Effects of encapsulated cinnamaldehyde on growth performance, intestinal digestive and absorptive functions, meat quality and gut microbiota in broiler chickens

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ABSTRACT: Essential oils are potential antimicrobial alternatives and their applications in animal feeds are limited due to their fast absorption in the upper gastrointestinal tract. This study investigated the effects of encapsulated cinnamaldehyde (CIN) at 50 mg/kg or 100 mg/kg on the growth performance, organ weights, meat quality, intestinal morphology, jejunal gene expression, nutrient digestibility, and ileal and cecal microbiota. A total of 320 male day-old broiler Cobb-500 chicks were randomly allocated to four treatments with eight pens per treatment (10 birds per pen): 1) basal diet (negative control, NC); 2) basal diet supplemented with 30 mg/kg avilamycin premix (positive control, PC); 3) basal diet with 50 mg/kg encapsulated CIN (EOL); 4) basal diet with 100 mg/kg encapsulated CIN (EOH). Despite birds fed EOH tended to increase ($P = 0.05$) meat pH at 24 h, all pH values were normal. Similar to PC group, meats from birds fed EOL and EOH showed a reduced ($P < 0.05$) Warner–Bratzler force shear (WBFS) compared to the NC group. The highest villus to crypt ratios (VH/CD; $P < 0.05$) were observed in broilers fed either EOL or EOH, with an average of 14.67% and 15.13% in the duodenum and 15.13% and 13.58% in the jejunum, respectively. For jejunal gene expressions, only six out of the 11 studied genes showed statistically significant differences among the dietary treatments. Gene expressions of cationic amino acid transporter 1 (CAT-1) and neutral amino acid transporter 1 (B^0AT-1) were upregulated in EOH-fed birds compared to PC and NC-fed birds ($P < 0.05$), respectively; while the expression of proliferating cell nuclear antigen (PCNA) was downregulated in EOL-fed birds when compared to NC birds ($P < 0.05$). Nonetheless, the expressions of cadherin 1 (CDH-1), zonula occludens 1 (ZO-1), and maltase-glucoamylase (MG) were all upregulated ($P < 0.05$) in EOH-fed birds compared to PC-fed birds. The apparent ileal digestibility (AID) of dry matter, crude protein, crude fat and of all 18 tested amino acids increased in EOL-fed birds ($P < 0.01$). Additionally, relative abundances (%) of ileal Proteobacteria decreased, while ileal and cecal *Lactobacillus* increased in EOH-fed birds ($P < 0.05$). In conclusion, dietary encapsulated CIN improved meat quality and gut health by reducing meat WBFS, increasing VH/CD in intestines, jejunal gene expressions, AID of nutrients and beneficial ileal and cecal microbiota composition.

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INTRODUCTION

Poultry products are essential components of a well-balanced human diet due to their nutritional richness in highly digestible proteins, B-group vitamins, and minerals (Marangoni et al., 2015). In Canada, it has been reported that broiler meats accounted for a large portion of total poultry products with over 130,000 metric tons in 2019 (Bedford, 2019). However, broiler could be a reservoir of pathogens such as *Eimeria* spp., *Clostridium perfringens*, nontyphoidal *Salmonella enterica* serovars, and extraintestinal pathogenic *Escherichia coli* (ExPEC) (Craven et al., 2001; Bergeron et al., 2012; Györke et al., 2013). These pathogens can be transmitted horizontally from feces or feathers to healthy birds through contaminated feeds, drinking water or beddings, and vertically from infected maternal breeders to their offspring (Liljebjelke et al., 2005). Additionally, due to the increased qualitative aspects of poultry meats, advanced management of poultry industry requires feed additives to promote growth performance, reduce incidences of infections, and improve meat quality (da Silva et al., 2017). Traditionally, antimicrobials were supplemented in feed as sub-therapeutic antimicrobial growth promoters (AGP) to prevent infections and improve performance (Wellenreiter et al., 2000). However, the overuse and misuse of antimicrobials have been linked to the development of antimicrobial resistances (Ventola, 2015). Thus, Chicken Farmers of Canada (CFC) eliminated category I antibiotics in 2014 and the preventive use of Category II antibiotics in 2018 and proposed to stop the use of category III antibiotics by end of 2020 (Chicken Farmers of Canada, 2020). However, withdrawal of AGP in feed may have adverse effects on broiler chicken such as reduction in performance and may pose food safety issues (Kumar et al., 2018). Consequently, it is necessary to explore antimicrobial alternatives such as probiotics, prebiotics, organic acids, and plant extracts for improving chicken production and health (Casewell et al., 2003; Osman et al., 2013).

Essential oils (EOs) are aromatic and volatile liquids extracted mainly from plants by steam distillation (Preedy, 2015). It has been reported that EOs from star anise oil, ginko biloba, and oregano had beneficial impacts on enhancing nutrient utilization, immunity, and liver antioxidant status in broilers (Galal et al., 2016; Ding et al., 2017; Ren et al., 2018). Encapsulated cinnamaldehyde and citral alone or in combination were shown to reduce necrotic enteritis (NE), improve chicken growth performance as bacitracin, and beneficially alter cecal microbiota compositions (Yang et al., 2020). However, limited studies were conducted on the effects of cinnamon oil on performance, meat quality, and intestinal histology and microbiota of broilers. Cinnamaldehyde (CIN; from *Cinnamonmum*) has been used for food flavorings and medications for many years without receiving much attention of their potential effects as antimicrobial alternatives on broilers (Burt, 2004; Nabavi et al., 2015). CIN powder had shown antimicrobial activities against some other pathogenic bacteria including *Listeria monocytogenes* and *Bacillus cereus* in laboratory media and rice cakes (Hong et al., 2013). It also demonstrated high antimicrobial efficacy against *C. perfringens*, *S. typhimurium* DT104, *E. coli* O157: H7, and enterotoxigenic *Escherichia coli* (ETEC) with little inhibition towards *Lactobacillus* and *Bifidobacterium* in vitro (Si et al., 2006; Si et al., 2009). However, CIN should not be applied directly to broilers as feed additives due to their instability during feed processing and gastric transition (Tian et al., 2016). In this study, the CIN was encapsulated by the soy protein polysaccharide reaction products to maintain its stability during prolonged storage,

feed processing, gastric transitions (Yang et al., 2015). It was hypothesized that encapsulated CIN can promote growth performance, gut health, and meat quality of broiler chickens. The objective of this study was to evaluate the effects of encapsulated CIN at 50 mg/kg or 100 mg/kg on growth performance, organ weight, meat quality and gut health of broilers.

MATERIALS AND METHODS

Preparation of Encapsulated Materials

CIN (catalogue no. W228613; ≥95% purity; $828/5 kg) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and encapsulated separately in a soy-derived product called soy protein-soy polysaccharide Maillard reaction product (SPPMP) by emulsification and spray drying technologies (20% CIN in the capsules) (Yang et al., 2020). Surmax 100 Premix (avilamycin premix, 100 g/kg) was purchased from Elanco Canada Co. Ltd (Guelph, Ontario, Canada).

Experimental Design

A total of 320 1-d-old male Cobb 500 broiler chickens obtained from a local hatchery in Manitoba (Carleton Hatchery, Grunthal, Manitoba) were housed in 32 pens with 10 birds per pen (University of Manitoba, Winnipeg, MB, Canada). The pens were randomly allocated to four dietary treatments (8 pens/treatment): 1) basal diet as negative control (NC); 2) basal diet with 30 mg/kg avilamycin premix as positive control (PC); 3) basal diet with encapsulated CIN at 50 mg/kg (EOL); 4) basal diet with encapsulated CIN at 100 mg/kg (EOH). The birds were fed a starter diet from d 1 to d 14, a grower diet from d 15 to d 28, and a finisher diet from d 29 to d 41. The diets were provided as mash form and formulated (Table 1) according to the nutritional recommendation by Cobb 500 guidelines (Cobb-Vantress Inc., 2012) and prepared in Glenlea Research Station (Manitoba, Canada), which were described by a previous study (Mogire et al., 2021). For diets in PC, EOL and EOH groups, antibiotic and EOs were added by replacing equal amounts of corn.

Animals and Management

All procedures involving birds in this experiment were approved by the Animal Care and Welfare Committee of University of Manitoba according to animal use protocol (# F18-024). The birds were weighed on day 1 and distributed into floor pens of identical size (81.5 inches × 59 inches = 4,808.5 square inches) in a deep litter system with a wood shaving floor. The size of floor pen was 2 m^2, and 4 cm of low floor straw were provided for chickens. Chickens were allowed ad libitum access to feed and drinking water (water cups were 6.5 inches in diameter and 2.25 inches deep) during the experiment. The temperature was maintained at 31°C from d 1 to d 3, 30 °C from d 3 to d 7, 28 °C from d 7 to d 14, 25 °C from d 21 to d 28, 24 °C from d 28 to d 35, and 22 °C from d 35 to d 41. The lighting program during the study was as follows: 24L:0D from d 0 to d 3, 22L:2D from d 4 to d 7 and 18L:6D in the

Table 1 Ingredient compositions and nutrient contents of starter (d 1–14), grower (d 15–28), and finisher (d 29–41) diets for broiler chickens (g/kg, as-fed basis, otherwise indicated)

	Inclusion in basal diet		
Ingredients	Starter	Grower	Finisher
Corn	522.29	529.38	563.00
Soybean meal	305.00	261.00	225.00
Corn gluten meal	35.00	35.00	35.00
Wheat	25.00	30.00	30.00
Canola meal	25.00	30.00	30.00
Soy oil	22.60	43.80	45.80
Corn DDGS	20.00	30.00	30.00
Limestone	15.00	13.00	13.00
Vitamin premix[a]	10.00	10.00	10.00
21% Monocalcium phosphate	9.00	7.00	5.00
Mineral premix[b]	5.00	5.00	5.00
99% DL-methionine	2.65	2.37	2.05
Lysine-HCl	2.25	2.46	2.31
Threonine	0.71	0.49	0.34
Xylanase 8000G[c]	0.20	0.20	0.20
Phytase 5000G[d]	0.30	0.30	0.30
Calculated composition			
ME (kcal/kg)	3000.00	3150.00	3200.00
CP	223.00	208.00	194.00
Ca	8.60	7.40	7.00
Total P	6.00	5.40	5.20
SID Lys	11.8	11.0	10.0
SID Met	6.80	5.50	5.00
SID Met + Cys	8.80	8.40	7.70
SID Thr	7.80	7.40	6.70

[a]Provided per kilogram of diet: vitamin A, 8,255 IU; vitamin D3, 3,000 IU; vitamin E, 30 IU; vitamin B12, 0.013 mg; vitamin K3, 2.0 mg; niacin, 41.2 mg; choline, 1300.5 mg; folic acid, 1.0 mg; biotin, 0.25 mg; pyridoxine, 4.0 mg; thiamine, 4.0 mg; calcium pantothenic acid, 11.0 mg; riboflavin, 6.0 mg.

[b]Provided per kilogram of diet: manganese, 70.0 mg; zinc, 80.0 mg; iron, 80.0 mg; iodine, 0.5 mg; copper, 10 mg; selenium, 0.3 mg.

[c]Xylanase 8000 G: 8,000 U/g; Danisco Animal Nutrition, Marlborough, United Kingdom.

[d]Quantum blue 5000 G: 5,000 FTU/g; AB Vista, Plantation, FL, United States.

period from d 8 to d 39 and 23L:1D in the period of d 40 to d 41. The birds were handled according to the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

Growth Performance and Organ Weights

All individual birds were weighed on day 1 and allotted with similar initial body weight (BW; 48.3 ± 3.3g) in a randomized complete-block (RCB) design. Feed intake and BW were recorded weekly throughout d 1–41 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). Besides, any injured, malformed, or suffering bird was euthanized by carbon dioxide (CO_2) and confirmed by cervical dislocation. Euthanized birds were recorded to calculate mortality (%). On d 41, a total of 32 birds (one bird per pen) were sacrificed to collect heart, liver, spleen, and bursa. The relative organ weights (%) were calculated as follows:

$$\text{Relative organ weights (\%)} = \frac{\text{Organ weight (g)}}{\text{chicken BW (g)}} \times 100$$

Intestinal Morphology

Intestinal sections were collected from duodenum (0.5 cm, the middle of the descending duodenum), jejunum (1.5 cm, midway between entry of bile ducts and Meckel's diverticulum), and ileum (0.5 cm, mid-ileum at the Meckel's diverticulum) on d 41 from 32 random-selected euthanized birds (1 bird per pen) for morphology. The collected sections were flushed gently using ice-cold physiological saline solution, dehydrated with alcohol, fixed in 10% neutral buffered formalin, and embedded in paraffin. Approximately 6 µm of each section was cut, mounted on slides, deparaffinized in xylene, rehydrated and stained with hematoxylin and eosin (H&E) for histology analysis (Fischer et al., 2008). Villus height (VH) and crypt depth (CD) were measured by Carl Zeiss MicroImaging equipped with a computer-assisted morphometric system (Carl Zeiss Ltd, Göttingen, Germany) and calculated villus/crypt ratio (VH/CD).

Gene Expression

The jejunum from 32 random-selected euthanized birds (one bird per pen) were flushed with phosphate buffered saline (PBS) and stored in liquid nitrogen immediately in 15 mL tubes.

Total RNA was extracted from jejuna using Trizol reagents according to manufacturer's protocol. The extracted RNA concentrations were measured by a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada). The RNA quality was visually checked by 1% agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from RNA using iScriptTM cDNA Synthesis kit (Bio-Rad, Mississauga, ON, Canada). Quantitative Real-time PCR (RT-PCR) of genes, including cationic amino acid transporter (CAT-1), neutral amino acid transporter (B^0AT-1), glutamate transporters excitatory amino acid carrier 1 (EAAC-1), cysteine/glutamate antiporter (xCT), sodium-dependent glucose transporter 1 (SGLT-1), peptide transporter 1 (PepT-1), maltase-glucoamylase (MG), zonula occludens 1 (ZO-1), cadherin 1 (CDH-1), claudin 1 (CLDN-1), and proliferating cell nuclear antigen (PCNA) were determined by iQ SYBR Green Supermix (Bio-Rad) in a CFX Connect Real-Time PCR Detection System (Bio-Rad). The detailed primer information was described previously (Lu et al., 2020). The cycling conditions were 95 °C for 3 min, 40 cycles at 95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. Relative gene expressions were calculated using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001).

Apparent Ileum Nutrient Digestibility

An indigestible analytical marker, 0.3% chromium oxide (Cr_2O_3), was added to the mash feed at the last 4 d of the trial for analysis of apparent ileal digestibility (AID) of dry matter (DM), crude protein (CP), crude fat (CF), and amino acids (AA). Briefly, approximately 1 kg of diets in each treatment were collected and kept in a cold room at 4 °C. Ileal digesta were collected from four birds per pen (pool digesta) on d 41 and then freeze-dried. The dried digesta samples were kept in airtight bags and stored at 22 °C for further analysis. Before analyzing, the dried digesta and feedstuff were finely ground by a grinder (CBG5 Smart Grind; Applica Consumer Products, Inc., Shelton, CT, USA).

The DM was measured by Official Methods of Analysis (AOAC, 2000; procedure # 934.01), CP was analyzed by a Leco NS 2000 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA) and calculated based on nitrogen content (CP = nitrogen × 6.25), and CF was determined using ANKOM Extraction System. Samples for AA analysis were prepared by acid hydrolysis according to the method of AOAC (2006; procedure # 994.12). Samples for methionine and cysteine analysis were oxidized

with performic acid (AOAC, 2006; procedure # 985.18) before acid hydrolysis. Samples for tryptophan analysis were determined by the method of Commission Directive (2000) after hydrolyzing with barium hydroxide octahydrate for 20 h at 110 °C. The AA were analyzed using an Amino Acid analyzer (SYKAM, Germany). The Cr content was measured by inductively coupled plasma spectrometer (VarianInc., Palo Alto, CA, USA):

$$\text{AID}\,(\%) = (1 - \frac{\text{ND} \times \text{CF}}{\text{NF} \times \text{CD}}) \times 100$$

where ND is the nutrient concentration in digesta; CF is the chromium concentration in feed; NF is the nutrient concentration in diet; CD is the chromium concentration in digesta.

Genomic DNA Extraction and 16S Ribosomal RNA Gene Sequencing

Ileal and cecal digesta were collected from four birds per pen (pooled digesta) at d 41 and stored in −80 °C for genomic DNA extraction using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Toronto, Canada). The quantity and quality of DNA were determined by Nanodrop 2.0 and 1.0% agarose gel electrophoresis, respectively. The sequencing of the 16S ribosomal RNA gene (16S rRNA) were prepared according to Illumina 16S Metagenomics Sequencing Library Preparation Guide Rev. B and sequenced using a MiSeq instrument (Illumina). Briefly, the amplicon library of V3-V4 hypervariable region (444 bp) was amplified and sequencing libraries were prepared as previously described (Yang et al., 2021). A 600-cycle v3 reagent kit (Illumina, MS1023003) was used to sequence after pooling equimolar quantities of each sample together. The sequencing data was analyzed by Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2018). Briefly, 300 bp paired-end reads were processed with DADA2 to denoise reads, remove chimeric sequences and singletons, join paired-ends and de-replicate sequences to produce unique amplicon sequence variants (ASVs) (Callahan et al., 2016). Taxonomic classification of the resulting feature table was performed with VSEARCH and the Greengenes 99% OTU sequences as reference (McDonald et al., 2012; Rognes et al., 2016). ASVs were discarded if they had fewer than 10 instances across all samples, were present in fewer than two samples, or were not assigned taxonomy at the phylum level. Multiple sequence alignment of ASV representative sequences was performed with MAFFT and a rooted phylogenetic tree constructed with FastTree (Katoh et al., 2009; Price et al., 2009). Core diversity analysis was performed using a sampling depth of 10,000 sequences to plot taxonomic relative abundances, calculate alpha-diversity metrics, and generate dissimilarity matrices based on Bray-Curtis, Jaccard, and UniFrac distances, which were used for principal coordinate (PCoA) analyses.

Meat Quality

The birds were fed finisher diet until d 49 for collecting breast meat (*Pectoralis major* muscle) for meat quality analysis as described previously (Lu et al., 2020). The breasts from euthanized birds (four birds per pen) were carefully split, deboned, and trimmed off extra-muscular fat and connective tissues without damaging the exposed surface. The split breasts were placed on white Styrofoam trays (foam meat tray, 8.25 × 5.75 × 1, Pack. All Manufacturing Inc, Rockland, ON, Canada) containing soaking pads and covered with oxygen permeable polyvinyl chloride films (PVC; 037242 PUR Value Polyvinylchloride Standard Meat Films, AGL, Richmond Hill, Ontario, Canada). The trays with breast samples were placed on a retail display cabinet (Model MI, Husmann) at 2 °C under LED lighting (light emitting diodes; Acuity Brands Dimmable Rigid 30-LED Light Strip Board HTG S7 - 94v-0 – 4000k) with an intensity about 1240 lx. The cabinet was rotated every 24 h to minimize temperature and lighting variations of the machine.

White striping (WS) and woody breast (WB) were evaluated visually and scored by a well-trained technician at a processing plant. The WS was scored as normal (0; no distinct white lines), moderate (1; visible white lines with <1 mm thick), and severe (2; large white lines 1–2 mm thick) as previously described (Kuttappan et al., 2012). The WB was scored as normal (0; fillets are flexible), mild (1; hard in the cranial region but flexible), moderate (2; hard throughout but flexible in mid to caudal region), and severe (3; extremely hard and rigid throughout from cranial region to caudal tip) based on tactile evaluation (Tijare et al., 2016).

Breast pH values at 24 h and 96 h post-slaughter were recorded by a waterproof meter (HI 99163, HANNA Instruments, Carrollton, TX). Meat color was measured at three locations by a colorimeter (Chroma Meter CR-410, Minolta Canada Inc., Mississauga, ON) using CIELAB systems for determining lightness (L^*), redness (a^*), and yellowness (b^*). Dripping loss (%) was measured

as previously described (Wang et al., 2016). Briefly, a small piece (approximately 5 g) of each breast sample on the caudal portion of the breast was cut and weighed. The pieces were hung perpendicularly to the ground in a flat-bottomed volumetric flask without touching the surface of the flask. The samples were suspended at 2 °C and final weights were measured after 48 h to calculate dripping loss (%):

$$\text{Dripping loss (\%)} = \frac{\text{weight before suspension (g)} - \text{weight after suspension (g)}}{\text{weight before suspesion (g)}} \times 100$$

Myofibrillar fragmentation index (MFI) was measured as previously described (Culler et al., 1978). Firstly, a 4 g of breast sample was minced at 2 °C, suspended in 40 mL of cold MFI buffer (100 mM KCk, 20 mM KPO_4, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, pH 7.0), and homogenized for 30 s. The homogenate was transferred into a 50 mL sterilized tube to centrifuge at 2 °C with a speed of 1000 × *g* for 15 min (Thermo Scientific Sorvall RC6 plus Centrifuge). After centrifugation, the pellet was re-suspended by 10 mL cold MFI buffer and mixed by a vortex mixer. The mixture was poured through a strainer to remove connective tissues. Then, the protein concentration of each suspension was determined using bovine serum albumin (BSA) as the standard. Briefly, 0.25 mL suspension was added in tubes with 0.75 mL MFI buffer and 4 mL of biuret reagent. Then the suspension was placed in the dark for 30 min at room temperature. Simultaneously, serial dilutions of BSA were made to generate a standard curve by measuring the optical density (OD) at 540 nm ($OD_{540\,nm}$) using a spectrophotometer (GENESYS 30 visible spectrophotometer). The protein concentration of each suspension was determined by OD_{540nm} measurements using the BSA standard curve. Finally, based on the protein concentration, the suspension was diluted to 8 mL of 0.5 mg protein per mL solution and mixed well. The MFI was calculated after the $OD_{540\,nm}$ determination (Culler et al., 1978):

$$\text{MFI (mg/mL)} = 200 \times \text{Absorbance}$$

After the measurements of WS, WB, pH, color, dripping loss (%), and MFI, the rest of the breast samples were cut into five pieces, vacuum packed (6″ × 10″ FlairPak Vacuum Pouch, Flair Flexible Packaging Corporation, Canada/USA), and stored in a −40 °C freezer for analyzing cooking loss (%) and Warner–Bratzler Shear Force (WBSF). Frozen breast samples were thawed overnight in a cold room at 2 °C, sealed in plastic bags, and cooked in a water bath at 85 °C. A thermometer was inserted immediately in breast samples to monitor the internal temperatures until they reached 75–78°C and the cooking times were recorded. Then breast samples were moved from the water bath to the cold room (2 °C) for 2 h cooling. The cooled samples were weighted to calculate cooking loss (%):

$$\text{Cooking loss (\%)} = \frac{\text{weight before cooking (g)} - \text{weight after cooking (g)}}{\text{weight before cooking (g)}} \times 100$$

The cooked samples were placed in the cold room at 2 °C overnight and were moved to room temperature for 30 min to measure WBSF by an analyzer (TA-XT Plus, Texture Technologies). Before analysis, the analyzer was calibrated with a 2 kg weight using a 10 kg loading cell. Then five rectangular strips (2–4 cm long, 1 cm wide, 1 cm height) were cut along the fiber by a ruler and a knife. The strips were placed in the analyzer with fiber perpendicular to the blade to record WBSF values (kg).

Data Analysis

The experiment was analyzed as complete random design (CRD) and pens were considered as experimental unit. The growth performance, organ weight, intestinal morphology, ileal digestibility, and gene expressions obtained in each treatment were evaluated using PROC ANOVA followed by the Tukey's multiple comparison test (SAS 9.4) with the model: $Y_{ij} = \mu + T_i + e_{ij}$, where μ is the total means, T_i is the fixed treatment effects, e_{ij} is residual of the model. The relative abundance of microbial taxa and diversity, and meat quality including meat color (a^*, b^*, L^*), pH, purge loss, cooking loss, MFI, and shear force were analyzed using PROC MIXED followed by the Tukey's multiple comparison test (SAS 9.4). Chi-square analysis was analyzed using PROC FREQ to check differences in the distribution of severity scores in WS and WB. A *P*-value of 0.05 was used to declare significance.

RESULTS

Growth Performance, Organ Weights

Results demonstrated that encapsulated CIN at 50 mg/kg or 100 mg/kg had no significant effects on BW, ADFI, and FCR compared to the controls for either each growing stage or for the overall feeding

Table 2. Effects of encapsulated cinnamaldehyde at either 50 mg/kg or 100 mg/kg in feed on growth performance of broiler chickens

	Treatments[b]					
Items[a]	NC	PC	EOL	EOH	SEM[c]	*P*-value
Starter (d 1–14)						
BW (14 d, g)	514.83	501.14	505.03	497.36	4.802	0.63
ADG, g	33.32	32.34	32.62	32.07	0.337	0.61
ADFI, g	44.99	46.07	46.63	45.42	0.401	0.51
FCR, g/g	1.35	1.43	1.43	1.42	0.015	0.19
Grower (d 15–28)						
BW (28 d, g)	1749.33	1784.13	1728.14	1696.89	15.793	0.26
ADG, g	88.18	91.64	87.37	85.68	1.067	0.25
ADFI, g	118.64	119.03	118.70	116.37	1.152	0.85
FCR, g/g	1.35	1.30	1.36	1.36	0.011	0.12
Finisher (d 29–41)						
BW (41 d, g)	3274.39	3319.32	3305.02	3185.67	34.625	0.54
ADG, g	116.54	118.09	121.3	114.52	2.146	0.74
ADFI, g	185.56	182.63	186.18	179.37	2.348	0.75
FCR, g/g	1.60	1.55	1.54	1.57	0.015	0.54
Overall (d 1–41)						
ADG, g	76.57	77.88	77.54	74.70	0.825	0.54
ADFI, g	110.06	108.87	110.66	108.12	0.982	0.81
FCR, g/g	1.44	1.40	1.43	1.45	0.010	0.32
Morality, %	16.25	10	12.5	10	-	-

[a]BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

[b]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde.

[c]SEM, standard error of the mean.

trial (Table 2). Similarly, no significant differences were noted between treatments for the weights of heart, liver, spleen, and bursa.

Intestinal Morphology, Jejunal Gene Expression, and Ileal Digestibility

Feeding EOL or EOH to birds, had no significant effect on VH and CD of duodenum jejunum and ileum of d 41 old broilers. However, EOL and EOH feeding increased ($P < 0.05$) VH/CD compared to NC and PC birds, with average increases by EOL and EOH respectively being 14.67% and 15.13% in the duodena and 15.13% and 13.58% in the jejuna (Figure 1).

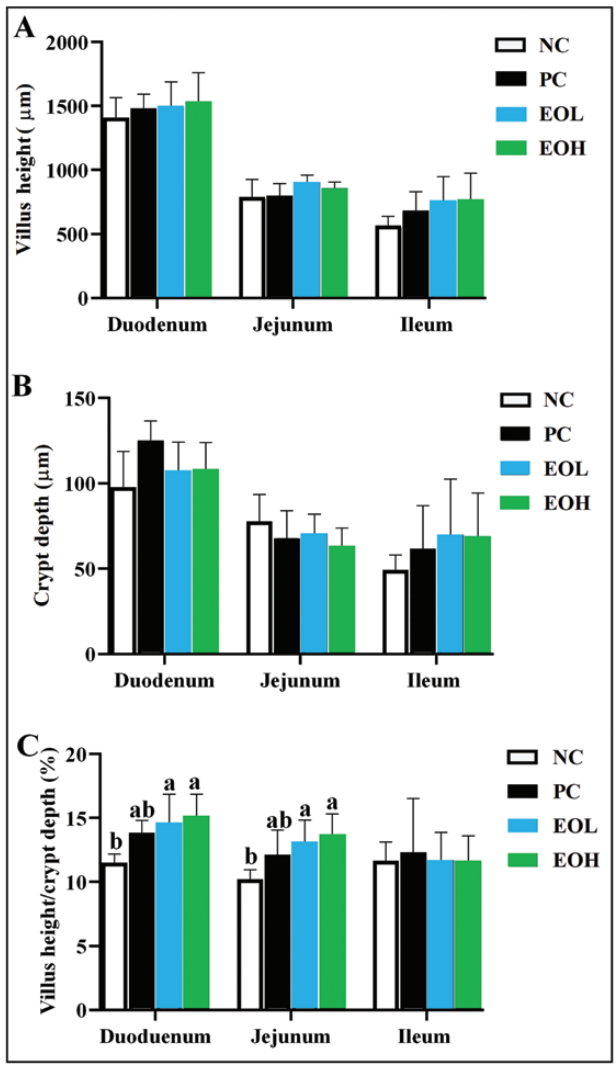

Figure 1. Effects of encapsulated cinnamaldehyde on villus height (A), crypt depth (B) and ratio of villus height and crypt depth (C) on duodenum, ileum, and jejunum in broilers. NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde. Significant differences are indicated by letters (a, b).

For jejunal gene expression, 6 of the 11 studied genes, including CDH-1, PCNA, ZO-1, B^0AT-1, CAT-1, and MG showed significant ($P < 0.05$) effects of dietary treatments. Birds fed EOL or EOH increased ($P < 0.05$) expressions of CDH-1 and B^0AT-1 compared to PC and NC, respectively. The PCNA expression was significantly downregulated ($P < 0.05$) with EOL supplemented diet compared to NC treatment. The ZO-1 expressions

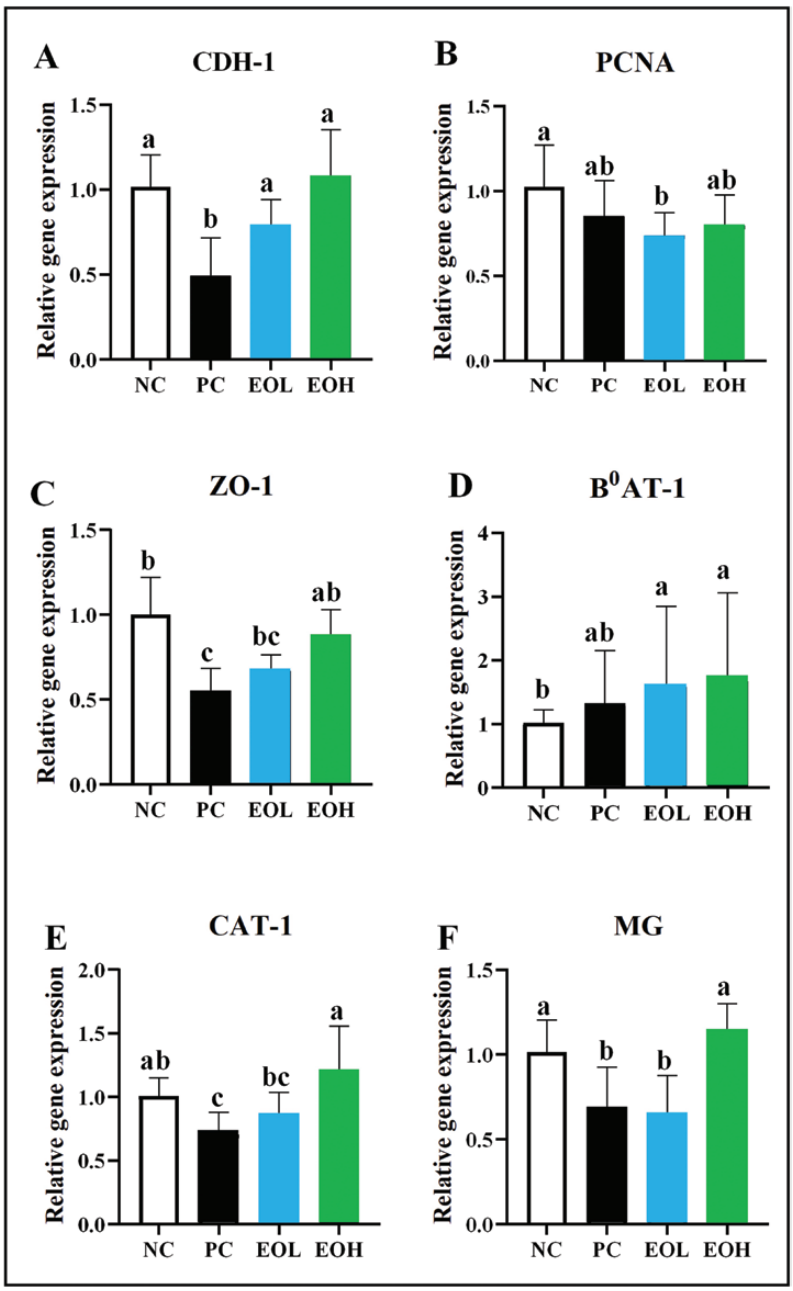

Figure 2. Effects of encapsulated cinnamaldehyde on jejunal gene expressions of CDH-1 (A), PCNA (B), ZO-1 (C), B^0AT-1 (D), CAT-1 (E) and MG (F). NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde; CDH-1, cadherin 1; PCNA, proliferating cell nuclear antigen; ZO-1, zonula occludens 1; B^0AT-1, neutral amino acid transporter; CAT-1, cationic amino acid transporter; MG, maltase-glucoamylase. Significant differences are indicated by letters (a, b, c).

were significantly upregulated in EOH treatments compared to PC ($P < 0.05$) but not different from NC treatment. Nonetheless, EOH fed birds showed a higher expression ($P < 0.05$) of CAT-1 and MG compared to PC (Figure 2).

The AID of DM, CP, CF, and 18 amino acids (alanine, arginine, aspartate, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylamine, proline, serine, threonine, tryptophan, tyrosine, and valine) were improved in birds fed EOL compared to NC birds ($P < 0.05$). However, when birds were fed EOH, no increases in AID of DM and CP were observed, and only eight amino acids (alanine, arginine, glutamine, isoleucine, leucine, phenylamine, tyrosine, and valine) showed a higher ($P < 0.05$) AID than NC birds. Additionally, as shown on Table 4, birds on PC showed an increased ($P < 0.01$) AID of CF and digestibility of 13 amino acids (alanine, arginine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylamine, threonine, tyrosine, and valine) compared to NC birds.

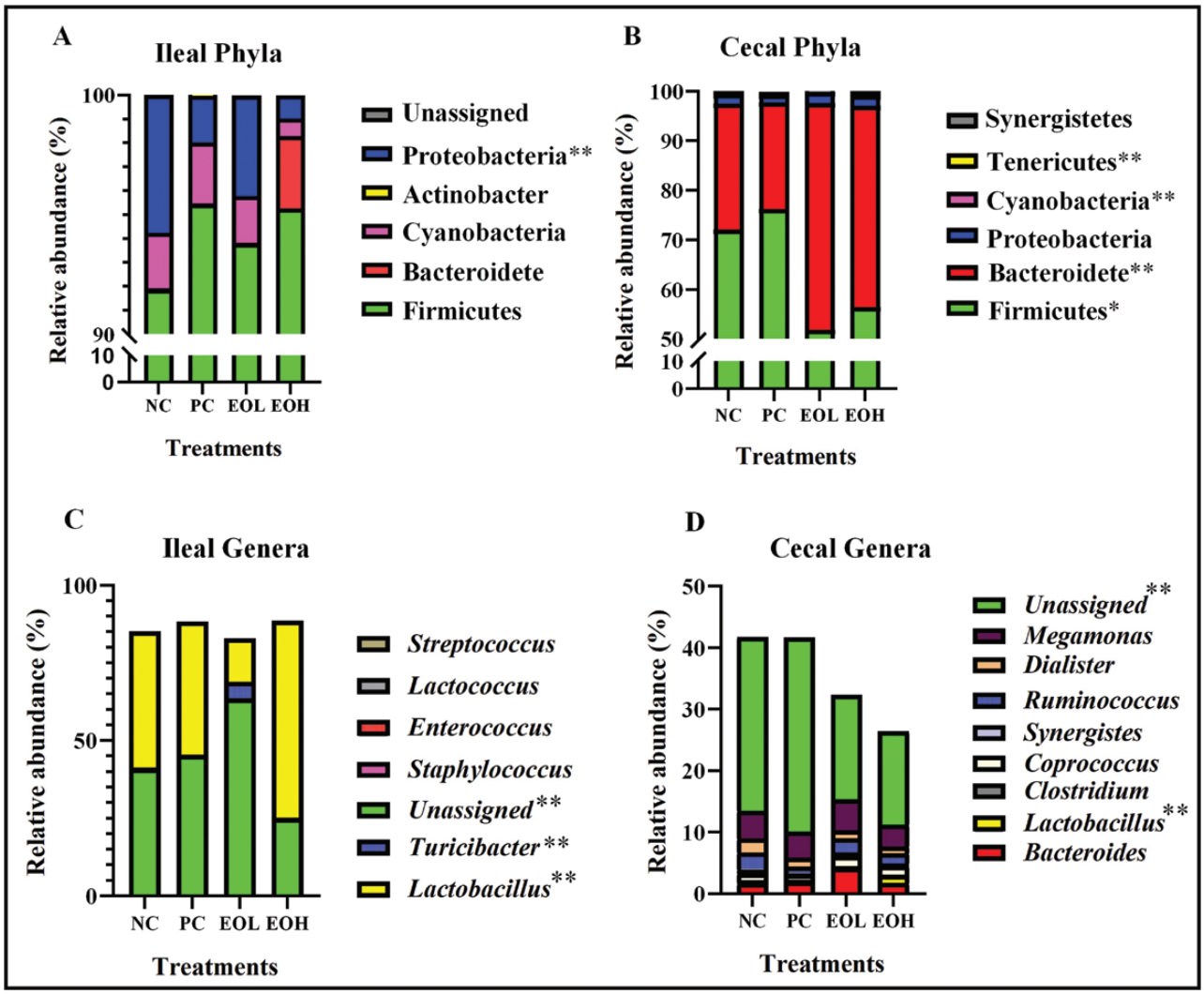

Figure 3. Relative abundance of ileal phyla (A) and major (>1% relative abundance) genera (C) and cecal phyla (B) and major (>1% relative abundance) genera (D) in birds treated with 30 mg/kg avilamycin or encapsulated cinnamaldehyde. NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde. Asterisks indicate significant statistically differences (one asterisk means a significance level of 0.05; two asterisks mean a significance level of 0.01).

Ileal and Cecal Microbiota

Four bacterial phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Cyanobacteria) with greater than 1% relative abundance were observed in both ileal and cecal digesta (Figure 3). A lower relative abundance of Proteobacteria was observed in ileal digesta of EOH-fed birds compared to NC birds ($P < 0.01$) while a higher proportion of Bacteroidetes were observed in cecal digesta of EOL- and EOH-fed birds than in those of NC and PC birds ($P < 0.01$). At the genus level, ileal and cecal digesta from EOH-fed birds showed the highest relative abundance of *Lactobacillus* population ($P < 0.01$) compared to other treatments, but a higher relative abundance of *Turicibacter* was observed in ileal digesta in EOL-fed birds ($P < 0.01$) than those in NC, PC and EOH groups. No significant differences were observed between treatments for alpha diversity (richness and diversity) including Chao1, Shannon, and Simpson indices (Table 5). Principal Coordinates Analysis (PCoA) of the microbiota according to weighted UniFrac phylogenetic distances showed that the majority of samples from ileal (blue) and cecal (red) digesta clustered separately ($P < 0.01$). In ileal samples, EOL was separated from NC ($P < 0.05$), PC ($P < 0.05$), and EOH ($P < 0.01$). In cecal samples, EOL was separated from NC ($P < 0.05$) and PC ($P < 0.05$). However, there were no significant differences between NC, PC, and EOH in both ileal and cecal samples (Figure 4).

Meat Quality

No treatment effects were found on cooking time (min), purge loss, dripping loss, pH values

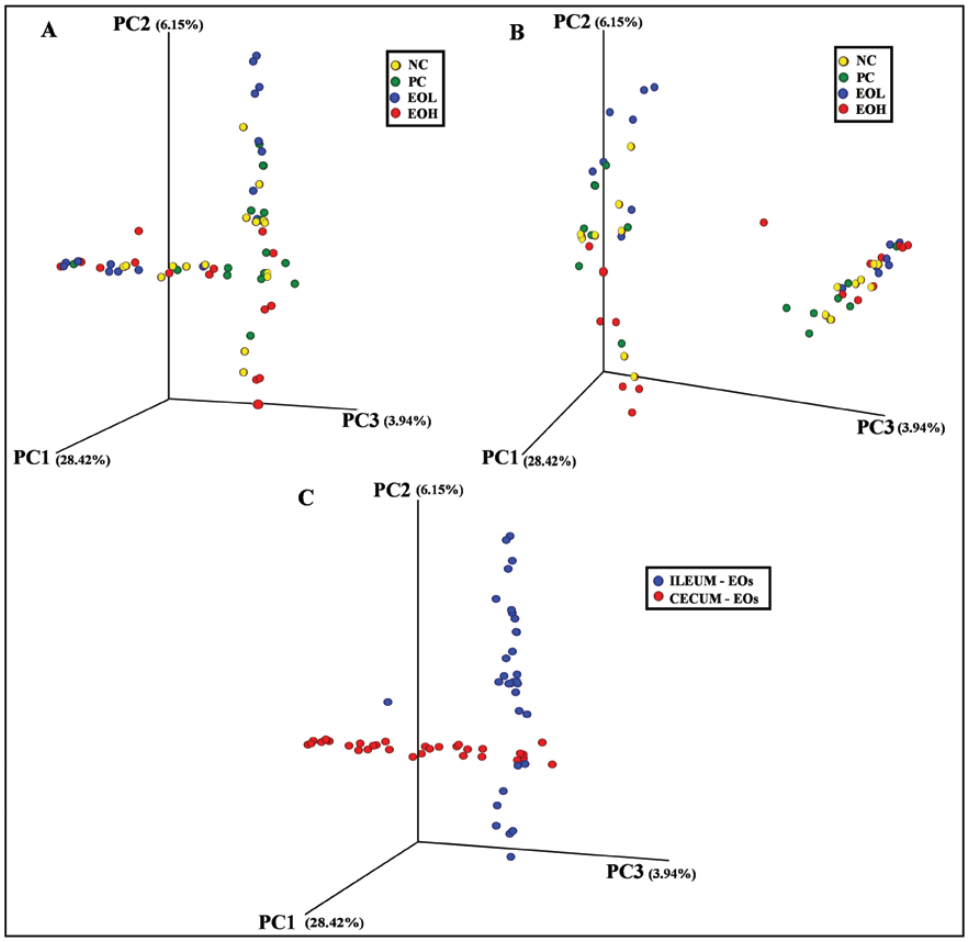

Figure 4. The 3D principal coordinate analysis (PCoA) graph shows the variation among distance matrixes (weighted UniFrac) of ileal (A) and cecal (B) microbiota alone or together (C) in birds treated with avilamycin premix or encapsulated cinnamaldehyde. Percentages shown are percentages of variation explained by the PC1 (28.42%), PC2 (6.15%), and PC3 (3.94%). NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde; EOs, essential oils.

(96 h), color (L^*, a^*, b^*), white striping (WS), and woody meat (WB). Despite meat pH at 24 h post slaughter broilers fed EOH tended to be higher ($P = 0.05$) than other treatments, all pH values were at the normal range. However, birds fed in PC and EOH treatments yielded ($P < 0.05$) the WBSF (kg) in breast meat compared to NC birds (Table 3).

DISCUSSION

A recent study found that dietary supplementations of encapsulated CIN (100 mg/kg) improved growth performance, reduced gut lesions caused by *Eimeria* spp. and *C. perfringens*, and modulated cecal microbiota in broiler chickens that received coccidiosis vaccine (Yang et al., 2020). In the present study, no vaccination was applied and a lower dosage of CIN (50 mg/kg) was selected in addition to the 100 mg/kg to evaluate their effects on performance and gut health parameters including some organ's weight, meat quality, intestinal morphology, jejunal gene expression, and ileal nutrient digestibility. Avilamycin (30 mg/kg), which is another common antibiotic besides bacitracin (55 mg/kg) used in broiler chicken production to prevent NE lesions caused by *C. perfringens*, was supplemented in diets as the PC (CFIA, 2020).

Data showed no significant improvement of BW, ADG, and FCR by inclusion of either CIN (50 or 100 mg/kg) or 30 mg/kg avilamycin in feeds. The result was inconsistent with a previous study (Yang et al., 2020) which may be due to the raising conditions since it has been reported that growth performance of broilers could be altered by different broiler-housing conditions (Mesa et al., 2017). The high mortality rate in the present study could be ascribed to a high rate of culling birds due to injuries according to the guidelines (CCAC, 2009). High mortality could be explained by high BW (>3,000 g in average at d 41) in this study which could have increased the incidences of injuries

Table 3. Effects of encapsulated cinnamaldehyde at either 50 mg/kg or 100 mg/kg in feed on breast meat quality of broilers

Variables[b]	NC[a]	PC	EOL	EOH	SEM[c]	*P*-value
WBSF (kg)	1.95[a]	1.46[b]	1.59[ab]	1.33[b]	0.16	0.04
Cooking loss (%)	23.11	23.63	23.24	23.04	0.85	0.96
Cooking time (min)	37.52	37.96	35.85	35.8	1.47	0.31
$pH_{24\ h}$	6.12[a]	6.18[ab]	6.20[ab]	6.21[b]	0.078	0.05
$pH_{96\ h}$	5.41	5.61	5.56	5.52	0.316	0.54
Dripping loss (%)	1.34	1.26	1.29	0.78	0.37	0.69
*L**	58.68	59.86	58.49	59.62	0.57	0.23
*a**	11.52	10.91	11.56	11.09	0.24	0.14
*b**	18.69	19.06	17.56	18.02	0.45	0.09
MFI (mg/mL)	39.54	37.99	40.28	37.54	0.91	0.69
WS % (*n*)						
WS scores	0.19	0.32	0.33	0.27	0.06	0.52
Normal	95.24 (20)	90.91 (20)	87.10 (27)	89.29 (25)	–	0.88
Moderate	4.76 (1)	9.09 (2)	12.9 (4)	10.71 (3)	–	–
Severe	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	–	–
WB % (*n*)						
WB scores	0.08	0.27	0.35	0.34	0.08	0.28
Normal	100 (21)	86.36 (19)	80.65 (25)	85.71 (24)	–	0.38
Mild	0.00 (0)	13.64 (3)	16.13 (5)	14.29 (4)	–	–
Moderate	0.00 (0)	0.00 (0)	3.23 (1)	0.00 (0)	–	–
Severe	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	–	–

[a]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde.

[b]WBSF, Warner−Bratzler shear force (kg); WS, white striping; WB, woody breast; .*L**, lightness; *a**, redness; *b**, yellowness; MFI, Myofibril Fragmentation Index.

[c]SEM, standard error of the mean.

Table 4. Effects of encapsulated cinnamaldehyde at either 50 mg/kg or 100 mg/kg in feed on dry matter, crude fat, crude protein, and amino acid digestibility

	Treatments[b]					
Item[a]	NC	PC	EOL	EOH	SEM[c]	*P* value
DM	71.26[b]	71.96[b]	76.21[a]	69.39[b]	0.514	<0.01
CF	81.51[b]	87.18[a]	89.70[a]	86.36[a]	0.765	<0.01
CP	76.88[b]	79.83[b]	84.03[a]	79.14[b]	0.632	<0.01
Ala	78.12[b]	84.78[a]	87.25[a]	82.94[a]	0.905	<0.01
Arg	79.51[c]	87.27[ab]	89.76[a]	85.49[b]	0.915	<0.01
Asp	74.10[bc]	79.15[ab]	81.35[a]	77.11[ab]	0.933	0.03
Cys	70.25[b]	79.00[a]	80.15[a]	73.32[ab]	1.188	<0.01
Glu	82.42[c]	87.23[ab]	88.94[a]	86.13[ab]	0.680	<0.01
Gly	71.16[c]	77.75[ab]	80.20[a]	75.10[abc]	1.035	0.01
His	54.48[bc]	62.91[a]	67.68[a]	59.54[ab]	1.301	<0.01
Ile	64.74[c]	80.48[ab]	82.47[a]	78.87[ab]	1.547	<0.01
Leu	78.24[c]	85.54[ab]	89.12[a]	84.67[b]	0.867	<0.01
Lys	82.60[c]	87.88[ab]	89.15[a]	83.12[bc]	0.791	<0.01
Met	89.65[b]	92.10[ab]	94.38[a]	90.38[b]	0.546	0.01
Phe	77.12[c]	84.62[ab]	88.13[a]	83.64[b]	0.874	<0.01
Pro	79.57[b]	83.89[ab]	86.36[a]	81.92[ab]	0.812	0.02
Ser	75.55[b]	80.95[ab]	83.11[a]	79.10[ab]	0.862	0.01
Thr	65.94[b]	76.35[a]	78.78[a]	71.39[ab]	1.374	<0.01
Trp	78.26[b]	80.70[ab]	84.81[a]	79.82[ab]	0.869	0.04
Tyr	73.11[c]	84.79[ab]	88.45[a]	83.16[b]	1.212	<0.01
Val	62.35[c]	79.13[ab]	84.27[a]	77.32[b]	1.701	<0.01

[a]DM, dry matter; CF, crude fat; CP, crude protein.

[b]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde.

[c]SEM, standard error of the mean.

(Martins et al., 2016). Notably, birds fed EOL and EOH had a relative lower mortality than those in NC. In poultry barns, enteric diseases of broilers including coccidiosis and NE could be the main cause of high mortality (Christaki et al., 2004; Cooper et al., 2013). In this study, the lower mortality in birds fed EOL and EOH may suggest that enteric infections could have been controlled by supplementation of CIN at 50 or 100 mg/kg, when compared to the previous study (Yang et al., 2020).

Any abnormal changes to organ weights including heart, liver, spleen, and bursa are indicators of chicken health disorders (Bowes and Julian, 1988). No changes in bursa and spleen weights may suggest no disorders were generated after feeding CIN since bursa and spleen could get larger due to inflammation (Cazaban et al., 2015). No differences in liver and heart weights could suggest that CIN may not be toxic to birds since toxicity could be one of major causes of abnormal liver and heart weight gains (Zaefarian et al., 2019), which was also consistent with results from a previous study when birds were fed with CIN powder (Najafi and Torki, 2010). However, toxic assessment is required in the further study.

Intestinal morphology, gene expression, nutrient digestibility, and gut microbiota, are four major parameters to reflect gut health. In this study, the higher VH/CD in duodena and jejuna detected in broilers fed EOL and EOH indicated potential of CIN in improving nutrient digestion, absorption, and gut barrier function. Higher VH/CD could explain increased AID of DM, CP, CF, and AAs when birds were fed EOL or EOH. The improvement of AID in broiler chickens was also reported in a previous study when birds were fed with mixtures of oregano, CIN, and pepper oils (Hernandez et al., 2004). Interestingly, lower AID of DM, CP, and AAs such as lysine and tryptophan were shown in birds fed EOH compared to EOL.

Table 5. Summary of alpha-diversity measurements of microbiota in ileum and caecum of broilers treated with avilamycin premix or cinnamaldehyde

		Treatments[a]					
α-diversity	Gut segments	NC	PC	EOL	EOH	SEM[b]	*P* value
Observed OTUs	Ileum	32.14	32.86	32.13	34.14	1.490	0.97
	Cecum	100.50	89.17	92.75	110.29	4.952	0.82
Chao1	Ileum	33.29	37.75	38.14	34.60	1.854	0.79
	Cecum	100.14	93.63	94.57	110.13	6.200	0.78
Shannon	Ileum	4.38	4.50	4.39	4.73	0.079	0.43
	Cecum	6.27	6.28	6.03	6.12	0.071	0.82
Simpson	Ileum	0.94	0.94	0.94	0.95	0.003	0.39
	Cecum	0.98	0.98	0.98	0.98	0.001	0.91

[a]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 mg/kg avilamycin premix; EOL, birds fed 50 mg/kg encapsulated cinnamaldehyde; EOH, birds fed 100 mg/kg encapsulated cinnamaldehyde.

[b]SEM, standard error of the mean.

This may be due to high concentrations of chemical compounds (aldehyde) in EOH, which can block lysine and tryptophan residuals from digestive enzymes (Rawel et al., 2002). Another interesting finding in the current study was that mRNA expressions of some proteins for nutrient absorption, gut barrier integrity, and DNA repair were altered by EOL or EOH. In this study, higher mRNA expressions for B^0AT-1 (compared to NC) and CAT-1 (compared to PC) in birds fed EOH were observed, suggesting improvements of neutral amino acids and cationic amino acids absorption (Gilbert et al., 2007). Additionally, higher MG expressions in birds fed EOH compared to PC and EOL indicated higher maltase-glucoamylase expressions for carbohydrate digestion (Diaz-Sotomayor et al., 2013). Since lower AID of DM was observed in birds fed EOH compared to EOL in this study, upregulated the mRNA expression of MG in birds fed EOH could be a compensatory feedback to maximize carbohydrate digestion (Ebrahimi et al., 2015). Tight junctions are multi-protein complexes that regulate ion and water transportations and prevent entry of harmful substances such as pathogens and endotoxins (Pitman and Blumberg, 2000). A recent study on IPEC-J2 cells in our lab reported that thymol oil could enhance intestinal barrier function by increasing the gene expression of ZO-1 (Omonijo et al., 2019). The present study suggested that birds fed EOH could improve gut barrier function when compared to birds fed PC. Additionally, lower mRNA expressions of PCNA may suggest alleviated pathogenic inflammations in birds fed EOL. This is because the mRNA expression of PCNA was higher in animals with inflammations compared to those without inflammations (Manohar and Acharya, 2015). For ileal microbiota, lower phylum Proteobacteria and higher genus *Lactobacillus* indicated that broilers fed EOH may possess ability to control the growth of pathogenic bacteria. This is because phylum Proteobacteria includes pathogenic genera such as *Salmonella* and *Campylobacter*, which are associated with inflammatory disorders in hosts (Moon et al., 2018), while many species in genus *Lactobacillus* such as *L. acidophilus* are beneficial bacteria (Azad et al., 2018). For cecal microbiota, higher phylum Bacteroidetes suggested increased carbohydrate degradation and propionate synthesis via succinate pathway in birds supplemented with CIN, and higher genus *Lactobacillus* demonstrated improvements of beneficial bacterial populations (Glendinning et al., 2019). The current results differ from the previous study in which that genus *Lactobacillus* was found to be more abundant in the cecum of broilers on citral, but not on CIN compared to birds fed a basal diet (Yang et al., 2020). Additionally, no significant differences of alpha diversity suggest that broilers fed EOL or EOH had minor effects on richness and diversity of microbiota. The PCoA results showed that diversity of ileal microbiota was significantly different from cecal microbiota, which is consistent with a recent study (Rios-Covian et al., 2017).

Increased AID, improved intestinal morphology, enhanced expressions of nutrient transporters, and altered ileal and cecal microbiota in birds fed EOL or EOH did not seem to promote growth performance in this study. This may because other factors such as genetics, management, and environment can affect growth performance of broilers (Craig et al., 2016). Additionally, in comparison to the recommendation in Cobb 500 guidelines (Cobb-Vantress Inc., 2012), the FCR values in each treatment was lower than the provided in

the recommendation, indicating the growth performance in the present study was reaching the optimum genetic potentials and there is little room for improvement.

In addition to the importance of bird growth performance and gut health, post-slaughtering meat quality is essential in ensuring consumers' satisfaction and could be judged by parameters including color, pH, water holding capacity (WHC), and scores of myopathies (Baracho et al., 2006). Since muscle myopathies such as WS and WB are frequently observed in heavy birds (>3,500 g live weight), four birds per pen were fed finisher diet until d 49 for investigating effects of CIN on breast meat quality (Kuttappan et al., 2013; Mogire et al., 2021). Because the fast-growing birds may be exposed to heat and pre-slaughtering stress in current intensive poultry farming system that often reduces meat quality (Sandercock et al., 2009), we tested the effects of CIN on breast meat quality of broiler chickens in the present study. Meat color is a critical parameter affecting consumer selection of deboned and skinless raw meats in markets (Qiao et al., 2001). In this study, all meat color parameters were normal and no color changes were found in breast meat from broilers fed EOL and EOH compared to NC and PC. This may be due to antioxidant compounds (aldehyde) in CIN, which could maintain meat color by preventing further oxidation of lipids and myoglobins due to air exposure (Kanani et al., 2017). Additionally, no changes on dripping loss (%) and cooking loss (%) may suggest that feeding birds with EOL or EOH did not have adverse effects on WHC of meat during storage, thawing and cooking, reflecting no alternations to meat juiciness (Bowker, 2017). Despite the observation of a higher pH value in EOH compared to NC at 24 h post slaughter, no pH differences among treatments were detected at 96 h. This result was in line with lack of alternations in meat color, cooking loss (%) and dripping loss (%) among treatments since any abnormal meat pH could cause alterations of these parameters (Mir et al., 2017). Interestingly, a similar study did not detect changes in pH, cooking loss (%) and dripping loss (%) in broiler chickens fed cinnamic bark powder at 200 mg/kg (Logaranjani, 2014). Myopathies on breast meat, such as WB and WS, reflect histological stress including lipolysis, fibrosis, necrosis, and myo-degeneration (Kuttappan et al., 2012; Kuttappan et al., 2013). In this study, we did not observe any differences of WB and WS scores among treatments. This may be due to better management and relatively lower stock density at the research barn used in the current study, compared to commercial farms (Kuttappan et al., 2016). Thus, a future study with higher bird numbers and a density comparable to commercial farms is required to test effects of CIN on WB and WS in broiler chickens. Additionally, the WBSF and MFI are another two meat quality parameters in predicting tenderness (Lyon and Lyon, 1990). The lower WBSF in birds treated with EOH compared to NC, suggested that CIN may have potential to enhance meat tenderness, which is consistent with the results obtained in a previous study when birds were fed with CIN (Gomathi et al., 2018).

In conclusion, this study indicated that encapsulated CIN in broiler feed could promote meat tenderness by reducing WBSF and gut health by improving AID, intestinal morphology and microbiota, and expressions of nutrient transporters. The impacts of CIN on growth performance were not significant probably due to reaching their optimum performance. Future studies are necessary to conduct a complete assessment to investigate the toxicity, safety, and economic impacts of encapsulated CIN in broiler chickens.

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Conflict of interest statement. None declared.

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