Letters in Animal Biology

Journal homepage: www.liabjournal.com

Dietary supplementation of a microencapsulated essential oil-organic acid blend with and without a protease affects performance, nutrient accretion, cecal microbiota, and circulatory biomarker concentrations in male broiler chickens

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Article info

Received: 13 May 2021
Received in revised form: 21 June 2021
Accepted: 22 June 2021
Published online: 23 June 2021

Keyword:

Broiler chickens
Caecal bacteria
Essential oil
Nutrient accretion
Organic acids
Protease

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Abstract

The objective of the present study was to investigate the effects of supplementation of a combination of a microencapsulated essential oil-organic acid (EO-OA) blend in diets of broiler chickens in absence or presence of an exogenous protease on performance and serum biomarker concentrations indicating small intestinal mucosal integrity. In a 42-days feeding trial, 800 male Cobb broiler chickens were divided into 8 treatments (10 replicates/treatment, n = 10 per replicate). The dietary treatments, formulated by supplementing the EO-OA and the protease enzyme to the basal diet, were, therefore, control(C), EO-OA 150 mg/kg diet (T1), EO-OA 300 mg/kg diet (T2), EO-OA 300 mg/kg diet up to 28 d followed by 3000 mg/kg diet till harvest (T3), C + protease 125 mg/kg diet (T4), T1 + protease 125 mg/kg diet (T5), T2 + protease 125 mg/kg diet(T6), T3 + protease 125 mg/kg diet (T7). The objective of adding the EO-OA at 3000 mg/kg diet in the T3 and T7 groups during the finishing stage was to ascertain if at a plethoric level of supplementation, the EO-OA could provide additional benefits when broiler chickens are exposed to several environmental and physiological stressor stimuli. EO-OA and protease had insignificant effect on body weight and feed conversion ratio. Protease alone increased carcass fat accretion during 14 - 42 d (P = 0.05). Serum D-lactate decreased when EO-OA in diet increased (P = 0.017) at 14 d. Irrespective of the dietary EO-OA level, serum D-lactate at 42 d decreased in the birds fed with the protease supplemented diets (main effect protease P = 0.025). *Clostridium perfringens* in caecal digesta at 42 d decreased due to protease supplementation (P = 0.049). Numbers of both *Escherichia coli* and *Campylobacter jejuni* in cecal digesta decreased by dietary EO-OA (P = 0.0001). Protease supplementation tended to decreased numbers of *E. coli* (P = 0.053) while significantly decreasing that of *C. jejuni* (P = 0.043). In this study EO-OA, with or without the protease, showed several beneficial effects which included reduction of potential pathogens in caeca, and a better nutrient accretion. The findings also revealed the possibility of modulating the intestinal microbiota through application of exogenous protease. The EO-OA and protease combinations may, therefore, be explored as an effective tool for growth promotion of broiler chickens in the post-antibiotic era.

1. Introduction

Increasing pressure to reduce the use of antimicrobial growth promoters (AGPs) in food animals makes it imperative to find alternatives to provide consumers with safe food without compromising the productivity of animals. The AGPs are used mainly to facilitate growth and feed conversion, which work mostly through modulating the small intestinal microbiota. Several dietary alternatives have been tried to replace or reduce AGPs in diets. Essential oil (EO) from phytogenic sources (PFA), organic acids (OA) and enzymes are some of these alternative strategies which are likely to offer potential benefits during the post-AGP era. Essential oils are concentrated volatile aromatic compounds obtained from plants with diverse positive effects on feed intake, nutrient digestibility, immune status, and antioxidative properties (Amerah et al. 2011; Karadas et al. , 2014, Wati et al. 2015; Yang et al. 2015). The controlled pathogen load through EO supplement- ation reportedly contributed to healthy microbial metabolites in the small intestine, improved intestinal integrity, and protection against enteric disease (Kirkpinar et al. 2011; Erhan et al. 2012; Hong et al. 2012; Zeng et al. 2015). Perhaps the most studied alternative to AGP is the OA (Polycarpo et al. 2017) which enhance performance through improvements of digestive enzymes activities, pancreatic secretions, and changes in the villus height and depth in the small intestine (Yang et al. 2016). Organic acids exert a direct bactericidal effect by penetrating

into the bacterial cells followed by disruption of bacterial metabolism (Suryanarayana et al. 2006). Several studies revealed that the addition of OA to broiler chicken diets improved weight gain (Nourmohammadi and Afzali 2013), increased feed intake (Moghadam et al. 2006), and improved feed conversion ratio (Abdel-Fattah et al. 2008). The OA and their blends inhibit the growth of potential gut pathogens like *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. (Engberg et al. 2000; Ricke, 2003; Dibner and Richards 2005; Garcia et al. 2007) and favour the growth of *Lactobacillus* (Nava et al. 2009) resulting in improved performance. A synergistic effect of EO and OA is expected when they are used together in a feeding regime owing to the overlapping effects they elicit when added individually (Cerisuelo et al. 2014; Thibodeau et al. 2014). In combination, EO and OA control gut microflora population more effectively (Luckstadt 2005) facilitating greater antibacterial activities in gut lumen (Manzanilla et al. 2004; Zhou et al. 2007) compared to when administered individually.

Ileal bypass protein is fermented by putrefactive bacteria in the caecum and produce many harmful and toxic compounds, like amines, indoles, phenols, cresol, and ammonia, which in high concentrations may adversely effects performance of the birds. Reducing the level of ileal bypass protein could potentially reduce the level of toxic metabolites in the caecum (Smith et al. 2005). Ideally, dietary protein should be easily digestible, microbial protein in the small intestine should be at the minimum and endogenous protein secretion used for defence is not exacerbated (Apajalahti and Vienola 2016). If the protein is highly digestible and amino acids are largely absorbed in the upper small intestine, where bacterial growth is suppressed by addition of other feed additives like EO and OA, the proportion of nutrients captured by the host should be higher. Therefore, exogenous protease enzymes which promote protein digestion are also likely to provide a competitive advantage to the chicken, offering less growth potential for amino acid-dependent bacteria (Apajalahti and Vienola 2016). It may not be inappropriate to assume that supplementation of protease should potentiate the action of EO and OA especially by curbing the unwarranted growth of bacteria, some of which might be potential pathogens, at the distal part of the small intestine. However, to achieve this objective, one of the challenges is to deliver the highly reactive and volatile EO and OA at the targeted site of action. Microencapsulation of EO and OA may provide a solution to this challenge by improving their stability and delaying their dissociation thus carrying them to the specific sites of absorption (Yang et al. 2016; Yang et al. 2019). The present study aimed to ascertain the effects of a microencapsulated EO-OA at different levels of inclusion along with a protease enzyme in diet on zootechnical performances, nutrient accretion and integrity of small intestinal mucosal cells measured through concentrations of specific biomarkers (anti *E. coli* lipopolysaccharide and D-lactate) in serum of male broiler chickens.

2. Materials and Methods

2.1 Dietary treatments

The experiment, which lasted for 42 d, involved feeding of a corn-soybean-meat and bone meal-based control diet (C) devoid of any gut acting growth promoter and exogenous protease enzyme to the birds and supplementation of this diet either with a combination of EO and OA or EO-OA combination and a protease enzyme. The treatment diets were formulated by supplementing the control diet with the microencapsulated EO+OA combination (obtained from Jefo Nutrition Inc., Quebec, Canada) at the rate of 150 mg/kg (T1), 300 mg/kg (T2) and 300 mg/kg up to 28 d of age followed by 3000 mg/kg till harvest (T3). Another four diets (T4 to T7) were formulated by supplementing the diets C to T3 with an exogenous protease at 125 mg/kg (obtained from Jefo Nutrition Inc., Quebec, Canada). The OA present in this blend included fumaric, sorbic, malic, and citric acids and the EOs were thymol, vanillin, and eugenol. The protease used in the current study was a commercial protease obtained from fermentation extracts of a naturally present novel bacterium where one protease unit hydrolyses azocasein to produce an absorbance at 440 nm equivalent to the action of one unit of a standard protease assayed under identical conditions (30 min, pH 7.7, 40 °C).

2.2 Experimental layout and general bird husbandry

The trial was conducted with 800 male Cobb chicks for 42 days following a completely randomized block design. The chicks were procured from a commercial hatchery and raised throughout the study period on litter in pens. Each treatment consisted of 10 pens with 10 chicks in each pen (n = 100 per treatment). A three-stage feeding comprised of starter (1-14 d), grower (15-28 d), and finisher (29-42 d) diets was followed. The ingredient composition, calculated and analysed nutrient composition, are presented in Table 1. All diets were in pelleted form and supplemented with a phytase {(a Buttiauxella phytase expressed in *Trichoderma reesei* fungus with declared phytase activity of 5000 FTU/kg) (Axtra Phy, Danisco Animal Nutrition, Marlborough, UK)} and the matrix of calcium, available phosphorus, and sodium of the phytase was considered during diet formulation. However, carbohydrate degrading enzymes, AGPs, and other gut acting growth promoters like prebiotics and probiotics were not used to avoid confounding effects from these additives. Chemical anticoccidial agent (diclazuril 0.5%) was used for coccidia control. All diets contained an animal protein (meat and bone meal) at a fixed quantity (5%) and the level of crude protein in the diets was kept at a comparatively

higher level. The birds had *ad libitum* access to drinking water. Identical management practices were followed throughout the experiment for all the dietary groups. All chicks received feed within 12 h of hatch and were exposed to continuous lighting program during the first week of age. Lighting period was reduced to 20 h a day thereafter. Vaccinations included the Infectious Bronchitis administered at 0 d of age (IB Ma5), Newcastle Disease live vaccine (Clone 30) on the 5th and 20th d of age, and Infectious Bursal Disease vaccine at 12 d of age (228E; Int plus).

2.3 Measurement of performance traits

Body weight (BW) was recorded weekly by pens at the same time of a day without fasting (0800 h). A measured quantity of feed was offered to each pens daily in two equal divisions and the cumulative feed intake (FI) was calculated weekly by subtracting the quantity of feed left in each pen from the total quantity of the feed offered. The *ad libitum* access to feed was ensured throughout the experiment. Average daily body weight gain (ADG) and average daily feed intake (ADFI) were calculated for the respective feeding periods which corresponded to 1-14 d, 15-28 d, 29-42 d, and 1-42 d and both the ADG and ADFI data for the corresponding periods were reported. Feed conversion ratio (FCR, ADFI/ADG) for the periods mentioned above were also reported. Mortality was recorded as and when it happened, and the weight of the dead birds was considered to adjust the FCR. Overall liveability was calculated during 1-42 d and European Productivity Index (EPI) was calculated according to the following formula:

$$EPI = [(100 - \text{mortality}) \times (\text{mean BW/age}) \times 100]/ FCR.$$

2.4 Nutrient accretion in carcass

The protein and fat accretion in carcass between 14 and 42 d of age was measured according to Samanta et al. (2009). It was assumed that the birds would reach a stable body composition within 14 d and so this age was considered to obtain the base line value. At 14 d, 10 birds were selected randomly (one bird from a pen) from each of the dietary treatments which were weighed and killed humanely by mechanical stunning followed by exsanguination and the same process was repeated at 42 d. The carcass was frozen at -20 ^{0}C and the breast muscles (including the pectoralis major and pectoralis minor) was used for analysis of nitrogen (N) and crude fat. The muscle was minced, homogenised in a tissue homogenizer, and the homogenised samples were finally analysed for N and crude fat (AOAC 1984). Nitrogen and CP content (N x 6.25) was determined using the Kjeldahl distillation procedure. For estimation of crude fat, moisture free dry meat samples were ground to pass through a 0.5 mm sieve and extracted with petroleum ether for 24 h with ether being changed every 8 h. All values concerned with the chemical composition of meat were expressed on a fresh weight (FW) basis. Protein and fat accretion were determined as a difference between their total content at 14 d and 42 d.

2.5 Enumeration of selected bacterial species in caecal contents

At 42 d, 1 bird from each pen was selected randomly and killed by mechanical stunning followed by exsanguination. The ceca were separated, washed repeatedly with sterile phosphate buffer saline (PBS) to remove the tissue debris, cut open through an incision, and the digesta was collected in sterile vials by applying gentle digital pressure for enumeration of *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens,* and *Campylobacter jejuni* (Muthusamy et al. 2011). The digesta was stored at 4oC till analysed. Approximately 1 g digesta samples were homogenised with double the volume of PBS in ice. The homogenised samples were decimally diluted with sterile PBS. The diluted sample (1 ml) was plated and cultured on specific media (Hi Media Laboratories, Mumbai, India) at 37oC for 36 h. The plates of *C. perfringens* were incubated anaerobically for 36-48 h. The visible colonies were enumerated in a colony counter and the numbers were expressed as Log10 colony forming units (cfu)/g.

2.6 Analyses of serum biomarkers

Serum concentrations of bacterial metabolites, D-lactate, and endotoxin lipopolysaccharide (LPS) were determined at 14 and 42 d. Whole blood was collected from 10 randomly selected birds (one from each pen) per treatment on each occasion through brachial venepuncture. The serum was separated by clotting the blood at room temperature and preserved at -20 ^{0}C. D-lactate was determined using a D-lactate colorimetric assay kit (Abcam PLC., Cambridge, United Kingdom) in a 96 well microplate reader at 570 nm wavelength (Bio Tek 800 TS micro plate reader, Vermont, USA) while LPS assay was carried out following double antibody sandwich ELISA method using a commercially available chicken specific anti-*E. coli*-LPS kit (Abcam PLC., Cambridge, United Kingdom) in the same equipment. Standard curves were generated and the results obtained were extrapolated to derive the concentrations of the markers using the best fit equation generated through an online data analysis tool (*www.MyCurveFit.com*).

2.7 Statistical Analysis

For BW, ADG, FI, ADFI, FCR and EPI, data were pooled by pens and those related to protein and fat accretion in carcass, caecal microbiology and serum biomarker levels, the individual observation from a single pen was considered as an experimental unit. The levels of dietary EO-OA and that of the protease used in the study were used as the main

Table 1 Ingredients composition (g/kg) and calculated nutritive values of the basal diets

Ingredients (g/kg)	Starter 1-14 d	Grower 15-28 d	Finisher 29-42 d	Calculated nutritive values (g/kg) unless stated otherwise	Starter 1-14 d	Grower 15-28 d	Finisher 29-42 d
Maize	562	580	616	ME Kcal/kg	2950	3050	3100
Soybean meal[1]	330	306	270	Crude protein	235.2	225.1	210.3
De-oiled rice bran	14.5	14.5	14.5	Digestible amino acids			
Meat-bone meal[2]	50.0	50.0	50.0	Lysine	13.2	12.0	11.0
Rice bran oil	21.0	30.0	32.0	Methionine	5.80	5.50	4.80
Di-calcium phosphate	5.40	3.50	3.00	Met + Cys	9.20	8.80	8.00
Limestone powder	3.30	3.00	2.60	Threonine	8.50	8.00	7.40
DL-Methionine	2.70	2.50	2.30	Tryptophan	2.50	2.30	2.20
L-lysine HCl	2.30	1.70	1.50	Arginine	15.4	14.6	13.5
L-threonine	0.70	0.70	0.50	Isoleucine	9.00	8.60	8.00
Salt	2.50	2.50	2.50	Valine	10.0	9.60	9.00
Sodium-bi-carbonate	1.50	1.50	1.00	Calcium	9.00	8.40	8.00
Trace mineral premix[3]	0.50	0.50	0.50	Available P	4.80	4.50	4.20
Choline chloride 60%	1.00	1.00	1.00	Sodium	2.40	2.20	2.00
Vitamin Premix[4]	1.00	1.00	1.00	Potassium	9.80	9.30	8.70
Toxin Binder	1.00	1.00	1.00	Chloride	2.50	2.40	2.20
Phytase 5000[5]	0.10	0.10	0.10	Choline mg/kg	1850	1800	1750
Diclazuril 0.5%	0.50	0.50	0.50	Crude fibre	30.0	28.0	27.0
Test products were added replacing an equivalent amount of de-oiled rice bran				Crude fat	50.0	55.0	60.0

[1] Analysed crude protein content was 495 g/kg
[2] Analysed crude protein content was 433 g/kg
[3] Contained manganese 60 mg, iron 30 mg, zinc 50 mg, copper 10 mg, selenium 0.5 mg, chromium 0.4 mg (all as yeast protein chelates), iodine 4.0 mg (as potassium iodide)
[4] Each kg contained vitamin A 13.5 MIU, vitamin D3 4.5 MIU, vitamin E 60 g, vitamin K3 3.5 g, vitamin B1 3.5 g, vitamin B2 8.0 g, vitamin B6 3.5 g, vitamin B 12 0.02 g, biotin 0.145 g, pantothenic acid 14.5 g, folic acid 2.25 g, niacin 60 g (DSM Nutritional Products India P Ltd., Mumbai, India); 5Buttiauxella phytase from Danisco Animal Nutrition, Marlborough, UK (Axtra Phy 5000).

factors and accordingly the data were analysed with a 4 (levels of EO-OA, 0, 150, 300 and 300/3000 mg/kg) x 2 (levels of protease, 0 and 125 mg/kg) factorial design through multivariate analysis of variance in the general linear model of SPSS (v 26.0). The main effects and interactions were calculated, and the results were expressed as mean and pooled standard error of mean. The effects of supplementation of the EO-OA and that of the protease was separated and presented as independent effects while the interaction between these two factors were presented as an overall "diet" effect. Probability of $P < 0.05$ was expressed as significant and that of $P < 0.1$ was described as trend. Whenever, a significant difference was observed the mean were further separated by Tukey's B test.

3. Results

3.1 Performance traits

Although BW in all the treated groups was 10 to 20 g higher than that in the control group (Table 2), EO-OA and protease independently had insignificant effect on these parameters ($P > 0.05$). The EO-OA blend decreased FI at 28 d (main effect EO-OA $P = 0.0001$) and ADFI during 15-28 d (main effect EO-OA $P = 0.001$) when supplemented at the rate of 300 mg/kg diet till 28 d and 3000 mg/kg diet thereafter as compared with the control group (Table 3). There was significant EO-OA*protease interaction on FI at 28 d ($P = 0.0001$) and ADFI during 15-28 d ($P = 0.0001$) with significantly lower values being observed in the T3 and T7 groups (both of which were supplemented with the protease and the higher levels of EO-OA) compared to the control. Protease alone, however, did not affect FI (main effect $P > 0.05$). Owing to the difference in FI during 15-28 d, FCR varied during that period (Table 4). Irrespective of the protease effect, EO-OA, at 300/3000 mg/kg diet improved FCR at 28 d as compared with the groups receiving EO-OA at 0 and 150 mg/kg diet (main effect EO-

OA P = 0.017). However, FCR was similar across all the levels of EO-OA during 29-42 d (main effect EO-OA P = 0.958) and the difference was just numerical when the data was pooled over the period of 1-42 d (main effect EO-OA P = 0.105). Protease improved FCR during 1-14 d (main effect protease P = 0.026) and 15-28 d (main effect protease P = 0.025) but not during 29-42 d (main effect protease P = 0.13) leading to a blunted response when the data was pooled together from 1-42 d (main effect protease P = 0.886). When supplemented together with the protease, the EO-OA at 300/3000 mg/kg improved (EO-OA*protease P = 0.018) FCR in the T7 group during 15-28 d compared to the control and T1 group. Liveability was similar (P > 0.05 for both the main effects and the interaction) across the treatments and so was the productivity index (Table 4) though the latter was numerically superior in all the treated groups irrespective of their levels of inclusions and the difference was as high as 5% which is commercially quite substantial.

3.2 Accretion of N and fat in carcass

Carcass N and fat contents (Table 5) at 42 d were refractory to the EO-OA levels in diet (main effect EO-OA P = 0.786 for N and P = 0.215 for fat) which had insignificant effect on their accretion during 1-42 d as well (main effect EO-OA P = 0.796 for N and P = 0.085 for fat). Protease increased N content in carcass at 14 d (main effect protease P = 0.001) but not on 42 d (main effect protease P = 0.262). Total carcass N tended to increase due to protease at 14 d (main effect protease P = 0.061) but not at 42 d (main effect protease P = 0.389). Protease decreased fat content (main effect protease P = 0.011) and total fat (main effect protease P = 0.013) in carcass at 42 d. The effect of protease supplementation on N accretion during 14-42 d was insignificant (P > 0.05) though the amount of fat deposited (main effect protease P = 0.023) either as a whole and that expressed relative to per kg BW gain (main effect protease P = 0.021) decreased by supplemental protease in diet. The interaction effect indicated that at 14 d, N in carcass increased by protease as revealed by higher carcass N in T4 to T7 groups (P = 0.004). Fat content in carcass was higher in the T3 group as that in the control, T5 and T6 groups (P = 0.041) at 42 d and so was the total fat in carcass (P = 0.035). Total fat accretion (P = 0.035) and that as a function of BW gain during 14-42 d (P = 0.042) was higher in the T3 group as compared with the control and the other protease supplemented groups albeit with no definite trend.

Table 2 Weekly body weight and average daily gain body weight of the experimental birds during 1-42 d of age[1]

Treatments	Weekly body weight (g)						Average daily gain in body weight (g)			
	7-d	14-d	21-d	28-d	35-d	42-d	1-14 d	15-28 d	29-42 d	1-42 d
Main effect: EO-OA										
0 mg/kg	234	599	1110	1753	2396	2868	39.6	82.4	79.7	67.2
150 mg/kg	235	596	1109	1758	2395	2874	39.4	83.0	79.7	67.4
300 mg/kg	235	599	1112	1766	2398	2876	39.6	83.4	79.3	67.4
300/3000 mg/kg[2]	234	596	1109	1754	2394	2879	39.4	82.7	80.4	67.5
P- value	0.904	0.895	0.991	0.888	0.999	0.987	0.894	0.876	0.946	0.987
Main effect: Protease										
0 mg/kg	235	597	1108	1748	2395	2874	39.5	82.2	80.4	67.4
125 mg/kg	234	598	1112	1767	2397	2875	39.5	83.6	79.1	67.4
P-value	0.370	0.898	0.586	0.135	0.887	0.956	0.926	0.103	0.31	0.961
Overall diet effects[3,4]										
Control	235	596	1106	1741	2390	2861	39.4	81.8	80.0	67.1
T1	235	596	1107	1746	2393	2876	39.4	82.1	80.7	67.4
T2	237	602	1111	1761	2399	2877	39.9	82.8	79.7	67.5
T3	234	594	1106	1743	2396	2880	39.3	82.1	81.2	67.5
T4	234	602	1113	1765	2402	2875	39.8	83.1	79.3	67.4
T5	234	596	1111	1769	2397	2871	39.4	83.8	78.8	67.3
T6	234	596	1114	1771	2396	2875	39.4	83.9	78.9	67.4
T7	233	597	1112	1765	2392	2878	39.5	83.4	79.5	67.5
Pooled SEM	0.8	2.0	4.3	6.5	8.5	10.6	0.14	0.41	0.63	0.25
P- value	0.892	0.748	0.997	0.98	0.988	0.989	0.747	0.998	0.982	0.989

[1] Means of 10 replicate pens in each dietary group (N = 10 birds in a single replicate up to 14 d; 9 birds from 15-42 d)

[2] Supplemented at the rate of 300 mg/kg diet up to 28 d and 3000 mg/kg diet from 29 d till harvest

[3]Control: EO-OA 0 mg, protease 0 mg, T1: EO-OA 150 mg/kg; T2: EO-OA 300 mg/kg; T3: EO-OA 300 mg/kg till 28 d followed by 3000 mg/kg till 42 d; T4: Control + Protease 125 mg/kg; T5: T1 + protease 125 mg/kg; T6: T2 + Protease 125 mg/kg; T7: T3 + Protease 125 mg/kg

[4] Represents means separated through one way ANOVA

Table 3 Weekly cumulative feed intake and average daily feed intake of the experimental birds during 1-42 d of age[1]

Treatments	Weekly cumulative feed intake (g)						Average daily feed intake (g)			
	7-d	14-d	21-d	28-d	35-d	42-d	1-14 d	15-28 d	29-42 d	1-42 d
Main effect: EO-OA										
0 mg/kg	180	597	1206	2207^{c}	3284	4298	42.6	115^{c}	149	102
150 mg/kg	180	590	1215	2196^{bc}	3282	4264	42.2	115^{bc}	1486	102
300 mg/kg	180	591	1206	2171^{ab}	3276	4241	42.2	113^{ab}	148	101
300/3000 mg/kg[2]	180	586	1189	2142^{a}	3258	4234	41.8	112^{a}	149	101
P- value	0.983	0.403	0.47	0.0001	0.913	0.18	0.402	0.001	0.723	0.179
Main effect: Protease										
0 mg/kg	181	594	1201	2187	3279	4259	42.5	114	148	101
125 mg/kg	179	588	1207	2172	3271	4259	42.0	113	149	101
P-value	0.223	0.167	0.591	0.132	0.783	0.972	0.167	0.303	0.47	0.971
Overall diet effects[3,4]										
Control	182	599	1196	2222^{d}	3302	4302	42.8	116^{d}	149	102
T1	182	594	1213	2215^{cd}	3299	4268	42.4	116^{d}	147	102
T2	180	598	1218	2170^{ab}	3284	4242	42.7	112 ^{abc}	148	101
T3	181	587	1177	2140^{a}	3231	4226	41.9	111^{a}	149	101
T4	179	595	1217	2192^{bcd}	3266	4294	42.5	114^{cd}	150	102
T5	179	587	1217	2178^{abc}	3266	4259	41.9	114^{bcd}	149	101
T6	180	585	1194	2173^{abc}	3268	4240	41.8	113^{abcd}	148	101
T7	179	584	1201	2144^{a}	3285	4242	41.8	111^{a}	150	101
Pooled SEM	0.7	2.3	5.8	5.7	13.6	11.1	0.17	0.3	0.7	0.3
P- value	0.951	0.571	0.601	0.0001	0.938	0.633	0.571	0.0001	0.942	0.632

[1] Means of 10 replicate pens in each dietary group (N = 10 birds in a single replicate up to 14 d; 9 birds from 15-42 d)
[2] Supplemented at the rate of 300 mg/kg diet up to 28 d and 3000 mg/kg diet from 29 d till harvest
[3] Control: EO-OA 0 mg, protease 0 mg, T1: EO-OA 150 mg/kg; T2: EO-OA 300 mg/kg; T3: EO-OA 300 mg/kg till 28 d followed by 3000 mg/kg till 42 d; T4: Control + Protease 125 mg/kg; T5: T1 + protease 125 mg/kg; T6: T2 + Protease 125 mg/kg; T7: T3 + Protease 125 mg/kg
[4] Represents means separated through one way ANOVA

3.3 Serum analytes

The effects of EO-OA and protease on serum D-lactate and anti *E. coli* LPS concentrations were variable (Table 6). EO-OA decreased serum D-lactate in a dose dependent manner at 14 d (EO-OA $P = 0.017$) but not at 42 d (EO-OA $P = 0.254$). Activity of LPS was refractory to EO-OA at both 14 d and 42 d (EO-OA $P > 0.05$). Protease, irrespective of the of the levels of EO-OA in diet, had insignificant effect on serum D-lactate (main effect $P = 0.952$) at 14 d though it decreased the same at 42 d (protease $P = 0.025$). However, protease had little effect on serum LPS at 14 d and 42 d (protease $P > 0.05$). The EO-OA*protease interaction with regard to serum D-lactate activity was significant at 14 d ($P = 0.04$) and tended to be significant at 42 d ($P = 0.065$). The trend indicated that EO-OA at a higher dose level (300/3000 mg/kg) in the T3 group without or with the protease decreased D-lactate in serum as compared to the control group. The EO-OA*protease interaction regarding serum LPS was not significant at either points ($P > 0.05$).

3.4 Bacterial population in caeca

EO-OA decreased cecal *E. coli* and *C. jejuni* (EO-OA $P = 0.0001$) with the latter apparently showing a dose dependent effect (Table 7). The effect of EO-OA on *C. perfringens*, and *Lactobacillus* was not significant ($P > 0.05$). Protease, irrespective of the level of EO-OA in the diets, decreased *C. perfringens* ($P = 0.049$) and *C. jejuni* ($P = 0.044$), tended to decrease *E. coli* ($P = 0.053$) and increased *Lactobacillus* ($P = 0.045$) in the caeca. Number of *C. perfringens* was higher in the control group as compared to that in the T5 and T7 groups ($P = 0.033$). Supplementation of EO-OA either alone or in combination with protease decreased the numbers of *E. coli* ($P = 0.0001$) and *C. jejuni* ($P = 0.0001$) as compared to that in the control group. It was further revealed that supplementation of EO-OA at incremental dose levels and combining the higher dose level (300/3000 mg/kg) with protease decreased the numbers of *C. perfringens, E. coli* and *C. jejuni*. *Salmonella* were not detected up to a dilution factor of 4 and further lowering the dilution did not give rise to counts more than 10 CFU/g and that too in an inconsistent manner and hence was not reported.

Table 4 Feed conversion ratio, liveability, and European productivity index in experimental birds during 1-42 d[1]

Treatments	Feed conversion ratio				Liveability (1-42 d)	EPI (1-42 d)
	1-14 d	15-28 d	29-42 d	1-42 d		
Main effect: EO-OA						
0 mg/kg	1.08	1.40^{c}	1.88	1.52	92.0	414
150 mg/kg	1.07	1.39^{bc}	1.86	1.51	93.0	422
300 mg/kg	1.07	1.36^{ab}	1.88	1.50	93.0	425
300/3000 mg/kg[2]	1.06	1.35^{a}	1.86	1.49	92.5	424
P- value	0.330	0.017	0.958	0.105	0.974	0.83
Main effect: Protease						
0 mg/kg	1.08	1.39	1.85	1.51	92.5	421
125 mg/kg	1.06	1.36	1.89	1.51	92.8	422
P-value	0.026	0.025	0.13	0.886	0.888	0.859
Overall diet effects[3,4]						
Control	1.09	1.43^{c}	1.86	1.53	91.0	407
T1	1.08	1.41^{bc}	1.83	1.51	94.0	428
T2	1.07	1.36^{ab}	1.87	1.50	93.0	425
T3	1.07	1.35^{ab}	1.84	1.49	92.0	423
T4	1.07	1.38^{abc}	1.90	1.52	93.0	421
T5	1.06	1.36^{ab}	1.90	1.51	92.0	417
T6	1.06	1.35^{ab}	1.89	1.50	93.0	425
T7	1.06	1.34^{a}	1.89	1.50	93.0	426
Pooled SEM	0.003	0.007	0.015	0.004	0.88	4.7
P- value	0.279	0.018	0.876	0.455	0.995	0.97

[1] Means of 10 replicate pens in each dietary group (N = 10 birds in a single replicate up to 14 d; 9 birds from 15-42 d)
[2] Supplemented at the rate of 300 mg/kg diet up to 28 d and 3000 mg/kg diet from 29 d till harvest
[3] Control: EO-OA 0 mg, protease 0 mg, T1: EO-OA 150 mg/kg; T2: EO-OA 300 mg/kg; T3: EO-OA 300 mg/kg till 28 d followed by 3000 mg/kg till 42 d; T4: Control + Protease 125 mg/kg; T5: T1 + protease 125 mg/kg; T6: T2 + Protease 125 mg/kg; T7: T3 + Protease 125 mg/kg
[4] Represents means separated through one way ANOVA

4. Discussion

The present experiment was conducted to ascertain if a blend of EO and OA supplemented at varying levels could promote the growth performance of broiler chickens under normal conditions of rearing and whether the presence of protease could enhance this response. The inclusion levels of the EO-OA used in this study were increased in a graded manner and the objective was to ascertain the optimum inclusion level to achieve the maximum benefit in terms of performance. In T3, the combination of 300 mg/kg up to 28 d and 3000 mg/kg from 29 d till harvest was used. The higher dose was applied during the finisher stage when the growth is extremely fast and the feed intake is at its peak, exposing the birds to a severe challenge of proper absorption of nutrients in order to support the growth. Hence, a higher dietary EO-OA level was presumed to facilitate nutrient absorption. Protease was added as an adjunct to the EO-OA and the objective was not to study the effects of the protease per se rather the combined effect of EO-OA and protease, if there is any, was the subject of interest in this experiment.

Further, the experiment was conducted without imposing any enteric challenges. Although for proper evaluation of gut acting growth promoters, it is important to disturb intestinal homeostasis (Stefanello et al. 2020) because in absence of such challenges, they can hardly enhance performance (Bedford, 2000). However, pathogens invading the GIT under challenged conditions may exacerbate or derogate the effects of gut modulating agents like EO, OA and feed enzymes. In order to develop strategies to help broiler chickens reach their maximum growth potential, it is important to have a clear understanding of the mechanisms involved in the functionality and health of the intestine when such additives are used in absence of enteric challenges.

Trials assessing the effect of EO, OA or their blends in broiler chickens yielded variable effects in performance and nutrient digestibility (Botsoglou et al. 2002; Lee et al. 2003; 2004; Hernandez et al. 2004; Cross et al. 2007) mainly because the effects depend on the quality and quantity of the EO used. In many cases, the detailed information on the blends used is not always reported, probably due to the fear

Table 5 Nitrogen (N) and fat accretion in carcass (*Pectoralis major* & *Pectoralis minor* muscles) during 14 and 42 d of age[1]

Treatments	14 d				42 d				N accretion		Fat accretion	
	Nitrogen		Fat		Nitrogen		Fat		Total	g/kg gain	Total	g/kg gain
	g/kg	Total (g)	g/kg	Total (g)	g/kg	Total (g)	g/kg	Total (g)	g	14-42 d	g	14-42 d
Main effect: EO-OA												
0 mg/kg	36.6	21.9	7.66	4.59	38.8	111	8.71	25.0	89.4	39.4	20.4	9.00
150 mg/kg	36.8	21.9	7.09	4.23	38.9	112	9.18	26.4	89.7	39.4	22.1	9.73
300 mg/kg	36.9	22.1	7.09	4.24	39.0	112	9.19	26.5	90.1	39.6	22.2	9.74
300/3000 mg/kg[2]	36.9	22.0	7.02	4.18	39.1	113	9.45	27.2	90.7	39.7	23.0	10.08
P- value	0.206	0.889	0.129	0.118	0.786	0.769	0.215	0.182	0.796	0.870	0.085	0.110
Main effect: Protease												
0 mg/kg	36.5	21.8	7.34	4.38	38.8	112	9.46	27.2	89.7	39.4	22.8	10.01
125 mg/kg	37.1	22.1	7.09	4.24	39.1	112	8.81	25.3	90.2	39.6	21.1	9.26
P-value	0.001	0.061	0.254	0.28	0.262	0.389	0.011	0.013	0.582	0.478	0.023	0.021
Overall diet effects[3,4]												
Control	36.3^{a}	21.6	7.65	4.56	38.6	111	8.59^{a}	24.5^{a}	88.8	39.2	20.0^{a}	8.84^{a}
T1	36.5^{ab}	21.8	7.28	4.33	38.6	111	9.65^{ab}	27.7^{abc}	89.3	39.2	23.4^{bc}	10.28^{bc}
T2	36.6^{abc}	22.1	7.24	4.36	39.0	112	9.69^{ab}	27.9^{bc}	90.0	39.6	23.5^{bc}	10.34^{bc}
T3	36.7^{abc}	21.8	7.19	4.27	39.1	113	9.91^{b}	28.6^{c}	90.8	39.7	24.3^{c}	10.61^{c}
T4	36.9^{bc}	22.2	7.67	4.62	39.0	112	8.83^{ab}	25.4^{abc}	90.0	39.6	20.8^{ab}	9.15^{ab}
T5	37.1^{c}	22.1	6.91	4.12	39.1	112	8.71^{a}	25.0^{ab}	90.1	39.6	20.9^{ab}	9.18^{ab}
T6	37.1^{c}	22.1	6.94	4.13	39.1	112	8.69^{a}	25.0^{ab}	90.2	39.6	20.9^{ab}	9.15^{ab}
T7	37.1^{c}	22.2	6.85	4.09	39.2	113	9.00^{ab}	25.9^{abc}	90.6	39.7	21.8^{abc}	9.56^{abc}
Pooled SEM	0.07	0.08	0.035	0.066	0.01	0.5	0.013	0.31	0.46	0.25	0.39	0.167
P- value	0.004	0.612	0.373	0.344	0.908	0.941	0.041	0.035	0.978	0.981	0.035	0.042

[1] Means of 10 replicate pens in each dietary group (N = 10 birds in a single replicate up to 14 d; 9 birds from 15-42 d)
[2] Supplemented at the rate of 300 mg/kg diet up to 28 d and 3000 mg/kg diet from 29 d till harvest
[3] Control: EO-OA 0 mg, protease 0 mg, T1: EO-OA 150 mg/kg; T2: EO-OA 300 mg/kg; T3: EO-OA 300 mg/kg till 28 d followed by 3000 mg/kg till 42 d; T4: Control + Protease 125 mg/kg; T5: T1 + protease 125 mg/kg; T6: T2 + Protease 125 mg/kg; T7: T3 + Protease 125 mg/kg
[4] Represents means separated through one way ANOVA

of intellectual property rights violation. The variations in responses might also be associated with the type of the EO present in the blend and their potential synergistic, additive, or counteractive effects. Other factors affecting the response to the supplementation of EO are the hygienic conditions in which the animals are kept, the background health condition of the flock, and the type of ingredients present in the diets. Tiihonen et al. (2010) reported that EO comprising of thymol and cinnamaldehyde improved BW gain in broiler chickens and the authors correlated the beneficial effects through a change in gut microbiota population especially a rise in Lactobacillus population.

A similar effect is expected with OA (Nava et al. 2009) although a positive effect on small intestinal microbiology may not always translate to enhanced performance owing possibly to the absence of real enteric challenges. Performa-nce of the birds in this study indicated that the diets were adequate in nutrients and there was no dietary "stress' per se that could compromise the defence mechanisms of the birds. Cowieson and Kluenter (2019) suggested that it is unlikely to get any significant response from the gut acting growth promoters including EO and OA as well as enzymes like proteases, unless the diets are nutritionally compromised, and the bird's health is challenged. Plausibly this explains the nonsignificant differences obtained in the current experiment with regard to BW and other performance parameters. In an earlier experiment, Stefanello et al. (2020) observed significant difference with regard to BW, FCR as well as digestibility of N and energy when broiler chickens were supplemented with the same EO-OA blend against a *Clostridium perfringens* challenged control although with the similar product Liu et al. (2017) did not find any effect on BW recorded at 42 d of age. These authors also reported non-

Table 6 Serum concentrations of D-lactate and *E. coli* LPS endotoxin in experimental birds measured at 14 and 42 d of age[1]

Treatment	D-lactate µmol/mL		LPS endotoxin EU/L	
	14 d	42 d	14 d	42 d
Main effect: EO-OA				
0 mg/kg	2.91[b]	2.75	43.3	39.7
150 mg/kg	2.79[ab]	2.61	41.3	37.4
300 mg/kg	2.70[ab]	2.54	42.1	37.8
300/3000 mg/kg[2]	2.58[a]	2.48	41.7	37.8
P- value	0.017	0.254	0.51	0.53
Main effect: Protease				
0 mg/kg	2.74	2.71	42.4	37.5
125 mg/kg	2.75	2.48	41.8	38.8
P-value	0.952	0.025	0.512	0.243
Overall diet effects[3,4]				
Control	3.01[c]	3.02	46.0	40.6
T1	2.80[b]	2.74	40.7	36.0
T2	2.72[b]	2.61	42.3	36.9
T3	2.45[a]	2.46	40.7	36.6
T4	2.81[b]	2.48	40.6	38.7
T5	2.79[b]	2.47	41.8	38.7
T6	2.68[b]	2.47	42.0	38.7
T7	2.71[b]	2.51	42.8	39.1
Pooled SEM	0.037	0.050	0.14	0.42
P- value	0.04	0.065	0.142	0.415

[1] Means of 10 replicate pens in each dietary group (N = 10 birds in a single replicate up to 14 d; 9 birds from 15-42 d)
[2] Supplemented at the rate of 300 mg/kg diet up to 28 d and 3000 mg/kg diet from 29 d till harvest
[3] Control: EO-OA 0 mg, protease 0 mg, T1: EO-OA 150 mg/kg; T2: EO-OA 300 mg/kg; T3: EO-OA 300 mg/kg till 28 d followed by 3000 mg/kg till 42 d; T4: Control + Protease 125 mg/kg; T5: T1 + protease 125 mg/kg; T6: T2 + Protease 125 mg/kg; T7: T3 + Protease 125 mg/kg
[4] Represents means separated through one way ANOVA
LPS: Lipopolysaccharide; EU: Endotoxin unit

significant effect of the EO-OA blend on BW and FCR up to 21 d of age although during 22-42 d, FCR was reported to be improved by the treatment. These observations corroborate to a large extent with the present findings and also supports the hypothesis that EO-OA based feed additives elicit greater responses when the birds approach maturity and hence the possibility of enteric infections remain there till 21 to 28 d of age unless a coverage with AGP is there in diet or the farm sanitation is made as perfect as possible.

The insignificant effect of protease alone on performance was intriguing though not unusual considering the nature of the diet used. The performance of the control group is reiterated here in order to indicate towards the nutrient adequacy of the basal diet. Earlier experiments (Simbaya et al. 1996; Odetallah et al. 2003; 2005) and a couple of meta-analysis-based reviews (Cowieson and Roos 2014; 2016) suggested that the effects to be obtained by a supplemental protease depends on the source and digestibility of the protein meal, the age of the birds, and digestibility of the dietary amino acids. As the amino acid digestibility of the basal diet improves, the likely response from the protease should decrease. The dietary ingredients used in this study, though not analysed per se for amino acid digestibility, might have had provided little room for the protease to elicit its effect through digestibility enhancement and the effect which was expected here might be referred to an "extra-proteinaceous" effect that relates to intestinal resilience.

In an earlier experiment, Wati et al. (2015) indicated that supplementation of an EO blend improved N retention in broiler chickens challenged with Salmonella and E. coli. Reports describing effects of EO and OA on nutrient accretion and body composition are not amply available and widely variable, respectively. In the present experiment, the effects of EO-OA were subtly visible only with respect to total fat accretion that tended to increase when the EO-OA was supplemented at 3000 mg/kg diet during 29-42 d. The data suggested that carcass fat content was more sensitive to the dietary treatments mostly at a later age as compared with N. The results indicated that carcass fat tended to increase due to supplementation of EO-OA or decrease significantly under the influence of protease. The significant interaction effect further suggested that when supplemented together with protease, EO-OA at any level of inclusion elicited better response than when it was supplemented alone. The current data clearly indicated beneficial effect of protease in improving the efficiency of energy utilization by increasing protein deposition in carcass mediated probably through a decrease in fat deposition. This hypothesis is bolstered by the absence of any significant EO-OA*protease interaction on protein deposition in contrast to the fat deposition.

Serum D-lactate concentration, which is a metabolite of carbohydrate fermenting bacteria, is considered to be a reliable biomarker for intestinal permeability in pigs and chickens (Gilani et al. 2016). Leaking epithelial junctional complexes also allow passage of bacteria-derived macromolecules, such as the LPS from gram-negative bacteria (Chen et al. 2015). In the healthy intestine, LPS is not leaking through the paracellular pathway. It is internalized in the epithelial cells and detoxified by the epithelial cell alkaline phosphatase (Guerville and Boudry 2016). Elevated plasma D-lactate concentrations are found to be associated with increased intestinal permeability and bacterial translocation in pigs (Kurundkar et al. 2010), laying hens (Lei et al. 2013), and broilers (Wu et al. 2014) while low D-lactate levels are indicative of decreased intestinal permeability (Xun et al. 2015). In the present experiment, supplementation of both EO-OA and protease has been found

Table 7 Caecal bacterial counts (log_{10} colony forming units, CFU per g of caecal contents) at 42 d of age[1]

Treatments	*Clostridium perfringens*	*Salmonella* spp.	*Lactobacillus* Spp.	*Escherichia coli*	*Campylobacter jejuni*
Main effect: EO-OA					
0 mg/kg	1.08	1.40[c]	1.88	1.52	92.0
150 mg/kg	1.07	1.39[bc]	1.86	1.51	93.0
300 mg/kg	1.07	1.36[ab]	1.88	1.50	93.0
300/3000 mg/kg[2]	1.06	1.35[a]	1.86	1.49	92.5
P- value	0.330	0.017	0.958	0.105	0.974
Main effect: Protease					
0 mg/kg	1.08	1.39	1.85	1.51	92.5
125 mg/kg	1.06	1.36	1.89	1.51	92.8
P-value	0.026	0.025	0.13	0.886	0.888
Overall diet effects[3,4]					
Control	1.09	1.43[c]	1.86	1.53	91.0
T1	1.08	1.41[bc]	1.83	1.51	94.0
T2	1.07	1.36[ab]	1.87	1.50	93.0
T3	1.07	1.35[ab]	1.84	1.49	92.0
T4	1.07	1.38[abc]	1.90	1.52	93.0
T5	1.06	1.36[ab]	1.90	1.51	92.0
T6	1.06	1.35[ab]	1.89	1.50	93.0
T7	1.06	1.34[a]	1.89	1.50	93.0
Pooled SEM	0.003	0.007	0.015	0.004	0.88
P- value	0.279	0.018	0.876	0.455	0.995

1 Means of 10 replicate pens in each dietary group (N = 10 birds in a single replicate up to 14 d; 9 birds from 15-42 d)
2 Supplemented at the rate of 300 mg/kg diet up to 28 d and 3000 mg/kg diet from 29 d till harvest
3 Control: EO-OA 0 mg, protease 0 mg, T1: EO-OA 150 mg/kg; T2: EO-OA 300 mg/kg; T3: EO-OA 300 mg/kg till 28 d followed by 3000 mg/kg till 42 d; T4: Control + Protease 125 mg/kg; T5: T1 + protease 125 mg/kg; T6: T2 + Protease 125 mg/kg; T7: T3 + Protease 125 mg/kg
4 Represents means separated through one way ANOVA
ND = not detected at dilution factor of 104 or more/ values obtained with lower dilutions were < 10 CFU and hence ignored

to decrease the concentration of D-lactate in serum. Judged by a lower serum concentration of fluorescein isothiocyanate-dextran in the groups treated with the same EO-OA blend, Stefanello et al. (2020) reported that it was possible to reduce mucosal leakage in the gastrointestinal tract of broiler chickens challenged with *C. perfringens*. The findings suggest towards the protective action this EO-OA blend may exert on the mucosal structure even under enteric challenge conditions. However, the gastrointestinal mucosa is always exposed to some pro-inflammatory stimuli from the dietary ingredients or soiled litter. Hence, the possibility of mild dysbacteriosis cannot be ruled out. Although it is difficult to say if the degree of dysbacteriosis may lead to any significant mucosal damage to cause translocation of bacteria and their metabolites, the present observations corroborate the findings of Stefanello et al. (2020) indicating the beneficial role of EO-OA and protease on maintaining the integrity of gastrointestinal mucosa. Cowieson and Roos (2016) suggested that by reducing the flow of undigested and endogenous proteins to the caudal gut, exogenous proteases may sequester the pro-inflammatory stimuli associated with proteinaceous anti-nutrients. This may result in better tensile strength of mucosal membranes and tight junction integrity. The present study corroborates this postulation where bacterial metabolites like LPS and D-lactate were on a lower side with the protease supplemented groups. Enzymes like protease shifts the site of digestion to the anterior intestinal segment affecting the population of the putrefactive organisms at the hind gut by reducing the supply of substrates, and thereby starving the microbiome (Bedford 2000). Though the effect of the dietary treatments on nutrient digestibility was not estimated in the present study, the reduction in the number of certain bacteria in the caecal contents, some of which might be potential pathogens, indicated that EO-OA and protease, either alone or in combination might have had exerted some starving effects on them.

The spectrum of activity of EO against different bacteria varies and they may be equally effective against the gram

negative and gram-positive bacteria (Helander et al. 1998; Smith-Palmer et al. 1998; Chao et al. 2000). It is possible to impede the proliferation of *C. perfringens* with the EO obtained from thymol, eugenol, curcumin, piperin, carvacrol and cinnamaldehyde (Jamroz et al. 2003; Mitsch et al. 2004; McReynolds et al. 2009; Wati et al. 2015). However, the EO might show selectivity in their spectrum of activity and the effects might be dose dependent as well. For example, thymol or cinnamaldehyde at an inclusion level of 50 mg/l reportedly decreased the growth of E. coli but not that of Bifidobacteria and Lactobacilli (Ouwehand et al. 2010). In the present study also the EO-OA did not affect the numbers of *C. perfringens* in caeca though significant reduction in numbers of E. coli and *C. jejuni* was there which corroborated the hypothesis that especially the EO used in the blend might be selective in their spectrum of activity. Helander et al. (1998) found that *E. coli* and *Salmonella* were extremely sensitive to cinnamaldehyde and thymol which corroborates the present observations. Wati et al. (2015) reported significant reduction in numbers of *Salmonella* and *E. coli* in caecal contents of broiler chickens challenged with a mixed infection of the above-mentioned bacteria and supplemented with a blend of EO. Beneficial effects of EO on *Lactobacillus* numbers have been reported earlier in pigs (Manzanilla et al.2006; Castillo et al. 2006; Ahmed et al. 2013) and poultry (Tiihonen et al. 2010; Wati et al. 2015) though in the present study *Lactobacillus* numbers did not change due to EO-OA supplementation. If growth of bacterial species like *Lactobacillus* and *Bifidobacterium* can be augmented by reducing the numbers of potential pathogens like *Salmonella* and *E. coli*, then it would be possible to achieve a more stable gut milieu which can enhance nutrient digestibility. However, *Lactobacillus* numbers were found to be refractory to EO-OA and even to the combination of EO-OA and protease. This might be one of the factors that did not allow the performance traits of the treated groups to take an edge over the control group. Several plant extracts and herbal derivatives have proven activity against C. jejuni (Kurekci et al. 2013; Kurekci et al. 2014) and the present findings suggested that supplementation of plant derived EO may effectively improve the quality of meat and meat products by reducing the number of C. jejuni (Karagoz Emiroğlu et al. 2010).

Protease supplementation decreased *C. perfringens* numbers in this study which is in accordance with the hypothesis by several authors (Bedford 2000; Bedford and Cowieson 2009; Cowieson and Roos 2014; Cowieson and Roos 2016) on starving of potential pathogens in the hind gut due to supplementation of enzymes including protease. The inhibitory effect of the protease was found to be extrapolated to *E. coli* and *C. jejuni*, which correlates well with the serum concentration of D-lactate and bacterial LPS. However, the inhibitory effect of the protease on the potential pathogens along with an encouraging effect on growth of *Lactobacillus* was not translated into growth and FCR of the birds. It could be important to not only reduce the amount of ileal bypass protein but also to reduce the supply of soluble carbohydrates resistant to ileal digestion to the hind gut to improve performance through gut microbiota modulation by enzyme application (Apajalahti and Vienola 2016). Achieving these two goals together only can reduce caecal putrefaction. In the current experiment, the diets were devoid of any non-starch polysaccharide degrading enzyme and the possibility of accumulation of starch resistant to pre-caecal digestion in diet cannot be ruled out. Serum LPS concentration, which indirectly indicates towards the bacterial population in the gut as a whole, did not suggest any substantial decrease in the number of bacteria by any of the dietary treatments. A significant improvement in animal performance can be achieved only when the total number of bacteria is reduced to downregulate the bacteria-induced protective mechanisms like secretion of mucin, production, and proliferation of epithelial cells and stimulation of host defence mechanism, all of which lead to reduced nutrient consumption and endogenous losses (Lochmiller and Deerenberg 2000). Seemingly, the effects of the dietary treatments did not reach the threshold, probably due to a lack of critically enough pro-inflammatory stimulus to spare nutrients, which would otherwise be "wasted" in the host defence mechanism and utilized in BW gain. This could be the most likely explanation for the performance traits not differing between the dietary treatments.

5. Conclusions

It was concluded from the present investigation that supplementation of the EO-OA subtly affected BW and FCR though it decreased the numbers of potential pathogens in caeca. Lower concentrations of bacterial metabolites in blood corroborated the above. A higher dose of EO-OA (300 mg/3000 mg/kg) had more beneficial effects. Protease improved protein content in carcass of the young birds and decreased total fat and fat accretion in the adults. Protease also decreased the numbers of potential pathogens in the caeca. Overall, the experiment suggested that supplementation of the EO-OA blend, and a protease had several beneficial effects in broiler chickens in terms of reducing the numbers of potential pathogens in caeca and nutrient accretion in carcass and hence these combinations may be explored as an effective tool for growth promotion in the post-antibiotic growth promoter era.

Declarations

Funding: The authors acknowledge the financial support received from Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada to carry out the experiment.

Conflict of interests: Amongst the authors, MAK Chowdhury and Glenmer Tactacan are employees of Jefo

Inc., the funding agency of the project, and they acted as the technical supervisors of the experiment. Agrivet Research and Advisory P Ltd acted as the research partner of the project funded by Jefo Inc., and the authors belonging to Agrivet do not have any competing interest and declare further that they have no financial and personal relationships with other people or organizations that can inappropriately influence the work presented here. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Ethics approval: The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes [and feed legislation, if appropriate].

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Citation

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