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Milk Kefir as a Natural Probiotic, Individually or in Combination with Organic Acids in Broiler Chickens: Influence on the Immune-Related Gene Expression, Intestinal Morphology, Microbiota Activity, and Serum Biochemistry

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Abstract

The objective of this study was to examine the influence of the addition of milk kefir (MK), as a natural probiotic to drinking water, individually or in combination with organic acids (OA), on the growth performance, intestinal microbial counts and morphology, serum metabolites and immune status in broiler chickens. Two hundred and fifty one-day-old broiler chicks (Ross 308) were randomly distributed into 4 treatment groups including a control group, and the control group with MK (2% of drinking water), OA (350 mL/1000 L of water), or MK+OA. At d 35, Body weight gain, feed intake, and feed conversion ratio were recorded. At the end of the experiment (d 35), five birds per treatment were selected to determine the bacterial population, serum biochemical metabolites, and intestinal morphology. According to the results, water supplemented with MK increased the mRNA levels of IFN-γ and IFN-β at 35 d in the jejunum ($P < 0.05$). OA supplementation significantly increased the gene expression of IL-6 ($P < 0.05$). On the other hand, the gene expression of IL-12 increased in broilers fed the control diet ($P < 0.05$). The broiler chickens that received MK+OA showed an improvement in feed conversion ratio ($P < 0.05$) compared with OA and control groups. The addition of MK+OA resulted in a higher relative weight of thigh in broiler chickens compared with the MK group ($P < 0.05$), while the relative weight of spleen was lower in OA and MK treatments compared with the MK+OA group ($P < 0.05$). The results also indicated that the addition of MK+OA decreased the ileocaecal *E. coli* population compared to other treatments ($P < 0.05$). Conversely, the birds that received MK+OA had a higher viable count of ileocaecal lactobacilli ($P < 0.05$) compared with the OA group. In conclusion, the combination of OA with MK had beneficial effects on the performance, intestinal immune-related genes, and gut microbiota activity of broiler chickens.

Introduction

Nowadays, there is an increased public concern about the risk of developing cross-resistance of pathogens to in-feed antibiotics in broiler chickens (Rezaeipour *et al.,* 2016).To protect the health and enhance the growth performance of broiler chickens and following the ban of in-feed antibiotics in the poultry industry, using suitable alternatives including probiotics, prebiotics, or organic acids has become popular. The use of organic acids in the diet may damage vehicle surfaces (corrosion) due to moisture absorption and volatility of these acids (Zhu *et al.,* 2014). Therefore, it can be expected that the use of organic acids in drinking water can prevent some of these problems. It has been observed that the addition of organic acids in drinking water has beneficial influences on feed efficiency (Eftekhari *et al.,* 2015), nutrient digestibility (Ragaa and Korany 2016), intestinal morphology (Eftekhari *et al.,* 2015), and microbial population (Chaveerach *et al.,* 2004) in

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broiler chickens. The mode of action of organic acids in the gastrointestinal tract of broiler chickens has been demonstrated in several studies (Roofchaei *et al.,* 2019; Zhu *et al.,* 2014). Briefly, the bacterial cell wall is permeable to organic acids. Therefore, as these compounds enter the bacterial cell, the acidity increases which in turn disrupts the normal function of the bacterium and reduces protein synthesis. (Hedayati *et al.,* 2014).

One of the alternative compounds for growth-promoting antibiotics in poultry is probiotic microorganisms that improve the population of beneficial bacteria in the gastrointestinal tract. It is a well-documented fact that probiotics enhance growth performance (Mountzouris *et al.,* 2010), microbiota activity (Yu *et al.,* 2008), and nutrient digestibility (Li *et al.,* 2008) in broiler chickens. The suggested mechanisms of action of probiotics include gene manipulation, increasing beneficial bacterial strains, and synergistically acting components (Awad *et al.,* 2009). Kefir is an acidic milky product obtained via fermentation of milk using a starter culture that contains a complex mixture of lactic acid bacteria and yeasts (Sarkar, 2008). According to Toghyani*et al.* (2015), Kefir is a milk production fermented by the action of *Lactobacillus, Streptococcus*, and yeasts. Furthermore, it contains polysaccharides, fat, protein, lactic acid, vitamins, and minerals (Magalhães*et al.,* 2011). It is reported that oral administration of kefir had positive effects on the growth performance and meat quality of broiler chickens (Cho *et al.,* 2013). Also, there is a lack of information about the effects of natural probiotics such as kefir and acidified drinking water on the relative gene expression of IL-6, IL-12, IFN-β, and IFN-Y in the jejunum of broiler chickens. We hypothesized that kefir products and acidified drinking water might modulate the immune-related gene expression in chicks. In addition, change in intraluminal pH levels in the small intestine can affect its microflora since certain bacteria are sensitive to pH, e.g. *E. coli, Salmonella spp., Listeria monocytogenes, Clostridium perfringens,* while others are not, e.g. *Bifidobacteria, Lactobacillus spp*(Eftekhari *et al.,* 2015). Therefore, it is hypothesized that a combination between kefir and organic acids in broiler diet may improve gut ecosystem and growth performance.

Therefore, the present study aimed to investigate the influence of an acidifier individually or its mixture with milk kefir (as a natural probiotic) in drinking water on the performance, carcass traits, gut morphology, microbiota counts, serum metabolites, and immune-related gene expression in broiler chickens.

Materials and Methods

Birds and experimental treatments

Two hundred and fifty one-day-old broiler chicks (Ross 308) were provided from a local hatchery and randomly allocated into 4 treatments with 5 replicates of 15 birds per each. The birds were raised in floor pens which were equipped with individual feeders and nipple drinkers and bedded with a layer of wood shavings. A constant lighting program was performed for all experimental groups during a period of 35 days. All diets were in pellet form with different sizes according to the bird's age. The experimental treatments were normal drinking water (as a control group) and the normal drinking water supplemented with either 2% of milk kefir (MK), 350 mL/1000 L of a commercial acidifier containing a mixture of organic acids (OA), and a combination of MK and OA. The acidifier product (AGROCID, Belgium) contained formic acid, propionic acid, lactic acid, ascorbic acid, and citric acid. Feed and water were provided *ad libitum* to the birds. Table 1 indicates the ingredients and chemical composition of the basal diet.

Gene expression

At the end of the experiment, one chick per pen, selected at random, was sacrificed by cervical dislocation, and 1 cm of the upper jejunum (approximately 10 cm below bile duct entrance into distal duodenum) was collected, and snap-frozen in liquid nitrogen. About 30 mg of powdered tissues were used to extract RNA using Tripure isolation reagent (Roche, Switzerland). Isolated RNA integrity was determined by gel electrophoresis, and its quality and quantity were measured by a Nanodrop spectrophotometer (TC 100, USA). For cDNA synthesis1µl of total RNA was reverse-transcribed to cDNA using a gene-specific stem-loop primer (Fattahi *et al.,* 2017), in the presence of 1µl (200 U) RevertAid Transcriptase (Thermo Fisher Scientific, USA), 5× reaction buffer, 100mM dNTPs (Qiagen, Germany) in a final volume of 20 µl, by incubation at 25℃ for 10 min, 42 °C for 1 hr, and 70 °C for 10 min.

The qPCR reactions were performed using Step One Real-Time PCR (Applied Biosystems, USA). Specific cDNA level was quantified by TaqMan universal probe, universal reverse primer, and specific forward primers for each cDNA Hot Star-Taq DNA polymerase (Qiagen, Germany) were used for evaluation of gene expression in 20µl reaction volume containing specific and universal primers and, probe 10X PCR buffer, $MgCl_2$ (25 mM), dNTPs (10 Mm) and Rox. The amplification protocol consisted of an initial denaturation at 95 °C for 15 min followed by 45 cycles, each including 15 s denaturation at 95 °C, and 1 min annealing at 60 °C. The RNA level of GAPDH was used to calibrate all other RNA levels. The sequence of the TaqMan probe was [FAM] 5' TGGATGTGTCTGCGGCGTTTTATCAT 3' [BHQ-1] and the sequence of reverse primer was 5' GTATCCAGTGCTGCGACCGT 3'. The sequences of IL-6, IL-12, IFN-β, IFN-Y, and GAPDH primer pairs are listed in Table 2.

Table 1. The ingredients and chemical composition of basal diet.

Item	Starter	Grower	Finisher
	d 1 to 10	d 11to 24	d 25 to 35
Ingredients (g/kg)			
Corn grain	557	578.1	620
Soybean meal (440 g CP/kg)	376	352.1	303
Soybean oil	18.6	29.1	40.1
Oyster shell	13.6	11.1	10.2
Dicalcium phosphate	18.1	16.2	14.7
Common salt	1.5	2.0	1.5
Sodium bicarbonate	3.5	2.7	2.5
Vitamin premix[1]	2.5	2.5	2.5
Mineral premix[2]	2.5	2.5	2.5
DL-Methionine	3.2	2.5	2.1
L-Lysine-HCl	2.0	1.0	0.8
Choline chloride	0.5	-	-
L-Threonine	1.0	0.2	0.1
Chemical composition			
Metabolizable energy (Kcal/kg)	2950	3050	3150
Crude protein (%)	21.94	20.90	19.07
Calcium (%)	1.02	0.87	0.79
Available Phosphorous (%)	0.49	0.46	0.40
Sodium (%)	0.20	0.18	0.16
Lysine (%)	1.39	1.25	1.11
Methionine + Cystine (%)	1.03	0.92	0.83
Threonine (%)	0.94	0.83	0.75

[1] Provides per kilogram of diet: (Starter:13,000 IU vitamin A; 5,000 IU vitamin D_3; 80 IU vitamin E; 3.2 mg menadion; 3.2 mg thiamine; 8.6 mg riboflavin; 65 mg niacin; 5.4 mg pyridoxine; 17 µg vitamin B_{12}; 20 mg pantothenic acid; 2.2 mg folic acid; 0.3 mg biotin; 1700 mg choline chloride; and 9.4 mg antioxidant.), (Grower: 11,000 IU vitamin A; 4,500 IU vitamin D_3; 65 IU vitamin E; 3 mg menadion; 2.5 mg thiamine; 6.5 mg riboflavin; 60 mg niacin; 4.3 mg pyridoxine; 17 µg vitamin B_{12}; 18 mg pantothenic acid; 1.9 mg folic acid; 0.25 mg biotin; 1600 mg choline chloride; and 8.85 mg antioxidant.), (Finisher: 10,000 IU vitamin A; 4,000 IU vitamin D_3; 55 IU vitamin E; 2.2 mg menadion; 2.2 mg thiamine; 5.4 mg riboflavin; 45 mg niacin; 3.2 mg pyridoxine; 11 µg vitamin B_{12}; 15 mg pantothenic acid; 1.6 mg folic acid; 0.2 mg biotin; 1500 mg choline chloride; and 8.25 mg antioxidant.).

[2] Provides per kilogram of diet: 120 mg Mn; 110 mg Zn; 20 mg Fe; 16 mg Cu; 1.25 mg I; and 0.3 mg Se.

Table 2. Sequences of primers used in stem-loop Taqman assay

The genes	Primer sequence 5' ------->3'
IL-6	**Specific forward primer** CCAGAAATCCCTCCTCGCCAATC
	R-Stem Loop IL-6 GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACG ACCTCACGGTCTTC
IL-12B	**Specific forward primer** AACTACACCTGCCTGTCTGCTAAG
	R-Stem Loop IL-6 GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACG ACCCCATTGGAGTC
IFN-β	**Specific forward primer** CCTTCAGAATACGGCTCCACCTC
	R-Stem Loop IL-6 GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACG ACGATGGCTGCTTG
IFN-Y	**Specific forward primer** AGTCAAAGCCGCACATCAAACAC
	R-Stem Loop IL-6 GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACG ACTCACGCCATCAG
GAPDH	**Specific forward primer** GCACGCCATCACTATCTTCCAG
	R-Stem Loop IL-6 GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACG ACCGCTTAGCACCA

Growth performance
To determine the growth performance variables including the body weight gain, feed consumption, and feed conversion ratio (FCR), all broiler chickens of each replicate pen and their feed intake were weighed at 10, 24, and 35 d of age. Subsequently, FCR was calculated by dividing feed intake by body weight gain of each pen. At the end of the trial (35 d of age) and to evaluate the carcass characteristics, 2 broiler chickens per pen were selected, individually weighed, and killed by cervical dislocation. Immediately, the weight of the thigh and breast as the major parts of the carcass, and the weight of the liver, pancreas, spleen, and the length of the intestine as internal organs were determined. The obtained data were presented as a percentage of live weight.

Microbial population
To determine the microbial population, at the end of the experimental period, 5 broilers were selected and slaughtered for each treatment. Then, 5 g of the contents of the ceca region was collected under sterile conditions. The samples were immediately transferred to the laboratory for evaluation of viable cell counts of *Escherichia coli, Lactobacilli,* and total bacterial count. Briefly, dilution of the samples was performed from 1/10 to $1/10^7$ using sterilized physiological saline solution (NaCl 85%) and in the range. These diluted samples were then cultured on specific media. Eosin methylene blue agar (Merck, Darmstadt, Germany) and de Man, Rogosa, Sharpe agar (Merck, Darmstadt, Germany) cultures were used for *Escherichia coli* and *Lactobacilli*, respectively. *Escherichia coli* were cultured on associated media at 37°C for 24 h, while *Lactobacilli* bacteria were counted after incubation for 48 to 72 h at 37°C.To determine the total bacterial count, a standard plate count agar (Merck) was used. Then, the obtained data from each strain were reported as the log 10 of colony-forming units (CFU) per g of sample.

Jejunum morphology
A 2-cm piece of the middle of the jejunum from 6 broiler chickens per treatment was excised for morphometric analysis. The jejunum is the part of the intestine between the end of the duodenum and Meckel's diverticulum. The jejunum samples were immediately placed into the tubes containing 10% formalin to prevent tissue damage. In the next step, 0.5 cm of each sample was embedded in paraffin and stained using eosin blue. The 10 longest and straightest villi and associated crypts were recorded in each sample.

Serum metabolites
On day 35 (end of the experimental period), to measure the serum biochemical parameters, the blood samples were taken from 5 broilers for each treatment via the wing vein. In the next step, the obtained samples were centrifuged at 5000 × g for 5 minutes at 23 °C. The isolated serum was then used to measure the serum concentrations of glucose, cholesterol, triglycerides, and high-density lipoprotein-cholesterol (HDL-c) by spectrophotometer (Shimadzu, Kyoto, Japan) using commercial kits (Pars Azmoon Company, Tehran, Iran).

Statistical analysis
The data obtained in this study were statistically analyzed based on a completely randomized design using one-way analysis of variance in PROC ANOVA using SAS software (SAS, 1999). Mean comparison between experimental treatments was performed using the Tukey test at $P < 0.05$.

Results
Gene expression
Figure 1 shows the expression of studied genes relative to GAPDH as a reference gene in different groups. Results indicated that supplementing water with MK as well as OA has more regulatory effects on studied genes, except for IL-12. Chicken fed with MK+OA had lower expression of IFN-γ, IFN-β, Il-6, and Il-12 genes rather than the control group in the jejunum. Those chicken who received MK had higher levels of IFN-γ and IFN-β ($P < 0.05$). Administration of OA significantly increased the expression of the IL-6 gene ($P < 0.05$).

Growth performance and carcass characteristics
The effects of the experimental treatments on the growth performance of broiler chickens are presented in Table 3. The experimental treatments had no significant effect on the body weight gain and feed intake in broilers. However, the broilers who received MK in combination with OA showed an improvement in feed conversion ratio ($P < 0.05$).

The results of carcass characteristics are shown in Table 3. The relative weight of the thigh and spleen were affected by the treatments ($P < 0.05$). According to these results, the supplementation of MK in combination with OA in drinking water increased the relative weight of the thigh and spleen in broiler chickens.

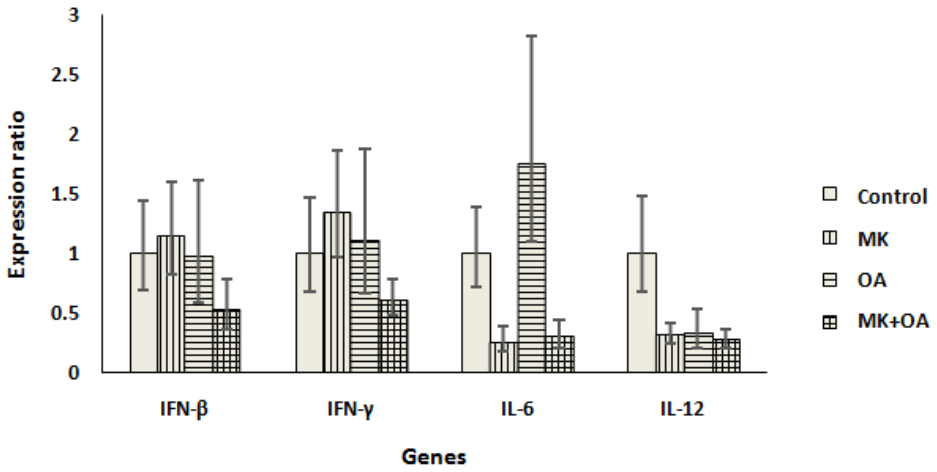

Figure 1. Expression ratio (mean ± standard error) of interferon beta (IFN-β), interferon-gamma (IFN-γ), interleukin 6 (IL-6), and interleukin 12 (IL-12) genes relative to GAPDH gene in jejunum samples of 35-day-old broiler chickens in different dietary groups ($P < 0.05$). MK: milk kefir; OA: organic acids.

Table 3. Effects of milk kefir and organic acids on weight gain, feed intake, feed conversion ratio (FCR), and carcass characteristics (g/100 g body weight of bird) in broiler chickens

Parameters	Treatments				*SEM*	*P*-value
	Control	OA	MK	OA+MK		
Weight gain (g/bird/d)	52.35	50.97	51.78	52.90	1.17	0.33
Feed intake (g/bird/d)	93.24	90.64	90.45	90.80	0.87	0.21
FCR	1.78^{a}	1.77^{a}	1.74^{ab}	1.71^{b}	0.02	0.04
Breast	33.13	33.89	33.41	33.51	0.57	0.82
Thigh	20.48^{ab}	20.64^{ab}	18.98^{b}	21.60^{a}	0.69	0.03
Liver	2.41	2.25	2.17	2.27	0.07	0.21
Spleen	0.11^{ab}	0.10^{b}	0.10^{b}	0.12^{a}	0.004	0.02
Carcass	60.36	59.29	57.52	62.33	1.23	0.10

^{ab}Means with different superscripts in each row are statistically significant ($P< 0.05$).
OA: organic acids
MK: milk kefir
SEM: standard error of the mean

Microbial population and intestinal morphology

Data for the ileal population of *E. coli*, *Lactobacilli,* and total bacterial count are presented in Table 4. The total viable count of bacteria including obligate and facultative did not affect by the experimental treatments. The addition of OA in combination with MK decreased the *E. coli* population compared to the other treatments ($P<0.05$). On the other hand, a comparison of means obtained from the experiment showed that the birds that received OA in combination with MK had a higher viable count of *Lactobacilli*. The results of intestinal morphology showed no statistical differences among treatments(Table 4).

Table 4. Effects of dietary treatments on the ileocecal microbial population and intestinal morphology in broiler chickens

Items	Treatments				*SEM*	*P*-value
	Control	OA	MK	OA+MK		
E. coli($\log_{10}$ cfu/g)	9.12^{a}	9.00^{a}	8.92^{a}	8.06^{b}	0.12	0.001
Lactobacilli($\log_{10}$ cfu/g)	7.40^{ab}	7.18^{b}	7.32^{ab}	7.68^{a}	0.13	0.03
Total count ($\log_{10}$ cfu/g)	10.18	10.11	10.04	9.98	0.14	0.33
Villus length(µm)	1109	1181	1121	1166	30.96	0.33
Villus width(µm)	136.72	142.75	140.65	137.97	3.61	0.65
Crypt depth(µm)	244.50	248.48	248.33	231.54	7.37	0.35
Villus length/villus width	8.11	8.27	7.97	8.45	0.67	0.21

$^{a, b}$ Means with different superscripts in each row are statistically significant ($P< 0.05$).
OA: organic acids
MK: milk kefir
SEM: standard error of the mean

Serum metabolites

The results of the effects of dietary treatments on the serum metabolites in broiler chickens are shown in Table 5. According to the results, all serum biochemical parameters did not influence by the experimental treatments.

Table 5. Effects of dietary treatments on the blood metabolitesin broiler chickens.

Items	Treatments					
	Control	OA	MK	OA+MK	*SEM*	*P*-value
Glucose (mg/dL)	224.1	218.7	218.5	238.0	10.11	0.51
Cholesterol (mg/dL)	135.2	129.0	131.1	133.4	3.22	0.59
Triglyceride (mg/dL)	67.75	66.01	71.1	67.50	4.01	0.35
HDL-c (mg/dL)	76.50	80.25	77.25	76.40	3.21	0.71

OA: organic acids
MK: milk kefir
SEM: standard error of the mean
HDL-c: high-density lipoprotein-cholesterol

Discussion

This research was conducted to investigate the effects of the addition of Kefir and/or an acidifier to broilers' diet on the expression of interferon-gamma (IFN-γ) and interferon beta (IFN-β), interleukin 6 (IL-6), and 12 (IL-12). The immunological role of chicken gut-associated lymphoid tissue (GALT) is important for reducing the incidence of broiler enteric diseases (Bai *et al.,* 2013). The activation of the immune system, inflammatory reactions, and consequently the production of cytokines are stimulated by a variety of factors, including viral, bacterial, or parasitic infections, cancer, inflammation, or interaction between T cells and antigens (Ghareeb *et al.,* 2013). Interleukins are a subclass of cytokines that regulate immune responses. In the present experiment, MK supplemented diet increased the mRNA levels of IFN-γ and IFN-β at 35 d in the jejunum of chickens. On the other hand, OA administered to chickens, significantly increased the expression of the IL-6 gene. In parallel with these findings, Adhikari and Kim (2017) reported that lactic acid bacteria increased the production of IFN-γ in the intestinal epithelium of broiler chicken. Also, Gaddee*t al.* (2017) indicated an elevated expression of the IL6 gene in the ileum of chickens supplemented with *Bacillus subtilis*-based probiotic. Diet supplemented with *Lactobacillus Plantarum* P-8 increased levels of IFN-γ and IL-12 transcripts in the jejunum of broiler chickens on day 14 (Wang*et al.,* 2015). In a previous report, the expression of the IFN-γ gene was up-regulated in cecal tonsil cells after treatment with *Lactobacillus acidophilus* DNA (Brisbin *et al.,* 2008). The present results indicated that supplementing water with both kefir and acidifier had regulatory effects on the studied genes. This kind of water supplement might have some positive influence on the chicken intestinal microbial population, which was confirmed with lower *Coliforms* and higher Lactobacilli jejunal populations in the current study. The presence of probiotics in the digestive system is directly related to the important physiological changes such as decreased luminal pH, secretion of bioactive peptides, reduction of pathogenic bacteria and also preventing the adhesion of these invasive bacteria to the epithelial cells (Otutumi*et al.,* 2012). On the other hand, it has been reported that altered gene expression of cytokines such as IFN-γ and IL-12 in the poultry gut-associated lymphoid tissues is related to probiotics (Haghighi *et al.,* 2008). Cytokines such as IL-12 and IFN-γ are important in cell-mediated response against intracellular pathogens and macrophage activation (Palamidi*et al.,* 2016). It is suggested that the decreased cytokine levels may be associated with *L. acidophilus* protective effects on the chicken gut health and inflammation amelioration (Li *et al.,* 2018).

In the present study, broiler chickens that received OM+MK treatment had a better feed conversion ratio from 1 to 35 d of age than the other groups. The beneficial effects of organic acids on the growth performance of broiler chickens have been studied by some authors (Hashemi *et al.,* 2012; Eftekhari*et al.,* 2015; Ragaa and Korany 2016). Furthermore, Toghyani *et al.* (2015) and Cho *et al.* (2013) reported that the addition of milk kefir in drinking water or as oral administration improved the broiler performance. However, the possible interaction additive effects of organic acids in combination with a natural probiotic such as kefir on the broiler growth have not been fully demonstrated. It is well documented that the positive effect of organic acids on the growth performance of broiler chickens may be firstly related to the alteration of viable counts of bacteria in the gastrointestinal tract. Considering this mechanism, the potential of nutrients absorption may be enhanced in the intestinal epithelial cells of broiler chickens. Furthermore, Kandir and Yardimci (2015) observed an improvement in ducks' growth performance treated by the addition of kefir in their drinking water. Therefore, it is suggested that the addition of OA in combination with MK has the potential to increase the growth performance of broiler chickens.

It is well documented that the gut microbial population such as *Lactobacilli* has a major role in

the health, immunity, nutrition, and physiological status of broiler chickens. On the other hand, the negative effect of some pathogenic bacteria such as Coliform on the infectious enhancing was observed. According to the results of the present experiment, the addition of OA in combination with MK increased the cecal population of *Lactobacilli* while the viable cell counts of *E. coli* declined. These results are supported by Eftekhari*et al.* (2015) who reported that the addition of organic acids to drinking water improved the intestinal population of *Lactobacilli* in broiler chickens. Furthermore, the addition of MK had an inhibitory effect on harmful bacteria like *Coliforms* in laying hens (Yenice*et al.,* 2014). The range of gut acidity (pH) is one of the most important factors to microbial competition and following the suppression of harmful bacteria such as *E.coli* and *Coliforms* in poultry (Sirikene*t al.,* 2003). However, it was observed that the use of kefir as a probiotic additive did not have a considerable effect on the pH of the small intestine, while this decrease in pH was significant in the large intestine of laying hens (Yenice*et al.,* 2014). Therefore, it is concluded that the alteration of microbiota activity in broiler chickens following the use of OA may be due to its effect on intestinal acidity.

Conclusion

According to the present study, it can be concluded that the synergistic effects of the combined use of acidifier and kefir (as a natural probiotic) in the drinking water of broiler chickens are manifested in terms of improvement of growth performance, intestinal microbial population, and immune-related genes expression.

Declaration of conflicting interests

The authors declare that they have no conflict of interest.

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