



Dietary *Caesalpinia sappan* Bark Extract Improves Intestinal Morphology and Modulates Immune- and Antioxidant-Related Gene Expression in Broilers

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ABSTRACT

Broiler chickens provide a sustainable and accessible protein source, but the use of antibiotics has prompted concerns about antimicrobial resistance and residual contamination. Therefore, this study evaluated dietary supplementation with *Caesalpinia sappan* bark extract (CSB) on intestinal morphology and immune- and antioxidant-related gene expression in broilers. A total of 256 Ross 308 broiler chickens were randomly allocated to four groups receiving diets supplemented with 0, 150, 300, or 600 mg/kg CSB. Growth performance, small intestinal histology, and immune- and antioxidant-related gene expression were evaluated at the end of the 35-day rearing period. CSB supplementation did not alter body weight or average daily gain, whereas feed intake and feed conversion ratio (FCR) increased at higher inclusion levels ($p < 0.05$). CSB improved several intestinal morphology parameters, including villus height and villus height-to-crypt depth ratio ($p < 0.05$). In the jejunum, higher CSB inclusion levels were associated with increased CAT and GPx expression and decreased TNF- α and IL-12 expression ($p < 0.05$). Correlation analysis showed associations between intestinal morphology and antioxidant- and immune-related gene expression. In conclusion, dietary CSB supplementation improved intestinal morphology and modulated jejunal antioxidant- and immune-related transcriptional responses. However, the higher inclusion levels that produced the clearest molecular responses also increased FCR. Thus, the present study does not establish a single optimal CSB dose, and further dose-response studies are required to balance intestinal and molecular responses with feed efficiency.

Keywords: broiler chickens; *Caesalpinia sappan* Bark extract; gene expression; intestinal morphology

INTRODUCTION

Broiler chickens (*Gallus gallus domesticus*), also known as meat chickens, are birds reared for meat and have creamy white plumage. After years of crossbreeding and selection, broiler chickens have been developed for efficient meat production (Neeteson *et al.*, 2023). Over the years, broiler selection has focused on improving the feed conversion ratio (FCR), growth rate, meat yield, and meat quality. This selection has resulted in chickens with a low FCR, rapid growth rate, and tender meat that is accepted worldwide (Maharjan *et al.*, 2021). The rapid expansion of the poultry industry has been driven primarily by advancements in genetics, nutrition, and health management. Antibiotics were traditionally employed as growth promoters (antibiotic growth promoters; AGPs) to control intestinal health. However, indiscriminate use of antibiotics has created serious global concerns on antimicrobial resistance

and tissue residues. Such problems not only impaired the physiological state of birds but also posed major zoonotic hazards as resistant diseases might be transmitted to humans through the food chain, making clinical treatments ineffective (Tang *et al.*, 2017).

Following restrictions on the use of AGPs, including the European Union ban in 2006, research has increasingly focused on sustainable natural feed additives with favorable safety profiles, including nutraceuticals and phytobiotics. Concerns regarding antibiotic residues and antimicrobial resistance have further encouraged the poultry industry to explore non-antibiotic nutritional strategies, such as probiotics, prebiotics, phytogenic compounds, and essential oils, for inclusion in feeding programs (Oladokun & Adewole, 2020). Plant-derived bioactive compounds have received particular attention because of their potential to support physiological functions and improve the nutritional and functional characteristics

of animal products (Biswas *et al.*, 2024; Chaiwang *et al.*, 2026).

Among phytogetic candidates, *Caesalpinia sappan* bark extract (CSB) has received increasing research interest. CSB contains phenolic bioactive compounds, including brazilin, with reported antioxidant, anti-inflammatory, and antibacterial properties. These characteristics provide a rationale for investigating CSB as a functional feed additive that may influence intestinal structure, oxidative status, and inflammatory signaling in poultry (Longchuphon *et al.*, 2024).

Recent poultry research has increasingly focused on functional phytogetic additives that may support intestinal health, oxidative stability, and immune regulation (Huanhong *et al.*, 2025a). Dietary *C. sappan* extract has also been investigated in laying hens, with reported responses in flock health and productivity (Longchuphon *et al.*, 2024). However, information on CSB supplementation during the broiler production cycle remains limited. The present study was therefore designed to evaluate growth performance, intestinal morphology, and transcriptional expression of selected immune- and antioxidant-related genes, providing additional evidence for the potential application of CSB as a phytogetic feed additive in broiler production.

MATERIALS AND METHODS

Plant Material and Chemical

CSB, extracted with distilled water, was supplied by Specialty Natural Product Co., Ltd (Chonburi, Thailand; product code HE-EP01-CAE). The additive used in this study was a CSB product extracted with water and dried to powder before being included in the diet. Therefore, supplementation levels were expressed as milligrams of CSB powder per kilogram of diet. The bioactive profile and the antioxidant capacity of the product were characterized by total phenolic content, phenolic compounds detected by high-performance liquid chromatography HPLC and antioxidant assays as below.

Antioxidant Analysis

The antioxidant capacity of CSB was evaluated by ferric-reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays, according to the protocol reported by Norkeaw *et al.* (2022). Standard curves were established using Trolox solutions (0–15 mM) prepared in 80% methanol. Absorbance values for all assays were recorded using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA).

Phytochemical Analysis

The total phenolic content was determined according to the method described by Norkeaw *et al.* (2022). A mixture containing 50 μ L of distilled water, 12.5 μ L of Folin-Ciocalteu reagent, and 12.5 μ L of

aqueous CSB was prepared. After a 6-min reaction, 100 μ L of distilled water and 125 μ L of 10% (w/v) sodium carbonate were added. The mixture was incubated at room temperature for 90 min. The absorbance was measured at 750 nm using a microplate reader (SpectraMax M3; Molecular Devices, CA, USA). Gallic acid at various concentrations was used to construct a standard calibration curve. The total phenolic content was expressed as mM gallic acid equivalents per gram of sample (mM GAE/g sample) (Sopian *et al.*, 2024). The polyphenol profile of CSB was analyzed using HPLC/UV (HPLC LC-20A; Shimadzu, Japan). One gram of CSB powder was extracted with 100 mL of 80% (v/v) methanol. The mixture was shaken at 60 °C for 1 h at 120–150 rpm in a shaking incubator and filtered through Whatman No. 1 filter paper. The filtrate was diluted to 100 mL with 80% methanol and filtered through a 0.45- μ m membrane filter before analysis. An aliquot of 5 μ L of each sample was introduced to the HPLC apparatus. Chromatography was carried out on a Raptor ARC-18 column (150 mm $\times$ 4.6 mm ID $\times$ 2.7 μ m; Restek Corporation, Bellefonte, PA, USA). The temperature of the column was kept at 30 °C. The mobile phase was composed of (A) water with 5 mM ammonium formate and 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The flow rate was 1 ml/min. The gradient elution programme was as follows: 0–5 min, 25% A and 75% B; 5–15 min, linear gradient from 25% to 60% A; 15–20 min, linear gradient from 60% to 90% A; 20–23 min, hold at 90% A; and 23–25 min, return to initial conditions (25% A) for column re-equilibration. The detection wavelength was set at 228 nm (Sopian *et al.*, 2024). The concentrations reported from HPLC represent concentrations in the prepared analytical extract solution.

Ethical Approval

The research was conducted according to the Manual for the Care and Use of Farm Animals for Research and Teaching. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (Agricultural), Faculty of Agriculture, Chiang Mai University, approved no. AG02004/2566.

Animal and Diets

In total, 256 one-day-old male broilers (Ross 308) were enrolled in the study. During the initial 3-day brooding phase, all broilers were managed under a communal brooding system and received a standard basal diet for physiological acclimatization. On day 4, the broilers were allocated to four dietary treatments using a completely randomized design. Each treatment group comprised eight replicate pens, with eight broilers per pen. The experimental diets consisted of a basal control diet and three diets supplemented with CSB powder at 150, 300, and 600 mg/kg diet, respectively. Commercial starter and finisher basal diets were used according to the feeding phases shown in Table 1. CSB powder was top-dressed and mixed

Table 1. Chemical composition of the commercial broiler diet

Chemical composition (%)	Starter diets (1-21 d)	Finisher diets (22-35 d)
Dry matter	89.08	88.97
Moisture	10.92	11.03
Crude protein	19.6	20.9
Ether extract	5.00	4.62
Ash	6.08	4.16
Crude fiber	4.97	5.03
Gross energy (Cal/g)	3991.55	3857.1

into the basal diet according to the treatment level. The starter diet was provided from day 4 to day 21, and the finisher diet was provided from day 22 to day 35. The entire 35-day trial was conducted in an evaporative housing system. The broilers were maintained in floor pens (120 × 100 × 50 cm) bedded with dry rice husks. Feed and water were provided ad libitum using feeding troughs and nipple drinkers, respectively. The first week was kept with a 24-h light regime, and then a 16-h light:8-h dark photoperiod was used for the rest of the experiment. Environmental parameters and husbandry procedures were the same in all experimental units.

Productive Performance

The trial was started by recording initial body weight, then body weight was recorded weekly for each pen throughout the study. Average daily feed intake (ADFI) was calculated on the basis of daily feed consumption for each pen. From these primary data, the key performance indicators like average daily gain, FCR, and weight gain from starter, finisher, and overall growth periods were calculated. These parameters were used for the evaluation of overall nutritional efficacy of the experimental diets.

Intestinal Morphology

At the end of the 35-day feeding trial, 3 broiler chickens per replicate were sampled for tissue collection. Vital organs and parts of three segments of the small intestine (duodenum, jejunum and ileum) were obtained. The weight of each organ was measured. The tissues were fixed in 10% neutral buffered formalin, processed through dehydration, clearing and paraffin impregnation. Tissues were embedded in paraffin and sectioned at 4.5 µm using a rotary microtome. Samples were deparaffinized and stained with hematoxylin and eosin (H&E). Villus morphology and crypt depth (CD) were assessed using a compound microscope equipped with a digital video camera. The images of the intestinal tissues were then analyzed by an image analyzer to determine villus height (VH; µm), villus width (VW; µm), CD (µm), and the ratio of villus height to crypt depth (VH/CD).

Immune- and Antioxidant-Related Gene Expression

At the end of the 35-day experiment, jejunal tissues were collected from 3 chickens per treatment group and

preserved in RNAlater solution for RNA extraction. The jejunum was selected for transcriptional analysis because it is a major site of nutrient absorption and represents an important region for assessing mucosal responses to dietary interventions. RNA was extracted from 50 mg of tissue with the PureLink RNA Mini Kit (Invitrogen). RNA concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The specific primers for the immune-related genes (tumour necrosis factor alpha, TNF-α; interleukin-1 beta, IL-1β; interleukin-10, IL-10; interleukin-12, IL-12) and antioxidant-related genes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx) were adopted from Huanhong *et al.* (2025b) and are shown in Table 2, with β-actin as the housekeeping gene.

Quantitative real-time PCR (qPCR) was performed using a CFX Connect Real-Time PCR System and iTaq Universal SYBR Green Supermix. Expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method and a standard curve according to Longchuphon *et al.* (2024).

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance in SPSS (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean. Differences between treatment means were evaluated with Duncan's multiple range test, and a $p < 0.05$ was considered statistically significant. Orthogonal polynomial contrasts were performed to determine if the CSB supplementation effects were linear, quadratic, or cubic. The relationships between the intestinal morphology parameters and the expression of immune- and antioxidant-related genes were investigated using principal component analysis (PCA) in the ggfortify package in R. The Pearson correlation coefficients between the parameters of intestinal morphology and the expression levels of immune- and antioxidant-related genes were visualized in the form of a heatmap using the R package heatmap.

RESULTS

Proximate Composition

The starter diet (days 1–21) contained 89.08% dry matter, 10.92% moisture, 19.6% crude protein, 5.00% ether extract, 6.08% ash, 4.97% crude fiber, and 3991.55 Cal/g gross energy (Table 1). The finisher diet (days 22–35) contained 88.97% dry matter, 11.03% moisture, 20.9% crude protein, 4.62% ether extract, 4.16% ash, 5.03% crude fiber, and 3857.1 Cal/g gross energy. These data represent the analyzed composition of the commercial basal diets rather than a manually formulated ingredient matrix.

Phenolic Compounds and Antioxidant Activities of *Caesalpinia sappan* Bark Extract

The bioactive profile and antioxidant capacity of the extract are summarized in Table 3. The analysis

Table 2. Primer sequences (5'→3'), amplicon sizes, and accession numbers for immune- and antioxidant-related genes analyzed by quantitative real-time PCR in broiler chickens

Target gene	Primer sequences (5'→3')	Product size (bp)	Accession No.
Housekeeping gene	F: CTGGCACCTAGCACAATGAA R: ACATCTGCTGGAAGGTGGAC	109	X00182.1
Immune-related gene			
IL-1 β	F: GTGAGGCTCAACATTGCGCTGTA R: TGTCCAGGCGGTAGAAGATGAAG	244	Y15006
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGGTACTCCTCGAT	103	NM_001004414.4
IL-12	F: CTGAAGGTTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	NM213588
TNF- α	F: TGCTGTCTATGACCGCC R: CTTTCAGAGCATCAACGCA	229	NM204267
Antioxidants-related gene			
SOD	F: GCCACCTACGTGAACAACCT R: AGTCACGTTTGATGGCTTCC	146	NM204211
CAT	F: CCACGTGGACCTCTTCTGT R: AAACACTTTCGCCTTGCAGT	160	NM001031215
GPx	F: CAGCAAGAACCAGACACCAA R: CCAGGTTGGTTCTTCTCCAG	99	GQ502186.2

Note: IL-1 β = interleukin 1 beta; IL-10= interleukin 10; IL-12= interleukin 12; TNF- α = tumor necrosis factor alpha; SOD= superoxide dismutase; CAT= catalase; GPx= glutathione peroxidase.

Table 3. Phenolic compounds and antioxidant activities of *Caesalpinia sappan* bark extract (CSB)

Variables	Contents
Bioactive compounds	
Total phenol content (mM GAE/g)	141.35 $\pm$ 13.80
Brazilin (mg/g extract)	70.53 $\pm$ 1.45
Quercetin (mg/g extract)	1.56 $\pm$ 0.00
o-Coumaric (mg/g extract)	0.55 $\pm$ 0.02
Ferulic acid (mg/g extract)	0.45 $\pm$ 0.00
Naringin (mg/g extract)	8.34 $\pm$ 0.53
Rosmarinic acid (mg/g extract)	7.61 $\pm$ 0.03
Antioxidant activities	
DPPH (mM TE/g)	752.69 $\pm$ 1.35
ABTS (mM TE/g)	923.75 $\pm$ 17.68
FRAP (mM Fe ²⁺ /g)	469.82 $\pm$ 18.19

Note: Values are expressed as mean $\pm$ standard deviation (n=4). 2,2-diphenyl-1-picrylhydrazyl; ABTS; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalent; TE: Trolox equivalent.

revealed that the total phenolic content reached 141.35 $\pm$ 13.80 mM GAE/g. Among the detected phytochemicals, brazilin was the predominant constituent (70.53 $\pm$ 1.45 mg/g extract), followed by naringin (8.34 $\pm$ 0.53 mg/g extract) and rosmarinic acid (7.61 $\pm$ 0.03 mg/g extract). Lower concentrations of quercetin (1.56 $\pm$ 0.00 mg/g extract), o-coumaric acid (0.55 $\pm$ 0.02 mg/g extract), and ferulic acid (0.45 $\pm$ 0.00 mg/g extract) were detected.

Regarding antioxidant activity, the extract demonstrated strong radical-scavenging activity, with DPPH and ABTS values of 752.69 $\pm$ 1.35 and 923.75 $\pm$ 17.68 mM TE/g, respectively. Moreover, the ferric-reducing antioxidant power was 469.82 $\pm$ 18.19 mM Fe²⁺/g, reflecting its substantial electron-donating and reducing capacity.

Productive Performance

The effects of CSB supplementation on broiler growth performance are summarized in Table 4. Over the entire experimental period, initial body weight, final body weight, and average daily gain were not significantly influenced by the dietary treatments. By contrast, feed intake increased with increasing levels of CSB, as evidenced by significant linear and quadratic responses in ADFI ($p < 0.05$). Broilers receiving the CSB300 and CSB600 diets exhibited higher feed consumption than those fed the basal diet ($p < 0.05$). Nevertheless, the increased feed intake was not accompanied by improved feed efficiency; the FCR increased with increasing CSB inclusion, showing significant linear, quadratic, and cubic effects ($p < 0.05$).

Intestinal Morphology

Duodenal VH (DVH) exhibited significant quadratic and cubic responses ($p < 0.05$), with a gradual increase as CSB levels increased (Table 5 and Figure 1). Jejunal VH (JVH) was significantly affected by linear, quadratic, and cubic effects ($p < 0.05$), with the greatest value observed in the group receiving 150 mg/kg CSB. Similarly, ileal VH (IVH) showed significant linear, quadratic, and cubic responses ($p < 0.05$), with the highest value recorded at 300 mg/kg CSB.

Duodenal VW (DVW) was not significantly affected by CSB supplementation, with no significant linear, quadratic, or cubic responses. Similarly, jejunal VW (JVW) showed no significant dose-response effects. In contrast, ileal VW (IVW) exhibited a significant linear response, with the greatest values observed in birds receiving 300 and 600 mg/kg CSB.

CD was significantly affected by CSB in the duodenum, jejunum, and ileum, exhibiting linear,

Table 4. Productive performance of broiler chickens fed diets supplemented with *Caesalpinia sappan* bark extract (CSB)

Variables	Treatments				SEM	p value		
	CON	CSB150	CSB300	CSB600		Linear	Quadratic	Cubic
Initial body weight, g	65.71	65.89	65.66	65.04	0.22	0.22	0.32	0.53
Final body weight, g	1505.31	1550.73	1498.94	1522.83	12.99	0.89	0.94	0.51
ADG, g/day	44.99	46.4	44.79	45.56	0.36	0.91	0.95	0.51
ADFI, g/broiler/day	82.07 ^b	86.00 ^{ab}	88.60 ^a	89.48 ^a	0.9	0.00	0.01	0.02
FCR	1.83 ^b	1.86 ^{ab}	1.99 ^a	1.97 ^a	0.02	0.02	0.49	0.22

Note: CON = basal diet without CSB, CSB150 = 150 CSB mg/kg added to the diet, CSB300 = 300 CSB mg/kg added to the diet, CSB600 = 600 CSB mg/kg added to the diet. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio. ^{a,b} Means within a row with different superscripts differ significantly ($p < 0.05$).

Table 5. Intestinal morphology of broiler chickens fed diets supplemented with *Caesalpinia sappan* bark extract (CSB)

Variables	Treatments				SEM	p value		
	CON	CSB150	CSB300	CSB600		Linear	Quadratic	Cubic
Duodenum								
VH	881.85 ^b	971.06 ^a	988.49 ^a	1022.43 ^a	10.75	0.002	0.046	0.474
VW	164.96	153.63	124.52	147.63	4.51	0.06	0.19	0.13
CD	333.96 ^a	259.79 ^b	215.31 ^c	177.04 ^d	4.64	<0.001	0.80	0.67
VH/CD ratio	2.98 ^d	4.17 ^c	5.26 ^b	6.52 ^a	0.11	<0.001	0.19	0.42
Jejunum								
VH	769.59 ^b	894.87 ^a	795.79 ^b	792.20 ^b	11.31	0.24	0.03	0.007
VW	146.8	146.59	139.52	153.89	2.87	0.70	0.10	0.50
CD	223.60 ^a	183.24 ^b	164.76 ^{bc}	161.52 ^c	3.77	<0.001	0.12	0.31
VH/CD ratio	3.76 ^b	5.52 ^a	5.28 ^a	5.33 ^a	0.12	<0.001	0.01	0.37
Ileum								
VH	666.66 ^b	821.95 ^a	826.80 ^a	708.40 ^b	9.87	0.001	<0.001	0.16
VW	136.57 ^b	135.95 ^b	153.04 ^a	164.79 ^a	2.66	<0.001	0.42	0.50
CD	232.55 ^a	171.68 ^{bc}	185.16 ^b	159.81 ^c	3.16	<0.001	0.001	<0.001
VH/CD ratio	3.16 ^b	5.18 ^a	4.94 ^a	4.78 ^a	0.11	<0.001	<0.001	0.01

Note: VH = Villus height (μm), VW = Villus width (μm), CD = Crypt depth (μm), VH/CD ratio = Villus height/Crypt depth, CON = basal diet without CSB, CSB150 = 150 CSB mg/kg added to the diet, CSB300 = 300 CSB mg/kg added to the diet, CSB600 = 600 CSB mg/kg added to the diet. ^{a,b,c,d} Means within a row with different superscripts differ significantly ($p < 0.05$).

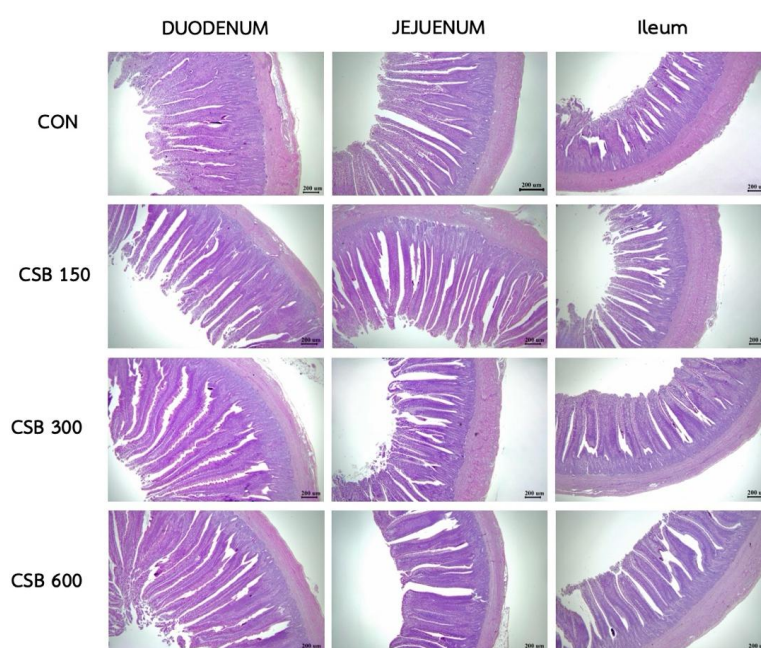


Figure 1. Intestinal morphology in broiler supplemented with *Caesalpinia sappan* bark extract (CSB) based on hematoxylin and eosin staining observed under 40x magnification. CON = basal diet without CSB, CSB150 = 150 CSB mg/kg added to the diet, CSB300 = 300 CSB mg/kg added to the diet, CSB600 = 600 CSB mg/kg added to the diet. Reference scale bar = 200 μm .

quadratic, and cubic responses ($p < 0.05$). In all intestinal segments, the control group had the highest CD values. By contrast, chickens receiving 600 mg/kg CSB showed the lowest CD throughout the small intestine.

The VH/CD ratio was affected by CSB level in a linear, quadratic, and cubic manner ($p < 0.05$). The ratio tended to increase with increasing levels of CSB inclusion. Significant linear, quadratic, and cubic effects were also observed in both the jejunum and ileum ($p < 0.05$), and chickens fed 150 mg/kg CSB had the highest VH/CD ratio.

PCA of Intestinal Morphology

Figure 2 shows the PCA used to evaluate the effect of different CSB levels on intestinal health and molecular defence mechanisms. PC1 and PC2 explained 49.47% of the total variance of the intestinal morphology analysis. The biplot showed a distinct separation between the control and the supplemented CSB groups. The control group was positively correlated with the variables ICD, JCD, and DCD. However, the CSB600 group showed strong correlations with DVHCD, DVH, DVW, and JVHCD, indicating that increased CSB supplementation significantly enhanced these intestinal structure parameters.

Immune- and Antioxidant-Related Gene Expression

Figures 3 and 4 show the expression of antioxidant- and immune-related genes in jejunal tissue of broilers after dietary supplementation with CSB. To examine the dose-response relationship of CSB inclusion levels, we performed additional orthogonal polynomial contrasts.

The transcript of antioxidant-related genes presented different responses to increasing levels of dietary CSB, as shown in Figure 3. CAT expression was significantly increased with increasing CSB

supplementation, with a significant linear effect ($p < 0.05$) and no quadratic or cubic response. A similar trend was observed for GPx, which showed a significant linear increase with increasing CSB levels ($p < 0.05$), indicating that higher CSB supplementation increased antioxidant defence mechanisms in the jejunal tissue. In contrast, SOD expression did not demonstrate a significant linear, quadratic, or cubic response to CSB supplementation. However, numerically higher values were observed in the CSB-supplemented groups than in the control group.

The expression patterns of immune-related genes are shown in Figure 4. The pro-inflammatory cytokines TNF- α and IL-12 decreased significantly as dietary CSB levels increased, showing a significant linear response ($p < 0.05$), whereas quadratic and cubic effects were not significant. This pattern suggests that CSB supplementation may attenuate inflammatory responses in the intestinal mucosa. Meanwhile, the expression levels of IL-1 β and IL-10 were not significantly affected by CSB supplementation, as no significant linear, quadratic, or cubic trends were observed, despite minor numerical variations among the treatment groups.

PCA of Gene Expression

Regarding the gene expression data (Figure 5), the first two principal components accounted for approximately 67.13% of the total variance (PC1: 41.61% and PC2: 25.52%). The results showed a strong positive correlation between the higher supplementation levels (CSB300 and CSB600) and the expression of antioxidant genes, including SOD, GPx, and CAT. Specifically, CSB600 was closely associated with CAT expression, whereas CSB300 was more closely associated with SOD and GPx expression. Conversely, the control group was positively associated with cytokine and immune-related markers, including IL-10, IL-12, and TNF- α .

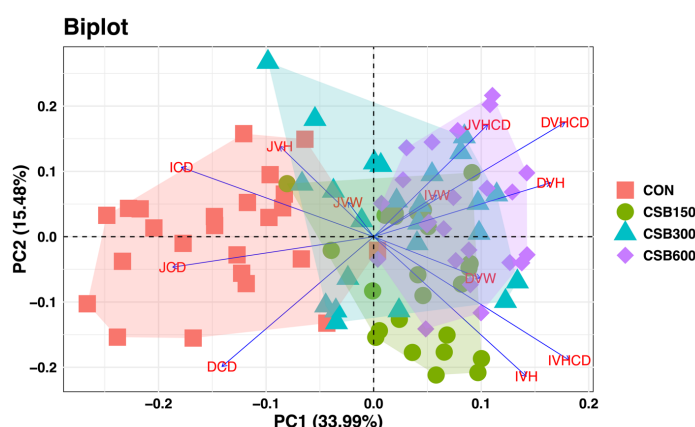


Figure 2. The chemometric PCA biplots of the intestinal morphology in different varieties of *Caesalpinia sappan* bark extract (CSB). CON = basal diet without CSB, CSB150 = 150 CSB mg/kg added to the diet, CSB300 = 300 CSB mg/kg added to the diet, CSB600 = 600 CSB mg/kg added to the diet, DCD = Duodenum crypt depth, DVH = Duodenum villus height, DVW = Duodenum villus width, Duodenum villus height : crypt depth, JCD = Jejunum crypt depth, JVH = Jejunum villus height, JVD = Jejunum villus depth, JVDW = Jejunum villus width, Jejunum villus height : crypt depth, ICD = Ileum crypt depth, IVH = Ileum villus height, IVW = Ileum villus width, Ileum villus height : crypt depth.

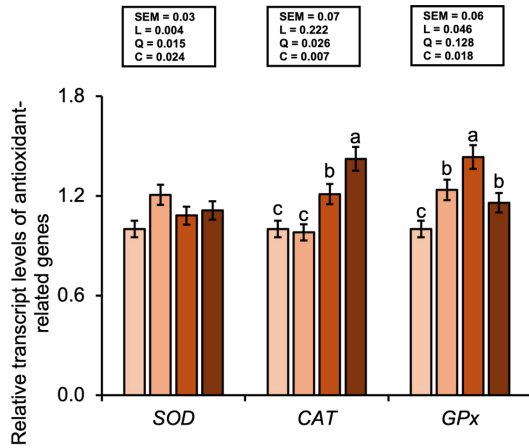


Figure 3. Relative transcript levels (means $\pm$ S.D.) of antioxidant-related genes for superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) from jejunum tissue of chicken at 35 days following diet supplementation with CSB ranging from 0 to 600 mg/kg diet. CON = basal diet without CSB, CSB150 = 150 CSB mg/kg added to the diet, CSB300 = 300 CSB mg/kg added to the diet, CSB600 = 600 CSB mg/kg added to the diet. ^{a,b,c} Means within a row with different superscripts differ significantly ($p < 0.05$). Note: $\square$ CON, $\square$ CSB150, $\square$ CSB300, $\square$ CSB600

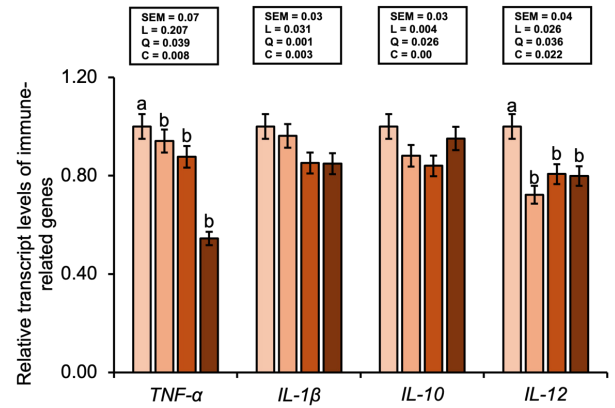


Figure 4. Relative transcript levels (means $\pm$ S.D.) of immune-related genes for tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-12 (IL-12), and interleukin-10 (IL-10) from jejunum tissue of chicken at 35 days following diet supplementation with *Caesalpinia sappan* bark extract (CSB) ranging from 0 to 600 mg/kg diet. CON = basal diet without CSB, CSB150 = 150 CSB mg/kg added to the diet, CSB300 = 300 CSB mg/kg added to the diet, CSB600 = 600 CSB mg/kg added to the diet. ^{a,b} Means within a row with different superscripts differ significantly ($p < 0.05$). Note: $\square$ CON, $\square$ CSB150, $\square$ CSB300, $\square$ CSB600

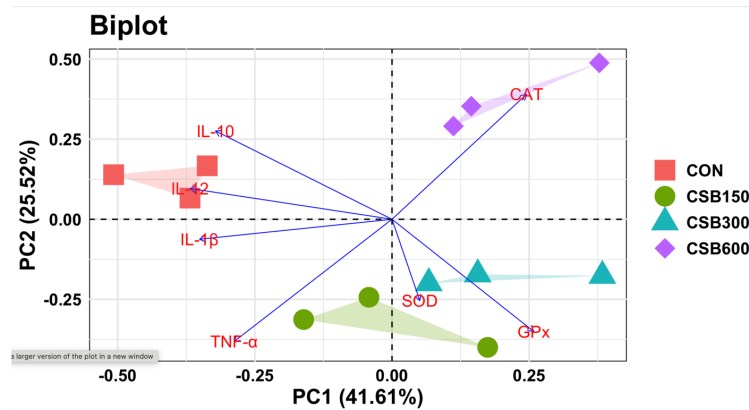


Figure 5. The chemometric PCA biplots of the gene expression in different varieties of CSB. CON= basal diet without CSB, CSB150= 150 CSB mg/kg added to the diet, CSB300= 300 CSB mg/kg added to the diet, CSB600= 600 CSB mg/kg added to the diet, CAT= catalase, SOD= superoxide dismutase, and GPx= glutathione peroxidase, IL-1 β = interleukin-1 β , IL-12= interleukin-12, TNF- α = tumor necrosis factor- α , and IL-10= interleukin-10.

Pearson Correlation Analysis

Pearson correlation analysis showed significant associations between intestinal morphology and gene-expression variables (Figure 6). CAT expression was positively correlated with DVHCD and IVW and negatively correlated with DCD and JCD. SOD expression was positively correlated with DVW and negatively correlated with ICD. TNF- α and IL-1 β were positively associated with DCD and negatively associated with DVHCD, while IL-12 was associated with ICD. These correlations indicate statistical relationships between intestinal structural characteristics and antioxidant- and immune-related transcriptional responses but do not establish causal or mechanistic links.

DISCUSSION

Phenolic Compounds and Antioxidant Activities of *Caesalpinia sappan* Bark Extract

The phytochemical investigation revealed that CSB has a high amount of phenolics (141.35 $\pm$ 13.80 mM GAE/g), indicating good radical scavenging capacity. Among the ingredients, brazilin was the most abundant bioactive compound, followed by naringin and rosmarinic acid, while o-coumaric acid, ferulic acid, and quercetin were present in lower concentrations. This increase in phenolics, especially brazilin, is in agreement with the findings of Arjin *et al.* (2021), who found ethanolic fractions of *Caesalpinia sappan* produce highly concentrated phytochemicals capable of

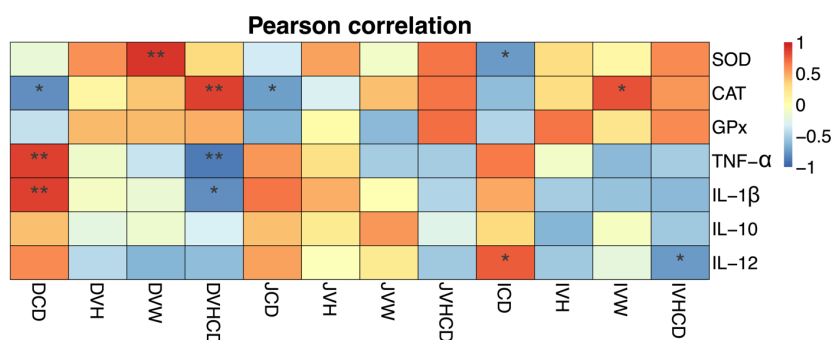


Figure 6. Pearson correlation coefficients among intestinal morphology variables and immune- and antioxidant-related gene-expression variables. DCD, duodenal crypt depth; DVH, duodenal villus height; DVW, duodenal villus width; DVHCD, duodenal villus height-to-crypt depth ratio; JCD, jejunal crypt depth; JVH, jejunal villus height; JWV, jejunal villus width; JVHCD, jejunal villus height-to-crypt depth ratio; ICD, ileal crypt depth; IVH, ileal villus height; IVW, ileal villus width; IVHCD, ileal villus height-to-crypt depth ratio; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1 beta; IL-10, interleukin-10; IL-12, interleukin-12. The asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$).

promoting cellular defence. These polyphenols are unlikely to function in isolation but rather in synergy to achieve the high antioxidant capabilities reported for DPPH, ABTS and FRAP experiments. Mechanistically, such a phytochemical profile provides a mechanistic rationale for the systemic and physiological responses of broilers fed dietary CSB, consistent with larger data that polyphenol-rich plant extracts enhance tissue redox stability in chicken (Biswas *et al.*, 2024; Chaiwang *et al.*, 2026).

Productive Performance

Dietary CSB supplementation had no significant effect on body weight or average daily gain, whereas ADFI increased with increasing CSB inclusion levels. This response may be associated with the aromatic characteristics of phytochemical supplements, which can influence feed palatability and voluntary feed intake (Abd El-Hack *et al.*, 2022). However, the increased feed intake was not accompanied by a corresponding improvement in body weight gain. Consequently, FCR increased at higher CSB supplementation levels, indicating reduced feed efficiency. These findings suggest that the additional feed consumed by birds receiving higher levels of CSB was not efficiently translated into body weight gain.

Interestingly, although CSB supplementation improved several intestinal morphological parameters and altered antioxidant- and immune-related gene expression, these responses did not result in improved growth performance. This finding suggests that improvements in selected intestinal and molecular indicators do not necessarily translate into enhanced productive performance under the present experimental conditions. The specific mechanisms underlying the increased feed intake and FCR at higher CSB supplementation levels could not be determined from the parameters measured in the present study and therefore warrant further investigation.

Furthermore, the final body weight observed in the present study was lower than the Ross 308 performance objectives under optimal commercial conditions (Aviagen, 2022). This difference may be attributable to several factors, including experimental housing, handling and sampling procedures, local environmental conditions, and tropical heat load, as heat stress is known to impair growth efficiency and alter nutrient utilization in broiler chickens (Emami *et al.*, 2021; Nawab *et al.*, 2018).

Intestinal Morphology

CSB supplementation in the diet improved several parameters of intestinal morphology. VH was higher in the duodenum, jejunum, and ileum, but CD was lower in all intestinal segments than in the control group. Moreover, supplementation with CSB increased the VH/CD ratio, suggesting improved intestinal integrity and absorptive capacity. Also, residues of *Cannabis sativa* improved intestinal morphology and ileal VH/CD ratio in broilers (Sopian *et al.*, 2024).

However, CSB supplementation did not show favourable effects on all measures of intestinal morphology. The highest DVW was in the control group, and JWV did not differ between dietary treatments. The results indicated that CSB supplementation had a selective effect on the intestinal structural parameters and did not improve all the morphological characteristics.

The antioxidant capacity of phenolic compounds from CSB, especially brazilin, may be related to the improvement of intestinal architecture. Oxidative stress in the intestinal microenvironment causes enterocyte injury and increases epithelial turnover, which decreases the efficiency of nutrient absorption. Antioxidant properties of CSB may have decreased oxidative damage and the biological cost of continuous epithelial repair, so more energy could be used for maintenance and development of healthy villous structures (Longchuphon *et al.*, 2024).

PCA of Intestinal Morphology

The principal component analysis (PCA) of intestinal morphology (Figure 2) clearly distinguished the control group from the CSB-supplemented treatments, with PC1 and PC2 collectively capturing 49.47% of the total variance. Notably, the control group exhibited a distinct clustering alongside crypt depth parameters across all intestinal segments (ICD, JCD, and DCD). In gastrointestinal physiology, deeper crypts often signify heightened epithelial turnover, a compensatory mechanism triggered by mucosal irritation or pathogen challenge that drains energetic resources away from muscular growth (Osho & Adeola, 2020).

Conversely, inclusion of CSB at 600 mg/kg (CSB600) shifted the morphological profile toward enhanced absorptive architecture, displaying strong positive correlations with DVH, DVW, DVHCD, and JVHCD. The observed expansion in duodenal villus dimensions directly expands the functional surface area for nutrient uptake and brush-border enzymatic activity (Gilani *et al.*, 2021). Furthermore, elevated villus height-to-crypt depth ratios (VH:CD) in both the duodenum and jejunum underscore a well-preserved enterocyte lining with minimal cell turnover costs. Together, these structural adaptations indicate that dietary CSB600 fortifies mucosal integrity, effectively converting metabolic energy from tissue repair toward improved growth performance and feed efficiency in animals (Ducatelle *et al.*, 2018).

Immune- and Antioxidant-Related Gene Expression

Dietary CSB supplementation was associated with increased expression of the antioxidant-related genes CAT and GPx in jejunal tissue. The linear responses of these transcripts with increasing CSB inclusion suggest a dose-related transcriptional response. Because the present study measured mRNA expression rather than antioxidant enzyme activity or oxidative-stress biomarkers, these findings should be interpreted as transcriptional evidence rather than direct demonstration of enhanced antioxidant capacity. Such responses may be relevant in fast-growing broilers, in which high metabolic activity can increase oxidative pressure in intestinal tissues (Syamsunarno *et al.*, 2021).

Conversely, CSB supplementation did not significantly affect SOD expression, but numerically higher values were obtained in the CSB-treated groups than in the control group. The enhanced antioxidant responses obtained in the present study may be related to phenolic components of CSB, especially brazilin and related compounds with free radical scavenging activity (Tasuksai *et al.*, 2025). Similar improvements in intestinal health and physiological responses have been seen in broilers supplemented with other phenolic-rich phytochemical extracts such as coffee pulp extract (Huanhong *et al.*, 2025a).

CSB supplementation was also associated with lower jejunal expression of the pro-inflammatory cytokines TNF- α and IL-12 as inclusion increased.

This transcriptional pattern is consistent with potential modulation of inflammatory signalling by phytochemical compounds in poultry (Hassan *et al.*, 2019). However, IL-1 β and IL-10 were not significantly affected, indicating that the observed response was gene-specific rather than a generalized suppression of immune-related transcription.

PCA of Gene Expression

The principal component analysis of intestinal gene expression (Figure 5) revealed a distinct segregation between treatment groups, with PC1 and PC2 accounting for 67.13% of the total variance. A clear divergence was observed between the control and CSB-supplemented groups regarding cellular defence and immune modulation. The control group clustered closely with pro-inflammatory and regulatory cytokines (IL-12, TNF- α , and IL-10), indicating a basal level of immune activation or low-grade mucosal inflammation that demands energetic trade-offs at the expense of growth performance (Mishra & Jha, 2019; Surai *et al.*, 2019).

In contrast, the higher CSB inclusion levels were associated with a transcriptional profile characterized by antioxidant-related genes. CSB300 was aligned with SOD and GPx, whereas CSB600 was closely associated with CAT expression. These PCA relationships are exploratory and should not be interpreted as direct evidence of enzyme activity, Nrf2 activation, or protection against oxidative damage. Further studies measuring antioxidant enzyme activities, oxidative-stress biomarkers, and relevant signalling pathways are required to clarify the underlying mechanisms.

Pearson Correlation Analysis

Pearson correlation analysis identified associations between intestinal structural variables and antioxidant- and immune-related gene expression (Figure 6). For example, CAT expression was positively correlated with DVHCD and IVW and inversely correlated with DCD and JCD, whereas SOD expression was positively associated with DVW and negatively associated with ICD. TNF- α and IL-1 β were positively associated with DCD and negatively associated with DVHCD. These findings indicate coordinated statistical patterns between morphology and transcriptional responses; however, correlation does not demonstrate that changes in intestinal architecture caused the observed gene-expression responses or vice versa.

CONCLUSION

Dietary CSB supplementation improved several intestinal morphology parameters and modulated selected antioxidant- and immune-related gene transcripts in the jejunum, while body weight and average daily gain were unchanged. Higher inclusion levels (300-600 mg/kg) produced clearer antioxidant- and anti-inflammatory transcriptional responses but were accompanied by increased FCR, whereas 150 mg/

kg improved several intestinal morphology measures without a clear molecular advantage over the control. These findings demonstrate a dose-dependent trade-off between biological responses and feed efficiency, highlighting the need for further dose-response studies to determine an appropriate CSB inclusion level that provides beneficial intestinal and molecular responses without compromising productive performance.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

AI-assisted tools were used only for language and grammar editing.

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