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Impact of dietary polyphenols from shredded, steam-exploded pine on growth performance, organ indices, meat quality, and cecal microbiota of broiler chickens

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ABSTRACT

The chicken's gastrointestinal tract is home to complex and diverse microbial communities that can be manipulated to enhance health and productivity. Although polyphenols have recently attracted the attention of researchers due to their potent antioxidant capabilities, their impact on the gut microbiota remains largely unexplored. Hence, in this study, we conducted a comprehensive analysis of the effects of dietary supplementation with polyphenol-rich extract from shredded, steam-exploded pine particles **(PSPP)** on growth, meat quality, and gut microbial dynamics in broiler chickens. Supplementation of PSPP was found to significantly improve birds' FCR until the third week of the trial but only marginally affected meat quality. Based on meta-taxonomic analyses of the cecal microbiotas of broilers fed increasing concentrations of PSPP, dietary PSPP modulated the composition of the cecal microbiota of the birds with a concomitant increase of Bacteroidetes and a decrease in the Firmicutes population. Similar trends were observed for the proportions of *Alistipes* and *Faecalibacterium* at the genus level. Additionally, 43 unique bacterial species were detected in the cecal microbiome of birds fed with PSPP. However, microbial diversity did not vary significantly among treatment groups. A particularly interesting finding was the specialization observed in the microbiome of birds receiving PSPP supplementation. Microbial co-occurrence network analyses revealed substantial modifications in their network structure when compared to control birds. Families like Rikenellaceae and Eubacteriaceae were notably absent, and the number of microbial interactions was drastically lower in the PSPP-fed group. Microbial taxa modeling revealed that the impact of increasing dietary PSPP levels primarily affected genus-level taxa, showing a decreasing trend. Overall, this offers compelling evidence that continuous PSPP supplementation may not only alter the composition of intestinal microbes but also have a profound effect on the interactions among different microbial species. Conversely, PSPP had minimal effects on broilers' performance and meat quality.

Introduction

It is widely acknowledged that the gut microbiota has a crucial impact on regulating host health and resilience (Dittoe et al., 2018). The interactions between the microbiota and the host are incredibly complex and can be influenced by several factors (Chong, et al., 2018). For instance, previous studies have shown that variations in the microbiota composition can be related to different host characteristics, environment, and diet (Xu, et al., 2015, 2020; Lkhagva, et al., 2021). Among the various animal species, chickens stand out due to their crucial role in the

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poultry industry and possess one of the most dynamic and extensively studied microbiomes (Oakley, et al., 2014).

The gastrointestinal tract **(GIT)** of chickens consists of the crop, proventriculus, gizzard, duodenum, jejunum, ileum, ceca, rectum, and cloaca. Each has a distinct function in the digestion of feed and microbial dynamics vary widely across its sections (Yeoman, et al., 2012; Oakley, et al., 2014). For example, the cecum, home to the richest anaerobic microorganisms, plays a vital role in nutrient fermentation (Ebeid, et al., 2022). This microbial ecosystem is closely linked to nutrient metabolism, immune modulation, and overall health (Borda-Molina, et al., 2018). Indeed, gut microbes can combat pathogens either via competitive exclusion or through the activation of the immune system (Grant, et al., 2018; Huang, et al., 2019). It has been shown that modifying or supplementing the diet of chickens was effective in reducing the prevalence of specific avian pathogens such as *Salmonella spp., Campylobacter spp., Clostridium spp.*, and *Escherichia/Shigella spp* (Eeckhaut, et al., 2016; Yitbarek, et al., 2018; Reuben, et al., 2019).

Owing to their multifaceted benefits, phytogenic feed supplements are gaining importance in the poultry industry (Abdelli, et al., 2021). An array of phytogenic feed additives have been used in poultry production such as thyme essential oil (Hoffman-Pennesi, et al., 2010), rosemary and sage (Lopez-Bote, et al., 1998), green tea (Sarker, et al., 2010), ginger (Kairalla, et al., 2022), and garlic (Elbaz, et al., 2021). The *modus operandi* of these phytogenic compounds is mainly via the production of secondary metabolites (Batiha, et al., 2020). Polyphenols are such substances of secondary metabolites usually synthesized as a result of physiological and environmental stressors (Rasouli, et al., 2017). These diverse, bioactive, and pervasive categories of secondary plant metabolites are of considerable interest mainly due to their biological properties (El Gharras, 2009).

For instance, inflammations and morbidity were decreased in White Leghorn chickens gavage-fed polyphenol extracts from *Terminalia chebula* and *Punica granatum and* challenged with *E.coli* (Zhong, et al., 2014). Dietary supplementation of epigallocatechin gallate improved the growth and overall antioxidant status of heat-stressed broiler chickens (Luo, et al., 2018). It is therefore clear that polyphenols inclusion in the diet could promote broiler performances, health, immune functions, and the general well-being of the birds (Abdel-Moneim, et al., 2020). However, the specific impact of polyphenol-rich extracts on the chicken cecal microbiome remains a topic of active investigation. Hence, in the current study, we aimed to provide a thorough description of how dietary polyphenol-rich extract from shredded, steam-exploded pine particles **(PSPP)** might modulate the cecal microbial communities in broilers and also assess its potential impact on other parameters, such as growth, blood biochemicals, organ indexes, and meat quality.

Material and methods

The Institutional Animal Care and Use Committee of Gyeongsang National University (GNU-200916-C0057) approved all the experimental procedures for this study.

Preparation of dietary supplements

The preparation of PSPP was done in a series of steps. Initially, steam-exploded pine particles **(SPP)** were prepared by exploding pinewood chips of approximately $2 \times 2 \times 0.5$ cm^3 with steam at 200 °C for 11.5 min. These particles were used in our previous study (Goel, et al., 2021a, 2021b, 2022b). Further, PSPP was extracted in 74 % ethanol with an extract ratio of 1/46 (w/v) at 90 °C for 240 min.

Experimental birds and housing

One-day-old mixed-sex Indian River broiler chicks were obtained from a local hatchery in Hapcheon, Korea. On the same day, the chicks were individually tagged, weighed, and randomly assigned ($n = 216$) to one of three treatment groups, each containing twelve replicates of six birds per cage. Commercial starter, grower, and finisher feed (Nonghyup Feed, Seoul, Republic of Korea) were obtained and supplemented with 0, 0.5, or 1 % PSPP prepared from pine wood. The feed composition is provided in Table S1. The total polyphenol content was measured as 23.7 g per L of PSPP, and was 0 mg, 118 mg, and 235 mg per kg of feed in the CON group, PSPP_0.5, and PSPP_1, respectively. Each diet was stored in a plastic drum and prepared every week or as required. The birds were housed in a room with controlled environmental conditions, including smart temperature and humidity control. The birds were reared until the 28th day of age and were provided with 23 h of lighting and 1 h of dark. Feed and water were provided *ad libitum* throughout the experiment. As recommended, the temperature in the first three days was maintained at 34°C and gradually decreased to 22°C by the end of the experiment. Individual body weight and feed intake were recorded weekly to calculate average daily feed intake **(ADFI)**, average daily gain **(ADG)**, and feed conversion ratio **(FCR)**, and mortality was recorded daily if it occurred.

Sample collection and measurements and plasma biochemical analysis

At the end of the experiment, six birds from each group were randomly selected for euthanasia using a carbon dioxide chamber on the 28th day of age. Blood samples were collected from the birds through a cardiac puncture after euthanasia in a heparinized vacutainer for further plasma processing. The blood was centrifuged at 2,000 x *g* at 4°C for 10 min to separate plasma, which was then immediately stored at −20 °C until use. Internal organs, including the liver, gizzard, bursa, and spleen, were dissected and weighed. Their weights were expressed as both absolute and relative to their body weight. The length of the duodenum, jejunum, ileum, and cecum was also recorded and presented as both absolute and relative to body weight. Additionally, both pouches of the cecum were tied at the ileocecum junction, excised carefully, measured, and frozen for 16S rRNA high-throughput sequencing analysis. Plasma metabolite concentrations were measured using a VetTest Chemistry Analyzer (IDEXX Co., Ltd., Westbrook, ME, USA) with a dry-slide technology, following the manufacturer's guide. Briefly, 300 uL of plasma was injected into the machine concomitant with prepackaged panels (glucose, total protein, triglycerides, and cholesterol) and bird-specific reference ranges provided by the manufacturer. Results were obtained within approximately 6 min of sample loading. The analyzer was connected to the IDEXX VetLab Station (IDEXX Co., Ltd., Westbrook, ME, USA) for data management and reporting. Concerning samples for meat quality, breast muscles were excised within 10 min post-mortem and were followed by the removal of all obvious fat and connective tissues. Each muscle part was then divided into four equal portions, and their weights were recorded before packing them separately in moisture-impermeable polyethylene bags that were sealed for subsequent analysis.

Meat quality analysis (proximate analysis, pH, cooking loss, and thiobarbituric acid reactive substance)

The proximate composition of the meat samples, including moisture, crude fat, and ash contents, was determined using the Association of Official Analytical Chemists **(AOAC)** 2000 methods as previously described (Seong, et al., 2015). Briefly, moisture content was obtained as a result of subtracting weight before and after drying the sample in a dry oven at 102°C for 24 h. The Kjeldahl method was employed to determine crude protein content. Ash content was measured by the classic dry-ashing technique at 550°C. Crude fat was measured as previously described (Folch, et al., 1957). All the parameters were run four times for each sample, and the average value was used.

The breast meat color parameters were determined using a chroma meter (CR-400, Minolta, Tokyo, Japan) calibrated with a standard white plate ($Y = 93.5$, $X = 0.3132$, and $y = 0.3198$). A total of eight readings

were collected from the center of each sample. According to the Commission International on Illumination **(CIE)**, the measured colors were expressed as CIE L*, lightness; CIE a*, redness; and CIE b*, yellowness. The chroma angle was calculated as $(a^{*2} + b^{*2})^{0.5}$, while the hue angle was calculated as $\tan^{-1}(b^*/a^*)$, respectively.

In addition, pH was measured immediately after sampling. Sample (3 g) was homogenized for 30 s using a polytron homogenizer (T25 basic, IKA Labortechnik. Selangor, Malaysia) with 27 mL of deionized water. The pH was measured using a portable pH meter (MP3230, Mettler-Toleda, Greinfensee, Switzerland). Before pH measurement, the pH meter was calibrated with standard buffers at pH 4.01, 7.0, and 9.21, at 25°C. For each sample, an average of four measurements was recorded. Cooking loss was calculated as the difference in weight before and after cooking.

For the thiobarbituric acid reactive substance **(TBARS)** analysis, approximately 5 g of each sample was mixed with 15 mL of 0.1 M phosphate buffer (pH 7.0) and homogenized for 2 min. Then, 1 mL of supernatant was transferred into a glass tube and combined with 50 μL of butylated hydroxytoluene (BHT; 7.2 % in ethanol) and 1.95 mL of a thiobarbituric acid **(TBA)**/trichloroacetic acid **(TCA)**/HCl solution (0.375 % TBA, 15 % TCA, and 0.25 N HCl). The mixture was incubated at 90°C for 15 min in a shaking water bath, followed by cooling to room temperature. After filtration through Whatman filter paper No. 1 (#WM.1001070, HANA biolab, Hanam-si, Gyeonggi-do, Republic of Korea), absorbance was measured at 532 nm using a UV–Vis spectrophotometer (G1115AA, Agilent Technologies, USA) against a blank containing 2 mL of TBA/TCA/HCl solution in 1 mL of distilled water. TBARS values for different samples were determined as mg of malonaldehyde **(MDA)** per kg of the sample, using a standard curve constructed with 1,1,3,3-tetramethoxypropane.

Metagenomic sequencing analysis of the cecal microbiota

The DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) was employed to extract total genomic DNA from the content of cecal samples. Six birds were randomly selected from each treatment, and the extracted DNA samples were quantified using Quant-IT PicoGreen (Invitrogen, Waltham, MA, USA). Subsequently, a 16S metagenomic sequencing library was constructed using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, San Diego, CA, USA). The Illumina platform was utilized for sequencing the library, and Macrogen, Inc. (Seoul, Korea) carried out the process. The FASTP program was then executed to perform quality profiling, adapter trimming, and read filtering using default parameters. Paired-end reads with a length of 400-500 bp were selected, and FLASH (v1.2.11) software (Magoc, et al., 2011) was used to assemble them into a single sequence. Thereafter, the CD-HIT-EST program (Li, et al., 2012) was employed to determine the number of operational taxonomic units **(OTUs)** with a 97 % sequence identity cutoff. The BLAST+ (v2.9.0) program (Madden, 2013) was used to check taxonomic similarity against the reference database (NCBI 16S Microbial), and an identical coverage of less than 85 % was identified as not defined. Finally, QIIME software (v1.9) (Bolyen, et al., 2018) was used to evaluate the OTU abundance and taxonomic information of the microbes.

Ecological and statistical analysis

In this study, all statistical analyses were conducted using R environment version 4.2.2 and R studio (Team, 2010). Before analysis, the homoscedasticity and normality of distribution assumptions were verified using Levene and Shapiro-Wilk's test (Schultz, 1985; Royston, 1992). As a result, normally distributed data such as growth parameters, organ indexes, blood parameters, and meat quality parameters of breast muscle characteristics were analyzed using a one-way ANOVA followed by a Tukey HSD test when significant differences were detected. Furthermore, polynomial regression analysis was carried out to determine any linear or quadratic trends associated with increasing levels of PSPP supplementation.

The following procedures were used for analyzing microbiome data: alpha diversity metrics were determined for the cecal microbial community, including OTUs, Chao1, Shannon, inverse Simpson, and Good's coverage. Due to the violation of the normality assumption, the non-parametric Kruskal-Wallis test (McKight, et al., 2010) followed by the pairwise Wilcoxon rank sum test with Benjamini-Hochberg correction (Rosner, et al., 2005) was used for analyzing alpha diversity metrics. Unweighted and weighted Unifrac distances were calculated as beta diversity metrics and non-metric dimensional scaling **(NMDS)** was used to visualize differences in microbial community structure based on these metrics (Kruskal, 1964). A permutational multivariate analysis of dispersion **(PERMDISP)** was conducted to assess the homogeneity of multivariate dispersion among dietary treatment groups (Bakker, 2024), followed by a permutational multivariate analysis of variance **(PERMANOVA)** to detect potential differences in beta diversity across treatment groups (Anderson, 2017). The taxonomic distribution of cecal samples was visualized using stacked bars, and Venn diagrams were built to represent the relationships and intersections between microbial taxa of dietary treatments. Linear discriminant analysis **(LDA)** effect size **(LEfSe)** was used to identify differentially ($|$ LDA effect size $| > 2.0$) abundant taxa among treatments and detect biological biomarkers of PSPP supplementation (Tharwat, et al., 2017). A Spearman correlation heatmap (Wissler, 1905) was constructed to detect the association between the top 20 microbial species abundance and meat quality indices. Co-occurrence microbial networks were constructed for the control and PSPP-supplemented diets to visualize and analyze interactions among microbial taxa specific to each environment (Liu, et al., 2023). Microbial taxa modeling was subsequently conducted to elucidate the impact of increasing doses of PSPP on the abundance of specific microbial taxa within the cecal microbial community. The effect of PSPP doses was utilized to model genus-level taxa using total sum scaling **(TSS)** log2 linear regression as previously described by Barnett, et al. (2021). This procedure employs a two-step transformation, initially converting count data into proportions, followed by applying a log2 transformation to these proportions. Subsequently, the PSPP dose is employed as a regressor to evaluate potential linear relationships between genus-level taxa and increasing doses of PSPP.

All the aforementioned analyses set the statistical significance threshold at $p < 0.05$. One-way ANOVA, Kruskal-Wallis, and pairwise Wilcoxon rank sum tests were performed using the base R functions. NDMS, PERMDISP, and PERMANOVA were carried out using the "vegan" (Dixon, 2003) package. Finally, the "phyloseq" (McMurdie, et al., 2013), "VennDiagram" (Chen, et al., 2011), "igraph" (Csardi, 2013), and "microViz" (Barnett, et al., 2021) packages were employed for microbial composition analysis, Venn diagrams, microbial network analysis, Spearman heatmap, LEfSe, and taxa modeling respectively.

Results

Dietary PSPP led to marginal effects on growth performances, organ indexes, plasma biochemical parameters, and meat quality

During the initial week of the trial, ADG and FCR from both PSPP-supplemented groups exhibited significant differences from the control group ($p < 0.01$), with a quadratic decrease ($p = 0.038$) observed in FCR values as PSPP doses increased (Table 1). In the second week of the trial, only FCR from the PSPP_0.5 group demonstrated significant improvement ($p < 0.01$) compared to the control group, accompanied by a significant quadratic trend ($p < 0.01$). Conversely, the third week of the trial revealed significant improvement ($p = 0.036$) in the PSPP_1 group compared to the control group, with a linear increase observed ($p < 0.01$). Analysis of growth parameters over the entire trial duration indicated that only the PSPP_0.5 group exhibited a marginally significant improvement ($p = 0.023$) in FCR compared to the control group,

Table 1
Effect of different doses of dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) on the growth performances of broiler chickens.

Parameters	Treatments			p - value		
	CON	PSPP_0.5	PSPP_1	ANOVA	Lin	Quad
BW (g) day 0	43.2 ± 0.25	43.51 ± 0.23	43.49 ± 0.23	0.675	0.405	0.656
BW (g) day 28	1718 ± 2.09	1762 ± 20.66	1762 ± 23.4	0.253	0.153	0.399
Day 0-7						
ADG	21.7^{a} ± 0.41	22.8^{b} ± 0.29	23.3^{b} ± 0.34	0.008	0.524	0.624
ADFI	22.3 ± 0.34	22.51 ± 0.3	23.06 ± 0.21	0.154	0.057	0.660
FCR	1.03^{b} ± 0.008	0.99^{a} ± 0.007	0.99^{a} ± 0.009	0.001	0.003	0.038
Day 7-14						
ADG	47.6 ± 1.01	49.1 ± 0.59	48.83 ± 0.43	0.306	0.707	0.768
ADFI	61 ± 0.98	61.46 ± 0.57	63.15 ± 0.49	0.097	0.039	0.491
FCR	1.28^{b} ± 0.011	1.25^{a} ± 0.009	1.29^{b} ± 0.004	0.006	0.514	0.002
Day 14-21						
ADG	72.3 ± 1.3	73.56 ± 0.85	75.17 ± 1.1	0.186	0.577	0.851
ADFI	101.5 ± 1.29	101.92 ± 1.24	102.17 ± 0.81	0.925	0.692	0.966
FCR	1.41b ± 0.014	1.39ab ± 0.012	1.36a ± 0.011	0.036	0.008	0.934
Day 21-28						
ADG	97.7 ± 1.83	100.03 ± 2.58	98.11 ± 1.22	0.666	0.425	0.944
ADFI	142.2 ± 2.45	145.66 ± 2.96	143.76 ± 1.91	0.610	0.648	0.379
FCR	1.46 ± 0.011	1.46 ± 0.017	1.48 ± 0.012	0.566	0.280	0.673
Day 0-28						
ADG	59.81 ± 0.90	61.38 ± 0.94	61.36 ± 0.51	0.301	0.182	0.425
ADFI	81.74 ± 1.11	82.89 ± 1.16	83.04 ± 0.62	0.606	0.357	0.684
FCR	1.29 ± 0.01b	1.27 ± 0.01a	1.28 ± 0.01ab	0.023	0.108	0.026

Chickens were fed with diets containing 0 % (control), 0.5 %, and 1 % polyphenolic extract during the first four weeks of age. Data show mean ± SE ($n = 6$). Means with different superscripts (a,b) within a row are significantly different. Abbreviation: ADG, average daily weight gain; ADFI, average daily feed intake, Bw, body weight; Lin, linear; Quad: quadratic.

associated with a significant quadratic decrease. Organ indexes were also analyzed to detect any variation linked to dietary treatments (Fig. 1). Increasing levels of PSPP resulted in a linear decrease ($p < 0.05$) in relative cecal weight, while other parameters, such as absolute organ weight and blood biochemicals, were not significantly affected by the dietary treatments. Concerning meat quality parameters, the proximate composition and color of the breast muscle were unaffected by the PSPP treatment (Tables 2 & 3). Similar trends were observed regarding the physicochemical properties of the breast muscles, with only the pH and TBARS exhibiting significant differences (Table 4). Specifically, lower pH values ($p = 0.024$) were recorded in the PSPP_0.5 group compared to the PSPP_1 group. Additionally, a quadratic decrease ($p = 0.028$) was associated with increasing levels of PSPP supplementation. TBARS values were significantly higher ($p < 0.01$) in the PSPP_1 group compared to both CON and PSPP_0.5 groups, demonstrating a quadratic increasing trend ($p < 0.01$).

Dietary PSPP modulated cecal microbiome composition but not diversity

A microbial community analysis was carried out to determine the differences in bacterial composition among dietary treatments. Two sets of separate visualizations were created, one set showing all three treatments and the other set highlighting the differences between control samples and PSPP diets (Fig. 2A). At the phylum level, 5 phyla were the most represented, including Firmicutes, Bacteroidetes, Candidatus Melainabacteria, Proteobacteria, and Actinobacteria. Firmicutes and Bacteroidetes were the predominant phyla, accounting for over 95 % of the total microbial community in all samples, and the four most abundant genera were *Alistipes, Faecalibacterium, Ligilactobacillus*, and *Mediterraneibacter*.

Microbial composition analysis also revealed differential relative abundances of taxa across treatments, with the relative abundance of Bacteroidetes increasing and that of Firmicutes decreasing with the inclusion of PSPP in the diet. Similar trends were observed for the proportions of *Alistipes* and *Faecalibacterium* genera when PSPP was supplemented. A relatively small number of bacterial species were exclusively associated with our dietary treatments (Fig. 2B). Specifically, 16, 18, and 17 species were only found in the CON, PSPP_1, and PSPP_0.5 groups, respectively. However, when considering the supplementation of PSSP, a much higher number of bacterial species were uniquely detected in the cecal microbiome of birds that were fed PSPP (43) compared to the control group (16). LEfSe analysis was conducted to identify the taxa that were differentially abundant between the treatments and to identify specific biomarkers of PSPP supplementation (Fig. 3). The results showed that two species (*Alistipes putredinis* and *Blautia schinkii*) were attributed to the PSPP treatments, while four species (*Sporobacter termitidis, Anaerostipes butyraticus, Defluviitalea raffinosedens, and Faecalicoccus acidiformans*) were attributed to the control treatment (Fig. 3D).

Kruskal-Wallis test was conducted to evaluate several alpha diversity metrics, including OTUs, Chao1, Shannon, inverse Simpson, and Good's coverage. None of these metrics were significantly different across the groups (Fig. 4). Similarly, beta diversity analysis by NMDS did not visually show any separation or specific clusters linked to dietary treatment, but PERMANOVA of PSPP diet indicated tendencies in p-values (Fig. 5C, $p = 0.058$ and Fig. 5D, $p = 0.063$) when using unweighted Unifrac distances. The PERMDISP procedure also revealed homogeneity of dispersion among the control and treatment groups, regardless of the distance matrix employed.

Dietary PSPP resulted in the specialization of the cecal microbiome

The top 20 most abundant microbial species were selected, and the potential association between their relative abundance and meat quality traits was tested using the Spearman correlation test. A visual representation of their prevalence, abundance, and correlation coefficient is displayed in Fig. 6. While abundance values were highly subjected to variations, prevalence was generally above 0.8, suggesting that these species were present in almost every sample. The correlation ranged from −0.5 to 0.7, with a single significant association found between *Gemmiger formicilis* and the total fat content of the pectoralis major of broilers.

A microbial co-occurrence network analysis was carried out to detect the interaction between cecal microbial taxa and to examine how dietary treatment might influence these connections. Two separate networks were constructed using control samples and PSPP-supplemented samples respectively (Fig. 7). These networks were obtained using prevalence filtering with a threshold of 0.7 and then selecting strong and significant correlations with a p-value less than or equal to 0.05 and an absolute value of the correlation greater than or equal to 0.6. From an initial network obtained from the control sample containing 94 species with 1062 interactions, dietary PSPP supplementation resulted in a network with a slightly reduced number of microbial taxa (82) and notably fewer interactions (322). The number of negative correlations among species was drastically reduced in the PSPP-based network. Interestingly, the number of families present in each network was only reduced by 2, with the absence of Rikenellaceae and Eubacteriaceae. Both networks were also dominated by the same families, which were

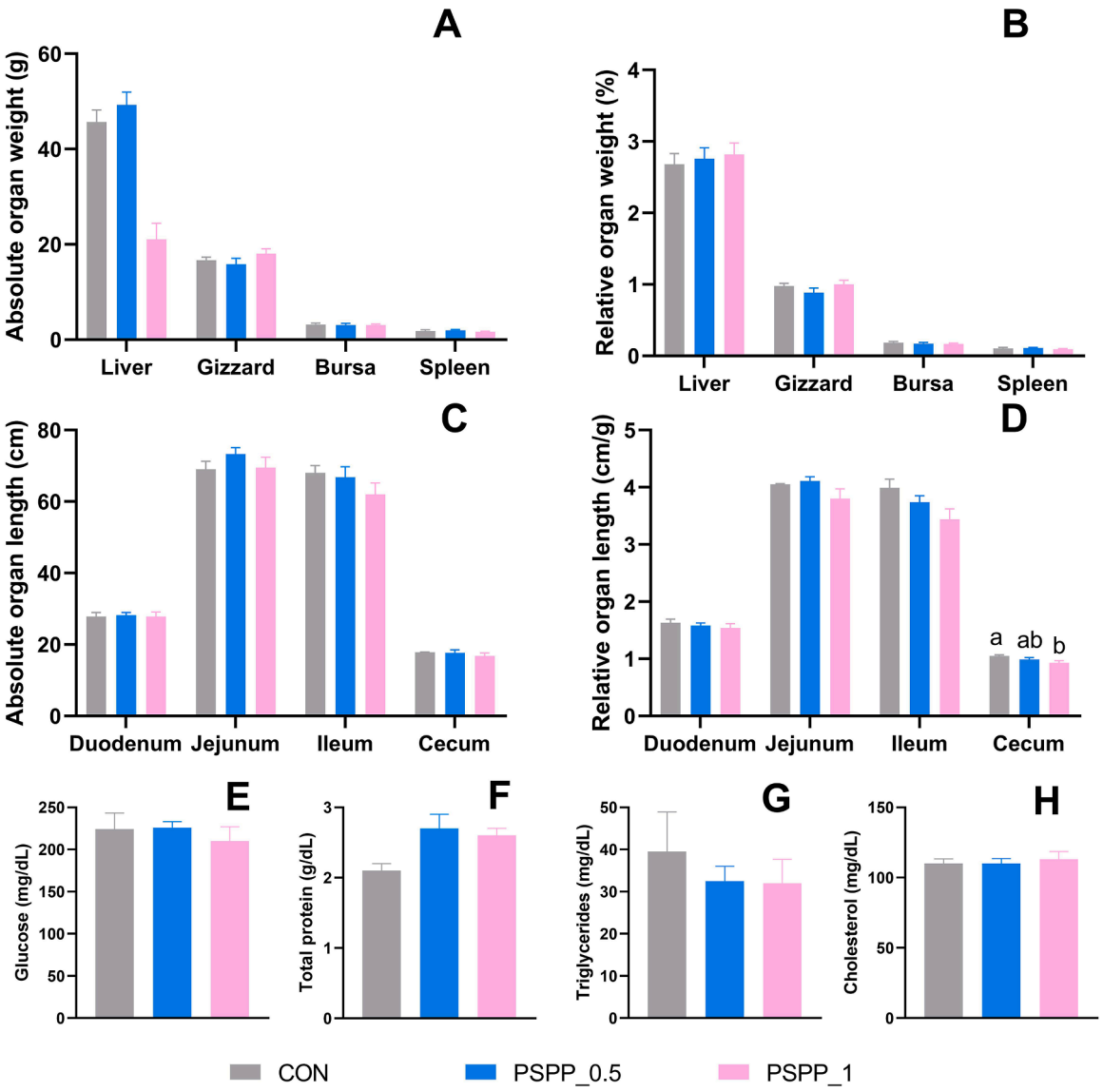

Fig. 1. Effect of different doses of dietary polyphenolic extract supplementation on organ indexes (A to D) and blood biochemicals (E to H) of broiler chickens. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

Table 2
Effect of different doses of dietary polyphenolic extract supplementation on the proximate composition of breast muscle of broiler chickens.

Parameters	Treatments			p-value		
	CON	PSPP_0.5	PSPP_1	ANOVA	Lin	Quad
Crude Fat	1.53 ± 0.38	1.30 ± 0.16	1.69 ± 0.20	0.584	0.662	0.353
Ash	22.17 ± 0.13	22.21 ± 0.13	22.24 ± 0.12	0.933	0.706	0.997
Crude Protein	21.91 ± 0.46	21.88 ± 0.45	21.63 ± 0.28	0.868	0.625	0.822
Moisture	75.51 ± 0.19	75.66 ± 0.35	75.56 ± 0.29	0.929	0.885	0.726

Chickens were fed with diets containing 0 % (control), 0.5 %, and 1 % polyphenolic extract during the first four weeks of age. Data show mean ± SE ($n = 6$).

Table 3
Effect of different doses of dietary polyphenolic extract supplementation and storage period on breast muscle color of broiler chickens.

Parameters	Treatments			p-value		
	CON	PSPP_0.5	PSPP_1.0	p-value	Lin	Quad
Lightness	52.43 ± 1.47	54.04 ± 0.83	54.67 ± 0.63	0.322	0.138	0.709
Redness	4.88 ± 0.71	5.27 ± 0.63	5.95 ± 0.49	0.48	0.224	0.852
Yellowness	6.73 ± 2.75	6.74 ± 2.75	7.84 ± 3.20	0.231	0.136	0.384
Chroma	8.44 ± 0.59	8.60 ± 0.85	9.97 ± 0.45	0.219	0.11	0.457
Hue angle	55.00 ± 4.05	52.20 ± 2.29	53.01 ± 2.21	0.793	0.634	0.627

Chickens were fed with diets containing 0 % (control), 0.5 %, and 1 % polyphenolic extract during the first four weeks of age. Data show mean ± SE ($n = 6$). Means with different superscripts (a,b) within a row are significantly different ($p < 0.05$).

Lachnospiraceae and Oscillospiraceae.

In our datasets, models were built to assess the impact of the PSPP level of supplementation on microbial abundance (Fig. 8). The models showed significant associations essentially at the genus level. PSPP supplementation was predicted to decrease the abundance of specific

Table 4
Effect of different doses of dietary polyphenolic extract supplementation and storage period on physicochemical properties of breast muscle in broiler chickens.

Parameters	Treatment			p-value		
	CON	PSPP_0.5	PSPP_1.0	p-value	Lin	Quad
pH	5.78 ± 0.02ab	5.82 ± 0.03b	5.72 ± 0.02a	0.024	0.111	0.028
Cooking Loss	13.52 ± 1.51	14.79 ± 1.18	10.55 ± 1.07	0.083	0.14	0.095
TBARS	0.06 ± 0.00a	0.05 ± 0.00a	0.08 ± 0.01b	0.002	0.036	0.005
Shear Force	0.62 ± 0.07	0.64 ± 0.08	0.62 ± 0.11	0.973	0.995	0.817

Chickens were fed with diets containing 0 % (control), 0.5 %, and 1 % polyphenolic extract during the first four weeks of age. Data show mean ± SE ($n = 6$). Means with different superscripts (a,b) within a row are significantly different ($p < 0.05$).

microbial taxa, such as *Desulfosporosinus, Faecalicoccus, Anaerostipes,* and especially *Faecalibacterium*. Conversely, higher dietary levels of PSSP were predicted to increase the proportion of *Pseudoflavonifractor, Anaerotruncus, Murimonas, Kineothrix,* and *Anaerotignum*. Overall estimates of downregulated taxa appeared to be greater (in absolute value) than those of upregulated taxa. The results were found to be robust, with the prevalence of significantly modulated taxa consistently greater than 0.7, indicating that they were present in at least 70 % of the analyzed samples.

Discussion

The current study is a follow-up to our previously conducted trial in which we assessed the impacts of incorporating low levels of SPP on the health of broilers. The key difference between these trials lies in the nature and refinement of the supplement. Goel et al. (2021a) utilized steam-exploded wood chips, resulting in a carbohydrate-rich powder, primarily to assess corn substitution levels in poultry diets. In this study, we employed a significantly refined supplement. We introduced a secondary extraction step using 74 % ethanol at a 1/46 (w/v) ratio, at 90°C for 240 min. This process yielded a liquid supplement enriched in polyphenols. Furthermore, our current research extended beyond growth performance to investigate the impact of this polyphenol-rich supplement on meat quality and detailed microbiota-related variables, including microbial network analysis and microbial taxa modeling, which were not studied in our previous work.

Our results show that dietary polyphenols can influence feed utilization in broilers. Specifically, the birds that were fed PSPP at 1 % had consistently better FCR compared to the control group. Furthermore, feed intake was similar across all treatments, which is consistent with a previous report that polyphenol-rich grape seed supplementation in birds resulted in improved FCR without changing feed intake (Abu Hafsa, et al., 2018). It is possible that the improved FCR observed after polyphenol supplementation is due to their ability to protect the intestinal mucosa, which limits peristaltic activity in digestive disorders (Gagnière, et al., 2017; Abu Hafsa and Ibrahim, 2018). For example, tannins may reduce intestinal movement, leading to better absorption of nutrients and improved feed efficiency (Ismail, et al., 2003). However, we did not observe any noticeable effects on growth, organ indexes, blood parameters, or meat quality. In the context of evaluating the potential effects of novel feed additives in broilers, it is crucial to ensure that basic health parameters remain within a comparable range to the control treatment. For instance, the supplementation of probiotics in broiler chickens was found to enhance the performance of the birds without inducing significant alterations in blood biochemical parameters such as total protein over 6 weeks (Alkhalf, et al., 2010). Comparable findings were also reported by Cho, et al. (2014), who observed similar triglyceride levels between control and phytogenic feed additive supplemented groups after a 35-day trial. As the birds were reared under recommended environmental conditions and not subjected to health challenges, changes in blood biochemical parameters may not necessarily be anticipated. Furthermore, it has been demonstrated that organ indices are also less likely to be affected by the supplementation of feed additives at low doses or when the nutritional values of the experimental diets are not substantially different. For instance, no significant changes were observed between the broiler chicken groups fed increasing levels of infertile eggs below 4 % as a replacement of fish meal in the control diet (Choi, et al., 2021). Similarly, others have reported no significant effects of phytobiotics supplementation with a maximum dose of 4 % on vital organs, namely the spleen, liver, kidney, lungs, and heart in broilers (Sultana, et al., 2023). As anticipated, our results aligned with previous findings and suggested that polyphenol supplementation may marginally enhance FCR without any adverse effects. Further, the breast meat quality of the broilers was marginally affected by PSPP supplementation. Indeed, PSPP supplementation did not affect their proximate composition, color values, cooking losses, and shear force. This resonates with a previous study, in which supplementation of polyphenols extracted from olive oil industry waste up to 165.0g/kg did not affect these parameters (Branciari, et al., 2017). Overall, besides subtle changes in muscle pH and TBARS, the current dietary treatments did not provide evidence for its use as a meat quality enhancer.

In the current trial, dietary PSPP modulated the microbial composition but not the diversity in the cecum of broilers. Microbial composition refers to the presence of specific types of microorganisms in a given microbial community (Kim, et al., 2017). It focuses on identifying and quantifying the different microbial taxa within a sample. On the other side, microbial diversity provides a more holistic understanding of the complexity and richness of the microbial population (Hughes, et al., 2001). This includes not only the type of microorganism but also their relative abundances as well as their variety within the community. While alpha diversity indices (richness and evenness) did not significantly vary across dietary treatments, the Good's coverage was above 0.99, indicating that the sequencing depth covered 99 % of all OTUs at a 97 % similarity. Likewise, beta diversity analyses (PERMANOVA, PERMDISP) and NMDS did not reveal any diet-induced microbial community diversity changes. Although diet is generally considered one of the prime factors for microbial diversity (Chen, et al., 2014; Xu and Knight, 2015; Dahl, et al., 2020), the birds in this trial were fed similar levels of macronutrients, including protein and fat, and were reared under identical conditions. Thus, these factors may have explained the similarities in the cecal microbial diversity observed. Previous studies on the effects of other polyphenol-rich supplements on broiler cecal microbiota mainly reported changes in microbial composition but not diversity (Li, et al., 2020; Mahmoudi, et al., 2022). Therefore, phenolic compounds may be more likely to modify microbial composition rather than diversity. Furthermore, changes in microbial diversity may require a longer timescale or the action of a potent component, such as antibiotics, to occur quickly (Elokil, et al., 2020).

A dynamic balance between beneficial and harmful bacteria should be maintained in the GIT of healthy birds to maintain homeostasis (Dittoe, et al., 2018; Grond, et al., 2018; Bodawatta, et al., 2022). Our results showed that Firmicutes and Bacteroidetes were the most dominant phyla across all treatments. Our previous and other studies have consistently reported similar trends (Xiao, et al., 2017; Goel, et al., 2021b, 2022a, 2022c; Goel, et al., 2023). Bacteroidetes are gram-negative bacteria that provide energy to their host by fermenting originally indigestible polysaccharides (Thomas, et al., 2011; Johnson, et al., 2017). On the other hand, Firmicutes are gram-positive bacteria and the main producers of butyrate (Magne, et al., 2020). In the current trial, birds fed diets supplemented with PSPP had higher proportions of Bacteroidetes compared to the control group, regardless of the level of

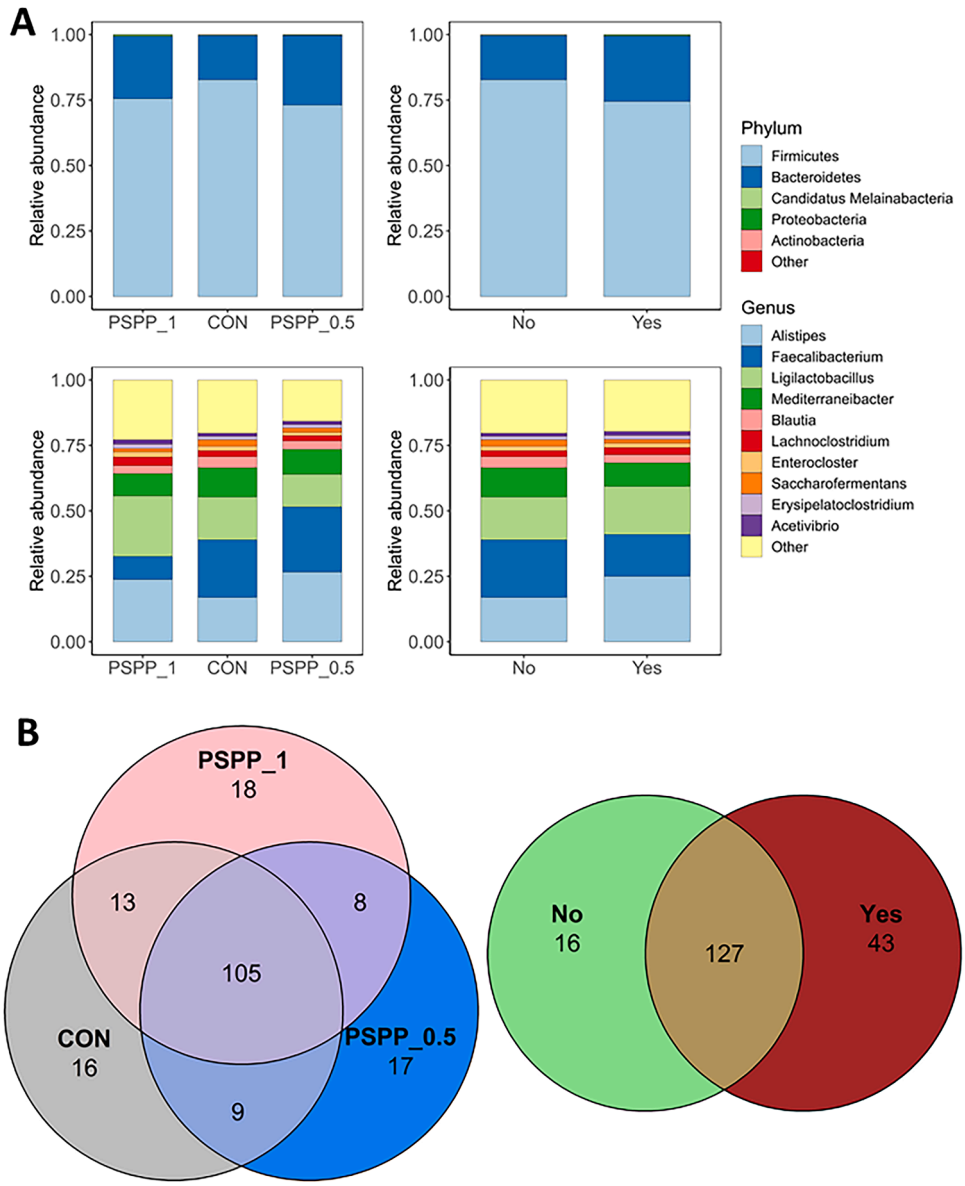

Fig. 2. Mean cecal taxonomic profiles composition associated with dietary treatments or PSPP supplementation in broilers (A) and Venn diagrams at the species level of broiler's cecal microbiota (B). For the stacked bar plots, the top 5 phyla and top 10 genus are presented. In both figures, No (CON only) and Yes (PSPP_0.5 and PSPP_1 combined) refer to the supplementation or not of PSPP. In the Venn diagrams, the circles show the cecal microbiomes as determined by only those species that were ubiquitous for the dietary treatments or the PSPP supplementation. Bacterial species within overlapping areas were common to the corresponding treatments. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

inclusion. Polyphenol ingestion in chickens affected the Firmicutes/Bacteroidetes ratio by mainly increasing the relative abundance of Bacteroidetes (Iqbal, et al., 2020). For example, a diet containing polyphenol-rich mulberry byproducts was found to lead to higher Bacteroidetes proportions in both cloacal and cecal microbial communities (Chen, et al., 2019). In broilers, healthy individuals tended to have similar proportions of Firmicutes and Bacteroidetes (Clavijo, et al., 2018). On the other hand, an increase in the Firmicutes/Bacteroidetes ratio was associated with a disruption in fatty acid metabolism leading to obesity (Yadav, et al., 2021). Therefore, the alterations in microbial phylum induced by PSPP supplementation may be associated with reduced production of volatile fatty acids in the cecum of the birds. However, further investigations are necessary to draw definitive conclusions.

Analysis of cecal samples at the genus level revealed an increase in *Alistipes* abundance. *Alistipes* are rod-shaped gram-negative anaerobic bacteria commonly found in the healthy human gut (Parker, et al., 2020). There is also growing evidence implying that *Alistipes* represent the majority of the genus in the cecum of broilers (Li, et al., 2022). Dietary supplements can modulate their population. Indeed, dietary rhamnolipids, for example, enhanced immunity and improved intestinal barrier functions in broilers, which was correlated with an increased proportion of *Alistipes* (Zhang, et al., 2022). The same study also suggested that *Alistipes* may have a protective role in inflammatory bowel disease, as a negative correlation was found between their relative abundance and intestinal mucosal concentrations of pro-inflammatory interleukins. While correlation does not always imply causation, it can be inferred that the regulatory effect of dietary PSPP on the *Alistipes* cecal population is likely to be beneficial for the host.

The LEfSe analysis of the microbiota community in the birds revealed the presence of *Blautia schinkii*, which was a biomarker of the PSPP treatment, particularly the PSPP_1 treatment. *Blautia schinkii* is an anaerobic bacterial species with probiotic characteristics that are found in the feces and intestines of mammals (Liu, et al., 2021). The species,

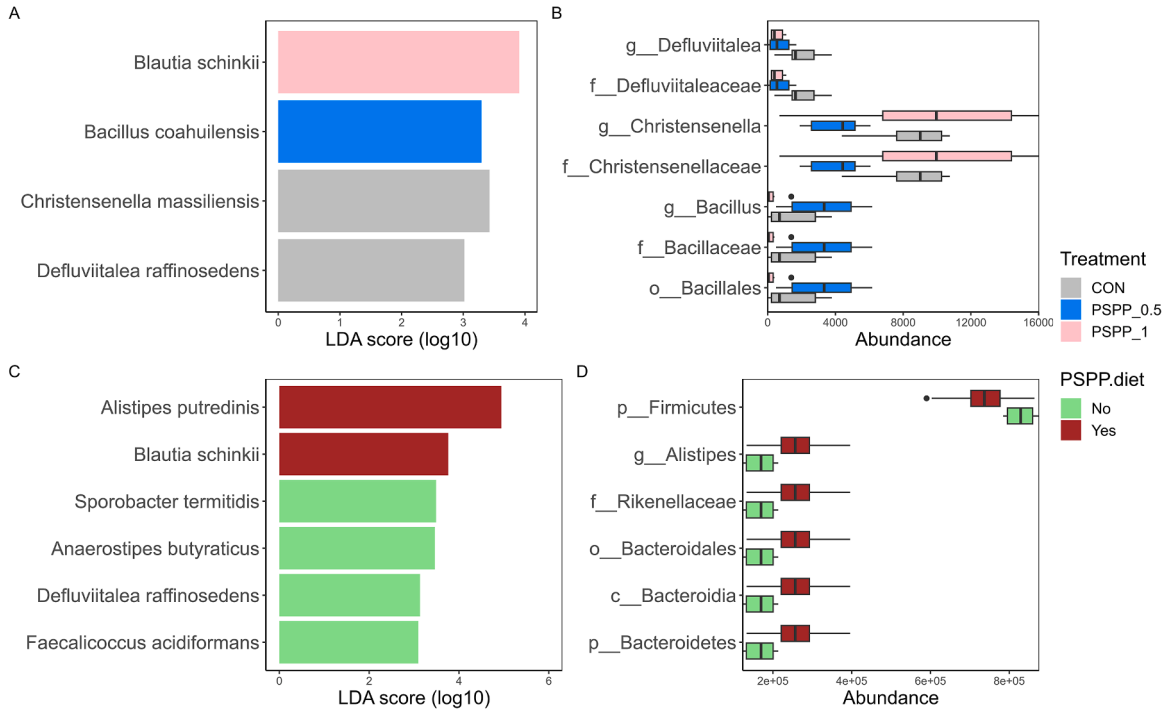

Fig. 3. Linear discriminant analysis combined effect size measurements (LEfSe) analysis of cecal microbiota in broilers based on the dietary treatments (A and B) or PSPP supplementation (C and D). Visualizations were presented using boxplots or bar plots according to the taxonomic ranks. The taxa represented are significantly different ($p < 0.05$; | LDA effect size | > 2.0) and play an important role in their respective treatments. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

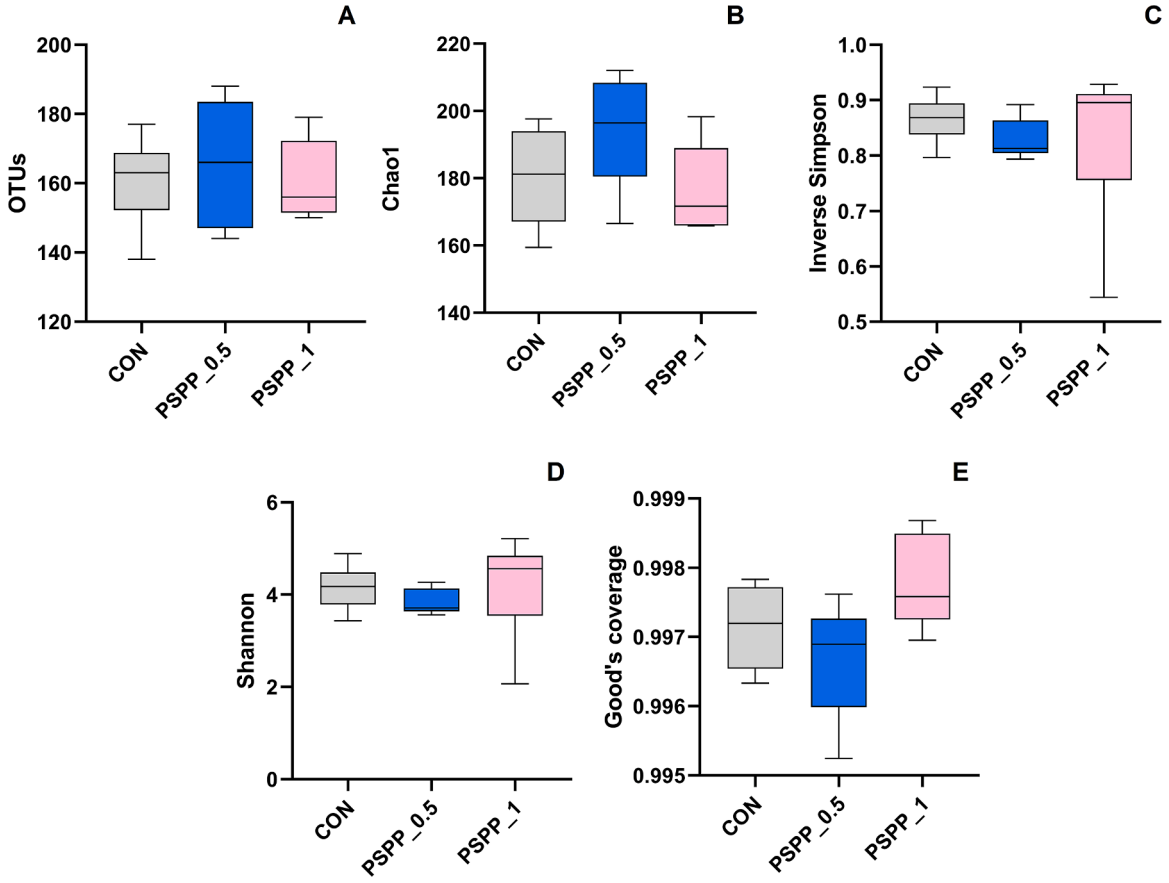

Fig. 4. Boxplots presenting the distribution of alpha diversity indices of broiler's cecal samples. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

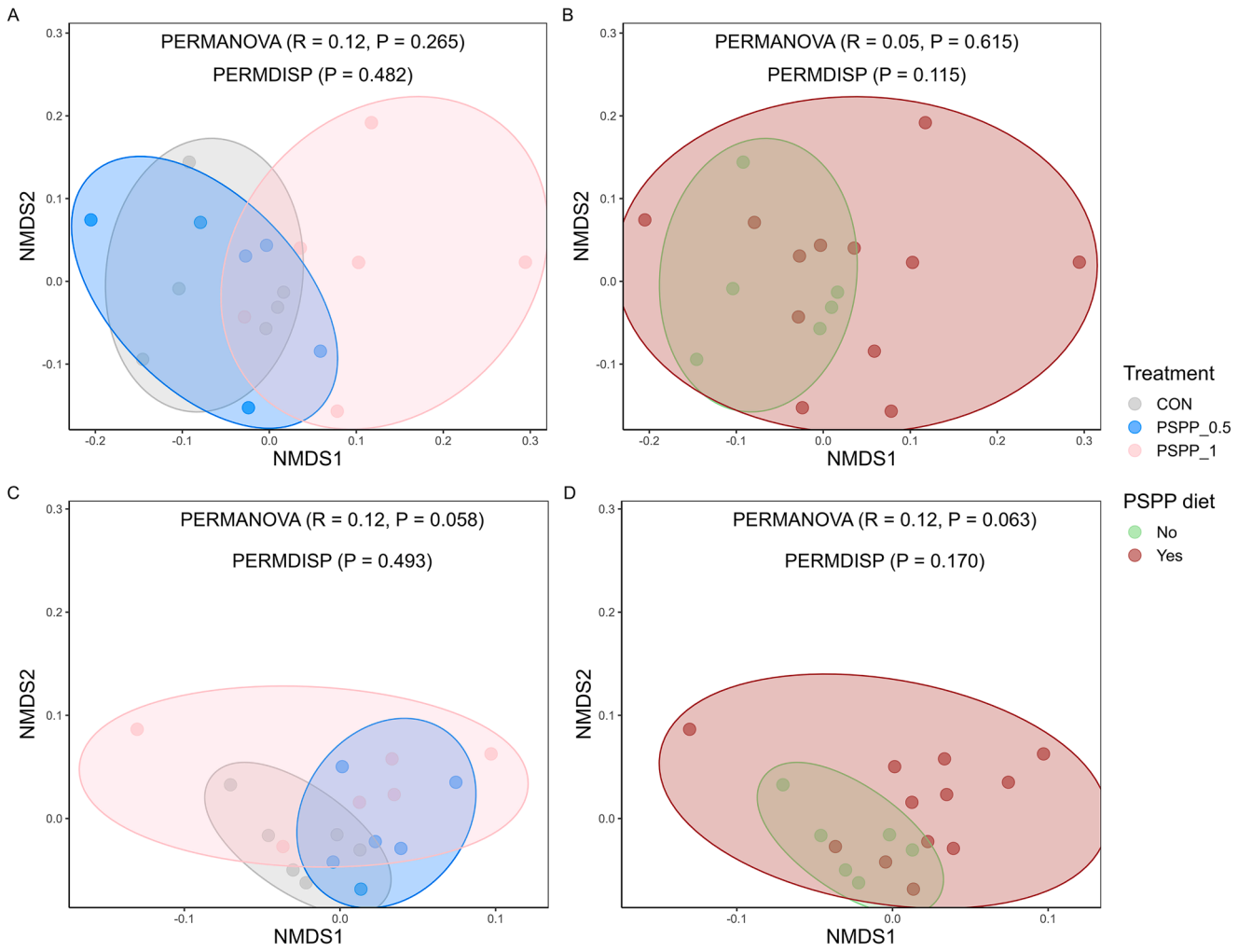

Fig. 5. Non-metric multidimensional scaling (NMDS) of broiler's cecal microbiota associated with dietary treatments of PSPP supplementation. NMDS was performed based on the measurement of the unweighted (A and B) and weighted (C and D) Unifrac distances. Colored dots indicate samples and were colored with regard to treatments. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

isolated from cattle rumen, is known for its prominent carbohydrate metabolism ability (Sabino, et al., 2019) and possesses a gene cluster that encodes sactipeptide (Liu, et al., 2021), a class of bacteriocins with antibacterial properties against pathogenic microorganisms like *Listeria monocytogenes, Clostridium perfringens*, and *Escherichia coli* (Chen, et al., 2021). This interaction between polyphenol-rich compounds and gut microbiota was previously studied, with wine polyphenols found to promote the growth of *Bifidobacteria* and *Lactobacilli* while inhibiting *Clostridia* (Requena, et al., 2010). Hence, the prevalence of *Blautia schinkii* in birds fed PSPP may promote the development of immunity and disease resistance.

Human microbiology has recently focused on identifying the "core microbiome," which refers to species found in every individual (Sekelja, et al., 2011). This concept has now been applied to livestock animals (Videnska, et al., 2014; Xue, et al., 2018). Unlike these trends, a higher number of bacterial species are ubiquitous in the PSPP treatment. Previous studies demonstrated that polyphenolic compounds can affect the microbial composition of the gut and signaling pathways (Wan, et al., 2021; Xie, et al., 2023). In particular, polyphenols have a strong antioxidant capacity (Khanizadeh, et al., 2008; Faller, et al., 2010), protecting the epithelial wall and mucosa while also influencing the gastrointestinal environment (Graziani, et al., 2005; Wang, et al., 2022). Indeed, phenolic compounds foster the growth of microbial communities through both prebiotic effects and antimicrobial actions (Viveros, et al., 2011; De Nardi, et al., 2016). Based on these facts, the suppression of pathogenic bacteria concomitant with the support of beneficial bacteria through prebiotic effects may have contributed to the higher number of unique taxa belonging to the PSPP treatment.

The results of the current study also highlight that dietary inclusion of PSPP resulted in the specialization of the cecal microbiome of the birds. PSPP supplementation led to the adaptation of a few taxa in carrying out specific metabolic activities and interacting with their host environment. These findings were corroborated by the results of the Spearman heatmap correlation, the co-occurrence network analysis and the microbial taxa modeling. A positive correlation between the cecal population of *Gemmiger formicilis* and the fat content of broiler meat was detected. Interestingly, this was the only significant correlation detected in our study. *Gemmiger formicilis* is a gram-negative bacterial species observed in the caecum of poultry and is well known for its ability to produce butyric acid and lactic acid from glucose (Gossling, et al., 1975; Salanitro, et al., 1976). Previous studies in humans also showed increased populations of this bacterium in obese individuals following a Westernized diet. These subjects had both a higher body mass index and a significantly disturbed serum lipid profile (Hur, et al., 2022). While the exact relationship between *Gemmiger formicilis* and lipid deposition is not currently understood, the available evidence suggests that further investigation is warranted.

Microbial co-occurrence networks can be constructed to explore the connections within microbial communities in a specific environment (Williams, et al., 2014; Connor, et al., 2017). Indeed, microbial

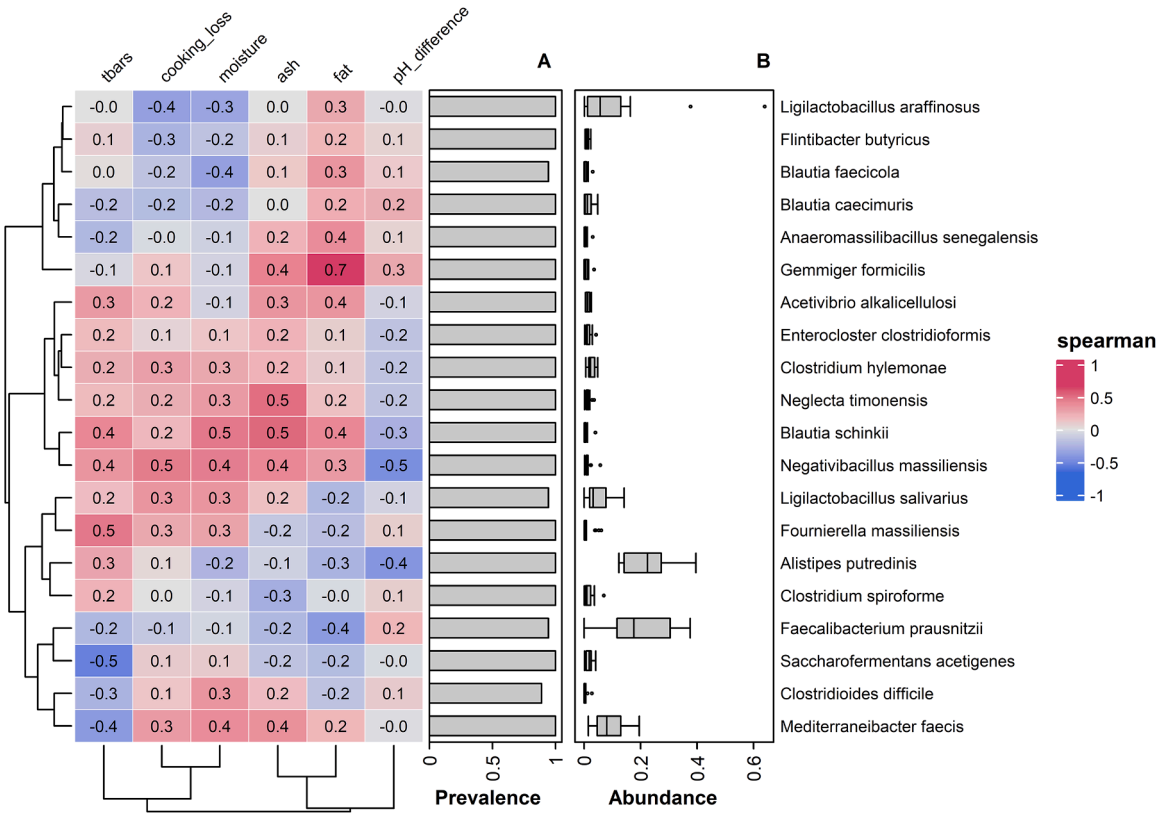

Fig. 6. Spearman correlation heatmap between the top 20 cecal microbial species and meat quality parameters. A red color indicates a positive correlation, a blue color indicates a negative correlation and a grey color indicates no correlation. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

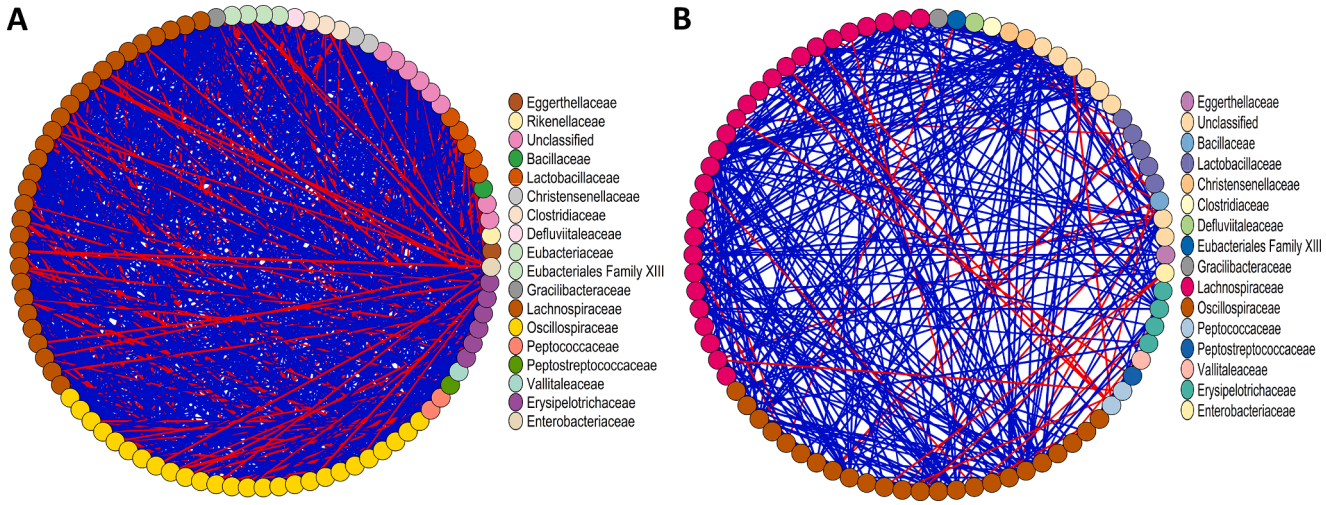

Fig. 7. Microbial co-occurrence network of broilers' cecal microbiota at the family level. The network describes microbial interactions and patterns of samples belonging to the control (A) or PSPP-supplemented (B) groups. The networks were built based on Spearman's rank correlation. The significance was set at $p < 0.05$, with a threshold of the absolute value of the correlation greater than 0.6. Vertexes (circles) represent microbial species while edges (segments) describe correlations between microbes. Positive correlations are in blue while negative correlations are in red. The birds were provided with 0, 0.5, or 1 % dietary polyphenol-rich extract from shredded, steam-exploded pine particles (PSPP) for 28 days.

co-occurrence networks can be used to predict hub species and potential species interactions (Ma, et al., 2020a; Ji, et al., 2022). In this study, obvious differences in network structure were apparent between the control samples and PSPP-supplemented diets. PSPP supplementation not only reduced the number of co-occurring OTUs but also drastically limited the interactions within the OTUs. Interestingly, bacteria belonging to the Rikenellaceae and Eubacteriaceae were completely absent from the PSPP-based network. Also, the proportion of negative correlations in the network was substantially reduced. In microbial network analysis, competition between microbial species is indicated by negative correlations, while symbiosis is portrayed by positive correlations (Barberan, et al., 2012; Ncho, et al., 2023). The current result suggests that as PSPP is added to the diet, microbes become more specialized in their functions by limiting their interactions with each

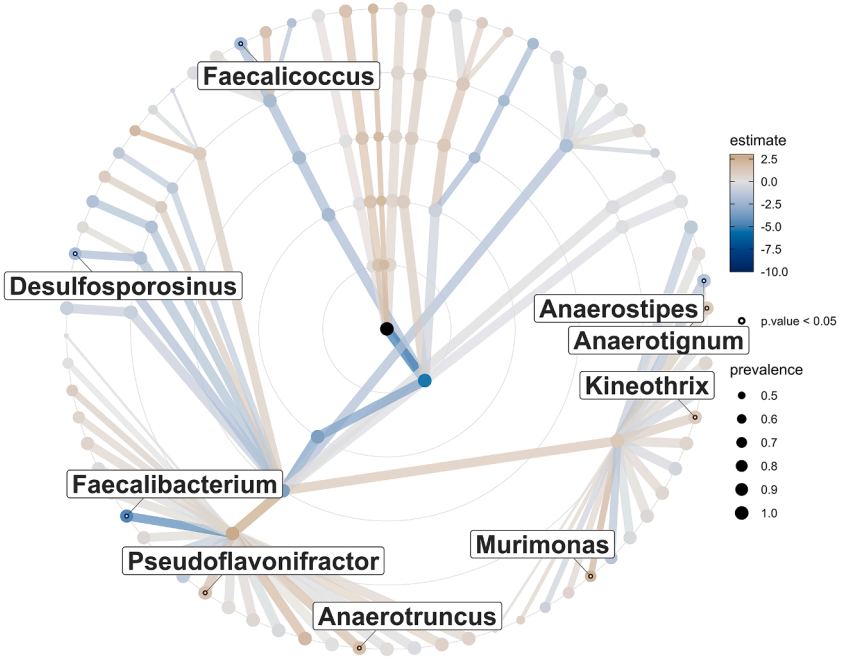

Fig. 8. Tree showing associations between microbial taxa relative abundances and dietary PSPP inclusion levels. Taxon models are organized by rank, from the central root node from the phyla around the center to the Genera in the outermost ring. Positive estimates are displayed in brown while negative estimates are in blue. Only taxa significantly affected by increasing levels of PSPP were annotated in the tree.

other, which can lead to the suppression of certain taxa in the network. Microbial communities were interconnected through metabolic connections, ranging from small scales involving just a couple of symbiotic organisms to larger scales encompassing the entire global ecosystem with complex trophic relationships (Ponomarova, et al., 2015). Numerous research studies have demonstrated that the exchange of metabolites is a viable strategy for the collective success of these groups (Morris, et al., 2013; McNally, et al., 2014). These metabolic interactions often play a crucial role in enhancing emergent capabilities at the community level, such as facilitating biodegradation, promoting faster growth, or increasing virulence, due to the efficient division of labor among community members (Lykidis, et al., 2011; Pande, et al., 2014). However, under significant changes in the nutrient composition of the environment, species with limited metabolic pathways may be prompted to share metabolites, leading to competition (Brown, et al., 2009). Previous evidence also suggests that it is not uncommon for microbial cells to secrete numerous enzymes, scavenging molecules, and signals that can inhibit the growth of other cells in their surroundings (Brown, et al., 2009; Foster, et al., 2012). Hence, the modified interaction within the species detected in the PSPP-fed group may explain not only the reduction in the proportion of Firmicutes but also the deletion of the families Rikenellaceae and Eubacteriaceae in the network. These two bacterial taxa were found to be significantly increased in the fecal microbiota of quails-fed silkworm meal, which is known to be rich in protein and lipids (Dalle Zotte, et al., 2021). In addition, polyphenols were known to regulate lipid metabolism through the microbiome (Ma, et al., 2020b), and might inhibit the absorption of dietary fats in the digestive tract or enhance fat oxidation. Thus, PSPP polyphenols may explain the absence of these bacterial families in the networks.

In this study, the microbial taxa modeling approach was used due to its versatility and accuracy in modeling noisy and non-normal data from microbiome data (Mallick, et al., 2021). Microbial taxa abundance modeling revealed that only genus-level taxa were significantly associated with increasing levels of PSPP supplementation. Negative estimates were found to be greater in absolute value compared to positive estimates, indicating that higher levels of PSPP tend to reduce bacterial taxa abundance rather than increase it. Specifically, a significant reduction in the *Faecalibacterium* genera was observed as PSPP supplementation levels increased. Interestingly, *Faecalibacterium* was found to be the second most abundant genera across all samples included in our dataset. *Faecalibacterium* are microbial taxa playing a pivotal role in maintaining gut health mainly via the production of energy to the colonic mucosa (Miquel, et al., 2013) and regulating gene expression, inflammation, differentiation, and apoptosis in host cells (Rabiei, et al., 2019; Auger, et al., 2022). For instance, dietary supplementation of a vitamin cocktail in broilers was found to substantially increase the abundance of this bacterial taxon (Luo, et al., 2013), which was accompanied by noticeable improvements in growth and survivability. Therefore, reducing the abundance of bacteria belonging to the *Faecalibacterium* genus, one of the primary butyrate producers, in response to increased PSPP levels is likely to be detrimental. Indeed, the current findings suggest that while PSPP may have beneficial effects on the gut microbiota, maintaining supplementation levels within an optimal range is crucial to prevent dysbiosis.

Although the current trial allowed the assessment of the effects of PSPP in broilers, it is important to acknowledge the limitations associated with the study. One limitation may be attributed to the sample size used to assess meat quality traits. While previous studies focusing on microbiota analysis used a similar number of replicates, meat quality parameters may have benefited from a higher number of replicates. Indeed, in this trial, we could detect significant differences in parameters such as pH and TBARS; however, the majority of proximate composition, color, and physicochemical properties of the breast muscle did not display significant changes across treatments. Although there is no certainty that a higher number of replicates would have led to the identification of significant differences, this should also be taken into consideration while interpreting the strength of the correlation between meat quality traits and the major microbial species identified in cecal samples. Another limitation may reside in the fact that the breast muscles were excised and collected without the keel bone from the birds immediately after euthanasia. This process could have affected the development of postmortem changes in the muscle during the conversion to meat. However, this study did not analyze differences in slaughter methods but rather analyzed breast meat from chickens fed

under the same raising conditions and using the same slaughtering and analytical methods. Therefore, other variables were minimized and it is judged that they do not affect the purpose of this study, which is to evaluate the effect of feed. Nevertheless, it should also be noted that the quality characteristics measured in this study do not represent industry standards and therefore should be interpreted with caution.

Conclusion

In broilers, polyphenols have multiple health benefits and can be used to shape intestinal microbiomes when supplemented. Our findings provide evidence that PSPP could effectively modulate composition, but did not result in changes in the diversity of the cecal microbiome of chickens. Substantial increases in Bacteroidetes and *Alistipes* microbial taxa were linked to PSPP supplementation. Increasing dietary levels of PSPP led to mainly reduction of specific genus-level taxa with *Faecalibacterium* being the most affected. In addition, microbial co-occurrence networks were drastically different between the control and the PSPP-supplemented groups with lower microbial interaction being the most significant changes. Finally, PSPP was found to significantly reduce the feed conversion rate of the birds until the third week of the trial but had only a marginal effect on meat quality. These findings suggest that further studies are warranted to fully understand the complex relationship between polyphenols and the avian gut ecosystem.

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CRediT authorship contribution statement

Chris Major Ncho: Writing – original draft, Formal analysis, Investigation, Data curation, Methodology. **Vaishali Gupta:** Writing – original draft, Formal analysis, Investigation, Data curation, Methodology. **Akshat Goel:** Data curation, Methodology. **Chae-Mi Jeong:** Data curation, Methodology. **Ji-Young Jung:** Methodology. **Si-Young Ha:** Methodology. **Jeong-Uk Eom:** Methodology. **Han-Sul Yang:** Methodology. **Jae-Kyung Yang:** Conceptualization, Resources. **Yang-Ho Choi:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The sequencing data of cecal microbiota have been deposited into the Sequence Read Archive (SRA) database of NCBI and is available on BioProject Accession Number PRJNA952334.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105088.

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