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Original Research Article

Rosemary leaf powder improves egg quality, antioxidant status, gut barrier function, and cecal microbiota and metabolites of late-phase laying hens

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ABSTRACT

This study sought to determine the effects of rosemary leaf powder (RP) on laying performance, egg quality, serum indices, gut barrier function, and cecal microbiota and metabolites of late-phase laying hens. A total of 84 "Jing Tint 6" laying hens at 65-week old were randomly divided into 2 groups and fed either a basal diet (CON) or a basal diet supplemented with 0.3% RP. Our study revealed that RP improved the Haugh unit and decreased yolk n-6/n-3 polyunsaturated fatty acid (PUFA) ratio of laying hens, increased serum superoxide dismutase (SOD), jejunal activities of SOD and catalase (CAT), and jejunal zonula occludens-1 (*ZO-1*) expression, as well as decreased serum tumor necrosis factor-α (TNF-α) level and jejunal *TNF-α* mRNA expression. Rosemary leaf powder markedly enhanced ($P < 0.05$) cecal abundances of Rikenellaceae, *Rikenellaceae_RC9_gut_group*, and *Turicibacter*, tended to promote ($P = 0.076$) butyrate concentration, and reduced ($P < 0.05$) cecal abundances of Erysipelatoclostridiaceae, Sutterellaceae, Fusobacteriaceae, Campylobacteraceae, *Sutterella*, *Campylobacter*, and *Fusobacterium*, which were closely linked with Haugh unit, yolk n-6/n-3 PUFA ratio, serum SOD and TNF-α. In addition, RP altered the metabolic functions of cecal microbiota and enhanced the abundances of butyrate-synthesizing enzymes, including lysine 2,3-aminomutase, β-lysine 5,6-aminomutase, and 3-oxoacid CoA-transferase. Together, 0.3% RP has the potential to enhance egg quality by partially modulating serum antioxidant status, jejunal barrier function, and cecal microbiota structure and metabolites, indicating that RP could be considered a promising feed additive to promote the production performance of late-phase laying hens.

1. Introduction

Eggs are a comparatively cheap source of nutrients that provide sufficient amounts of vitamins, minerals, and high-quality protein (Zhou et al., 2021). The productivity and egg quality of laying hens frequently decrease in the final stages of production when intensive farming is used (Yang et al., 2022a). Many stressors frequently affect late-phase laying hens, including decreased activities of antioxidant enzymes, excessive accumulation of reactive oxygen species (ROS), imbalance of redox process, and impaired reproductive

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function, which could result in a lower Haugh unit and a shorter shelf-life of eggs (Wang et al., 2018; Zhang et al., 2023a,b). Furthermore, aging causes digestive dysfunction, immunological imbalance and a disturbance of the gut microbiome in laying hens (Feng et al., 2021). Over the past few decades, antibiotics have frequently been added to feed to reduce stress response and improve poultry production (Castanon, 2007). However, this inappropriate use of antibiotics has led to several issues like antibiotic resistance and outbreaks of foodborne illness. In order to increase poultry output, it is critical to investigate promising feed resources.

Several medicinal plants have been approved as natural antioxidant sources. *Rosmarinus officinalis* L. is a medicinal herb that belongs to the Lamiaceae family. Rosemary has antioxidant (Martínez-Tomé et al., 2022), anti-inflammatory (Satoh et al., 2022), antibacterial (Ertas et al., 2022), anti-apoptotic (Sánchez-Marzo et al., 2020), and anti-cancer effects (Moore et al., 2016). Rosemary contains bioactive components, including phenolic acids (rosmarinic acid, chlorogenic acid, caffeic acid, and ferulic acid), diterpenoid phenols (carnosic acid and carnosol), and flavonoids (kaempferol, quercetin, and rutin) (Chang et al., 2008; Maldini et al., 2016; Moreno et al., 2006) that appear to regulate inflammatory responses and carbohydrate-metabolizing enzymes (Akbari et al., 2022). Several plants have been successfully used as feed additives, both dried and powdered. In 2018, China legalized the use of dried stems, leaves, or flowers of rosemary as feed additives. In broilers (Loetscher et al., 2013; Rostami et al., 2017; Yesilbag et al., 2011) and turkeys (Botsoglou et al., 2007), the use of rosemary leaves increased meat quality and significantly reduced lipid oxidation. However, the benefits of rosemary leaves used in laying hens on production efficiency, egg quality, and gut health are not well understood. Therefore, in order to provide a theoretical foundation for the use of rosemary leaves in late-phase laying hens, this study sought to ascertain the effects of rosemary leaf powder (RP) on laying performance, egg quality, serum indices, gut barrier function, cecal microbiota, and metabolites.

2. Materials and methods

2.1. Animal ethics statement

Our study was reviewed and granted by the Institutional Animal Care and Use Ethics Committee of China Agricultural University (Beijing, China; No. AW42601202-1-1).

2.2. Experimental animals and design

A total of 84 healthy 65-week-old "Jing Tint 6" laying hens (average laying rate = 79.25% ± 3.41%) were randomly allocated into 2 groups with 6 replicates and 7 birds per replicate: (1) CON group: basal diet; (2) RP group: basal diet + 0.3% RP. The adaptation period in this study lasted 1 week, and the official trial lasted 8 weeks. All experimental birds were obtained from Gu'an Songhe Poultry Breeding Co., Ltd. (Hebei, China) and raised in wire-floored cages with free access to mash feed and water. Before the official experiment, egg production was examined to confirm that there was no statistical difference between the two treatments. The temperature in the room was kept at around 23 °C. In mid-May, the plant material of rosemary 'Blaulippe' was collected from the Fuyang Base of the National Aromatic Plant Germplasm Resource Bank (Anhui, China). Firstly, fresh rosemary leaves were picked, washed, and dried in the shade. Dried rosemary leaves were then crushed and sifted for laying hens' feed. Rosemary leaf powder contained 4.88% crude protein, 42.60% crude fiber, 15.22% crude fat, 1.28% calcium, and 0.07% phosphorus on dry matter basis. The contents of carnosic acid and carnosol as the main antioxidant compound in the ethanolic extract of dry rosemary leaves were 13.27% and 13.20%, respectively. Table S1 shows the ingredients and nutritional content of the basal diet based on China National Feeding Standard of Chicken (NY/T 33-2004). Crude protein (method 990.03), calcium (method 968.08), and phosphorus (method 985.01) contents were determined according to the Association of Official Analytical Chemists (AOAC, 2006). The values of metabolizable energy and standardized ileal digestible amino acids were calculated by referring to metabolizable energy, amino acid contents and their standardized ileal digestibility of each feed material provided in the China Feed Database (https://www.chinafeeddata.org.cn/). Feed intake was weighed weekly in our trial and egg weight and production were recorded daily.

2.3. Sample collection

Blood samples (5 mL) were taken from the wing veins of laying hens on d 28 and 56, and centrifuged at 3000 × *g* for 10 min. Serum samples were collected and kept at −20 °C for examination of serum parameters. On d 56, 3 eggs were retrieved from each replicate to analyze egg quality, and 2 eggs were randomly chosen from each replicate to separate egg yolk for analysis of fatty acid profile. Cervical dislocation was used to slaughter birds (1 bird per replicate). The jejunal mucosa was lightly scraped after death to analyze gut barrier function, and cecal contents were collected for analyzing microbiota and metabolites.

2.4. Egg quality and yolk fatty acids

Haugh unit, yolk color, and albumen height were analyzed by an Egg Analyzer (EA-01). Eggshell strength was analyzed by an Egg Force Reader (EFR-01). Eggshell thickness was analyzed by an Egg Shell Thickness Gauge (ESTG-1). These above instruments were obtained from Israel Orka Food Technology Ltd. (Bountiful, UT, USA). The fatty acids in egg yolk were measured using the technique reported by Zhang et al. (2023a,b). Briefly, 200 mg of lyophilized yolk was combined with 1 mL of internal standard (1 mg/mL C11 fatty acid methyl ester), 1 mL of n-hexane, and 4 mL of methanolic HCl solution. After cooling, the mixture was kept at 80 °C for 2.5 h and then combined with 5 mL of 7% potassium carbonate. The collected supernatant was used to detect yolk fatty acids using a gas chromatograph (6890 series, Agilent Technologies, Wilmington, DE).

2.5. Serum parameters and jejunal antioxidant status

Colorimetric kits were used to measure serum parameters, such as total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, glucose (GLU), triglyceride (TG), total cholesterol (TC), total protein (TP), and albumin (ALB). Mucosal samples were homogenized in saline solution (1:9, wt:vol) before being centrifuged at 2500 × *g* for 10 min. The collected supernatant was used to detect the antioxidant capacity (T-AOC, CAT, GSH-Px, and SOD) of the jejunal mucosa using commercial kits. The kits were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

2.6. Gene expression of inflammatory factors, tight junction proteins, and mucin-2 (MUC-2)

Total RNA was isolated from the jejunal mucosa using an EASYspin RNA Mini Kit (Aidlab Biotechnologies, Co., Ltd., Beijing, China). Reverse transcription was performed using the HiScript III

1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Jiangsu, China). Quantitative real-time PCR (qRT-PCR) was carried out using the Mx3000P system (Agilent StrataGene). The relative mRNA expressions of targeted genes were calculated according to the $2^{-\Delta\Delta CT}$ method. Primers used in this study are shown in Table S2.

2.7. Cecal short-chain fatty acids (SCFA)

The contents of cecal SCFA, such as acetate, propionate, butyrate, etc., were determined using the technique published by Zhang and Piao (2022). Briefly, cecal digesta (0.5 g) was combined with 8 mL of ultrapure water. After centrifugation at 3000 × *g* for 5 min, the supernatant was diluted 50 times and filtered through 0.22-μm membrane. A high performance ion chromatograph (DIONEX ICS-3000, Thermo Fisher, Waltham, MA, USA) was used to examine the SCFA profile of cecal digesta. The cecal SCFA profile was reported as milligram per gram of digesta.

2.8. Cecal microbial community

A Stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) was used to extract whole genomic DNA from cecal digesta. The V3 to V4 region of the 16S rRNA gene was amplified using the primers 338F (5′-ACTCCTRCGGGAGGCAGCAG-3′) and 806R (5′-GGACTACCVGGG-TATCTAAT-3′). The samples were separated on a 2% agarose gel electrophoresis and recovered with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). On the Illumina MiSeq platform, the purified amplicons were pooled and paired-end sequenced. UPARSE software was used to cluster operational taxonomic units (OTU) based on 97% sequence similarity. The RDP Classifier determined the taxonomy of each OTU representative sequence with a confidence level greater than 70%. To compare the β-diversity of cecal microbiota between the two groups, principal coordinate analysis (PCoA) analysis based on the Bray–Curtis distance matrix algorithm was employed. Differences in the cecal microbiota were detected using linear discriminant analysis effect size (LEfSe) analysis (linear discriminant analysis [LDA] score > 2). PICRUSt2 was used to predict the metabolic functions (carbohydrate metabolism and amino acid metabolism) of cecal microbiota.

2.9. Statistical analysis

All data was analyzed using SAS 9.4 (SAS Inst. Inc., Cary, NC). To find significant differences between the CON and RP groups, the independent sample *t*-test procedure was performed. For determining the relative abundance of cecal bacteria at the family and genus levels, the Wilcoxon rank-sum test was used. Spearman's correlation test was used to identify relationships between serum parameters, egg quality, SCFA, and cecal microbiota. Differences in the predictive metabolic functions of cecal microbiota were analyzed by STAMP using Welch's *t*-test. $P < 0.05$ denoted a significant difference, and $0.05 \leq P < 0.10$ denoted a tendency.

3. Results

3.1. Production performance and egg quality

As demonstrated in Table 1, no significant differences were observed for egg production, egg weight, egg mass, average daily feed intake, and feed conversion ratio. However, as compared to the CON group, dietary supplementation with 0.3% RP substantially enhanced ($P < 0.05$) Haugh unit of laying hens (Table 2).

Table 1
Effects of rosemary leaf powder on laying performance of laying hens.[1]

Item	CON	RP	SEM	*P*-value
1 to 4 wk				
Egg production, %	74.74	82.35	3.441	0.247
Egg weight, g	57.39	58.10	0.690	0.470
Egg mass, g/d per bird	44.40	47.76	2.342	0.423
ADFI, g/d per bird	108.23	107.27	1.347	0.641
FCR, g/g	2.53	2.25	0.151	0.323
5 to 8 wk				
Egg production, %	77.11	75.58	4.086	0.800
Egg weight, g	58.33	58.28	0.570	0.946
Egg mass, g/d per bird	44.75	46.38	2.017	0.604
ADFI, g/d per bird	108.13	109.04	1.356	0.647
FCR, g/g	2.47	2.36	0.121	0.575
1 to 8 wk				
Egg production, %	75.93	75.12	5.224	0.915
Egg weight, g	57.96	58.16	0.620	0.826
Egg mass, g/d per bird	44.58	47.07	2.094	0.467
ADFI, g/d per bird	108.18	108.15	1.183	0.989
FCR, g/g	2.49	2.30	0.131	0.420

ADFI = average daily feed intake; FCR = feed conversion ratio.
CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder.
[1] Values are represented as mean and SEM, $n = 6$.

3.2. Yolk fatty acids

As shown in Table 3, RP significantly decreased ($P < 0.05$) the contents of C12:0, C15:0, C18:2 n-6c and n-6 polyunsaturated fatty acid (PUFA) in the yolk, and tended to decrease C14:1 ($P = 0.060$) and n-6/n-3 PUFA in the yolk ($P = 0.071$) compared with the CON group.

3.3. Serum parameters

Compared with the CON, dietary RP supplementation enhanced ($P < 0.05$) serum SOD activity, and reduced ($P < 0.01$) serum TNF-α level on d 56 (Fig. 1). However, no significant differences were observed for serum contents of GLU, TG, TC, TP, ALB, and globulin (GLB) between the two groups.

3.4. Jejunal antioxidant status and barrier function

Compared with the CON, dietary RP supplementation tended to increase jejunal SOD level ($P = 0.071$) and significantly increased ($P < 0.05$) jejunal CAT activity (Fig. 2A). Dietary RP supplementation tended to decrease ($P = 0.067$) jejunal *IL-6* mRNA abundance and significantly decreased ($P < 0.05$) jejunal *TNF-α* mRNA abundance (Fig. 2B). No difference was observed for jejunal interferon-γ (*IFN-γ*) mRNA abundance. Compared with the CON, the mRNA level of zonula occludens-1 (*ZO-1*) in the jejunal mucosa of the RP group tended to be markedly increased ($P = 0.063$; Fig. 2C).

3.5. Cecal SCFA profile

Figure 3 depicts the cecal SCFA profile. The cecal SCFA profile differed considerably between the CON and RP groups (Fig. 3A). Compared with the CON, cecal butyrate concentration in the RP group tended to be substantially higher ($P = 0.076$) (Fig. 3B). Cecal butyrate concentration was positively correlated with Haugh unit ($P < 0.01$) and serum SOD activity, and negatively correlated with C15:0 in yolk and serum TNF-α content according to Spearman's correlation analysis (Fig. 3C).

Table 2
Effects of rosemary leaf powder on egg quality of laying hens.[1]

Item	CON	RP	SEM	*P*-value
Yolk color	7.61	7.94	0.399	0.587
Albumen height, mm	4.40	4.76	0.332	0.527
Haugh unit	58.55	67.91	2.554	0.029
Eggshell strength, kgf	3.78	4.03	0.132	0.216
Eggshell thickness, mm	0.31	0.33	0.008	0.135

CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder.
[1] Values are represented as mean and SEM, $n = 6$.

Table 3
Effects of rosemary leaf powder on yolk fatty acid profile (g/100 g) of laying hens.[1]

Item	CON	RP	SEM	*P*-value
C12:0	0.003	0.002	0.0002	0.029
C14:0	0.19	0.17	0.010	0.107
C15:0	0.02	0.01	0.002	<0.001
C16:0	13.57	13.41	0.258	0.839
C18:0	4.28	5.25	0.183	0.146
C21:0	0.03	0.04	0.003	0.309
SFA	18.10	18.88	0.399	0.583
C14:1	0.05	0.04	0.005	0.060
C16:1	1.89	1.57	0.132	0.127
C18:1 n-9c	10.67	11.52	0.269	0.221
MUFA	12.61	13.12	0.256	0.403
C18:2 n-6c	7.91	6.78	0.368	0.026
C18:3 n-3	0.28	0.29	0.016	0.849
C20:2	0.12	0.14	0.007	0.179
C20:3 n-6	0.11	0.12	0.007	0.527
C20:4 n-6	1.10	1.25	0.048	0.246
C22:6 n-3	0.55	0.63	0.029	0.324
PUFA	10.07	9.21	0.419	0.121
n-6 PUFA	9.12	8.16	0.385	0.049
n-3 PUFA	0.82	0.92	0.043	0.436
n-6/n-3 PUFA ratio	11.13	9.32	0.369	0.071
UFA/SFA	1.26	1.20	0.038	0.302

SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; UFA = unsaturated fatty acid.
CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder.
[1] Values are represented as mean and SEM, $n = 6$.

3.6. Cecal microbial structure and community

As shown in Fig. 4A, no significant differences were observed for the α-diversity indices of cecal microbiota between the CON and RP groups. PCoA analysis showed that RP tended to significantly change ($P = 0.070$) the β-diversity of cecal microbiota compared with the CON (Fig. 4B). This result was also shown by partial least squares-discriminant analysis (PLS-DA). Bacteroidota and Firmicutes were the dominant phyla, accounting for 90% (Fig. 4C). Down to the family level, the predominant bacteria were Bacteroidaceae, Lachnospiraceae, Rikenellaceae, Lactobacillaceae, Ruminococcaceae, Muribaculaceae, unclassified_o__Bacteroidales, Oscillospiraceae, Prevotellaceae, norank_o__Clostridia_UCG-014, and so on (Fig. 4D). At the genus level, the predominant genera were *Bacteroides*, *Rikenellaceae_RC9_gut_group*, *Ruminococcus_torques_group*, *Lactobacillus*, *Faecalibacterium*, *Phascolarctobacterium*, *unclassified_o__Bacteroidales*, *unclassified_f__Lachnospiraceae*, *norank_f__Muribaculaceae*, *norank_f__norank_o__Clostridia_UCG-014*, and so on (Fig. 4E). LEfSe analysis revealed different bacterial taxa among the two treatments (Fig. 5A and B). At the family level, a great abundance of Erysipelatoclostridiaceae, Campylobacteraceae, Sutterellaceae, Fusobacteriaceae, and Flavobacteriaceae in the CON group, and Rikenellaceae and norank_o__Saccharimonadales in the RP group were detected. At the genus level, *Campylobacter*, *Fusobacterium*, *Angelakisella*, *Parasutterella*, *Sutterella*, *norank_f__Flavobacteriaceae*, and *unclassified_f__Sutterellaceae* in the CON group, and *Rikenellaceae_RC9_gut_group*, *Turicibacter*, and *norank_f__norank_o__Saccharimonadales* in the RP group were detected.

3.7. The correlations among differential cecal microbiota, SCFA, egg quality, and serum parameters

At the family level (Fig. 5C), Rikenellaceae was negatively correlated with C15:0 in yolk and serum TNF-α concentration, and positively correlated with Haugh unit, cecal butyrate, and serum SOD. Erysipelatoclostridiaceae was negatively ($P < 0.05$) correlated with Haugh unit and serum SOD, and positively correlated with C18:2 n-6c and n-6 PUFA in yolk and serum TNF-α concentration. Campylobacteraceae was negatively ($P < 0.05$) correlated with Haugh unit, cecal butyrate, and serum SOD, and positively correlated with n-6 PUFA in yolk and serum TNF-α concentration. Sutterellaceae was negatively ($P < 0.05$) correlated with Haugh unit and serum SOD, and positively ($P < 0.05$) correlated with C18:2 n-6c and n-6 PUFA in yolk and serum TNF-α concentration. Fusobacteriaceae was negatively ($P < 0.05$) correlated with Haugh unit, cecal butyrate, and serum SOD, and positively ($P < 0.05$) correlated with C15:0, C18:2 n-6c, and n-6 PUFA in yolk and serum TNF-α concentration. Down to the genus level (Fig. 5D), *Rikenellaceae_RC9_gut_group* was positively ($P < 0.05$) correlated with Haugh unit and cecal butyrate. *Turicibacter* was negatively ($P < 0.05$) correlated with C15:0, C18:2 n-6c, n-6 PUFA and n-6/n-3 PUFA in yolk and serum TNF-α concentration, and positively ($P < 0.05$) correlated with serum SOD activity. *Campylobacter* was negatively ($P < 0.05$) correlated with Haugh unit, cecal butyrate, and serum SOD, and positively correlated with n-6 PUFA in yolk and serum TNF-α concentration. *Sutterella* was negatively ($P < 0.05$) correlated with Haugh unit and serum SOD activity, and positively ($P < 0.05$) correlated with C18:2 n-6c and n-6 PUFA in yolk and serum TNF-α concentration. *Fusobacterium* was negatively ($P < 0.05$) correlated with Haugh unit, cecal butyrate, and serum SOD, and positively ($P < 0.05$) correlated with C15:0, C18:2 n-6c, and n-6 PUFA in yolk and serum TNF-α concentration.

3.8. Metabolic functions of cecal microbiota

As demonstrated in Fig. 6A, compared with the CON, the abundance of function genes related to ascorbate and aldarate metabolism tended to decrease ($P = 0.050$), whereas the abundance of function genes connected with fructose and mannose metabolism tended to increase ($P = 0.076$) in the RP group. As demonstrated in Fig. 6B, when compared to the CON, the abundance of function genes associated with tryptophan metabolism and lysine degradation significantly decreased ($P < 0.05$) and the abundance of function genes associated with valine, leucine and isoleucine degradation tended to decrease ($P = 0.063$) in the RP group, whereas the abundance of function genes associated with phenylalanine, tyrosine and tryptophan biosynthesis tended to enhance ($P = 0.098$) in the RP group. The abundance of function genes related to butyrate-synthesizing enzymes was reported in Fig. 6C. Compared with the CON, the abundance of 3-oxoacid CoA-transferase and lysine 2,3-aminomutase markedly increased ($P < 0.05$), and the abundance of β-lysine 5,6-aminomutase tend to enhance ($P = 0.053$) in the RP group.

4. Discussion

Consumers' purchasing decisions are significantly influenced by the internal quality of eggs, including the egg white's quality and the yolk's fatty acid profile. Haugh unit is a critical metric for assessing albumen quality and shelf life that is determined based

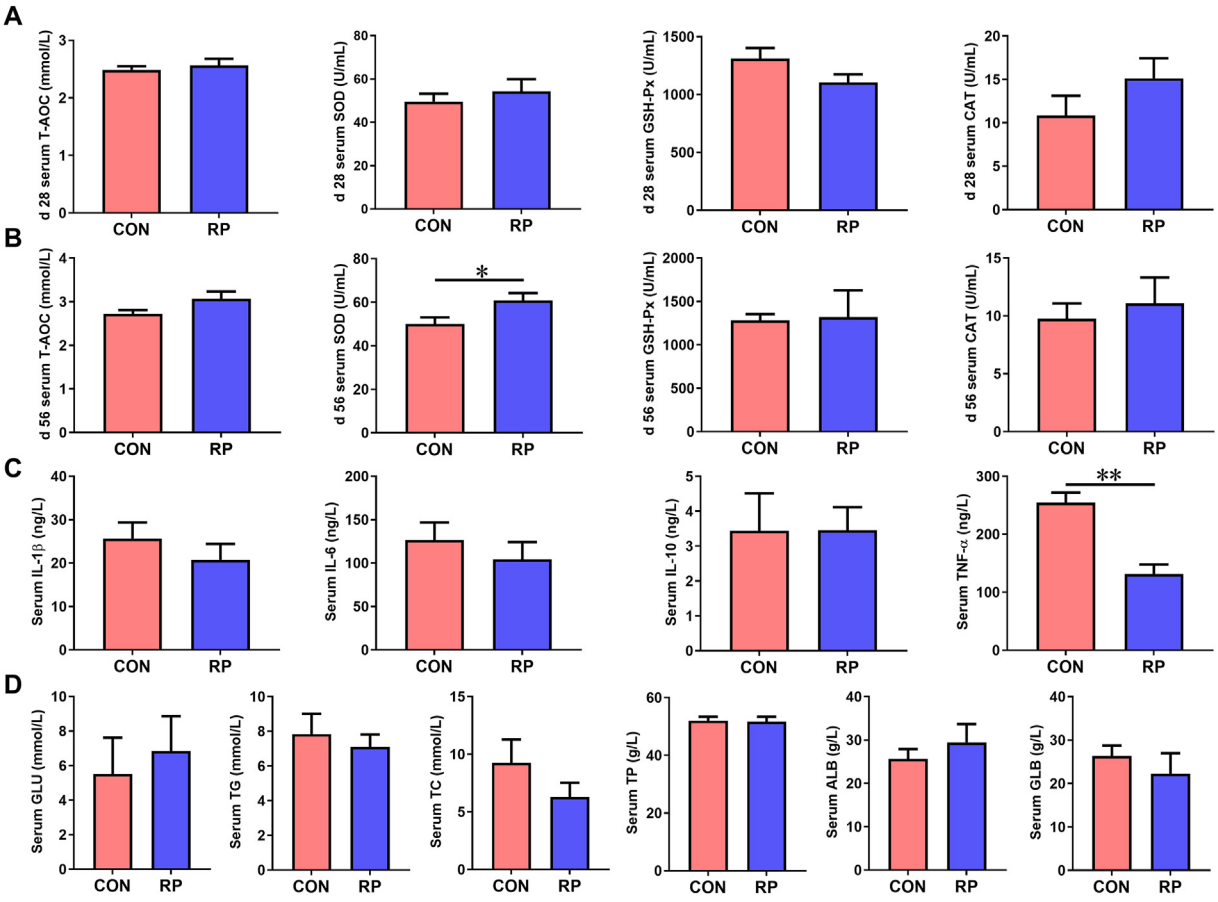

Fig. 1. Serum indices of laying hens. (A) Antioxidant status on d 28. (B) Antioxidant status on d 56. (C) Inflammatory cytokines on d 56. (D) Serum biochemical parameters on d 56. T-AOC = total antioxidant capacity; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; TNF-α = tumor necrosis factor-α; GLU = glucose; TG = triglyceride; TC = total cholesterol; TP = total protein; ALB = albumin; GLB = globulin. CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder. Values are represented as mean ± SEM, $n = 6$. * Denotes $P < 0.05$; ** denotes $P < 0.01$.

on the thickness of the egg white and the egg's weight (Eisen et al., 1962; Wang et al., 2018). The current study showed that 0.3% RP significantly improved the Haugh unit of laying hens as compared to the CON group, indicating that RP was beneficial for enhancing egg white quality and extending the shelf life of eggs. According to earlier research, dietary intake of saturated fatty acid (SFA) usually increases the incidence of type 2 diabetes and worsens insulin resistance (Fu et al., 2021). The PUFA in eggs is essential to human health, with anti-inflammatory properties and the ability to lower blood fats, protect blood vessels and prevent cancer. Importantly, an appropriate n-6/n-3 PUFA ratio in food offers significant advantages for human health. A low n-6/n-3 PUFA ratio (or higher n-3 PUFA) has inhibitory effects on the etiology of several diseases, including osteoporosis, autoimmune disorders, and cardiovascular disease (Zhang et al., 2021a). Compared to the CON group in this study, 0.3% RP decreased the n-6/n-3 PUFA ratio and the n-6 PUFA concentration of laying hens' egg yolks. As a result, the current study revealed that RP might be a promising feed additive for enhancing particular egg quality traits and raising customer acceptance. We hypothesized that the functional properties of rosemary, such as its antioxidant and anti-inflammatory properties (Martínez-Tomé et al., 2022), as well as its modification of the intestinal barrier and microbiota (Liu et al., 2022; Yang et al., 2021) may be responsible for these positive benefits. Given this, we further analyzed the changes in serum antioxidant status, immunity, intestinal barrier, gut microbiota and metabolism of late-phase laying hens.

According to earlier studies, oxidative stress has a clear destructive effect on intestinal tissue. Serum antioxidant status is a measure of an animal's resistance to oxidative damage, and high antioxidant enzyme activity is an effective countermeasure (Zhang et al., 2021b, 2022a). According to Bai et al. (2018), SOD was thought to be the body's first line of defense against the buildup of oxidative radicals. SOD could catalyze the conversion of superoxide radicals to hydrogen peroxide, and hydrogen peroxide is then further broken down into water and oxygen molecules by GSH-Px and CAT to reduce oxidative damage. In the current study, serum SOD activity and jejunal activities of SOD and CAT of laying hens were increased in the RP group, suggesting that 0.3% RP improved antioxidant enzyme activity in laying hens, which may help reduce intestinal oxidative damage and remove excess ROS accumulation. The pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6, etc.) usually result in inflammation and impair tight junction protein of the intestinal epithelium (Bruewer et al., 2003). In contrast to the CON group, RP significantly decreased the levels of serum TNF-α and the mRNA expression of jejunal *TNF-α*, as well as tended to increase the mRNA expression of jejunal *ZO-1*, suggesting that 0.3% RP helped reduce the inflammatory response in late-phase laying hens. This increased the abundance of tight junction protein and facilitated gut barrier function. Collectively, the addition of 0.3% RP could reduce oxidative damage and improve gut barrier function by increasing antioxidant enzyme activity and regulating the secretion of inflammatory cytokines, which is conducive to improving egg quality in laying hens at the late stage of production.

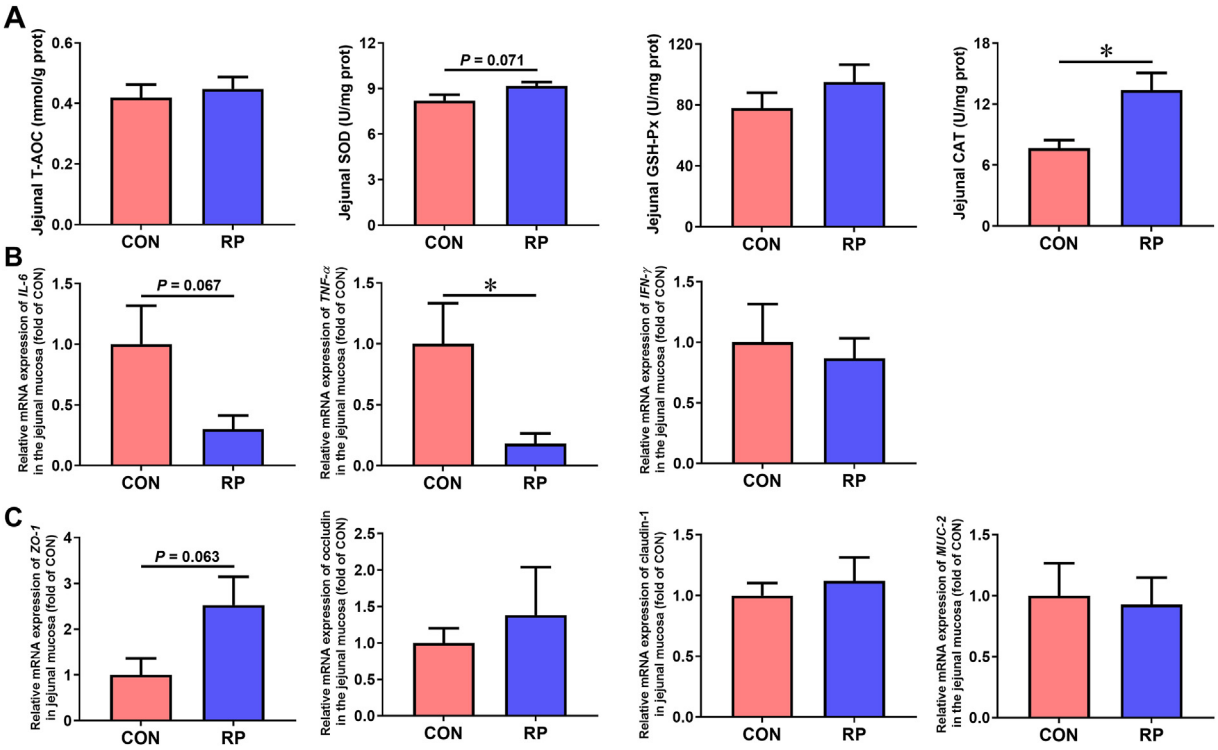

Fig. 2. Jejunal function of laying hens. (A) Antioxidant status. (B) The mRNA expression of inflammatory cytokines. (C) The mRNA expression of gut barrier-related genes. T-AOC = total antioxidant capacity; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; *IL-6* = interleukin-6; *TNF-α* = tumor necrosis factor-α; *IFN-γ* = interferon-γ; *ZO-1* = zonula occludens-1; *MUC-2* = mucin-2. CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder. Values are represented as mean ± SEM, $n = 6$. * Denotes $P < 0.05$.

The host's intestinal homeostasis and health depend on the intestinal microbiota. An important metabolite of microbial fermentation in the hindgut is SCFA, particularly butyrate, which has been shown to alleviate intestinal oxidative stress, inhibit inflammatory response, improve gut barrier function, and mediate microbial regulation of host metabolism and immune function (Feng et al., 2018; Hamer et al., 2008; Koh et al., 2016). Additionally, modifications in the composition of gut microbiota typically result in changes to intestinal SCFA levels (Zhang and Piao, 2022). Importantly, it has been established that gut bacteria and metabolites play a role in controlling egg quality. According to recent studies (Liu et al., 2021; Xu et al., 2023; Zhou et al., 2022), gut bacteria may have a possible regulatory function in halting the deterioration in egg quality of laying hens. Gut microbiota and SCFA indirectly participated in modulating egg quality by the microbiota-gut-liver/brain-reproductive tract axis (Dai et al., 2022). SCFA could interact with intrinsic enteric neurons and intestine-innervating vagal and spinal afferents to affect the secretion of estradiol, which modulates the formation of albumen in the oviducal magnum and finally achieves an increase in egg-white quality. Additionally, it has been noted that the gut microbiota could modify the fatty acid profile of the egg yolk in Japanese quail, including lowering C14:1 and C16:1 and boosting C18:0 (Furuse et al., 1992). According to several studies (Khong et al., 2014; Zhang et al., 2022b), sodium butyrate improved the quality of laying hens' eggs by promoting eggshell strength and increasing yolk color. Our findings demonstrated that the β-diversity of cecal microbiota in the RP groups was different from the control. Compared with CON group, RP tended to increase butyrate concentration in the cecum, which was consistent with higher abundances of butyrate-synthesizing enzymes, such as lysine 2,3-aminomutase, β-lysine 5,6-aminomutase, and 3-oxoacid CoA-transferase. The LEfSe analysis showed that 0.3% RP supplementation markedly increased the cecal abundances of Rikenellaceae, *Rikenellaceae_RC9_gut_group* and *Turicibacter* and decreased the cecal abundances of Erysipelatoclostridiaceae, Fusobacteriaceae, Campylobacteraceae, *Campylobacter* and *Fusobacterium*. These findings demonstrated that 0.3% RP could alter the microbial structure of cecum and further change the cecal SCFA profile. Rikenellaceae is closely linked to the formation of colonic butyrate and has been shown to be an effective treatment for experimental colitis in mice caused by dextran sulfate sodium (Huang et al., 2019; Yang et al., 2022b). A genus of Rikenellaceae called *Rikenellaceae_RC9_gut_group* could produce SCFA from dietary fibers in the hindgut (Gao et al., 2022). It has been demonstrated that the abundance of *Turicibacter* is decreased in obesity and irritable bowel syndrome (Jung et al., 2016; Zhuang et al., 2018). *Turicibacter* is a probiotic bacteria that may change intestinal motility patterns and stimulate the production of intestinal SCFA (Li et al., 2022). According to Yu et al. (2023), the levels of several Erysipelatoclostridiaceae species, including the potential pathogen *Erysipelatoclostridium*, are greater in mice with colitis and positively linked with TNF-α concentration. Animal health issues are associated with Campylobacteraceae, which has been found to diminish the intestinal mucosal layer and cause proliferative enteritis (Deng et al., 2022). Acetate, butyrate, and total SCFA levels in the cecum have all been found to be adversely linked with *Campylobacter* abundance. *Campylobacter* is susceptible to the bactericidal effects of SCFA, particularly butyrate (Fan et al., 2022). According to Reshef et al. (2015), Fusobacteriaceae and *Fusobacterium* are intimately linked to the development of an inflammatory response and elevated disease activity in colitis patients. The abundances of Rikenellaceae and *Rikenellaceae_RC9_gut_group* in the cecum were shown to be positively linked with butyrate concentration and Haugh unit in this study, according to Spearman's correlation test. The amount of cecal

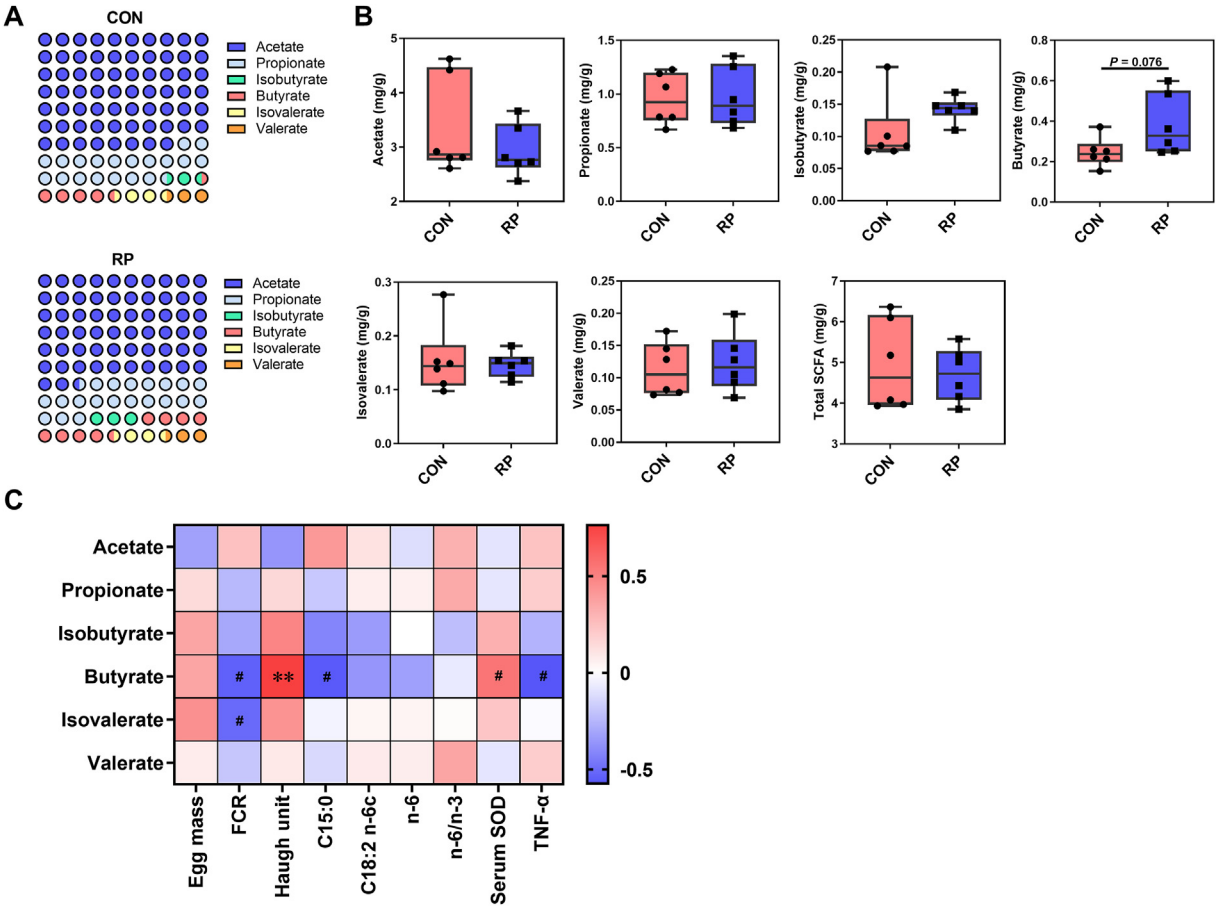

Fig. 3. Cecal short-chain fatty acids (SCFA) profile. (A) SCFA compositional proportion. (B) SCFA contents. (C) Spearman's correlation analysis. CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder. Values are represented as mean ± SEM, $n = 6$. # Denotes $0.05 \leq P < 0.1$; ** denotes $P < 0.01$.

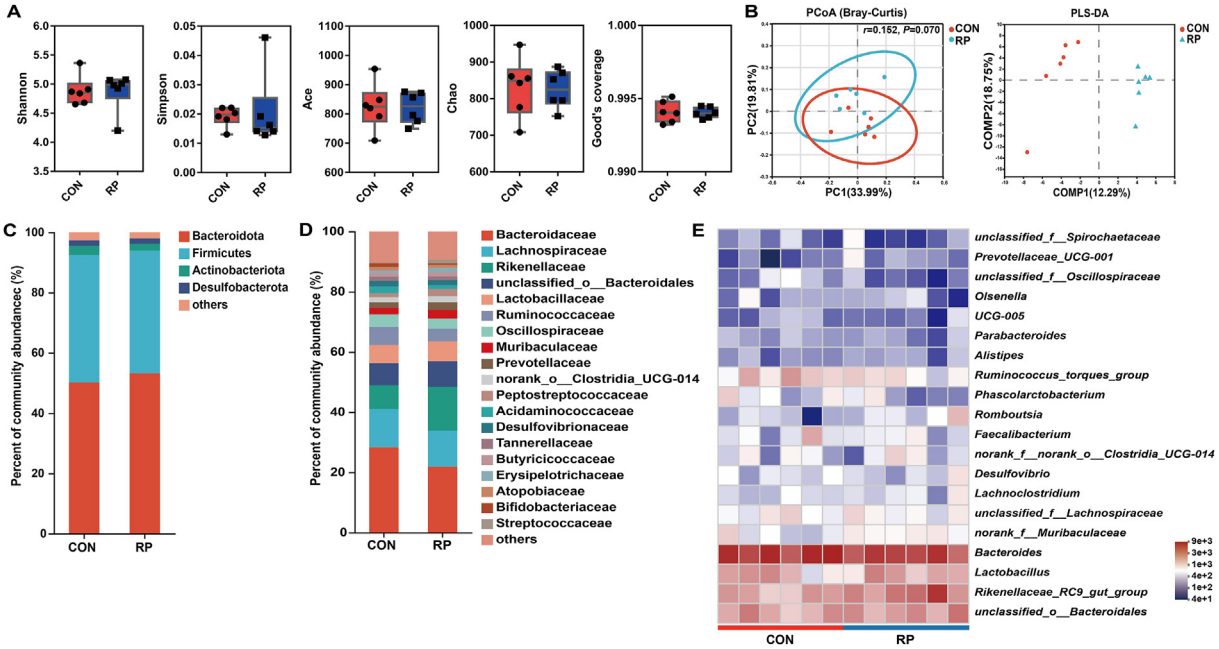

Fig. 4. Cecal microbiota structure and composition. (A) The α-diversity. (B) The β-diversity. Microbiota composition at the phylum (C), family (D), and genus (E) levels. PCoA = principal coordinate analysis; PLS-DA = partial least squares-discriminant analysis. CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder. Values are represented as mean ± SEM, $n = 6$.

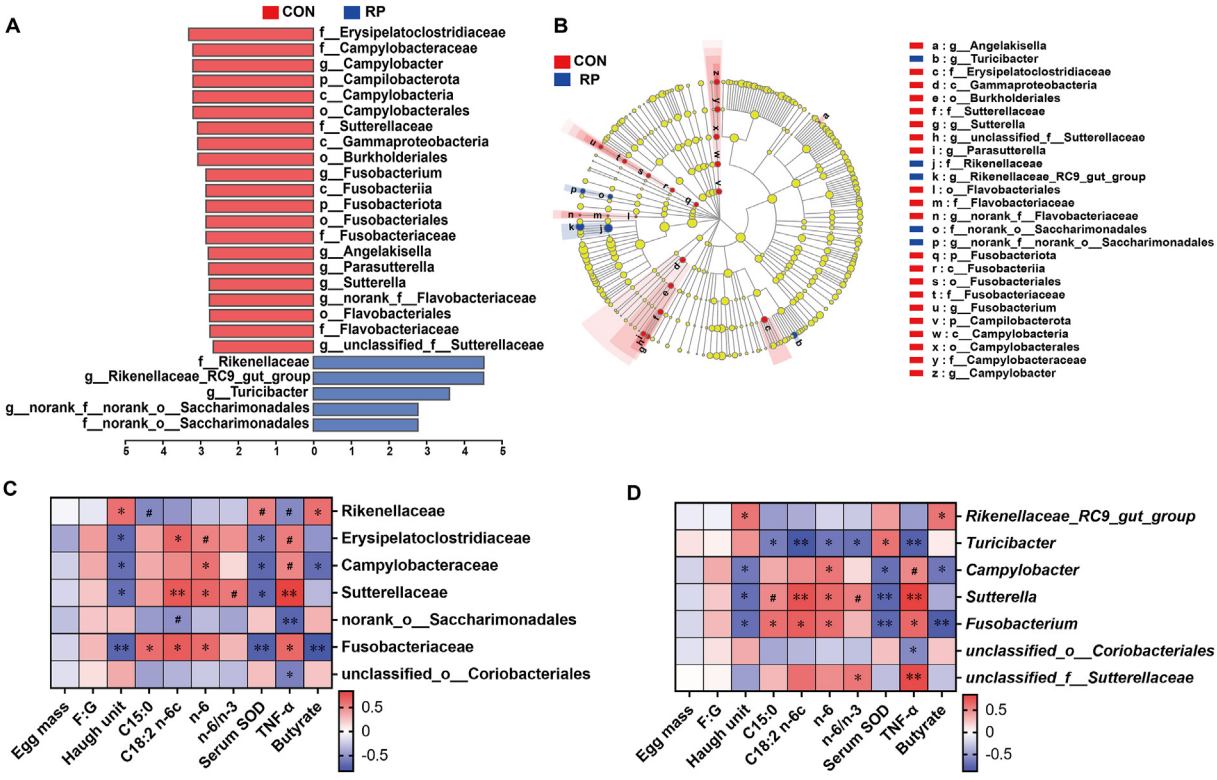

Fig. 5. Differential cecal microbiota. (A) Linear discriminant analysis (LDA) score distribution. (B) Linear discriminant analysis effect size (LEfSe) cladogram. Spearman's correlations analysis at the family (C) and genus (D) levels. CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder. Values are represented as mean ± SEM, $n = 6$. # Denotes $0.05 \leq P < 0.1$; * denotes $P < 0.05$; ** denotes $P < 0.01$.

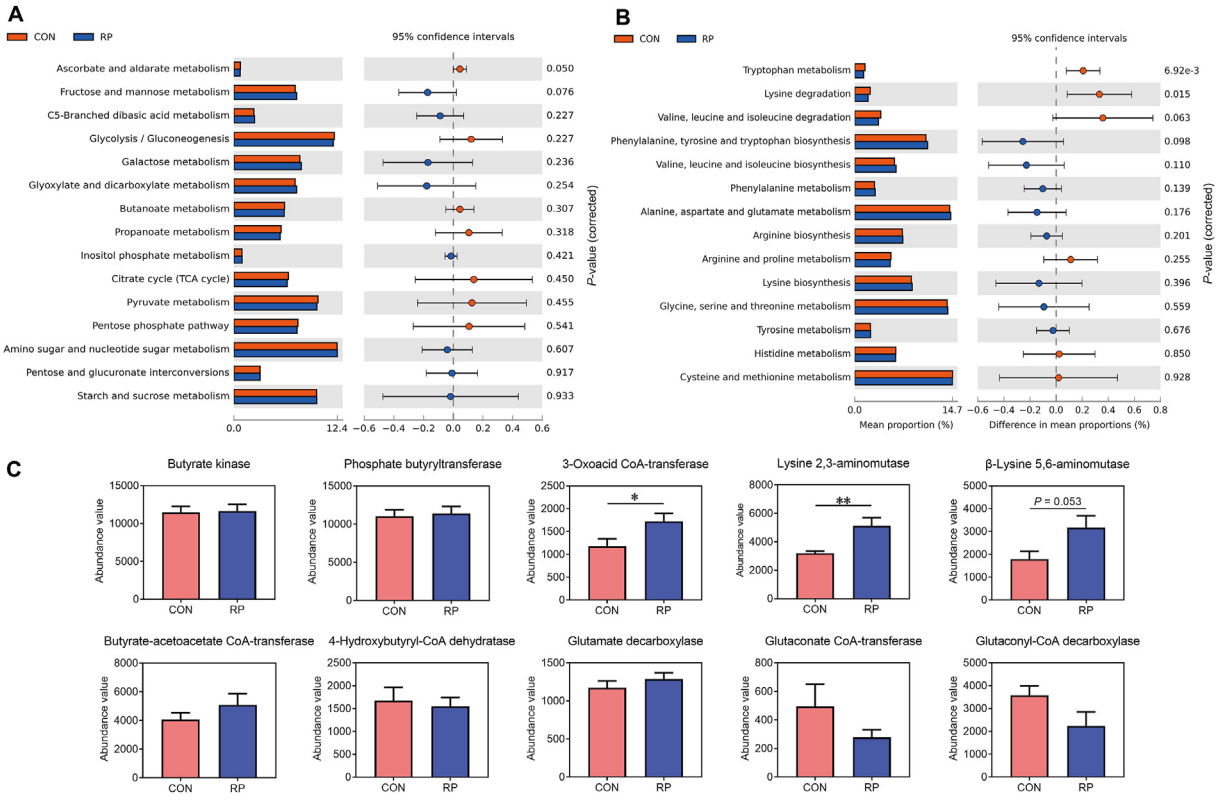

Fig. 6. Prediction on metabolic functions of cecal microbiota. (A) Carbohydrate metabolism. (B) Amino acid metabolism. (C) Abundance values of butyrate-synthesizing enzymes. CON group, basal diet; RP group, basal diet + 0.3% rosemary leaf powder. Values are represented as mean ± SEM, $n = 6$. * Denotes $P < 0.05$; ** denotes $P < 0.01$.

Turicibacter was negatively correlated with C18:2 n-6c, n-6 PUFA and n-6/n-3 PUFA in yolk and serum TNF-α content, and positively related to serum SOD activity. The amount of cecal Erysipelatoclostridiaceae was markedly negatively correlated with Haugh unit and serum SOD, and positively correlated with C18:2 n-6c and n-6 PUFA in yolk and serum TNF-α concentration. The cecal abundances of Campylobacteraceae and *Campylobacter* were negatively correlated with Haugh unit, serum SOD and butyrate, and positively correlated with n-6 PUFA in yolk and serum TNF-α concentration. The cecal abundances of Sutterellaceae and *Sutterella* were markedly negatively correlated with Haugh unit and serum SOD, and positively correlated with C18:2 n-6c and n-6 PUFA in yolk and serum TNF-α concentration. The cecal abundances of Fusobacteriaceae and *Fusobacterium* were markedly negatively correlated with butyrate, Haugh unit, and serum SOD, and positively correlated with C18:2 n-6c and n-6 PUFA in yolk and serum TNF-α concentration. The aforementioned findings demonstrated a direct relationship between the alterations in cecal microbiota composition caused by RP and higher butyrate concentration, an enhanced Haugh unit, better serum antioxidant enzyme activity, and an increase in inflammatory cytokines. Notably, our study also showed that cecal butyrate content was positively correlated with Haugh unit and serum SOD, and negatively correlated with serum TNF-α concentration, which further demonstrated that higher butyrate level caused by 0.3% RP could reduce intestinal oxidative stress and promote intestinal barrier function. Cecal butyrate, a metabolic indicator of intestinal microbiota, was also found to be closely related to an improvement in egg quality in the RP group. Together, dietary supplementation with 0.3% RP could alter the intestinal microbiota and control the formation of cecal butyrate, which helps enhance antioxidant activity and reduce inflammatory injury in laying hens, so as to boost egg quality at the late stage of production.

5. Conclusion

In conclusion, 0.3% RP has the potential to improve egg quality by modifying the status of serum antioxidants and intestinal health, including elevating serum SOD activity, decreasing serum TNF-α content and jejunal *TNF-α* mRNA abundance, and altering cecal microbiota community, cecal abundances of butyrate-synthesizing enzymes and cecal butyrate content. These findings shed light on the regulatory effects of rosemary on egg quality and gut health in late-phase laying hens.

Author contributions

Lianhua Zhang: Investigation, Methodology, Formal analysis, Data curation, Software, Conceptualization, Funding acquisition, Writing – original draft preparation, Writing – review & editing. **Junwei Ge:** Methodology, Data curation, Conceptualization. **Fei Gao:** Investigation, Validation. **Min Yang:** Investigation, Validation. **Hui Li:** Resources. **Fei Xia:** Resources. **Hongtong Bai:** Resources, Supervision. **Xiangshu Piao:** Supervision. **Zhiying Sun:** Supervision. **Lei Shi:** Supervision, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.02.003.

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