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Effect of Butyric Acids Glycerides and Eugenol on Growth Performance, Intestinal Morphology and Bacteriological Examination in Broilers under Necrotic Enteritis Challenge

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Abstract

A total of 210 one-day-old male Ross 308 broilers were used to investigate the effect of butyric acids glycerides (BAG) and eugenol (EU) on growth performance, intestinal morphology, blood metabolites and bacteriological examination in broilers under necrotic enteritis challenge**.** Dietary treatments consisted of 2 supplemental BAG levels (0 and 0.2%) and 3 EU levels (0, 500, and 1000 ppm) in a 2×3 factorial arrangement with five replicates and six birds in each group. The treatment groups were as follows: 1) basal diet, negative control group (NC); 2) basal diet + *C. perfringens*, positive control group (PC); 3) PC + 0.2% butyric acid glycerides (BAG); 4) PC + 500 ppm of eugenol (EU500); 5) PC + 1000 ppm of eugenol (EU1000); 6) PC + 500 ppm of eugenol +0.2% butyric acid glycerides (EU500+BAG); 7) PC + 1000 ppm of eugenol +0.2% butyric acid glycerides (EU1000+BAG). The addition of EU1000 and EU1000+BAG in the diet of challenged chickens increased the body weight compared to the PC group ($P < 0.05$). Also, adding EU at 500 or 1000 ppm + 0.2% BAG in the diet significantly increased the digestibility of dry matter and organic matter of challenged birds compared to the NC and PC control groups. The lowest cholesterol was observed for the challenged groups that supplemented EU1000+BAG compared to NC and PC controls. Supplementation in the diet with high or low-level EU and 0.2% BAG increased the carcass weight compared to the PC group ($P < 0.05$). The Thymus, spleen, and bursa of Fabricius weight in the PC group decreased compared to the NC birds ($P < 0.05$). Counts of the *Lactobacillus* subgroup were not affected by treatments, but *C. perfringens* in NC birds was higher compared to the PC group ($P < 0.05$). Generally, our results showed that adding 1000 ppm EU+0.2% BAG effectively controlled experimental Eimeria and *C. perfringens* coinfection.

Introduction

One of the common diseases in the poultry industry is necrotic enteritis (NE), which causes economic losses of about 6 billion dollars annually (Wade and Keyburn, 2015a). This disease is caused by *C. perfringens*, which is a gram-positive, spore-forming anaerobe bacterium (Sokale *et al.*, 2019). NE occurs in broilers between 2 and 6 weeks of age and common symptoms in broiler chickens include daily weight loss, increased mortality and feed conversion ratio (Wade and Keyburn, 2015a). Such products obtained from infected chicken cause serious problems in human health (Wade and Keyburn, 2015b).

Coccidiosis is one of the important predisposing factors for NE in chickens that is caused by protozoa of the *Eimeria* genus (Santiani *et al.*, 2023). Proliferation and colonization of *C. perfringens* due to intestinal epithelial layer damage can be facilitated by coccidiosis (Miska and Fetterer, 2017).

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In the last decades, antibiotics have been widely used as growth stimulants to stabilize the microbial population of the digestive tract, improve performance and diminish intestinal pathogen concentration and incidence of enteric diseases such as NE. As a result of increasing concerns regarding antibiotic resistance in animal and poultry tissues and then in humans, their use was banned (Melaku *et al.,* 2021). Several compounds such as probiotics, prebiotics, enzymes, essential oils, organic acids etc., have been widely considered in poultry nutrition as alternatives to antibiotic growth promoters (Caly *et al*., 2015; Seifi *et al.,* 2018).

Butyric acid (BA) is an organic acid (OA) with a short four-carbon chain, which through direct stimulation of the growth of enterocytes increases density, improves intestinal integrity, inhibits the growth of pathogenic agents, and increases the villus height and, as a result, the absorption of nutrients in chicken's increases (Melaku *et al*., 2021; Makowski *et al*., 2022). It has been reported that BA in free form has an unpleasant odor; 60% of it is absorbed in the upper part of the digestive tract, and less than 1% reaches the lower part of the small intestine (Wu *et al*., 2018). Coating butyric acid increases its absorption and allows it to reach the lower parts small intestine and exert positive effects (Smith *et al*., 2012). It has been reported that butyric acid glycerides (BAG) directly enter the intestine, are more efficient than free BA salts, and improve the microbial population of broilers by reducing the pH (Sadeghiyan *et al*., 2023)

Essential oils (EOs) are volatile compounds with a slight molecular weight and biological activities synthesized in different plant organs (Jain *et al.,* 2022). EOs extracted from plants have antibacterial, antiviral, antifungal, and antioxidant properties, as well as immunomodulation effects, reduce blood lipids, and stimulate the digestive system of poultry (Bouhaddouda *et al.,* 2016; Micciche *et al.,* 2018). Also, EOs improve nutrient digestibility and growth performance of poultry (Abu Isha *et al.* 2018; Torki *et al*. 2021). EU is a polyphenolic complex as an antimicrobial active substance found in clove essential oil (*Syzgium aromaticum*) which has antioxidant, anti-inflammatory and antibacterial properties (El-Maati *et al.,* 2016). In addition, EU acts as an appetite and digestion stimulant and has a positive effect on the microbial population, the integrity of the intestinal mucosal barrier and the function of the immune system in poultry (Mandey, 2022; Ibrahim *et al.,* 2022; Zhao *et al.,* 2022).

In recent years, the combination of OAs and plant EOs has been widely used and has had significant effects (Iqbal *et al*., 2021; Vinolya *et al.,* 2021). OAs can complete the effect of EOs through the synergism of antibacterial and bactericidal activities (Pham *et al*., 2020). We hypothesized that this combination of EOs and OAs could be used to improve the growth and gut health and control NE infection in broiler chickens. Therefore, in this study, we assessed the effects of different levels of EU with the combination of BAG on growth performance, nutrient digestibility, blood metabolites, carcass characteristics, jejunal morphology, and cecal microbial population in broilers under necrotic enteritis challenge.

Materials and Methods

Experimental design and Husbandry

A total of 210 one-day-old male (Ross 308) broiler chicks were obtained from a local hatchery and then were randomly divided into seven treatments with five replications of 6 birds in each. The treatment groups were as follows:1) basal diet, negative control group (NC), 2) basal diet + *C. perfringens*, positive control group (PC), 3) PC + 0.2% BAG (BAG); 4) PC + 500 ppm of EU (EU500); 5) PC + 1000 ppm of EU (EU1000); 6) PC + 500 ppm of EU+0.2% BAG (EU500+BAG); 7) PC + 1000 ppm of EU +0.2% BAG (EU1000+BAG). Clove oil (Eugenol as ≥86% purity) was purchased from the ayat essence company in Tehran, Iran. Butyric acid was obtained from Sanadam Pars company. The manufacturer of this product is Silo (SILO Additives Ind. Co. Italy) in Italy. The brand name of the product is BABY-C_4, which has a metabolic energy of 7 kcal/kg. This product contained 25-35% monoglycerides in the 1 or 3 positions, 50-55 % diglycerides in the 1 or 3 positions and 15-25% triglyceride. This composition is produced in two forms: powder and liquid. In this study, the powder form was used. The trial was conducted during 1-42 days of age. The diets included a starter (1 to 10 days of age), grower (11 to 22 days of age), and finisher (23 to 42 days of age). They were formulated according to Ross 308 (Aviagen. 2019) strain catalog recommendations (Table 1). During the experimental period, the feed and water were offered *ad libitum*. The lighting system of 16 h light/8 h darkness was imposed throughout the experimental period. The temperature of the room was set at 32°C for the first three days and then reduced until it reached 21°C. This temperature was maintained until the end of the 42-d experiment.

Necrotic enteritis challenge

At d 22, all treatments except the NC group were orally gavaged with a 10-fold dose of a commercial live attenuated vaccine (Paracox-5) field strains of *Eimeria* spp. Oocysts in 1 mL dose consisting of sporulated *E. tenella* (1500), *E. necatrix* (1000), *E. maxima* (1000), and *E. acervulina* (500) as described by *C. perfringens* challenged according to Gholamiandehkordi *et al.* (2007) with some modifications.

Table 1. Ingredients and calculated composition of experimental diets

Ingredients	Starter (1-10 d)	Grower (11-22 d)	Finisher (23-42 d)
Corn	26.68	19.50	17.87
Wheat	26.04	38.92	28.19
Soybean meal (CP 44%)	38.20	32.49	44.69
Soybean oil	4.37	4.88	5.13
Limestone	1.18	0.93	0.92
Di-calcium phosphate	1.99	1.89	1.91
Common salt	0.38	0.37	0.34
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25
DL-Methionine	0.37	0.30	0.28
L-Lysine HCl	0.29	0.22	0.17
Chemical compassion			
AME_n (kcal/kg)	2975	3050	3100
Crude protein (%)	22.56	20.81	19. 37
Ca (%)	1.04	0.91	0.90
P-available (%)	0.49	0.45	0.44
Na (%)	0.18	0.17	0.17
Arginine (%)	1.40	1.25	1.13
Lysine (%)	1.41	1.22	1.07
Methionine (%)	0.69	0.59	0.55
Methionine + cysteine (%)	1.05	0.93	0.87

[1] A kilogram of premix included the following: vitamin A, 9000 *IU*; vitamin D_3, 2000 *IU*; vitamin E, 36 mg; vitamin k_3, 2 mg; vitamin B_1, 1.75 mg; vitamin B_2, 6.6 mg; Calcium pantothenate, 9.8 mg; Niacin, 29.65 mg; vitamin B_6, 2.94 mg; vitamin B_9, 1 mg; vitamin B_{12}, 0.015 mg; Choline chloride, 250 mg and antioxidant, 1 mg

[2] Manganese,99.2; Zinc,84.7; Iron,50 mg; Copper, 10 mg; Iodine, 0. 99 mg; Selenium, 0.2 mg

Four d after d 26, 27, and 28 infected chickens were continuously inoculated orally with *C. perfringens* type A PTCC No: 1765 (1 mL/chick/ two times a day 3×10^{-8} CFU/mL via 1 ml syringe each time except those in the NC group. Similarly, broiler chickens in the NC group (unchallenged) received 1 mL/chick of sterile PBS (Phosphate-buffered saline).

Growth performance

Average body weight (BW), average daily feed intake (ADFI), average daily gain (ADG) and Feed efficiency (FE) were calculated for 22 and 42 days (Li *et al*., 2023).

Nutrient digestibility

At 40 to 42 days, fresh fecal samples were collected from 2 birds of each pen (one male and one female). Then, the stored fecal samples at -20℃ dried at 60℃ for 72 h. To determine the digestibility of dry matter (DM), organic matter (OM), ash, and ether extract (EE), chromium oxide (0.3%) as an indigestible marker was added to the bird's diet for seven days before fecal collection. The content of chromic oxide in the samples was measured according to Fenton and Fenton (1979). The apparent digestibility (AD) of nutrients was calculated according to the following equation:

$$AD = 100 \times [1 - (\%\ Cr_2O_3 \text{ in diet} / \%\ Cr_2O_3 \text{ in feces}) \times (\%\ \text{excreta nutrient} / \%\ \text{dietary nutrient})]$$

Blood metabolites

At 35 days, the blood samples were obtained from the wing vein for the determination of blood biochemical markers. Blood samples were centrifuged to obtain serum for 15 min at 3000 rpm (VISION Model VS-15000 CFN II Made in South Korea). Serum samples were kept in tubes at −20 °C until analyzed. Serum biochemical markers (Total cholesterol (TCHO), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-c)) were measured by using commercial kits (Ziest Chem, Iran) by an automatic clinical chemistry analyzer (Spectrophotometer, UNICO 2100, USA). Also, low-density lipoprotein cholesterol (LDL-c) and very low-density lipoprotein cholesterol (VLDL-c) were determined (mg/dl) as follows (Friedewald *et al*., 1972):

$$LDL\text{-}c = TCHO - HDL\text{-}c - TG/5.0$$

$$VLDL\text{-}c = Triglycerides/5$$

Carcass characteristics

On day 32, two birds from each replicate were randomly slaughtered after four h fasting for carcass processing. The feathers, heads, shanks, and viscera were removed, and the remaining carcasses were weighed immediately to determine the hot carcass yield and then dissected. Data collected from carcass weight, abdominal fat, liver, and cut parts (breast and thigh) were recorded as relative to BW (g of organ/kg of BW) (Park *et al*., 2021).

Jejunal morphology

Briefly, the middle part of jejunum from 2 birds per pen samples was cut off (approximately 2 cm) on day 32 and placed immediately in 10% buffered formalin carefully without chyme. The samples were serially dehydrated by transferring through alcohols and embedded in paraffin wax. Slices of Paraffin sections (5 μm) mounted on glass slides using a microtome (RM-1512, Leica microsystems., Austria) mounted on glass slides and stained with hematoxylin-eosin staining. Images were taken using a light microscope (Olympus BX51, Japan) coupled with Spot Photo software (SPOT 3.1; Diagnostic Instruments, Sterling Heights, MI, USA). A total of 45 measurements were conducted for the three intact well-oriented villi per cross-section of each five birds per treatment. The villus height (VH), villus width (VW), crypt depth (CD), thickness of muscle layer (ML), villus height to crypt depth ratio (VH/CD), and villus area (VS) were observed and measured (Sakamoto *et al*., 2000).

DNA extraction, 16S rDNA gene sequencing, and analysis

For bacteriological examination, on day 32, the digesta from the cecum were collected and subsequently placed into a sterile 1.8 ml Cryogenic Tube (QC LAB), and snap frozen in a liquid nitrogen solution then stored at -80°C until microbial quantitative real-time PCR (RT-PCR) analyses as described by Du *et al.* (2015). Briefly, genomic DNA was isolated from 100 mg digesta of each sample using a commercial DNA stool kit (Viragene, Tehran, Iran) according to the manufacturer's instructions. Primer sequences of *C. perfringens* and *Lactobacillus* spp used are given in Table 2. For real-time PCR amplification of the bacterial targets from cecal contents, 20 μL of reaction mixture containing 10 μL of Ampliqon SYBR Green PCR Master Mix High ROX (Denmark), 2 μL of diluted cDNA, 4 μL of PCR-grade water, and 2 μL of each primer was prepared.

The thermocycler program was: 1 cycle of 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 56 °C for 20 s, and 72 °C for 25 s. Quantitative RT-PCR with a 7500-fluorescence detection system (Applied Biosystems, Foster City, CA, USA) was used to determine genomic DNA from cecal samples.

The following formula calculated DNA copy numbers according to Lee *et al.* (2006):

DNA (copy) = 6.02×10^{23}(copy/mol) × DNA amount (g)/ DNA length (dp) × 660 (g/mol/dp)

Table 2. 16s rDNA real-time PCR primers used to quantify intestinal bacteria

Target	Primer sequence (5′–3′) [a]	Amplicon size (bp)	Reference
Lactobacillus subgroup	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	341	Du *et al.* (2015)
Clostridium perfringens	F: AAAGATGGCATCATCATTCAAC R: TACCGTCATTATCTTCCCCAAA	279	Du *et al.* (2015)

Economic efficiency

A simplified analysis of the economic efficiency of broiler production was performed based on the final BW of birds, feed intake, and the average price of diets in January 2024 (Makowski *et al*., 2022). Total costs were calculated based on the assumption that feed costs accounted for 70% of total costs.

Statistical analysis

All data were analyzed using analysis of (GLM) procedure (SAS Institute, 2001). Significant differences between treatments were determined using Tukey's test for the comparison differences of means ($P < 0.05$). The statistical model for data analysis was as below: $Y_{ij} = \mu + A_i + e_{ij}$ Where: Y_{ij} = the measured value for each observation (data), μ = mean, A_i = treatment effect, and e_{ij} = experimental error

Results

Growth performance

The results related to the effect of experimental treatments on the growth performance of *C. perfringens*-challenged chickens are shown in Table 3. The addition of EU1000 and BAG + 1000 ppm of EU in the diet of challenged chickens increased the BW compared to the PC group ($P < 0.05$), But no significant difference was observed compared to the NC group. Challenged chickens fed with EU1000, EU500+BAG, and EU1000+BAG had ADFI and ADG compared to the PC group ($P < 0.05$), but no significant effect was observed compared to the NC group. FE was not affected by the experimental treatments.

Nutrient Digestibility

The results related to the effect of experimental treatments on the digestibility of challenged chickens are shown in Table 4. The results showed that adding levels of 500 and 1000 ppm of EU with and without BAG in the diet of challenged chickens increased the digestibility of DM, OM, and CP compared to the PC group ($P < 0.05$). However, the use of BAG did not have a significant effect on the digestibility of DM, OM, and CP. Supplementing the diet of challenged chickens with 1000 ppm EU with and without BAG significantly increased EE digestibility compared to the PC group ($P < 0.05$). Also, except for BAG treatment, other experimental treatments increased the digestibility of EE compared to the NC group. Chickens receiving 1000 ppm EU with and without BAG and also EU500 increased ash digestibility compared to the PC group ($P < 0.05$).

Table 3. Effects of dietary BAG and EU supplementation on growth performance of *C. perfringens* challenged broilers in different feeding phases

Treatment	BW22 (g)	BW42 (g)	ADFI (g/d)	ADG (g/d)	FE (g/g)
NC	782.1	2273.3^{ab}	164.5^{a}	82.8^{abc}	0.50
PC	781.0	2018.6^{b}	139.2^{b}	72.3^{c}	0.51
BAG	799.7	2180.6^{b}	143.9^{b}	78.3^{bc}	0.55
EU500	754.2	2294.2^{ab}	141.3^{b}	81.2^{abc}	0.54
EU1000	770.5	2380.4^{a}	158.8^{a}	87.8 ^{ab}	0.54
EU500+BAG	814.1	2277.0^{ab}	158.4^{a}	85.5^{ab}	0.54
EU1000+BAG	760.2	2388.1^{a}	161.6^{a}	90.4^{a}	0.56
SEM[1]	9.62	18.89	1.40	0.99	0.006
P-value	0.462	0.008	<0001	0.003	0.067

a-b-c: Mean values within the same column with different superscripts differ significantly ($P < 0.05$). NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides.
[1] standard error of means.

Table 4. Effects of dietary treatments on digestibility coefficients (%) of nutrients in broilers on day 42 of age.

Treatment	DM	OM	CP	EE	Ash
NC	57.77^{b}	57.96^{b}	61.00^{c}	60.04^{c}	34.92^{c}
PC	59.64^{b}	60.38^{b}	61.78^{c}	63.65^{bc}	34.65^{c}
BAG	59.68 ^{b}	59.79^{b}	63.97^{bc}	69.64^{ab}	42.20^{bc}
EU500	67.01^{a}	67.33^{a}	71.04^{a}	63.25^{bc}	34.96^{c}
EU1000	66.27^{a}	67.11^{a}	68.97^{ab}	72.72^{a}	48.71^{ab}
EU500+BAG	68.52^{a}	67.99^{a}	71.57^{a}	70.92^{ab}	57.26^{a}
EU1000+BAG	68.99 ^{a}	68.906^{a}	72.96^{a}	72.14^{a}	56.83^{a}
SEM[1]	0.45	0.40	0.76	0.97	1.45
P-value	0.001	0.001	0.001	0.004	0.001

a-b-c: Mean values within the same column with different superscripts differ significantly ($P < 0.05$). NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides.
[1] standard error of means.

Blood metabolites

The results of blood metabolites are presented in Table 5. The chickens of EU1000, EU500+BAG, and EU1000+BAG groups reduced TCHO and LDL-c concentrations compared to the PC group ($P < 0.05$). TG concentration in chickens (challenged) receiving BAG, EU and their combination had a significant decrease compared to the NC group ($P < 0.05$), but no difference was observed with the PC group. All the experimental treatments, compared to the PC group except the NC group, decreased the VLDL-c concentration ($P < 0.05$). Blood HDL-c concentration was not affected by experimental treatments ($P > 0.05$).

Table 5. The blood parameter (mg/dl) in response to additive treatments in *C. perfringens*-challenged broilers on day 32.

Treatment	TCHO	TG	HDL-c	VLDL-c	LDL-c
NC	120.49^{a}	80.13^{a}	49.23	14.52^{ab}	50.59^{ab}
PC	118.82 ^{a}	72.60^{ab}	53.71	16.02^{a}	55.23^{a}
BAG	116.15^{ab}	68.41^{b}	52.34	13.68^{b}	45.56^{b}
EU500	116.59^{ab}	67.37^{b}	52.88	13.47^{b}	50.24^{ab}
EU1000G	111.59^{bc}	65.69^{b}	53.37	13.14^{b}	49.64^{ab}
EU500+BA	110.92^{bc}	65.69^{b}	51.86	13.14^{b}	45.92^{b}
EU1000+BAG	107.58^{c}	65.90^{b}	50.98	13.18^{b}	43.42^{b}
SEM[1]	0.52	0.79	0.44	0.15	0.60
P-value	0.0001	0.008	0.315	0.008	0.001

a-b-c: Mean values within the same column with different superscripts differ significantly ($P < 0.05$). NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides. TCHO: Total cholesterol, TG: Triglyceride, HDL-c: High-density lipoprotein cholesterol, LDL-c: Low-density lipoprotein cholesterol, VLDL-c: Very Low-density lipoprotein cholesterol.
[1]: standard error of means.

Carcass characteristics

The results related to the effect of experimental treatments on carcass characteristics of *C. perfringens*-challenged chickens are shown in Table 6. No significant differences were observed using different levels of EU with and without BAG on the carcass, breast, thigh, liver, and abdominal fat weight of challenged chickens compared to the NC and PC groups.

Table 6. Eviscerated Carcass characteristics (% of BW) in challenged broilers fed diets containing the different levels of BAG (%) and EU (ppm) on day 32

Treatment	Carcass	Breast	Thigh	Liver	Abdominal fat
NC	63.290[a]	26.88	27.39	2.10	0. 97
PC	57.51[b]	25.61	25.99	2.50	0.86
BAG	58.12[ab]	23.81	25.47	2.47	0.89
EU500	60.41[ab]	25.81	25.75	2.41	0.79
EU1000	61.42[ab]	26.09	26.50	2.37	0.81
EU500+BAG	58.85[ab]	24.16	25.68	2.28	0.73
EU1000+BAG	60.54[ab]	23.95	24.64	2.22	0.78
SEM[1]	0.56	0.43	0.30	0.03	0.03
P-value	0.041	0.192	0.190	0.174	0.665

a-b-c: Mean values within the same column with different superscripts differ significantly ($P < 0.05$). NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides.
[1] standard error of means.

Jejunal morphology

Results of intestinal morphology are presented in Table 7. The results showed that the PC group had lower VH, VH/CD, and VS compared to the NC group ($P < 0.05$). The addition of BAG and EU and their combination decreased CD compared to the PC group ($P < 0.05$). The use of BAG and EU and their combination increased VH/CD in challenged chickens compared to the PC group ($P > 0.05$). However, there was no significant difference in morphology ML and VW between experimental treatments ($P > 0.05$).

Table 7. Jejunal morphological in challenged broilers fed diets containing the different levels of BAG and EU on day 32

Treatment	VH (μm)	VW (μm)	CD (μm)	ML (μm)	VH/CD	VS (mm^2)
NC	1143.40 [a]	112.40	230.60[b]	250.00	4.96[a]	0.12[a]
PC	1024.40 [b]	103.40	318.80[a]	231.80	3.22[b]	0.10[b]
BAG	1133.00 [a]	110.20	233.20[b]	243.40	4.86[a]	0.12[ab]
EU500	1113.20 [a]	111.00	239.80[b]	237.60	4.64[a]	0.12[ab]
EU1000	1123.80 [a]	112.20	246.60[b]	232.60	4.56[a]	0.12[ab]
EU500+BAG	1138.00 [a]	113.80	239.80[b]	243.60	4.75[a]	0.12[a]
EU1000+BAG	1139.80 [a]	114.20	239.00[b]	243.40	4.77[a]	0.13[a]
SEM[1]	4.66	1.82	2.22	2.16	0.04	0.002
P-value	<.0001	0.578	<.001	0.120	<.001	0.030

a-b-c: Mean values within the same column with different superscripts differ significantly ($P < 0.05$). NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides. villus height (VH), villus width (VW), crypt depth (CD), thickness of muscle layer (ML), Villus height to crypt depth ratio (VH/CD), villus surface area (VS)
[1] Standard error of means.

Bacteriological examination

The results related to the effect of experimental treatments on the bacteriological examination of challenged chickens are shown in Table 8. No significant differences were observed using different levels of EU with and without BAG on *lactobacillus* and *C. perfringens* populations compared to the PC group. However, the population of *C. perfringens* in NC chickens was higher compared to the challenged chickens even with different levels of EU and BAG ($P < 0.05$).

Economic efficiency

Simplified economic evaluation (Table 9) showed that the weighted average price of the control diet ($ 356 per t) and Price per kg of live weight was $ 1.76. The feed cost/kg live weight in broilers receiving EU1000 and EU1000+BAG were higher than other experimental treatments. Total costs, including feed cost and indirect costs, were lower by $ 0.05 and 0.07 per kg live weight in group BAG and EU500 than in the positive control group respectively. As a result, revenue per kg live weight was higher in group BAG

and EU500 than in the other groups ($ 0.34 and 0.36 vs. 0.29). The profit resulting from the addition of the tested feed additives to broilers diets reached $ 0.163 per bird in group BAG, and $ 0.246 per bird in group EU500 compared to the positive control group. But the broiler chickens receive EU500+BAG. EU1000, and EU1000+BAG groups have loss $ 0.247, 0240 and 0.367, respectively, compared to the positive control group.

Table 8. Cecal *Lactobacillus* and *Clostridium perfringens* counts log10 (copy/g digesta) of challenged broiler chickens fed diets supplemented with different levels of BAG and EU on d 32.

Treatment	Cecal microbiota	
	Lactobacillus	*Clostridium perfringens*
NC	2.97	2.07[b]
PC	2.89	4.78[a]
BAG	2.57	2.22[b]
EU500	2.78	2.33[b]
EU1000	2.88	2.32[b]
EU500+BAG	2.90	2.38[b]
EU1000+BAG	3.11	2.35[b]
SEM[1]	0.10	0.17
P-value	0.832	0.011

a-b-c: Mean values within the same column with different superscripts differ significantly (P <0.05). NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides.
[1] standard error of means.

Table 9. Economic evaluation (per bird).

Specification	NC	PC	BAG	EU500	EU1000	EU500+BAG	EU1000+BAG
Price per kg of live weight, $/kg					1.76		
Cost of BAG $/kg					2.77		
Cost of EU $/L					44		
Average cost of diets, $/kg	0.356	0.356	0.362	0.381	0.406	0.387	0.412
Farm Performance							
Live weight, kg	2.28	2.01	2.19	2.30	2.39	2.28	2.39
Feed consumed, kg	6.92	5.84	6.04	5.93	6.68	6.66	6.80
Financial Performance ($)							
Feed price, kg	0.356	0.356	0.362	0.381	0.406	0.387	0.412
Feed cost/bird	2.463	2.079	2.186	2.259	2.713	2.577	2.802
Feed cost/kg live weight	1.08	1.03	0.99	0.98	1.17	1.13	1.17
Total cost/bird	3.518	2.97	3.122	3.227	3.876	3.681	4.002
Total cost/kg live weight	1.54	1.47	1.42	1.40	1.62	1.61	1.67
Revenue/kg	1.76	1.76	1.76	1.76	1.76	1.76	1.76
Revenue/bird	4.012	3.537	3.854	4.048	4.206	4.012	4.206
Margin/kg live weight	0.22	0.29	0.34	0.36	0.14	0.15	0.09
Margin/bird	0.501	0.582	0.745	0.828	0.335	0.342	0.215
Difference (compared to the positive control)			0.163	0.246	-0.247	-0.240	-0.367

NC= Negative control; PC= basal diet C. *perfringens*, positive control; BAG= PC + 0.2% butyric acid glycerides; EU500= PC + 500 ppm of eugenol; EU1000= PC + 1000 ppm of eugenol; EU500+BAG= PC + 500 ppm of eugenol +0.2% butyric acid glycerides and EU1000+BAG= PC + 1000 ppm of eugenol +0.2% butyric acid glycerides.

Discussion

Growth performance

In the present study, the addition of EU1000 and EU1000+BAG in the diet of challenged chickens increased the BW compared to the PC group. Also, the chickens of EU1000, EU500+BAG, and EU1000+BAG groups had higher ADFI and ADG compared to the PC group (Table 3). It has been reported that BA reduces the feed intake of poultry, unlike propionates and acetates (Namkung *et al*., 2011). The reduction of feed intake in the effects of using butyric acid can be caused by the strong taste of organic acid, loss of appetite and decrease in palatability (Adil *et al*., 2010). In this regard, Moquet (2018), reported that the use of 1 g/kg of different butyrate derivatives had an anorexic effect in broiler chickens. Also, Józefiak *et al.* (2010) observed that the inclusion of benzoic acid at 0.2% depressed the growth of broiler chickens. However, Panda *et al.* (2009) reported that BA could be used to improve the growth performance of broilers.

Regarding the effects of the EOs, it has been reported that adding EOs at 200 or 500 mg/kg could be beneficial to BWG and FE in *C. perfringens*-challenged birds (Pham *et al*., 2022). Eid *et al.* (2018) reported that a concentration of 0.125% of clove oils was able to improve the growth performance of infected broiler chickens completely. About the mixed effects of plant OAs and EOs, Stamilla *et al.* (2020) investigated the effects of 5 g/kg of encapsulated blends of OAs and EOs as a natural alternative compound for broiler chicken's improved growth performance and gut healthiness either in terms of morphology or of microbiology. Grilli *et al.* (2013) stated that microencapsulation of a blend of OAs (propionic and sorbic acid) and EOs (eugenol and thymol) at levels 0.1 or 0.3% improved the growth performance of broilers due to their antibacterial action. Giannenas *et al*. (2014) reported that a combination of OAs and EOs has synergistic effects. Furthermore, EU can disturb lipid fraction and damage the integrity of bacteria cell membranes in a short period, causing leakage of the cell contents and direct uptake of hydrogen ions from the extracellular environment (Jeyakumar and Lawrence., 2021). The antibacterial and antioxidant properties of EOs and the bactericidal effect of OAs may also be the possible reasons for enhanced broiler performance (El-Shenway and Ali, 2016; Elnaggar and El-Maaty, 2017).

Nutrient digestibility

In our results, adding EU500 and EU1000 with and without BAG in challenged broilers improved the digestibility of DM, OM, and CP compared to the PC group ($P < 0.05$). Also, the chickens of the EU1000 and EU1000+BAG groups increased the digestibility of EE compared to the PC group (Table 4). In this regard, Stefanello *et al.* (2020) reported that a 300 g/t protected blend of organic acids (fumaric, sorbic, malic, and citric acids) and essential oils (thymol, vanillin, and eugenol) improved nutrient digestibility in challenged broilers.

Gao *et al.* (2019) showed that the 150, 200, or 250 mg/kg encapsulated blends of OAs (citric acid and sorbic acid) and EOs (thymol and vanillin) increased activity of digestive enzymes and could result in improved growth performance of broilers. The EOs have been shown to possess antimicrobial properties, which may help to control the growth and colonization of pathogenic bacteria in the gut of broiler chickens. This can reduce inflammation and competition with pathogenic microbes, which in turn can improve feed efficiency by promoting better nutrient digestion and absorption (Nava *et al*. 2009; Adil *et al*. 2011). In the present study, the use of eugenol essential oil reduced the number of *C. perfringens* populations in the intestines of broiler chickens. Basmacioğlu Malayoğlu *et al* (2010) reported that oregano EOs (250 and 500 mg/kg) with or without enzymes have improved crude protein digestibility by increased chymotrypsin activity in the digestive system. Jang *et al.* (2004) reported that an increase in the digestive enzyme activities, pancreas, and intestinal mucosa with the combination of OAs and EOs could be the explanation for better nutrient digestibility.

Blood metabolites

The chickens of EU1000 and EU500+BA and EU1000+BA groups reduced TCHO and LDL-c concentrations compared to the PC group. TG and VLDL-c in chickens (challenged) receiving treatments had a significant decrease compared to the control groups (Table 5). Similar to the results of the present experiment, in the research conducted by Kamal and Raga (2014) and Deepa *et al*. (2017), the reduction of TCHO and LDL-c levels without affecting the HDL-c level was shown by adding different forms of BA. Hussein *et al.* (2019) reported that LDL-c levels in broilers reduced in response to supplementation of clove oil (0.75–1.5 ml/kg diet). The blood serum triglyceride level was reduced by supplementing the glycerides of butyric acid in the diet of broiler chickens by the amount of 0.2% and 0.25% in the diet (Dehghani Tafti and Jahanian, 2016), which was consistent with the results obtained in this study. Supplementation of clove oil (0, 0.5, 1, 1/5%) and Tulsi (0, 2, 3, 4%) improved serum-biochemical profile by reducing TCHO and LDL-c levels in broilers (Sultana *et al.,* 2023). Harb *et al.* (2019) suggested that EU due to its antioxidant properties can reduce serum TCHO levels and inhibit lipogenesis in the liver. Ibrahim *et al.* (2022) reported that with the inclusion of 250 and 400 mg/kg levels of EU nanoemulsion in a broiler diet, there was hypolipidemic activity and reduced TCHO and LDL-c concentrations. LDL-c was decreased by feeding EO 0.1g/kg and OA 1g/kg in combination (Iqbal *et al*., 2021). The concentrations of cholesterol in serum in broiler chickens received EOs ajwain (seed oil; 300 mg/kg diet), clove (bud oil; 600 mg/kg diet) and cinnamon (bark oil; 400 mg/kg diet) decreased due to the inhibitory effects of its active compounds on 3-hydroxy 3-methylglutaryl reductase enzyme (HMG-CoA) (Chowdhury *et al*., 2018). The previous research reported that EOs and OAs decreased the serum LDL-c concentration and HDL-c remained unchanged (Yildirim *et al*., 2018).

Carcass characteristics

Our study indicated that EU and BAG treatment did not affect the carcass, breast, thigh, liver, and abdominal fat weight of challenged chickens in the PC group (Table 6). Leeson *et al.* (2005) reported that breast weight and carcass weight were improved in chickens fed with a mixture of BA (mono, di, and

triglyceride) at doses of 2000 or 4000 mg/kg. In another experiment, Mahdavi and Torki (2009) reported that the addition of BA to the diet of broiler chickens compared to the control group had no significant difference in the relative weight of breast, thigh, abdominal fat, or liver.

Falaki *et al* (2016) reported that adding EOs to the diet of broiler did not affect the percentages of abdominal fat, gizzard, and liver. Also, in agreement with this finding, no significant effects were observed on carcass traits by broilers fed clove oil (Azadegan Mehr *et al*. 2014). Contrary to the results of this study, Yang *et al.* (2022) reported that abdominal fat was numerically reduced in d-28 chickens fed with a combination of microencapsulated sodium butyrate (1 g/kg diet) and forskolin (5, 10, or 25 mg/kg). These researchers have reported that because forskolin and butyrate both decrease appetite and lipogenesis therefore, by showing a synergistic effect, they have reduced feed intake and decreased abdominal fat deposition. Some researchers reported that by use of OAs and EOs, no significant effect on carcass characteristics of broilers was observed (El-Shenway and Ali, 2016; Ozsoy *et al.*, 2017; Gomathi *et al.*, 2018).

Jejunal morphology

In the present study, the VH increased in chickens fed with BAG and EU and their combination, while the CD decreased due to the use of additives in the diet of challenged chickens compared to the PC group (Table 7). It has been reported that OAs decrease the CD and increase the VH/CD of the crypt, which indicates the positive effect of OAs on intestinal health (Mustafa *et al*., 2021). BAG can stimulate the cell differentiation of intestinal epithelium (Kang *et al*., 2011). Also, BAG reduces the inflammatory response by reducing the growth of pathogenic bacteria and improving the integrity of the intestinal mucosa (Zou *et al.*, 2019). Kumar *et al*. (2021) showed that the addition of EU and garlic microencapsulated products can improve intestinal integrity and increase the number of mucin-producing goblet cells as a defense response to birds against necrotic infection. EOs act as an antioxidant agent to protect villi from oxidative damage and increase the hardening of tight junctions (El-Baroty *et al.*, 2010). Also, EOs strengthen mature enterocytes, which improves absorption capacity due to increased VH and decreased CD (Hesabi Nameghi *et al.*, 2019). According to our findings, Jerzsele *et al*. (2012) reported that in broiler chickens infected with *C. perfringens* and receiving diets containing sodium butyrate and volatile oil (ginger and carvacrol) protected with vegetable fat, the VH/CD ratio increased. It is stated that improved intestinal morphology may be due to the anti-inflammatory and anti-oxidation mechanism of essential oils and organic acids (Du *et al*., 2016; Gao *et al*., 2019). Also, it has been reported that OAs provide the necessary energy for the regeneration and repair of intestinal epithelial cells and improve intestinal health by preventing the proliferation of pathogens (Kriss *et al*., 2018).

Bacteriological examination

In the current study, we found a significant increase in *C. perfringens* count in the cecum of the birds in the PC group compared with those in the NC groups and other treatments ($p < 0.05$). Feeding EU and BAG decreased *C. perfringens* count compared with the PC group (Table 8). OAs in the cecum of broiler chickens affect the number of bacteria (Dhama *et al*., 2014). M'Sadeq *et al* (2015) reported that simple monocarboxylic acids such as BAG possess a strong antimicrobial activity. Due to their lipophilic nature, OAs can pass through the bacterial cell membrane and kill the bacterial cell by reducing the intracellular pH (Nava *et al*., 2009). The hydrophobic activity of eugenol causes the penetration of the lipopolysaccharide part of the cytoplasmic bacterial cell membrane and the destruction of the integrity of the membrane structure, which ultimately leads to the leakage of intracellular materials of the bacteria (De Souza *et al*., 2015). It has been reported that EOs such as thymol, eugenol, curcumin, piperine, carvacrol, and cinnamaldehyde reduce the proliferation of *C. perfringens* bacteria in the intestines of broilers (Jamroz *et al*., 2003; Mitsch *et al*., 2004; McReynolds *et al*., 2009). EU + BAG groups were effective in reducing the C. *perfringens* count in the cecum (Table 8). Researchers reported that supplementation of feed with OAs and EOs has been used to inhibit bacterial growth (Teissedre and Waterhouse, 2000; Lambert *et al*., 2001). EOs cause an increase in the permeability of the bacterial cell membrane and facilitate the entry of OAs into the bacterial cell cytoplasm, causing a decrease in pH and disruption of sensitive bacteria activity such as *E. coli, Salmonella*, and *C. perfringens* (Oviedo-Rondón *et al*., 2010; Smyth *et al*., 2018).

Economic efficiency

In the present study, the profit resulting from the addition of the tested feed additives to broiler diets reached $ 0.07 per bird (group BAG) and $ 0.05 per bird (group EU500) in compeer to the positive control group. Similar observations were made by other authors, who found that organic acids added to poultry diets improved economic efficiency (Menconi *et al.* 2014; Onunkwo *et al.* 2021). Ramota *et al.* (2018) reported that the inclusion of essential oil increased feed intake and consequently average total weight of poultry. But, such inclusion increased the average total cost of feed in production. However, the final economic results

depended on the feed prices and live body weight of broilers in an experimental period.

Conclusion

The results of our study revealed that the addition of EU and BAG could effectively ameliorate gut injury caused by NE infections. Also, the addition of EU with and without BAG increased and Jejunal morphological and the digestibility of DM, OM and, CP. The EU1000 mg/kg with and without BAG can be valuable feed additives in broiler nutrition, but according to an economic estimate in this study, it was not economical.

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