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Effect of Thymol + Carvacrol by Next Enhance 150® on Intestinal Development of Broiler Chickens Fed CMC Containing Diet

Research Article

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ABSTRACT

A 3 × 2 factorial experiment was conducted to investigate the effect of thymol + carvacrol (0, 100 and 200 mg/kg) on ileal microbial population and jejuna and ileal histomorphology of broilers fed Carboxy Methyl Cellulose (CMC: 0 and 2%) containing diet. Each of the 6 dietary treatments was replicated 5 times with 12 chicks each from 0 to 42 days of age. There was no interaction effect of CMC and thymol + carvacrol in any measured parameters. At d 42, the ileal population of lactobacilli and Bifidobacteria were greatest (P<0.05) in birds fed 200 mg/kg thymol + carvacrol but 100 mg/kg of additive had no significant difference with two others treatment. The *E. coli* population was significantly decreased in birds fed 100 and 200 mg/kg thymol + carvacrol. Supplementation of diet with CMC enhanced (P<0.05) ileal count of *E. coli* and reduced ileal counts of Lactobacilli and Bifidobacteria as compared to those in chicks fed control diet. Data showed that supplementation of diet with CMC decreased (P<0.05) intestinal histomorphology including jejunal and ileal villus height (VH), villus height to crypt depth (VH:CD) and increased jejunal and ileal villus width (VW), crypt depth (CD) at 21 and 42 days of age as compared to those in chicks fed control diet. Inclusion of 100 and 200 mg/kg thymol + carvacrol improved VH, villus surface (VS), VH:CD and muscular layer (MSL) of jejunum at 21 and 42 days of age and also increased ileal VH, VH:CD, MSL and GC at d 21 and MSL and GC at 42 days of age. Supplementation of diet with CMC decreased (P<0.05) jejunal histomorphology including VH, VH:CD and increased (P<0.05) VW and CD at 21 and 42 days of age as compared to those in chicks fed control diet. At d 21, dietary CMC decreased (P<0.05) VH and increased (P<0.05) VW but it did not affect the rest of ileal histomorphological parameters. At d 42, CMC supplementation significantly affected some ileal histomorphological parameters in terms of increasing VW and CD and decreasing VH and VH:CD but the other parameters were not influenced by CMC.

KEY WORDS carvacrol, CMC, histomorphology, microbial population, thymol.

INTRODUCTION

The water-soluble, non-starch polysaccharides (NSP) existing in ryegrass, wheat and barley are believed to be responsible for the reduction of growth performance and digestibility of lipids, protein and starch in broiler chickens fed these feedstuffs (Rodriguez *et al*. 2012). On the basis of a literature review, Stef *et al*. (2009) proposed that dietary soluble NSP inhibit nutrient absorption in broiler chickens, not only by raising the viscosity of the digesta but also by increasing bacterial fermentation. Addition of antibiotic growth promoters to the diet of broiler chickens can reduce the inhibitory effect of NSP on nutrients absorption (Basmacioglu *et al*. 2010). NSP generally are readily fer-

mentable (Santos *et al.* 2008), and thus it is difficult to assess separately the antinutritive effects of viscosity and fermentability. The effect of viscosity *per se* can be studied by the use of non-fermentable carboxy methyl cellulose (CMC) preparations. Recently, the concerns about possible antibiotic residues and disease resistance have aroused great caution in the usage of antibiotics in the poultry industry. The ban on the use of antibiotics as feed additives has been accelerated and the use of phytogenic compounds, i.e. essential oils, has gained momentum for their potential role as natural alternatives to antibiotic growth promoters in poultry nutrition (Scheuermann *et al.* 2009). The primary mode of action of essential oils and aromatic plants appears to arise from stabilizing feed hygiene and beneficially affecting the ecosystem of gastrointestinal microflora by controlling potential pathogens (Franz *et al.* 2010). Thymol and carvacrol, the main bioactive components of thyme essential oil have considerable antimicrobial and antifungal activity (Ghasemi-Pirbalouti *et al.* 2011). These two components crosses the bacterial cell wall, where after it may interact with periplasmic enzymes and, after penetration into the lipid-rich interior of the bacterial cytoplasmic membrane, may interact with membranal proteins and cause a back-flow of protons across the membrane, thus affecting the cellular activities powered by the proton motive force (Russell and Copra, 1990). The manipulation of gut functions and microbial habitat of domestic animals with feed additives has been recognized as an important tool for improving growth performance and feed efficiency (Viveros *et al.* 2011). It has also been reported that feed additives may be more efficient when the dietary nutrients level or availability is less than optimum (Torres-Rodriguez *et al.* 2005).

Plant-derived supplements, such as carvacrol, cinnamaldehyde, and capsicum oleoresin, have been noted to have an effect on stimulating the production and secretion of mucin in the intestine, thereby possibly impairing the adhesion of pathogens and having a positive effect on the gut of the chicken (Jamroz *et al.* 2006). Further benefits include influences on nutrient digestibility, nutrient absorption, intestinal morphology and a stabilizing effect on the intestinal microbiota (Windisch *et al.* 2008). Oregano essential oil and its active component, carvacrol, were found to be most potent and proved bactericidal. Thymol and its isomer carvacrol, components derived from thyme and oregano plants are classified as monoterpene phenols and have already proven their antimicrobial effect *in vitro* (Ouwehand *et al.* 2010). Numerous reports exist about the antibacterial effects of *Origanum vulgare*, *Piper nigrum*, *Syzygium aromaticum* and *Thymus vulgaris*, and the essential oil components thymol, carvacrol and eugenol against *Clostridium sporogenes* (Dorman and Deans, 2000) and other bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* (Cosentino *et al.* 1999). However, all these tests were done *in vitro* with only a limited number of tests performed in poultry (Losa and Kohler, 2001). Although the antimicrobial and antioxidative properties of plant oils are well known and confirmed in numerous studies, there is only slight evidence on intestinal histomorphology in broiler chickens fed on diets supplemented with active components of essential oils and CMC. The objective of the present experiment was to study the interaction effect of 100 and 200 mg/kg thymol + carvacrol (Next enhance® 150) on intestinal development of broiler chickens under standard and challenged gut conditions of broilers.

MATERIALS AND METHODS

Birds, management and experimental design

A total of 360 day-old male Ross 308 broilers were obtained from a local commercial hatchery and raised over a 42-d experimental period. The chicks were housed on wire floors in an environmentally controlled building. The experiment was performed as a completely randomized design in a factorial arrangement (3×2) with 5 replicates of 12 chicks each. Factors included 3 levels (0, 100 and 200 mg/kg) of Next Enhance150® (1:1 thymol: carvacrol; Novus international Inc., Missouri, USA) and 2 levels (0 and 2%) of CMC were added to the basal diet from first day of age. Throughout the study, the birds were brooded following standard temperature regimens, which gradually decreased from 32 to 21 ˚C and standard humidity regimens for first 3 days 70% and then 60% under a 23L:1D cycle. All starter, grower and finisher diets were formulated (Table 1) to meet the nutrient requirements according to Ross-308 rearing guideline (Aviagen, 2007).

Data collection

At 21 and 42 days of age, two birds per replicate were randomly selected, euthanized by cervical dislocation. The ileum was assigned from Meckel's diverticulum to ileo-caecal junction. Briefly, the small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction). The ileums were excised and contents were collected by gently squeezing into tubes. Digesta were pooled with a replicate, put on ice until they were transported to the laboratory for enumeration of microbial population. One gram of ileal contents was homogenized in 9 mL sterile water. Each sample was serially diluted. Using these diluted subsamples, Lactobacillus was enumerated on De Man-Rogosa-Sharpe (MRS) agar and *E. coli* was counted on Mac Conkey (MC) agar after incubated

at 37 ˚C in an anaerobic chamber for 48 h and in an aerobic chamber for 24 h, respectively (Guban *et al*. 2006).

Table 1 Composition of experimental diets

	Starter (1-10 d)	Grower (11-24 d)	Finisher (25-42 d)
Ingredients, g/kg			
Corn	53.20	55.88	57.25
Soybean meal, 44% CP	38.41	34.90	33.31
Wheat bran	2.02	2.02	2.02
Vegetable oil	2.08	3.60	4.10
Limestone	1.30	1.10	1.04
Dicalcium phosphate	1.65	1.40	1.31
Salt	0.42	0.42	0.40
HCl-Lys	0.21	0.08	0.00
DL-Methionine	0.15	0.10	0.07
Threonine	0.06	0.00	0.00
Vit. premix[1]	0.25	0.25	0.25
Min. premix[2]	0.25	0.25	0.25
Calculated composition (%, Unless otherwise noted)			
ME, kcal/kg	2850	2970	3020
CP	22.14	20.74	19.82
Calcium	1.00	0.85	0.8
Available phosphorus	0.47	0.42	0.40
Sodium	0.18	0.18	0.17
Lysine	1.35	1.17	1.03
Methionine	0.48	0.42	0.39
Methionine+Cystine	1.01	0. 90	0.81
Threonine	0.89	0.78	0.70

[1] Vitamin mix per kilogram of diet: vitamin A (*trans*-retinyl acetate): 10000 IU; vitamin D_3 (cholecalciferol): 3500 IU; vitamin E (DL-α-tocopheryl acetate): 60 mg; vitamin K (menadione): 3 mg; Thiamine: 3 mg; Riboflavin: 6 mg; Pyridoxine: 5 mg; vitamin B_{12} (cyanocobalamin): 0.01 mg; Niacin: 45 mg; Pantothenic acid (D-calcium pantothenate); 11 mg; Folic acid: 1 mg; Biotin: 0.15 mg; Choline chloride: 500 mg and Ethoxyquin (antioxidant): 150 mg.
[2] Mineral mix per kilogram of diet: Fe: 60 mg; Mn: 100 mg; Zn: 60 mg; Cu: 10 mg; I: 1 mg; Co: 0.2 mg; and Se: 0.15 mg.
ME: metabolizable energy and CP: crude protein.

The population of Bifidobacterium in ileal samples was determined using the standard laboratory method (Ibrahim and Salameh, 2001). Briefly, ileal samples (10 g) were diluted with 90 mL sterilized 0.1% peptone water and homogenized using Stomacher 400Lab System 4 (Seward, Norfolk, UK) for 2 min and 100 ml of appropriate dilution was surface plated onto modified BIM 24 agar (Ibrahim and Salameh, 2001). The level was determined at the serial dilution of 10-5. Plates were incubated at 37 ˚C for at least 3 days.

These birds were also considered for assessing the jejunal and ileal histomorphology. The mid part of jejunum and ileum were excised for morphological analysis. Samples of jejunum and ileum (3-cm segments) were obtained at its midpoint and immersed in a 10% buffered formalin solution for 72 h. Then they were excised and washed by physiological saline. The samples were treated in a tissue processor apparatus and embedded in paraffin wax (Bancroft and Gamble, 2002). Transverse sections were cut (2 µm) using a micrometer, placed on a glass slide and stained with hematoxylin and eosin, and analysed under a light microscope to determine morphometric indices. Morphological parameters were measured using the Image Pro Plus v 4.5 software package. The following measured morphometric variables were included: villus height (VH) was measured from the top of the villus to the top of the lamina propria; villus width (VW) was measured at half height; villus surface area (VS) was measured using the formula: $(2\pi) \times (VW/2) \times (VH)$; crypt depth (CD) was measured from the base upwards to the region of transition between the crypt and villus; VH:CD ratio; mucosa layer thickness (MCL); muscular layer thickness (MSL) and number of goblet cells (GC) (Sakamoto *et al.* 2000; Aptekmann *et al.* 2001). The mean from 10 villus per sample was used as the average value for further analysis.

Bioactive component analysis

To extract the active components from the feed and thymol + carvacrol, 4 g of grinded feed samples were weighed into a centrifuge tube with 2.5 mL of water, 1 mL of carvacrol as internal standard (200 mg/L) and 1 mL of ethanol. The calibration samples were prepared from control feed and supplemented with standard solutions of carvacrol and thymol at five different concentrations (5, 10, 20, 40 and 100 mg/L in ethanol) or unsupplemented ethanol as a blank. The samples were mixed and allowed to stand for 15 min. Then, 12 ml of diethyl ether was added; the samples were shaken for 16 h and centrifuged at 15000 × g for 5 min. To analyze the extracts, 1 mL of each supernatant was injected into the gas chromatograph with flame ionization detector (FID). Gas chromatographic analyses were performed using a GC PU 4500 system (Shimadzu GC solution) equipped with a flame ionization detector and E30 (30 m×0.32 mm ID, 5% phenyl methyl silicone; phase thickness 0.5 mm) capillary column. The column temperature ranged from 80 ˚C to 202 ˚C increments of 8 ˚C per minute. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. Sample injection was carried out in split less mode at 200 ˚C with split less time of 1 min using a sample injection volume of 0.5 µL. Temperature of the detector was 202 ˚C. Oven temperature was maintained initially at 80 ˚C for 2 min, then raised at a rate of 8 ˚C / min to 125 ˚C and maintained for 10 min, then raised at a rate of 25 ˚C / min to 200 ˚C and maintained for 10 min. The 5 concentration linear calibration curves were calculated using the internal standards. Using the peak heights the concentrations (mg/kg) of the analysts in the samples were calculated from the calibration curves.

Statistical analysis

The experiment was carried out as a completely randomized design with treatments arranged factorially. Data were

analyzed as a 3 × 2 factorial arrangement (3 levels of thymol + carvacrol and 2 levels of CMC) using PROC GLM of SAS (SAS, 2001). Data were analyzed considering the pen of birds as an experimental unit.

RESULTS AND DISCUSSION

Chemical composition of plant extracts and diets

Calculated and analysed carvacrol and thymol contents of Next enhance150® (mg/kg) are shown in Table 2. Analysis of the Next enhance150® by gas chromatography revealed the components to be: 54.13% carvacrol and 45.87% thymol.

Table 2 Calculated and analyzed carvacrol and thymol contents of the experimental diets (mg/kg)

	Calculated		Analyzed	
Experimental diets	**Carvacrol**	**Thymol**	**Carvacrol**	**Thymol**
Control	-	-	-	-
CMC	-	-	-	-
NE100	54.13	45.87	51	40.5
NE200	108.24	91.74	104.4	87.9
CMC + NE100	54.13	45.87	49	42
CMC + NE200	108.24	91.74	100.3	90

Control: contained no CMC or Next Enhance150®; CMC: Control + 2% CMC; NE100; 100 mg/kg Next Enhance150®; NE200: 200 mg/kg Next Enhance150®; CMC + NE100: 2% CMC+100 mg/kg Next Enhance150® and CMC + NE200: 2% CMC+200 mg/kg Next Enhance150®.

Intestinal microbial population

Ileal microbial population (log CFU/g of digesta) in broilers fed Carboxy Methyl Cellulose (CMC) and thymol + carvacrol at 42 days of age are shown in Table 3. There was no interaction effect of CMC and thymol + carvacrol in any measured parameters. At d 42, the ileal population of Lactobacilli and Bifidobacteria were greatest (P<0.05) in birds fed 200 mg/kg thymol + carvacrol but 100 mg/kg of additive had no significant difference with two others treatment. The *E. coli* population was significantly decreased in birds fed 100 and 200 mg/kg thymol + carvacrol. Supplementation of diet with CMC enhanced (P<0.05) ileal count of *E. coli* and reduced ileal counts of Lactobacilli and Bifidobacteria as compared to those in chicks fed control diet.

Intestinal histomorphology

Effect of feeding corn-based diets containing Carboxy Methyl Cellulose (CMC) and / or thymol + carvacrol on jejunal histomorphology of broilers at 21 and 42 days of age are shown in Tables 4 and 5. Data showed that supplementation of diet with CMC decreased (P<0.05) jejunal histomorphology including VH, VH:CD and increased (P<0.05) VW and CD at 21 and 42 days of age as compared to those in chicks fed control diet. Villus surface (VS), mucosa layer thickness (MCL), muscular layer thickness (MSL) and number of goblet cells (GC) were not affected by feeding CMC.

Table 3 Ileal microbial population (log CFU/g of digesta) in broilers fed Carboxy Methyl Cellulose (CMC) and thymol + carvacrol at 42 days of age

Treatments	*Lactobacillus*	*Bifidobacterium*	*E. coli*
Carboxy methyl cellulose (CMC), %			
0	8.43^{a}	8.52^{a}	6.53^{b}
2	8.22^{b}	8.43^{b}	6.97^{a}
SEM	0.026	0.027	0.077
Thymol + carvacrol (NE), mg/kg			
0	8.24^{b}	8.41^{b}	7.16^{a}
100	8.37^{a}	8.47^{ab}	6.89^{a}
200	8.48^{a}	8.56^{a}	6.21^{b}
SEM	0.032	0.033	0.094
P-value			
CMC	0.001	0.030	0.008
NE	0.003	0.015	0.001
CMC × NE	0.119	0.099	0.629

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

At d 21, thymol + carvacrol ameliorated (P<0.05) some jejunal histomorphology like VH, VS, VH:CD, MCL, MSL and GC but it did not affect VW and CD compared to the control diet.

Levels of 100 and 200 mg/kg thymol + carvacrol did not differ in all jejunal morphological parameters at d 21. At d 42, 200 mg/kg thymol + carvacrol enhanced (P<0.05) VH, VS, VH:CD and MSL compared to those fed 100 mg/kg additive or control diet and also the difference between 100 mg/kg thymol + carvacrol and the control diet was significant in VH. No interaction effect between CMC and thymol + carvacrol was observed in any of measured morphological parameters.

Effect of feeding corn-based diets containing Carboxy Methyl Cellulose (CMC) and / or thymol + carvacrol on ileal histomorphology of broilers at 21 and 42 days of age are shown in Tables 6 and 7.

At d 21, dietary CMC decreased (P<0.05) VH and increased (P<0.05) VW but it did not affect the rest of ileal histomorphological parameters. Adding phytogenic product into the basal diet increased (P<0.05) VH, VH:CD, MSL and GC whereas VW, VS, CD and MCL were not affected by phytogenic product. Inclusion of 100 or 200 mg/kg thymol + carvacrol was not differing in any ileal affected parameters at 21 days of age. At d 42, CMC supplementation significantly affected some ileal histomorphological parameters in terms of increasing VW and CD and decreasing VH and VH:CD but the other parameters were not influenced by CMC. Inclusion of 200 mg/kg phytogenic product increased (P<0.05) VH and VH:CD compared to other treatments and inclusion of 100 or 200 mg/kg of that enhanced (P<0.05) MSL and GC compared with those fed control diet.

Table 4 Effect of feeding corn-based diets containing Carboxy Methyl Cellulose (CMC) and / or thymol + carvacrol on jejunal[1] histomorphology of broilers at 21 days of age

	Morphological parameters							
Main effect	**VH[2] (µm)**	**VW[2] (µm)**	**VS[2] (µm^2)**	**CD[2] (µm)**	**VH:CD[2]**	**MCL[2] (µm)**	**MSL[2] (µm)**	**GC[2]**
Carboxy methyl cellulose (CMC), %								
0	1189^a	155^b	577,548	151^b	7.87^a	1394	4.11	292
2	1142^b	167^a	599,556	160^a	7.15^b	1399	4.11	289
SEM	14.32	3.88	15,067	1.41	0.130	10.29	6.00	4.61
Thymol + carvacrol (NE), mg/kg								
0	1062^b	159	530,323^b	154	6.94^b	1365^b	373^b	249^b
100	1200^a	167	627,539^a	158	7.62^a	1423^a	430^a	306^a
200	1236^a	157	607,795^a	155	7.96^a	1401^{ab}	430^a	316^a
SEM	17.54	4.75	18,453	1.72	0.159	12.61	7.35	5.65
P-value								
CMC	0.032	0.030	0.315	0.005	0.001	0.718	0.992	0.678
NE	0.001	0.316	0.003	0.243	0.008	0.014	0.001	0.001
CMC × NE	0.640	0.207	0.353	0.744	0.798	0.505	0.153	0.129

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

[1] The small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction).

[2] VH: villus height; VW: villus width; VS: villus surface area; CD: crypt depth; VH:CD: villus height to crypt depth ratio; MCL: mucosa layer; MSL: muscular layer and GC: number of goblet cells.

SEM: standard error of the means.

Table 5 Effect of feeding corn-based diets containing Carboxy Methyl Cellulose (CMC) and / or thymol + carvacrol on jejunal[1] histomorphology of broilers at 42 days of age

		Morphological parameters							
Main effect		**VH[2] (µm)**	**VW[2](µm)**	**VS[2] (µm^2)**	**CD[2](µm)**	**VH:CD[2]**	**MCL[2](µm)**	**MSL[2](µm)**	**GC[2]**
Carboxy methyl cellulose (CMC), %									
0		1343^a	231^b	971183	213^b	6.32^a	1447	4496	340
2		1263^b	248^a	984720	225^a	5.61^b	1437	502	340
SEM		13.34	4.01	20212	1.44	0.070	11.44	4.82	3.65
Thymol + carvacrol (NE), mg/kg									
0		1221^c	246	942800^b	219	5.59^b	1442	464^b	339
100		1297^b	232	944235^b	220	5.88^b	1454	515^a	337
200		1392^a	240	1046818^a	217	6.42^a	1459	516^a	343
SEM		16.34	4.91	24755	1.77	0.086	14.01	5.91	4.48
P-value									
CMC		0.005	0.006	0.641	0.001	0.001	0.557	0.397	0.974
NE		0.001	0.150	0.011	0.446	0.001	0.480	0.001	0.702
CMC × NE		0.069	0.537	0.421	0.204	0.023	0.641	0.009	0.658
Interactions									
CMC	NE								
0	0	1290	237	961610	210	6.15	1437	452	338
	100	1309	227	933571	216	6.07	1459	505	340
	200	1429	227	1018368	212	6.75	1443	531	341
2	0	1151	255	923990	228	5.04	1447	476	339
	100	1284	237	954900	225	5.70	1448	526	334
	200	1355	252	1075269	223	6.08	1415	503	345
SEM		23.11	6.948	35009	2.510	0.122	19.82	8.361	6.344

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

[1] The small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction).

[2] VH: villus height; VW: villus width; VS: villus surface area; CD: crypt depth; VH:CD: villus height to crypt depth ratio; MCL: mucosa layer; MSL: muscular layer and GC: number of goblet cells.

SEM: standard error of the means.

Intestinal microbial population

Antimicrobial activity has been recognized as the major beneficial effect of essential oils on poultry production, although the exact antimicrobial mechanism has not been fully revealed.

In the present study inclusion of thymol + carvacrol increased ileal counts of lactobasillus and bifidobacterium and decreased *E. coli* count at 42 days of age.

Table 6 Effect of feeding corn-based diet containing Carboxy Methyl Cellulose (CMC) and / or thymol+ carvacrol on ileal[1] histomorphology of broilers at 21 day of age

	Morphological parameters							
Main effect	**VH[2] (µm)**	**VW[2] (µm)**	**VS[2] (µm^2)**	**CD[2] (µm)**	**VH:CD[2]**	**MCL[2] (µm)**	**MSL[2] (µm)**	**GC[2]**
Carboxy methyl cellulose (CMC), %								
0	949	119^b	377003	105	9.07	1069	367	244
2	912^b	131^a	353571	104	8.81	1068	367	237
SEM	9.24	3.88	13054	1.088	0.128	5.97	6.00	4.91
Thymol + carvacrol (NE), mg/kg								
0	880^b	123	340263	104	8.47^b	1060	328^b	204^b
100	955^a	131	392038	105	9.13^a	1078	386^a	256^a
200	956^a	121	363560	104	9.21^a	1067	386^a	261^a
SEM	11.31	4.76	15988	1.33	0.157	7.31	7.35	6.02
P-value								
CMC	0.009	0.032	0.220	0.490	0.167	0.868	0.922	0.350
NE	0.002	0.316	0.099	0.929	0.007	0.235	0.001	0.001
CMC×NE	0.744	0.207	0.245	0.183	0.271	0.728	0.153	0.065

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

[1] The small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction).

[2] VH: villus height; VW: villus width; VS: villus surface area; CD: crypt depth; VH:CD: villus height to crypt depth ratio; MCL: mucosa layer; MSL: muscular layer and GC: number of goblet cells.

SEM: standard error of the means.

Table 7 Effect of feeding corn-based diet containing Carboxy methyl cellulose (CMC) and / or thymol + carvacrol on ileal[2] histomorphology of broilers at 42 day of age

	Morphological parameters							
Main effect	**VH[2] (µm)**	**VW[2] (µm)**	**VS[2] (µm^2)**	**CD[2] (µm)**	**VH:CD[2]**	**MCL[2] (µm)**	**MSL[2] (µm)**	**GC[2]**
Carboxy methyl cellulose (CMC), %								
0	1184^a	177^b	656578	160^b	7.39^a	1137	498	273
2	1111^b	201^a	701640	189^a	5.88^b	1134	499	272
SEM	15.71	3.99	17911	1.40	0.106	12.34	5.80	5.44
Thymol + carvacrol (NE), mg/kg								
0	1107^b	188	650653	173	6.47^b	1104	461^b	241^b
100	1137^{ab}	194	691245	177	6.47^b	1149	517^a	288^a
200	1198^a	185	695430	174	6.96^a	1152	517^a	288^a
SEM	19.24	4.89	21936	1.726	0.130	15.12	7.10	6.67
P-value								
CMC	0.004	0.005	0.092	0.001	0.001	0.865	0.912	0.822
NE	0.011	0.434	0.304	0.243	0.023	0.064	0.001	0.001
CMC × NE	0.099	0.286	0.084	0.744	0.073	0.633	0.111	0.068

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

[1] The small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction).

[2] VH: villus height; VW: villus width; VS: villus surface area; CD: crypt depth; VH:CD: villus height to crypt depth ratio; MCL: mucosa layer; MSL: muscular layer and GC: number of goblet cells.

SEM: standard error of the means.

Our results were in consistent with the findings of Jamroz and Kamel (2002) who report that the dietary herbal treatment results in lower *E. coli* counts comparing to the control group. It is shown that phytogenics modulated the intestinal microflora composition via the reduction of coliforms at 14 day of age and the beneficial fortification of gut microflora with purportedly beneficial members such as the *Lactobacillus* and *Bifidobacterium* at 42 day of age (Mountzouris *et al*. 2011). Cross *et al*. (2002) and Jang *et al*. (2007) also reported a reduction of *coliform* counts in birds supplemented with thyme oil or commercial blend of essential oils including thymol.

A blend of capsicum, cinnamaldehyde and carvacrol lowered the number of *E. coli* and *C. perfringens* (Jamroz *et al*. 2003). Tellez *et al*. (1993) and Orndorff *et al*. (2005) in chickens and Vicente *et al*. (2007) in laying hens also showed the prophylactic effect of capsaicin on *S. enteritidis*. However, Cross *et al*. (2007) showed no effect of herbs or their essential oils on the intestinal microbial populations. Inclusion of plant extracts in diets may therefore affect gut microflora and although the chemical composition of the extracts appears to be important in obtaining the optimal effects. It is speculated that the antimicrobial property of essential oils in birds can be influenced by the basal diet

and environmental conditions. Some studies showed a clear reduction of *Clostridium perfringens* colonization and proliferation in the jejunum and caecum of broilers fed with a mixture of thymol, eugenol, curcumin and piperin or carvacrol and thymol (Mitsch *et al*. 2004). The authors inferred that the effects of the products were due partly to a direct inhibition of the pathogens. However, digestive enzymes induced by essential oils could also increase nutrient digestibility and improve the regulation and stabilization of the gut microbiota. Inactivation of *C. perfringens* by digestive enzymes, such as trypsin (Baba *et al.* 1992), could also explain why colonization of these bacteria was reduced in the chickens gut by the essential oils. Similar results were presented by Sims *et al*. (2004) who demonstrated that *C. perfringens*-challenged broilers receiving a commercial blend of essential oils had less intestinal lesions and performed better than control birds.

Numerous *in vitro* studies demonstrated that thymol and carvacrol, displayed antimicrobial activity against intestinal microbes such as *Clostridium perfringes*, *Salmonella typhimurium* and *E. coli*. Antimicrobial action of essential oils is mediated by lipophilic property to perforate the bacterial membrane, which releases membrane components from the cells to the external environment (Helander *et al*. 1998). On the other hand, in an *in vivo* study, it seemed that the effect of essential oils on gastrointestinal microflora is not consistent, even though essential oils have been generally recognized as antimicrobial agents. Therefore, it was speculated that the *in vivo* antimicrobial property of essential oils in birds can be influenced by basal diet and environment conditions. Most essential oils exert their antimicrobial effect by damaging the bacterial cell wall, denaturating and coagulating proteins. They change the permeability of the cytoplasmic membrane to hydrogen (H^+) and potassium (K^+) ions, causing the interruption of essential cell processes, such as electron transport, protein translocation, phosphorylation steps and other enzyme dependent reactions, resulting in a loss of chemiosmotic control of the affected cell, leading to cell death (Veldhuizen *et al.* 2006). Changes in the permeability of bacteria' cell wall membranes are due to the lipophilic characteristic of essential oils, which accumulate in the membrane. The external membrane of gram-negative bacteria contains lipopolysaccharides, forming a hydrophilic surface. This hydrophilia creates a barrier to the permeability of hydrophobic substances, such as essential oils (Dorman and Deans, 2000). This explains the resistance frequently observed in gram-negative bacteria to the antimicrobial effect of some essential oils (Chao *et al.* 2000).

It is thought that the changes in membrane permeability may not be the direct cause of bacterial cell death, as the studies conducted by Trombetta *et al*. (2005) revealed that monoterpenes can cross the lipid bilayer, penetrate the cell and interact in specific sites, exerting their antimicrobial activity intracellularly. Other effects are shown by substances such as carvacrol, which prevents the synthesis of flagellin, causing bacteria / cells to be a flagellate and therefore non-motile. Such cells are significantly less able to adhere to the epithelial cells, which renders potentially pathogenic strains of bacteria non-infective (Burt *et al.* 2007).

In the present study, CMC elevated ileal population of *E. coli* and also reduced bifidobacterium and lactobacillus population. CMC is a non-fermentable, viscous fiber that raises the viscosity of intestinal chyme and lowers growth performance in broiler chickens which is explained by growth of pathogens and depressed digestibility of macronutrients (Smits *et al*. 1997). This observation corroborates earlier study (Smit *et al.* 1997). Smits *et al*. (1998) reported that inclusion of CMC in broiler's diet increased total aerobic and anaerobic microbial counts in the intestine digesta. Increasing Enterobacteriaceae counts in the caeca of birds fed CMC is probably because of viscous digesta. It has been shown that dietary cereals leads to high intestinal viscosity enhance enterobacteria (Hubener *et al*. 2002).

The results obtained by CMC disagree with the report of Shakouri *et al.* (2006) who indicated no effect of 3% of CMC on the number of anaerobic bacteria in the digesta of proximal parts (duodenum plus jejunum) of intestine. It may be because of high level of included CMC (3% of the diet) that resulted in high viscosity in the posterior parts of gastrointestinal tract and created a good environment by reducing digesta flow for bacterial activity in this part of the gut.

Intestinal histomorphology

The results of this study showed that thymol + carvacrol supplementation improved some intestinal histomorphological parameters of broilers at 21 and 42 d of age. Inclusion of 100 and 200 mg/kg thymol + carvacrol improved VH, VS, VH:CD and MSL of jejunum at 21 and 42 days of age and also increased ileal VH, VH:CD, MSL and GC at d 21 and MSL and GC at 42 days of age. These results are in agreement with those reported by Reisinger *et al.* (2011); due to supplementation of diets with a blend of essential oils from oregano, anise and citrus peel had the most notable effects on mid-ileum morphology, causing an increase in crypt depth as well as an increase in the total number of goblet cells. Other observation was reported by Jamroz *et al*. (2006), who observed quantitative increases in the number of goblet cells and in mucin secretion at the surface of the villi in the jejunum when feeding broilers a mixture of carvacrol, cinnamaldehyde and capsicum oleoresin.

In contrary, some studies reported that there was no significant effect in the VH, CD and VS due to feeding 200 ppm of plant extract, based on a blend of oregano, cinnamon and pepper essential oils and 5000 ppm of hydroalcoholic plant extract from sage, thyme and rosemary leaves.

Intestinal mucosa status and their microscopic structure might be good indicators of the response in the intestinal tract to active substances in feeds. Few reports have documented the effect of dietary polyphenols or related phenolics on the localized intestinal growth and function in broiler chickens and the contribution to changes in performance (Viveros *et al*. 2011). Improving intestinal health in the poultry industry is of a great importance to achieve target growth rates and feed efficiency (Montagne *et al*. 2003). Antimicrobial agents such as essential oils or their active components are known to reduce the intestinal microbial load, which in turn reduces the presence of toxins that are associated with changes in intestinal histomorphology, such as shorter villi and deeper crypts (Xu *et al*. 2003). Addition of 2% of CMC into the diet increased jejunal and ileal CD, VW and decreased VH and VH:CD at 21 and 42 days of age. These findings are in line with those of Jin *et al*. (1994), Klurfeld (1999) and Dhalke *et al*. (2003) indicating fibers can affect gastro-intestinal tract by affecting villi height and crypt depth in gastro intestinal tract. In contrary, Sarikhan *et al*. (2010) reported that supplementation of insoluble fiber to the diet enhanced VH and villi to crypt (V:C) but it had no effect on CD.

The intestinal mucosal architecture can reveal useful information on the intestinal function. Increasing villi height suggests an increased surface area for greater absorption of available nutrients (Awad *et al*. 2008). Also increase in villi to crypt ratio is related to an increase in digestion and absorption (Montagne *et al*. 2003).

In conclusion, for most parameters, it is shown that there is no interaction between bioactive component of essential oils and CMC. So it can be concluded that essential oils did not perform better under conditions of high viscosity. Thymol + carvacrol modified gut health by increasing non-pathogen bacteria and decreasing pathogens and subsequently improved intestinal structure like VH, VS, VH:CD and MSL. CMC changed intestinal histomorphology that may partly be contributed to changing the physico-chemical conditions of the gut and possibly the changing of the microflora.

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