Buffered formic acid and a monoglyceride blend coordinately alleviate subclinical necrotic enteritis impact in broiler chickens

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ABSTRACT The objective of this study was to evaluate the effect of 2 different doses of a partially buffered formic acid product (Amasil NA; 61% formic acid, 20.5% sodium formate), and a monoglyceride blend of short- and medium-chain fatty acids (BalanGut LS P) on necrotic enteritis (**NE**) infected broilers in terms of performance, intestinal microbial population and short-chain fatty acids concentrations in the gastrointestinal tract. A total of 528-day-old as hatched Ross 308 broilers were allocated to 48 pens with 11 birds in each pen. Six dietary treatments applied in the study were: T1) nonsupplemented diet (Control); T2) antibiotic supplemented diets; T3) and T4) high (Starter: 0.5%; Grower and Finisher: 0.5%) and low (Starter: 0.3%; Grower and Finisher: 0.2%) dose of Amasil NA; and groups T5) and T6) high (Starter: 0.3%; Grower and Finisher: 0.2%) and low dose (Starter: 0.3%; Grower: 0.15%; Finisher: 0.075%) of (BalanGut LS P). All birds in this study were fed starter (d 0-10), grower (d 11−24) and finisher (d 25−35) diets and challenged with NE. To induce subclinical NE, oral administrations of *Eimeria* oocysts (d 9) followed by inoculation of *Clostridium perfringens* strains (d 14 and 15) were applied. Results showed that birds fed the high dose of Amasil NA, had a higher feed conversion ratio (**FCR,** $P < 0.05$) compared to the nonsupplemented group during the starter period. Antibiotic supplementation reduced FCR during the grower ($P < 0.001$), finisher ($P < 0.05$) and overall ($P < 0.001$) periods of the experiment. Both levels of BalanGut LS P and low levels of Amasil NA enhanced overall FCR ($P < 0.05$) compared to the birds in the nonsupplemented group. Compared to the nonsupplemented group, high levels of Amasil NA and low levels of BalanGut LS P improved FCR in the finisher stage ($P < 0.05$). On d 16, cecum digesta of birds fed with antibiotic supplemented diets showed a significantly lower number of *C. perfringens* ($P < 0.001$) compared to the nonsupplemented and high level of BalanGut LS P group. *Bacillus* ($P < 0.01$) and *Ruminococcus* numbers were significantly lower in the birds fed with high level of Amasil NA ($P < 0.05$) compared to the antibiotic supplemented diets. High doses of Amasil NA, showed the highest propionate concentration in the cecum ($P < 0.001$). The study suggests that supplementation of BalanGut LS P and Amasil NA at different feeding phases may achieve optimal performance improvement in broilers under NE challenge.

Key words: broiler, monoglycerides, microbiota, necrotic enteritis, organic acids

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INTRODUCTION

Necrotic enteritis (**NE**) is caused mainly by the proliferation of *Clostridium perfringens* in chickens' gut. Due to the concerns regarding the potential risk of antibiotic resistance, use of antibiotics in animal feed has been banned or limited in different parts of the world, leading to increased occurrences of enteric diseases such as NE in poultry. The emergence of intestinal diseases has forced the poultry industry to pursue alternatives to antibiotics (Dahiya et al., 2006) and current approaches are based on adding functional ingredients to feed, which can reduce the need for antibiotics and other medication, to control NE and maintain chicken performance. Liquid short-chain organic acids can protect feed from microbial and fungal demolition (Kum et al., 2010) and improve chicken performance due to their antimicrobial benefits (Adil et al., 2010).

Short-chain organic acids, in their undissociated form, can freely pass through the outer and plasma membrane of microorganisms (Walter and Gutknecht, 1984). Upon entry, these compounds dissociate into the slightly alkaline cytoplasm, which causes a release of protons that

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lower the internal pH (Roe et al., 2002). The dissociation of organic acids in the cell results in the reduction of intercellular pH, which can lead to activity inhibition of important microbial enzymes and nutrient transport systems that can, in turn, inhibit the proliferation of the bacteria (Huyghebaert et al., 2011). Supplementation of short-chain organic acids has been shown to reduce the number of pathogenic bacteria such as *Salmonella, Campylobacter, E. coli* and *Clostridium* in the chicken intestine (Wang et al., 2010; Naseri et al., 2012; Sultan et al., 2015; Ragaa and Korany, 2016). Further, short-chain organic acids promote proteolytic enzyme activity and nutrient digestibility, intensify pancreatic secretions and encourage digestive enzyme activity (Papatsiros et al., 2013).

Glycerides are made up of a glycerol molecule esterified with up to 3 fatty acids. By esterifying one fatty acid with a glycerol molecule, a monoglyceride is formed. Under standard esterification conditions, monoglycerides tend to prefer the 1-monoglyceride form rather than the 2-monoglyceride form. Medium-chain monoglyceride have shown promising effects on feed efficiency, health improvement, growth performance capacity and immune status of broilers (Fortuoso et al., 2019; Valentini et al., 2020; Amer et al., 2021). Monoglycerides are natural compounds which, alongside their nutritional values of lipids, act against gram-positive and gram-negative bacteria at concentrations at which no evidence for harm to the body has been found (Mansour and Millière, 2001; Thormar et al., 2006). It is believed that esterification of fatty acids will increase the antibacterial activity of that fatty acid (Kabara, 1984; Batovska et al., 2009). Due to the amphipathic properties of monoglycerides, these compounds show a membrane-lytic action, which can cause bacterial membrane destabilization and pore formation. Membrane-destabilizing activity causes increased cell permeability and cell lysis, leading to cell death (Yoon et al., 2018). Monoglycerol compounds have shown to reduce numbers of *Salmonella typhimurium* (Tosi et al., 2016) *Campylobacter jejuni* (Hilmarsson et al., 2006), *E. coli* (Thormar et al., 2006) and *C. perfringens* (Skřivanová et al., 2014) in chickens.

The objective of the present study was to assess the effectiveness of 2 different doses of 2 additives, an organic acid mixture of formic acid and sodium formate (**Amasil NA;** 61% formic acid, 20.5% sodium formate), and a synergic combination of a monoglyceride blend (mono- di and triglyceride with a majority of 1-monoglycerides) (**BalanGut LS P**), on the performance and gut health of broilers challenged with NE. We hypothesized that these additives could positively alter the gut environment and improve NE-infected broiler chicken performance.

MATERIALS AND METHODS

Animal Ethics

The following experimental protocol was approved by the Animal Ethics Committee (Authority No.: AEC17-027) of the University of New England, Armidale, NSW, 2351, Australia. The protocol was carried out in accordance with the guidelines specified in the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition 2013.

Experimental Design and Diets

A total of 528-day-old Ross 308 chicks (as hatched) were obtained on the day of hatching from Baiada Hatchery in Tamworth, NSW. The average weight of chickens upon arrival was 38 g and they all were vaccinated against infectious bronchitis and Marek's disease in the hatchery. On arrival, birds were feather sexed, and 11 birds (5 males and 6 females) were allocated to all 48 pens, measuring 75 × 120 cm. Wood shavings were used as bedding material to a depth of approximately 7 cm in each pen. Pens were equipped equally with a tube feeder and cup drinkers. Feed and water were provided ad libitum. The lighting, relative humidity and temperature followed Ross 308 strain (Aviagen., Ross Broiler Management Manual. 2014) guidelines.

All diets were based on wheat and soybean meal and pelleted at 65 to 70°C. Feed in the starter stage was given as crumbles (approximately 1.5−3 mm in length and diameter), pellets in the grower and finisher stage had a 3-mm diameter with a 6 and 8 mm length, respectively. Diets were formulated to meet nutrient profiles of the Ross 308 specifications (Aviagen, 2014) and diet compositions are presented in Table 1.

Dietary Treatments

Except for antibiotic supplementation, the additives used in this study were added at different doses throughout the stages of chicken growth. Table 2 shows the dosages of additives used at 3 phases of feeding in each treatment.

NE Challenge Model

On d 9, all birds were subjected to oral gavage of 1 mL vaccine Eimeria strains (Bioproperties Pty Ltd., Sydney, Australia) including phosphate-buffered saline suspension of approximately 5,000 oocysts each of *Eimeria acervulina* and *Eimeria maxima*, and 2,500 oocysts of *Eimeria brunetti*. Primary poultry isolates of *C. perfringens* (EHE-NE18) containing the toxin NetB (Keyburn et al., 2008) were obtained from CSIRO Livestock Industries, Geelong, Australia. and was incubated overnight at 39 °C in 100 mL of sterile thioglycollate broth (USP alternative; Oxoid) followed by subsequent overnight incubation of 1 mL of the previous culture in 100 mL of cooked meat medium (Oxoid), and then in 700 mL of thioglycolate broth (USP alternative; Oxoid) containing starch (10 g/L) and pancreatic digest of casein (5 g/L) to obtain the challenge inoculum. After preparation of the inoculums, 1 mL of fresh inoculums containing

Table 1. Composition and nutrient contents of diets in three growing phases (Starter 0-10, Grower 11-24 and finisher 25-35).

Ingredients %	Starter	Grower	Finisher
Wheat	62	68	71
Soybean meal (45.7%)	28	25	21
Canola meal	3.00	1	1.08
Meat and bone meal	2.61	2.01	2.00
Cottonseed oil	1.00	1.01	2.00
Limestone	1.00	0.97	0.85
Salt	0.157	0.171	0.157
Na bicarbonate	0.14	0.20	0.19
Sand	0.50	0.50	0.50
Vitamins premix[1]	0.075	0.075	0.075
Trace mineral premix[2]	0.100	0.100	0.100
Choline Cl 60%	0.036	0.029	0.023
Phytase[3]	0.020	0.020	0.020
Kynofos 21P/16Ca	0.128	0.80	-
L-lysine HCl 78.4	0.362	0.341	0.03
DL-methionine	0.270	0.241	0.20
L-threonine	0.19	0.17	0.13
TIO2	-	0.50	0.50
Calculated nutrients			
ME (kcal/kg)	3,050	3100	3,200
Crude protein %	23.8	21.7	21.2
Crude fat %	3.04	3.02	4.0
d-Lysine %	1.280	1.15	1.03
d-Methionine %	0.57	0.51	0.46
Calcium %	0.96	0.87	0.79
Phosphorus available %	0.480	0.43	0.480
Sodium %	0.162	0.18	0.18
Chloride %	0.230	0.23	0.23

[1]Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg;thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg;pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg;cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2]Trace mineral premix supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg;Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg;cereal-based carrier, 128 mg; mineral oil, 3.75 mg.4 Zinc bacitracin (Albac 150) was purchased from Ridley AgriProducts,(Tamworth, NSW, and Australia).

[3]Phytase (Natuphos E 5000 FTU/g).

approximately 10^8 CFU/mL *C. perfringens* was inoculated to the chickens on d 14 and 15.

Bird Weight, Feed Intake, and Feed Conversion ratio

Pen body weight (**BW**) and feed intake (**FI**) were recorded on d 0, 10, 24, and 35 and used to calculate mean bird weight gain, FI, and feed conversion ratio (**FCR**). The FCR was corrected for mortality by adding the weight of dead chickens back to the pen BW within each period.

Lesion Scoring and Sample Collection

On d 16, two birds (1 male and 1 female) were randomly selected from each pen, electrically stunned and euthanized by cervical dislocation to perform postmortem analyses, digesta collection and intestinal lesion scoring. For short-chain fatty acid (**SCFA**) analysis, ileal and cecal contents from the same two birds, were collected into 50 mL plastic containers separately and stored at −20°C. For bacterial population quantification, approximately 1 g of cecal digesta was collected in a 2 mL Eppendorf tube, snap-frozen in liquid nitrogen, and stored at −20°C until required for DNA extraction. All sections from the small intestine (duodenum, jejunum and ileum) of the same 2 birds were excised for lesion scoring. Lesion scoring system was ranged from 0 to 4 based on previously reported studies, which is 0: No gross lesions, 1: thin-walled or friable small intestine, 2: focal necrosis or ulcerations, 3: larger patches of necrosis and 4: severe, extensive necrosis (Prescott et al., 1978).

Ileal and Cecal SCFA Analysis

The cecal and ileal SCFAs were measured according to a method described (Jensen et al., 1995). Briefly, approximately 1 g of cecal content (1−1.5 g ileal content) was weighed, and 1 mL of internal standard (0.01 M ethylbutyric acid) was added, thoroughly mixed and followed by centrifugation at 15,000 × *g* at 5°C for 20 min. One mL of the supernatant was transferred to an 8 mL vial, and 0.5 mL of concentrated HCl (36%) and 2.5 mL of diethyl ether were added and thoroughly mixed by vortexing for 30 s, followed by centrifugation at 1000 × *g* at 5°C for 15 min. Four hundred microliters of the resulted supernatant was then mixed with 40-μL N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide. The sample was vortexed and heated (at 80°C) for 20 min. The vial was kept in room temperature for 48 h before being analyzed on a Varian CP3400 CX gas Chromatograph (Varian Analytical Instruments, Palo Alto, CA).

Cecal Bacteria Quantification

DNA was extracted from the frozen cecal digesta samples following the method described by Kheravii et al. (2017). Approximately 60 mg of freshly defrosted cecal content and 300 mg of glass beads (0.1 mm) were placed in a 2 mL Eppendorf tube. Then 300 μL Qiagen

Table 2. Experimental design.

Group No.	Group name	Products name	Amount added in different growth stages (%)
T1	Nonsupplemented	-	-
T2	Antibiotic	Zinc bacitracin and Salinomycin	0.033% and 0.05%, respectively, in all stages.
T3	Low dose- buffered formic acid	Amasil® NA	Starter: 0.3; Grower and Finisher: 0.2
T4	High dose- buffered formic acid	Amasil® NA[1]	Starter: 0.5; Grower and Finisher: 0.5
T5	Low dose- monoglyceride blend	BalanGut™ LS P	Starter: 0.3; Grower: 0.15; Finisher: 0.075
T6	High dose- monoglyceride blend	BalanGut™ LS P[2]	Starter: 0.3; Grower and Finisher: 0.2

[1]Amasil® NA and BalanGut™.

[2]LS P were provided by BASF Australia Ltd.

Lysis Buffer (270 μL DXL and 30 μL digestive enzymes) was added to the samples and mixed using a vortex mixer. The samples were then transferred to bead beater mill (Retsch GmbH & Co, Haan, Germany), and cells were disrupted at a frequency of 30/s for 5 min. Samples were then incubated in a heating block at 55°C for 2 h followed by centrifugation at 20,000 × g for 5 min. Reagents (DXB, DXW, DXF, and DXE) were placed in their specific locations in the robotics machine together with 200 μL of the lysate transferred automatically into loading block. Then 400 μL of the binding buffer (DXB) was added to the 200 μL lysate and incubated for 6 min, and then 500 μL of the lysed samples were transferred into capture columns and vacuumed at 30 kPa for 3 min. Then, 600 μL DXW was transferred to the capture columns and vacuumed for 30 kPa for 2 min, 600 μL DXF was transferred to the columns and vacuumed at 35 kPa for 1 min, and DNA was dried by vacuuming again at 25 kPa for 5 min. Finally, an elution block was used to elute the extracts by the addition of 60 μL DXE and the samples were vacuumed at 30 kPa for 2 min. The resulting DNA samples were measured on a Nanodrop 8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) for assessment of quantity and purity. DNA with ratios of A260/A280 and A260/A230 being >1.8 were considered of high purity and were stored at −20°C.

The extracted cecal DNA was diluted 20 times in nuclease-free water, and the quantitative real-time PCR was performed to quantify 6 bacterial groups with a real-time PCR system Rotorgene 6000 (Corbett, Sydney, Australia). The SYBRGreen containing Mix (Sensi-Mix SYBR No-Rox, Bioline, Sydney, Australia) was used for the quantification of *Bacillus,* Enterobacteriaceae*, Bifidobacteria, Ruminococcus, Lactobacillus, Bacteroides, Salmonella* and *C. perfringens.* The 16S rRNA primers are shown in Table 3. The quantified bacteria are expressed as log10 (genomic DNA copy number)/g digesta.

Data Analysis

All the data derived in this study were checked for normal distribution prior to performing statistical analysis. Data with normal distribution were subjected to one-way ANOVA analysis as a completely randomized design, using the General Linear Model procedure of SPSS statistics version 22 (IBM Corporation, Armonk, NY). Differences between mean values per pen were determined using Tukey's multiple range test at the level of $P < 0.05$. Performance data were analyzed for the treatment effect with male percentage (corrected for mortality) as a covariate. The intestinal lesion scores data were analyzed by the nonparametric Kruskal-Wallis test as the data were not normally distributed.

RESULTS

Broiler Performance

The broiler performance results in the three different growth stages of chickens are presented in Table 4. In the starter period (d 0−10), the birds fed diets with high level of Amasil NA (T4) mixture showed a significantly higher FCR ($P < 0.05$) compared to nonsupplemented group (T1), antibiotic supplemented (T2) and low level- BalanGut LS P (T5) groups. In the grower period (d 11−24), BW gain of birds fed with the antibiotic supplemented diets was higher than all other treatments ($P < 0.05$). The lowest FCR ($P < 0.001$) in the grower period was observed in the high level- BalanGut LS P (T6) and antibiotic supplemented group (T2) compared to other treatments. FI was not significantly affected by different additives ($P > 0.05$). In the finisher stage (d 24−35), birds in the antibiotic group (T2) showed a significantly lower FCR compared to all other groups. Birds fed with the high level- Amasil NA (T4) and low level- BalanGut LS P (T5) groups showed a significantly lower FCR ($P < 0.05$) compared to the

Table 3. Primers used for the qPCR analysis of different bacteria groups.

Target group	Primer/probe sequence (5'-3')	Amplicon length, bp	Annealing temperature, °C	Reference
Bacillus spp.	F-GCA ACG AGC GCA ACC CTT GA R-TCA TCC CCA CCT TCC CC GGT	92	63	(Zhang et al., 2015)
Bacteroides spp.	F-GAG AGG AAG GTC CCC CAC R-CGC TAC TTG GCT GGT TCA G	108	63	(Layton et al., 2006)
Bifidobacterium spp.	F-GCG TCC GCT GTG GGC R-CTT CTC CGG CAT GGT GTT G	106	63	(Requena et al., 2002)
Clostridium perfringens	F- GCA TAA CGT TGA AAG ATG G R- CCT TGG TAG GCC GTT ACC C TaqMan probe: 5'-FAM-TCA TCA TTC AA C CAA AGG AGC AAT CC-TAMRA-3'	120	60	(Wise and Siragusa, 2007)
Lactobacillus spp.	F-CAC CGC TAC ACA TGG AG R-AGC AGT AGG GAA TCT TCC A	186	63	Wise and Siragusa (2007)
Ruminococcus spp.	F-GGC GGC YTR CTG GGC TTT R-CCA GGT GGA TWA CTT ATT GTG TTA A	157	63	(Ramirez-Farias et al., 2008)
Salmonella	F-GT TTC CTGCGG TAC TGT TAA TT R- AGA CGG CTG GTA CTG ATC GAT A A	67	56	(Bartosch et al., 2004)
Enterobacteriaceae	F-CGG YCC AGA CTC CTA CGG G R-TTA CCG CGG CTG CTG GCA C	190	63	(Bartosch et al., 2004)

Abbreviations: qPCR, real time quantitative PCR; F, forward primer; R, reverse primer.

Table 4. Performance response of NE challenged broilers to diets containing either antibiotics, organic acid mixture, or monoglycerides in different growth periods of the experimental trials.

		Starter (day 0−10)			Grower (day 11−24)			Finisher (day 25−35)			Overall (day 0−35)		
Treatment[1]	Additive and dose	BW	FI	FCR	BW	FI	FCR	BW	FI	FCR	BW	FI	FCR
T1	-	239	266	1.113^{b}	807^{b}	1153	1.429^{a}	1063	1750	1.646^{a}	2117	3144	1.485^{a}
T2	Antibiotic	248	274	1.106^{b}	903^{a}	1166	1.291^{b}	1102	1711	1.555^{c}	2244	3112	1.389^{c}
T3	Amasil® NA- Low	249	279	1.117^{ab}	836^{b}	1172	1.397^{a}	1059	1712	1.598^{ab}	2188	3184	1.456^{b}
T4	Amasil® NA- High	246	279	1.133^{a}	837^{b}	1219	1.455^{a}	1108	1770	1.589^{b}	2140	3177	1.484^{a}
T5	BalanGut™ LS P- Low	251	278	1.108^{b}	813^{b}	1145	1.414^{a}	1093	1751	1.559^{b}	2190	3154	1.440^{b}
T6	BalanGut™ LS P- High	250	280	1.119^{ab}	822^{b}	1152	1.380^{b}	1126	1753	1.604^{ab}	2184	3163	1.449^{b}
SEM		3.32	3.84	0.005	17.2	20.4	0.021	20.4	27.4	0.019	31.41	45.09	0.010
P-value		0.172	0.149	0.024	0.013	0.219	<0.001	0.743	0.320	0.040	0.104	0.329	<0.001

[a,b,c] means in a column not sharing a common letter are significantly different ($P < 0.05$).

Abbreviations: BW, body weight gain; FI, feed intake; FCR, feed conversion ratio.

[1]T1: No additive, T2: Salinomycin (0.050%) and zinc bacitracin (0.033%); T3: buffered formic acid - low dose (Starter: 0.3 %; Grower and finisher: 0.2%); T4: buffered formic acid- high dose (Starter: 0.5%; Grower and finisher: 0.5%); T5: monoglyceride blend − low dose (Starter: 0.3%; Grower: 0.15%; Finisher 0.075%).; T6: monoglyceride blend − high dose (Starter: 0.3%; Grower and finisher: 0.2 %).

nonadditive group. No difference was observed for FI and BW in this period ($P > 0.05$).

The overall performance (d 0−35) showed that birds fed with antibiotics supplemented diets (T2) had the lowest FCR compared to all other groups ($P < 0.001$). Meanwhile, broilers fed with diets supplemented with low levels of Amasil NA (T3) and both levels of BalanGut LS P (T5 and T6) had a significantly lower FCR compared to the nonsupplemented group (T1) ($P < 0.001$). High level of Amasil NA (T4) did not significantly alter overall performance traits compared to the nonsupplemented group ($P > 0.05$). Weight gain and FI were not affected by different types of additives compared to the negative control group in the 0−35−day period ($P > 0.05$).

Intestinal Lesion Score

The presence of lesions in different sections of the intestine for all sampled birds is shown in Figure 1. Different types of additives did not significantly affect intestinal lesions compared to those fed with the nonsupplemented diet.

Mortality due to NE

Table 5 shows the mortality due to NE, which occurred within 4 d following the challenge on d 14. No significant differences of NE mortality were observed between birds treated with different doses of the supplement. However, the birds in the nonsupplemented group had a numerically higher mortality compared to the diets supplemented with additives.

Cecal Bacterial Population

Table 6 illustrates the abundance of cecal bacteria groups on d 16. The genomic DNA copy numbers of *Bacillus spp.* observed in the high levels of Amasil NA

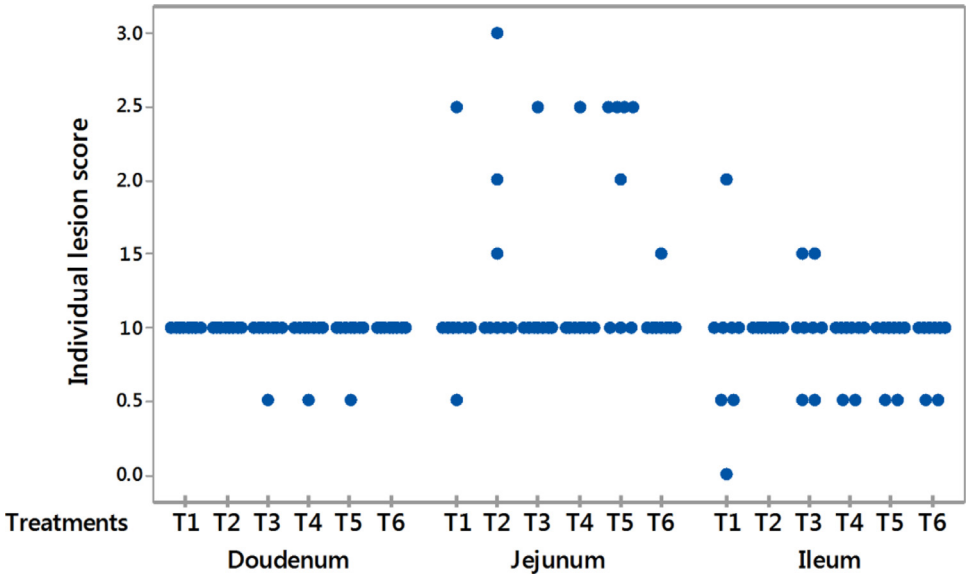

Figure 1. Intestinal lesion score at d 16 for all sampled birds. T1: No additive, T2: Salinomycin (0.050%) and zinc bacitracin (0.033%); T3: buffered formic acid - low dose (Starter: 0.3 %; Grower and finisher: 0.2%); T4: buffered formic acid- high dose (Starter: 0.5%; Grower and finisher: 0.5%); T5: monoglyceride blend − low dose (Starter: 0.3%; Grower: 0.15%; Finisher 0.075%).; T6: monoglyceride blend − high dose (Starter: 0.3%; Grower and finisher: 0.2 %).

Table 5. Mortality due to NE following challenge of the birds.

Treatment[1]	Additive and dose	Mortality (%)
T1	-	4.55
T2	Antibiotic	0
T3	Amasil® NA- Low	1.14
T4	Amasil® NA- High	1.14
T5	BalanGut™ LS P- Low	1.25
T6	BalanGut™ LS P- High	0
SEM		0.92
P-values		0.164

[1]T1: No additive, T2: Salinomycin (0.050%) and zinc bacitracin (0.033%); T3: buffered formic acid - low dose (Starter: 0.3 %; Grower and finisher: 0.2%); T4: buffered formic acid- high dose (Starter: 0.5%; Grower and finisher: 0.5%); T5: monoglyceride blend − low dose (Starter: 0.3%; Grower: 0.15%; Finisher 0.075%).; T6: monoglyceride blend − high dose (Starter: 0.3%; Grower and finisher: 0.2 %).

(T4) group was significantly lower compared to the birds fed diets supplemented with antibiotic (T2) and both levels of BalanGut (T5 and T6). Furthermore, the number of *Lactobacillus* and *C. perfringens* numbers in the high levels of BalanGut (T6) was significantly higher compared to the birds fed with the antibiotic diets (T2). *Ruminococcus* spp. population in the cecal content of birds fed with high levels of Amasil NA (T4), was significantly lower than birds fed either levels of BalanGut (T5 and T6) ($P < 0.001$). Antibiotics significantly reduced *C. perfringens* ($P < 0.001$) population in the cecum compared to the nonsupplemented group (T1). No significant difference was detected in Enterobacteriaceae, *Bacteroides* spp., *Bifidobacterium* spp. and *Salmonella* populations between treatments.

Ileal and Cecal SCFAs

The results of cecal and ileal SCFAs on d 16 are presented in Tables 7 and 8, respectively. Results are presented as the level relative to the total SCFA (%). The challenged birds fed diets supplemented with high levels of Amasil NA (T4) had a higher percentage of caecal propionic acid compared to all other groups ($P < 0.001$). Different type and/or doses of feed additives did not affect ($P > 0.05$) on the amount of caecal formate, acetate, isobutyrate, butyrate, isovalerate, lactate and succinate compared to the non-supplemented group. Birds fed with high levels of the buffered formic acid (Amasil NA) (T4) had a significantly higher percentage of valerate compared to birds fed with antibiotic (T2) and high levels of monoglyceride blend (BalanGut LS P) (T6) supplemented diets. The results indicated that ileal SCFA contents were not affected by any treatment ($P > 0.05$) (Table 8).

DISCUSSION

In the present study, the effects of 2 levels of the buffered formic acid (Amasil NA) and 2 levels of a monoglyceride blend (BalanGut LS P) were evaluated and compared with an antibiotic supplemented and nonsupplemented diet in broilers under a subclinical NE infection. Results show that both levels of the monoglyceride blend (BalanGut LS P) enhanced overall FCR compared to the birds in the nonsupplemented diets. In the grower and finisher period, antibiotics reduced FCR compared to the nonsupplemented groups. Further, the low levels of Amasil NA and low levels of BalanGut LS P improved FCR in the finisher stage, compared to the nonsupplemented group where they had no significant difference with the antibiotic group. Antibiotic supplemented group, together with the low levels of BalanGut LS P, showed a significantly higher number of *Ruminococcus* in the cecum.

The constituents of the Amasil NA used in this study are approximately 61% formic acid and 20.5% sodium formate. In the present study, although high levels of Amasil NA did not show a significant effect on the performance of birds, the positive effects of the low-level supplementation of this product are observed with reduced FCR in the finisher stage of this trial. Similarly, Ragaa et al. (2016) found an improvement in feed efficiency when adding 5 g/kg formic acid to broiler diets. A meta-analysis of organic acids in broiler diets has shown that organic acids improve FCR and increase the activity of digestive enzymes, pancreatic secretions and gastrointestinal mucous in broilers (Polycarpo et al., 2017). Emami et al. (2017) reported improvement in chicken growth performance using an organic acid blend, which was accompanied with improved nutrient digestibility. Similarly, improvement in FCR and protein accretion was reported by Samanta et al. (2009) by using an organic acid blend in broiler feed. However, the current results illustrate that the highest FCR in the starter and grower period were observed in birds fed with diets supplemented with high levels of Amasil NA. It has been previously reported that high levels of organic acids could have an adverse effect on broiler performance (Esmaeilipour et al., 2012; Günal, 2013). Increased levels of organic acids can disturb the acid-base balance and metabolic acidosis, which can negatively alter performance (Mroz et al., 1997). The administered levels of Amasil NA at a high level might have had an irritating effect on the digestive tracts in the young chicken (up to d 10), which may be the reason for higher FCR observed in this group at earlier stages. High levels of citric acid in broiler diets have shown to reduce nutrient digestibility and disrupted liver function of the birds (Nourmohammadi and Khosravinia, 2015). It has been suggested that high acidic diets can act as stressors causing dysfunction of internal organs (e.g., kidney, liver, heart, and skeletal muscle). These parameters have not been evaluated in the present study, but can be suggested as an underlying reason for the altered performance of these groups of birds.

The fatty acid glyceride product applied in this experiment is a mixture of monoglycerides (C4, C8, and C10) and has total glycerides of butyric and medium-chain fatty acid content of ~ 45%). Monobutyrin and some of the fatty acids in this product can be effective on the reduction of *E. coli* and *C. perfringens* (Namkung et al., 2011; Skřivanová et al., 2014). In the present study, the

Table 6. Response of cecal bacteria (log10 cfu) population in NE challanged broilers to diets containing either antibiotics, different levels of organic acid mixture, and different levels of 1-monoglycerides at d 16.

Treatment[1]	Additive and dose	*Bacillus*	*Enterobacteriaceae*	*Lactobacillus*	*Rumincoccus*	*Bacteriodes*	*Bifidobacterium*	*Salmonella*	*Clostridium perfringens*
T1	-	8.54^{bc}	10.0	9.71^{ab}	9.49^{bcd}	7.54	8.70	7.40	12.9^{a}
T2	Antibiotic	9.21^{ab}	9.85	9.51^{bc}	9.72^{abc}	7.80	9.02	6.77	11.3^{b}
T3	Amasil® NA- Low	8.57^{bc}	9.99	9.68^{ab}	9.44^{cd}	6.81	8.75	7.43	12.3^{ab}
T4	Amasil® NA- High	8.24^{c}	9.78	9.71^{ab}	9.20^{d}	7.40	8.70	6.26	12.5^{ab}
T5	BalanGut™ LS P- Low	9.03^{b}	9.70	9.83^{ab}	9.80^{ab}	8.19	8.87	7.66	12.2^{ab}
T6	BalanGut™ LS P- High	9.14^{ab}	9.55	9.33^{a}	9.68^{abc}	8.50	8.78	7.14	12.7^{a}
SEM		0.123	0.127	0.050	0.050	0.263	0.061	0.210	0.301
P-value		0.005	0.244	0.012	0.001	0.226	0.598	0.487	0.001

[ab,c] means in a column not sharing a common letter are significantly different ($P < 0.05$).

[1]T1: No additive, T2: Salinomycin (0.050%) and zinc bacitracin (0.033%); T3: buffered formic acid - low dose (Starter: 0.3 %; Grower and finisher: 0.2%); T4: buffered formic acid- high dose (Starter: 0.5%; Grower and finisher: 0.5%); T5: monoglyceride blend − low dose (Starter: 0.3%; Grower: 0.15%; Finisher 0.075%).; T6: monoglyceride blend − high dose (Starter: 0.3%; Grower and finisher: 0.2 %).

addition of both levels of fatty acid glyceride product improved the overall feed efficiency compared to the nonsupplemented group. Similar to our results, monobutyrin has previously shown to improve FCR in broilers (Antongiovanni et al., 2011). Monobutyrin, a large component of the monoglyceride product used in this study, is a 1-monoglyceride resulting from the formal esterification of butyric acid with one of the primary hydroxy groups of glycerol. Free butyric acid can be immediately absorbed by the upper digestive tract (Pituch et al., 2013), and the majority of this substance cannot reach the intestine, where it exerts its main functions. In this regard, butyric acid glycerides do not have this limitation and can only be released under the action of lipase (Namkung et al., 2011), which enables this substance to reach the intestine and exert its positive effects there.

Compared to fatty acids, monoglycerides bear the distinction of not having ionizable functional groups across relevant pH conditions, and hence, are nonionic molecules with neutral electrical charge properties and some degree of polarity (Yoon et al., 2018). As mentioned previously, approximately half of the fatty acid glyceride composition is monobutyrin, which has shown to play a significant role in stimulating tissue development and could contribute to early gut development in chicks (Dobson et al., 1990). Butyrate is known to be a major energy source for enterocytes and is involved in maintaining gut mucosal health, and plays a key role in enhancing epithelial cell proliferation and differentiation and in improving intestinal absorption (Canani et al., 2011). Further, butyrate has shown to have anti-inflammatory properties and the potential to stimulate the immune system (Chen et al., 2018). Early access of chickens to additives that improve gut development can enhance immune responses in *C. perfringens* infections (Ao et al., 2012).

The microbiota resident in the gastrointestinal tract plays a key role in nutrition and immune responses in animals (Prenderville et al., 2015). Day 16 evaluation of bacteria populations in the cecum show that birds fed with diets supplemented with low levels of fatty acid glyceride mixture along with the antibiotic-supplemented group showed a significant increase in the number of *Ruminococcus* and *Bacillus* numbers compared to the birds with nonsupplemented diets. *Ruminococcus* is one of the dominant bacteria prevalent in the ceca (Park et al., 2017), and these species are typical butyrate-producing bacteria (Louis et al., 2004). *Bacillus* species are known to improve broiler growth and feed utilization efficiency (Harrington et al., 2016), and enhance host immune responses and protect birds against pathogens (Jayaraman et al., 2013). Yang et al. (2018) observed an increase in the number of cecal Ruminococcaceae family when supplementing butyrate glycerides to broiler diets. The higher number of *Bacillus* and *Ruminococcus* bacteria observed in the low levels of fatty acid

Table 7. Response of caecal SCFAs (%) of NE challenged broilers to diets containing either antibiotics, different levels of Amasil® NA, and different levels of BalanGut™ LS P at d 16.

		Short-chain fatty acid (%)									
Treatment[1]	Additive and dose	Formate	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Lactate	Succinate	Total (μmol/g)
T1	-	0.15	46.2	5.19^{b}	1.32	18.9	0.95	1.25^{ab}	18.2	4.62	116
T2	Antibiotic	0.23	54.7	3.23^{b}	0.81	22.6	0.39	0.72^{b}	14.9	5.58	196
T3	Amasil® NA- Low	0.19	57.4	4.83^{b}	1.69	19.6	1.26	1.39^{ab}	10.1	4.81	121
T4	Amasil® NA- High	0.10	56.1	8.35^{a}	1.84	18.9	1.57	1.86^{a}	7.3	4.87	102
T5	BalanGut™ LS P- Low	0.075	57.8	4.05^{b}	1.19	20.3	0.66	1.29^{ab}	11.2	4.73	127
T6	BalanGut™ LS P- High	0.36	57.0	3.61^{b}	0.76	17.7	0.49	0.76^{b}	14.3	4.98	157
SEM		0.088	5.288	0.765	0.343	2.057	0.297	0.220	6.405	1.410	23.59
P-value		0.434	0.669	0.001	0.227	0.739	0.169	0.013	0.915	0.998	0.323

[a,b] means in a column not sharing a common letter are significantly different ($P < 0.05$).

[1]T1: No additive, T2: Salinomycin (0.050%) and zinc bacitracin (0.033%); T3: buffered formic acid - low dose (Starter: 0.3 %; Grower and finisher: 0.2%); T4: buffered formic acid- high dose (Starter: 0.5%; Grower and finisher: 0.5%); T5: monoglyceride blend − low dose (Starter: 0.3%; Grower: 0.15%; Finisher 0.075%).; T6: monoglyceride blend − high dose (Starter: 0.3%; Grower and finisher: 0.2 %).

Table 8. Response of ileal SCFAs (%) of challenges broilers with NE to diets containing either antibiotics, and two different levels of Amasil® N, and BalanGut™ LS P at d 16.

		Short-chain fatty acid (%)							
Treatment[1]	Additive	Formate	Acetate	Isobutyrate	Butyrate	Isovalerate	Lactate	Succinate	Total (μmol/g)
T1	-	0.63	5.49	0.52	1.14	0.68	91.0	0.58	23.4
T2	Antibiotic	0.49	4.08	0.72	0.10	0.89	94.8	0.09	15.1
T3	Amasil® NA- Low	1.79	7.58	0.17	2.34	0.31	87.0	0.78	27.2
T4	Amasil® NA- High	0.82	3.34	0.40	0.57	0.51	94.0	0.32	24.4
T5	BalanGut™ LS P- Low	0.77	6.45	0.41	2.06	0.47	89.6	0.23	21.4
T6	BalanGut™ LS P- High	0.31	2.74	0.21	0.63	0.35	95.6	0.20	29.5
SEM		0.402	1.558	0.166	0.643	0.160	2.491	0.164	4.069
P-value		0.330	0.301	0.407	0.195	0.265	0.199	0.099	0.216

[1]T1: No additive, T2: Salinomycin (0.050%) and zinc bacitracin (0.033%); T3: buffered formic acid - low dose (Starter: 0.3 %; Grower and finisher: 0.2%); T4: buffered formic acid- high dose (Starter: 0.5%; Grower and finisher: 0.5%); T5: monoglyceride blend − low dose (Starter: 0.3%; Grower: 0.15%; Finisher 0.075%).; T6: monoglyceride blend − high dose (Starter: 0.3%; Grower and finisher: 0.2 %).

glyceride mixture, similar to the effect of antibiotic supplementation could mean an improved gut environment and better feed utilization. The highest concentration of propionate was observed in the birds fed with the high levels of the organic acid mixture. Propionate is a fatty acid that shows an inhibitory effect on microbial biomass and high concentrations of this fatty acid usually cause severe problems in the anaerobic digestion process (Li et al., 2017). The higher level of this fatty acid in the cecal contents of the birds fed with high doses of the Amasil NA group might be a reason for the lower number of *Bacillus* and *Ruminococcus* observed in these birds.

Overall, in the current study, both doses of BalanGut LS P enhanced the overall feed efficiency of birds compared to the nonsupplemented group. High doses (0.2%) of monoglyceride supplementation appear beneficial during the grower stage (d 10−24), which is mainly the NE challenge period. This could mean that BalanGut LS P could potentially amoliate the effect of The NE challenge. Low doses of BalanGut LS P (0.075%) led to improved birds' performance at the finisher phase. On the other hand, Amasil NA supplementation at high dose (0.5%) or low dose (0.3−0.2 %) did not show the beneficial effect at starter and grower phase. In contrast, higher dose deteriorated FCR at the starter phase. However, lower-level supplementation of this product improved feed efficiency compared to the non-supplemented group during the finisher phase. These results show the benefits of buffered formic acid and monoglyceride blends in broilers under NE challenge if applied during appropriate feed phases. We speculate that supplementation of these 2 products in combination at different feed phases may achieve optimal performance improvement in broilers under NE challenge. Further research is warranted to determine the ideal dose of each product at each growth stage, to achieve optimal performance enhancement under NE challenge at different stages of broiler growth.

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DISCLOSURES

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence our work, 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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