Original Research Article

Potential of a mixture of eugenol and garlic tincture to improve performance and intestinal health in broilers under necrotic enteritis challenge

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ABSTRACT

Plant extracts (PE) are gaining increased attention as potential alternatives to in-feed antimicrobials (AM) due to their known antimicrobial activities. This study was conducted to examine the potential of PE, a microencapsulated product composed of eugenol and garlic tincture as an alternative to AM-agent on performance and intestinal health in broilers under necrotic enteritis (NE) challenge. A total of 960 day-old mixed-sex Cobb 500 chicks were randomly distributed to 48-floor pens with 6 treatments replicated 8 times with 20 birds each. The 6 treatments were as follows: UC, unchallenged control; CC, challenged control; PE, challenged group plus PE; AM, challenged group plus AM; FAP, challenged group plus a full dose of AM with PE; HAP, challenged group plus a half dose of AM with PE in starter, grower and finisher phases. Birds in the challenged groups were inoculated with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14. The body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), and livability of birds were compromised, and intestinal lesions and mortality were increased ($P < 0.05$) by NE challenge, illustrating a successful clinical NE challenge. Birds fed AM had higher BWG and FI, and lower FCR, mortality, and intestinal lesions compared to the CC group ($P < 0.05$). Birds fed PE had improved FCR ($P < 0.05$) and livability (5.8%) in an overall period compared to the CC group. On d 16, PE supplementation reduced ileal lesion scores in only male birds ($P < 0.05$). Birds fed PE had decreased *Eimeria maxima* and *Eimeria acervulina* oocyst counts in caecal content ($P < 0.05$). Birds fed PE had decreased *Escherichia brunetti* and total oocyst counts in caecal content, and *E. acervulina* oocyst counts in ileal content in only female birds ($P < 0.05$). On d 35, PE supplementation reduced variation of BW in both male and female birds and increased yellowness (b* value, 14.4%) in the thigh. These findings suggest the potential of PE supplementation in diets to improve the performance and intestinal health of birds under clinical NE as indicated by improved FCR, livability, uniformity, reduced ileal lesions, oocyst counts and increased skin yellowness. However, the protective effect of PE may not be apparent in the presence of AM in the feed.

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1. Introduction

Necrotic enteritis (NE) is an economically important and devastating enteric bacterial disease widespread in the fast-growing broiler flocks. It is primarily caused by NetB producing strains of *Clostridium perfringens*, a Gram-positive spore-forming anaerobic ubiquitous bacterium causing sub-clinical or clinical disease together with one or more predisposing factors such as Eimeria spp. and fish meal, etc. (Keyburn et al., 2008; Moore, 2016).

The sub-clinical form of NE can reduce body weight gain (BWG), feed intake (FI), digestibility, increase feed conversion ratio (FCR), intestinal lesions, and diarrhoea, whereas the clinical form can cause sudden flock mortality ranging from 2% to 10% in mild cases, and up to 50% in severe cases (Kaldhusdal et al., 2001; Immerseel et al., 2004). The total cost of NE to the world poultry industry has been predicted to be over US$6 billion per annum due to reduced BWG, impaired FCR, increased mortality, and cost associated with disease control and management strategies (Wade and Keyburn, 2015). Traditionally, antimicrobials (AM) have been applied to control NE. However, due to the development of AM-resistance in bacteria and public health concerns over the use of in-feed AM, the application of AM has been banned or restricted in the poultry feed industry in many parts of the world leading to increased incidence of NE (Kocher and Choct, 2008; Kaldhusdal et al., 2016). Thus, there is a growing interest in the search for viable alternatives to in-feed AM to combat enteric diseases such as NE in the post-AM era.

Plant-derived bioactive compounds, as natural feed additives, have been used as alternatives to in-feed AM for years to improve the growth performance and health of broilers (Windisch et al., 2008; Puvača et al., 2013; Kothari et al., 2019). Some bioactive compounds derived from plants, spices and herbs are known to have antimicrobial, anti-coccidial, antifungal, antiviral, and anti-oxidative properties (Prasad and Sharma, 1981; Brenes and Roura, 2010; Mohiti-Asli and Ghanaatparast-Rashti, 2015; Lillehoj et al., 2018). Garlic (*Allium sativum*) and garlic metabolites have these properties and can be characterised by their important bioactive compounds such as, sulphuric compounds, diallyl sulphide, allin, allicin, S-allyl cysteine and ajoene (Kumar and Berwal, 1998; Khan et al., 2012). Researchers have shown that the nature-identical phenolic compound, eugenol (4-allyl-2-methoxyphenol) has shown these effects as well (Devi et al., 2010; Pramod et al., 2010; Lillehoj et al., 2018). The dietary addition of plant extracts (PE) including garlic and eugenol may be able to modulate intestinal microbiota and reduce pathogenic bacterial load via antimicrobial activities, reduce *Eimeria* spp. oocysts via anticoccidial activities, increase immune cells and reduce oxidative stress through immunomodulatory, and antioxidative effects (Si et al., 2009; Applegate et al., 2010; Brenes and Roura, 2010; Abou-Elkhair et al., 2014). They might also play a significant role to increase nutrient digestibility via their positive impacts on digestive enzymatic activities, thus improved growth performance (Platel and Srinivasan, 2004; Jamroz et al., 2005). Literatures have shown that diets supplemented with plant-derived products in different forms could replace AM and maintain growth performance in birds infected with coccidiosis and NE (Mitsch et al., 2004; Brenes and Roura, 2010; Mohiti-Asli and Ghanaatparast-Rashti, 2015; Eid et al., 2018; Oh et al., 2018; Adhikari et al., 2020). Among different plant-derived products, garlic and eugenol supplementation in diets alone or in combination with various PE positively affected growth performance, reduced oocyst counts, and improved intestinal health (Giannenas et al., 2003; Issa and Omar, 2012; Kim et al., 2013; Kirubakaran et al., 2016; Sidiropoulou et al., 2020). Further, a recent study suggests the beneficial effects of a microencapsulated product composed of eugenol and garlic tincture on BWG and FCR under a subclinical NE (Pirgozliev et al., 2019). In contrast, reviews compiled with several studies did not observe beneficial effects of PE on performance parameters and intestinal health (Demir et al., 2003; Windisch et al., 2008; Wallace et al., 2010; Pirgozliev et al., 2018). The inconsistent findings observed in field studies could be attributed to the variations in the origin, type, composition, form, inclusion level, health status, and the heterogeneity of experimental conditions, thus warrants further research. Moreover, despite the positive effects of PE in broilers under subclinical infection, the potentials of PE to ameliorate the negative effects under more severe diseased conditions such as clinical NE, are not well-documented.

It was hypothesised that dietary addition of PE, a microencapsulated product composed of eugenol and garlic tincture may improve the performance and intestinal health of birds under severe diseased conditions. The current study was designed to evaluate the efficacy of PE on performance, mortality, intestinal lesions, *Eimeria* oocyst counts, skin pigmentation and uniformity in broilers subjected to clinical NE challenge. The potential of PE in mitigation of clinical NE effects on performance and intestinal health was compared against an AM-agent. In addition, it was hypothesised that the combination of PE and AM may exert synergistic effects in protecting birds against NE. Therefore, this study was also designed to evaluate the effects of PE in combination with full and half dosages of AM in broilers under NE challenge and to compare their efficacy against an AM-agent without PE supplementation.

2. Materials and methods

2.1. Animal ethics

The experimental procedures applied in the current study were approved by the Animal Ethics Committee of University of New England, Armidale, NSW 2351, Australia (AEC18-116). The experiment was conducted following the guidelines set for the care and use of laboratory animals for scientific purposes accredited by the Australian Bureau of Animal Health (NHMRC, 2013).

2.2. Design and husbandry

A total of 960 one-day-old Cobb 500 mixed-sex chicks were obtained from Baiada hatchery in Tamworth, NSW, Australia. Birds were weighed upon arrival (initial body weight, 43.2 g) and randomly allocated to 6 treatments in 48-floor pens measuring 75 cm × 120 cm, based on a completely randomised design (CRD). Each of the 6 treatments had 8 replicate pens with 20 birds per pen. The birds were randomly distributed to the pens without knowing the sex initially, thus the sex ratios were different from pen to pen. The sex of the birds was identified at an earlier age by feather DNA sexing using high resolution melting curve analysis (England et al., 2020). Birds were housed in a climate-controlled facility with softwood shavings as litter. Each pen was stocked with 3 nipple drinkers and a tube feeder. Feed and freshwater were provided ad libitum. Temperature, relative humidity, and lighting were maintained following Cobb 500 guidelines (Cobb500, 2018b).

2.3. Dietary treatments

Six treatments in a CRD were comprised of one unchallenged treatment group as control and 5 challenged treatment groups to examine the efficacy of PE, a microencapsulated product composed of eugenol and garlic tincture in broilers under clinical NE challenge as shown in Table 1. The treatments were: UC, unchallenged control, without additive or in-feed antimicrobial (AM); CC, challenged control, without additives or in-feed AM; PE, challenged group plus additive plant extract containing 10% eugenol and 10% garlic tincture at 100 part per million (ppm) (ADMi|Pancosma SA, A-One Business Center, CH-1180 Rolle, Switzerland); AM, challenged group plus AM containing 50 ppm each active compound of narasin and nicarbazin (Maxiban 72, Elanco US Inc., Indiana, USA); FAP, challenged group plus a full dose of AM with PE; HAP, challenged group plus a half dose of AM with PE in starter, grower and finisher phases. All diets were formulated based on wheat, soybean meal, sorghum, and meat and bone meal where the feed additives and

Table 1
Treatment groups with additives applied in this study.

Treatments[1]	Additives	Inclusion level; starter (d 0 to 9), grower (d 9 to 21) and finisher (d 21 to 35) phases, ppm	Necrotic enteritis challenge[2]
UC	–	–	Unchallenged
CC	–	–	Challenged
PE	Plant extract	100	Challenged
AM	Antimicrobial	50 of narasin and nicarbasin	Challenged
FAP	AM full dose + PE	50 + 100	Challenged
HAP	AM half dose + PE	25 + 100	Challenged

ppm = part per million.
[1] UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbasin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE.
[2] Challenged birds were orally gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14.

phytase were formulated with nutrient and the matrix values respectively as shown in Table 2. Synthetic pigments, carophyll red (canthaxanthin) at 2 ppm and carophyll yellow (apo-ester) at 35 ppm were added as micro-ingredients and their matrix values were considered in the formulation as indicators for the skin pigmentation measurements. Prior to feed formulation, the nutrient contents of feed ingredients were determined using near-infrared spectroscopy (NIRS, Evonik AminoProx, Essen, Germany). Cold pelleted diets were fed in the starter phase (d 0 to 9; crumbled), grower phase (d 9 to 21), and finisher phase (d 21 to 35) and followed Cobb 500 feeding standards and nutrient specifications for broilers (Cobb500, 2018a).

Table 2
Experimental diet composition and nutrients (as-fed basis, presented as percentage unless declared otherwise).

Item	Starter phase (d 0 to 9)	Grower phase (d 9 to 21)	Finisher phase (d 21 to 35)
Ingredients			
Wheat	41.6	43.4	44.9
Sorghum	26.0	27.0	31.0
Soybean meal	26.8	22.3	17.4
Meat and bone meal	2.00	3.30	2.60
Canola oil	0.70	1.30	2.10
Limestone	0.97	0.78	0.77
Dicalcium phosphate 18P/21Ca	0.42	–	–
Salt	0.12	0.08	0.08
Sodium bicarbonate	0.32	0.28	0.26
Vitamin premix[1]	0.09	0.09	0.09
Mineral premix[2]	0.08	0.08	0.08
Choline chloride 70%	0.06	0.07	0.06
L-lysine HCl	0.39	0.36	0.32
D, L-methionine	0.25	0.23	0.23
L-threonine	0.19	0.13	0.12
Phytase	0.01	0.01	0.01
Carophyll red (Canthaxanthin), ppm	2.0	2.0	2.0
Carophyll yellow (Apo-ester), ppm	35.0	35.0	35.0
Titanium di-oxide (TiO$_2$)	–	0.50	–
Nutrient composition[3]			
AME, kcal/kg	2,985	3,050	3,150
Crude protein	22.5	21.4	19.2
Crude fat	2.70	3.40	4.20
Crude fiber	2.92	2.80	2.67
Digestible Arg	1.28	1.18	1.01
Digestible Lys	1.22	1.12	0.96
Digestible Met	0.54	0.50	0.48
Digestible Met + Cys	0.91	0.85	0.80
Digestible Trp	0.25	0.23	0.20
Digestible Thr	0.83	0.73	0.66
Digestible Val	0.92	0.86	0.76
Non starch polysaccharides, insoluble	9.91	10.3	11.8
Calcium	0.90	0.84	0.76
Phosphorus available	0.45	0.42	0.38
Sodium	0.22	0.20	0.19
Potassium	0.90	0.82	0.73
Chloride	0.22	0.20	0.19
Linoleic acid 18:2	1.07	1.22	1.39
Choline, mg/kg	1,700	1,700	1,550

ppm = part per million; AME = apparent metabolisable energy.
[1] Vitamin premix provided the following per kilogram diet: vitamin A, 12,000,000 IU; vitamin D, 5,000,000 IU; vitamin E, 75 mg; vitamin K, 3 mg; cyanocobalamin, 0.016 mg; folic acid, 2 mg; riboflavin, 8 mg; pyridoxine, 5 mg; biotin, 0.25 mg; thiamine, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg and antioxidant ethoxyquin, 50 mg.
[2] Mineral premix provided the following per kilogram diet: Cu sulfate, 16 mg; Mn sulfate, 60 mg; Mn oxide, 60 mg; I (iodide), 0.125 mg; Se (selenite), 0.3 mg; Fe sulfate, 40 mg; Zn oxide and sulfate, 100 mg.
[3] Ingredients were measured using near-infrared spectroscopy (NIRS, Evonik AminoProx, Germany).

2.4. Necrotic enteritis challenge

The NE challenge model was employed in the current study following the previous report (Wu et al., 2014; Rodgers et al., 2015) where field strains of *Eimeria* spp. oocysts were used as a predisposing factor and *C. perfringens* as a principal causative agent to induce NE. In brief, challenged birds were orally gavaged with 1 mL *Eimeria* spp. containing 5000 oocysts of both *Eimeria acervulina* and *Eimeria maxima*, and 2500 oocysts of *Escherichia brunetti* (Eimeria Pty Ltd., Ringwood, VIC, Australia). On d 14, challenged birds were orally gavaged with 1 mL *C. perfringens* (EHE-NE18) containing approximately 10^8 CFU (CSIRO Livestock, Geelong, VIC, Australia). Simultaneously, birds in the unchallenged group were orally gavaged with 1 mL phosphate-buffered saline (PBS) on d 9 and sterile medium on d 14.

2.5. Performance measurement and sampling

Pen weight and FI were recorded on d 0, 9, 21, and 35. On d 35, individual body weight (BW) of birds was recorded to determine flock uniformity. The coefficient of variation (CV) of BW of birds in each treatment group was calculated to determine flock uniformity. The weight of dead birds was recorded daily and the FCR was adjusted accordingly. Necropsy was carried out to examine the cause of deaths. All the dead, sampled, and birds left on d 35 were opened to determine the sex by visual inspection of testes.

2.6. Sampling and intestinal lesion scoring

On d 16, randomly selected 4 birds (2 males and 2 females) close to the average pen BW weight from each pen were weighed, electrically stunned by using an electric stunner (JF poultry equipment, Weltevreden Park, South Africa), and euthanised to collect ileal and caecal digesta samples and perform post mortem analysis. All the sampled birds were scored for intestinal lesions in the duodenum, jejunum, and ileum by visual examination following a previously reported lesion scoring system (Keyburn et al., 2006; Shojadoost et al., 2012). The 7-point scale lesion scoring was performed, where 0 illustrated no lesions and 6 emerged as the most severe and macroscopic intestinal lesions.

2.7. Eimeria oocyst counts

On d 16, the ileal and caecal contents from 2 males and 2 females per pen were collected separately in 2 mL Eppendorf tubes and stored at 4°C for *Eimeria* spp. oocyst counts and oocysts were counted within 5 d. One hundred milligram of faecal samples were weighed out and diluted with 900 μL saturated salt solution (relative density, 1.3). Samples were vortexed to mix and left for 1 h in the fridge to allow oocysts to float and sample debris to settle. Then, 600 μL saturated salt solution was added to the Whitlock chamber (Whitlock universal slides, JA Whitlock & Co., NSW 2122, Australia) and 150 μL of diluted samples were pipetted from the top of the samples and added to the Whitlock chamber. Samples were counted using a 40 × lens, and species were determined by size and morphology. The data were expressed as oocyst/gram with the counts in the chamber multiplied by 100 as the dilution factor.

2.8. Skin pigmentation

Two birds (one male and one female) per pen were randomly selected for skin pigment measurements on d 35. Birds were stunned, decapitated and bled for 180 s. After that, the carcasses were carried to the scalding tanks filled with hot water at a temperature of 54 to 55°C. The carcasses were kept in hot water for 120 s and then plucked in a plucking machine with rotating rubber fingers. Birds were kept in a cold room (4°C) for 18 to 20 h and skin pigment was measured by using a reflectance colorimeter. The CIE (l'Eclairage, 1978) system of the colour profile, lightness/luminosity (L*), redness (a*), and yellowness (b*), was measured by a reflectance colorimeter (Minolta Chroma Meter CR-400, Konica Minolta Inc., Osaka, Japan).

2.9. Data analysis

All the data generated in this study were examined for normal distribution before statistical analysis. The performance data were analysed as a completely randomised design using JMP 14.0 (SAS Institute, Cary, NC, USA), where the pen was served as an experimental unit ($n = 48$). The significant differences between means were separated by the Least Significant Difference test. Performance data were analysed for the treatment effect with male percentage (corrected to dead birds) as a covariate. The means were considered significantly different when *P*-value was <0.05, and declared a tendency to be different with $0.05 < P < 0.10$.

Eimeria oocyst counts data were analysed after square root transformation but the original values are shown in tables and figures. Data collected from male and female birds separately were subjected to 2-way ANOVA analysis as a 6×2 factorial arrangement to assess the main effects of the experimental treatment and sex, and interaction of experimental treatment × sex. The intestinal lesion scores and NE-caused mortality data were analysed by the non-parametric Kruskal−Wallis test as the data were not normally distributed.

3. Results

3.1. Performance and uniformity

The effects of NE challenge and PE on growth performance are shown in Table 3. One-way ANOVA analysis showed significant differences in the following measurements: BWG from d 9 to 21 ($P < 0.001$), 21 to 35 ($P < 0.001$) and 0 to 35 ($P < 0.001$), FI on d 9 to 21 ($P < 0.001$), 21 to 35 ($P < 0.001$) and 0 to 35 ($P < 0.001$), FCR from d 9 to 21 ($P < 0.001$), 21 to 35 ($P = 0.026$) and 0 to 35 ($P < 0.001$), and livability from d 9 to 21 ($P < 0.001$) and 0 to 35 ($P < 0.001$).

In the starter phase (d 0 to 9), BWG, FI, FCR, and livability were not different among treatment groups ($P > 0.05$).

In the grower phase (d 9 to 21), the NE challenge significantly decreased FI, BWG and livability, and increased FCR in the CC group compared to the UC group indicating the successful NE challenge of the birds. The supplementation of PE significantly improved FCR compared to the CC group. Birds in the PE group showed a numeric improvement of livability by 3.9% compared to the CC group. Compared to the AM group, PE fed birds had higher FCR and lower FI, BWG, and livability. Furthermore, birds fed FAP had similar BWG, FI, FCR, and livability, and birds fed HAP had lower FI, BWG, and higher FCR compared to the AM group. Livability was not different between these treatment groups.

In the finisher phase (d 21 to 35), the effect of the NE challenge on FI, BWG, FCR, and livability did not continue. The supplementation of PE significantly reduced FCR compared to the CC group. Compared to the AM group, birds fed PE had lower FCR, FI, and BWG, but similar livability. Birds fed FAP and HAP had similar BWG, FI, FCR, and livability compared to the AM group.

In an overall study period (d 0 to 35), FI, BWG, FCR, and livability were negatively affected by the NE challenge. However, the supplementation of PE significantly reduced FCR compared to the CC group. Birds in the PE group showed a numeric improvement of livability and improved livability by 5.8% compared to the CC group.

Table 3
Effects of PE and NE challenge on the performance in broilers at different phases.[1]

Item	UC	NE challenged[2]					SEM	P-value
		CC	PE	AM	FAP	HAP		
Starter phase (d 0 to 9)								
BWG, g	220	212	213	215	211	213	2	0.139
FI, g	256	255	256	254	256	254	2	0.913
FCR	1.180	1.190	1.204	1.189	1.204	1.207	0.008	0.126
Livability, %	98.8	98.8	100	98.8	97.5	99.4	1	0.289
Grower phase (d 9 to 21)								
BWG, g	736[a]	497[d]	501[d]	671[b]	670[b]	569[c]	8	<0.001
FI, g	1,021[a]	870[bc]	835[c]	994[a]	978[a]	896[b]	13	<0.001
FCR	1.388[e]	1.734[a]	1.688[b]	1.472[d]	1.458[d]	1.575[c]	0.010	<0.001
Livability, %	100[a]	81.8[c]	85.7[bc]	100[a]	98.5[a]	91.6[ab]	2	<0.001
Finisher phase (d 21 to 35)								
BWG, g	1,293[c]	1,299[c]	1,312[bc]	1,386[a]	1,384[a]	1,359[ab]	13	<0.001
FI, g	2,299[b]	2,319[b]	2,220[c]	2,413[a]	2,392[a]	2,344[ab]	24	<0.001
FCR	1.760[a]	1.747[ab]	1.712[c]	1.741[ab]	1.728[bc]	1.726[bc]	0.010	0.026
Livability, %	99.1	100	97.8	100	99.0	98.8	1	0.640
Overall period (d 0 to 35)								
BWG, g	2,262[a]	2,008[c]	2,015[c]	2,270[a]	2,261[a]	2,136[b]	18	<0.001
FI, g	3,575[ab]	3,439[c]	3,320[d]	3,661[a]	3,622[a]	3,494[bc]	33	<0.001
FCR	1.581[e]	1.681[a]	1.645[b]	1.613[cd]	1.602[de]	1.636[bc]	0.007	<0.001
Livability, %	98.1[a]	83.1[c]	88.8[bc]	98.8[a]	95.6[a]	93.8[ab]	2	<0.001

BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio; NE = necrotic enteritis; PE = plant extract.
[a–d] Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 20 birds per replicate and 8 replicates per treatment.
[1] UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE.
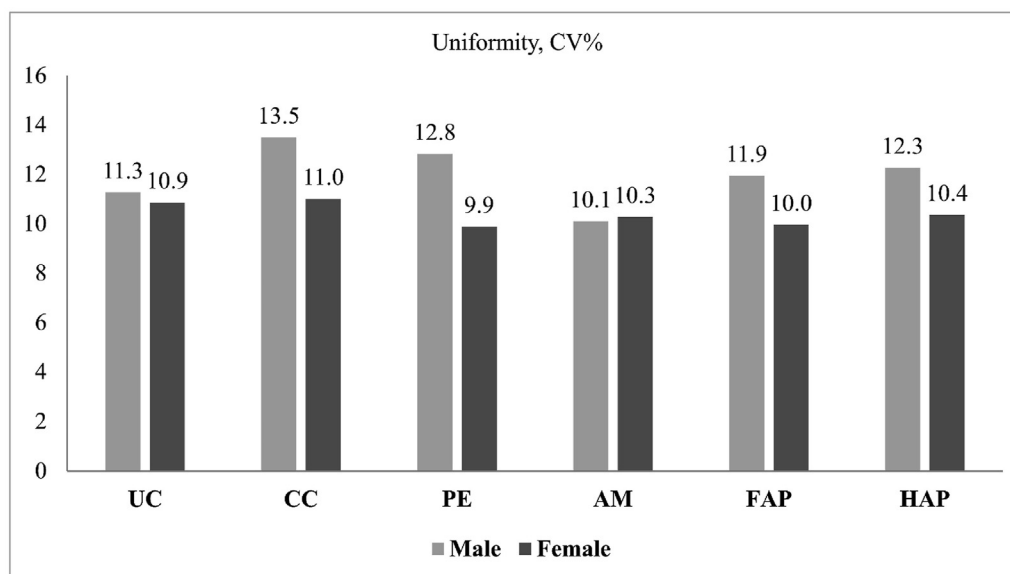[2] Challenged birds were orally gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14.

Compared to the AM group, birds fed PE had higher FCR and lower FI, BWG, and livability but birds fed FAP had similar BWG, FI, FCR, and livability. Birds fed HAP had similar FCR and livability but lower FI and BWG compared to the AM group.

The effects of NE challenge and PE on flock uniformity on d 35 are shown in Fig. 1. The order of CV of BW according to the treatment groups was AM < UC < FAP < HAP < PE < CC in male birds and PE < FAP < AM < HAP < UC < CC in female birds. In male birds, AM group had the lowest CV (10.1%) and in female birds, the PE group had the lowest CV (9.9%) whereas the CC group had the highest CV in both male and female birds (respectively 13.5% and 11.0%).

3.2. Necrotic enteritis lesion

The effects of NE challenge and PE on duodenal, jejunal and ileal lesion scores on d 16 are presented in Fig. 2A, B, and C, respectively. Non-parametric Kruskal—Wallis test showed that the intestinal lesion scores in the duodenum ($P = 0.031$), jejunum ($P = 0.001$) and ileum ($P = 0.004$) were significantly different. The NE challenge significantly increased lesion scores in both male and female birds except in the duodenum of the CC group. Compared to the CC group, supplementation of PE reduced ileal lesion scores in male birds but not different in female birds. Compared to the AM group,

Fig. 1. Effects of PE and necrotic enteritis (NE) challenge on flock uniformity on d 35. UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE. Challenged birds were orally gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14.
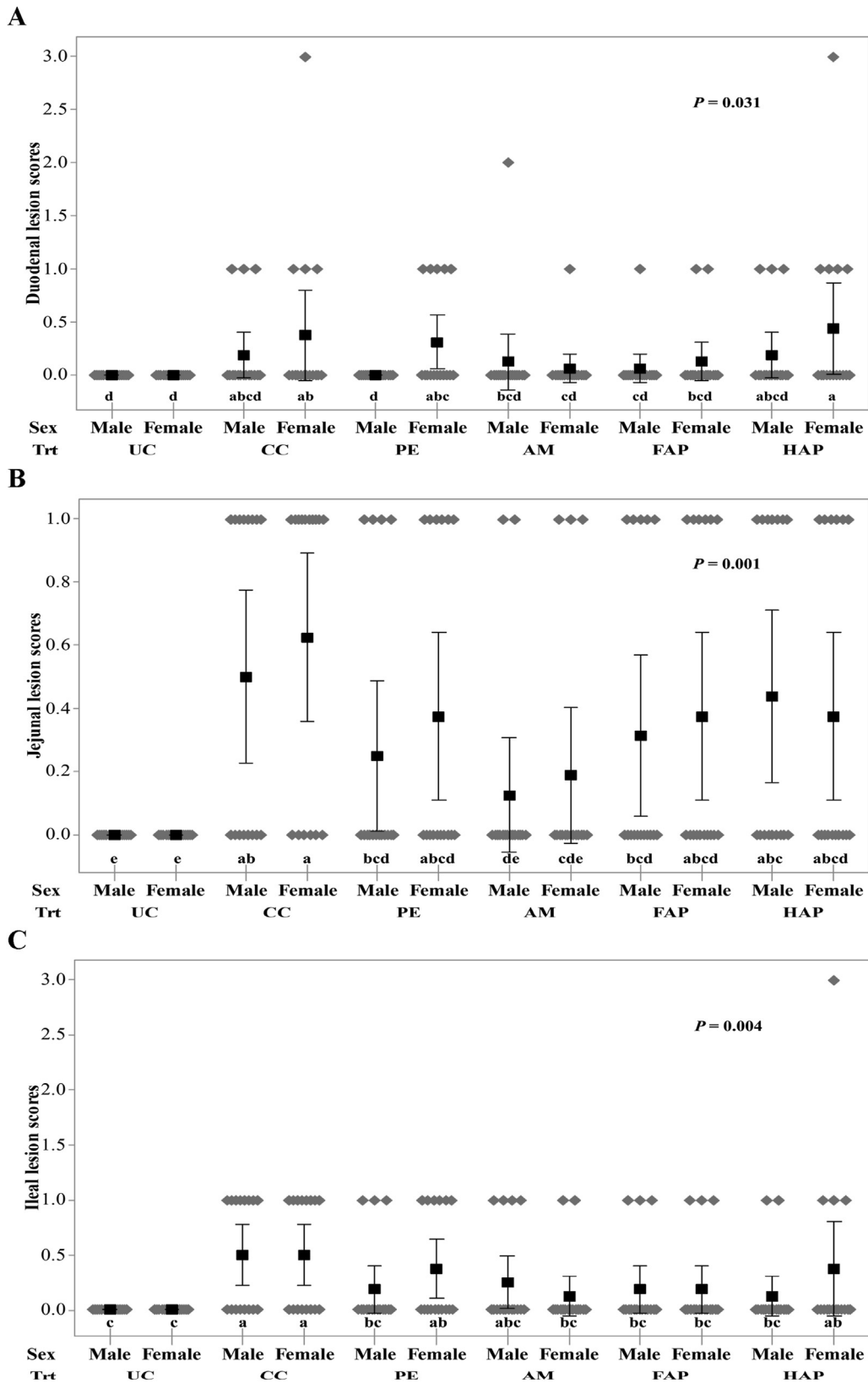
Fig. 2. Effects of PE and necrotic enteritis (NE) challenge on intestinal lesions on d 16: (A) duodenum, (B) jejunum, and (C) ileum. UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE. Challenged birds were orally gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14. [a–c] Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 2 males and 2 females birds per replicate and 8 replicates per treatment.

birds fed PE and FAP had similar duodenal, jejunal and ileal lesion scores in both male and female birds. Birds fed HAP had higher duodenal lesions in female birds and jejunal lesions in male birds compared to the AM group whereas no difference was observed in ileal lesions in both male and female birds.

3.3. Necrotic enteritis caused mortality

The effects of NE challenge and PE on mortality caused by NE are presented in Fig. 3. Non-parametric Kruskal−Wallis test indicated that the mortality due to NE was significantly different ($P < 0.001$). The NE challenge significantly increased mortality in both male and female birds. Mortality was not significantly different in PE supplemented birds compared to the birds in the CC group although had a numeric reduction in PE fed birds. Birds fed PE had higher mortality compared to the AM group. Birds fed FAP had similar mortality in both male and female birds compared to the AM group. Birds fed HAP had higher mortality in female birds compared to the AM group, whereas no difference was observed in male birds.

3.4. Eimeria oocyst counts and Clostridium perfringens loads

The effects of experimental treatment and sex on *Eimeria* oocyst counts on d 16 are shown in Table 4. Two-way ANOVA analysis demonstrated that the main effects of experimental treatment on *E. maxima* ($P < 0.001$) and *E. acervulina* ($P < 0.001$) oocyst counts in caecal content, *E. maxima* ($P < 0.001$) oocyst counts in ileal content, and sex on *E. maxima* oocyst counts in caecal content ($P = 0.038$). There was no interaction between experimental treatment and sex ($P > 0.05$).

The challenge increased *E. maxima* and *E. acervulina* oocyst counts in caecal content, and *E. maxima* and total oocyst counts in ileal content in the CC group. Birds supplemented with PE significantly reduced *E. maxima* and *E. acervulina* oocyst counts in caecal content compared to the CC group. Compared to the AM group, birds fed PE had higher counts of *E. maxima* and *E. acervulina* oocysts in caecal content whereas similar counts of *E. maxima* and total oocysts in ileal content. Birds fed FAP had similar counts of

Eimeria oocysts in the ileal and caecal content compared to the AM group. Birds fed HAP had higher *E. maxima* and *E. acervulina* oocyst counts in caecal content whereas similar counts of *E. maxima* and total counts of oocysts in ileal content compared to the AM group. Female birds had higher counts of *E. maxima* than male birds in caecal content ($P < 0.05$).

The interactions between experimental treatment and sex on *Eimeria* oocyst counts on d 16 are shown in Table 5. Two-way ANOVA analysis showed that there was an interaction between experimental treatment and sex on *E. brunetti* ($P < 0.001$) and total oocyst counts ($P = 0.033$) in caecal content, *E. acervulina* ($P = 0.001$) and *E. brunetti* ($P = 0.033$) oocyst counts in ileal content. All the additives decreased *E. brunetti* and total oocyst counts in caecal content compared to the CC group in female birds whereas in male birds, only AM and FAP addition decreased the *E. brunetti* and total oocyst counts but PE and HAP did not. Birds fed AM, FAP and HAP had decreased *E. brunetti* oocyst counts in ileal content compared to the CC group in female birds but no difference was observed in male birds. All the additives (PE, AM, FAP and HAP) decreased *E. acervulina* oocyst counts in ileal content compared to the CC group in female birds but not in male birds.

The microbiota results both in ileal and caecal contents have been reported in a previous paper(Kumar et al., 2021). The effects of NE challenge and PE on *C. perfringens* loads in ileal and caecal contents of broilers on d d16 are shown in Table 4. Briefly, the NE challenge significantly increased *C. perfringens* loads in the CC group compared to the UC group both in ileal and caecal contents. Birds fed PE had similar *C. perfringens* loads compared to the CC group, and had higher loads compared to the AM group both in ileal and caecal contents. Birds fed FAP had similar *C. perfringens* loads whereas the HAP group had higher *C. perfringens* loads compared to the AM group both in ileal and caecal contents.

3.5. Skin pigmentation

The effects of NE challenge and PE on skin pigmentation in broilers on d 35 are shown in Table 6. Two-way ANOVA analysis showed the main effects of experimental treatments on L*
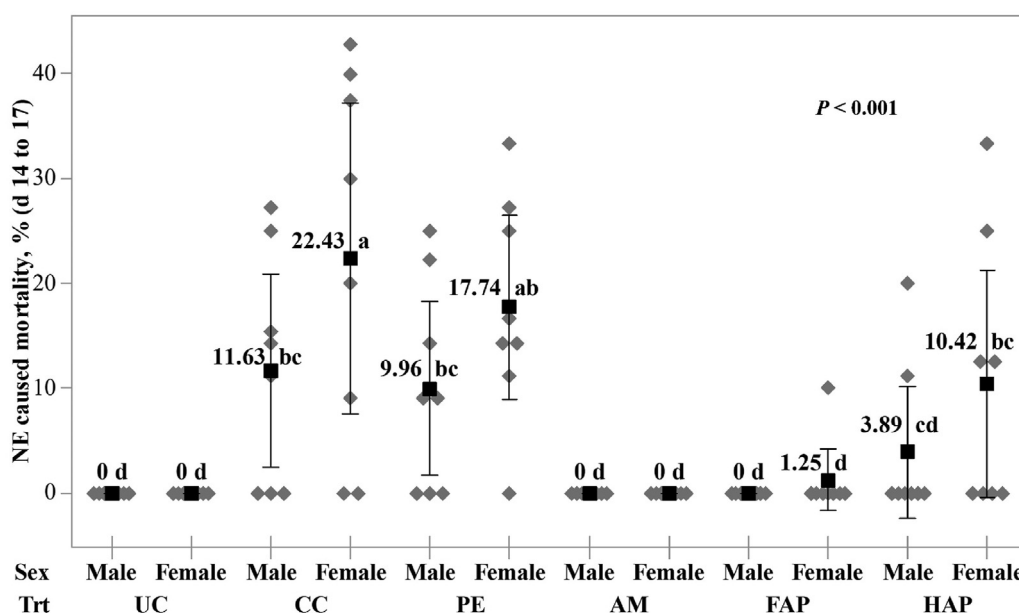

Fig. 3. Effects of PE and necrotic enteritis (NE) challenge on mortality on d 16. UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE. Challenged birds were orally gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14. [a − d] Values in a row with no common superscripts differ significantly ($P < 0.05$).

Table 4
Experimental treatment and sex as main effects on Eimeria oocyst counts in the caecal and ileal contents on d 16.[1]

Item	Caecal content − E. Maxima	Caecal content − E. Acervulina	Ileal content -E. Maxima	Ileal content -total counts	Ileal content-C. perfringens[2]	Caecal content -C. perfringens[2]
Treatment						
UC	0[d]	0[c]	0[b]	0[b]	3.94[c]	0.27[c]
CC	46,094[a]	25,181[a]	4,683[a]	10,999[a]	9.16[a]	9.62[a]
PE	19,289[b]	11,527[b]	6,679[a]	7,567[a]	8.94[ab]	9.29[a]
AM	1,531[cd]	265[c]	10,914[a]	11,937[a]	4.99[c]	2.89[b]
FAP	3,200[c]	349[c]	9,571[a]	10,214[a]	5.19[c]	3.87[b]
HAP	20,238[b]	5,782[b]	8,379[a]	9,453[a]	7.73[b]	8.80[a]
SEM	4,849	2,584	1,547	1,984	0.46	0.58
Sex						
Male	10,217[b]	5,403	6,737	7,778	6.50	5.44
Female	19,901[a]	8,965	6,732	8,945	6.81	6.14
SEM	2,800	1,492	893	1,145	0.27	0.33
P-value						
Experimental treatment	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Sex	0.038	0.185	0.507	0.507	0.412	0.136
Experimental treatment × Sex	0.142	0.172	0.924	0.924	0.557	0.538

[a – d] Values in a row with no common superscripts differ significantly (P < 0.05). Mean values are based on 4 birds per replicate and 8 replicates per treatment.
[1] UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE.
[2] The microbiota results in detail have been reported in a previous paper (Kumar et al., 2021).

Table 5
Interactions between experimental treatment and sex on Eimeria oocyst counts in the caecal and ileal contents on d 16.[1]

Sex	Treatment	Caecal content- E. Brunetti	Caecal content − total counts	Ileal content−E. Brunetti	Ileal content− E. Acervulina
Male	UC	0[d]	0[d]	0[e]	0[c]
	CC	3,186[bc]	46,211[b]	240[bcde]	1,558[b]
	PE	1,214[bcd]	23,071[bc]	89[de]	486[bc]
	AM	63[d]	1,888[cd]	671[ab]	958[bc]
	FAP	100[d]	3,233[cd]	100[cde]	771[bc]
	HAP	225[cd]	24,100[bc]	614[abc]	763[bc]
Female	UC	0[d]	0[d]	0[e]	0[c]
	CC	26,843[a]	126,368[a]	1,013[a]	9,463[a]
	PE	6,858[b]	46,633[b]	414[abcd]	788[bc]
	AM	125[cd]	1,893[cd]	29[e]	388[bc]
	FAP	225[cd]	4,190[cd]	14[e]	400[bc]
	HAP	250[cd]	28,414[bc]	43[e]	729[bc]
P-value					
Experimental treatment		<0.001	<0.001	0.001	<0.001
Sex		<0.001	0.013	0.415	0.457
Experimental treatment × Sex		<0.001	0.018	0.001	0.033

[a – e] Values in a row with no common superscripts differ significantly (P < 0.05). Mean values are based on 4 birds per replicate and 8 replicates per treatment.
[1] UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE.

(lightness) value in the upper breast (P = 0.006), L* (P = 0.024) and b* (yellowness) values (P = 0.024) in the thigh, and sex on b* value in the upper breast (P = 0.002), lower breast (P = 0.018) and thigh (P = 0.001). There was no interaction between experimental treatments and sex (P > 0.05) except for a* (redness) value in the lower breast (P = 0.047).

The NE challenge group without additives supplementation (CC group) had significantly lower L* and b* values in the thigh compared to the UC group. Supplementation of PE increased L* value in the thigh compared to the CC group. Although b* value in the thigh was not statistically different between PE and CC groups, PE addition led to the b* value in the thigh to the level statistically similar to the UC group and all the groups containing AM (AM, FAP and HAP). Birds fed PE had a higher L* value in the upper breast and thigh compared to the AM group. Birds fed FAP and HAP had similar L* value and b* value in the thigh whereas the HAP group had a higher L* value in the upper breast compared to the AM group. Female birds had a higher b* value compared to male birds in the

upper breast, lower breast, and thigh but no difference was observed in L* and a* values. The interaction between experimental treatment and sex on a* value in the lower breast is shown in Fig. 4. Birds fed PE had a lower a* value in the lower breast compared to the CC and AM groups in the female birds whereas, in male birds, PE fed birds had a higher a* value compared to the AM group but not different from the CC group.

4. Discussion

Dietary inclusion of PE has shown promising effects to improve performance and health in broilers challenged with subclinical NE (Puvača et al., 2013; Kothari et al., 2019; Adhikari et al., 2020). However, the data are limited in broilers under clinical NE challenge. The present study examined the potential of PE, a micro-encapsulated product composed of eugenol and garlic tincture to mitigate the detrimental effects of clinical NE on performance, intestinal lesions, mortality, Eimeria oocyst counts, and skin

Table 6
Effects of PE and NE challenge on the skin pigmentation (**L***, **a***, and **b*** values) in broilers on d 35[1].

Item	Upper breast			Lower breast			Thigh		
	L*	a*	b*	L*	a*	b*	L*	a*	b*
Treatment[2]									
UC	72.3[ab]	6.59	8.78	69.0	4.95	6.63	79.0[a]	4.59	8.27[a]
CC	70.9[bcd]	6.48	6.72	67.5	6.56	5.90	77.6[b]	4.99	6.58[b]
PE	71.8[abc]	6.32	7.50	68.7	5.69	6.26	79.0[a]	5.08	7.53[ab]
AM	69.9[d]	6.07	7.95	67.7	5.65	7.10	77.3[b]	4.31	8.22[a]
FAP	70.5[cd]	6.22	7.65	67.9	5.83	6.76	77.9[ab]	4.41	8.46[a]
HAP	72.5[a]	5.55	7.36	68.4	5.85	6.30	77.7[b]	4.61	7.55[ab]
SEM	0.6	0.41	0.51	0.6	0.41	0.51	0.4	0.40	0.42
Sex									
Male	71.1	6.03	6.99[b]	68.1	5.97	5.99[b]	78.2	4.44	7.12[b]
Female	71.5	6.38	8.33[a]	68.3	5.53	6.99[a]	78.0	4.89	8.41[a]
SEM	0.3	0.24	0.29	0.3	0.24	0.29	0.3	0.23	0.24
P-value									
Experimental treatment	0.006	0.550	0.120	0.359	0.182	0.630	0.024	0.705	0.024
Sex	0.356	0.306	0.002	0.599	0.195	0.018	0.575	0.175	0.001
Experimental treatment × Sex	0.386	0.624	0.763	0.651	0.047	0.893	0.228	0.399	0.560

PE = plant extract.

[a–c] Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 4 birds per replicate and 8 replicates per treatment.

[1] UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE.

[2] Colour: lightness/luminosity (L*), redness (a*), and yellowness (b*).
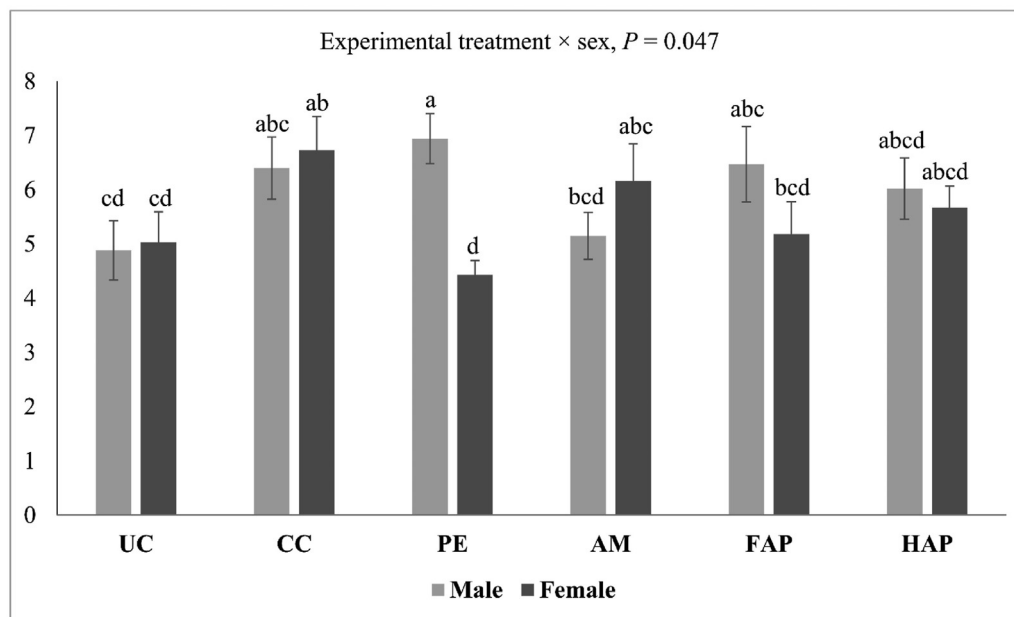
Fig. 4. Interaction between experimental treatment and sex on a* value in the lower breast on d 35. UC, unchallenged control; CC, challenged control; PE, plant extract; AM, antimicrobial (narasin and nicarbazin; 50 ppm each active compound); FAP, full dose of AM plus PE; HAP, half dose of AM plus PE. Challenged birds were orally gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14. [a–d] Values in a row with no common superscripts differ significantly ($P < 0.05$).

pigmentation in broilers. The reduced FI and BWG, increased FCR, intestinal lesions, and high mortality examined in NE challenged birds without supplementation, confirmed the successful induction of clinical NE challenge. Despite the severity of NE challenge conditions in the current study, AM treatments were able to protect birds against clinical NE as demonstrated by improved BWG and FCR, and reduced intestinal lesions and mortality. The results in the current study indicated that the supplementation of PE improved FCR, overall livability and uniformity, reduced ileal lesion scores in male birds and counts of *Eimeria* spp. in the intestine, and increased thigh luminosity and yellowness compared to the birds in the CC group. On the other hand, birds fed PE had shown similar intestinal lesions, *E. maxima* and total oocyst counts in ileal content, *Eimeria*

spp. counts in the small intestine compared to the AM-fed birds. These findings of the current study support the hypothesis that the supplementation of PE improves feed efficiency and helps to mitigate the negative impact of clinical NE challenge on intestinal health. However, in contrast to our anticipations, dietary supplementation of AM in combination with PE had similar results compared to the birds fed AM alone, and a half dose of AM in combination with PE had shown effects in between those of AM and PE. These results reject the hypothesis that the combination of PE and AM may exert synergistic effects in protecting birds against NE. In fact, a previous study reported that the birds fed anticoccidial in combination with oregano essential oil had no synergistic effects (Bozkurt et al., 2016), which is in agreement with the observations

in the current study despite of the difference in the composition of the additive from plants.

The dietary inclusion of PE either singly or blended with different forms have the potential to improve FCR, increase BWG, digestibility, and promote intestinal health status to protect birds against enteric diseases (Windisch et al., 2008; Kothari et al., 2019; Yadav and Jha, 2019; Adhikari et al., 2020). However, the mode of action of PE is yet to be determined and may vary due to different sources, compositions, and forms of active elements being used in diets. In general, the mechanism of action of PE is believed to be associated with their antimicrobial and antioxidative activities. The dietary addition of PE ameliorates the deleterious effects of enteric diseases on intestinal health via reduced pathogenic microbial loads, altered microbiota by antimicrobial activities resulted in stabilised microbial inhabitants (Mitsch et al., 2004; Lillehoj et al., 2018). The results observed in the current study indicated that birds supplemented with PE reduced FCR and increased livability by 5.8% considering the entire period of the study (d 0 to 35) compared to the birds without supplementation. Birds fed PE had a higher BW uniformity on d 35 in both male and female birds compared to the CC group, which showed better uniformity in birds fed PE. In addition, birds fed PE had significantly improved FCR and reduced mortality, although numerically, during the onset of NE (d 9 to 21) indicated the beneficial effects of PE supplementation in diets. Similar to our results, birds supplemented with a micro-encapsulated mixture of eugenol and garlic tincture showed better feed efficiency under a subclinical NE challenge (Pirgozliev et al., 2019). However, during the starter phase birds were not challenged, and thus, the effect of PE was not significant. Further, the objective of the study was to exam whether the PE shows an effect when the birds are challenged with NE. However, during the finisher phase, the challenged birds (CC group) recovered from NE and the performance caught up with unchallenged birds (UC group). Therefore, finisher phase did not show PE effect on growth. On the other hand, birds fed PE had lower FCR compared to the CC group indicating the beneficial effect of PE supplementation in the finisher phase. Improved feed efficiency and livability in PE supplemented group possibly are likely due to the promoted intestinal health status of the birds as indicated by reduced ileal and caecal Eimeria oocysts counts and reduced ileal lesion scores. It has been proven that birds challenged with field strains of Eimeria spp. oocysts led to intestinal damage and alter microbiota which is essential to predispose chickens to NE outbreak (Stanley et al., 2014; Wu et al., 2014). Positive effects of dietary supplementation of PE could be due to its suppressive effects on the overgrowth of Eimeria oocysts resulting in decreased intestinal damage, thus reduced the risk of chickens succumbing to NE. In fact, previous studies in broilers stated that PE (garlic and garlic metabolites) in diets reduced mortality under Escherichia coli challenge (Elmowalid et al., 2019) and decreased mortality, oocyst counts and intestinal lesions under Eimeria, and NE challenge (Kim et al., 2013; Lee et al., 2018; Ali et al., 2019), which is in agreement with the observations in the current study. Moreover, reduced NE effects of birds may have played a positive role on skin pigmentation as indicated by higher luminosity in the thigh, and yellowness in the breast and thigh compared to the CC group observed in this study. It has been reported that skin pigmentation is associated with enteric diseases such as coccidiosis and necrotic enteritis (Horst, 2020). Impaired mucosa and intestinal integrity caused by enteric diseases can reduce the absorption of pigment from the diet, which is transported through the blood and stored in tissues and skin (Tyczkowski et al., 1991; Luis and Oscar, 2020). Moreover, birds fed PE had similar intestinal lesions and oocysts counts compared to the AM group further confirmed the beneficial effects of PE supplementation in diets on intestinal health. In addition, it has been

shown that birds fed PE had improved intestinal integrity and increased mucin-secreting goblet cells (Kumar et al., 2021) which can further confirm the protective effects of PE supplementation to the birds against NE. Cumulatively, these findings demonstrated the beneficial effects of PE to reduce the severity of clinical NE challenge on performance and intestinal health as evidenced by improved feed efficiency, livability and uniformity reduced ileal lesions, oocyst counts, and increased skin yellowness.

Eimeria spp. oocyst in the excreta of the chickens is a good indicator of intestinal health status and the overgrowth of oocysts can negatively affect chickens as a predisposing factor of NE. Essentially, the administration of field strains of Eimeria spp. oocysts in the NE challenge model were to predispose chickens for the adequate infection by C. perfringens to induce a successful NE challenge. Eimeria spp. applied in the challenge model include E. brunetti, E. maxima, and E. acervulina can multiply in the intestinal environment, alter microbiota and damage the intestinal epithelial layer resulting in reduced FI, BWG, and nutrient digestibility (Dahiya et al., 2006; Stanley et al., 2014). The PE has long been used in poultry feed with proven efficacy on parasite control either by destroying sporozoites or by altering the wall formation of oocysts (Kim et al., 2013; Fatemi et al., 2015). The anti-inflammatory and immunomodulatory mechanism and antiparasitic activities of bioactive compounds of PE can defend against the infection caused by coccidia (Ali et al., 2019; Pop et al., 2019). The results observed in this study showed that birds fed PE had reduced oocyst counts at least some of Eimeria spp. in caecal and ileal contents. These findings are in agreement with previous studies that used essential oil containing garlic in broilers, where faecal oocyst counts were significantly reduced (Abou-Elkhair et al., 2014; Lee et al., 2018; Sidiropoulou et al., 2020). This suggests that the dietary supplementation of PE reduces Eimeria spp oocyst counts as a result reduces the incidence of clinical NE.

Skin pigmentation, specifically yellow skin, is an important trait in chickens from the consumer point of view and is attributed to a sign of health for chickens. It is influenced by several factors including diet ingredients and compositions, and chicken genetics, age, sex, and enteric health status (Bilgili et al., 1998; Petracci and Fletcher, 2002; Liu et al., 2008). Studies have reported that the absorption and deposition of pigments in the skin are affected by health conditions of birds which could influence digestion and absorption of nutrients from diets, such as in the cases of coccidiosis and NE (Tyczkowski et al., 1991; Horst, 2020). Enteric diseases damage intestinal mucosa and impair digestion and absorption of nutrients (Kaldhusdal et al., 2001; Immerseel et al., 2004; Williams, 2005), which might be the cause to decrease pigment deposition in the skin. This is supported by an earlier study reporting that xanthophyll concentrations in plasma significantly decreased in broilers infected with E. acervulina compared to the uninfected broilers (Hernández-Velasco et al., 2014). The results observed in the current study indicated that dietary supplementation of PE increased yellowness by 11.6%, 6.1%, and 14.4% in the upper breast, lower breast, and thigh, respectively. Similarly, a recent study suggested that birds fed blended additives including a phenolic compound had significantly increased skin yellowness in broilers compared to unsupplemented birds (Horst, 2020). Therefore, the increased yellowness in the breast and thigh by PE application indicates the beneficial effects of PE on bird health under the clinical NE challenged condition.

In conclusions, the present study demonstrated that the dietary inclusion of a microencapsulated product composed of eugenol and garlic tincture has the potential to improve performance and intestinal health, and reduce the severity of clinical NE challenge as illustrated by improved FCR, livability, uniformity and reduced intestinal lesions, ileal and caecal Eimeria oocyst counts, and

increased skin yellowness. However, as birds fed AM was more protective in controlling clinical NE, the combination of antimicrobial and PE failed to exert synergistic effects under the present conditions. Further investigations in similar experimental conditions with different dosages would provide a better understanding of their mode of action to combat severe enteric diseases such as NE in the post-AM era.

Author contributions

Alip Kumar: Study design, feed formulation, animal trial, laboratory analysis, statistical analysis and manuscript writing. **Nishchal K. Sharma**: Animal trial, lab analysis, statistical analysis and manuscript review. **Sarbast K. Kheravii**: Statistical analysis and manuscript review. **Chake Keerqin**: Study design, feed formulation and manuscript review. **Catherine Ionescu**: Data analysis and manuscript review. **Alexandra Blanchard**: Data analysis and manuscript review. **Shu-Biao Wu**: Coordinator, study design, statistical analyses, critical manuscript review.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and 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.

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