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| **Author** | Chris Major Ncho 1,2, Akshat Goel 1,2, Vaishali Gupta 1,3, Chae-Mi Jeong 1,3, Ji-Young Jung 2,4, Si-Young Ha 4, Jae-Kyung Yang 2,4 and Yang-Ho Choi 1,2,3,* |
| **Affiliation** | 1 Department of Animal Science, Gyeongsang National University, Jinju 52828, Korea<br>2 Institute of Agriculture and Life Sciences, Gyeongsang National University, Jinju 52828<br>3 Division of Applied Life Sciences (BK21 Plus Program), Gyeongsang National University, Jinju 52828, Korea<br>4 Department of Environmental Materials Science, Gyeongsang National University, Jinju 52828, Korea |
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5 **CORRESPONDING AUTHOR CONTACT INFORMATION**

| **For the corresponding author (responsible for correspondence, proofreading, and reprints)** | **Fill in information in each box below** |
|---|---|
| First name, middle initial, last name | Yang-Ho Choi |
| Email address – this is where your proofs will be sent | yhchoi@gnu.ac.kr |
| Secondary Email address | - |
| Address | 501 Jinju-daero, Gajwa-dong, Jinju 52828, Republic of Korea |
| Cell phone number | - |
| Office phone number | 055-772-1946 |
| Fax number | 055-772-1946 |

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

Running title: Dietary solubles from shredded, steam-exploded pine particles affects chickens' microbiome

Chris Major Ncho [1,2], Akshat Goel [1,2], Vaishali Gupta [1,3], Chae-Mi Jeong [1,3], Ji-Young Jung [2,4], Si-Young Ha [4], Jae-Kyung Yang [2,4] and Yang-Ho Choi [1,2,3,*]

[1] Department of Animal Science, Gyeongsang National University, Jinju 52828, Korea; major159@gnu.ac.kr (C.M.N.); genesakshat@gnu.ac.kr (A.G.); wjdcoa@gnu.ac.kr (C.-M.J.); vaishali2020@gnu.ac.kr (V.G.)

[2] Institute of Agriculture and Life Sciences, Gyeongsang National University, Jinju 52828, Korea; charmhanjjy@nate.com (J.-Y.J.); jkyang@gnu.ac.kr (J.-K.Y.)

[3] Division of Applied Life Sciences (BK21 Plus Program), Gyeongsang National University, Jinju 52828, Korea

[4] Department of Environmental Materials Science, Gyeongsang National University, Jinju 52828, Korea; hellohsy2@gmail.com

* Correspondence:

Yang-Ho Choi; Department of Animal Science, Gyeongsang National University, 501 Jinju-daero, Gajwa-dong, Jinju 52828, Korea; yhchoi@gnu.ac.kr; Tel.: 055-772-1946; Fax: 055-772-1949

## 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

# 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 structure) 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 (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 Inc., 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 (RH) 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 Co., 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 x 70 cm x 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 Co., Ltd., Franklin Lakes, NJ, USA). Plasma was obtained by centrifuging blood samples at 2,000 x 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 Co., Ltd., Westbrook, ME, USA) with a dry-slide technology [19].

**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, Inc., Seoul, Korea). The fastp program was then used 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.

149

150 **Ecological and statistical analyses**

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

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

170 Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was applied to determine the OTUs
171 most likely to explain differences ($p < 0.05$; | LDA effect size | > 2.0) between control and
172 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 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 ($p < 0.05$) 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

196 OTUs and Chao1 indices. Furthermore, the similar pattern observed in both indices was
197 indicating that the sequencing depth obtained was sufficient. In contrast, none of the other
198 indices (Shannon, inverse Simpson, and Good's coverage) were showing statistical differences
199 neither when SSPP was supplemented at 0.1% or 0.4%.

200 Beta diversity results are depicted in Fig. 2. In this trial, PERMDISP revealed that individual
201 community variation was greater ($p < 0.05$) in the control group (0% SSPP), while lower
202 variances were found in SSPP-supplemented groups (0.1% SSPP and 0.4% SSPP). In addition,
203 the NMDS plot using the Bray-Curtis distance matrix showed SSPP supplementation-related
204 clustering, with birds fed SSPP clustering closer to each other (Fig. 2B). Similarly, microbial
205 community structures were significantly different ($R = 0.31$, $p < 0.01$) when all birds fed SSPP
206 (0.1% SSPP and 0.4% SSPP) were compared to the control group based on ANOSIM.

207

208 **Microbial community composition**

209 As illustrated in Fig. 3 the relative abundance of OTUs from birds' cecal microbiota was
210 analyzed at different taxonomic levels. *Firmicutes* (56.6%) and *Bacteroidetes* (40.2%) together
211 were representative of the majority of the microbiome from birds' cecal samples in the current
212 trial (Fig. 3A). SSPP supplementation also led to an increase in *Bacteroidetes* relative
213 abundance (from 35.5% to 42.7%) while concomitantly decreasing *Firmicutes* proportions
214 (from 60.3% to 52.6%). Although minimal, there was a reduction in *Proteobacteria* relative
215 abundance (from 3.8% to 1.2%) with the supplementation of SSPP. The dominant classes across
216 treatment groups were *Clostridia* (45.4%), *Bacteroidia* (40.2%), and *Bacilli* (8.3%) (Fig. 3B).
217 The birds supplemented with SSPP had a higher abundance of *Bacteroidia* and a lower
218 abundance of *Bacilli* while the *Clostridia* abundance did not seem to be affected treatment-wise.
219 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.

**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

244 filtering and selecting strong and significant correlations ($p < 0.05$, | r |= 0.65) the correlation
245 network obtained had 43 nodes (species) and 35 edges (interactions). There was a striking
246 predominance of *Clostridia* in the network, while *Bacilli* and *Erysipelotrichia* were represented
247 in minority. Besides, *Tissirella* and *Bacterodia* had only single individuals in the network.
248 *Bacilli* appeared to be mostly connected between themselves while *Erysipelotrichia* were linked
249 to *Clostridia*. Two interesting subnetworks could be spotted (Fig. 6A) with the first one
250 composed of *Bacilli* and *Clostridia* while the second one regrouped *Clostridia* and
251 *Erysipelotrichia*. *Clostridia* were generally positively correlated with each other. Only a few
252 negative correlations were detected in the network and appeared to be evenly distributed among
253 intra-classes (3) and inter-classes (4) connections.

254

255 # Discussion

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

262 There were no significant differences in ADFI, final body weight, and ADG between control
263 and SSPP-fed birds. These results were in agreement with our previous study that evaluated the
264 effects of other particles (from 1% to 2% of inclusion) on broilers' growth [16]. Similarly, a
265 0.2% chestnut wood extract supplementation did not significantly increase growth performance
266 in broilers [28]. On the other side, a considerable improvement was seen in ADG, feed intake,
267 and FCR when chicks were supplemented (from 25 to 100g/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 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. *Defluviitalea 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]. *Vallitalea 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 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 *Butyricicoccus 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

343 bacteria stimulates enterocyte growth and reduces the invasion of pathogenic bacteria such as
344 *Salmonella* [57].

345 LEfSe also showed that *Ruminococcus albus*, *Christensella timonensis*, *Acetivibrio*
346 *alkalicellulosi*, and *Anaerotignum propionicum* were microbial biomarkers of dietary SSPP
347 inclusion. In LEfSe, the effect size can be translated as the magnitude difference of the
348 abundance [58]. Therefore, species with higher LDA scores in absolute values, rank higher for
349 biomarker relevance. *Ruminococcus albus* was the top-ranked biomarker (| LDA effect size | >
350 3.5) of SSPP supplementation. This particular specie was found to produce significant amounts
351 of albusin-B, a bacteriocin that is lethal to several other gram-positive bacteria [59]. Since
352 bacteria belonging to the phyla *Firmicutes* are gram-positive, it might therefore explain the
353 reduction in their proportion seen after including SSPP in the diet. Information related to
354 *Christensella timonensis, Acetivibrio alkalicellulosi,* and *Anaerotignum propionicum* remains
355 limited and scarce, especially concerning chicken. Indeed, only studies related to the discovery
356 and taxonomy of these microbial species are available [60, 61]. Thus, more specialized
357 experiments are needed to elucidate their potential roles in the chicken microbiome.

358 Interactions between bacteria and their environment contribute greatly to the balance in
359 microbial systems [62]. In the current study, we used microbial network analysis to gain
360 additional insights into the structure of the microbial communities. In a microbial network,
361 cooperative interactions or similar biological functions between species are positively
362 correlated whereas competition between species is negatively correlated [63]. From our current
363 results, the prevalence of positive correlations observed between species belonging to the class
364 *Clostridia*, was suggesting mutualistic relationships between such microbes. Similarly, the co-
365 culture of bacteria belonging to the *Clostridia* class has been shown to improve the yield of
366 cellulose fermentation [64, 65]. On the other side, negative correlations in the network were
367 found between *Erysipelotrichia, Bacteroidia* and *Clostridia*. A previous study [66] suggested

368 that some bacteria of the classes *Clostridia* and *Erysipelotrichia* consume sugar acids and sugar
369 alcohols to produce butyrate. Therefore, the anticorrelation between these species revealed in
370 the network may indicate competition for nutrient availability. Consistent with our previous
371 study [67], we detected a negative correlation between the only *Bacteroidetes* (*Bacteroides*
372 *fragilis*) and a *Firmicute* (*Acetanaerobacterium elongatum*) present in our network. While there
373 is a lack of studies reporting interactions between these two species, the negative correlation
374 can be largely explained by considering their phylum. In fact, a significant number of trials
375 have highlighted the negative correlation between these two phyla [15, 16, 68].

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

381

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588

589 # Tables and Figures

590 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 |

591

592

593

594 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 (%)** | | | |
| 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 |
| Total | 100 | 100 | 100 |
| **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) | 3050 | 3150 | 3200 |

595 [1] The feeds were purchased from Nonghuyp Feed, Inc. (Seoul, Korea), and their compositions
596 were provided by the company.
597 [2] Trace minerals and vitamins provided per kilogram of premix: vitamin A, 12,000,000 IU;
598 vitamin D3, 3,000,000 IU;vitamin E, 40,000 IU; vitamin K3, 2,000 IU; vitamin B1, 2,000 mg;
599 vitamin B2, 5,000 mg; vitamin B6, 3,000 mg; vitamin B12, 20 mg; niacin, 40,000 mg;
600 pantothenic acid, 10,000 mg; folic acid, 1000 mg; iron, 88,000 mg; copper, 72,600 mg; zinc,
601 60,000 mg; manganese, 66,000 mg; iodine, 990 mg; selenium, 220 mg; cobalt, 330 mg."
602

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

| Parameters | SSPP supplementation (%) | | | 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) | 1635.4 | 1680.7 | 1660.5 | 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 |

605 Values are presented as mean ± SEM (n = 12). Treatments are as follows: 0% SSPP, chicks fed
606 with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP;
607 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP. Abbreviations:
608 ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio

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

| Parameters | SSPP supplementation (%) | | | 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 |

611 Values are presented as mean ± SEM (n = 8). Treatments are as follows: 0% SSPP, chicks fed
612 with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP;
613 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

614

615

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

| Parameters | SSPP supplementation (%) | | | 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 |

618 Values are presented as mean ± SEM (n = 8). Treatments are as follows: 0% SSPP, chicks fed
619 with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP;
620 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

621

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

| Parameters | SSPP supplementation (%) | | | 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 |

624 Values are presented as mean ± SEM (n = 8). Treatments are as follows: 0% SSPP, chicks fed
625 with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of SSPP;
626 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

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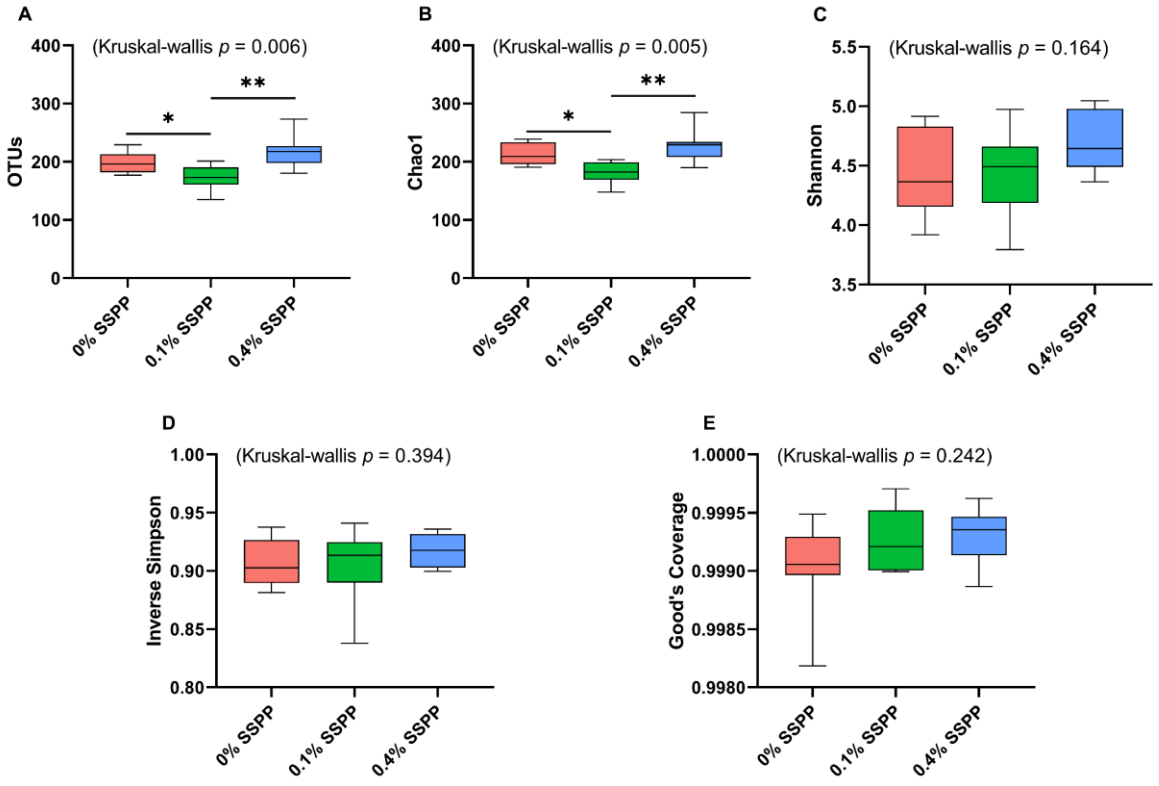


Fig. 1. Box plots representing the distribution of alpha diversity indices OTUs (A), Chao1(B), Shannon(C), Inverse Simpson (D), and Good's coverage (E) of broilers' cecal samples. Data were analyzed using the Kruskal-Wallis test followed by the Wilcoxon rank sum test (adjusted by the Benjamini-Hochberg 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 $p < 0.01$.

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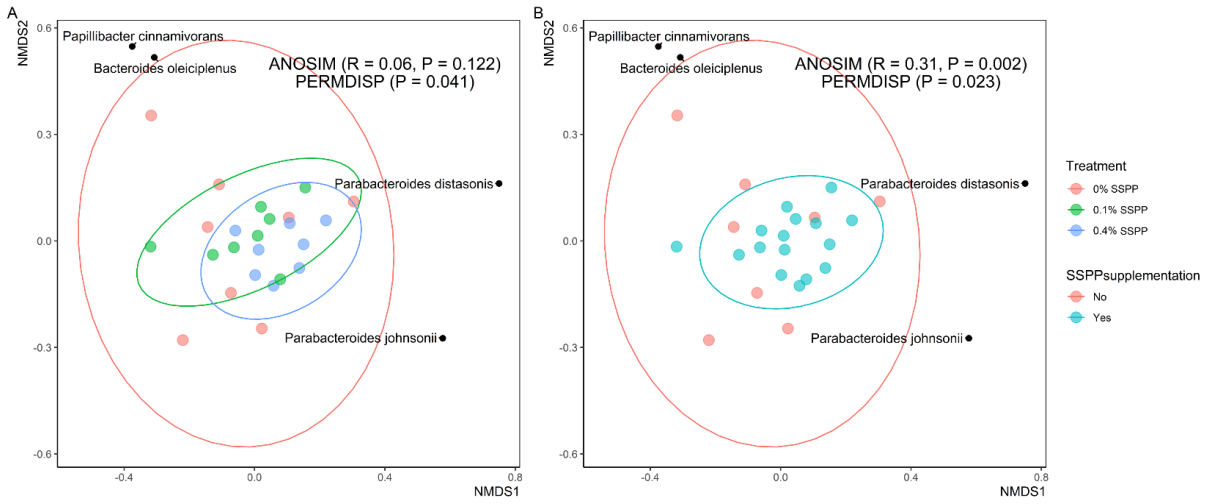


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646 Fig. 2. Non-metric multidimensional scaling (NMDS) plot issued from Bray-Curtis
647 dissimilarity matrix based on relative abundance data of cecal samples. Colored dots represent
648 cecal samples (biological replicates), while black dots are microbial species. Ellipses indicate
649 95% confidence intervals of multivariate t-distribution around centroids of the groupings with
650 treatments (A) and SSPP supplementation (B) as factors. PERMDISP ($p = 0.041$ (A); $p = 0.023$
651 (B)) revealed lower individual variation in the SSPP-supplemented group, and ANOSIM (R =
652 0.31, $p = 0.002$) indicated SSPP-related clustering. Treatments are as follows: 0% SSPP, chicks
653 fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented with 0.1% of
654 SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

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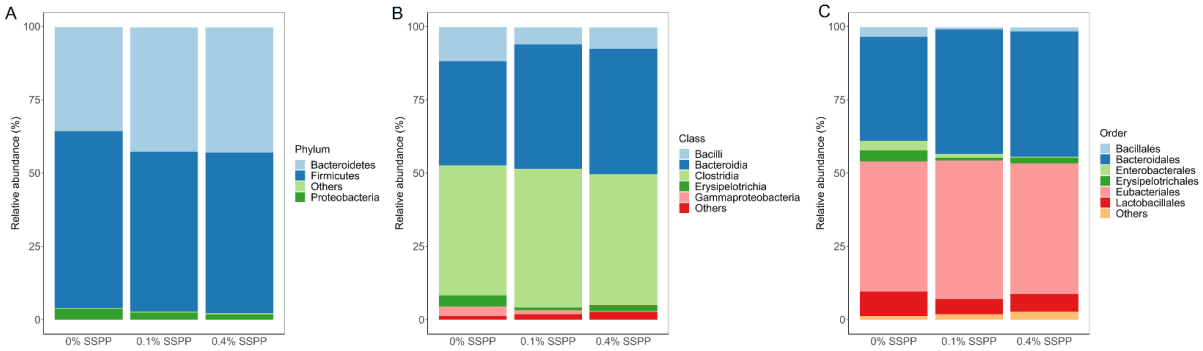


658

659 Fig. 3. Microbial composition of broilers' cecal samples. Relative abundances of major phyla
660 (A), classes (B), and orders (C) are depicted in stacked bar plots. Treatments are as follows: 0%
661 SSPP, chicks fed with the basal diet; 0.1% SSPP, chicks fed with the basal diet supplemented
662 with 0.1% of SSPP; 0.4% SSPP, chicks fed with the basal diet supplemented with 0.4% of SSPP.

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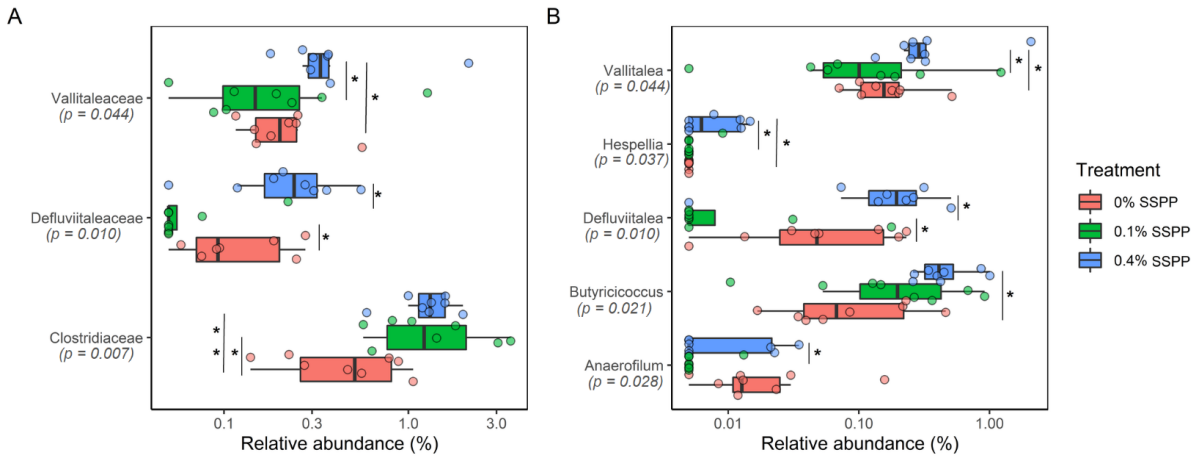


Fig. 4. Relative abundance of significantly different families (A) and genera (B) from broilers' cecal samples. Data were analyzed using the Kruskal-Wallis test followed by the Wilcoxon rank sum test (adjusted by the Benjamini-Hochberg method). For each experimental group, the samples represent biological 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 $p < 0.01$.

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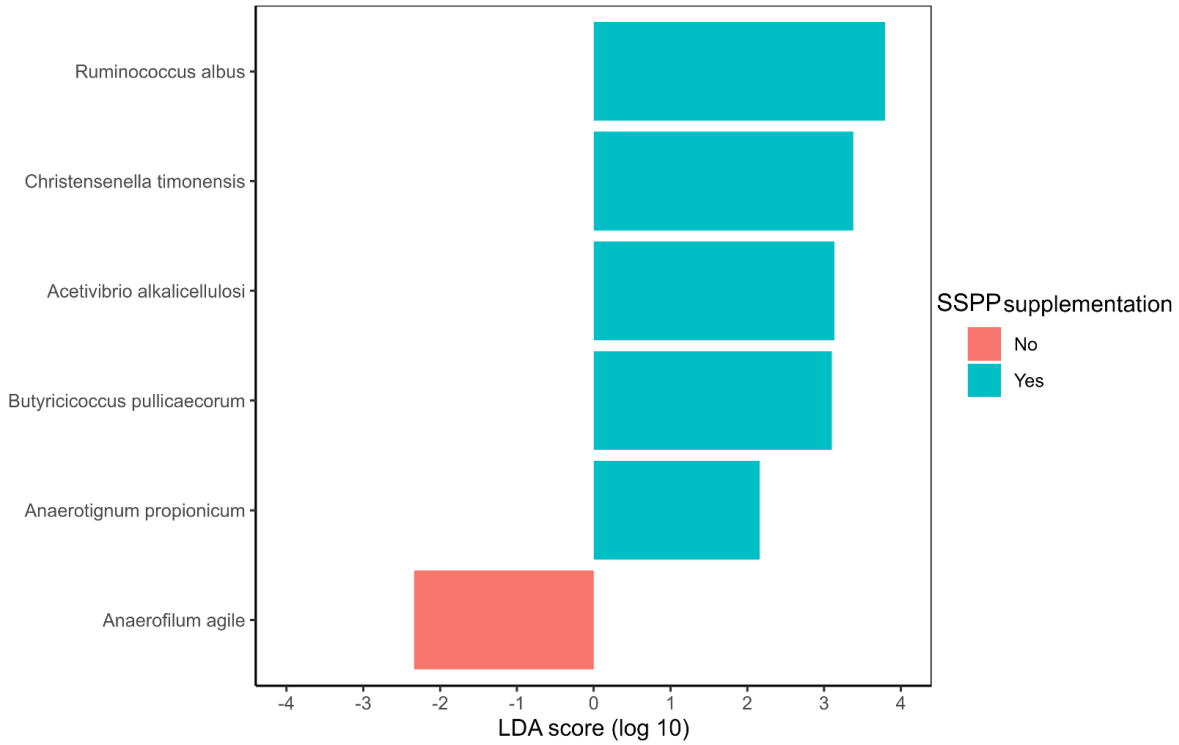


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682 Fig. 5. Linear discriminant analysis (LDA) combined effect size measurements (LEfSe)
683 analysis of cecal microbiota in broiler chickens. The species represented are statistically
684 significant ($p < 0.05$; | LDA effect size | > 2.0) and play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

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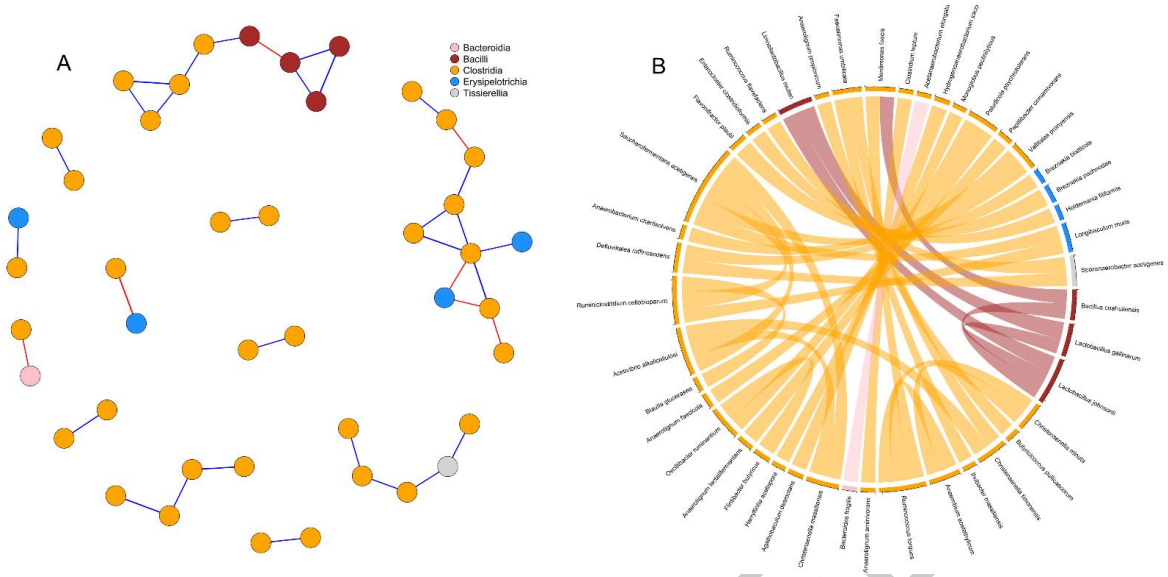


Fig. 6. Network pattern (A) and chord diagram (B) of broilers' cecal microbiota at the class level. Both network and chord diagrams network have been drawn based on Spearman's rank correlation. The significance was set at $p < 0.05$ and the threshold of correlation was set as $|r| = 0.65$. In the network (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.