RESEARCH ARTICLE

J Anim Sci Technol 2023;65(5):971-988
https://doi.org/10.5187/jast.2023.e15

JAST
Journal of Animal Science and Technology
pISSN 2672-0191 eISSN 2055-0391

Dietary supplementation of solubles from shredded, steam-exploded pine particles modulates cecal microbiome composition in broiler chickens

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Received: Oct 7, 2022
Revised: Jan 5, 2023
Accepted: Feb 1, 2023

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Abstract

This study evaluated the effects of supplementing solubles from shredded, steam-exploded pine particles (SSPP) on growth performances, plasma biochemicals, and microbial composition in broilers. The birds were reared for 28 days and fed basal diets with or without the inclusion of SSPP from 8 days old. There were a total of three dietary treatments supplemented with 0% (0% SSPP), 0.1% (0.1% SSPP) and 0.4% (0.4% SSPP) SSPP in basal diets. Supplementation of SSPP did not significantly affect growth or plasma biochemicals, but there was a clear indication of diet-induced microbial shifts. Beta-diversity analysis revealed SSPP supplementation-related clustering (ANOSIM: $r = 0.31$, $p < 0.01$), with an overall lower (PERMDISP: $p < 0.05$) individual dispersion in comparison to the control group. In addition, the proportions of the *Bacteroides* were increased, and the relative abundances of the families Vallitaleaceae, Defluviitaleaceae, Clostridiaceae, and the genera *Butyricicoccus* and *Anaerofilum* ($p < 0.05$) were significantly higher in the 0.4% SSPP group than in the control group. Furthermore, the linear discriminant analysis effect size (LEfSe) also showed that beneficial bacteria such as *Ruminococcus albus* and *Butyricicoccus pullicaecorum* were identified as microbial biomarkers of dietary SSPP inclusion ($p < 0.05$; | LDA effect size | > 2.0). Finally, network analysis showed that strong positive correlations were established among microbial species belonging to the class *Clostridia*, whereas *Erysipelotrichia* and *Bacteroidia* were mostly negatively correlated with *Clostridia*. Taken together, the results suggested that SSPP supplementation modulates the cecal microbial composition of broilers toward a "healthier" profile.

Keywords: Microbiome, Broiler, Solubles from shredded, Steam-exploded pine particles, Cecum, Growth

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Competing interests
No potential conflict of interest relevant to this article was reported.

Funding sources
This work was supported by the Forest Science Technology R&D Program (2020193C10-2022-BA01) provided by the Korea Forest Service (Korea Forestry Promotion Institute).

Acknowledgements
Not applicable.

Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Yang JK, Choi YH.
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Writing - review & editing: Ncho CM, Goel A, Gupta V, Jeong CM, Jung JY, Ha SY, Yang JK, Choi YH.

Ethics approval and consent to participate
All the experimental procedures for this study were approved by the Institutional Animal Care and Use Committee of Gyeongsang National University (GNU-200916-C0057).

INTRODUCTION

The gut microbiota plays a vital role in the physiological, metabolic, and nutritional uptake functions of its host [1]. The host usually accommodates and establishes a symbiotic relationship with the different bacterial species populating its digestive tract [2]. Further, the gut microbiota associates itself with the epithelial membrane of enterocytes to form a protective layer against pathogens [3]. Therefore, a clear definition of a "healthy" microbiota is important to help establish approaches for the modulation of its composition, which can result in improved host health and performance. In chickens, the distal part of the intestinal tract, namely the cecum and rectum, is inhabited by a greater number of microbial species than the proximal part, such as the crop, proventriculus, and gizzard [4].

A growing body of evidence suggests that diet is a major factor affecting the microbiome [5,6]. In fact, the non-digestible and unabsorbed portions of chicks' diets can serve as available fuel for microbial growth in the gut [7]. Insoluble fibers in general are used by microbes to produce short-chain fatty acids (SCFA) via fermentation [8]. These SCFA generated are widely considered to be having health-promoting effects. For instance, studies revealed that after purification, lignin and mannan oligosaccharides could be used as potential antibiotics and growth promoters in broiler chickens [9,10]. Besides, another dietary strategy used to positively modify the intestinal bacterial population resides in the use of phenolic compounds. Polyphenols are thought to be transformed by microbes into derived metabolites which can inhibit pathogens and activate the proliferation of the same beneficial microbes [11].

Plant-derived products have been extensively evaluated as feed additives, due to their potential effects on growth and health in livestock [12–14]. Indeed, researchers have been conducting trials using non-conventional dietary ingredients in poultry farming. As a result, novel products such as pine particles have been tested as feed additives in chickens under thermoneutral and heat stress conditions [15,16]. Besides, it was highlighted that the submission of dietary fiber-rich products to different extraction techniques could enhance the bioavailability of nutrients [17]. For instance, hot water extraction was used to transform spent mushroom substrate into a valuable feed additive in dairy cows [18]. Similarly, solubles from shredded, steam-exploded pine particles (SSPP) can be obtained after submitting pine particles to hot water extraction. The resulting SSPP is primarily composed of phenolic compounds, lignin, and carbohydrates.

In this study, we attempted to thoroughly assess the cecal microbiota and growth performance in broilers supplemented with SSPP. Considering the chemical composition of SSPP, we hypothesized that its dietary inclusion in broiler diets can enhance beneficial bacterial growth, resulting in an overall "healthier" microbiota. For doing so, birds were fed SSPP-supplemented diets from 8 to 28 days old. Thereafter, parameters such as growth performances, organ indexes, plasma biochemicals, and cecal microbiota characteristics (diversity and composition) were evaluated.

MATERIALS AND METHODS

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

Preparation of soluble from shredded, steam-exploded pine particles (SSPP)

SSPP was prepared via the explosion of pinewood chips (2 cm × 2 cm × 0.5 cm) with steamed water at 200°C for 11.5 min. The first stage resulting particles were used in our previously published studies [15,16]. The current SSPP used in this trial was obtained after mixing particles with water

https://doi.org/10.5187/jast.2023.e15

(ratio of 46:100 v/w) following extraction at 80°C for 213 min. The extract was then filtered through a Whatman filter paper, grade 2 (Z177601, Sigma-Aldrich, Seoul, Korea), and stored at 4°C until use. The chemical composition of SSPP obtained was the following: 4.9% phenolic compounds, 9.2% acid-insoluble lignin, and 75.2% carbohydrates (Table 1).

Experimental setup and birds housing

A total of 323 unsexed day-old Ross 308 broiler chicks were obtained from a commercial hatchery. Following standard rearing conditions, chicks were raised in a room containing H-type battery cage assemblies with 3 tiers and 7 cages per tier. The room environmental condition was set at 34 ± 1°C and 50% relative humidity for the first 3 days and then the temperature was decreased gradually to reach 22 ± 1°C on day 28. During the first seven days, the birds were fed ad libitum with a commercial starter diet (Nonghyup Feed, Seoul, Korea) in crumbled form. From day 8, chicks (n = 216) were allocated into three different treatment groups having similar body weights. The three dietary treatments were supplemented with 0% (0% SSPP), 0.1% (0.1% SSPP) and 0.4% (0.4% SSPP) SSPP in basal diets. The composition of the basal diets is shown in Table 2. During the whole trial, feed and water were provided ad libitium, and lightning conditions were 23 h of light and 1 h of dark. Each treatment had 12 cages containing 6 birds. The length, width, and height of the cages were 90 cm × 70 cm × 45 cm respectively. Average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were calculated based on weekly body weight and feed intake.

Blood, tissue sampling, and plasma biochemical parameters analysis

On day 28, at the end of the trial, 8 birds from each treatment were randomly selected and euthanized using CO_2, thereafter, blood and organ were sampled. Blood was drawn from a heart puncture and then collected into heparinized vacuum containers (#367874, BD, Franklin Lakes, NJ, USA). Plasma was obtained by centrifuging blood samples at 2,000×g for 10 min at 4°C, and was stored at −20°C for subsequent analysis. The duodenum, jejunum, ileum, liver, and spleen were sampled and weighed. Absolute and relative weights were calculated as organ indexes. In addition, the length of the duodenum, jejunum, ileum, and ceca were measured before being snap-frozen in liquid N_2 and stored at −80°C for further analysis.. Plasma metabolite concentrations were measured according to the manufacturer guide using a VetTest Chemistry Analyzer (IDEXX, Westbrook, ME, USA) with a dry-slide technology [19].

Table 1. The composition of solubles from shredded, steam-exploded pine particles (SSPP)

Composition	mg/mL	%
Total solid	216.7 ± 4.3	100
Ash	2.6 ± 0.1	1.2
Acid insoluble lignin	19.9 ± 0.6	9.2
Total phenolic compound	10.6 ± 0.3	4.9
Carbohydrate		
Glucose	19.5 ± 0.9	9.0
Arabinose	5.5 ± 0.6	2.5
Xylose	23.9 ± 0.7	11.0
Galactose	88.7 ± 2.1	40.9
Mannose	25.5 ± 0.6	11.8
Others		9.4

Table 2. The composition and nutrient levels of the basal diets[1)]

	Starter (0–7 days)	Grower (8–21 days)	Finisher (22–28 days)
Ingredients (%)	100	100	100
Corn grain	38.97	45.87	39.68
Wheat grain	15	15	25
Soybean meal (42.6% CP)	32	25.6	20.6
Corn gluten	3	2.64	3
Meat & bone meal	2	2	2.5
Animal fat	4	3.88	4.54
Salt	0.25	0.25	0.25
Tricalcium phosphate	1.3	1.04	0.86
Limestone	1.26	1.22	1.26
Sodium bicarbonate	0	0.02	0
L-Threonine	0.12	0.16	0.16
Lysine	1.23	1.44	1.32
DL-Methionine	0.33	0.34	0.29
Choline chloride (50.0%)	0.03	0.03	0.03
Premix[2)]	0.2	0.2	0.2
Phytase	0.05	0.05	0.05
Feed additive	0.25	0.25	0.25
Anti-coccidia	0.01	0.01	0.01
Calculated nutrients			
Crude protein (%)	23	20.5	19.5
Crude fat (%)	6.31	6.36	6.9
Crude fiber (%)	3.01	2.8	2.68
Crude ash (%)	5.99	5.34	5.02
Calcium (%)	1.01	0.9	0.86
Available phosphorus (%)	0.6	0.53	0.49
Digestible lysine (%)	1.43	1.24	1.09
Digestible methionine + cystine (%)	1.07	0.95	0.86
Copper (ppm)	82.21	81.04	80.78
Zinc (ppm)	100.27	96.63	97.33
Metabolizable energy (kcal/kg)	3,050	3,150	3,200

[1)]The feeds were purchased from Nonghuyp Feed (Seoul, Korea), and their compositions were provided by the company.

[2)]Trace minerals and vitamins provided per kilogram of premix: vitamin A, 12,000,000 IU; vitamin D_3, 3,000,000 IU;vitamin E, 40,000 IU; vitamin K_3, 2,000 IU; vitamin B_1, 2,000 mg; vitamin B_2, 5,000 mg; vitamin B_6, 3,000 mg; vitamin B_{12}, 20 mg; niacin, 40,000 mg; pantothenic acid, 10,000 mg; folic acid, 1,000 mg; iron, 88,000 mg; copper, 72,600 mg; zinc, 60,000 mg; manganese, 66,000 mg; iodine, 990 mg; selenium, 220 mg; cobalt, 330 mg.

DNA isolation and next-generation sequencing

The DNeasyPowerSoil 135 Kit (Qiagen, Hilden, Germany) was used to conduct the total genomic DNA of cecal samples according to the manufacturer's protocol. The cecal samples were randomly selected from 8 birds per treatment. The DNA samples obtained were quantified using Quant-IT PicoGreen (Invitrogen, Waltham, MA, USA). Thereafter, the 16S metagenomic sequencing library was constructed for metagenomic estimation using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, San Diego, CA, USA). Illumina platform was used for sequencing the library (Macrogen, Seoul, Korea). The fastp program was then used

https://doi.org/10.5187/jast.2023.e15

to perform quality profiling, adapter trimming, and read filtering. Sequences within the range of 400 to 500 bp were used and paired-end reads were assembled into one sequence using FLASH (v1.2.11) software. The CD-HIT-EST program was used to determine the number of operational taxonomic units (OTUs) with a 97% sequence identity cutoff. BLAST+ (v2.9.0) program was then used to check taxonomic similarity against the reference database (NCBI 16S Microbial). Identical coverage of less than 85% was identified as not defined. QIIME software (v1.9) was used to evaluate the OTU abundance and taxonomic information of the microbes.

Ecological and statistical analyses

In the current study, the cage was considered as the experimental unit. The experiment was performed in a completely randomized design with diet as a single factor. Downstream data wrangling, analysis, and visualization were performed using different packages of the R software v.4.1.0 (R core Team, 2021). Variables were tested for the homoscedasticity and normality assumption with Levene's and Shapiro-Wilk's tests respectively [20]. Data following a normal distribution, namely, growth performances, organ indexes, and plasma biochemical parameters were analyzed via a one-way ANOVA, followed by a Tukey post-hoc test when a significant *p*-value ($p < 0.05$) was found.

Alpha and beta diversity metrics were calculated for the estimation of microbial community diversity. Indices such as OTUs, Chao1, Shannon, inverse Simpson, and Good's coverage were calculated to estimate alpha diversity. Non-parametric Kruskal-Wallis test followed by a pairwise Wilcoxon rank sum test (adjusted via the Benjamini-Hochberg method) was performed to assess alpha diversity indices and relative abundance between treatment groups. Concerning beta diversity analysis, Non-Metric Dimensional Scaling (NMDS) based on Bray Curtis distance matrix was used for visualizations [21]. Furthermore, the homogeneity of multivariate dispersion among treatment groups was tested by conducting a permutational multivariate analysis of dispersion (PERMDISP) [22]. Thereafter, analysis of similarities (ANOSIM) was used to evaluate the effects of SSPP supplementation on microbiota composition variability between samples based on beta diversity distance matrices [23].

Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was applied to determine the OTUs most likely to explain differences ($p < 0.05$; | LDA effect size | > 2.0) between control and SSPP-supplemented diets. In addition, to uncover internal interaction within microbial communities, a correlation network analysis was executed [24].

One way-ANOVA, Kruskal-Wallis, and pairwise Wilcoxon rank sum tests were conducted using base R functions. NDMS, PERMDISP, and ANOSIM were conducted with envfit, betadisper, and anosim functions of the "*vegan*" package. Finally, the "*igraph*", and "*circlize*" packages were used to draw the microbial community network and chord diagram while the LEfSe algorithm was launched from the "*mothur*" software [25] via the R command line.

RESULTS

Growth performances, organ indexes, and plasma biochemical parameters

Table 3 presents the effects of increasing the concentration of dietary SSPP supplementation on growth performance parameters. Supplemental SSPP did not significantly affect ADG, ADFI, and FCR, regardless of inclusion level. In addition, there was no impact of SSPP supplementation on absolute and relative organ weights (Table 4). Similarly, absolute and relative organ lengths were not significantly modified by the inclusion of different SSPP levels in the diet of broilers (Table 5). As depicted in Table 6, plasma metabolites did not present any changes in their concentration

Table 3. Effects of supplementing diets with solubles from shredded, steam-exploded pine particles (SSPP) on broilers' growth performances[1)]

Parameters	SSPP supplementation (%)[2)]			SEM	*p*-value
	0	0.1	0.4		
Initial body weight (g)	184.8	185.8	186.1	0.21	0.369
Final body weight (g)	1,635.4	1,680.7	1,660.5	10.1	0.182
Day 8–14					
ADG (g)	44.39	44.65	43.78	0.33	0.594
ADFI (g)	58.07	59.29	58.93	0.32	0.277
FCR	1.31	1.33	1.35	0.005	0.093
Day 14–21					
ADG (g)	70.31	70.73	71.19	0.53	0.803
ADFI (g)	98.87	100.57	101.23	0.61	0.273
FCR	1.41	1.42	1.42	0.007	0.627
Day 21–28					
ADG (g)	92.72	98.17	94.29	1.14	0.133
ADFI (g)	138.5	143.87	140.9	1.27	0.228
FCR	1.5	1.47	1.5	0.008	0.225

[1)]Values are presented as mean ± SEM (n = 12).

[2)]Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Table 4. Effects of supplementing diets with solubles from shredded, steam-exploded pine particles (SSPP) on broilers' organs weight[1)]

Parameters	SSPP supplementation (%)[2)]			SEM	*p*-value
	0	0.1	0.4		
Absolute weight (g)					
Duodenum	8.21	8.07	8.04	0.25	0.960
Jejunum	16.59	15.99	17.5	0.55	0.557
Ileum	12.67	11.57	13.24	0.41	0.229
Liver	45.72	49.04	49.25	1.02	0.299
Spleen	1.68	1.81	1.75	0.11	0.871
Relative weight (%)					
Duodenum	0.528	0.504	0.509	0.01	0.789
Jejunum	1.074	0.998	1.098	0.03	0.420
Ileum	0.821	0.723	0.834	0.02	0.129
Liver	2.951	3.041	3.136	0.06	0.484
Spleen	0.109	0.112	0.111	0.006	0.982

[1)]Values are presented as mean ± SEM (n = 8).

[2)]Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

regardless of the inclusion of SSPP in the diet or not.

Microbial diversity

The alpha diversity results including OTUs, Chao1, Shannon, inverse Simpson, and Good's coverage are presented in Fig. 1. Compared to the control group (0% SSPP) a decrease ($p < 0.05$)

https://doi.org/10.5187/jast.2023.e15

Table 5. Effects of supplementing diets with solubles from shredded, steam-exploded pine particles (SSPP) on broilers' organs length[1)]

Parameters	SSPP supplementation (%)[2)]			SEM	*p*-value
	0	0.1	0.4		
Absolute length (cm)					
Duodenum	27.3	26.9	26.4	4.58	0.723
Jejunum	63.1	61.8	60.8	1.48	0.819
Ileum	62.1	61.5	65.6	3.53	0.539
Cecum	15.8	15.4	16.2	0.28	0.498
Relative length (cm/kg)					
Duodenum	17.6	16.7	16.8	0.37	0.559
Jejunum	41	38.4	38.6	1.09	0.579
Ileum	40.1	38.3	41.6	1.01	0.406
Cecum	10.2	9.6	10.3	0.24	0.523

[1)]Values are presented as mean ± SEM (n = 8).

[2)]Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

Table 6. Effects of supplementing diets with solubles from shredded, steam-exploded pine particles (SSPP) on broilers' plasma biochemicals[1)]

Parameters	SSPP supplementation (%)[2)]			SEM	*p*-value
	0	0.1	0.4		
Glucose (mg/dL)	252.4	251.4	258.8	4.56	0.790
Total protein (g/dL)	2.9	2.8	2.8	0.07	0.530
Triglycerides (mg/dL)	25.6	26.5	25.8	1.76	0.978
Cholesterol (mg/dL)	129.6	133	130.6	3.04	0.905

[1)]Values are presented as mean ± SEM (n = 8).

[2)]Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

in species richness was observed with low supplementation levels (0.1% SSPP). On the other side, the birds belonging to the 0.4% SSPP group presented an overall increase ($p < 0.01$) in species richness when compared to their 0.1% SSPP counterpart, as indicated by observed OTUs and Chao1 indices. Furthermore, the similar pattern observed in both indices was indicating that the sequencing depth obtained was sufficient. In contrast, none of the other indices (Shannon, inverse Simpson, and Good's coverage) were showing statistical differences neither when SSPP was supplemented at 0.1% or 0.4%.

Beta diversity results are depicted in Fig. 2. In this trial, PERMDISP revealed that individual community variation was greater ($p < 0.05$) in the control group (0% SSPP), while lower variances were found in SSPP-supplemented groups (0.1% SSPP and 0.4% SSPP). In addition, the NMDS plot using the Bray-Curtis distance matrix showed SSPP supplementation-related clustering, with birds fed SSPP clustering closer to each other (Fig. 2B). Similarly, microbial community structures were significantly different ($r = 0.31$, $p < 0.01$) when all birds fed SSPP (0.1% SSPP and 0.4% SSPP) were compared to the control group based on ANOSIM.

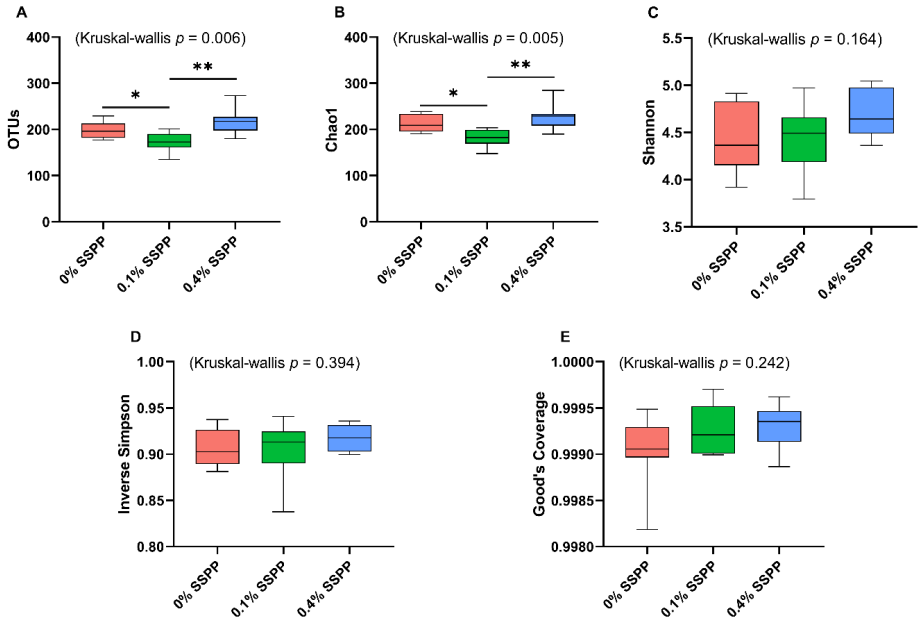

Fig. 1. Box plots representing the distribution of alpha diversity indices OTUs (A), Chao1 (B), Shannon (C), Inverse Simpson (D), and Good's coverage (E) of broilers' cecal samples. Data were analyzed using the Kruskal-Wallis test followed by the Wilcoxon rank sum test (adjusted by the Benjamini-Hochberg method). For each experimental group, the samples represent biological replicates (n = 8). Box plots indicate median values, quartiles as well as whiskers of the distribution of numerical data. Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP. * Indicates significance for the pairwise Wilcoxon rank sum test at $p < 0.05$. ** Indicates significance for the pairwise Wilcoxon rank sum test at $p < 0.01$. OTU, operational taxonomic units; SSPP, solubles from shredded, steam-exploded pine particles.

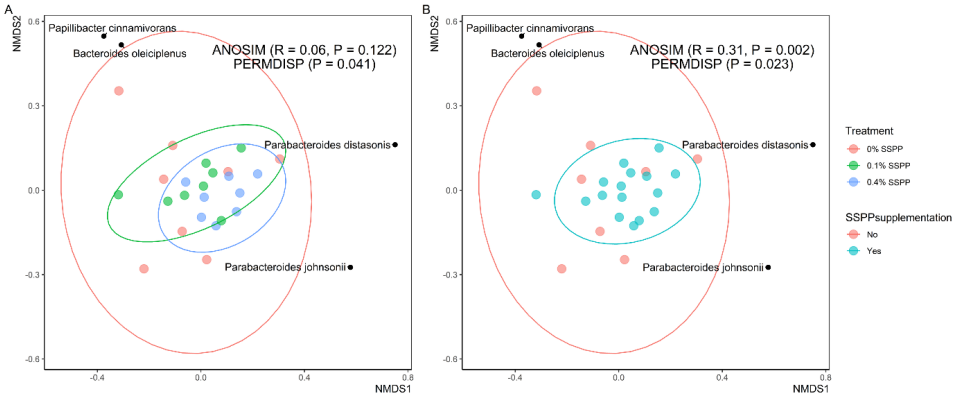

Fig. 2. Non-metric multidimensional scaling (NMDS) plot issued from Bray-Curtis dissimilarity matrix based on relative abundance data of cecal samples. Colored dots represent cecal samples (biological replicates), while black dots are microbial species. Ellipses indicate 95% confidence intervals of multivariate t-distribution around centroids of the groupings with treatments (A) and SSPP supplementation (B) as factors. PERMDISP ($p = 0.041$ (A); $p = 0.023$ (B)) revealed lower individual variation in the SSPP-supplemented group, and ANOSIM ($r = 0.31$, $p = 0.002$) indicated SSPP-related clustering. Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP. ANOSIM, analysis of similarities; SSPP, solubles from shredded, steam-exploded pine particles; PERMDISP, permutational multivariate analysis of dispersion.

https://doi.org/10.5187/jast.2023.e15

Microbial community composition

As illustrated in Fig. 3 the relative abundance of OTUs from birds' cecal microbiota was analyzed at different taxonomic levels. *Firmicutes* (56.6%) and *Bacteroidetes* (40.2%) together were representative of the majority of the microbiome from birds' cecal samples in the current trial (Fig. 3A). SSPP supplementation also led to an increase in *Bacteroidetes* relative abundance (from 35.5% to 42.7%) while concomitantly decreasing *Firmicutes* proportions (from 60.3% to 52.6%). Although minimal, there was a reduction in *Proteobacteria* relative abundance (from 3.8% to 1.2%) with the supplementation of SSPP. The dominant classes across treatment groups were *Clostridia* (45.4%), *Bacteroidia* (40.2%), and *Bacilli* (8.3%) (Fig. 3B). The birds supplemented with SSPP had a higher abundance of *Bacteroidia* and a lower abundance of *Bacilli* while the *Clostridia* abundance did not seem to be affected treatment-wise. More precisely, the relative abundances of *Bacteroidia* increased from 34.5% for 0% SSPP to 42.4% and 42.7% for 0.1% SSPP and 0.4% SSPP, respectively. On the other side, *Bacilli* proportions dropped from 11.7% to 7.4% and 5.9% for 0% SSPP, 0.1% SSPP and 0.4% SSPP, respectively. Order level microbiota analysis revealed that *Eubacteriales* (45.4%), *Bacteroidales* (40.2%), and *Lactobacilliales* (6.51%) which were unchanged, increased (from 35.5% to 42.7%), and decreased (from 8.5% to 5.1%) by SSPP supplementation, respectively, and accounted for the largest proportion of the microbial composition (Fig. 3C).

At lower taxonomic levels (family and genus), statistically, significant modifications were detected, with 0.4% SSPP group generally increasing abundance (Fig. 4). Indeed, *Vallitaleaceae, Defluviitaleaceae, and Clostridiaceae* had their proportion significantly increased ($p < 0.05$) by 0.4% SSPP when compared to either the 0.1% SSPP or 0% SSPP groups. In addition, the abundance of *Clostridiaceae* was also significantly higher at 0.1% SSPP in contrast to 0% SSPP. Similarly, the *Vallitalea*, *Hespellia*, *Defluviitalea*, *Butyricicoccus* and *Anaerofilum* genera were significantly more abundant in the 0.4% SSPP. On the other side, 0.1% SSPP led to a reduction of *Defluviitalea* when set against the 0% SSPP diet.

The identification of species potentially representing biomarkers of SSPP supplementation was conducted via LEfSe (Fig. 5). *Ruminococcus albus*, *Christensella timonensis*, *Acetivibrio alkalicellulosi*, *Butyricicoccus pulliceacorum*, and *Anaerotignum propionicum* were enriched ($p < 0.05$; | LDA effect size | > 2.0) in the cecum of birds fed SSPP supplemented diets Besides, LEfSe identified only *Anaerofilum agile* as a biomarker of the basal diet.

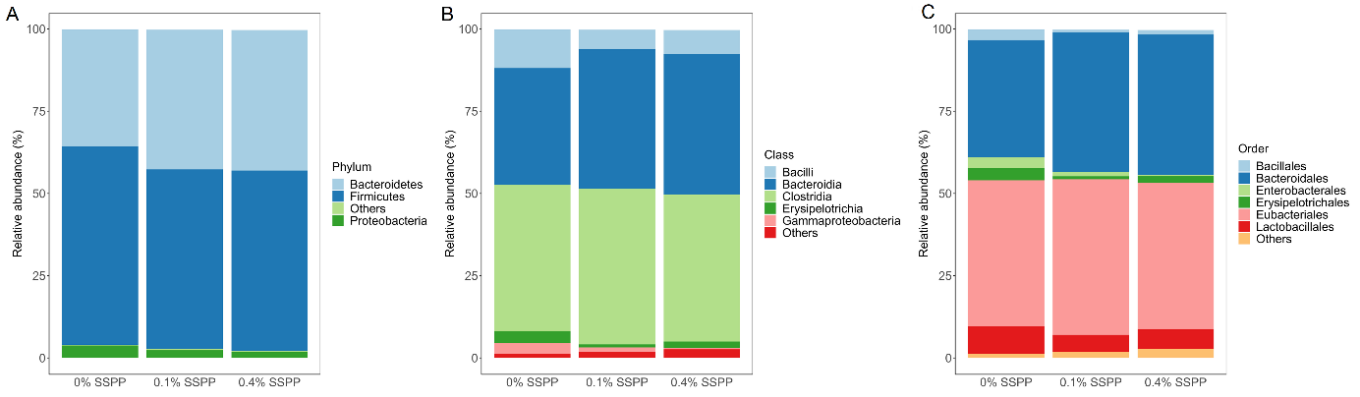

Fig. 3. Microbial composition of broilers' cecal samples. Relative abundances of major phyla (A), classes (B), and orders (C) are depicted in stacked bar plots. Treatments are as follows: SSPP, solubles from shredded, steam-exploded pine particles; 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

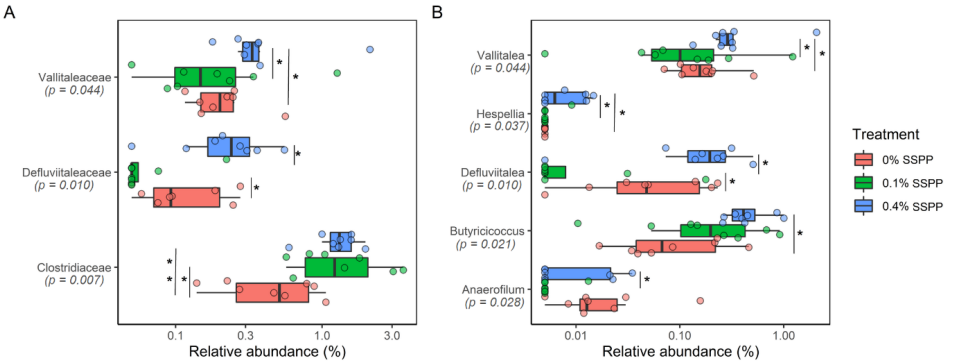

Fig. 4. Relative abundance of significantly different families (A) and genera (B) from broilers' cecal samples. Data were analyzed using the Kruskal-Wallis test followed by the Wilcoxon rank sum test (adjusted by the Benjamini-Hochberg method). For each experimental group, the samples represent biological replicates (n = 8). Box plots indicate median values, quartiles as well as whiskers of the distribution of numerical data. The p-values under each taxon were derived from the Kruskal-Wallis test. Treatments are as follows: 0% SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP. * Indicates significance for the pairwise Wilcoxon rank sum test at $p < 0.05$. ** Indicates significance for the pairwise Wilcoxon rank sum test at $p < 0.01$. SSPP, solubles from shredded, steam-exploded pine particles.

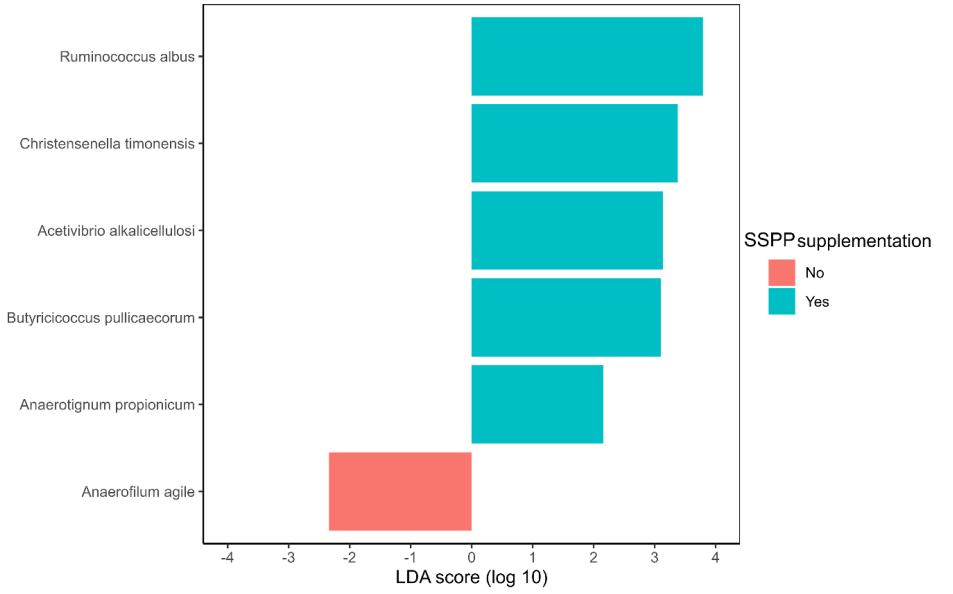

Fig. 5. Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) analysis of cecal microbiota in broiler chickens. The species represented are statistically significant ($p < 0.05$; | LDA effect size | > 2.0) and play an important role in the control (red) or SSPP-supplemented groups (green). SSPP, solubles from shredded, steam-exploded pine particles.

Microbial network patterns

Correlation network analysis was used to detect interactions between cecal microbial species of birds (Fig. 6). Simultaneous visualization from the network graph, and chord diagram is primordial to enhance the reader's understanding of the microbial interactions. After prevalence filtering and selecting strong and significant correlations ($p < 0.05$, | r |= 0.65) the correlation network obtained had 43 nodes (species) and 35 edges (interactions). There was a striking predominance of *Clostridia*

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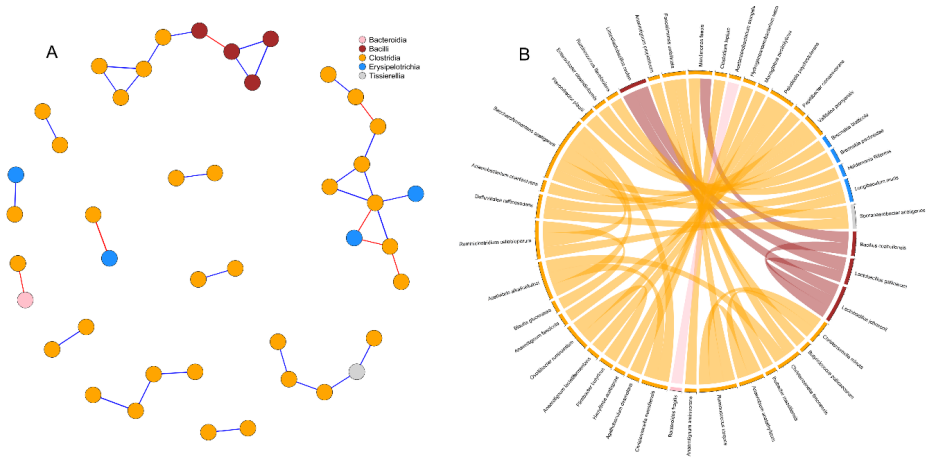

Fig. 6. Network pattern (A) and chord diagram (B) of broilers' cecal microbiota at the class level. Both network and chord diagrams network have been drawn based on Spearman's rank correlation. The significance was set at $p < 0.05$ and the threshold of correlation was set as $|r| = 0.65$. In the network (A), the vertexes (circles) correspond to microbial species while the weight of the edges (segments) represents correlations between microbes. Positive correlations are colored in blue while negative correlations are colored in red. In the chord diagram (B), sectors represent the same species described in the network while the thickness of the links is proportional to correlations.

in the network, while *Bacilli* and *Erysipelotrichia* were represented in minority. Besides, *Tissirella* and *Bacterodia* had only single individuals in the network. *Bacilli* appeared to be mostly connected between themselves while *Erysipelotrichia* were linked to *Clostridia*. Two interesting subnetworks could be spotted (Fig. 6A) with the first one composed of *Bacilli* and *Clostridia* while the second one regrouped *Clostridia* and *Erysipelotrichia*. *Clostridia* were generally positively correlated with each other. Only a few negative correlations were detected in the network and appeared to be evenly distributed among intra-classes (3) and inter-classes (4) connections.

DISCUSSION

The utilization of phytogenic feed additives in animal nutrition has been the subject of recent research due to their potential role in the growth and health of livestock [26]. Among these compounds, byproducts from the wood industry are gaining popularity [27,28]. Their relatively low price and availability make them suitable for their inclusion in broilers' diets [29]. The current study provides a detailed assessment of the growth, health, and cecal microbiota in broilers after SSPP supplementation.

There were no significant differences in ADFI, final body weight, and ADG between control and SSPP-fed birds. These results were in agreement with our previous study that evaluated the effects of other particles (from 1% to 2% of inclusion) on broilers' growth [16]. Similarly, a 0.2% chestnut wood extract supplementation did not significantly increase growth performance in broilers [28]. On the other side, a considerable improvement was seen in ADG, feed intake, and FCR when chicks were supplemented (from 25 to 100 g/kg of feed) with dietary charcoal obtained from the oak tree [30]. Therefore, it is easily understandable that in vivo trials focusing on the supplementation of byproducts from the wood industry have inconsistent effects on poultry performances. The disparity observed in the results within studies can be explained by their setups and designs. Even though dietary wood-derived products are well known for their relatively high

amount of fiber, carbohydrates, and phenolic compounds [31,32], factors such as source, extraction method, supplementation duration, and inclusion level have a greater influence on growth-related outcomes [33].

Although relatively simple to assess the organ index is a highly informative parameter that gives insights into the development status of the organ [34]. In the current study, we evaluated the relative weight and length of intestinal segments. None of the different organ indexes evaluated appeared to be affected by the inclusion of SSPP in broilers' diets. It is already acknowledged that improvement in intestinal development leads to enhanced nutrient absorption, which is reflected by higher growth [35]. Since chicks' body weight did not significantly differ among treatments, the observed results were therefore expected.

While our findings did not support any effects of SSPP supplementation on phenotypic parameters (growth and organ development), there was a clear indication of diet-induced microbial shifts. Other researchers also highlighted that feed additives and diet were usually associated with significant modifications in microbiome composition and diversity [36–38]. In the current trial, both the diversity and composition of the cecal microbiota were influenced by the inclusion of SSPP in the diet. SSPP supplementation significantly impacted not only richness indices (OTUs and Chao1) but also beta diversity. For example, a lower individual variation was observed in the SSPP-supplemented birds in comparison to the control birds, suggesting that regardless of the inclusion level, SSPP had the same effects on broilers' microbial community structure. Besides, it is critical to mention that the overall variations observed between controls and birds supplemented with SSPP were mostly caused by changes in relative abundance rather than taxonomic composition. Similar findings were also reported in our previously performed trials [15,16].

In chickens, the cecal microbiota considerably increases in complexity and composition in comparison to the proximal part of the gastrointestinal tract [39]. *Firmicutes* and *Bacteroidetes* are the two dominant phyla usually followed by *Proteobacteria* and *Actinobacteria* [40]. *Bacteroidetes* are gram-negative bacteria providing their host energy in the form of acetate and propionate through the fermentation of originally indigestible polysaccharides [41]. On the other side, *Firmicutes* are gram-positive bacteria and the main producer of butyrate [42]. In the current study, SSPP-supplementation was shown to induce an augmentation in *Bacteroidetes* abundance concomitant with a reduction in *Proteobacteria* proportions. Like most plant-based bioproducts, SSPP contains considerable amounts of lignin, phenolic compounds, and carbohydrates. It was suggested that polyphenols ingestion can lead to modifications in *Firmicutes* to *Bacteroidetes* ratios by mainly supporting the proliferation of *Bacteroidetes* [11]. Similarly, *Bacteroidetes* proportions were found to increase in cecal and cloacal samples from chickens fed polyphenol-rich mulberry byproducts [43]. Healthy broiler individuals tended to have a similar proportion of *Firmicutes* and *Bacteroidetes* while a higher *Firmicutes* to *Bacteroidetes* ratio was correlated with obesity and body fat accumulation [44]. Furthermore, an increase in *Proteobacteria* abundance may lead to compromised growth and inflammatory reactions [45,46]. Although SSPP supplementation is likely to be associated with overall health improvement in broiler chicks, the relatively short duration of inclusion (21 days) in the current trial might have limited the effectiveness of SSPP as we only saw a slight improvement in birds' body weight. Therefore, further studies evaluating the long-term supplementation of SSPP will be of great interest.

In agreement with other studies [47,48], the abundance of bacteria belonging to families *Clostridiaceae*, *Defluviitaleaceae*, and *Vallitaleaceae* was significantly affected by feed supplementation. *Clostridiaceae* are among the phylotypes responsible for the transformation of glucose, lactate, and succinate into butyrate [49]. Furthermore, starch cleavage enzyme production is one of the main characteristics of bacteria in this family [50]. *Clostridiaceae* relative abundance was not only

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significantly higher after SSPP supplementation, but the level of significance increased with higher SSPP doses. This finding suggests that SSPP inclusion in broilers' diets resulted in the proliferation of bacteria with amylolytic properties, indicating an overall higher metabolic activity of hydrolyzable components. The relative abundance of the genera *Defluviitaleaceae*, and *Vallitaleaceae* was also modified by SSPP supplementation, and the 0.4% SSPP group consistently showed the highest value. Interestingly, both genera were similarly affected, perhaps because they are close phylogenetic relatives with an estimated sequence similarity of about 89% [51]. In fact, in our datasets, each of these genera represented a single species which were *Defluviitalea raffinosedens* and *Vallitalea pronyensis* for *Defluviitalea* and *Vallitalea,* respectively. *D. raffinosedens* is a saccharolytic bacterium that acts synergistically with its cellulolytic counterpart to enhance cellulolysis by preventing feedback inhibition and improving the degradation process [52]. *V. pronyensis* is a fermentative anaerobic bacterium found in hydrothermal chimneys rich in alkaline compounds [53]. Interestingly, both species are thermophilic and thus can withstand temperatures ranging from 41°C to 122°C. As chicken core body temperature can reach 45°C under heat stress [54], the current results suggest that SSPP supplementation can be an appropriate approach against heat stress. Here, as described in another study [55], the relative abundance of the genus *Butyricicoccus* was significantly increased by dietary supplements. Furthermore, LEfSe identified *B. pulliceacorum* as a biomarker of SSPP supplementation (| LDA effect size | > 3). This particular species has recently been used as a probiotic in chickens [55] and humans [56] due to its resistance to bile, colonization efficiency, and butyrate production. From an intestinal health perspective, this type of butyrate-producing bacteria stimulates enterocyte growth and reduces the invasion of pathogenic bacteria such as *Salmonella* [57].

LEfSe also showed that *R. albus*, *C. timonensis*, *A. alkalicellulosi*, and *A. propionicum* were microbial biomarkers of dietary SSPP inclusion. In LEfSe, the effect size can be translated as the magnitude difference of the abundance [58]. Therefore, species with higher LDA scores in absolute values, rank higher for biomarker relevance. *R. albus* was the top-ranked biomarker (| LDA effect size | > 3.5) of SSPP supplementation. This particular specie was found to produce significant amounts of albusin-B, a bacteriocin that is lethal to several other gram-positive bacteria [59]. Since bacteria belonging to the phyla *Firmicutes* are gram-positive, it might therefore explain the reduction in their proportion seen after including SSPP in the diet. Information related to *C. timonensis, A. alkalicellulosi,* and *A. propionicum* remains limited and scarce, especially concerning chicken. Indeed, only studies related to the discovery and taxonomy of these microbial species are available [60,61]. Thus, more specialized experiments are needed to elucidate their potential roles in the chicken microbiome.

Interactions between bacteria and their environment contribute greatly to the balance in microbial systems [62]. In the current study, we used microbial network analysis to gain additional insights into the structure of the microbial communities. In a microbial network, cooperative interactions or similar biological functions between species are positively correlated whereas competition between species is negatively correlated [63]. From our current results, the prevalence of positive correlations observed between species belonging to the class *Clostridia*, was suggesting mutualistic relationships between such microbes. Similarly, the co-culture of bacteria belonging to the *Clostridia* class has been shown to improve the yield of cellulose fermentation [64,65]. On the other side, negative correlations in the network were found between *Erysipelotrichia, Bacteroidia* and *Clostridia*. A previous study [66] suggested that some bacteria of the classes *Clostridia* and *Erysipelotrichia* consume sugar acids and sugar alcohols to produce butyrate. Therefore, the anticorrelation between these species revealed in the network may indicate competition for nutrient availability. Consistent with our previous study [67], we detected a negative correlation between

the only *Bacteroidetes* (*Bacteroides fragilis*) and a *Firmicute* (*Acetanaerobacterium elongatum*) present in our network. While there is a lack of studies reporting interactions between these two species, the negative correlation can be largely explained by considering their phylum. In fact, a significant number of trials have highlighted the negative correlation between these two phyla [15,16,68].

In conclusion, SSPP supplementation strongly influenced the cecal microbiota of broilers without affecting growth performances. When birds were fed with SSPP, there was an overall shift in microbial community structure toward a "healthier" profile. However, the results also suggest that trials focusing on supplementing SSPP for a longer period and evaluating its effect in heat-stressed birds can lead to interesting findings.

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https://doi.org/10.5187/jast.2023.e15