of laying hens, eventually resulting in ovarian oxidative damage (Gu et al., 2021; Xu et al., 2023; Chen et al.,

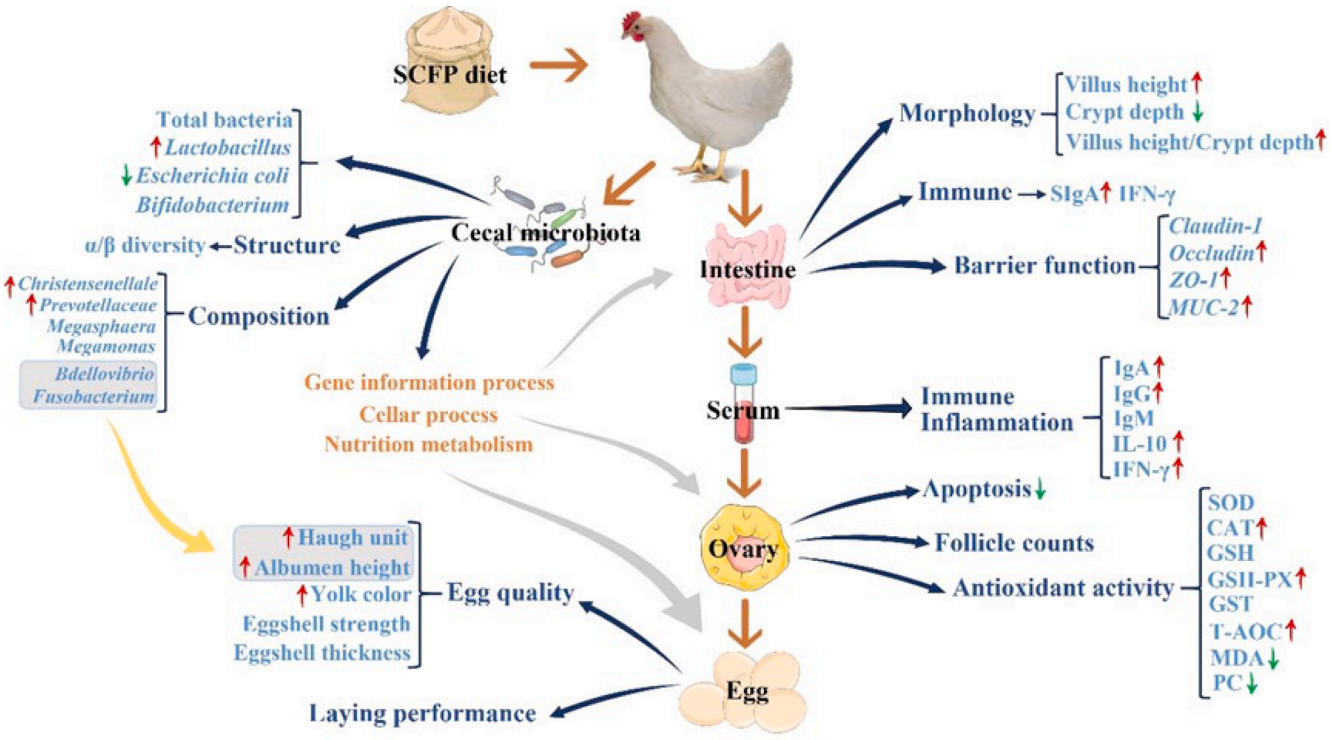

Fig. 8. SCFP improved egg quality by modulating intestinal health, ovarian function, and cecal microbiota.

2024). In this study, dietary addition of 750 or 1250 mg/kg SCFP increased ovarian CAT, GSH-PX, and T-AOC activities, and decreased the contents of oxidation products (MDA and PC) in post-peak laying hens. Similar to the beneficial roles of SCFP in alleviating oxidative stress, the previous research indicated that yeast culture could enhance antioxidant capacity and reduce oxidative damage of poultry (Liu et al., 2022; Qiu et al., 2024). Evidence exists that points out that *Saccharomyces cerevisiae* possesses robust antioxidant defense systems, and the oligosaccharides in SCFP have potential to scavenge free radicals (Assalve et al., 2024). Accordingly, these results indicate that dietary SCFP may strengthen ovarian antioxidant capacity of post-peak laying hens.

It is widely acknowledged that gut microbiota plays a crucial role in animial growth, productivity, reproductivity, and overall health. Gut microbiota should maintain a dynamic balance with stable microbial abundances for animal normal health (Lee and Hase, 2014). In the current study, SCFP significantly decreased *Escherichia coli* and increased *Lactobacillus* in post-peak laying hens. *Escherichia coli* and *Lactobacillus* are representative harmful and beneficial bacteria, respectively. A previous study reported reduced *Escherichia coli* colonization in birds fed with SCFP (Soren et al., 2024). Indeed, *Saccharomyces cerevisiae* is effective combats Gram-negative pathogens by breaking down bacterial membranes while preserving beneficial bacteria, and it can even act synergistically with *Lactobacillus* (Xu et al., 2021). The alteration of intestinal microbial diversity was observed in yeast culture-fed birds (Qiu et al., 2024). In addition, dietary yeast culture increased the relative abundance of *Bacteroidetes* and decreased the relative abundances of *Firmicutes* and *Proteobacteria* at the phylum level of laying hens (Liu et al., 2021). Nevertheless, this study found that dietary SCFP supplementation did not alter cecal microbiota diversity. According to 16S rRNA sequencing, *Bacteroidota, Firmicutes, Fusobacteriota*, and *Proteobacteria* were dominant across all treatment groups in post-peak laying hens, which is in agreement with the previous study believed that SCFP was beneficial to maintain the stability of microbial community (Tun et al., 2020). In order to further understand the differences of cecal microbial communities between the CON and SCFP groups, the LEfSe analysis revealed that *Christensenellales* and *Prevotellaceae* were biomarkers that enrich in cecum of laying hens from SCFP2 groups. Both *Christensenellales* and *Prevotellaceae* play an important role in promoting nutrient absorption and regulating intestinal health, which have been already considered as potential candidates for a new generation of probiotics (Precup and Vodnar, 2019; Waters and Ley, 2019). Moreover, the alteration of gut microbial composition and structure might result in the laying performance and egg quality (Tian et al., 2022). In the current study, the correlation analysis results indicated that *Bdellovibrio* and *Fusobacterium* were negatively associated with Haugh unit and albumen of eggs, *Megamonas* was negatively associated with the expression levels of *MUC-2, ZO-1*, and the content of CAT. *Bdellovibrio, Fusobacterium*, and *Megamonas* were all Gram-negative bacteria and closely related to bowel disorders (Zhou et al., 2018; Schultz Marcolla et al., 2024). Therefore, our results indicated that SCFP regulated the differential bacteria to more efficiently enhance the egg quality, intestinal barrier function and ovarian function of laying hens.

In addition to altering gut microbial composition and structure, modulating related metabolism path might be one of the potential reasons for improving egg quality in laying hens. The results of this study on the gut microbial predictive metabolic functions revealed that dietary SCFP supplementation significantly regulated energy metabolism, carbohydrate metabolism, and amino acid metabolism, which may explain that gut microbiota was participated in modulating egg quality via the intestine-liver-ovary axis (Dai et al., 2022). Consequently, these results provided the possible mechanism that dietary SCFP is absorbed in the intestine, altering gut microbial composition and structure, promoting nutrient absorption, subsequently improving intestinal health and ovarian function, ultimately enhancing egg quality in post-peak laying hens (Fig. 8).

Conclusion

In summary, dietary supplementation with 750 or 1250 mg/kg SCFP effectively improved egg quality by modulating intestinal health,

ovarian function, and cecal microbiota in post-peak laying hens. The results suggest that SCFP is a valuable feed additive for post-peak laying hens, with 1250 mg/kg SCFP showing the better effects. The present study provides a theoretical basis for applying *Saccharomyces cerevisiae* fermentation product in post-peak laying hens and a feasible strategy for improving egg quality.

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.2025.104979.

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