Poultry Science

Dietary honokiol supplementation improves antioxidant capacity, enhances intestinal health, and modulates cecal microbial composition and function of broiler chickens

Encun Du ,[*] Meihan Jiang,[*,†] Fang Chen,[*] Qiwen Fan,[*] Shuangshuang Guo,[*,†] Na Zhao,[*] Feng Jin,[*] Wanzheng Guo,[*] Shaowen Huang,[*] and Jintao Wei[*,1]

[*]*Hubei Key Laboratory of Animal Embryo and Molecular Breeding, Institute of Animal Science and Veterinary Medicine, Hubei Academy of Agricultural Sciences, Wuhan 430064, P. R. China; and* [†]*Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, P. R. China*

ABSTRACT Honokiol is a multifunctional polyphenol present in *Magnolia officinalis*. The effects of honokiol as a supplement in broiler chicken diets, and the underlying mechanisms, remain unclear. Therefore, the aim of the present study was to investigate the effects of honokiol on the growth performance, antioxidant capacity, and intestinal histomorphology of broiler chickens and to explore the underlying mechanisms. In total, 240 one-day-old broilers were randomly allocated to 5 dietary treatments, with 6 replicate pens and 8 birds per pen. Birds were fed a basal diet supplemented with 0 (blank control, **BC**), 100, 200, or 400 mg/kg honokiol (**H100, H200**, and **H400**), or 200 mg/kg bacitracin zinc (**PC**) for 42 d. The results showed that H200 and H400 increased body weight gain (**BWG**) and decreased feed conversion ratio (**FCR**) during the starter period ($P < 0.05$). H100 and H200 increased total superoxide dismutase (**T-SOD**) activity in the serum and decreased malondialdehyde (**MDA**) amount in the jejunum on d 42 ($P < 0.05$). Moreover, H100 increased villus height-to-crypt depth ratio in both the jejunum and ileum on d 21 ($P < 0.05$). PCR analysis showed that honokiol upregulated intestinal expression of *glutathione peroxidase* (***GSH-Px***) and downregulated intestinal expression of *inducible nitric oxide synthase* (***iNOS***) on d 42 ($P < 0.05$). The Shannon index, which represents the microbial alpha diversity, was reduced for the PC, H200, and H400 groups. Notably, honokiol treatment altered the cecal microbial community structure and promoted the enrichment of several bacteria, including *Firmicutes* and *Lactobacillus*. Higher production of short-chain fatty acids was observed in the cecal digesta of H100 birds, accompanied by an enriched glycolysis/gluconeogenesis pathway, according to the functional prediction of the cecal microbiota. This study provides evidence that honokiol improves growth performance, antioxidant capacity, and intestinal health of broiler chickens, possibly by manipulating the composition and function of the microbial community.

Key words: antioxidant capacity, gut microbiota, honokiol, intestinal health, short-chain fatty acid

2024 Poultry Science 103:103798
https://doi.org/10.1016/j.psj.2024.103798

INTRODUCTION

Over recent decades, strong artificial selection, high dietary energy input, and intensive poultry farming have remarkably increased the growth rate of broilers and significantly reduced the breeding costs for commercial breeders (Zuidhof et al., 2014). However, these activities exert a heavy metabolic burden and trigger oxidative stress and inflammatory reactions in broilers (Lauridsen, 2019), promoting dysbiosis of the intestinal microbiota (Furukawa et al., 2004; Goyette et al., 2007). Emerging evidence has shown that intestinal microbiota plays an important role in production performance, overall health, and resistance to microbial infections in poultry (Liu et al., 2023). With the removal of in-feed antibiotic growth promoters (**AGPs**), the development of alternative AGPs is highly desirable for improving intestinal health and enhancing disease resistance in the modern poultry industry (Gadde et al., 2017).

Owing to consumer preferences for natural products, phytogenic feed additives, such as plant extracts and essential oils, have attracted increased attention (Zeng et al., 2015). Houpu magnolia (*Magnolia officinalis*) is extensively cultivated in China, Japan, and Korea and

Received February 1, 2024.
Accepted April 22, 2024.

[1]Corresponding author: jintao001@126.com

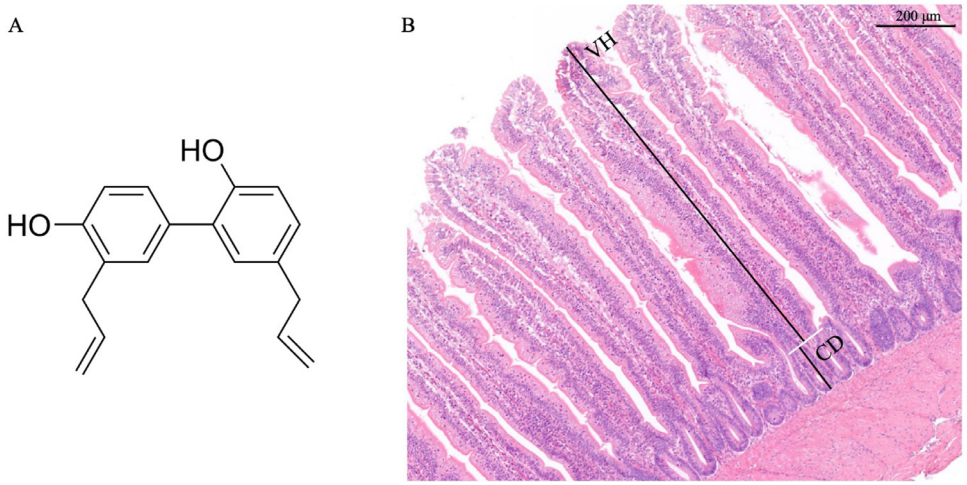

Figure 1. Chemical structure of honokiol (A) and morphometric measurement of the intestine (B). VH, villus height; CD, crypt depth. Bar: 200 μm.

has been used in traditional Chinese medicine for the treatment of digestive disorders, asthmatic cough, and depression (Niu et al., 2021). Honokiol (molecular formula $C_{18}H_{18}O_2$, chemical structure shown in Figure 1A) and its isomer, magnolol, are extracted from the root and stem bark of Houpu magnolia. Honokiol and magnolol are the major active components of magnolia extract (Lee et al., 2011). Previous studies have demonstrated that honokiol and magnolol have multiple functions, and possess antioxidant, anti-inflammatory, antibacterial, antidepressant, and anticancer properties (Niu et al., 2021).

Several recent studies have identified the positive effects of magnolia bark extract and magnolol as feed additives on the performance, antioxidant activity, and immunity of poultry diets (Oh et al., 2018; Du et al., 2021; Xie et al., 2022; Mo et al., 2023). In an experimental model of necrotic enteritis induced by co-infection with *Clostridium perfringens* and *Eimeria maxima*, supplementation with magnolia bark extract improved growth performance and decreased gut lesions of broiler chickens, partially by upregulating antioxidant enzyme transcript levels (Oh et al., 2018). Magnolol has also been reported to ameliorate the clinical symptoms of coccidiosis, enhance cecum health, and improve the growth performance of broiler chickens infected with *Eimeria tenella* (Mo et al., 2023).

However, the effects of honokiol in broiler chickens remain unclear. Considering that honokiol and magnolol are isomers and honokiol has an even stronger antioxidant capacity than magnolol (Zhao and Liu, 2011), it is hypothesized that honokiol supplementation may be an effective strategy to improve intestinal health and production efficiency in the modern poultry industry. Hence, the objective of the present study was to investigate the effects of various levels of honokiol supplementation on the growth performance, antioxidant capacity, and intestinal histomorphology of broiler chickens and to explore the underlying mechanisms. The potential of honokiol as a substitute for AGPs was studied by comparing its effects with antibiotics.

MATERIALS AND METHODS

Experimental Animals and Diets

The experimental protocol was approved by the Animal Care and Use Committee of Hubei Academy of Agricultural Sciences.

A total of 240 one-day-old male broiler chicks (Arbor Acre) with similar body weights (**BW**) were randomly allocated to 5 treatment groups, and each group consisted of 6 replicate pens (8 birds per pen). The 5 treatment groups included blank control (**BC**), positive control (**PC**), and 3 honokiol-treated groups (**H100, H200**, and **H400**, respectively). All chickens were fed starter (d 0−21) and grower (d 22−42) diets in mash form. Birds in group BC were fed antibiotic- and coccidiostat-free basal diets, which were formulated to meet or exceed the feeding standards of China for broilers (Ministry of Agriculture of the People's Republic of China, 2004). Diet composition and nutrient levels of the basal diets are shown in Table 1. Birds in the PC group were fed a basal diet supplemented with 200 mg/kg of an antibiotic additive (10% bacitracin zinc premix, Shandong Mingzheng Biotech Co., Ltd., Jining, China), whereas birds in the H100, H200, and H400 groups were fed a basal diet supplemented with 100, 200, and 400 mg/kg honokiol, respectively. Honokiol, with a purity greater than 98%, was extracted from Houpu Magnolia (manufactured by ChengDu ConBon Biotech Co., Ltd., Chengdu, China).

Animal Management

All chicks were reared on floor pens (1.0 m x 0.8 m) in an environmentally controlled facility at the research base of Hubei Academy of Agricultural Sciences. Birds had free access to food and water throughout the feeding trials. The birds received 24 h of light on the first day and then 23L:1D for the remaining days. The room temperature was maintained at 32°C for the first 3 d post-hatch, then gradually decreased by 0.5°C per day until

Table 1. Diet composition and nutrient levels of the basal diets (air-dry basis).

Ingredients (g/kg)	d 0−21	d 22−42	Calculated nutrient levels	d 0−21	d 22−42
Maize	555.3	610.1	Metabolizable energy (Kcal/kg)	2.89	2.97
Soybean meal	376.0	322.0	Crude Protein (%)	20.50	18.60
Soybean oil	25.0	29.0	Calcium (%)	1.01	0.90
Dicalcium phosphate	19.0	16.5	Available Phosphorus (%)	0.45	0.40
Limestone	12.4	11.4	Lysine (%)	1.15	1.00
Sodium chloride	3.5	3.5	Methionine (%)	0.50	0.40
Choline chloride (50%)	3.0	3.0	Threonine (%)	0.81	0.72
DL-Methionine (98%)	2.1	1.3			
L-Lysine hydrochloride (78%)	0.8	0.5			
L-Threonine(98.5%)	0.4	0.2			
Trace mineral premix [1]	2.0	2.0			
Vitamin premix [2]	0.2	0.2			
Santoquin	0.3	0.3			
Total	1,000.0	1,000.0			

[1]The trace mineral premix provided the following (per kilogram of diet): manganese, 100 mg; zinc, 75 mg; iron, 80 mg; copper, 8 mg; selenium, 0.25 mg; iodine, 0.35 mg.

[2]The vitamin premix supplied the following per kilogram of complete feed: vitamin A, 12,500 IU; vitamin D3, 2500 IU; vitamin E, 30 IU; vitamin K3, 2.65 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 4.5 mg; vitamin B12, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

reaching a final temperature of 22 to 24°C. The general health of all birds was assessed twice daily.

Growth Performance

All birds were weighed individually after their arrival from the hatchery to the experimental farm (initial body weight) and on d 21 and 35. The body weight gain (**BWG**) was also measured for each dietary group. The feed consumption of each pen was recorded to calculate the average feed intake (**AFI**) and feed conversion ratio (**FCR**).

Sample Collection

On d 21 and 42, one bird per replicate was selected, weighed, and slaughtered via jugular exsanguination. Blood samples were collected and centrifuged at 3,000 rpm for 15 min to separate the serum samples. The liver, spleen, and bursa of Fabricius were removed and weighed to determine organ indices. A segment of approximately 1 cm taken from the proximal part of the jejunum (between the bile duct entry and Meckel's diverticulum) was dissected, flushed with phosphate buffer solution, snap frozen in liquid nitrogen and stored at -80°C for mRNA expression analysis. Another 1-cm section from the proximal part of the jejunum and ileum (between the Meckel's diverticulum and ileocecal junction) was collected and fixed in 4% paraformaldehyde for morphological analysis. Jejunal mucosa, ileal mucosa, and cecal digesta were collected from the rest of the intestine and snap frozen in liquid nitrogen before storage at -20°C.

Antioxidant Capacity

The total superoxide dismutase (**T-SOD**) activity and the amount of malondialdehyde (**MDA**) in the serum and intestinal mucosal homogenate were measured using commercial assay kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China), according to the manufacturer's instructions.

Intestinal Histomorphology

For histological observations, 6 birds were used for each group (n = 6) and one section was evaluated for each bird. After fixation in 4% paraformaldehyde for 48 h, the intestinal tissues were embedded in paraffin and sectioned. Three sections (3 μm) from different locations on the intestinal segment were placed on every slide. The tissue sections were stained with hematoxylin and eosin. Ten complete and vertically-oriented intestinal villi from each slide were randomly selected to measure the villus height (**VH**), crypt depth (**CD**) using an Olympus optical microscope with a micro-image processing system (Shineso, Hangzhou, China). VH was measured from the tip of the villus to the villus-crypt junction, whereas CD was defined as the depth of invagination between the adjacent villi (Figure 1B).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from jejunal tissue using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After determining the purity and concentration on a spectrophotometer (NanoPhotometer N60, Implen Inc., München, Germany), 1 μg of total RNA was reverse-transcribed to complementary DNA by PrimeScript RT reagent kit (RR047A, Takara Biomedical Technology, Shiga, Japan). The transcript levels of *glutathione peroxidase* (***GSH-Px***), *superoxide dismutase 1* (***SOD1***), *inducible nitric oxide synthase* (***iNOS***), apoptosis-related genes (*Caspase3, Bcl2*, and *Bax*), and tight junction protein-related genes (*ZO1, Claudin1*, and *Occludin*) were expressed as the relative expression to β-actin gene using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for real-time PCR are presented in Table 2.

Table 2. Primers used for quantitative real-time PCR.

Targets	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
Antioxidant-related gene		
GSH-Px	TTGTAAACATCAGGGGCAAA	ATGGGCCAAGATCTTTCTGTAA
SOD1	AGGGGGTCATCCACTTCC	CCCATTTGTGTTGTCTCCAA
Inflammation-related gene		
iNOS	CCTGTACTGAAGGTGGCTATTGG	AGGCCTGTGAGAGTGTGCAA
Apoptosis-related gene		
Caspase3	TGGCCCTCTTGAACTGAAAG	TCCACTGTCTGCTTCAATACC
Bcl2	GATGACCGAGTACCTGAACC	CAGGAGAAATCGAACAAAGGC
Bax	TCCTCATCGCCATGCTCAT	CCTTGGTCTGGAAGCAGAAGA
Tight junction protein-related gene		
ZO1	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCATGGCTGGATC
Claudin1	CATACTCCTGGGTCTGGTTGGT	GACAGCCATCCGCATCTTCT
Occludin	ACGGCAGCACCTACCTCAA	GGGCGAAGAAGCAGATGAG
Housekeeping gene		
β-actin	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA

Abbreviations: GSH-Px, glutathione peroxidase; SOD1, superoxide dismutase 1; iNOS, inducible nitric oxide synthase; Bcl-2, B-cell lymphoma 2 gene; Bax, Bcl-2-associated X gene; ZO1, zona occluden 1.

Real-time PCR was performed using the TB Green Premix Ex Taq II (RR820A; Takara Biomedical Technology). Each 20 μL of the PCR reaction contained 10 μL of the supplied 2 × SYBR Green, 6 μL of nuclease-free water, 1 μL of each primer (0.5 μM final concentration each), and 2 μL of templates (the RT reaction mixture containing the original cDNA). The real-time PCR was run on a LightCycler 96 PCR System (Roche Life Science, Munich, Germany) with the following cycle parameters: an initial denaturation at 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s, followed by a melting curve ranging from 60°C to 95°C. The PCR efficiency of each primer pair was verified using the standard curve method. The PCR efficiency for each primer for each gene used in the present study was $100 \pm 5\%$ with an $R^2 \geq 0.99$.

DNA Extraction, PCR Amplification of 16S rRNA Gene and Sequencing

Genomic DNA was extracted from cecal microbiota using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The concentration of DNA was determined using spectrophotometry, and its quality was evaluated using 1% agarose gel electrophoresis. The microbial 16S rRNA gene was amplified using indexed and adaptor-linked universal primers (341F: ACTCCTACGGGAGGCAGCAG and 806R: GGACTACHVGGGTWTCTAAT) targeting the V3−V4 region. The PCR products were visualized using 2% agarose gel electrophoresis and purified using a Qiagen gel extraction kit (Qiagen Inc.). Purified amplicons were pooled at equal concentrations and sequenced using the pair-end method on an Illumina Miseq250 platform (Illumina Inc., San Diego, CA).

For the 16S rRNA gene analysis, the raw reads obtained were first merged into sequences based on the relationship between their overlaps, and poor or low-quality sequences were discarded. The obtained sequences were then aligned into operational taxonomic units (**OTUs**) and analyzed using the VSEARCH software version 1.9.6 (VSEARCH GitHub repository) based on 97% sequence similarity. The alpha diversity of the microbiota was estimated using the Chao1, Ace, Shannon, and Simpson indices in QIIME (version 1.9.1; https://qiime.org). Principal coordinate analysis (**PCoA**) based on the weighted UniFrac distance was performed to represent the beta diversity, which illuminates the species complexity of the microbial community. Analysis of similarity (**ANOSIM**) was used to determine significant differences between the 2 groups of samples. An R-value > 0.75 with a P-value < 0.05 denotes groups completely different from one another; 0.50 < R-value < 0.75 with a P-value < 0.05 denotes groups different from one another; 0.30 < R-value < 0.50 with a P-value < 0.05 denotes groups that tended to be different from one another; and an R-value < 0.30 denotes groups that were not different from one another. Representative OTU sequences were compared with the Silva_132 16S rRNA database (http://www.arb-silva.de/) using the RDP Classifier for taxonomic classification (at an 80% confidence threshold) at the kingdom, phylum, class, order, family, and genus levels.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (**PICRUSt**) was used to analyze the metagenomes, which were predicted based on the Greengenes database. The predicted microbial KEGG ortholog profiles were analyzed using t-tests.

Determination of Bacterial Metabolites

As important metabolites of intestinal flora, the concentration of short-chain fatty acids (**SCFA**) was analyzed using gas chromatography (Agilent 6890; Agilent Technologies, Palo Alto, CA) as described previously (García-Villalba et al., 2012).

Correlation Analysis

Spearman's rank correlations between the relative abundance of cecal bacteria and SCFA concentrations were analyzed using R software (R Foundation for

Table 3. Effect of various amounts of honokiol on the growth performance of broiler chickens.

Item	BC	PC	H100	H200	H400	SEM	*P* value		
							Treatment	Linear	Quadratic
Initial BW/g	46.7	46.8	46.7	46.8	46.7	0.1	0.969	0.837	0.745
d 0-21									
AFI/g	875.1	911.4	858.0	887.2	913.0	9.8	0.310	0.156	0.533
BWG/g	511.8[b]	572.8[ab]	534.6[ab]	587.8[a]	582.3[a]	10.5	0.084	0.012	0.222
FCR	1.72[a]	1.61[ab]	1.61[ab]	1.51[b]	1.57[b]	0.02	0.016	0.004	0.004
d 22-42									
AFI/g	2815.3	2929.7	2824.9	2844.0	2971.3	23.2	0.132	0.035	0.429
BWG/g	1470.1	1474.9	1417.5	1424.9	1479.3	15.4	0.585	0.702	0.183
FCR	1.92	1.99	2.00	2.00	2.02	0.02	0.525	0.192	0.425
d 0-42									
AFI/g	3690.3	3838.9	3682.8	3705.3	3883.9	30.5	0.111	0.039	0.295
BWG/g	1981.9	2037.9	1952.1	1982.5	2061.7	21.2	0.496	0.177	0.383
FCR	1.87	1.89	1.89	1.87	1.89	0.01	0.971	0.746	0.918

[a,b]Mean values within the same row not sharing a common superscript letter differ significantly ($P < 0.05$). Values are means of 6 replicates per treatment. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.Abbreviations: AFI, average feed intake; BW, body weight; BWG, average body weight gain; FCR, feed conversion ratio; SEM, pooled standard error.

Statistical Computing, Vienna, Austria) and the psych package. The correlations were then plotted using the pheatmap package in the R software.

Statistical Analysis

The results are presented as mean values and pooled standard errors (**SEM**). Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test, using SPSS software (version 20.0; IBM Inc., Armonk, NY). Polynomial contrasts were performed to determine the linear and quadratic responses of the defined characteristics to different honokiol concentrations. Differences were considered statistically significant at $P < 0.05$. A linear discriminant analysis (**LDA**) and LDA effect size (**LEfSe**) analysis (http://huttenhower.sph.harvard.edu/lefse/) were performed to identify biomarkers for each group. An LDA score of 4 was used to identify bacterial groups with statistical significance.

RESULTS

Growth Performance

The growth performance of broiler chickens is shown in Table 3. During d 0 to 21, birds fed the 200 or 400 mg/kg honokiol-supplemented diet demonstrated higher BWG ($P < 0.05$) and lower FCR ($P < 0.05$) than birds fed the non-supplemented diet. During d 0 to 21, the BWG of broilers increased linearly ($P = 0.012$) and the FCR decreased both linearly and quadratically ($P = 0.004$ and $P = 0.004$, respectively) with increasing supplemental honokiol levels.

Dietary treatment had no influence on the growth performance of broilers during d 22 to 42 or d 0 to 42 ($P > 0.05$), except for a linear increase in the AFI with increasing supplemental honokiol levels ($P = 0.035$ and $P = 0.039$, respectively). No significant difference in growth performance was found between the PC group and the honokiol-supplemented groups ($P > 0.05$).

Organ Indices

The addition of honokiol did not affect liver, spleen, or bursa of Fabricius indices before d 21 (Table 4, $P > 0.05$). With increasing supplemental honokiol levels, the bursa of Fabricius index increased linearly and quadratically on d 21 ($P = 0.017$ and $P = 0.007$, respectively). The bursa of Fabricius index of the H200 group was higher than that of the H100 group on d 21 ($P < 0.05$). On day 42, the liver index of H400 birds was lower than that of PC birds ($P < 0.05$).

Table 4. Effect of various amounts of honokiol on the organ indices of broiler chickens (%).

Item	BC	PC	H100	H200	H400	SEM	*P* value		
							Treatment	Linear	Quadratic
d 21									
Liver	3.33	3.53	3.35	3.91	3.83	0.10	0.203	0.030	0.337
Spleen	0.11	0.11	0.11	0.09	0.11	0.00	0.750	0.934	0.401
Bursa of Fabricius	0.26[ab]	0.29[ab]	0.23[b]	0.30[a]	0.25[ab]	0.01	0.025	0.017	0.007
d 42									
Liver	2.09[ab]	2.23[a]	1.95[ab]	1.97[ab]	1.95[b]	0.03	0.028	0.246	0.388
Spleen	0.19	0.21	0.17	0.18	0.15	0.01	0.166	0.713	0.416
Bursa of Fabricius	0.07	0.06	0.07	0.05	0.06	0.00	0.096	0.126	0.119

[a,b]Mean values within the same row not sharing a common superscript letter differ significantly ($P < 0.05$). Values are means of 6 replicates per treatment. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.Abbreviations: SEM, pooled standard error.

Table 5. Effect of various amounts of honokiol on the antioxidant capacity of broiler chickens.

							P value		
Item	BC	PC	H100	H200	H400	SEM	Treatment	Linear	Quadratic
T-SOD (U/mL for serum, U/mg protein for intestinal mucosa)									
d 21									
Serum	333.46[bc]	323.59[c]	395.90[ab]	409.09[a]	407.72[a]	11.48	0.020	0.025	0.069
Jejunum	310.07	371.55	335.60	329.31	327.72	9.47	0.347	0.640	0.462
Ileum	312.72	343.14	321.61	321.08	321.98	7.69	0.803	0.732	0.778
d 42									
Serum	355.74[b]	368.35[ab]	434.65[a]	432.69[a]	350.65[b]	12.77	0.028	0.201	0.001
Jejunum	403.83	372.56	445.16	413.67	437.7	18.35	0.737	0.699	0.831
Ileum	488.95	504.56	624.08	561.62	522.12	25.56	0.458	0.810	0.190
MDA (nmol/mL for serum, nmol/mg protein for intestinal mucosa)									
d 21									
Serum	6.55[a]	5.89[ab]	5.29[bc]	4.43[c]	4.49[c]	0.19	< 0.001	0.004	0.824
Jejunum	0.12	0.15	0.16	0.15	0.14	0.01	0.229	0.362	0.069
Ileum	0.16	0.16	0.17	0.15	0.15	0.01	0.634	0.217	0.907
d 42									
Serum	5.63	4.8	4.99	5.18	4.9	0.12	0.360	0.242	0.714
Jejunum	0.87[a]	0.71[b]	0.67[b]	0.64[b]	0.69[b]	0.03	0.048	0.100	0.028
Ileum	0.72	0.62	0.75	0.91	0.71	0.04	0.272	0.939	0.177

[a,b,c]Mean values within the same row not sharing a common superscript letter differ significantly ($P < 0.05$).Values are means of 6 replicates per treatment. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.Abbreviations: MDA, malondialdehyde; SEM, pooled standard error, T-SOD, total superoxide dismutase.

Antioxidant Capacity

T-SOD activity and MDA levels in the serum and intestinal mucosa are shown in Table 5. On d 21, higher serum T-SOD activity and lower serum MDA levels were observed in birds fed the 200 or 400 mg/kg honokiol-supplemented diet compared with birds fed the non-supplemented diet or the antibiotic-treated diet ($P < 0.05$). Serum T-SOD activity increased linearly ($P = 0.025$), whereas MDA decreased linearly ($P = 0.004$) with increasing honokiol levels on d 21.

Serum T-SOD activity exhibited a quadratic response to dietary honokiol levels on d 42 ($P = 0.001$), and higher serum T-SOD activity was found in the 100 and 200 mg/kg honokiol-supplemented groups than in the non-supplemented diet control group ($P < 0.05$).

For intestinal mucosa, the jejunal MDA amount was lower in all treated groups compared with the nonsupplemented diet control group on d 42 ($P < 0.05$). Jejunal MDA levels showed a quadratic response to dietary honokiol on d 42 ($P = 0.028$).

Intestinal Histomorphology

On d 21, compared to the non-supplemented diet control group, the VH of the ileum was significantly higher in the 100 and 200 mg/kg honokiol-supplemented groups ($P < 0.05$, Table 6), and the VH/CD ratios in both the jejunum and ileum were significantly higher in the 100 mg/kg honokiol-supplemented group ($P < 0.05$). In addition, the jejunal VH/CD ratio of birds fed the 100 mg/kg honokiol-supplemented diet was higher than

Table 6. Effect of various amounts of honokiol on the intestinal histomorphology of broiler chickens.

							P value		
Item	BC	PC	H100	H200	H400	SEM	Treatment	Linear	Quadratic
VH, μm									
d 21 Jejunum	957.20	946.77	973.87	1045.48	1015.18	23.43	0.649	0.379	0.411
d 21 Ileum	608.14[b]	659.90[ab]	725.45[a]	734.40[a]	675.18[ab]	14.96	0.040	0.039	0.021
d 42 Jejunum	1100.74	1031.23	1015.81	1056.95	1055.87	14.26	0.408	0.708	0.289
d 42 Ileum	772.35	703.82	734.76	678.28	781.32	18.74	0.366	0.696	0.137
CD, μm									
d 21 Jejunum	211.73	242.99	166.15	236.76	209.54	9.38	0.070	0.426	0.693
d 21 Ileum	125.24[b]	128.13[b]	128.76[b]	156.79[a]	126.41[b]	3.35	0.007	0.141	0.117
d 42 Jejunum	318.17[a]	258.41[b]	258.64[b]	267.68[ab]	286.95[ab]	7.97	0.080	0.890	0.063
d 42 Ileum	158.80	159.00	150.77	144.43	156.29	5.46	0.914	0.927	0.357
VH/CD, μm/μm									
d 21 Jejunum	4.82[b]	4.40[b]	6.54[a]	4.59[b]	5.15[b]	0.21	0.007	0.509	0.694
d 21 Ileum	5.00[b]	5.33[ab]	5.86[a]	4.82[b]	5.45[ab]	0.12	0.033	0.603	0.281
d 42 Jejunum	3.69	4.16	4.23	4.17	3.78	0.11	0.441	0.624	0.186
d 42 Ileum	5.09	4.90	4.99	4.90	5.38	0.16	0.883	0.442	0.562

[a,b]Mean values within the same row not sharing a common superscript letter differ significantly ($P < 0.05$). Values are means of 6 replicates per treatment. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.Abbreviations: CD, crypt depth; SEM, pooled standard error; VH, villus height.

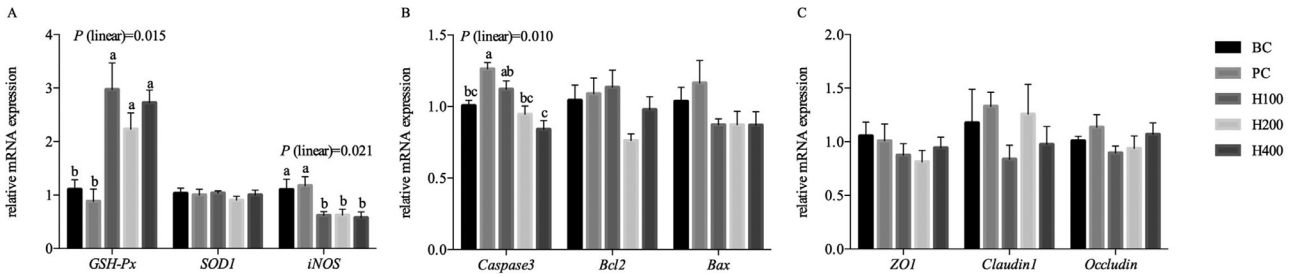

Figure 2. Effect of various amounts of honokiol on the jejunal gene expression of broiler chickens. Column charts not sharing a common lowercase letter differ significantly ($P < 0.05$). Data are presented as mean ± standard error (n = 6). Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.

that of birds fed the antibiotic-treated diet ($P < 0.05$). Ileal VH displayed both linear and quadratic responses to dietary honokiol levels on day 21 ($P = 0.039$ and $P = 0.021$, respectively).

On d 42, the CD of the jejunum was significantly decreased in the 100 mg/kg honokiol-supplemented group and the antibiotic-treated group compared to the non-supplemented diet control group ($P < 0.05$); however, there was no difference in the VH or VH/CD ratio ($P > 0.05$).

Intestinal Gene Expression

In contrast to the non-supplemented diet control and the antibiotic-treated groups, the honokiol-supplemented groups (H100, H200, and H400) showed up-regulated jejunal expression of *GSH-Px* ($P < 0.05$, Figure 2) and down-regulated gene expression of *iNOS* ($P < 0.05$) on d 42. *Caspase3* expression was up-regulated in the antibiotic-treated group ($P < 0.05$), whereas the 200 and 400 mg/kg honokiol-supplemented groups showed down-regulated *Caspase-3* expression compared with the antibiotic-treated group ($P < 0.05$). Furthermore, with increasing honokiol levels, *GSH-Px* expression was linearly up-regulated ($P = 0.015$), whereas *iNOS* and *Caspase 3* expression were linearly down-regulated ($P = 0.021$ and $P = 0.010$, respectively).

Honokiol supplementation did not influence the expression of *SOD1, Bcl2, Bax*, or tight junction protein-related genes in the jejunum ($P > 0.05$).

Cecal Microbiota

The α-diversity of cecal microbiota is shown in Figure 3A. Compared to birds fed the non-supplemented diet, the Shannon index of the cecal microbiota was lower in birds fed diets supplemented with antibiotic or with 200 or 400 mg/kg honokiol ($P < 0.05$). Shannon and Simpson indices decreased linearly with increasing honokiol supplementation ($P = 0.004$ and $P = 0.016$, respectively).

The PCoA (Figure 3B) showed that microbial communities of the honokiol-supplemented groups were clustered separately from those of the antibiotic-treated group, and principal coordinates 1 and 2 accounted for 45.68% and 12.81% of the total variation, respectively.

ANOSIM results showed that the cecal microbial community of the 400 mg/kg honokiol-supplemented group differed from that of the non-supplemented diet control group ($0.3 < R$-value < 0.5, $P < 0.05$, Table 7). The cecal microbial community in the 200 mg/kg honokiol-supplemented group differed from that in the antibiotic-treated group ($0.3 < R$-value < 0.5, $P < 0.05$). In addition, the cecal microbial communities in the 100 and 400 mg/kg honokiol-treated groups differed from that in the antibiotic-treated group ($0.5 < R$-value < 0.75, $P < 0.05$).

Bar-plot analysis of the microbial community showed that *Firmicutes* was dominant in all groups at the phylum level (Figure 4A). At the family level, *Lachnospiraceae* was dominant, followed by *Ruminococcaceae, Streptococcaceae, Methanobacteriaceae*, and *Oscillospiraceae* (Figure 4B).

LEfSe plots (Figure 5) revealed that *Oscillospiraceae* was significantly enriched in the non-supplemented diet control group, whereas *Methanobrevibacter* (from kingdom to genus) were significantly enriched in the antibiotic-treated group. *Firmicutes* and 3 groups of bacteria belonging to *Firmicutes* were significantly enriched in the 100 mg/kg honokiol-treated group: *Oscillospirales, Ruminococcaceae*, and *Lactobacillus* (from family to genus). *Rikenellaceae_RC9_gut_group* was significantly enriched in the 200 mg/kg honokiol-supplemented group, whereas *Lactobacillales* and *Streptococcus* (from family to genus) were significantly enriched in the 400 mg/kg honokiol-supplemented group.

A heatmap was created based on the top 35 third-level KEGG pathway classifications identified in the cecal samples (Figure 6A). The predictive functional profiles of the non-supplemented diet control and the 100 mg/kg honokiol-supplemented group were clustered together, while those of the 200 and 400 mg/kg honokiol-supplemented groups were clustered together. Simultaneously, the antibiotic-treated group was separated into one cluster with the other 4 groups. Compared with birds fed the non-supplemented diet, pathways related to glycolysis/gluconeogenesis and drug metabolism−other enzymes were increased in birds fed the 100 mg/kg honokiol-supplemented diet ($P < 0.05$), whereas pathways related to phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, and cell motility (e.g., bacterial motility proteins, bacterial chemotaxis,

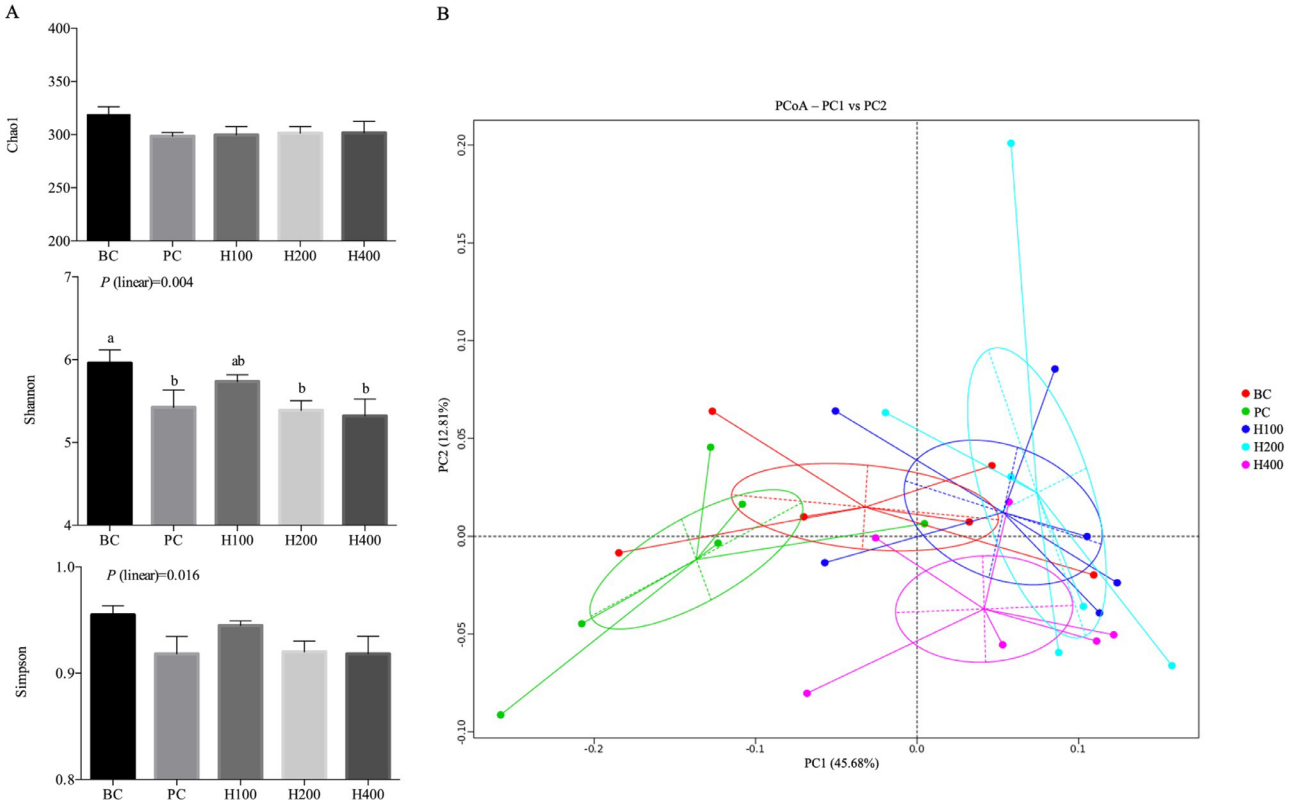

Figure 3. Effect of various amounts of honokiol on the cecal microbial community of broiler chickens. (A) Alpha diversity. Column charts not sharing a common lowercase letter differ significantly ($P < 0.05$). Data are presented as mean ± standard error (n = 6). (B) Principal coordinate analysis (PCoA) based on weighted UniFrac distance. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.

and flagellar assembly) were decreased ($P < 0.05$, Figure 6B). In contrast to birds fed the antibiotic-treated diet, birds fed the 100 mg/kg of honokiol supplemented diet displayed significantly elevated pathways related to carbohydrate metabolism (e.g., amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis, starch and sucrose metabolism, galactose metabolism), lipid metabolism (e.g. lipid biosynthesis proteins and fatty acid metabolism), and replication and repair (e.g., DNA repair and recombination proteins, chromosomes, homologous recombination, and DNA replication; $P < 0.05$) (Figure 6C). In contrast, pathways related to methane metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, and cell motility were decreased in the 100 mg/kg honokiol-supplemented group compared with the antibiotic-treated group ($P < 0.05$).

Bacterial Metabolites

As shown in Figure 7, the cecal concentrations of propionic acid, butyric acid, and total SCFAs were higher in birds fed the 100 mg/kg honokiol-supplemented diet than in birds fed the non-supplemented diet on d 42 ($P < 0.05$). The 100 mg/kg honokiol-supplemented group also showed enhanced production of propionic acid and total SCFAs, compared with the antibiotic-treated and 400 mg/kg honokiol-supplemented groups ($P < 0.05$). The highest concentration of acetic acid was found in birds fed the antibiotic-supplemented diet, which exceeded that of birds fed the non-supplemented and the 400 mg/kg honokiol-supplemented diets ($P < 0.05$).

Relationship Between the Bacterial Community and SCFAs

We identified 14 significant associations between genus abundance and SCFA concentrations (Figure 8). Spearman's correlation analysis showed that *CHKCI001*

Table 7. *R*- and *P*-values of pairwise comparison of the cecal microbial community using analysis of similarity (ANOSIM).[1]

R-values	BC	PC	H100	H200	H400
BC	0				
PC	0.030	0			
H100	0.107	0.632*	0		
H200	0.122	0.320*	0.011	0	
H400	0.487*	0.617*	0.282	-0.015	0

*Asterisk represents the pairwise comparison with a *P*-value < 0.05. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.

[1]*R*-value > 0.75 with a *P*-value < 0.05 denotes groups that are completely different from one another; 0.50 < *R*-value < 0.75 with a *P*-value < 0.05 denotes groups that are different from one another; 0.30 < *R*-value < 0.50 with *P*-value < 0.05 denotes groups that tend to be different from one another; and an *R*-value < 0.30 denotes groups that are not different from one another.

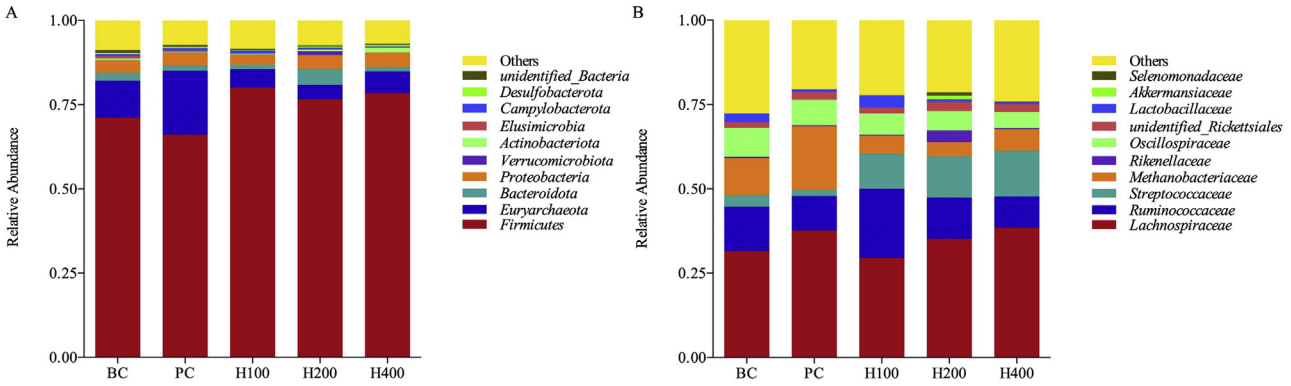

Figure 4. Effect of various amounts of honokiol on the cecal microbiol composition of broiler chickens. (A) Microbial composition at phylum level (n = 6). (B) Microbial composition at family level (n = 6). Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.

was positively correlated ($P < 0.05$) and *Romboutsia, UCG.005*, and *Christensenellaceae_R.7_group* were negatively correlated with valeric acid ($P < 0.05$). *Akkermansia* showed a negative correlation ($P < 0.05$) and *Lachnoclostridium* showed a positive correlation with acetic acid and total SCFAs ($P < 0.05$). *Megamonas, Shuttleworthia*, and *Erysipelatoclostridium* were positively correlated with isovaleric acid ($P < 0.05$). Among the 35 most abundant genera, *Spiroplasma* showed a positive correlation with isobutyric acid ($P < 0.05$), while *Erysipelatoclostridium* showed a negative correlation with propionic acid ($P < 0.05$).

DISCUSSION

Honokiol, a natural polyphenol, possesses remarkable antioxidant and anti-inflammatory properties (Niu et al., 2021). A previous study reported that the extract of *Magnolia officinalis* bark improved the AFI and BWG of broiler chickens infected with *Eimeria maxima*/*Clostridium perfringens*, at a dosage of 0.33 or 0.56 mg/kg (Oh et al., 2018). In addition, we previously reported an improved laying rate in hens fed 300 mg/kg honokiol in the late phase of the laying cycle (Chen et al., 2022). Similarly, the present study demonstrated linearly

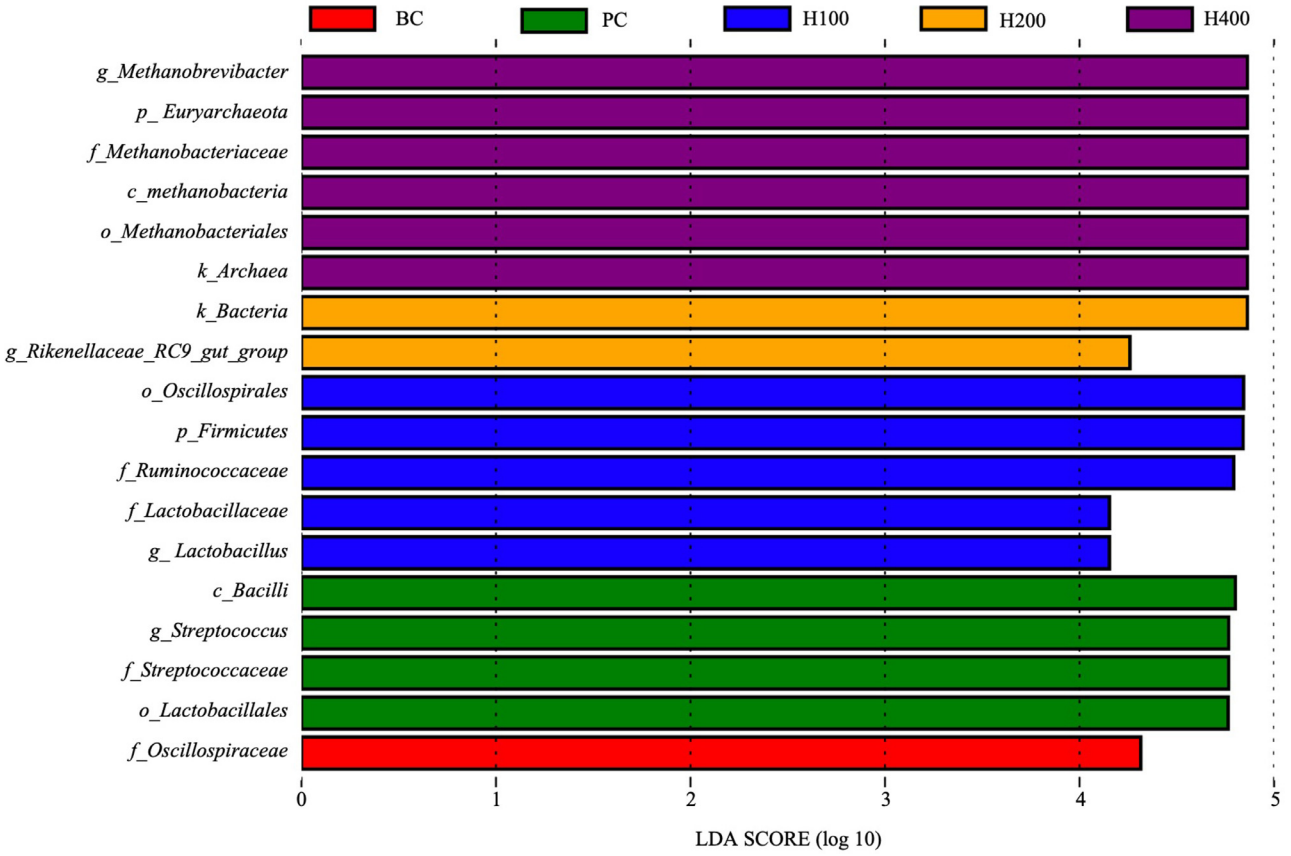

Figure 5. Comparison of the bacteria variations using LEfSe analysis. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol. LEfSe, linear discriminant analysis effect size.

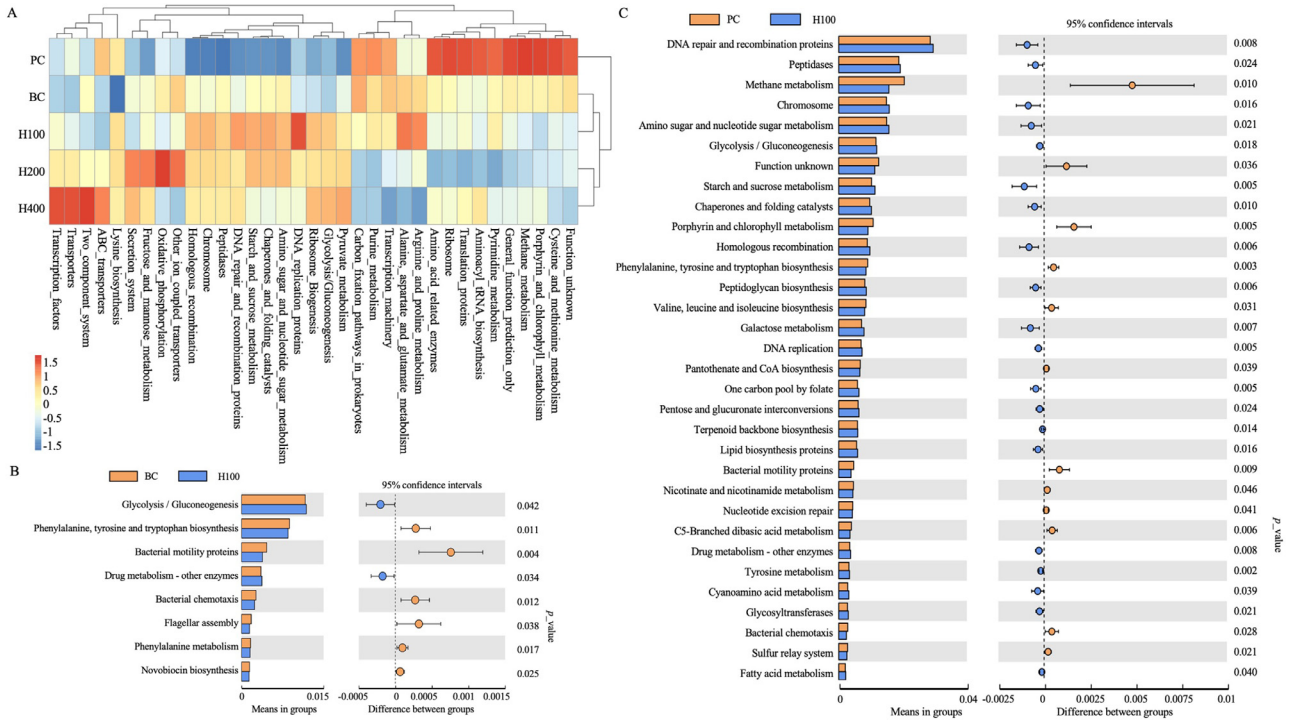

Figure 6. Predicted microbial function of the cecal microbiota. (A) The heatmap of predicted microbial function by PICRUSt based on the top 35 third-level classification KEGG pathways. (B and C) Comparisons of cecal microbial functions for different treatments. Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol. PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states.

increased BWG and feed efficiency (indicated by FCR) during d 0 to 21 in broiler chickens, implying a linearly improved capacity for digestion and absorption with increasing honokiol levels during the starter period. In addition, the growth performance of the honokiol-supplemented groups was similar to that of the AGP-supplemented groups. These data suggest that honokiol is a potential candidate for improving the growth performance of broiler chickens during the starter period.

Several studies have shown that honokiol has excellent antioxidant activity in in vitro and in vivo models of liver and kidney injury in rodents (Zhao and Liu, 2011; Xia et al., 2019). It can effectively reduce ONOO- and $^1O_2$, scavenge free radicals, and prevent lipid peroxidation (Niu et al., 2021). Moreover, the antioxidant activity of honokiol is reported to be 1000 times higher than that of α-tocopherol (Zhao and Liu, 2011). In an experimental model of necrotic enteritis, transcripts of the antioxidant enzymes SOD1, catalase, and heme oxygenase 1 increased in chickens fed diets supplemented with Magnolia bark extract (Oh et al., 2018). In accordance with previous studies, supplementation with various amounts of honokiol improved the antioxidant capacity of broiler chickens in the present study, as evidenced by increased T-SOD activity and decreased MDA content in the serum and intestinal mucosa.

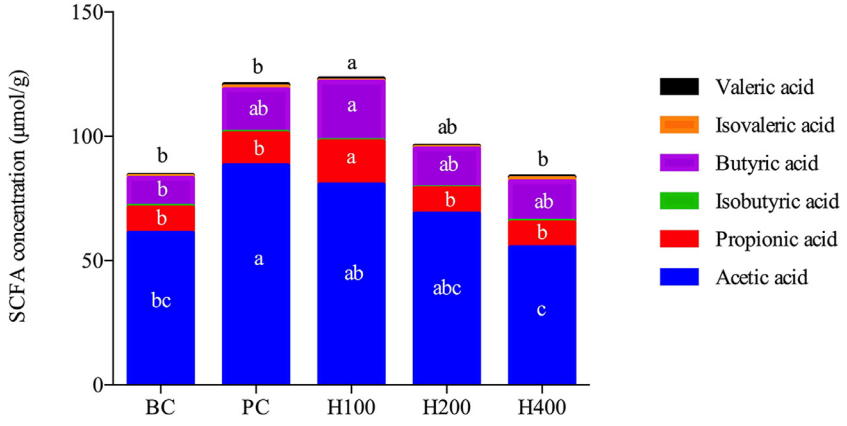

Figure 7. Effect of various amounts of honokiol on the concentrations of short-chain fatty acids in the cecal digesta of broiler chickens. Values are means ($n = 6$), and column charts not sharing a common lowercase letter differ significantly ($P < 0.05$). Dietary treatments are as follows: BC, basal diet; PC, basal diet + bacitracin zinc; H100, basal diet + 100 mg/kg honokiol; H200, basal diet + 200 mg/kg honokiol; H400, basal diet + 400 mg/kg honokiol.

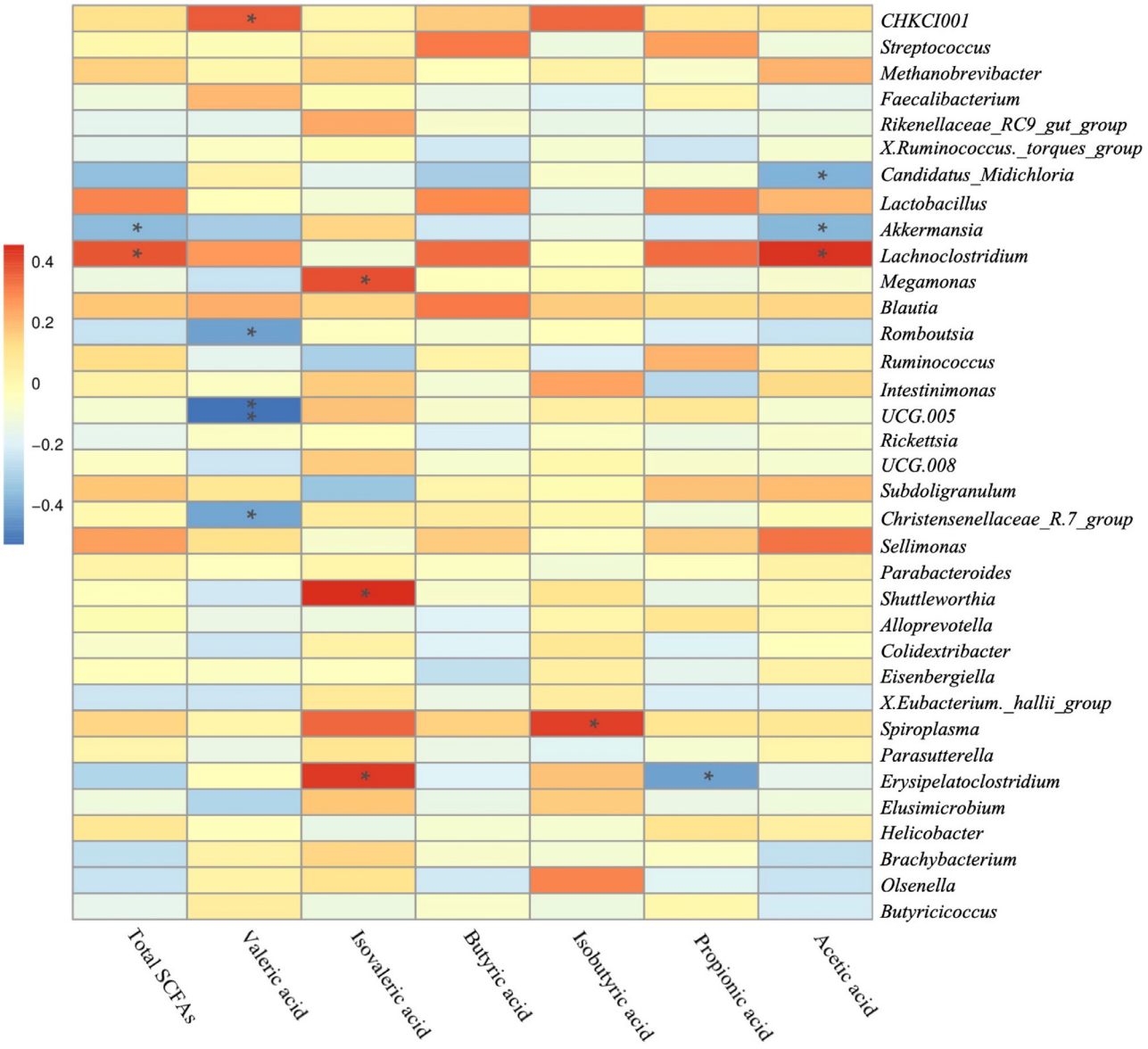

Figure 8. Correlation analysis between short chain fatty acid concentrations and relative abundance of the top 35 genera in the cecal digesta of broiler chickens. Colors refer to the degree of correlation. * $P < 0.05$, ** $P < 0.01$.

Moreover, jejunal gene expression of *GSH-Px* was upregulated in the honokiol-supplemented groups. Therefore, apart from its direct antioxidant effect, honokiol may play a favorable role against oxidative stress in broilers by enhancing the gene expression of antioxidant enzymes.

The VH, CD, and VH/CD ratio are regarded as sensitive indicators of the development and function of the gut (Zhao et al., 2020). Longer villi indicate increased intestinal surface area for nutrient digestion and absorption. In an enterotoxigenic *Escherichia coli* (**ETEC**)-induced diarrhea mouse model, both honokiol and magnolol were found to elongate intestinal VH and CD and reduce the number of goblet cells to protect the intestinal mucosa (Deng et al., 2018). In laying hens, honokiol and magnolol have also been reported to increase the VH in the jejunum and ileum (Chen et al., 2022). In the current study, honokiol consistently increased the ileal VH and VH/CD ratios of broilers on d 21. This might be one of the main reasons why dietary honokiol increased the BWG and decreased the FCR of broilers during the starter period. Houpu magnolia and its main components are well known for their preventive and therapeutic effects on digestive system diseases, such as regulation of gastrointestinal motility and gastrointestinal dysfunction (Niu et al., 2021). However, the specific mechanism by which honokiol and magnolol promote the development of intestinal villi remains unclear. It may involve a variety of factors, such as immunity, hormones, and intestinal flora, which require further study.

Nitric oxide (**NO**) demonstrates diverse biological activities including anti-inflammatory effects (Yu et al., 2015). It is generally accepted that iNOS is responsible for the increased NO synthesis in tissues during inflammatory processes (Keklikoglu et al., 2008). Hence, *iNOS* gene expression inhibitors should be considered potential anti-inflammatory agents. A previous study indicated that the administration of honokiol improved the

survival of rats with sepsis by inhibiting the production of NO and reducing the protein levels of iNOS in the kidneys (Li et al., 2014). Similarly, supplementation with various amounts of honokiol was found to down-regulate jejunal *iNOS* gene expression in the current study, indicating the inhibition of inflammation in the intestine of broilers. Apoptosis and proliferation are important mechanisms for updating epithelial cells to maintain normal construction and function of the intestine (Der Flier and Clevers, 2009). Meanwhile, abnormal apoptosis can lead to various diseases and intestinal dysfunctions. Our study showed a linear down-regulation of the pro-apoptosis gene, *Caspase-3*, with increasing honokiol levels, indicating an anti-apoptotic state of the jejunal epithelial cells. Honokiol inhibits hydrogen peroxide ($\mathbf{H_2O_2}$)-induced apoptosis in myoblasts by inhibiting the expression of *Bax, Caspase-3*, and *Caspase-9* (Park et al., 2019). Moreover, nanosized liposome-encapsulated honokiol efficiently attenuates cisplatin-induced chronic renal damage by reducing kidney inflammation, oxidative stress, and *Caspase-3*-associated cellular apoptosis (Liu et al., 2019). The intestinal histomorphology and gene expression results indicated that honokiol benefits intestinal construction and function in broilers.

The microbiota contributes to the development and maintenance of the intestinal epithelial barrier, nutrient absorption, productivity, immunity, and competition with pathogenic microorganisms (Honda & Littman, 2012). As stated earlier, oxidative stress and inflammation in the gut are closely related to the dysbiosis of intestinal microbiota and metabolic disorders (Furukawa et al., 2004; Goyette et al., 2007). To further explore the mechanism by which honokiol promotes intestinal construction and function in broilers, the intestinal microbiota and bacterial metabolites were analyzed. Results showed that *Firmicutes* was the dominant phylum in the cecal microbiota and *Firmicutes* was more abundant in the 100 mg/kg honokiol-treated group. *Firmicutes* plays an important role in polysaccharide decomposition and contribute to the maintenance of intestinal homeostasis and health (Ley et al., 2006). In addition, a reduction in the FCR of broilers is reportedly accompanied by an increased abundance of *Firmicutes* (Singh et al., 2012; Wang et al., 2017), which implies a positive correlation between *Firmicutes* abundance and feed efficiency. At the family level, enrichment of *Ruminococcaceae* and *Lactobacillaceae* (*Lactobacillus* genus in particular) was observed in chickens fed diets supplemented with 100 mg/kg honokiol. *Ruminococcaceae* are commonly found in animal intestines and metabolize indigestible cellulose and hemicellulose to SCFAs. *Lactobacillus*, a typical probiotic bacterium, is widely used in poultry production and reportedly protects intestinal integrity, modulates immune responses, and improves intestinal development (Li et al., 2018; Bogucka et al., 2019). It can be speculated that the enrichment of *Firmicutes, Ruminococcaceae* and *Lactobacillus* as a result of honokiol addition may also be involved in improved intestinal construction and function. *Rikenellaceae_RC9_gut_group* is an uncultivated bacterium. In the intestines of diarrheic goats, the relative abundance of *Rikenellaceae_RC9_gut_group* is significantly reduced compared with that in healthy goats (Wang et al., 2018). *Rikenellaceae* are associated with resistance to the development of colitis following cytotoxic T-lymphocyte-associated antigen-4 blockade and can limit inflammation by stimulating T-regulatory cell differentiation (Dubin et al., 2016). In the current study, the enriched *Rikenellaceae_RC9_gut_group* in the 200 mg/kg honokiol-treated group may also be associated with the inhibition of inflammation in the intestines of broilers. In addition, honokiol promoted the enrichment of the order *Lactobacillales* and genus *Streptococcus* at a high dose (400 mg/kg). The intestinal *Streptococcus* population exhibited high phenotypic variability. Although some can cause diseases, most *Streptococci* species are ecologically important components of the normal microbial flora of animals and humans, and are capable of producing butyrate (van den Bogert et al., 2013).

In the current study, the cecal microbiota structures of the honokiol-supplemented groups were clearly different from those of the antibiotic-treated group, and the biomarker of the antibiotic-treated group was *Methanobrevibacter*, a major methanogen found in human and animal intestines. By converting bacterial primary and secondary fermentation products, such as hydrogen and carbon dioxide, to methane, *M. smithii*, the predominant *Methanobrevibacter*, is essential for syntrophic metabolism within the intestinal microbial community and thus plays a crucial role in energy balance (Bang et al., 2014). One study reported that rats that gained more weight had more extensive intestinal colonization of *Methanobrevibacter smithii* than those that gained less weight (Mathur et al., 2013). Another species, *M. stadtmanae*, has pro-inflammatory properties and has been reported to increase the prevalence in inflammatory bowel disease (Blais Lecours et al., 2014). Whether the enhanced expression of *Caspase3* is related to the enrichment of *Methanobrevibacter* in broilers fed diets containing bacitracin-zinc remains to be studied.

Predicted functions of the cecal microbiota revealed an enriched glycolysis/gluconeogenesis pathway in the 100 mg/kg honokiol-treated group. The glycolysis/gluconeogenesis pathway in non-ruminant animals is closely related to SCFAs production (Yang et al., 2021). SCFAs are the main metabolites produced by intestinal bacteria. In addition to their long-known role as energy sources for intestinal epithelial cells, SCFAs play a potent role in maintaining intestinal barrier integrity, mucus production, and regulation of inflammatory responses (Silva et al., 2020). Results showed that dietary supplementation with 100 mg/kg honokiol increased the concentrations of propionic acid, butyric acid, and total SCFAs in the cecum, which partially explains the beneficial effects of honokiol on the intestinal health of broilers. In addition, honokiol supplementation increased the number of pathways related to carbohydrate metabolism, and replication and repair, compared to the antibiotic-treated group. These

pathways may play important roles in broiler health and productivity.

CONCLUSIONS

In conclusion, the results of our study indicate that dietary supplementation with honokiol could improve the growth performance of broilers during the starter period and enhance their antioxidant capacity and intestinal health. The beneficial effects of honokiol may, at least partially, be attributed to changes in the composition and function of the cecal microbial community. These findings suggest that honokiol may be an alternative to AGP for poultry production. Considering the changes in antioxidant capacity, intestinal histomorphology, gene expression, and cecal microbial community composition and function, we recommend 100 mg/kg honokiol supplementation in the diet of broiler chickens.

ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (32102579), the Natural Science Foundation of Hubei Province (2022CFB396), the Key Research and Development Plan of Hubei Province (2022BBA0014), and the project of Hubei Agricultural Science and Technology Innovation Center (2021-620-000-001-021).

DISCLOSURES

The authors declare no conflicts of interest.

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