EFFECT OF ESSENTIAL OIL BLEND ON INTESTINAL MORPHOLOGY, GUT MICROBIOTA AND IMMUNE RESPONSE OF BROILER CHICKENS

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Abstract

A study was conducted with the objectives to explore the effect of an essential oil (EO) blend containing cinnamon oil, clove oil and ajwain oil at equal ratio on intestinal morphology, gut microbiota and immune response of broiler chickens fed diet deficient in digestible amino acids. Total two hundred eighty eight (n = 288) day-old mixed sex Vencobb 400 broiler chickens were randomly divided into four experimental groups comprising of eight replicates each. Each replicate contained nine (9) birds. Birds were fed a positive control diet (PC), negative control diet (NC, 5% less digestible amino acids than PC), NC+ EO blend at 250 mg/kg diet (EO250), NC+ EO blend at 500 mg/kg diet (EO500) for 39 days. On day 39, one bird from each replicate was slaughtered for intestinal sample collection. The villi height in duodenum was significantly increased ($P < 0.05$) in EO500 group compared to PC and NC groups. The counts of *E coli*, *Lactobacillus* spp. and *Clostridium* spp. in precaecal digesta were not affected ($P > 0.05$) by dietary treatments. The antibody titers against Newcastle disease (ND) vaccine were higher ($P < 0.001$) in EOs supplemented groups in comparison to PC and NC groups. Supplementation of EO at 500 mg/kg diet increased ($P < 0.05$) catalase activity in serum, while superoxide dismutase (SOD) activity was not affected by dietary treatments. It is concluded that essential oils could be used to improve the gut morphology and immunity in broiler chickens.

Key words: Broiler chickens, Essential oil, Gut microbiota, Immune response, Intestinal morphology

INTRODUCTION

Essential oils (EOs) have attracted most attention in recent years in poultry industry because they can improve digestion, nutrient utilization, and may have a potential to promote nutrient sparing effect. Many studies have observed that EOs positively affect the morphology of small intestine, such as increased villus height and number of goblet cells (Jang *et al.*, 2007). Such effect on gut morphology

has been shown to improve nutrient digestibility and growth performance (Hernandez *et al.*, 2004). Essential oils may possess various biological properties, such as antimicrobials properties (Cowan, 1999; Ultee *et al.*, 1999), antioxidant and anti-inflammatory activities (Viuda-Martos *et al.*, 2010) and immunomodulating properties (Chowdhury *et al.*, 2018). Thus, EOs can be used as growth promoters in poultry production.

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Nevertheless, some studies reported negative or no effect of EOs on growth performance, intestinal morphology and gut microbiota (Muhl, 2007; Abildgaard *et al.*, 2010; Hafeez *et al.*, 2016). The inconsistent results were mainly attributed to dose, source and type of EOs and composition of EOs (Cross *et al.*, 2007; Buchanan *et al.*, 2008). *In-vitro* studies also suggested that combination of EOs has a greater effect than individual EOs alone, indicating a synergy between individual chemical of EOs (Burt, 2004; Lee *et al.*, 2004). Though there are substantial research works on EOs in broiler chickens, the studies on effect of EOs in poultry fed on diet deficient in nutrients are limited. We hypothesized that EOs blend may be capable of sparing nutrients by improving gut health leading to euobiosis. Therefore, the aim of the present study was to investigate the effect of EOs blend on gut microbiota, intestinal morphology, immunity and antioxidant status of broiler chickens fed on amino acids deficient diets.

MATERIALS AND METHODS

The Institutional Animal Ethics Committee of West Bengal University of Animal and Fishery Sciences, Kolkata, India approved the experimental procedures that were followed as per the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India.

Two hundred eighty eight (n=288) day-old mixed sex Vencobb 400 broiler chicks were randomly divided into four experimental groups comprising of eight replicates each. Each replicate contained nine birds. Corn and soybean based positive control (PC) diets were formulated to meet the requirements of the birds according to the breed specifications (Table 1). The negative control diets were prepared by reducing digestible amino acids content by 5% as compared to PC (Table 2). The dietary groups were consisted of 1) PC- basal diet without any additive; 2) NC- basal diet containing 5% less

digestible amino acids; 3) EO250- NC diet supplemented with EO blend at the rate of 250 mg/kg diet; 4) EO500- NC diet supplemented with EO blend at the rate of 500 mg/kg diet. The doses of the EOs were decided based on our *in vitro* study using pure pathogenic culture of *Escherichia coli* and *Salmonella gallinarum* isolated from chickens. Essential oils were obtained from a local store, Kolkata, India. A blend was made by cinnamon oil, clove oil and ajwain oil by mixing at equal ratio. No antibiotics were added to diets. The blend of EOs was mixed with the NC diet homogenously. The diet and water were provided *ad libitum*. The blended mash feed was packed in separate labeled HDPE (high density polythene) bags with inner lining. All the birds were vaccinated against Newcastle disease (ND) at 5 and 20 days of age, and infectious bursal disease (IBD) at 14 days of age.

Feed samples were analyzed for proximate composition (AOAC, 1995) and calcium (Talapatra *et al.*, 1940). At 28 and 35 days of experimental trial, blood sample (2 mL) was collected with the help of sterile syringes and needles by Vena brachialis puncture under the wing from one bird from each replicate, centrifuged and serum was stored in a deep freeze at -20°C for analysis of antibody titers and antioxidant status of the broiler chickens. The antibody titers against ND vaccine on day 28 and 35 were measured by using a commercial ELISA kit (x-OvO limited, United Kingdom). The activities of catalase (Beers and Sizer, 1952) and superoxide dismutase (SOD) (Misra and Fridovich, 1972) in serum samples were measured colorimetrically following standard procedures.

At day 39, one bird from each replicate was slaughtered by cervical disarticulation for measurement of intestinal morphology, bacterial count and lymphoid organs. The small intestine was removed and 2 to 3 cm sections of duodenum, jejunum (between the entry of bile duct and Mackel's diverticulum) and ileum were removed, rinsed with phosphate buffer saline (PBS) and cross

Table 1. Ingredient and nutrient composition of the positive control diets (digestible amino acids as per requirements)

	Starter (day 1 - 14)	Grower (day 15 - 28)	Finisher (day 29 - 39)
Ingredient composition (%)			
Maize	55.003	57.049	60.507
Soya bean meal	38.045	34.546	30.568
Veg. oil	2.772	4.456	5.116
Dicalcium phosphate	1.745	1.616	1.547
Lime stone phosphate	1.210	1.262	1.224
Salt	0.323	0.325	0.327
DL-Methionine	0.310	0.250	0.222
L-Lysin	0.283	0.197	0.188
L-Threonine	0.033	0.023	0.026
Toxin binder ¹	0.050	0.050	0.050
Sodium bi carbonate	0.100	0.100	0.100
Choline chloride	0.050	0.050	0.050
Organic trace mineral premix ²	0.050	0.050	0.050
vitamin primix ³	0.015	0.015	0.015
Antioxidant ⁴	0.010	0.010	0.010
Nutrient composition			
Metabolizable energy (kcal/kg) ⁵	3000	3125	3200
Crude protein (%) ⁶	22.50	21.00	19.50
Ether extract (%) ⁶	5.21	6.92	7.66
Calcium (%) ⁶	0.94	0.92	0.88
Dig. Lysine (%) ⁵	1.25	1.10	1.00
Dig. Methionine (%) ⁵	0.62	0.54	0.49
Dig. Methionine + Dig. Cysteine (%) ⁵	0.90	0.81	0.75
Dig. Threonine (%) ⁵	0.77	0.71	0.66

¹Nilttox™, Zeus Biotech Limited, Mysore, India; ²Contains (per kg): Zn proteinate- 50 g, Fe proteinate- 30 g, Cu proteinate- 10 g, Se proteinate- 0.5 g, Mn proteinate- 50 g, I- 4 g, Cu proteinate- 0.4 g (Zeus Biotech Limited, Mysore, India); ³Contains (per kilogram): vitamin A- 80,000,000 IU, vitamin D₃- 16,000,000 I.U, vitamin E- 64 g, vitamin K- 8 g, vitamin B₁- 6.4 g, vitamin B₂- 40 g, niacin- 96 g, pantothenic acid- 64 g, vitamin B₆- 12.8 g, folic acid- 6.4 g, vitamin B₁₂- 0.164 g and biotin- 0.24 g; ⁴Endox, Kemin Industries, Inc., USA, ⁵Calculated values; ⁶Analysed values

sectional lengths of 1 cm were fixed in 10% buffered formaldehyde (pH 7.2), followed by embedding in paraffin wax. The slides with the tissue sections were then stained with Delafield's Hematoxyline and Eosin, and mounted on

distrene plasticiser xylene (DPX) as per the protocol described by Kumar *et al.* (2017). The villi height and crept length were measured using an ocular micrometer (under a microscope fitted with a stage micrometer) and image analysis

Table 2. Ingredient and nutrient composition of the negative control diets (5% less digestible amino acids)

	Starter (day 1 - 14)	Grower (day 15 - 28)	Finisher (day 29 - 39)
Ingredient composition (%)			
Maize	58.707	60.528	63.638
Soya bean meal	34.804	31.499	27.772
Veg. oil	2.268	3.982	4.705
Dicalcium phosphate	1.780	1.649	1.577
Lime stone powder	1.214	1.266	1.227
Salt	0.324	0.326	0.365
DL-Methionine	0.293	0.232	0.202
L-Lysine	0.301	0.222	0.206
L-Threonine	0.035	0.022	0.032
Toxin binder ¹	0.050	0.050	0.050
Sodium bi carbonate	0.100	0.100	0.100
Choline chloride	0.050	0.050	0.050
Trace mineral premix ²	0.050	0.050	0.050
Vitamin primix ³	0.015	0.015	0.015
Antioxidant ⁴	0.010	0.010	0.010
Nutrient composition			
Metabolizable energy (kcal/kg) ⁵	3000	3125	3200
Crude protein (%) ⁶	21.38	19.95	18.53
Ether extract (%) ⁶	4.82	6.55	7.35
Calcium (%) ⁶	0.94	0.92	0.88
Dig. Lysine (%) ⁵	1.19	1.05	0.95
Dig. Methionine (%) ⁵	0.59	0.51	0.46
Dig. Methionine + Dig. Cysteine (%) ⁵	0.86	0.77	0.71
Dig. Threonine (%) ⁵	0.73	0.67	0.63

¹Nilttox™, Zeus Biotech Limited, Mysore, India; ²Contains (per kg): Zn proteinate- 50 g, Fe proteinate- 30 g, Cu proteinate- 10 g, Se proteinate- 0.5 g, Mn proteinate- 50 g, I- 4 g, Cu proteinate- 0.4 g (Zeus Biotech Limited, Mysore, India); ³Contains (per kilogram): vitamin A- 80,000,000 IU, vitamin D₃- 16,000,000 I.U, vitamin E- 64 g, vitamin K- 8 g, vitamin B₁- 6.4 g, vitamin B₂- 40 g, niacin- 96 g, pantothenic acid- 64 g, vitamin B₆- 12.8 g, folic acid- 6.4 g, vitamin B₁₂- 0.164 g and biotin- 0.24 g; ⁴Endox, Kemin Industries, Inc., USA, ⁵Calculated values;

⁶Analysed values

software (Biowizard 4.2, Dewinter Optical and New Delhi, India).

The pre-caecal contents of the intestine were aseptically collected from the birds after slaughter

on day 39 of the trial. After removal, the pre-caecal contents were stored at 4°C, and bacteriological enumeration was performed on the same day. One gram of the pre-caecal content was serially diluted (10-fold) with PBS. From 10⁻⁴ and 10⁻⁵

dilution, 10 µL was placed on eosin methylene blue (EMB) agar (HiMedia, Mumbai, India), Clostridial agar (HiMedia, Mumbai, India) and Lactobacilli Agar (HiMedia, Mumbai, India), for culture of *E. coli*, *Clostridium*, *Lactobacilli* spp., respectively. The plates were incubated aerobically at 37°C for 24 h except for *Lactobacillus* which was incubated for 48 h. Clostridial agar plates were incubated at 37°C anaerobically. The colonies for each pathogen were enumerated in a colony counter (hand held digital colony counter, LA663, Himedia Lab. Pvt. Ltd., Mumbai, India) and the numbers were expressed as Log₁₀ colony forming units (CFU) per gram of sample.

The data were analyzed by one way analysis of variance (ANOVA) using SPSS (1997) in a completely randomized design with a model containing treatments as the effect. Single pen was used as an experimental unit. Probability values of $P > 0.05$ were declared as significant and the values of $P > 0.05$ but ≤ 0.10 were declared as trend. When treatment effect was significant, the difference among the treatment mean were detected using Duncan's multiple range test.

RESULTS

Intestinal morphology and microbiota: Intestinal morphology is presented in Table 3. The villi height in duodenum was significantly increased in EO500 group compared to NC ($P < 0.05$), while no differences were observed among PC, EO250 and EO500 groups. A trend ($P < 0.1$) in greater villi height in jejunum was observed in EO500 group than other groups.

The villi height in ileum was not affected by treatments ($P > 0.05$). The crypt length in duodenum, jejunum and ileum was same across the groups. The VH/CD ratio was similar in all groups ($P > 0.05$). The pre-caecal bacterial count of birds was shown in Table 4. The counts of *E. coli*, *Lactobacillus* spp. and *Clostridium* spp. were not affected ($P > 0.05$) by dietary treatments.

Immune response and antioxidant enzymes:

Antibody titres against ND vaccine on day 28 were higher ($P < 0.001$) in EOs supplemented group in comparison to PC and NC groups, while the increment was higher in EO500 than EO250 (Table 5). On day 35, antibody titres were higher ($P < 0.01$) in EO500 than PC, NC, however no

Table 3. Effect of essential oil supplementation on intestinal morphology (µ) in broiler chickens

Attribute	Treatment				SEM	P- value
	PC	NC	EO250	EO500		
Duodenum						
Villi height (Vh)	1247 ^{ab}	1194 ^b	1247 ^{ab}	1343 ^a	19.55	0.049*
Crypt depth (CD)	68.68	68.09	73.02	70.46	1.743	0.572
Vh/CD ratio	18.92	18.27	17.27	19.23	0.516	0.568
Jejunum						
Vh	1028	989	1007	1109	18.02	0.085
CD	62.74	67.75	64.41	65.78	1.418	0.662
Vh/CD ratio	16.51	14.80	15.74	17.08	0.389	0.184
Ileum						
Vh	828	761	817	801	11.36	0.172
CD	56.04	57.28	61.34	64.93	1.453	0.115
Vh/CD ratio	14.99	13.61	13.65	12.50	0.453	0.295

* ($P \leq 0.05$), Means bearing different superscripts in the same row differ significantly

Table 4. Effect of supplementation of essential oil on viable bacteria numbers ($\log_{10}$ CFU/g) in precaecal content in broiler chickens

Precaecal bacteria	Treatment				SEM	P-value
	PC	NC	EO250	EO500		
<i>Escherichia coli</i>	5.66	5.41	5.73	5.84	0.100	0.522
<i>Lactobacillus</i> spp.	5.37	5.44	5.81	5.83	0.098	0.207
<i>Clostridium</i> spp.	5.88	5.84	5.95	5.62	0.085	0.571

Table 5. Effect of essential oil supplementation on antibody titre ($\log_{10}$) against Newcastle disease vaccine, weight of lymphoid organs and antioxidant enzyme activities in broiler chickens

Attribute	Treatment				SEM	P- value
	PC	NC	EO250	EO500		
Antibody titre						
day 28	877 ^c	994 ^c	1660 ^b	2620 ^a	172.83	0.001 ^{**}
day 35	2868 ^b	2593 ^b	3503 ^{ab}	4267 ^a	200.67	0.007 ^{**}
Lymphoid organs						
Liver (g)	50.03	46.99	47.59	51.60	0.956	0.295
Liver (% of SBW)	2.90	2.83	2.81	3.06	0.049	0.264
Spleen (g)	1.93	2.08	1.81	2.38	0.094	0.149
Spleen (%) of SBW	0.11	0.12	0.11	0.14	0.006	0.084
Bursa (g)	1.99	1.54	1.80	2.07	0.108	0.321
Bursa (% of SBW)	0.12	0.09	0.11	0.12	0.006	0.370
Antioxidant enzymes						
Catalase (U/mL)	9.83 ^b	15.99 ^b	23.02 ^{ab}	36.55 ^a	3.016	0.006 ^{**}
SOD(U/mL)	178.34	172.12	176.80	167.59	2.325	0.364

** ($P \leq 0.01$), Means bearing different superscripts in the same row differ significantly

differences were observed between EO250 and EO500 group. The weight and percent of slaughter body weight (SBW) of lymphoid organs was similar ($P > 0.05$) across treatments. (Table 5). However, a trend ($P < 0.1$) in higher percent of SBW of spleen was observed in EO500 group. The catalase activity was higher ($P < 0.01$) in EO500 than PC and NC, while no differences were observed between EO250 and EO500 groups. However, the SOD activity was similar ($P > 0.05$) among the groups (Table 5).

DISCUSSION

In this study, villi height of duodenum was

significantly increased by EO supplementation (500 mg/kg diet). A trend in greater villi height in jejunum due to supplementation of 500 mg EO blend/kg diet was also noted. The higher villi height leads to more surface area for absorption of nutrients which, in turn, results in better digestion and absorption of nutrients (Murugesan *et al.*, 2014). This result is similar with the result of Khattak *et al.* (2014) who reported that supplementation of a commercial feed additive (300 mg/kg) containing a blend of EO from basil, caraway, laurel, lemon, oregano, sage, tea, and thyme increased villi height of intestine. Similarly, Chowdhury *et al.* (2018) reported that

villi height of duodenum and jejunum was increased following supplementation of cinnamon oil at 300 mg/kg. The beneficial effects of EO blend on intestinal morphology might be associated with their anti-inflammatory effects, which in turn, protect the villi from oxidative damage (Du *et al.*, 2016).

The present study showed that the EO blend was ineffective to alter the precaecal bacterial counts. Chowdhury *et al.*, (2018) also reported that supplementation of cinnamon oil at 300 mg/kg diet, clove oil at 600 mg/kg diet or ajwain oil at 400 mg/kg diet did not alter the counts of *Clostridium* spp. and *Lactobacillus* spp., while count of *E. coli* was lowered. Similarly, Ahsan *et al.* (2018) observed that dietary supplementation of different levels of a commercial phytogenic feed additive containing cinnamon, cumin, peppermint oil, garlic oil, anise oil, fennel oil caused no differences in counts of *E. coli*, *Lactobacilli* and Coliforms. Most essential oils exert their *in vitro* antibacterial activity at dose level of 0.03 to 2% (Hammer *et al.*, 1999). However, EOs are supplemented in diet much lower levels than those required for *in vitro* antibacterial activity (Hafeez *et al.*, 2016). Therefore, the dose of EO blend in present study may not sufficient enough to exert antimicrobial activity.

Antibody titres against ND vaccine on day 28 and 35 were significantly higher owing to supplementation of EO blend at 500 mg/kg diet. This finding is similar as reported by Chowdhury *et al.*, (2018), where supplementation of cinnamon oil at 300 mg/kg diet, clove oil at 600 mg/kg diet or ajwain oil at 400 mg/kg diet increased antibody titres against ND vaccine in broiler chickens. Sadeghi *et al.* (2012) reported that dietary supplementation of cinnamon oil increased antibody titers against ND vaccine in broiler

chickens. Similarly, a commercial product containing mixture of oregano oil, carvacrol, thyme oil, eucalyptus oil, thymol and eucalyptol added at 0.5 mL/L of drinking water improved humoral immune response against ND vaccine (El-Shall *et al.*, 2020). Though the exert mechanism by which EOs stimulate immune response is largely unknown. It is postulated that the active component of EOs may act as additional ligands of Fc receptors of IgG molecules, which bind to IgG and stimulate the immune response (Nimmerjahn and Ravetch, 2010; Ahmed *et al.*, 2013).

The catalase activity was significantly increased by EO blend supplementation at 500 mg/kg diet, while SOD activity was not affected by EO blend supplementation. This result is in agreement with Gumus *et al.* (2017) who reported that supplementation of thyme essential oil at 150, 300 or 450 mg/kg diet increased serum catalase activity in Japanese quails. Lee *et al.* (2020) observed that supplementation of EO blend (an equal concentration of thymol and carvacrol) at 60 or 120 mg/kg diet did not affect serum catalase and SOD activities in broiler chickens. The antioxidant activity of EOs may be due to their ability to prevent lipid peroxidation, scavenging free radicals or, and in a few cases, chelating metal ions (Miguel, 2010).

Supplementation of EO blend containing cinnamon oil, clove oil and ajwain oil in equal proportion at 500 mg/kg to a diet deficient in digestible amino acids significantly increased villi height in duodenum, antibody titres against ND vaccine and serum catalase activity in broiler chickens. Therefore, it is concluded that essential oils could be used to improve the gut morphology and immunity in broiler chickens fed on amino acids deficient diet.

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