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Original Article

The Effects of Acidifier Inclusion in the Diet on Growth Performance, Gastrointestinal Health, Ileal Microbial Population, and Gene Expression in Broilers

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ABSTRACT

The purpose of this study was to investigate the effects of acidifier on broilers' performance. A total of 648-day-old broilers were assigned to four treatments (0, 1, 2, and 3 g/kg acidifier in the diet). In the grower period, the acidifier inclusion resulted in a higher average daily weight gain (ADWG) than in the control. On the 40th day of age, 3 g/kg of acidifier increased ADWG and average daily feed intake (ADFI). The highest ADWG was observed in the 3 g/kg of acidifier treatment for the whole period. Orthogonal contrast between acidifier and control indicated that cholesterol and high-density lipoprotein (HDL) concentration in the serum was lower in the acidifier-fed broilers. Inclusion of 2 and 3 g/kg of acidifier reduced Salmonella population in the ileum. Adding 2 and 3 g/kg acidifier to the diet increased crypt depth compared to other treatments. Weight and length of the tibia were also significantly increased by acidifier. The quadratic effect showed that the acidifier had a significant effect on the tibio-tarsal index. The mRNA expression of PPARγ and Toll-Like Receptor 4 (TLR4) genes in the ileum of broilers that were fed 3 g/kg acidifier was significantly higher than in other treatments. Fatty Acid-Binding Protein 1 gene showed a significant enhancement effect by the acidifier: with increasing levels of acidifier, its expression also increased. In conclusion, the acidifier improved the performance, upregulated the expression of ileal fatty acid-binding protein 1 (FABP1), TLR4, and PPARγ genes, as well as increased the tibia length, and reduced the Salmonella population in the ileum.

INTRODUCTION

One of the poultry industry's challenges is to exploit the utilization of special feed supplements to promote broiler performance and production efficiency (Chand *et al*., 2014; Khan *et al*., 2014). Currently, the use of acidifiers can increase feed quality and utilization, safety conditions, and production performance in poultry (Khan *et al*., 2013; Abudabos *et al*., 2016; Khan *et al*., 2016; Abudabos *et al*., 2017). Organic acids such as propionic, citric, fumaric, and formic have been recognized as acidifiers that have positive effects, including 1) decreasing the effects of dietary buffering capacity; 2) reducing the pH in broilers' diets and consequently decreasing intestinal surface pH; thus controlling pathogenic microflora in the digestive and respiratory organs; 3) increasing digestion and absorption of nutrients, resulting in enhanced nutrient availability; 4) increasing immune system reactions in broilers (Yesilbag *et al*., 2006; Park *et al*., 2009; Abudabos *et al*., 2014); and 5) preventing the growth of pathogenic microorganisms (Afsharmanesh & Pourreza, 2005). Additionally, at low pH levels, the un-dissociated form of the acidifiers is able to passively diffuse through the cell membrane of pathogenic bacteria and mold. Once inside

the cell, they separate to form hydrogen ions, which reduces the pH value of the bacterial cell. This results in $RCOO^-$ (carboxylate) ions being produced from the acid, which can interrupt the cell's normal function and protein synthesis. Acidification of the intestine stimulates enzyme activity and improves nutrient digestion and mineral absorption processes (Hedayati *et al*., 2014).

Moreover, the undissociated forms of acidifiers penetrate the phospholipid membrane of bacterial cells and are then separated into cations and anions. Acidifiers disrupt the neutral pH of the bacterial cytosol, inhibiting microbial growth by disrupting ATP levels in organic phosphate reactions and oxidative phosphorylation (Hedayati *et al*., 2014). Organic acids such as butyric acid have a direct anti-microbial effect by penetrating microbial cells and disrupting microbial metabolism (Suryanarayana *et al*., 2012). Acidifiers and their blends prevent the growth of potential intestinal pathogens, including *E. coli*, *Salmonella* infections, and *Campylobacter* jejuni (Engberg *et al*., 2000; Ricke, 2003; Dibner *et al*., 2005; Garcia *et al*., 2007), and support the growth of *Lactobacillus* (Nava *et al*., 2009), resulting in better growth performance in broilers. Organic acids have been shown to increase nutrient metabolism and improve performance in broiler chickens due to their antimicrobial effects against a wide range of enteric pathogens (Huyghebaert *et al*., 2011). Moreover, organic acids assist in protecting broiler chickens from pH-sensitive pathogens, and enhancing their immune system physiology, as the intestinal microbiota is associated with immune responses in chickens. The beneficial effects of acidifier (Emami *et al*., 2017) or fiber supplements (Sadeghi *et al*., 2015) on the enteric microbiota also improve the immune reaction of broiler chickens. All of the mentioned mechanisms can significantly contribute to the positive effects of acidifiers in livestock efficiency. Therefore, acidifiers can contribute to healthy and nutritious bird products for people.

Peroxisome proliferator-activated receptors gamma (PPARγ) are a component of the nuclear receptor group and are involved in lipid metabolism as the main regulator of adipose tissue. PPARs- are a type of receptor that is responsible for this function (Royan *et al*., 2016). Therefore, PPARγ is a significant transcriptional agent during adipogenesis. PPARs also play a crucial role in insulin sensibility (Chistiakov et al., 2010), lipid retention, energy loss, and adipokine secretion, making them the main regulators of adipose-tissue generation and function (Dahlman & Arner, 2010). In broiler adipose tissues, the expression level of PPARγ is high and is related to lipid accumulation (Mandrup *et al*., 1997). This suggests that PPARγ plays a crucial role in regulating lipid accumulation in the abdominal fat pad of broilers (Sato *et al*., 2009). Fatty acids are synthesized by hepatic cells in broilers and are the initial site of fat storage is adipose tissue (Fouad *et al*., 2014). Toll-like receptors (TLRs) are a type of transmembrane-spanning proteins that act as sentinels of tissue damage, mediate inflammatory responses to aseptic tissue injury, discriminate self from non-self antigens, identify molecules unique to microbes, and trigger appropriate immune responses. (Marsh *et al*., 2009). In response to stress, signal passage is activated by TLR4 (Zhou *et al*., 2005; Xiang-Hong *et al*., 2011). Molecular genetics is one way to enhance growth in breeding by utilizing key genes that control lipid deposition. One group of proteins associated with both extracellular and intracellular lipid metabolism are fatty acid-binding proteins (FABPs) (Wang, *et al*., 2009; Liu *et al*., 2015). FABPs such as heart-type fatty acid-binding proteins (H-FABP or FABP3) are functional genes that relate to energy consumption that uses fat as a source (Wang *et al*., 2007; Tyra *et al*., 2012). The transportation of fats to specific sections within the cell occurs through FABPs, a process that includes lipid droplets for storage, the endoplasmic plexus for signaling, trafficking, and membrane synthesis, the nucleus for the regulation of lipid-mediated transcriptional programs via nuclear hormone receptors, and the mitochondria or peroxisome for oxidation (Furuhashi *et al*., 2008). Li *et al*. (2013) reported a negative correlation between intramuscular lipids in the leg and breast of broilers and the mRNA expression of H-FABP.

However, the mode of operation of acidifiers in poultry has not been fully elucidated in the literature. This limited understanding may limit the usage of acidifiers in diets. Therefore, more research is required to determine the effects of acidifiers on gastrointestinal health through changes in the expression of inflammatory genes. We hypothesized that acidifiers could be effective in improving the expression of genes implicated in the absorption of fatty acids in the ileum and their metabolism under the influence of acidic and inflammatory conditions of the intestine. Therefore, the objective of this study was to investigate the effect of acidifier inclusion as a feed additive in the diet on growth performance, immune response, gastrointestinal tract traits, and gene expression involved in nutrient absorption and inflammatory signaling in the ileum of broilers.

MATERIALS AND METHODS

Source of the blended acidifier

The blended acidifier used in the current experiment was provided by a commercial company (Sepehr Makian Fartak). Its composition was 15% fumaric acid (99.62 % Purity, FIC, China), 20% citric acid (100% Purity, Jovain, Iran), 5% lactic acid (85.1% Purity, Henan, China), 10% propionic acid (99.5 % Purity, Merck, Germany), and 10% acetic acid (98% Purity, FIC, China) (acetic acid salts). Furthermore, 40% vermiculite was applied as a carrier (Lidoma, Iran).

Animals and experimental design

The study was conducted at the research farm of the Isfahan University of Technology, Iran. Guidelines for the care and use of animals were approved by the agency of investigations on principles, procedure and welfare, and were in line with the FASS (2010). A total of 648 day-old straight-run Ross 308 broiler chickens were divided into 4 treatments based on initial body weight. Treatments comprised 6 replicates, with 27 broilers in each replicate, in a completely randomized design (CRD). They were floor-reared (120x100 cm) in separate clean and disinfected pens, as recommended by the Ross-308 management guide (Aviagen, 2014). All broilers had access to feed and water *ad libitum*. Chickens were fed basal broiler diets formulated to meet or exceed Aviagen (2014) recommendation on the nutrient requirements for broiler chickens. A 24-h lighting diet was carried out during the first 3 days, and 23 h of lighting with 1 h of darkness was used from 4 days of age onward. The current research was divided into three phases: starter (0 to 10 days of age), grower (11 to 24 days of age), and finisher (25 to 40 days of age). Dietary treatments consisted of a corn-soybean meal-based diet in the mash physical form, and the basal diet was supplemented with 1, 2, and 3 g/kg of blended acidifier.

Growth Performance

The broilers were weighed individually at 10, 24, and 40 days of age, and the feed intakes of broilers were recorded by pen. The average daily feed intake (ADFI), average daily weight gain (ADWG), feed conversion ratio (FCR), coefficient of variation (CV), European production efficiency factor (EPEF), and bird uniformity were calculated (Aviagen, 2014). Mortalities were counted, and their body weight (BW) was recorded for FCR adjustment.

European Production Efficiency Factor (EPEF):

EPEF = {viability (%) x BW (kg)/age (d) x FCR (kg feed/kg gain)} x 100

Organ weight and ileum pH

On day 40 of age, 6 bird/treatment was randomly selected, weighed, and euthanized by Co_2 asphyxiation. The absolute weights of the liver, spleen, bursa of Fabricius, and thymus were recorded using a high precision scale and expressed as %BW. Subsequently, a digital pH meter (Testo 205-Germany) was directly inserted into the ileum digesta of the same broilers (the electrode was placed in a 4 cm incision made near the ileocecal junction, as described by (Teuchert, 2014), while avoiding direct contact of the pH electrode with the gut wall. The pH was measured and recorded in duplicate. Once all the readings were taken, the probe was rinsed with distilled water. The mean of the 2 readings per site of the ileum was then calculated and recorded.

Blood biochemical parameters

At 40 days of age, 2 broilers per replicate were randomly selected, and 2 mL of blood from their wing veins was drawn using a sterilized syringe. Serum was obtained by centrifugation of the blood samples at 3000 × g for 10 min at 4 °C and stored at -20°C for further analysis. The serum concentration of aspartate aminotransferase (AST), alanine amino transferase (ALT), total protein, albumin, triglyceride (TG), cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were measured by an enzymatic method based on Pars Azmun Kits (Alcyon 300. USA device). Serum globulins were also calculated by subducting the serum albumin levels from the total serum protein, as described by Gupta et al. (2005).

Jejunal morphology

For the morphology analysis, 1cm of the middle part of the jejunum tissue was sampled from one bird in each replicate. It was then flushed with distilled water to remove the digesta content and fixed in 10% buffered formalin. After dehydration and infiltration with solidified paraffin wax, a 6 µm cross-section was made using a microtome (Sakura SRM 200, Tokyo, Japan), which was then placed on a glass slide, and stained with hematoxylin-eosin using standard histological techniques. The tissue slides were then analyzed with a light microscope (Olympus, CX31, Shinjuku, Tokyo, Japan), and the villus height (VH), crypt depth (CD), villus width (VW), and muscular layer thickness were

measured for each segment using image-analysis software (ImageJ 1.52v). Jejunal morphometric variables were measured from 2 sections per bird, with a minimum of 20 villi and 20 crypts per section. Data from the VH and CD were used to obtain the VH/CD ratio. The villus surface area (VSA) was calculated using the formula: VSA= $\pi \times$ (villus width) $\times$ (villus height), as described by Sakamoto *et al*. (2000).

Microbial population in the ileum

Samples of the ileum digesta were collected on day 40 of age. For ileal microflora determination, samples were taken in 10-ml sterile falcons under flame conditions, placed on ice, immediately transferred to a laboratory for culture, and then cooled until incubation. One g of each sample was used for serial dilutions by phosphate-buffered saline (PBS), vortexed, and 0.1 ml of each sample was dispensed and extended on selective media in petri dishes. The total bacteria population was cultured on plate count agar culture medium in 10^{-5} and 10^{-6} dilutions, and coliform bacteria were cultured in 10^{-4} and 10^{-5} dilutions on MacConkey agar medium. These bacterial populations were enumerated for 24 hours in an incubator at 37 °C. The *Lactobacillus* population was cultured in de Man Rogosa Sharpe (MRS) agar medium at 10^{-4} and 10^{-5} dilutions. After 48 hours in an anaerobic incubator at 37°C, the number of colonies was counted. All microbial species mentioned were recognized with the original medium (Condalab, Madrid, Spain). As described by Andreatti Filho *et al*., (2007), *Salmonella* was grown for the duration of a night in Tryptic Soy Broth (TSB) at 37°C. Condensations of *Salmonella* were certified by spread-plating on Xylose Lys Deoxycholate (XLD) agar plates (Andreatti Filho *et al*., 2007).

In order to evaluate the effectiveness of the acidifier in reducing *Salmonella* bacteria in vitro conditions, the basic feed of broilers was autoclaved without an acidifier. Five replications were carried out for each treatment. For each repetition, 2 g of feed was mixed with 5 mL of PBS in sterile tubes. Then 0.5 mL of *Salmonella enteritidis* containing 8 x 10^3 CFU/mL was added to each tube. According to the treatments, 1, 2, and 3 g/kg of acidifier were also added to them. All tubes were vortexed for 5 seconds and incubated at 37°C. After 6 hours, the tubes were again vortexed for 5 seconds and cultured on an XLD culture medium to count the population of *Salmonella enteritidis* bacteria. The plates were incubated for 24 hours at 37°C and the number of colonies was counted.

Antibodies response against sheep red blood cell (SRBC)

Sheep blood samples were collected to provide Sheep Red Blood Cell (SRBC) injection suspension and poured into tubes containing EDTA. Samples were washed three times with PBS, and then the suspension of 5% SRBC was made in PBS. The procedure was performed under sterile conditions (Belali *et al*., 2021). Then, the SRBC was injected into broilers at 25 and 32 days, and blood sampling was performed 7 days following each injection. The blood sample obtained in each treatment was heat-inactivated (at 56°C for 30 min) and IgM was examined for total, mercaptoethanol-sensitive (MES), and mercaptoethanol-resistant IgG anti-SRBC antibodies (Delhanty & Solomon, 1966; Yamamoto & Glick, 1982; Qureshi & Havenstein, 1994). Total serum antibody titers to SRBC were specified by a hemagglutination trial, as described by Cheema *et al*. (2003). In summary, serum at a level of 50 µm was added to the initial column of a 96-well plate with a V-shaped bottom, in an adequate value of PBS, and the solution was incubated for 30 min at 37°C. A serial dilution was produced (1:2), and 2% SRBC suspension at the level of 50 was added to the whole well. Total antibody titers were read after incubation at 37°C for 30 min. Then, to estimate MES (IgM) response, instead of PBS alone, 50 µL of 0.01 M mercaptoethanol in PBS was used. The IgM titer was calculated using the contrast between total and IgG titer.

Tibia traits

The left tibia bone of euthanized broilers (one bird per replicate) was removed and stored at -20°C at 40 days of age. All samples were analyzed to determine morphological characteristics (weight, length, diaphysis diameter, tibial modular canal diameter, wall thickness, tibio-tarsal index, and robusticity index), mechanical properties (elasticity coefficient, shear and tension stress), and biochemical properties (dry matter and ash content). Firstly, soft tissues and fats were separated from the bones and then heated in boiling water (100°C) for 10 min. The femoral head was removed from the left leg of the broilers and dried in an oven. The length and diameter of the diaphysis were measured in the central part of the bone in both perpendiculars by a digital caliper. Subsequently, the bone weight-to-length ratio was calculated as the tibia weight divided by its length (Seedor *et al*., 1991). After breaking the bone, the wall thickness was measured by a digital caliper in the central part of the bone, both vertically and parallel to the direction of the applied force.

Instron was used to measure the shear stress, tension stress, and modulus of elasticity (Santam, STM 20, Iran), as described by (Kocabagli, 2001). The diameter of the medullar channel of the tibia was measured by subtracting the thicknesses of the medial and lateral walls from the diameter at the diaphysis. Ash and dry matter of the tibia were measured according to the methods that were described by AOAC (2005). The robusticity and the tibio-tarsal indexes were assessed using the following formulas:

Tibio-tarsal index = diaphysis diameter – medullary canal diameter / diaphysis diameter × 100 (Barnett & Nordin, 1960):

Robusticity index = bone length / cubic root of bone weight (Riesenfeld, 1972).

The RNA extraction of the ileal tissue

At the end of the experiment (40 days of age), two centimeters of the ileum tissue of 3 broilers from each pen were sampled for RNA extraction. The samples were washed with distilled water, immediately frozen in fluid nitrogen and kept at -80 °C. Tissue samples (40 mg) were mixed with liquid nitrogen in a sterile mortar and then crushed. The cells were broken by the addition of one ml of Trizol (Sinaclon, Tehran, Iran) and vortexed intensely for 40 seconds. After this, 200 µl of chloroform were added and centrifuged for 15 min (12,000 rpm at 4°C). Further purification steps were conducted matching the kit instructions. The RNA sample was DNase-treated by RNase-free DNase I (Sinaclon, Tehran, Iran) to eliminate genomic DNA contamination. The total RNA concentration and purity were assessed by measuring absorbance at 260 nm and determining the A260/A280 ratio utilizing NanoDrop (Thermo Scientific). RNase-free water at the level of 50 ml was used for RNA extraction, subsequently being stored at -80°C until use in future molecular analysis (Huang *et al*., 2016; Royan *et al*., 2016; Parada *et al*., 2018; Xu *et al*., 2020).

Real-Time Quantitative RT-PCR (qRT-PCR) analysis

The quantitative reverse transcription-PCR (qRT-PCR, ABI StepOne™ Real-Time PCR System - Thermo Fisher Scientific) was used for determination of the expression of candidate genes (qRT-PCR, ABI StepOne™ Real-Time PCR System - Thermo Fisher Scientific), to test the fold change of the selected genes. RealQ Plus 2x Master Mix Green was used for the reaction (Amplicon). Based on the gene sequences, primers were designed and NCBI Blast primer was blasted and synthesized economically (TAG Co., Copenhagen, Denmark; Table 1). The whole RNA using cDNA synthesis® RT reagent Kit was used to synthesize complementary DNA (cDNA) (Sinaclon). 25 ng of cDNA per sample was utilized as a template in a final reaction volume of 25 µL, adhering to the manufacturer's guidelines. The thermal cycle profile was as follows: a primary denaturation stage at 95 °C for 10 min, following 40 periods containing the denaturation stage at 95 °C for 30 s, and an annealing and expanse stage at 60 °C for 30 s. The reference gene was the *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* gene. Each trial was conducted in triplicate and replicated three times separately. The triplicate PCRs were averaged by the period threshold (Ct) values, and the comparative $2^{-\Delta\Delta CT}$ method was performed to the relative quantification of the transcript levels (Huang *et al*., 2016; Royan & Navidshad, 2016; Parada *et al*., 2018; Xu *et al*., 2020).

Table 1 – Composition and calculated analysis of the experimental diets, as-fed basis (g/kg).

	Starter (0 -10 d)	Grower (11 - 24 d)	Finisher (25 - 40 d)
Corn	481.20	557.25	610.40
Soybean meal	397.80	357.20	308.30
Soybean oil	35.70	30.10	31.50
Corn gluten meal	40.00	15.00	15.00
Salt	1.80	2.20	2.30
$NaHCO_3$	2.50	2.10	2.00
Di-calcium phosphate	21.50	19.10	17.10
Limestone	9.20	8.50	5.90
Vitamin premix[a]	1.00	1.00	1.00
Mineral premix[b]	1.00	1.00	1.00
L-Lysine-HCL	2.80	1.80	1.70
DL-Methionine	3.30	2.90	2.50
L-Threonine	1.20	0.90	0.70
Choline chloride	1.00	0.80	0.50
Calculated Analysis			
Metabolizable energy (kcal/kg)	3000	3100	3150
Crude protein[c]	230.0	209.0	188.2
Lysine SID	12.8	11.5	10.2
Methionine SID	6.4	5.8	5.4
Methionine + cysteine SID	9.5	8.7	8.0
Threonine SID	8.6	7.7	6.8
Valine SID	9.6	8.7	8.1
Calcium	9.6	8.7	7.8
Available phosphorus	4.8	4.3	3.9

[a]Provided per kilogram of diet: 12000 IU Vit A, 5000 IU Vit D_3, 80 IU Vit E, 3.2mg Vit K, 3.2 mg Vit B1, 8.6 mg Vit B_2, 65 mg niacin, 20 mg pantothenic acid, 4.3 mg Vit B_6, 0.22 mg biotin, 2.2 mg folic acid, 0.017 mg VitB_{12}.

[b]provided per kilogram of diet: 16 mg copper, 1.25 mg iodine, 20 mg iron, 120 mg manganese, 0.3 mg selenium, 110 mg zinc.

[c]Feed amino acids were formulated based on SID values (standardized ileal digestible).

Statistical analysis

The GLM model (General Linear Model) was used to analyze all data with analysis of variance in a completely randomized design (CRD). Means were compared with Tukey's test at a 5% probability level ($p \leq 0.05$). Orthogonal polynomial contrasts were computed for the levels-response effect of the acidifier (linear and quadratic). The mean comparison among treatments was conducted by orthogonal contrast (control vs. acidifier).

RESULTS

Growth performance

Experimental treatments (Table 2) did not affect ADWG, ADFI, FCR, body weight uniformity and CV from 0 to 10 days of age. Also, neither orthogonal contrast between the control and acidifier supplement nor linear and quadratic effects showed any significant differences in ADWG, ADFI, FCR, body weight uniformity, or CV in the first phase (0 to 10 days of age).

Table 2 – Effect of acidifier inclusion in the diet on the growth performance of broilers from 0 to 10 days of age.

Treatments	ADWG [1] (g/d/b)	ADFI[2] (g/d/b)	FCR[3]	Uniformity (%)	CV[4]
Control	22.891	25.795	1.125	70.372	10.285
Acidifier					
1 g/kg	23.650	25.958	1.098	75.308	8.805
2 g/kg	23.424	26.148	1.116	70.988	9.736
3 g/kg	22.808	25.581	1.121	69.138	9.551
SEM	0.352	0.380	0.131	3.609	0.420
p-value[5]					
Treatment	0.289	0.754	0.497	0.705	0.130
Control vs. Acidifier	0.334	0.820	0.408	0.752	0.072
Linear	0.767	0.794	0.888	0.651	0.507
Quadratic	0.065	0.348	0.241	0.395	0.138

[1]Average daily weight gain. [2]Average daily feed intake. [3]Feed conversion ratio. [4]Coefficient of variation. [5]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

The addition of the acidifier improved the ADWG of broilers (Table 3) compared to the control diet from 11 to 24 days of age. Orthogonal contrasts between acidifier and control diets showed that the addition of acidifier increased ADWG. From 11 to 24 days of age, the ADWG of broilers increased (linear, $p<0.0001$; quadratic, $p=0.018$) with increasing levels of acidifier inclusion in the diet. Orthogonal contrast analysis of the data from 11 to 24 days of age showed that the inclusion of acidifiers increased ADFI. Also, FCR and BW uniformity were linearly improved ($p<0.05$) by supplementing the diet with increasing levels of acidifier. Broilers fed with acidifier had lower body weight CV than those fed the control diet. Also, the CV for body weight linearly decreased with the acidifier levels.

Table 3 – Effect of acidifier inclusion in the diet on the growth performance of broilers from 11 to 24 days of age.

Treatments	ADWG [1] (g/d/b)	ADFI[2] (g/d/b)	FCR[3]	Uniformity (%)	CV[4]
Control	60.485[c]	87.667	1.450	67.902	10.836[a]
Acidifier					
1 g/kg	64.165[b]	91.218	1.422	72.840	9.270[ab]
2 g/kg	67.643[a]	92.280	1.367	76.543	9.093[ab]
3 g/kg	67.245[ab]	91.635	1.363	77.780	7.861[b]
SEM	1.309	1.519	0.034	3.372	0.547
p-value[5]					
Treatment	<0.0001	0.17	0.139	0.202	0.011
Control vs. Acidifier	<0.0001	0.032	0.069	0.061	0.004
Linear	<0.0001	0.071	0.027	0.041	0.002
Quadratic	0.018	0.182	0.688	0.593	0.767

[1]Average daily weight gain. [2]Average daily feed intake [3]Feed conversion ratio. [4]Coefficient of variation. [5]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

As observed in Table 4, experimental treatments had a significant effect on ADWG, ADFI, and FCR from 25 to 40 days of age (p<0.05); when broilers fed with 3 g/kg of acidifier had greater ADWG, ADFI and FCR (p<0.05). Quadratic effects also showed that ADWG increased along with the inclusion levels of acidifier at the finisher period. Feeding the acidifier linearly increased ADFI from 25 to 40 days of age (p= 0.031).

Table 4 – Effect of acidifier inclusion in the diet on the growth performance of broilers from 25 to 40 days of age.

Treatments	ADWG [1] (g/d/b)	ADFI[2] (g/d/b)	FCR[3]	Uniformity (%)	CV[4]
Control	100.975[b]	175.944[b]	1.743[b]	66.048	12.191
Acidifier					
1 g/kg	100.143[b]	175.912[b]	1.756[b]	58.025	12.430
2 g/kg	100.616[b]	175.440[b]	1.746[b]	54.936	15.188
3 g/kg	104.532[a]	184.705[a]	1.770[a]	69.751	9.770
SEM	1.232	1.998	0.014	6.323	1.682
p-value [5]					
Treatment	0.007	0.039	0.046	0.341	0.191
Control vs. Acidifier	0.884	0.460	0.260	0.489	0.890
Linear	0.155	0.031	0.407	0.779	0.555
Quadratic	0.005	0.051	0.073	0.086	0.108

[1]Average daily weight gain. [2]Average daily feed intake. [3]Feed conversion ratio. [4]Coefficient of variation. [5]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. p<0.05; Tukey's pairwise test.

The results (Table 5) showed that ADWG was affected by the experimental treatments through the entire experimental period (p<0.05). Broilers fed 3 (g/kg) of acidifier had the highest ADWG (p<0.05). The addition of the acidifier linearly enhanced overall ADWG, ADFI, and EEF (p<0.05).

Table 5 – Effect of acidifier inclusion in the diet on the growth performance of broilers from 0 to 40 days of age.

Treatments	ADWG [1] (g/d/b)	ADFI[2] (g/d/b)	FCR[3]	Survival rate	EPEF[4]
Control	67.278[b]	107.510	1.598	96.913	414.410
Acidifier					
1 g/kg	67.958[ab]	108.380	1.595	98.766	427.601
2 g/kg	67.736[ab]	109.010	1.609	98.150	419.570
3 g/kg	70.558[a]	111.550	1.580	99.383	450.160
SEM	0.871	1.078	0.014	1.195	9.276
p-value [5]					
Treatment	0.038	0.081	0.586	0.519	0.060
Control vs. Acidifier	0.127	0.102	0.844	0.194	0.108
Linear	0.014	0.016	0.511	0.218	0.027
Quadratic	0.196	0.448	0.368	0.798	0.359

[1]Average daily weight gain. [2]Average daily feed intake. [3]Feed conversion ratio. [4]European production efficiency factor. [5]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. p<0.05; Tukey's pairwise test.

Organ weight and ileum pH

The relative weight of organs is shown in Table 6. Broilers that were fed 3 (g/kg) of acidifier showed a higher relative weight of the thymus, which significantly differed only from the control diet (p<0.05).

The addition of 3 g/kg of acidifier decreased the pH of the ileum. Orthogonal contrast between control and acidifier indicated that liver and thymus weight had a significant increase, and that the pH of the ileum was decreased by acidifier inclusion. Furthermore, with increasing acidifier levels, relative liver weight linearly increased, and the ileum pH linearly decreased at the finisher period. The quadratic response when comparing the weight of inner organs showed that only the relative weight of the liver (p=0.009), bursa of Fabricius (p=0.031), and thymus (p=0.014) had significant differences.

Table 6 - Effect of acidifier inclusion in the diet on the relative weight of organs (%BW) and ileum pH of broilers at 40 days of age.

Treatments	Liver	Bursa of Fabricius	Thymus	Spleen	pH
Control	1.642	0.102	0.180[b]	0.078	6.218
Acidifier					
1 g/kg	1.671	0.103	0.250[ab]	0.089	6.273
2 g/kg	1.729	0.129	0.240[ab]	0.082	6.186
3 g/kg	1.836	0.141	0.331[a]	0.103	5.485
SEM	0.150	0.014	0.026	0.012	0.209
p-value[1]					
Treatment	0.806	0.157	0.005	0.474	0.053
Control vs. Acidifier	0.018	0.080	0.002	0.351	0.006
Linear	0.001	0.573	0.101	0.849	0.035
Quadratic	0.009	0.031	0.014	0.452	0.086

[1]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

Blood biochemical parameters

According to the results shown in Table 7, among the biochemical blood factors, cholesterol and HDL levels were affected by experimental treatments ($p<0.05$). The concentration of cholesterol and HDL was the highest in the control treatment compared to other treatments, and it was the lowest in the treatment supplemented with 3 g/kg of acidifier. Orthogonal contrast between acidifier supplements and control indicated that cholesterol, TG and HDL levels were significantly decreased by the use of acidifier in the diet. At the same time, the linear effect showed that the amount of cholesterol, TG, HDL and LDL decreased linearly with acidifier inclusion ($p=0.001$, $p=0.043$, $p=0.0005$ and $p=0.019$ rep). The experimental treatments did not have a significant effect on other blood biochemical parameters.

Table 7 – The effect of acidifier inclusion in the diet on the blood parameters of broilers at 40 days of age.

Treatments	AST[1] (IU/l)	ALT[2] (IU/l)	TP[3] (g/dl)	Albumin (g/dl)	Globulin (g/l)	Alb:glob[4] (g/l)	Cholesterol (mg/dl)	TG[5] (mg/dl)	HDL[6] (mg/dl)	LDL[7] (mg/dl)
Control	275.33	24.16	2.53	1.10	1.43	0.78	143.00[a]	130.50	63.00[a]	40.51
Acidifier										
1 g/kg	293.17	22.66	2.67	1.28	1.38	0.96	109.00[ab]	97.83	46.17[b]	47.50
2 g/kg	296.00	21.60	2.66	1.20	1.46	0.83	103.60[b]	77.40	44.80[b]	30.89
3 g/kg	285.50	25.33	2.63	1.23	1.40	0.90	95.33[b]	89.50	39.17[b]	24.81
SEM	19.856	2.441	0.127	0.073	0.097	0.082	8.908	14.841	3.941	4.022
p-value[8]										
Treatment	0.884	0.718	0.872	0.390	0.945	0.442	0.006	0.103	0.002	0.064
Control vs. Acidifier	0.487	0.735	0.425	0.126	0.868	0.256	0.001	0.023	0.001	0.009
Linear	0.711	0.825	0.613	0.358	0.957	0.577	0.001	0.043	0.001	0.019
Quadratic	0.484	0296	0.537	0.332	0.959	0.546	0.164	0.147	0.171	0.137

[1] Aspartate aminotransferase; [2] Alanine transaminase; [3]Total protein; [4]Albumin to globulin Ratio; [5]Triglyceride; [6]High-density lipoprotein; [7]Low-density lipoprotein; [8]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

Antibodies response against sheep red blood cells (SRBC)

The results of the antibodies generation against sheep red blood cells titer are shown in Table 8. As the results show, the experimental treatments did not affect the total and specific antibody production titers in any of the experimental phases. Nevertheless, the total and specific antibody production titers increased in the secondary SRBC compared to the primary SRBC. Also, with increasing acidifier levels, IgM levels decreased linearly in the primary period ($p=0.017$).

Ileum Microbial population

The results of Table 9 show that the addition of 1, 2, or 3 g/kg acidifier had no significant effect on the total microbial, *Lactobacillus,* and coliforms population in the

Table 8 - Effect of acidifier inclusion in the diet on SRBC[1] (log 2) of broilers at 40 days of age.

	Primary SRBC			Secondary SRBC		
Treatments	Total	IgG	IgM	Total	IgG	IgM
Control	3.50	2.00	1.50	4.83	2.83	2.00
Acidifier						
1 g/kg	3.33	1.83	1.50	4.50	3.00	1.50
2 g/kg	2.67	1.50	1.17	4.67	3.17	1.50
3 g/kg	2.50	1.83	0.67	4.00	2.33	1.67
SEM	0.424	0.419	0.244	0.716	0.508	0.345
p-value[2]						
Treatment	0.292	0.860	0.808	0.858	0.685	0.709
Control vs. Acidifier	0.189	0.573	0.183	0.597	1.000	0.278
Linear	0.068	0.662	0.017	0.475	0.564	0.525
Quadratic	1.000	0.558	0.318	0.818	0.337	0.346

[1]Sheep Red Blood Cell. [2]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

Table 9 – Effect of acidifier inclusion in the diet on the microflora of the broilers' ileum and the Salmonella population in the feed (Log10 CFU/g).

	Ileum			Feed
Treatments	Total bacteria	*lactobacillus*	coliforms	*salmonella*
Control	8.352	7.963	6.701	2.097[a]
Acidifier				
1 g/kg	8.275	8.036	6.498	2.013[a]
2 g/kg	8.274	7.901	6.461	1.873[b]
3 g/kg	8.157	8.234	6.424	1.871[b]
SEM	0.148	0.198	0.146	0.042
p-value[1]				
Treatment	0.829	0.667	0.554	0.042
Control vs. Acidifier	0.502	0.686	0.170	0.407
Linear	0.388	0.454	0.201	0.513
Quadratic	0.897	0.521	0.574	0.717

[1]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet. [ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

ileum. However, the population of *Salmonella* bacteria in the ileum of broilers fed with a diet containing 2 and 3 g/kg of acidifier significantly decreased compared to broilers in the control group ($p<0.05$).

Jejunal morphology

Results related to intestinal tissue morphology are reported in Table 10. The crypt depth was significantly increased in treatments supplemented with both 2 and 3 g/kg of acidifier compared to other treatments ($p<0.05$). There was no significant difference between experimental treatments in terms of height, width, and area of jejunum villi.

Orthogonal contrast between control and acidifier diets indicated that dietary acidifier increased the villus surface area and crypt depth. Also, acidifier inclusion in the diet linearly increased the crypt depth, villus height and villus surface area ($p=0.049$, $p=0.006$ and $p=0.016$, respectively).

Tibia traits

Acidifier addition did not affect morphometric (weight, length, density, weight index, medullary, diaphyseal diameter, canal diameter, tibio-tarsal index, and bone robusticity index) and biochemical (dry matter and ash content) parameters of the tibia (Table 11). However, orthogonal contrast between the acidifier-supplemented treatments compared to the control showed acidifier treatments caused an increase in bone length. The acidifier supplement inclusion level had a marginal quadratic effect on the tibio-tarsal index ($p=0.042$).

Gene expression

Modifications in FABP1 mRNA expression in the ileal tissue

The expression of Fatty Acid-Binding Protein*1* (*FABP1*) gene had a significant increase when acidifier

Table 10 – Effect of acidifier inclusion in the diets on jejunal morphology (µm) of broilers at 40 days of age.

Treatments	VH[1]	CD[2]	VH: CD	VW[3]	Muscular layer thickness	Villus surface area (mm^2)
Control	991.82	91.33[b]	10.80	86.87	252.04	0.269
Acidifier						
1 g/kg	1081.66	93.40[b]	11.62	87.52	265.43	0.298
2 g/kg	1101.64	105.43[a]	10.47	89.93	289.08	0.311
3 g/kg	1162.17	107.52[a]	10.81	91.61	276.42	0.332
SEM	56.904	4.431	0.321	3.439	22.185	0.017
p-value[4]						
Treatment	0.236	0.036	0.104	0.751	0.683	0.107
Control vs. Acidifier	0.075	0.047	0.667	0.487	0.342	0.037
Linear	0.049	0.006	0.433	0.292	0.340	0.016
Quadratic	0.799	0.997	0.461	0.881	0.564	0.812

[1]Villus height. [2]Crypt depth. [3]Villus width. [4]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

Table 11 – Effect of acidifier inclusion in the diet on tibia traits of broilers at 40 days of age.

	Acidifier (g/kg)				*p*-value[1]				
	Control	1	2	3	Treatment	Control vs. Acidifier	Linear	Quadratic	SEM
Weight, (g)	6.88	7.46	7.23	7.94	0.160	0.089	0.053	0.836	0.321
Length, (mm)	98.46	102.55	101.63	103.37	0.189	0.043	0.072	0.477	1.622
Density (g/cm^3)	9.07	9.35	8.99	9.84	0.161	0.327	0.135	0.320	0.277
Weight: length (mg/mm)	69.92	72.736	70.98	76.83	0.238	0.219	0.099	0.545	2.463
Diaphysis diameter, (mm)	9.07	9.35	8.99	9.84	0.161	0.327	0.135	0.320	0.277
Medullary canal diameter, (mm)	7.45	8.00	7.73	8.14	0.367	0.144	0.177	0.809	0.289
Robusticity Index	5.17	5.25	5.27	5.18	0.589	0.408	0.899	0.183	0.060
Tibio-tarsal index	17.89	14.72	14.04	17.16	0.208	0.138	0.663	0.042	1.447
Ash%	45.59	44.22	44.87	45.59	0.712	0.683	0.711	0.315	0.882

[1]Treatment: General effects of treatment; Control vs Acidifier: contrasting broilers without acidifier versus with acidifier; Linear: linear effects of increasing level of acidifier inclusion; Quadratic: quadratic effects of increasing level of acidifier inclusion in the diet.

[ab]Values within a column followed by different superscripts are significantly different. $p<0.05$; Tukey's pairwise test.

was added to the diet. Therefore, the use of 3 g/kg of acidifier increased FABP1 gene expression in comparison to the control (Figure 1A). Orthogonal contrast between the acidifier-supplemented and control showed that the expression of the *FABP1* gene was increased in the acidifier group (Figure 2A).

Modifications in mRNA expression rates of TLR4 and PPARγ in the ileal tissue

In the ileum tissue, the toll-like receptor 4 (*TLR4*) mRNA expression was significantly increased by dietary supplementation with 3g/kg acidifier compared to the control at 40 days of age (Figure 1B). Orthogonal contrast showed that *TLR4* mRNA expression was increased by the acidifier in comparison to the control (Figure 2B).

Therefore, the PPARγ mRNA expression in the ileum of broiler was significantly higher by using 3 g/kg acidifier when compared to other treatments at 40 days of age (Figure 1C). Also, orthogonal contrast showed that the *PPARγ* mRNA expression was increased with the use of acidifier in comparison to the control group (Figure 2C).

DISCUSSION

Growth performance

The performance of broilers was not affected by the acidifiers at starter period. Khalil *et al*. (2020) demonstrated that the live body weight, body weight gain, and feed conversion ratio of broilers ($p<0.05$) were not significantly affected by diets containing acidifier (1 ml/L through drinking water) at 14 days of age (Khalil *et al*., 2020). The coefficient of variation (CV) of body weight was significantly decreased among broilers fed with acidifier compared to the control diet ($p<0.05$) in the grower period. Data scatter of the body weight CV showed that broilers fed 3 g/kg acidifier treatment had less scatter and more uniformity compared to the control. It was also shown that this diet could cause a

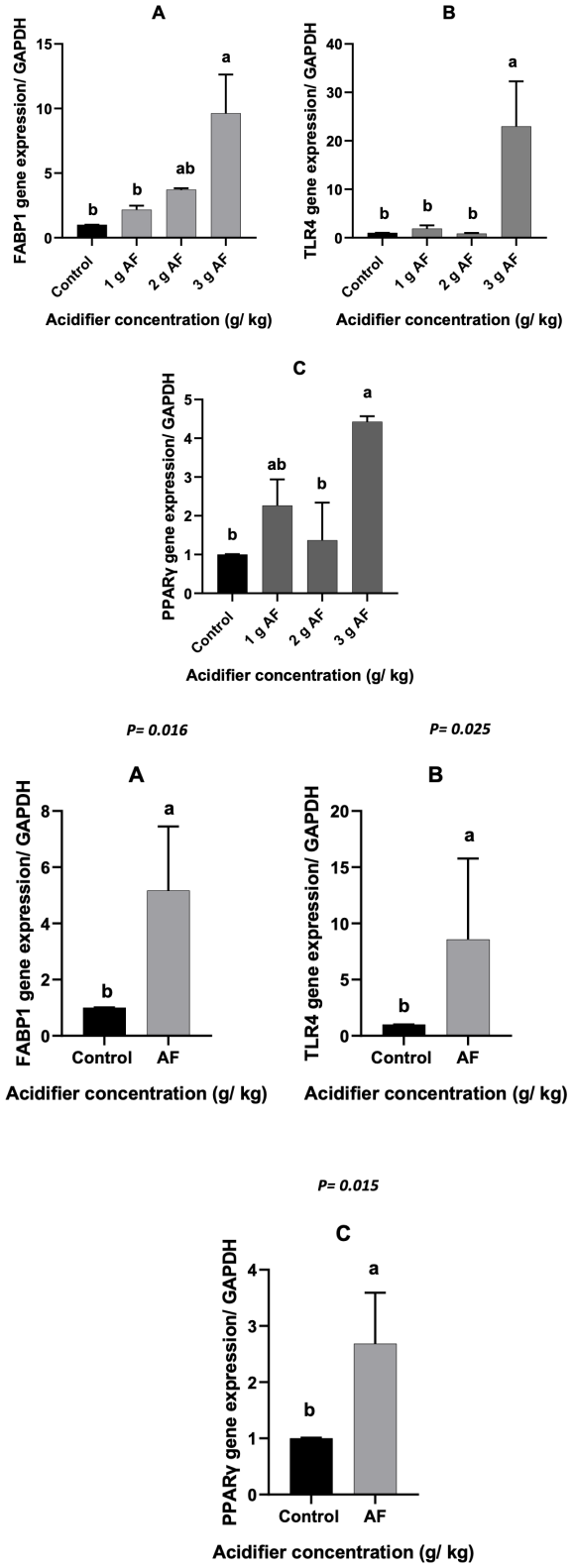

Figure 1 – Toll-like receptor 4, PPARγ (Peroxisome proliferator-activated receptor-γ), and FABP1 (fatty acid-binding protein1) mRNA expression in the ileum of broiler chickens at 40 days of age.

Total RNA (30 µg) obtained from broiler ileum. The results are shown as optional units. Bars indicate SE of the mean values (n = 3).

AF: acidifier.

a,b, c The same letters do not differ significantly ($p<0.05$).

higher ADWG in broiler chickens at the grower period. Similarly, Brzoska *et al*. (2013) reported that increasing levels of acidifier significantly enhanced the body weight of broilers at 21 and 42 days of age ($p \leq 0.01$). The level of use, duration, and route of acidifier administration were different in the present study in comparison to previous reports (Zhang *et al*., 2012; He *et al*., 2020; Khalil *et al*., 2020) that found body weight to be higher in chickens fed with acidified rations through the whole period. Our study indicates that the addition of acidifier blend supplements had a positive influence on performance, which is in agreement with previous field trials conducted by (Samanta *et al*., 2010).

Dietary acidifiers can decrease the pH of the diet and of broilers' gut digesta, which depend on both the pH status of the intestine and the pKa value of the specific acidifiers (Kim *et al*., 2005). As expected, increasing the level of acidifiers in the diet can decrease the pH of the diet in a dose-dependent manner (Kil *et al*., 2011). Eventually, the pH of digesta was decreased in different segments of the intestine by the addition of acidifiers to bird diets. Kim *et al*, (2014) indicated that the levels of pH were greatly reduced by acidifiers in the upper segments of the intestine as compared to the lower segments of the gastrointestinal tract (duodenum, jejunum, ileum, and cecum). Decreased pH in the upper portion of the intestine increased nutrient digestibility, which can improve the utilization of nutrients in the diet (Kim *et al*., 2015). The reduction in gastric pH activated pepsinogen and other zymogens in the stomach by regulating stomach acidity to the optimal level for their action (Jongbloed *et al*., 2000), resulting in increased digestion of proteins and other nutrients. Dietary acidification can positively affect growth performance through acidity reduction of the diet and gut, eliminating harmful microbes that are sensitive to low pH or selectively enhancing *Lactobacillus* (Jongbloed *et al*., 2000). Moreover, acidic digestion can slow stomach emptying, providing more time for the digestion of nutrients in the intestine (EA, 1994).

Organ weight and ileum pH

The relative weight of the thymus was only affected by the inclusion of 3 g/kg acidifier compared to the control diet. Pearlin *et al* (2020) indicated that supplementing acidifier at an inclusion level of 3, 6, and 9 g/kg of the diet had no significant effect on the relative weights of carcass, leg and breast muscles, liver, and gizzard (Pearlin *et al*., 2020). A previous study indicated that broiler chickens that were fed

diets supplemented with acidifiers (butyric acid, formic acid) had higher thymus weight (Al-Mutairi *et al*., 2020). Moreover, they indicated that the lymphoid organ weight and immunity were improved by acidifier inclusion at 42 days of age. Acidifier supplementation (1 ml per liter) had no significant effects on the carcass traits (relative weight of breast, thigh, liver, heart, and gizzard) of broiler chickens (Heidari *et al*., 2018).

In our study, the weight of the thymus was increased by the acidifier. Better immune response and disease resistance were reported by the addition of acidifiers to the broiler diet. Regarding this, Katanbaf *et al*, (1989) indicated that the use of acidifiers in the diet causes beneficial immunological progress due to relative organ weight increment (Katanbaf *et al*., 1989). Furthermore, Al-Mutairi *et al*. (2020) reported that lymphoid organ weight was increased by diet acidification at 42 days of age, indicating improved immunity.

Mikulski *et al*. (2008) studied the physiological and growth performance effects of adding acidifiers, acidifiers with essential oils, or herbal extracts to diets on male turkeys, reporting a significant decrease in the pH of the crop digesta, but no effect on the pH of caecal digesta. Paul *et al* (2007) indicated no significant difference was found in the pH of several sections of the gastrointestinal tract (crop, proventriculus, gizzard, duodenum, jejunum, and ileum) because of the addition of different acidifiers (Paul *et al*., 2007). Previous studies (Izat *et al*., 1990; AG, 1991; Hernandez *et al*., 2006) detected no significant differences on gut pH following the supplementation of 53.5 % propionic acid and formic acid. Paul *et al* (2007) concluded this is due to the strong buffering capacity of birds' gastrointestinal tracts.

Blood biochemical parameters

According to the results, cholesterol and HDL levels were the highest in the control treatment compared to other treatments, and it was the lowest in the broilers fed with 3 g/kg of acidifier. Brzoska *et al*. (2013) reported no significant differences were obtained for blood plasma parameters (including glucose, total protein, total cholesterol and HDL cholesterol) when broilers were fed with diets containing acidifier. On the other hand, similar to these results, Khalil *et al*. (2020) showed that the serum LDL and total cholesterol levels were reduced by acidifier inclusion in the diet compared to the control, and HDL was improved without altering triglyceride values. Powell (2000) indicated that bile acids can cause the expanding disintegration of cholesterol, and as a result they may decrease cholesterol levels, while micelle formation suppresses the low pH of digesta content. Also, Soltan (2008) reported this could be associated with a desirable environment in the gastrointestinal tract due to the feeding of acidifiers, which might have helped to digest and absorb more nutrients such as nitrogen and calcium. Engberg *et al*. (2000) reported that acidifiers in the diet significantly reduced serum levels of cholesterol, total lipid, or low-density lipoprotein (LDL). However other researchers reported that the use of an acidified diet for broilers had no effect on total protein and cholesterol blood values (Midilli *et al*., 2004). Hajati *et al.* (2018) observed that the addition of an acidifier to the diet significantly increased serum total protein and albumin levels, as well as AST activity; while it did not cause differences on the other evaluated serum factors, including cholesterol, HDL, triglyceride, VLDL, total lipid concentrations, and ALT activity. Our results when examining enzymes involved in liver and kidney functions showed that the liver and kidney function of broilers might not have been influenced by the addition of an acidifier. This result is similar to the study of Kamal *et al*. (2014) and Adil *et al* (2010), but differed from the findings of Viveros *et al*, (2002), which indicated that acidifier supplementation enhanced serum ALT and AST activity rates (Brenes *et al*., 2003; Adil *et al*., 2010; Khalil *et al*., 2020).

Antibodies response against sheep red blood cells (SRBC)

In our study, the total and specific antibody production titers were not affected by the experimental treatments in any of the experimental periods. Similar to this study, it is reported that the immune response and microbial population broilers were not changed by acidifiers (Heidari *et al*., 2018). Hedayati *et al.* (2014) reported that acidifiers caused no significant difference in antibody titers against Newcastle disease Virus (NDV), inflammatory bowel disease (IBD), and avian influenza (AI). Also, Eftekhari *et al*. (2015) showed that acidifiers in drinking water had no effect on the immune function and antibody titer against NDV of broilers. Sarica *et al*. (2005) reported that the mode of function of feed additives is mainly related to competitive elimination and prohibition of growth and reproduction of pathogens. However, researchers reported that acidifiers could improve immune responses. The density of lymphocytes in lymphoid tissues was increased by feeding 0.5% citric acid to broilers, which led to improvements in non-special immunity (Haque *et al*., 2010).

Microbial population of the ileum

Acidifier treatments had no effect on the total microbial, *Lactobacillus* and coliforms populations in the ileum. While the *Salmonella* population was significantly reduced for 2 and 3 g/kg acidifier levels when compared to the control. Moreover, in line with our results, Heidari *et al*. (2018) reported that intestinal bacterial population such as *Lactobacilli* and *E. coli* were not affected by acidifier treatments in broilers at 24 and 42 days of age. A larger population of pathogenic microbes in the digestive tract of broilers often causes decreased performance. Acidifiers, through the physicochemical case of the outside environment and the physiological conditions of the organism, can have antibacterial mechanisms (Ricke, 2003).

Pathogenic bacteria reproduce in the intestine and damage the intestine villus. Nutrient absorption may consequently decrease with intestinal membrane thickening due to cell multiplication, thus impairing the performance of broilers. The proposed continuous antibacterial mechanism can be explained in various stages (Mani-López *et al*., 2012). Through the pH-mediated reduction in bacterial competition for host nutrients, dietary acidifiers can inhibit the proliferation of pathogenic bacteria. Most pH-sensitive bacteria have minimal reproduction under pH 5, while acid-resistant bacteria survive. The unseparated form of acidifiers can interpenetrate freely into the semi-penetrable membrane of the microbial cell, after which it will separate and release protons (H+), consequently causing a lower pH inside the bacteria cell. A stressful environment is created by low pH, thus reducing cellular function and bacterial multiplication. Finally, the enzymatic responses of glycolysis indicator conveyancing and nutrient transportation of the bacteria are prevented, leading to energy depletion to restore the pH to its baseline level (Mroz *et al*., 2006). Sending out excess protons also demands the use of cellular adenosine triphosphate (ATP). This may cause a discharge of cellular energy and cell death. It has also been considered that acidifiers intervene with cytoplasmic membrane structures and intercellular conveyors as a result of variations in electrical gradients across the cell membrane, which may also be lethal to pathogenic bacterial cells. Bacterial membranes are disturbed by the trapped anions of the acid shift, which are toxic to the metabolism of the cell (Russell, 1992). Acidifiers have the potential to eradicate bacteria through the reduction of intestinal pH and by reverting to their undissociated formation, so that acid resistant bacteria including *Lactobacillus sp*. and *Bifidobacterium sp*. may also suffer from the imbalance between external and internal cellular pH. It is possible that the acid anions are neutralized by the higher rate of internal cell potassium in Gram-positive bacteria (Russell & Diez-Gonzalez, 1997). Coliform bacteria or *Salmonella* are also more susceptible to lower pH compared to lactic acid-generating bacteria or *Lactobacilli* in the intestine, so that dietary acidifiers may have a lower effect on the latter than the former (Kim *et al*., 2009). Thus, acidifiers support the growth of broiler intestinal microflora and therefore improve the condition of the gastrointestinal tract, preventing the growth and development of pathogenic microflora (*Salmonella*, *E. coli*, and others), and pathogenic fungi in the diet and raw materials used in animal feed. The pH and microbial load in the gut of the birds are decreased, the absorption of nutrients is increased, weight gain is improved, and the incidence of digestive disorders is decreased. This eventually leads to an enhancement in the general resistance of the bodies of broiler chickens, enhancing the growth rate and safety of the poultry (Syrovatko, 2021).

Feed is an important factor in *Salmonella* transmission on the farm (Williams, 1981). When broilers are fed with *Salmonella* contaminated diet, the intestine is colonized and *Salmonella* enters the ambiance (Hinton, 1988). Assuming that the entry of Salmonella bacteria is hindered by the acids, it was postulated that incorporating acidifiers into the diet could potentially decrease the incidence of contamination in broiler chickens. It can be concluded that occupation and virulence gene expression of *Salmonella* may be affected by acidifiers (Lawhon *et al*., 2002; Immerseel *et al*., 2004; Gantois *et al*., 2006), and that the normal amount of the acidifiers might play an important role by reducing *Salmonella* colonization. If feed combinations are modified for short-chain fatty acid generation in the caeca, managers and producers could have an efficient and very low-cost method to control *Salmonella*. It can be concluded that the *Salmonella* population and likely that of other potentially infectious bacteria were reduced by the addition of acidifiers, which can have an advantageous effect on the quality of the broilers.

Jejunal morphology

The results of this study showed that crypt depth was significantly increased in the treatments supplemented with 2 and 3 g/kg of acidifier compared to the other treatments. Similar to these results, Heidari *et al*. (2018) showed that acidifiers caused a significant increase in duodenal and jejunal crypt depth in the broilers as

compared to the control treatment at 42 days of age. Panda *et al.* (2009) showed that duodenal crypt depth was improved by butyrate supplementation (2, 4, or 6 g/kg) in broiler diets. In other experiments, Sabour *et al*. (2019) showed that villus height in broilers was higher with 1 g/kg acidifiers supplementation (Sabour *et al*., 2019). In contrast, Adil *et al*. (2010) reported that 30 g/kg butyric acid, 30 g/kg fumaric acid, or 20 g/kg fumaric acid remarkably enhanced villus height in the duodenum, jejunum, and ileum of broilers.

Many researchers have showed that acidifier supplementation have positive effects on villus height, width, and surface of the gut. Experiments show that acidifiers can significantly improve the surface and villus height in the duodenum, jejunum, and ileum of broilers (Rodríguez-Lecompte *et al*., 2012). Leeson *et al*. (2005) and Panda *et al.* (2009) indicated that villus height and crypt depth in the duodenum were enhanced in broilers by adding butyrate to the diet, regardless of the concentrations (0.2%, 0.4%, or 0.6%).

In some experiments on the acidification of the diet of broilers, gut indicators are used as the main factor to check the health status of the intestine (Garcia *et al*., 2007; Eftekhari *et al*., 2015). According to reports in the literature, when the length of intestinal villi increase, the adsorbent area may be enhanced in the small intestine (Eftekhari *et al*., 2015). Garcia *et al* (2007) studied the mechanism of the activity of acidifiers on the gut morphology and reported that the intestinal microbial load was decreased by acidifiers, and that the presence of toxins was decreased by the variation in the gut morphology of broilers. When a disturbance in the normal microflora or the gut epithelium occurs by pathogenic materials, the permeance of this natural barrier may change, therefore accelerating the offensive of infectious bacteria, causing a correction of the metabolism i.e., capability to digest and absorb nutrients, which causes persistent inflammatory responses in the gut mucous membrane (Khan, 2013). Finally, villus height and digestible and absorbing capacities are decreased, but the cell turnover is increased by the addition of an acidifier. In this case, enteric colonization and infected activity were reduced by acidifiers, therefore inflammatory response declined at the enteric epithelium, and the villus height and action of secretion, digestion, and absorption of feed nutrients was improved (Pelicano *et al*., 2005).

Tibia traits

Orthogonal contrast test between acidifier and control treatments showed that the weight and length of bone were significantly increased by acidifiers. The higher value of the tibio-tarsal index indicates a higher level of mineralization in the bone, whereas a lower robusticity index indicates a stronger structure of bone (Mutuş *et al*., 2006).

Mineral chelators containing acidifiers compete positively with phytate by forming soluble compounds with minerals and other nutrients in the lumen of the intestine (Boling *et al*., 2000). The soluble complex that results from this action is easily absorbed by the body, thus increasing nutrient utilization. Snow *et al*. (2004) claimed that dietary supplementation with acidifiers in a diet with 0.39% available phosphorus versus the 0.50% recommended by NRC (1994) enhanced weight gain and mineral utilization in broilers (Snow *et al*., 2004). Boling *et al*., (2000) considered that acidifiers, being a potent chelator of calcium, may form complexes with calcium and reduce its ability to bind phytate, thereby causing phytate to be less constant and more sensitive to endogenous enzymes.

Idachaba *et al*. (2018) reported that all parameters measured (tibia length, weight, ash, calcium, phosphorus, bone density, and leg deformity) showed significant differences with the use of acidifier. This is due to acidifiers increasing phytate hydrolysis by improving the accessibility of minerals for skeletal progression. In the study conducted by Idachaba *et al*. (2018), it was observed that broiler chickens fed with 0.3% acidifier exhibited significantly higher bone density compared to other treatment groups. Moreover, the groups supplemented with 0.1%, 0.2%, and 0.4% acidifiers showed similar results, all of which were significantly superior to the control group.

Gene expression

The expression of fatty acid-binding protein 1 (*FABP1*) and toll-like receptor4 (*TLR4*) genes showed a significant increase with the use of acidifier; increasing levels of acidifier (2 and 3 g/kg), led its expression to also increase. Moreover, *PPARγ* mRNA expression was significantly higher using a 3 g/kg acidifier as compared to the other treatments at 40 days of age.

Changes in *FABP1* mRNA expression levels in the ileal tissue

*FABP*s are a group of intracellular proteins that is extremely expressed in several tissues, with plenty of tissues including more than only *FABP*. Absorption of long-chain fatty acids into enterocytes is controlled by the *FABP1* gene, which is mostly expressed in the enteric epithelial tissue (Banaszak *et al*., 1994; Prows

et al., 1995). *FABP1* is also extremely expressed in hepatocytes, and to a lesser content in the kidneys, lungs, and pancreas (Storch & Corsico, 2008). *FABP2* and *FABP1* have been suggested to operate as intracellular fatty acids (FA) transporters, probably targeting FAs to various subcellular segments and/or metabolic passages relevant to their comparative affinity and selectivity for various ligands. *FABP1* has a similar affinity for saturated and unsaturated FAs (Richieri *et al*., 1994), attaching to two FAs and also other ligands like haem class, sterols, monoacylglycerols (MG), acyl-CoAs, lysophospholipids and endocannabinoids (Huang *et al*., 2016). *FABP1* interrelates with hepatocyte nuclear factor 4α (*HNF4α*), likely mediating inflammatory passages in the intestine and liver (McIntosh *et al*., 2013). In our study, it was shown that increasing levels of acidifier could increase the expression of the *FABP1* mRNA gene in the ileal region. Therefore, it was hypothesized that by lowering the pH level of the intestine, the solubility of fat (and eventually micelle formation) would be reduced in the intestine. Moreover, hydrogen ions generated by low pH can cause the expression of transporter genes such as *FABP1* in epithelial cells, and increase the absorption of fatty acids in the intestine. As a result, birds increase the expression rate of the *FABP1* mRNA gene to compensate for this condition, thus increasing the level of fat absorption in the intestine.

Changes in mRNA expression of TLR4 and PPARγ in the ileal tissue

TLR4 is involved in the recognition of intestinal microorganisms through the attachment to internal or external bacterial productions. The *TLR4* gene is significant for improving damaged enteric tissues (Fukata & Abreu, 2007). Toll-like receptors (TLRs) are related to the defense against pathogens and protection of homeostasis by signaling induced passageways. The existence of conserved microbial structures in the environment is recognized by TLRs, which guide the response of eukaryotic cells. While *TLR2* mostly identifies the bacterial cell wall portions of Gram-positive bacteria, *TLR4* identifies Gram-negative ones (Paul *et al*., 2013). The activity of the transcription factor *NF-κB* is the main signaling aim of the TLRs, and can be a key modulator of immune and inflammatory reactions (Zhang & Ghosh, 2001). Unlike the results of this research, Palamidi *et al*. (2016) indicated that *TLR2*, *TLR4,* and *NF-kB* ileal mucosa expressions were not affected by acidifiers (1 g/kg diet) in diet formulations (Palamidi *et al*., 2016). A probable explanation for this might be that the acidifier-based formulation coverage had no effect on the ileal microorganisms. It is recognized that *NF-κB* signaling is not ubiquitinated or extended by most commensal microbes, and that *TLR4* expression profiles stay low in a healthful digest tract and assist in intestine homeostasis (O'Hara & Shanahan, 2006; Cario, 2010). Moreover, it has been reported that mucin turnover was possibly affected by the instigation of mucin gene expression by gut microorganisms (Smirnov *et al*., 2005).

The majority of experiments on broilers investigate the communication between metabolic passageways, genes, and nutrients. The energy source in the body is maintained as fatty acids, the main functions of which are the composition of plasma membranes, genes adjustment and production of various metabolites. Nuclear-type hormone receptors are described as ligand-activated transcript agents, dependently or independently regulated by a number of genes required in inflammatory signaling and fat metabolism. Peroxisome proliferator-activated receptors (PPARs) are segments of singular transcript agents of nuclear hormone receptors (Royan & Navidshad, 2016). PPARs are one of the main regulators in the progress of adipocytes and fat metabolism (Royan & Navidshad, 2016). PPARs have three various isoforms (α, β/δ, γ) (Michalik *et al*., 2006). Adipogenesis is one of the important processes that illustrate the function of PPARγ as a hub gene. The expression of many genes involved in adipogenesis is stimulated by *PPARγ,* which is a central gene moderator in adipose tissue. Based on lipid metabolism experiments with chicken, it is our theory that *PPARγ* conducts regulative pathway reactions (Royan & Navidshad, 2016). *PPARγ* is expressed in plenty of cell groups, such as adipocytes, endothelial cells, smooth muscle cells, epithelial tissue cells, and numerous other tissues (Law *et al*., 2000; Padula, *et al*., 2000; Spiegelman, 1997). Agonists such as fats, 14-prostaglandin J2, prostaglandin D2 metabolite 15-deoxy-12, and thiazolidinedione have been greatly used in experiments that aimed to show the activity of *PPARγ* in mammalians. This resulted in adipogenesis (Spiegelman, 1997) and insulin sensitization (Lehmann *et al*., 1995) through the operation of PPAR, while these alone are not related to PPARs (Spiegelman & Flier, 1996). It has been reported that *PPARγ* agonists prevent fat metabolism and the production of inflammatory cytokines (Spiegelman, 1998) in peripheral monocytes and macrophages (Spiegelman & Flier, 1996). This information showed that *PPARγ* has a important role in fat storage, energy metabolism, and cell dissociation.

In our experiment, the use of 3 g/kg acidifier remarkably enhanced the expression of the *TLR4*, *FABP1* and *PPAR*γ mRNA in the intestine. Considering that the expression of inflammatory genes increases during inflammation due to the presence of harmful bacteria in the intestine, it can be inferred that acidifiers reduce the acidity of the internal contents of harmful bacteria by reducing the pH of the gastrointestinal tract, which can cause their elimination. Therefore, fatty acid binding proteins (FABPs), which are involved in the extracellular and intracellular metabolism of fats, have better conditions to transport fatty acids for cellular metabolism (Wang *et al*., 2009; Liu *et al*., 2015). The presence of harmful bacteria (such as C. *perfringens*) also causes damage to intestinal tissues (Shojadoost *et al*., 2012), with acidifiers reducing the pH to prevent the growth of these bacteria. Therefore, it can be concluded that the population of harmful intestinal bacteria is reduced by adding acidifiers, which can improve digestion and absorption of nutrients, including fats (Elbayoumi *et al*., 2014). This may increase the expression of the *FABP1* gene. As a result, the population of invasive agents decreases in the small intestine, which may change the expression level of inflammatory factors such as the *TLR4* and metabolic factors such as *FABP1* and *PPAR*γ mRNA. According to these results, it can be stated that the expressions of these three genes are linked together through positive feedback.

CONCLUSION

In summary, the present study showed that acidifier supplementation in the diet improved the performance of broilers in the grower and finisher periods, resulting in a reduced Salmonella population, increased length of the tibia, increased expression of FABP1 (Fatty Acid-Binding Proteins), PPARγ, and toll-like receptors (TLRs) mRNA inflammatory genes. Therefore, the utilization of 3 g/kg of acidifier can be useful to improve the performance of broiler chickens.

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CONFLICT OF INTEREST

There is no conflict of interest of any sort in this work.

ETHICS APPROVAL

Animal welfare statement

The authors confirm that they have adhered to the animal welfare statement in this manuscript, and they confirm that all of the EU standards for the protection of animals and/or feed legislation have been met. The only exception was for stock density. In this case, the final body weight was set to be less than 30kg/m^2, which was lower than that mentioned in council directive 2007/43/EC of June 28, 2007. We also confirm that we have followed the animal welfare guide, as described by FASS (2010). All animal care and experimental procedures were approved by the animal policy and welfare committee of Isfahan University of Technology. This study also followed the ARRIVE guidelines.

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