

Performance and gut health status of broilers fed diets supplemented with two graded levels of a monoglyceride blend

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ABSTRACT The present study was undertaken to evaluate the efficacy of 2 graded levels (0.03 and 0.05% of diet) of a monoglyceride blend containing butyric, caprylic, and capric acids in broilers' diet for optimizing gut structure and animal growth performance. For this purpose, a total of 210, one-day-old male Ross 308 broiler chicks were randomly allocated to 3 experimental treatments using 7 replicates each and 10 birds/replicate. The treatment groups involved supplementation of blend of short and medium chain fatty acids at the level of 0, 0.03, and 0.05% of the diet for 42 d. The incorporation of mixes of monoglycerides into broilers' diet linearly improved BWG between d 0 and 21 ($P = 0.034$). At the end of trial, however, no significant changes were observed in performance indexes (BWG, FI, FCR). Jejunal morphometric parameters (villus height, crypt depth, and their ratio) remained unaltered with the monoglyceride supplementation on d 21. The results further showed that monoglycerides supplementation increased the goblet cell counts along the jejunal

villi ($P = 0.034$) and crypt regions ($P = 0.022$), as well as it effectively modulated the mRNA abundances of tight junction protein (ZO-1, $P = 0.033$) and nutrient transporters (SGLT, PePT1; $P = 0.005, 0.023$, respectively) in the jejunum. Moreover, the downregulation in mRNA abundance of TNF α ($P = 0.030$) was observed with the monoglyceride supplementation. The SCFAs analysis of cecal contents showed no notable differences with monoglyceride blend supplementation when compared to the unsupplemented group. Collectively, high goblet cell numbers in the jejunum along with downregulation of the mRNA abundances of pro-inflammatory cytokines, upregulation of tight junction proteins, and nutrient transporters showed favorable responses of low doses of monoglycerides blend in broiler feeding. Further studies should be conducted in different rearing conditions to examine the effectiveness of such low levels of a monoglyceride blend in the modulation of gut structure, its functionality and animal performance.

Key words: short- & medium chain fatty acids, intestinal histomorphology, tight junction proteins, cytokines, nutrient transporters

2022 Poultry Science 102:102359
<https://doi.org/10.1016/j.psj.2022.102359>

INTRODUCTION

In livestock production, poultry enterprises have a mega share in producing quality protein for human consumption. Such contribution would not be possible if it was not for the genetic improvements in the poultry industry; however, these modifications also brought many challenges along with it. For many years, antibiotic growth promoters (AGPs) assisted the farmers to

prevent various infections as well as to cope with performance efficiency (Gomez-Osorio et al., 2021). Nevertheless, with the implementation of antibiotic free production, the livestock industry started investing in alternative feed additive strategies mainly targeting the gut health and making it resilient to harmful pathogens.

The diversified gut microbial communities and their role in warding off different diseases and health preservation has been revealed by a great amount of scientific literature, suggesting a robust link between stable functioning of gut ecosystem and animal performance (Perumalla et al., 2012; Liao et al., 2020). Gut microbiota and their metabolites (short-chain fatty acids, SCFAs) interact in a complex way with the intestinal morphological structure improving its functionality (Calik and Ergün, 2015). SCFAs can regulate the bacterial

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Received September 12, 2022.

Accepted November 15, 2022.

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virulence via reducing the severity and span of different enteric infections; moreover, they can promote the intestinal barrier integrity by enhancing the tight junctions' function (Pérez-Reytor et al., 2021).

Previous studies have demonstrated that dietary supplementation of different blends containing short and medium chain fatty acids (MCFAs) protected the gut function in broilers against necrotic enteritis challenge, which also subsequently translated into better performance results (Gharib-Naseri et al., 2021; Kumar et al., 2021a,b). Scientific data also unveiled the role of dietary SCFAs and MCFAs in the regulation of intestinal health in animals. Studies showed improvements in intestinal architecture of broilers fed diets supplemented with different fatty acid blends (Bedford et al., 2018; Amer et al., 2020). Apart from being the immediate energy source for the intestinal epithelium, these fatty acids have been shown to greatly impact the intestinal inflammatory responses and immune function (Liu, 2015). There is great evidence implicating therapeutic role of dietary short and/or medium chain fatty acids by regulating the chemical mediators of inflammatory responses critical in maintaining immune quiescence, thus, leading to healthier and stable gut environment (Tan et al., 2014; Çenesiz and Çiftci, 2020). Literature also showed SCFAs induced strengthening of the intestinal tight junction proteins, thus, further enhancement of the intestinal barrier function and prevention of a digestive condition known as 'leaky gut syndrome' (Tan et al., 2014; Onrust et al., 2015).

Due to all these beneficial effects, the usage of short and medium chain fatty acids in poultry diets is gaining popularity and various fatty acid products are available in the livestock market solely or in different combinations. Along with chemical composition, the pKa value, form and molecular weight are one of the driving factors determining the efficacy of these products in animal nutrition (Patten and Waldroup, 1988). Butyrate is one of the most widely studied supplemental SCFAs in animal diets, and reports revealed that efficacy of dietary fatty acids could be enhanced when using their monoglyceride forms (Rimoldi et al., 2018; Çenesiz and Çiftci, 2020), thus avoiding their digestion in the proximal part of the digestive system. Potential nutritional and physiological effects of MCFAs, similar to SCFAs, have also been widely documented (Zentek et al., 2011; Çenesiz and Çiftci, 2020) making them a possible candidate as alternative to AGPs.

The present study aimed to explore the efficacy of 2 graded levels (0.03 and 0.05% of diet) of a commercial blend containing monoglycerides of butyric, caprylic, and capric acids in broilers' diet on gut structure and performance. We hypothesized that, low-dose regimens of the blend of short- and medium chain fatty acid would exert positive effects on intestinal histomorphology, markers of cell proliferation and apoptosis, mRNA abundance of intestinal tight junction proteins, intestinal nutrient transporters, and cytokines ultimately resulting in better performance.

MATERIALS AND METHODS

Birds and Management

A total of 210, one-day-old male Ross 308, broiler chicks were obtained from a commercial hatchery (Beypiliç, Bolu, Turkey). Upon arrival, all chicks were orally vaccinated against coccidiosis. In the experiment, birds were randomly allocated to 3 experimental groups using seven replicates each and ten birds/replicate. Diets (except control) were supplemented with two graded levels of a monoglyceride blend (BalanGut LS P, supplied by BASF SE, Ludwigshafen, Germany) as 0.03 and 0.05% of the diet. Birds were housed in a controlled environment for 42 d. Ambient temperature was gradually decreased from 33°C at first day to 22°C after 3 wk of age and remained constant thereafter. The relative humidity of the house during the experiment was 50 ± 5%. The house was artificially ventilated and lighting schedule was 24L:0D for first 3 d, reduced further to 23L:1D from d 4 to 7 and then kept constant at 20L:4D thereafter. All experimental procedures were approved by the Animal Ethics Committee of the Ankara University.

The starter, grower, and finisher diets were based on the corn-soybean meal and were offered to birds from 0 to 14, 14 to 28, and 28 to 42 d of age, respectively (Table 1). All diets were formulated to meet or exceed Aviagen, 2019 nutrient recommendations. Each pen was equipped with a plastic feeder and an automatic nipple drinker with fresh wood shavings as litter. Water and diets (in mash form) were provided ad libitum throughout the experimental period. All chicks were weighed individually, and feed

Table 1. Ingredients and composition of basal diet (as-fed basis).

Ingredient, g/kg	Starter	Grower	Finisher
Corn	54.73	57.23	61.34
Soybean meal, CP 48%	38.00	34.72	30.00
Vegetable oil	2.75	4.00	5.00
Monocalcium phosphate	1.80	1.65	1.42
Limestone	1.35	1.20	1.10
Sodium bicarbonate	0.20	0.20	0.20
L-Lysine sulphate (55%)	0.28	0.20	0.18
DL-Methionine (98%)	0.33	0.28	0.26
L-Threonine	0.11	0.07	0.05
Sodium chloride	0.25	0.25	0.25
Vitamin premix ¹	0.10	0.10	0.10
Mineral premix ²	0.10	0.10	0.10
Total	100	100	100
Chemical composition (Calculated)			
Dry matter, %	88.05	88.12	88.14
Crude protein, %	23.00	21.52	19.54
AME _n , kcal/kg	3000	3109	3217
Lysine, %	1.44	1.30	1.15
Dig. Lysine, %	1.28	1.15	1.02
Methionine + cysteine, %	1.07	0.98	0.90
Dig. Methionine + cysteine, %	0.95	0.87	0.80
Threonine, %	1.00	0.90	0.80
Dig. Threonine, %	0.86	0.77	0.68
Calcium, %	0.96	0.87	0.78
Available phosphorus, %	0.48	0.44	0.39

¹Provided per kilogram of complete diet: vitamin A, 15,000 IU; vitamin D3, 5,000 IU; vitamin E, 100 mg; vitamin K3, 3 mg; thiamin, 5 mg; riboflavin, 8 mg; pyridoxine, 5 mg; pantothenic acid, 16 mg; niacin, 60 mg; folic acid, 2 mg; biotin, 200 µg; vitamin B12, 20 µg.

²Provided per kilogram of complete diet: Cu, 16 mg; I, 1.5 mg; Co, 500 µg; Se, 350 µg; Fe, 60 mg; Zn, 100 mg; Mn, 120 mg; Mo, 1 mg.

intake (**FI**) was recorded at weekly intervals. Any mortality was removed and recorded (including bird weight) daily. Body weight gain (**BWG**), FI, and feed conversion ratio (**FCR**) were subsequently calculated to evaluate growth performance.

Sampling Procedures

At 21 d of age, two birds from each replicate were selected based on average pen body weight. Birds were euthanized by cervical dislocation and the intestinal tract was immediately removed. Tissue samples were obtained from the jejunum (middle section, 1 cm) for histomorphological and immunohistochemical analyses. A second sample was collected from the jejunum (middle section, 1 cm) and snap-frozen in liquid nitrogen to assess the mRNA abundances of some cytokines, tight junction proteins, and nutrient transporters related genes. Furthermore, ceca were ligated and aseptically removed from the gastrointestinal tract for short chain fatty acid analysis.

Morphological Measurements of the Jejunum and Goblet Cell Counting

Histomorphological examination of samples were performed on 5- μ m thick sections of 10% buffered formalin fixed, paraffin embedded tissue. Cross sections were stained with Masson's trichrome stain, as modified by Crossmon, in order to determine the jejunal morphometry (Culling et al., 1985). Villus height (**VH**) was measured from the top of the villus to the crypt mouth, and crypt depth (**CD**) was defined as the depth of the invagination between adjacent crypt mouths. A total of 10 well-oriented villi and crypts were randomly selected for histological measurements. Acidic and neutral mucin goblet cells were identified by staining with Alcian blue-periodic acid-Schiff reagent. Quantification of the number of goblet cells were performed on a site where there were at least three contiguous and intact crypts and villus in 5 random fields, a total of 10 crypts and villus being studied in each segment (Figure 1). Histological sections were examined under a light microscope (Leica DM 2500, Leica Microsystems GmbH, Wetzlar, Germany) and images were captured with a digital microscope camera (Leica DFC450, Leica Microsystems GmbH). The images were evaluated using the ImageJ software (US National Institutes of Health, Bethesda, MD).

Proliferating Cell Nuclear Antigen Staining

Proliferating jejunal cells were identified via immunohistochemistry by using antibody directed against proliferating cell nuclear antigen. Tissue sections were placed on poly-L-lysine microscope slides (Thermo Scientific, Braunschweig, Germany), which were incubated at 37°C overnight and de-paraffinized with xylene and rehydrated through graded alcohols. After deparaffinization

and rehydration step endogenous peroxidase activity was blocked by quenching with 3% hydrogenperoxide solution for 15 min. To remove the methylene bridges between proteins a heat induced epitope retrieval method performed with a sodium citrate (10 mM, pH 6). After cooling for 20 min at room temperature, tissue sections were washed with PBS and incubated with 10% normal goat serum for 30 min for protein blocking to prevent the nonspecific binding of antibody, followed by incubation with the primary antibody to proliferating cell nuclear antigen (**PCNA**) (MAB424, mouse anti PCNA monoclonal antibody, PC10 clone; EMD Millipore, Darmstadt, Germany) at dilutions of 1:100 overnight at 4°C. After incubation with the primary antibody, the tissue sections were washed with PBS and incubated with a biotinylated secondary antibody (Goat anti-mouse IgG, Invitrogen, Waltham, MA) for 30 min at room temperature. Background controls were included by replacing the primary antibodies with PBS. After a PBS wash, tissue sections were incubated using a streptavidin horseradish peroxidase kit (Histostain-Plus IHC Kit, HRP, broad spectrum, Invitrogen) for 30 min at room temperature. A final PBS wash was followed by incubation for color development with 3,3-diaminobenzidine tetrahydrochloride (DAB, Invitrogen) for 3 min at room temperature. Tissue sections were counterstained with Gill's hematoxylin, dehydrated in graded alcohols, applied to a coverslip using Entellan (Merck, Darmstadt, Germany), and examined with a Leica DM2500 light microscope. All images were captured with a digital camera (Leica DFC450) and processed with Image J (Figure 1). PCNA positive nuclei of total crypt epithelial cells on 10 different randomly selected intact crypts, regardless of the staining intensity, were counted as described by (Bologna-Molina et al., 2011).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay

Apoptotic jejunal cells were identified by terminal transferase deoxyuridine nick-end labeling (**TUNEL**) by using the ApoBrdU-IHC DNA Fragmentation Assay Kit (Biovision Inc., K403-50, Waltham, MA). The staining procedure was performed with slight modifications to the manufacturer's instructions. Tissue sections were deparaffinized to ddH₂O and then processed for antigen retrieval by heating in a microwave at 800 W in sodium citrate (10 mM, pH 6) for 10 min. Sections were washed and then incubated with the TUNEL reaction mixture for 45 min at 37°C in a dark, humidified chamber. The sections were then counterstained with Gill's (III) haematoxylin and cover slipped. A section was incubated with DNase to serve as the positive control (DNase RQ1). A section incubated without the terminal transferase served as the negative control. Cells were considered to be apoptotic if they stained positive (Figure 1) with chromogen and exhibited nuclear morphology consistent with that of apoptotic cells (chromatin clumping and condensation).

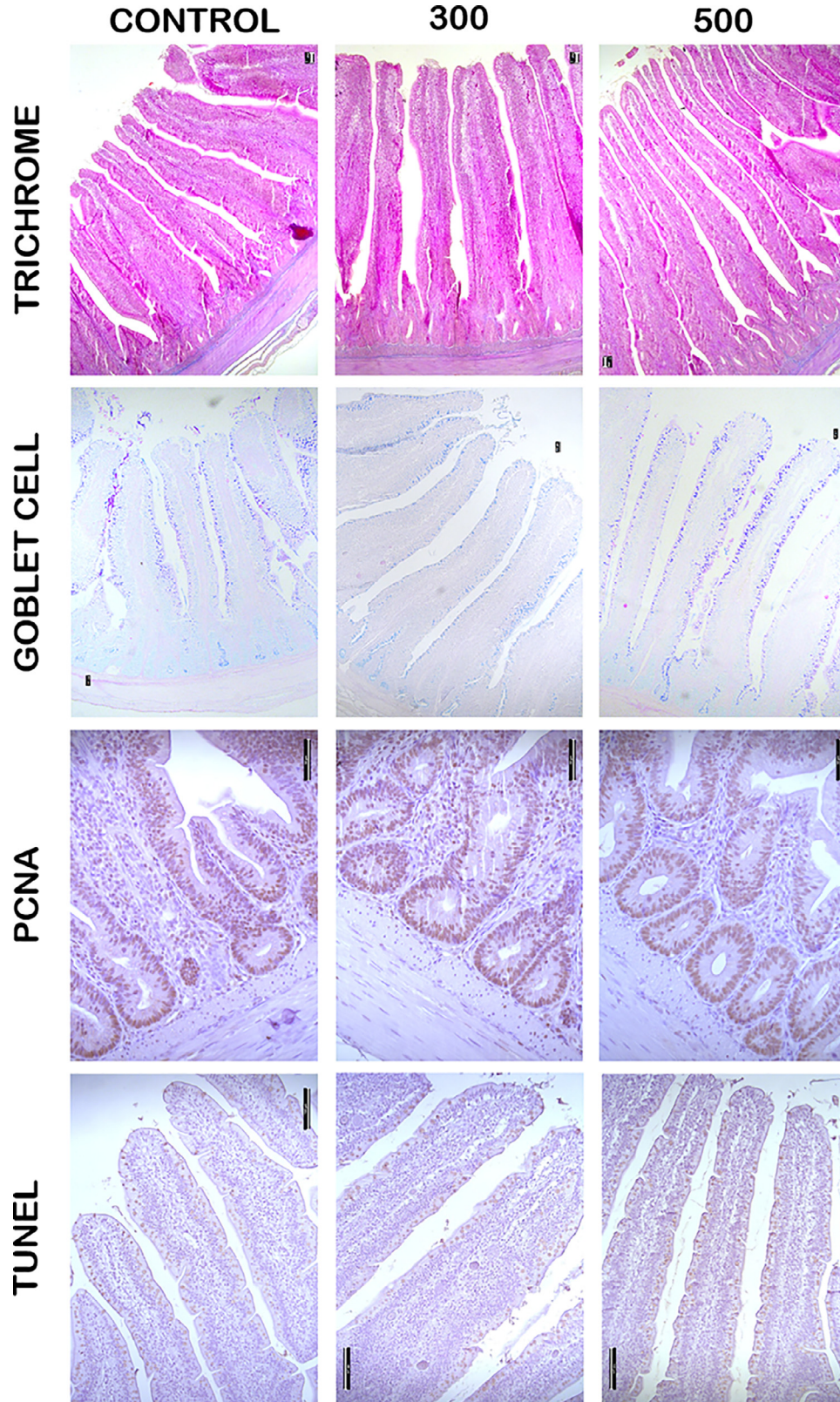


Figure 1. Representative light microscopy images of the jejunum on d 21. Masson's trichrome staining of jejunum sections. AB/PAS staining of jejunal goblet cells. Immunohistochemistry staining for determining the expression of PCNA and detection of late apoptosis by TUNEL assay in different groups. Scale Bars: 50 μ m.

Short Chain Fatty Acid Concentration

Frozen cecal contents were thawed at 4°C and diluted with 4-fold double-distilled water in sterile screw cap tubes. Cecal contents were homogenized and centrifuged

at 4,000 $\times g$ for 15 min at 4°C. One mL of supernatant was then transferred to Eppendorf tube and mixed with 0.2 mL of ice-cold 25% metaphosphoric acid solution. Subsequently, tubes were placed in an ice bath for 30 min and samples were centrifuged at 11,000 $\times g$ for

10 min at 4°C. Supernatants were analyzed by using gas chromatography (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with 30 m × 0.25 mm × 0.25 μm column (Stabilwax-DA, Restek, Bellefonte, PA) and a flame ionization (FID) detector to determine SCFA concentrations of cecal digesta. Injector port and FID temperatures were fixed at 230°C and 250°C, respectively. The temperature program was as follows: the initial temperature was held at 120°C for 4 min after injection, then programmed to increase at 4°C/min to 160°C, held there for 4 min. Helium was used as carrier gas. Injection volume was set to 1 μL and analyses were performed in duplicate. Total SCFA represents the sum of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate derivatives (Calik and Ergün, 2015).

Total RNA Extraction and Reverse Transcription

On d 21, two birds/pen were selected, euthanized, and jejunal sections (from the middle, 1 cm) were excised to assess the mRNA abundance of cytokines, tight junction proteins, and nutrient transporter related genes. Approximately 50 mg aliquot of jejunal tissues was weighed into a 2 mL microcentrifuge tube and homogenized in 900 μL TRI Reagent (Zymo Research, Irvine, CA) by a FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, CA). Total RNA was extracted from the homogenate using the Direct-zol RNA Miniprep Plus Kit (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. Total RNA concentration was determined at optical density (OD) of 260 (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA), and RNA purity was verified by evaluating the 260/280 OD ratios. RNA integrity was evaluated by gel

electrophoresis on 1.5% agarose gel in 0.5 × TAE buffer. After extraction, two micrograms of total RNA were used to synthesize first-strand cDNA using the One-Script Plus cDNA Synthesis Kit (Applied Biological Materials Inc., Richmond, BC, Canada) according to the manufacturer's recommendation and the cDNA was stored at -20°C.

Quantitative Real-Time PCR

The mRNA abundance of cytokines (IL-1β, IL-10, IL-17A, and TNFα), tight junction proteins (ZO-1, ZO-2, Occludin), and nutrient transporters (SGLT1, PePT1, and NaPi-IIb) related genes were determined by CFX Connect Real-Time PCR System (Bio-Rad Laboratories Inc., Hercules, CA) using BlasTaq 2X qPCR MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada). Details of primer sets are provided in Table 2. The cDNA was diluted 1:5 in nuclease-free water, and 4 μL of the diluted cDNA was added to each well of a 96-well plate. Next, 16 μL of real-time PCR master mix containing 10 μL of BlasTaq 2X qPCR MasterMix, 1 μL each of 10 μM forward and reverse primers and 4 μL of sterile nuclease-free water per reaction were added to each well for a final volume of 20 μL. During the RT-PCR reaction, samples were subjected to initial enzyme activation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 1 min. Product specificity was confirmed by analysis of the melting curves. The mRNA levels were analyzed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. Average mRNA abundance relative to GAPDH for each sample was calculated using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). The calibrator for each gene was the average ΔCt value from the control group.

Table 2. Sequences of primer pairs used for amplification of target and reference genes. For each gene, the primer sequence for forward (F) and reverse (R) (5'-3') primers, the amplicon size (bp) and the NCBI Accession number (Acc) used for the primer design are listed.

Gene ¹	Primer sequence	Size	Acc (Reference)
IL-1β	CCCGCCTTCCGCTACA CACGAAGCACTTCTGGTTGATG	66	NM_204524.1
IL-10	CGCTGTCACCGCTTCTTCA CGTCTCCTTGATCTGCTTGATG	63	NM_001004414.2
IL-17A	AGCTGGACCACAGCGTCAAC GGCGGAGGACGAGGATCT	57	NM_204460.1
TNFα	CCCATCCCTGGTCCGTAAC ATACGAAGTAAAGGCCGTC	77	MF000729.1
ZO-1	GGAGTACGAGCAGTCAACATAC GAGGCGCACGATCTTCATAA	101	XM_413773
ZO-2	GCGTCCCATCCTGAGAAATAC CTTGTTCACTCCCTTCCTCTTC	89	NM_204918
Occludin	CCGTAACCCCGAGTTGGAT ATTGAGGCGGTCTGTTGATG	214	NM_205128.1
PepT1	CCCCTGAGGAGGATCACTGTT CAAAAGAGCAGCAGCAACGA	66	NM_204365
SGLT1	GCCATGGCCAGGGCTTA CAATAACCTGATCTGTGC	71	NM_001293240
NaPi-IIb	ACCAGTA GGCTGGCATGACTTTCGTTG GGGTGAGGGGATAAGAACGC	107	NM_204474
GAPDH			

CTTAGGATACACAGAGGACCAGGTT64NM_204305GGTGGAGGAATGGCTGTCA

¹Abbreviations: GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IL, Interleukin; NaPi-IIb, type IIb sodium-phosphate cotransporter; PepT1, peptide transporter-1 (SLC15A1); SGLT1, sodium glucose transporter-1 (SLC5A1); TNFα, Tumor necrosis factor alpha, ZO, Zonula occludens.

Table 3. Effects of monoglycerides blend supplementation on broiler performance.¹

Item ³	Dietary treatments ²			SEM	P	P-value ⁴	
	Control	300	500			L	Q
0 to 14 d							
BWG (g)	395.1	393.5	399.1	3.00	0.753	0.608	0.588
FI (g)	511.9	514.1	524.3	4.09	0.438	0.234	0.650
FCR	1.294	1.306	1.314	0.01	0.674	0.383	0.942
0 to 21 d							
BWG (g)	838.2	861.0	881.8	8.37	0.099	0.034	0.955
FI (g)	1,158.5	1,168.5	1191.7	8.35	0.261	0.114	0.706
FCR	1.381	1.359	1.353	0.01	0.471	0.252	0.687
14 to 28 d							
BWG (g)	1,116.5	1,163.6	1181.4	17.03	0.287	0.131	0.685
FI (g)	1,610.3	1,644.4	1650.9	16.10	0.566	0.326	0.695
FCR	1.449	1.414	1.399	0.01	0.206	0.086	0.701
28 to 42 d							
BWG (g)	1,701.7	1,722.2	1,716.4	11.61	0.778	0.627	0.614
FI (g)	2,821.0	2,843.9	2,872.8	20.59	0.613	0.330	0.948
FCR	1.657	1.650	1.673	0.01	0.293	0.290	0.245
0 to 42 d							
BWG (g)	3,213.3	3,279.3	3296.9	25.38	0.386	0.196	0.659
FI (g)	4,943.1	5,002.4	5048.0	35.28	0.500	0.247	0.929
FCR	1.539	1.526	1.531	0.01	0.408	0.456	0.268
Mortality, %	1.43	2.86	4.29	-	0.509	-	-

¹Data represent mean values from 7 replicates per treatment.

²Control: birds fed a basal diet; 300: birds fed a basal diet supplemented with 300 mg/kg monoglycerides blend; 500: birds fed a basal diet supplemented with 500 mg/kg monoglycerides blend.

³Abbreviations: BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

⁴L: Linear, Q: Quadratic.

Statistical Analyses

Data were analyzed using the ANOVA procedure of the SPSS software, version 14.01 (SPSS Inc., Chicago, IL). One-way ANOVA was used to determine the effects of monoglycerides supplementation and significant means were separated using Tukey test. Polynomial contrasts were also applied to determine the linear and quadratic effects. Mortality rates were compared using a chi-square test. Statistical differences were considered significant at $P \leq 0.05$.

RESULTS

Broiler Performance and Mortality

Overall, all the birds showed optimum health signs and differences in the mortality were insignificant ($P = 0.509$) among treatments (Table 3). During the 42

d of production period, inclusion of blend of monoglycerides of butyric, caprylic, and capric acids in broilers' diets had no effect on performance parameters (BWG, FI, FCR) when compared to the control group ($P = 0.386, 0.500, 0.408$, respectively). However, data revealed linear dose response ($P = 0.034$) for BWG as well as a tendency ($P = 0.086$) of linear improvement in FCR during the 0 to 21 d and 14 to 28 d of production, respectively.

Jejunum Histomorphology, Goblet Cells Counts, and Immunohistochemistry

Supplementation of monoglyceride blend to broilers' diets at different levels (0.03 and 0.05%) did not affect the overall histomorphological features of jejunum, including villus height ($P = 0.871$), crypt depth ($P = 0.075$), and VH:CD ratio ($P = 0.554$; Table 4).

Table 4. Effects of monoglycerides blend supplementation on intestinal morphology of the jejunum on d 21.¹

Item ³	Dietary treatments ²			SEM	P	P-value ⁴	
	Control	300	500			L	Q
Villus height, μm	972.3	961.9	987.5	126.67	0.871	0.757	0.674
Crypt depth, μm	176.5	172.3	186.2	16.70	0.075	0.118	0.092
VH:CD ratio	5.56	5.59	5.31	0.72	0.554	0.379	0.527
Goblet cell, villus	117.1 ^{ab}	108.0 ^b	135.7 ^a	28.58	0.034	0.081	0.044
Goblet cell, crypt	39.40	43.13	46.94	8.59	0.071	0.022	0.988
PCNA positive cells	34.83	35.11	35.62	4.67	0.912	0.673	0.945
TUNEL positive cells	64.17	63.51	70.06	13.89	0.418	0.279	0.437

¹Data represent mean values from 14 birds per treatment.

²Control: birds fed a basal diet; 300: birds fed a basal diet supplemented with 300 mg/kg monoglycerides blend; 500: birds fed a basal diet supplemented with 500 mg/kg monoglycerides blend.

³Abbreviations: PCNA, proliferating cell nuclear antigen, TUNEL, transferase deoxyuridine nick-end labeling.

⁴L: Linear, Q: Quadratic.

Increased goblet cell count ($P = 0.034$) was observed along the jejunal villi when monoglyceride blend at the concentration of 0.05% was added to broiler diets, whereas a linear ($P = 0.022$) dose response was noted in goblet cell numbers in crypt region with monoglyceride blend supplementation (Table 4). The obtained results on PCNA immunohistochemistry in jejunal crypts showed no significant variations ($P = 0.912$) in PCNA-positive staining with the supplementation of monoglyceride based additive in broilers' diets (Table 4). In addition, no difference ($P = 0.418$) was found between treatments for the numbers of TUNEL-positive cells evaluated in the villi regions of the jejunum (Table 4).

Cecal Concentration of SCFAs

Supplementation of monoglyceride blend to broilers' diets did not influence ($P > 0.05$) the cecal concentrations of SCFAs either individually or in total albeit there was a linear increase ($P = 0.037$) observed in the valeric acid levels (Table 5).

mRNA Abundance of Cytokines, Tight Junction Proteins, and Nutrient Transporters in the Jejunum

The mRNA abundance of cytokines (IL-1 β , IL-10, IL-17A, and TNF α), tight junction proteins (ZO-1, ZO-2, Occludin) and nutrient transporters (SGLT1, PePT1, NaPi-IIb) related genes were examined and results are presented in Figures 2 and 3. Supplementation of monoglyceride blend into broilers' diets significantly lowered ($P = 0.030$) the mRNA abundance of TNF α in jejunal tissues. Results on TNF α mRNA levels also exhibited a trend of linear decrease ($P = 0.009$) with the increase in monoglycerides dosage. Furthermore, there was a linear tendency ($P = 0.069$) of lower IL-1 β mRNA abundance in the jejunum due to supplemented monoglyceride blend whilst no significant variations were noted for IL-17A ($P = 0.894$) and IL-10 ($P = 0.568$) mRNA abundances.

Jejunal ZO-1 mRNA levels increased linearly ($P = 0.033$) alongside a tendency ($P = 0.086$) for linear increment in ZO-2 mRNA in the jejunum of broilers fed increased levels of monoglyceride blend. There was no significant variation ($P = 0.537$) among groups for the occludin mRNA levels. With regards to nutrient transporters, a significant response to monoglyceride supplementation was observed in mRNA abundance of SGLT1 ($P = 0.005$) and PePT1 ($P = 0.023$) in jejunum; both of which also responded linearly to applied dosages ($P = 0.001, 0.011$, respectively). On the other hand, the mRNA levels of NaPi-IIb showed non-significant changes ($P = 0.155$), although there was a linear tendency ($P = 0.077$) of increment with the additive supplementation.

DISCUSSION

Over the years, the associated risks of antibiotic administration in livestock enterprises including growing antibiotic resistances in animals and humans as well as environmental pollution, triggered the poultry producers to invest in alternative feed additives in order to expand the animal performance up to its genetic potential. On many occasions, literature has showed beneficial effects of supplemental SCFAs and MCFAs or their blends on intestinal health modulation and performance enhancement in broilers. The current study is a continuation of that work and was undertaken with an objective to evaluate the potential usage of 2 dosages of a commercial blend containing monoglycerides of butyric, caprylic, and capric acids in broilers' diets.

Previously, it was corroborated that, opposed to unsupplemented groups, dietary addition of the same fatty acid blend at levels ranging from 0.1 to 0.5% significantly improved the FCR in broilers raised under sub-clinical or clinical necrotic enteritis challenges (Gharib-Naseri et al., 2021; Kumar et al., 2021a). In the present study, under unchallenged conditions, we only observed a linear improvement in BWG between d 0 and 21. However, by the end of 42-d production cycle, the performance indexes (BWG, FI, FCR) from the supplemental

Table 5. Effects of monoglycerides blend supplementation on short chain fatty acid composition ($\mu\text{mol/g}$ of digesta) of cecum on d 21.¹

Item	Dietary treatment ²			SEM	P	P-value ³	
	Control	300	500			L	Q
Acetate	44.07	49.10	48.81	1.60	0.363	0.233	0.437
Propionate	4.50	4.37	5.23	0.21	0.210	0.162	0.276
Butyrate	7.98	8.84	9.05	0.02	0.557	0.369	0.551
Isobutyrate	0.57	0.57	0.62	0.41	0.542	0.299	0.711
Isovalerate	0.52	0.60	0.61	0.02	0.155	0.078	0.425
Valerate	0.73	0.82	0.84	0.02	0.082	0.037	0.409
Total SCFA	58.37	64.29	65.14	1.95	0.310	0.162	0.541
Acetate, %	75.67	76.00	74.63	0.42	0.400	0.325	0.353
Propionate, %	7.68	7.08	8.37	0.38	0.393	0.463	0.250
Butyrate, %	13.51	13.75	13.74	0.04	0.689	0.978	0.391
Isobutyrate, %	0.99	0.91	0.98	0.42	0.968	0.832	0.891
Isovalerate, %	0.89	0.96	0.97	0.04	0.674	0.433	0.683
Valerate, %	1.26	1.30	1.31	0.03	0.738	0.450	0.862

¹Data represent mean values from 14 birds per treatment.

²Control: birds fed a basal diet; 300: birds fed a basal diet supplemented with 300 mg/kg monoglycerides blend; 500: birds fed a basal diet supplemented with 500 mg/kg monoglycerides blend.

³L: Linear, Q: Quadratic.

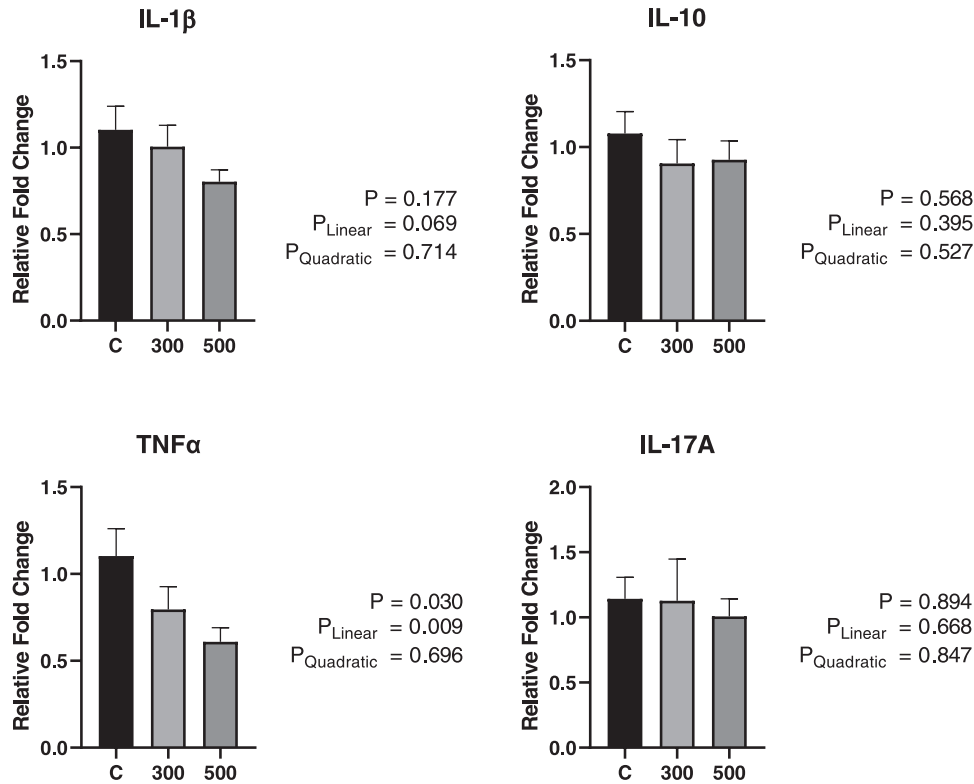


Figure 2. Effects of monoglycerides blend on relative mRNA abundance of IL-1 β , IL-10, TNF- α , and IL-17A in jejunum on d 21. The relative mRNA abundance of IL-1 β , IL-10, TNF- α , and IL-17A was determined by qPCR and analyzed by $2^{-\Delta\Delta Ct}$ method using control group as a calibrator. Data represent mean values from 14 birds per treatment (mean \pm SEM).

groups (0.03 and 0.05% of diet) did not reveal any significant differences when compared to the control group (Table 3). Consistent with our results, when compared with the control group, research showed no significant effect on overall growth performance of unchallenged broilers fed a mixture of fatty acid glycerides at levels ranging 0.05 to 0.5% of the diet (Bedford et al., 2018; Khatibjoo et al., 2018; Amer et al., 2020; Lin et al., 2022). In another study with broilers raised under necrotic enteritis challenge (Kumar et al., 2021b), significant improvement in feed efficiency was reported in response to the dietary supplementation with a blend of SCFAs and MCFAs (0.1–0.25%). The lack of prominent response in overall growth performance in this study, therefore, could mainly be attributed to the absence of disease challenge or low stress condition. In addition, the observed linear-dose responses for BWG in the first half of this trial showed the beneficial role of the product but it seemed to be frittered away in later half. One might advocate phase dependent effect of such additives in varied doses; however, more research is needed to gain further understanding.

The intestine is the primary digestive organ in animals, and it must harbor the environment not conducive to pathogenic proliferation. Intestinal morphological features that is, villus height, crypt depth, and their ratio are considered main representatives of its environment, and developments in intestinal villus-crypt axis are critical in determining the digestive and absorptive efficiency of this organ. In this study with broilers, when compared to the control group, the morphometric

measurements in the jejunal segment remained unaltered with the monoglycerides supplementation regardless of the dosage (Table 4). Similarly, under healthier conditions, some earlier studies did not show statistical changes in the intestinal villi and crypt characteristics of broilers fed diets supplemented with different short- and medium-chain fatty acid blends at levels ranging from 0.03 to 0.3% (Khatibjoo et al., 2018; Liu et al., 2021; Lin et al., 2022). In other reports, however, broilers fed fatty acid blends (0.2–0.5%) showed beneficial effects on intestinal structure (Amer et al., 2020; Dauksiene et al., 2021; Letlole et al., 2021), and such effects were more pronounced under disease challenge (Santos et al., 2019; Abdelli et al., 2020).

Further in the intestinal architecture, the goblet cells located in epithelium are one of the components engaged in the innate defense system as they provide a mechanical barrier in the form of mucin (Pearson and Brownlee, 2005). Any shift in mucin production dynamics could be detrimental to the absorptive and protective functions of the small intestine (Uni et al., 2003). The present study examined the stimulatory effects of dietary monoglyceride mixture on goblet cell counts in jejunum along the villi and crypt regions. Data showed positive dose-dependent changes in goblet cells distribution along the jejunal villi and crypt regions (Table 4). The linear effect was more obvious in crypt goblet counts. These results were in agreement with previous studies reporting alteration in the intestinal goblet cell counts with the supplementation of fatty acid blends to broilers (Amer et al., 2020; Letlole et al., 2021). However, some authors

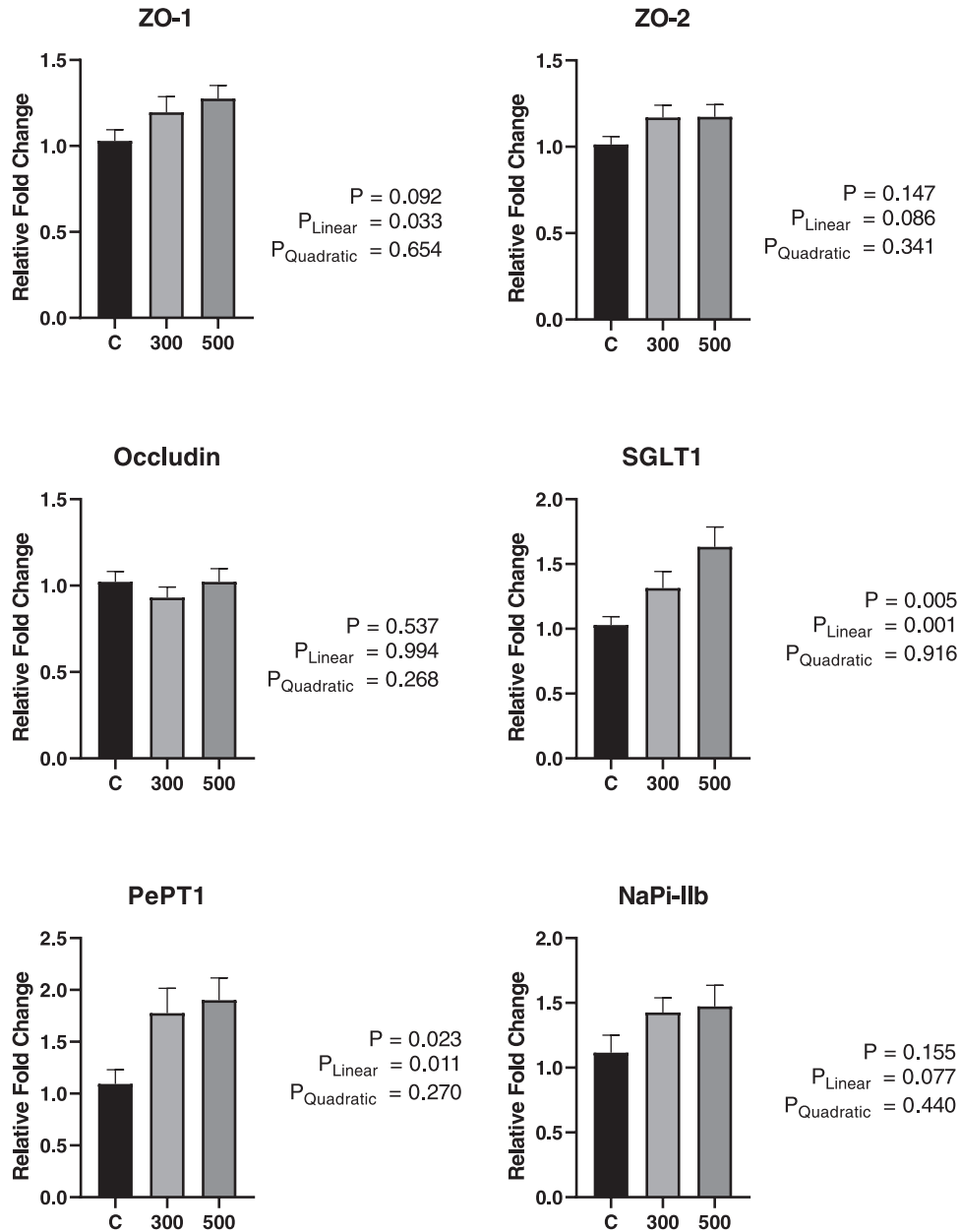


Figure 3. Effects of monoglycerides blend on relative mRNA abundance of ZO-1, ZO-2, Occludin, SGLT1, PePT1, and NaPi-IIb in jejunum on d 21. The relative mRNA abundance of ZO-1, ZO-2, Occludin, SGLT1, PePT1, and NaPi-IIb was determined by qPCR and analyzed by $2^{-\Delta\Delta Ct}$ method using control group as a calibrator. Data represent mean values from 14 birds per treatment (mean \pm SEM).

reported no treatment effect of fatty acid mixtures on goblet cell counts in the intestine of either healthy or stressed animals (Abdelli et al., 2020; Lin et al., 2022). Increased number of goblet cells in the jejunum may potentially imply good mucin production, hence improved protection against pathogen infiltration. Thus, monoglyceride blend under investigation might provide a modulatory factor promoting the goblet cell development in the small intestine.

The main ingredient in the fatty acid blend being studied was 4-carbon fatty acid, the butyrate. Butyrate supplementation has been recorded to offer multiple benefits to intestinal architecture including its role in enhancing epithelial cell proliferation (Canani et al., 2011). Therefore, for the assessment of the impact of monoglyceride blend on epithelial cell turnover, we also evaluated PCNA and TUNEL markers in the jejunal

tissue sections to visualize the balance between cell proliferation and cell death activities. Our data suggest that dietary addition of monoglyceride blend to broilers raised under unchallenged experimental model had no effect on metabolic fate of jejunal cells, as no significant variations were observed between cell proliferation and apoptosis markers (Table 4). Butyrate supplementation was reported to alleviate the intestinal injury in piglets via restraint in the apoptosis and enhancement in the gut junction integrity (Hou et al., 2014). In another study with piglets, cecal infusion of butyrate increased the intestinal cell proliferation while no effect on apoptosis of enterocytes was observed (Kien et al., 2007). However, these findings corroborate with jejunum villus and crypt morphometric measurements of the current study.

The gut junction integrity is another constitutive feature of animal health, any disruption to which decreases

the nutrient absorption along with increased invasion of the enteric pathogens (Emami et al., 2019; Suzuki, 2020). In the present study, dose-dependent upregulation of the mRNA levels of jejunal ZO-1 and ZO-2 in response to monoglyceride blend supplementation (Figure 3) could mean reduced gut permeability and pathogenic invasion, thus, revealing better gut health status. Coinciding with our results, opposed to unsupplemented group, the dietary addition of same monoglycerides blend to broiler diets upregulated the tight junction protein (ZO-1) while no difference was observed in the mRNA levels of jejunal occludin (Kumar et al., 2021a). In another study with broilers, a blend of MCFAs, organic acids and polyphenol alleviated the heat stress associated deleterious effects in jejunum by modulating the mRNA levels of tight junctions (ZO-1 and ZO-2) as well as nutrient transporters (Santos et al., 2019). Our study also showed modulation in the transporter genes of glucose (SGLT) and peptides (PePT1) in the jejunum with the supplementation of monoglyceride blend to broilers (Figure 3). Therefore, observed improvements in performance between d 0 and 21 could be attributable to upregulation of nutrient transporter. More follow-up studies are needed, especially under tough conditions that is, pathogen challenge, heat stress, feed restriction etc. to effectively understand the role of SCFA and/or MCFA in maintaining gut permeability and nutrient transportation when supplemented into broiler diets.

Animal gastrointestinal tract is not only a digestive organ, also it is the largest immunological organ (Denbow, 2015). In the present study, when compared to control group, the supplemental groups (0.03 and 0.05% of diet) revealed significant down-regulation of TNF α and a tendency of decrement in IL-1 β mRNA expression in the jejunum (Figure 2). It might suggest the anti-inflammatory effect of monoglycerides blend, which could modulate the inflammatory response notably through changes in the expression of pro-inflammatory cytokines. Previously, in a study with broilers, increment in serum IL-10 levels was reported when high (0.5%) rather than low dosage (0.1%) of MCFAs monoglycerides was administered (Amer et al., 2020), thus, also indicating a dose-dependent response.

The SCFAs analysis of cecal contents showed no notable differences with monoglyceride blend supplementation when compared to the unsupplemented group (Table 5) although higher quantities of valeric acid were accumulated in the cecum of supplemental groups showing a dose-dependent response. In another study with broilers, Gharib-Naseri et al. (2021) also published similar results stating no significant variations for cecal SCFAs among monoglycerides supplemental (0.075–0.3%) and unsupplemented groups. Contrary to these results, Dauksiene et al. (2021) found statistical increase in the concentrations of cecal acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids when broilers were fed diets supplemented with MCFAs (C₆-C₈-C₁₀-C₁₂) at the level of 0.2% of feed; they attributed these modifications to the changes in the cecum microbial profiles.

Collectively, early improvement in BWG, high goblet cell numbers in the jejunum along with downregulation of the mRNA levels of pro-inflammatory cytokines and up-regulation of tight junction proteins and nutrient transporters showed favorable responses of low doses of monoglycerides blend in broiler feeding. For future research, challenging environment should be considered which might show more pronounced impact of supplemental monoglycerides at these dosage rates than observed in this experiment.

ACKNOWLEDGMENTS

This study was supported by a BASF SE. BASF SE had no role in conducting the research, generating the data, interpreting the results or writing the manuscript.

DISCLOSURES

Authors declare that the current study was carried out in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

- Abdelli, N., J. F. Pérez, E. Vilarrasa, I. Cabeza Luna, D. Melo-Duran, M. D'Angelo, and D. Solà-Oriol. 2020. Targeted-release organic acids and essential oils improve performance and digestive function in broilers under a necrotic enteritis challenge. *Animals* 10:259.
- Amer, S. A., A. A-Nasser, H. S. Al-Khalafah, D. M. AlSadek, D. M. Abdel Fattah, E. M. Roushdy, W. R. Sherief, M. F. Farag, D. E. Altohamy, and A. A. Abdel-Wareth. 2020. Effect of dietary medium-chain α -monoglycerides on the growth performance, intestinal histomorphology, amino acid digestibility, and broiler chickens' blood biochemical parameters. *Animals* 11:57.
- Aviagen, R. 2019. *Ross 308 nutrition specifications*. Aviagen, Scotland (UK).
- Bedford, A., H. Yu, M. Hernandez, E. J. Squires, S. Leeson, and J. Gong. 2018. Effects of fatty acid glyceride product SILOhealth 104 on the growth performance and carcass composition of broiler chickens. *Poult. Sci.* 97:1315–1323.
- Bologna-Molina, R., P. Damián-Matsumura, and N. Molina-Frecher. 2011. An easy cell counting method for immunohistochemistry that does not use an image analysis program. *Histopathology* 59:801–803.
- Calik, A., and A. Ergün. 2015. Effect of lactulose supplementation on growth performance, intestinal histomorphology, cecal microbial population, and short-chain fatty acid composition of broiler chickens. *Poult. Sci.* 94:2173–2182.
- Canani, R. B., M. D. Costanzo, L. Leone, M. Pedata, R. Meli, and A. Calignano. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* 17:1519–1528.
- Çenesiz, A., and I. Çiftci. 2020. Modulatory effects of medium chain fatty acids in poultry nutrition and health. *World Poult. Sci. J.* 76:234–248.
- Culling, C. F. A., R. T. Allison, and W. T. Bar. 1985. *Cellular Pathology Technique*. 4th ed Butterworth and Co. Ltd., London, UK.
- Dauksiene, A., M. Ruzauskas, R. Gruzauskas, P. Zavistanaviciute, V. Starkute, V. Lele, D. Klupsaite, J. Klementaviciute, and E. Bartkiene. 2021. A comparison study of the caecum microbial profiles, productivity and production quality of broiler chickens fed supplements based on medium chain fatty and organic acids. *Animals* 11:610.
- Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in *Sturkie's Avian Physiology*. C. G. Scanes, ed. 6th ed. Academic Press, San Diego, CA.

- Emami, N. K., A. Calik, M. B. White, M. Young, and R. A. Dalloul. 2019. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. *Microorganisms* 7:231.
- Gharib-Naseri, K., S. K. Kheravii, L. Li, and S.-B. Wu. 2021. Buffered formic acid and a monoglyceride blend coordinately alleviate sub-clinical necrotic enteritis impact in broiler chickens. *Poult. Sci.* 100:101214.
- Gomez-Osorio, L. M., V. Yepes-Medina, A. Ballou, M. Parini, and R. Angel. 2021. Short and medium chain fatty acids and their derivatives as a natural strategy in the control of necrotic enteritis and microbial homeostasis in broiler chickens. *Front. Vet. Sci.* 8:773372.
- Hou, Y., L. Wang, D. Yi, B. Ding, X. Chen, Q. Wang, H. Zhu, Y. Liu, Y. Yin, J. Gong, and G. Wu. 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. *Br. J. Nutr.* 111:1748–1758.
- Khatibjoo, A., M. Mahmoodi, F. Fattahnia, M. Akbari-Gharaei, A.-N. Shokri, and S. Soltani. 2018. Effects of dietary short-and medium-chain fatty acids on performance, carcass traits, jejunum morphology, and serum parameters of broiler chickens. *J. Appl. Anim. Res.* 46:492–498.
- Kien, C. L., R. Blauwiel, J. Y. Bunn, T. L. Jetton, W. L. Frankel, and J. J. Holst. 2007. Cecal infusion of butyrate increases intestinal cell proliferation in piglets. *J. Nutr.* 137:916–922.
- Kumar, A., S. K. Kheravii, L. Li, and S. B. Wu. 2021a. Monoglyceride blend reduces mortality, improves nutrient digestibility, and intestinal health in broilers subjected to clinical necrotic enteritis challenge. *Animals* 11:1432.
- Kumar, A., M. Toghyani, S. K. Kheravii, L. Pineda, Y. Han, R. A. Swick, and S.-B. Wu. 2021b. Potential of blended organic acids to improve performance and health of broilers infected with necrotic enteritis. *Anim. Nutr.* 7:440–449.
- Letlolle, B. R., E. P. Damen, and C. Jansen van Rensburg. 2021. The effect of α -monolaurin and butyrate supplementation on broiler performance and gut health in the absence and presence of the antibiotic growth promoter zinc bacitracin. *Antibiotics* 10:651.
- Liao, X., Y. Shao, G. Sun, Y. Yang, L. Zhang, Y. Guo, X. Luo, and L. Lu. 2020. The relationship among gut microbiota, short-chain fatty acids, and intestinal morphology of growing and healthy broilers. *Poult. Sci.* 99:5883–5895.
- Lin, Q., Y. Liu, L. Li, M. Huai, Y. Wang, T. Lv, H. Zhao, G. Jiang, X. Wang, C. Liu, H. Qiu, and Q. Dai. 2022. Effects of a mixture of mono-glycerides of butyric-, capric-, and caprylic acid with chlor-tetracycline on the growth performance, intestine morphology, and cecal microflora of broiler birds. *Poult. Sci.* 101:101617.
- Liu, Y. 2015. Fatty acids, inflammation and intestinal health in pigs. *J. Anim. Sci. Biotechnol.* 6:1–9.
- Liu, T., C. Li, H. Zhong, and F. Feng. 2021. Dietary medium-chain α -monoglycerides increase BW, feed intake, and carcass yield in broilers with muscle composition alteration. *Poult. Sci.* 100:186–195.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) (-Delta Delta C) method. *Methods* 25:402–408.
- Onrust, L., R. Ducatelle, K. Van Driessche, C. De Maesschalck, K. Vermeulen, F. Haesebrouck, V. Eeckhaut, and F. Van Immerseel. 2015. Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. *Front. Vet. Sci.* 2:75.
- Patten, J., and P. Waldroup. 1988. Use of organic acids in broiler diets. *Poult. Sci.* 67:1178–1182.
- Pearson, J. P., and I. A. Brownlee. 2005. Structure and Function of Mucosal Surfaces. Pages 1–16 in *Colonization of Mucosal Surfaces*. J. P. Nataro, P. S. Cohen, H. L. Mobley and J. N. Weiser, eds. ASM Press, Washington DC.
- Pérez-Reytor, D., C. Puebla, E. Karahanian, and K. García. 2021. Use of short-chain fatty acids for the recovery of the intestinal epithelial barrier affected by bacterial toxins. *Front. Physiol.* 12:721.
- Perumalla, A., N. S. Hettiarachchy, and S. C. Ricke. 2012. Current perspectives on probiotics in poultry preharvest food safety. Pages 89–120 in *Direct-Fed Microbials and Prebiotics for Animals: Science and Mechanisms of Action*. T. R. Callaway and S. C. Ricke, eds. Springer, New York.
- Rimoldi, S., E. Gliozheni, C. Ascione, E. Gini, and G. Terova. 2018. Effect of a specific composition of short-and medium-chain fatty acid 1-Monoglycerides on growth performances and gut microbiota of gilthead sea bream (*Sparus aurata*). *PeerJ* 6:e5355.
- Santos, R. R., A. Awati, P. J. Roubos-van den Hil, T. van Kempen, M. H. G. Tersteeg-Zijderveld, P. A. Koolmees, C. Smits, and J. Fink-Gremmels. 2019. Effects of a feed additive blend on broilers challenged with heat stress. *Avian Pathol* 48:582–601.
- Suzuki, T. 2020. Regulation of the intestinal barrier by nutrients: the role of tight junctions. *Animal Sci. J.* 91:e13357.
- Tan, J., C. McKenzie, M. Potamitis, A. N. Thorburn, C. R. Mackay, and L. Macia. 2014. The role of short-chain fatty acids in health and disease. *Adv. Immunol.* 121:91–119.
- Uni, Z., A. Smirnov, and D. Sklan. 2003. Pre- and posthatch development of goblet cells in the broiler small intestine: effect of delayed access to feed. *Poult. Sci.* 82:320–327.
- Zentek, J., S. Buchheit-Renko, F. Ferrara, W. Vahjen, A. G. Van Kessel, and R. Pieper. 2011. Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. *Anim. Health Res. Rev.* 12:83–93.