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Dietary Supplementation of Different Levels of Phytogenic Feed Additive in Broiler Diets: The Dynamics of Growth Performance, Caecal Microbiota, and Intestinal Morphometry

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ABSTRACT

The present study was conducted to investigate the influence of different levels of dietary phytogenic feed additive (PFA) on growth performance, caecal microbiota, and intestinal morphology of broilers. A total of 480 Ross-308 one-day-old male broilers chicks (body weight 43±3 g) were randomly assigned to 32 replicate pens of four experimental groups, each experimental group consisting of 8 replicates (each replicate pen consisting of 15 chicks). A basal diet was formulated based on corn and soybean meal that was fed to the control group. Other dietary treatments received a commercial PFA at 100 mg/kg (PFA100), 125 mg/kg (PFA125), and 150 mg/kg (PFA150). Body weight gain, feed intake, and feed conversion rate of broilers were recorded on 1-21, 22-42, and 1-42 days of age. One bird was slaughtered on the 21st and 42nd days and caecal contents were aseptically collected. Jejunal tissue samples were also collected on the same days. Total aerobic bacteria, coliforms, *Escherichia coli*, and lactobacilli were counted in the caecal contents. Villus height, villus diameter, crypt depth, muscular thickness, and goblet cell number per villus were recorded. There was no difference among the dietary treatments for growth performance and caecal microbe populations at any phase. However, the dietary PFA supplementation increased the villus height, villus width, muscularis thickness, and reduced the crypt depth and goblet cell number per villus in broilers compared to those fed control diets. In conclusion, this study suggests that dietary supplementation of a PFA consisting of blend of different spices and essential oils did not improve growth performance and caecal microbial populations despite a positive improvement in the jejunal morphometry of broilers.

INTRODUCTION

Sub-therapeutic levels of antibiotic growth promoters (AGPs) remained in practice for more than 50 years to achieve the growth targets until questioned for growing concerns to antibiotic resistance (Kabir, 2009) and decreasing efficacy of antibiotics used for medical purposes (Dibner & Richards, 2005). New pathogens have emerged over the last 25 years, some of them prompted from animals, probably a consequence of irrational use of antibiotics. Most of the pathogenic organisms of medical importance seem to have crossed the species barrier from the animals (Ahsan *et al.*, 2016). The use of AGPs is, therefore, being repressed by public that resulted in a complete ban on the use of in-feed AGPs in 2006 by the EU (Vesna *et al.*, 2007). This has attracted the scientists' consideration to develop the alternatives to AGPs in animal nutrition. Phytogenic feed additives (PFAs) have recently gained the attention with their prospective among such alternatives to spare the use of AGPs (Murugesan *et al.*, 2015; Wati *et al.*, 2015). The botanical products with their source belonging to herbs, spices,

essential oils, or oleoresins are referred to as PFAs. Herbs and spices are commonly used as whole plant or their parts. Essential oils are secondary metabolites of odoriferous plants, that have higher biological functions compared with raw materials from which it is derived (Yitbarek, 2015). Usually, the essential oils are comprised of two main compounds i.e. terpenoids and phenylpropanes (Lee *et al.*, 2004). These phytogenic compounds consist of many bioactive biomolecules such as anethole, allicin, allyl-isothiocyanate, cineole, carvacrol, capsaicin, linalool, piperine, and thymol that possess numerous beneficial properties in poultry health and growth performance (Ruberto *et al.*, 2002; Burt, 2004; Puvaca, 2008; Windisch *et al.*, 2008; Yang *et al.*, 2009; Applegate *et al.*, 2010; Hippenstiel *et al.*, 2011; Puvaca *et al.*, 2013).

PFA have been known to improve the performance (Jamroz & Kamel, 2002; Ciftci *et al.*, 2005; Windisch *et al.*, 2008; Hong *et al.*, 2012), nutrient digestibility (Hernandez *et al.*, 2004; Amad *et al.*, 2011; Mountzouris *et al.*, 2011), digestive enzyme activity (Windisch *et al.*, 2008), and reduce the pathogenic bacterial population (Brenes & Roura, 2010; Reisinger *et al.*, 2011) in broilers. Despite these improvements and beneficial effects, some studies have reported no impact, or negative impact on performance, gut morphology, and gut microbiota (Muhl & Liebert, 2007; Abildgaard *et al.*, 2010; Hafeez *et al.*, 2016). Buchannan *et al.* (2008) reported that the wide range of composition of PFAs may improve or decline the growth performance of broilers. This may be attributed to the variations in the chemical compositions of PFAs that is governed by different factors including location or origin, plant species, growth stage, harvesting time, soil type, climate and stress condition, cultivation practices, fertilization, and irrigation (Daferera *et al.*, 2003; Yitbarek, 2015). Moreover, the composition of bioactive biomolecules in PFAs also varies depending on the parts of the plant used such as seeds, leaf, wood, or bark (Yitbarek, 2015). In addition, the biological properties of PFAs differ based on the source (plant) from which they are extracted (Lawrence ad Reynolds, 1984; Jang *et al.*, 2004; 2007). The composition of PFAs plays a very important role in determining the effectiveness when they are blended due to the fact that bioactive molecules may behave differently when fed as a blend compared to when fed alone (Jang *et al.*, 2004; 2007). Despite the substantial attention being given to use a combination of phytogenic extracts, the results have been inconsistent in terms of their efficacy. There is a need to optimize the dietary supplementation levels of PFAs. Therefore, we hypothesized that gradual increasing levels of a dietary phytogenic product containing a mixture of different essential oils and parts of plants that may help overcome the distorted responses of dietary PFAs. Probably, the inclusion levels of PFAs would be different for various criterions like maximum growth performance, better gut morphology and gut ecosystem, and microbiota. Hence, the aim of the present study was to evaluate the effect of different levels of dietary PFAs on growth performance, intestinal microbiota, and intestinal morphology of broilers.

MATERIALS AND METHODS

The present study was carried out at the poultry research and experimental unit of the Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın, Turkey. A written approval was obtained from the animal care and use committee of Adnan Menderes University prior to conduct the study (letter No. 64583101/2015/128).

Management of birds

The experimental area was thoroughly cleaned, fumigated, well heated at a constant temperature of 32 °C a week before the arrival of the chicks. A total of four hundred eighty male ROSS 308 one-d-old broiler chicks were obtained from a local commercial hatchery located nearby Aydın. The chicks ranging their body weight 43±3 g were randomly allocated to 32 replicate pens in four equal experimental groups, each consisting of 8 replicate pens with 15 chicks in each pen. The trial lasted for 42 days. Each replicate pen provided a floor space of 0.07 m^2/bird. Birds were allowed *ad libitum* access to feed through floor feeders during starter period (d 1 to 10) and tube feeders from d 11 and onwards. Each pen was equipped with three nipple drinkers. A deep layer of wood shavings with 6 to 8 cm height served as bedding material. Fluorescence lights were illuminated during the dark period of the day so as to provide a continuous light. The temperature was maintained at 32 °C in the first week that was reduced 0.5 °C per day onwards in order to attain a constant temperature of 24 °C. Relative humidity was maintained between 50 to 60% throughout the study period.

Experimental design and dietary treatments

Corn/soybean meal-based basal diets were formulated (Table 1) for starter (d 1-10), grower (d 11-24), and finisher (d 25-42) according to the

recommendations by Aviagen (2014). Basal diet without supplementation was randomly fed to one of the four experimental groups defined as control group. The other experimental groups were fed the basaldiet supplemented with the phytogenic product Digestarom® (BIOMIN Holding GmbH, Getzersdorf, Austria) at 100 mg/kg (PFA100), 125 mg/kg (PFA125), and 150 mg/kg (PFA150). Digestarom® is a powdery blend of essential oils, spices, and functional flavours. Each kg of Digestarom® contains cinnamon 20 g, cumin 20 g, peppermint oil 170 g, garlic oil 150 g, anise oil 50 g, fennel oil 40 g, and SiO_2 and NaCl as carrier.

Table 1 – Composition of basal diets for broiler's starter, grower, and finisher phases.

Ingredients	Starter	Grower	Finisher
		%	
Corn	55.63	56.15	33.50
Soybean meal (48% CP)	37.50	36.00	33.50
Vegetable oil	2.50	4.15	4.25
Limestone	0.89	0.85	0.80
Dicalcium phosphate	2.30	2.00	1.73
Salt	0.35	0.35	0.35
DL-Methionine	0.37	0.25	0.12
L-Lysine sulphate	0.21	-	-
Vitamin-mineral premix[1]	0.25	0.25	0.25
Nutrient Composition of Diets (%)			
Crude protein	21.75	20.89	19.92
Metabolizable energy (Kcal/kg) (Calculated)	2910	3029	3070
Crude fiber	3.47	3.39	3.30
Crude ash	5.70	5.33	4.95
Calcium	0.97	0.88	0.79
Available phosphorus	0.54	0.48	0.43
Digestible lysine (Calculated)	1.17	1.02	0.96
Digestible methionine (Calculated)	0.66	0.54	0.40

[1]Vitamin and mineral premix (per kg of diet): retinol acetate, 1706 mg; cholecalciferol, 41 mg; DL-α-tocopherol, 27 mg; menadione, 0.99 mg; cobalamin, 0.015 mg; folic acid, 0.8 mg: D-pantothenic acid, 15 mg; riboflavin, 5.4 mg; niacin, 45 mg; thiamine, 2.7 mg; D-biotin, 0.07 mg; pyridoxine, 5.3 mg; manganese, 90 mg; zinc, 83 mg; iron, 121 mg; copper, 12 mg; iodine, 0.5 mg; selenium, 0.3 mg

Growth performance

Weekly pen body weight (BW) and feed intake (FI) were recorded using an electrical weight balance with minimum accuracy of ±1 g. Body weight gain (BWG) and feed conversion ratio (FCR) were calculated for each pen. BW of the dead birds was added in the pen weight in order to adjust the FCR. BWG, FI, and FCR were reported for the phases comprising the days 1-21, 22-42, and 1-42.

Caecal microbe count

On d 21 and 42 of the experiment, one bird from each pen (8 birds per experimental group) was randomly selected for slaughtering. The birds were slaughtered in a clean slaughter room by cutting the jugular vein. Intestines were exposed following the removal of feathers. Caecal digesta samples were aseptically collected from one bird in each replicate (8 samples per treatment) slaughtered for sampling at d 21 and 42 of the experiment. For this purpose, the caeca were ligated and carefully hand-stripped into sterile plastic bags. In the present study, total aerobic bacteria, *Escherichia coli*, coliforms, and lactobacilli were enumerated in the caecal digesta samples according to the procedures previously described by Cengiz *et al.* (2012). The caecal digesta samples were appropriately stored at -80 °C. At the time of microbiological analysis, the samples were thawed and immediately 10-fold diluted with sterile ice-cold anoxic phosphate buffered saline followed by homogenisation in stomacher for 3 min (Bagmixer 100 Minimix, Interscience, Arpents, France). In order to count the bacterial populations, 10^{-1} to 10^{-7} serial dilutions were prepared in buffered peptone water followed by culturing of each dilution on to the selective media. Nutrient, de Man Rogosa Sharpe, violet red bile lactose, and MacConkey agar (Oxoid™, Hampshire, UK) were used for total aerobic bacteria, lactobacilli, coliform, and *E. coli* count, respectively. The culture plates were subsequently incubated at 37 °C for 24 h for total aerobic bacteria, *E. coli*, and coliforms. The culture plates intended for lactobacilli count were incubated at 30 °C in a microaerobic environment. The colony forming units (cfu) $\log_{10}$/g of digesta were counted based on the colony morphology and characteristics of the particular bacteria. *E. coli* colonies were further subjected to IMViC reactions for further confirmation.

Gut morphology

Meckel's diverticulum was identified as a benchmark, 2 cm portion of jejunum was dissected, washed in normal saline solution, and fixed in 10% neutral buffered formalin. Excessive fixation was removed by washing the tissues in running water followed by dehydration in ascending grades of alcohol as dehydrating agent. Dehydrating agent was cleared from tissues using pure xylene as clearing agent and infiltrated with melted paraffin wax as embedding agent. Afterwards, the jejunal tissues were embedded in paraffin blocks, six sections of 2 µm were cut from paraffin block of each sample using a microtome. The gap between the sections of each block was maintained 100 µm. Three of the six 2 µm sections were stained with hematoxylin and eosin after being mounted on the glass slides for histological analysis. In

order to enumerate the goblet cell number per villus, three remaining sections were stained with periodic Acid-Schiff (PAS) stain. The sections were observed under fluorescence microscope (Leica DMLB, Germany) coupled with digital imaging analysis system (Leica DC200, Germany). Villus height, villus diameter, crypt depth, and muscularis layer thickness were measured as 5 replications per section (15 measurements per bird) with the help of an image analysis program (Leica QWin Standard, Version 2.8, Germany). The goblet cell number per villus were counted manually in the middle 100 µm portion of the PAS-stained sections.

Statistical analysis

Shapiro-Wilk's test was applied to test the normality of the data. Non-normalized traits were transformed using log or square root transformation. Data were statistically analysed with one-way analysis of variance in a completely randomized design using a computer statistical software package SPSS (Version 17.0; SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was applied as a post-hoc test. Confidence interval was set at 95% (p<0.05). Results were presented as Mean ± SEM.

RESULTS

The present study revealed that varying levels of dietary PFAs had no significant effect on growth performance (Table 2) and gut microbe populations (Table 3). However, the gut morphology was affected in response to dietary PFAs on 21 and 42 days of age

Table 2 – Rearing characteristics of broilers in response to different dietary levels of PFAs (n=8).

Treatments	Body Weight Gain (g)			Feed Intake (g)			Feed Conversion Ratio		
	d 1-21	d 22-42	d 1-42	d 1-21	d 22-42	d 1-42	d 1-21	d 22-42	d 1-42
Control	1059±10.6	1834±46.7	2893±47.4	1377±11.1	3738±44.3	5115±52.0	1.30±0.01	2.05±0.05	1.77±0.02
PFA100	1066±5.80	1795±61.5	2860±62.9	1388±7.00	3817±74.1	5206±75.1	1.30±0.01	2.14±0.08	1.83±0.04
PFA125	1050±16.2	1758±57.6	2808±55.8	1357±11.2	3630±54.0	4986±61.2	1.30±0.02	2.08±0.05	1.78±0.02
PFA150	1057±10.3	1800±62.8	2856±68.8	1372±13.1	3723±49.0	5095±58.9	1.30±0.01	2.08±0.05	1.79±0.03
p-value	0.802	0.831	0.788	0.241	0.161	0.125	0.960	0.715	0.599

Table 3 – Total aerobes, coliforms, *E. coli*, and *Lactobacilli* count (log_{10}cfu/g) in caecaldigesta of broilers in response to different dietary levels of PFAs (n=8).

Treatments	Day 21				Day 42			
	Total Aerobes	Coliforms	*E. coli*	*Lactobacilli*	Total Aerobs	Coliforms	*E. coli*	*Lactobacilli*
Control	9.17±0.16	6.89±0.23	5.49±0.21	7.11±0.08	8.87±0.19	7.68±0.16	7.52±0.12	8.07±0.15
PFA100	9.14±0.10	7.00±0.20	4.76±0.82	7.20±0.26	8.49±0.20	7.46±0.31	7.29±0.32	8.43±0.13
PFA125	9.02±0.14	7.10±0.18	5.90±0.25	7.31±0.17	8.88±0.20	7.54±0.21	7.41±0.17	8.72±0.15
PFA150	8.93±0.15	6.62±0.38	4.44±0.83	7.29±0.28	8.76±0.26	7.95±0.33	7.83±0.34	8.49±0.23
p-value	0.616	0.589	0.274	0.883	0.551	0.585	0.492	0.110

(Table 4 and 5). Villus height was significantly higher at d 21 in broilers fed PFA150 diet compared to those fed the control diet (1086.47 vs 1019.69 µm; p<0.05). At d 21, villus diameter (207.02 µm) and crypt depth (155.63 µm) were significantly lower and higher (p<0.05) in broilers in the control group in comparison with other dietary treatments, respectively. Compared to the control group (149.97 µm), dietary PFAs supplementation increased (p<0.05) the muscularis thickness in PFA100 and PFA150 (166.40 and 163.18 µm, respectively) groups at d 21, whereas decreased in PFA125 group (139.22 µm; p<0.05). Goblet cell number was higher (p<0.05) in PFA100 and PFA125 (11.63 and 11.76, respectively) in comparison with control and PFA150 groups (10.81 and 10.37, respectively) at d 21 of age. At d 42, broilers fed PFA125 diets

Table 4 – Villus height (µm), villus diameter (µm), crypt depth (µm), muscularis thickness (µm), and goblet cell number per villus of broilers on d 21 of experiment in response to different dietary levels of PFAs (n=8).

Treatments	Villus Height	Villus Diameter	Crypt Depth	Muscularis Thickness	Goblet Cell Number
Control	1019.69±22.47^{b}	207.02±5.55^{c}	155.63±3.55^{a}	149.97±3.56^{b}	10.81±0.22^{b}
PFA100	1037.56±20.71^{ab}	238.98±5.75^{b}	143.44±2.44^{c}	166.40±4.59^{a}	11.63±0.26^{a}
PFA125	1074.96±20.75^{ab}	262.49±6.48^{a}	145.97±3.05^{ab}	139.22±3.25^{c}	11.76±0.24^{a}
PFA150	1086.47±11.27^{a}	235.86±5.50^{b}	153.97±2.71^{bc}	163.18±2.93^{a}	10.37±0.20^{b}
p-value	<0.05	<0.001	0.008	<0.001	<0.001

$^{a, b, c}$Means bearing different superscripts within the same column differ significantly.

Table 5 – Villus height (µm), villus diameter (µm), crypt depth (µm), muscularis thickness (µm), and goblet cell number per villus of broilers on d 42 of experiment in response to different dietary levels of PFAs (n=8).

Treatments	Villus Height	Villus Diameter	Crypt Depth	Muscularis Thickness	Goblet Cell Number
Control	1066.35±18.12^{b}	141.24±3.67^{b}	168.36±3.17^{b}	178.43±3.51^{b}	10.53±0.22^{b}
PFA100	987.80±19.78^{c}	188.86±6.75^{a}	171.55±3.69^{b}	201.08±4.97^{a}	11.59±0.26^{a}
PFA125	1155.43±21.20^{a}	173.13±8.88^{a}	166.10±4.09^{b}	204.81±5.95^{a}	10.51±0.28^{b}
PFA150	1017.70±18.68^{bc}	186.04±5.97^{a}	192.42±4.45^{a}	179.38±3.75^{b}	10.41±0.22^{b}
p-value	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b, c}$Means bearing different superscripts within the same column differ significantly.

had higher villus height compared to control group (1155.43 vs 1066.35 µm; $p<0.05$). Villus diameter and crypt depth were lowest and highest in control and PFA150 groups, respectively, compared to other dietary treatments at d 42 ($p<0.05$). Muscularis thickness increased significantly in PFA100 and PFA125 groups (201.08 and 204.81 µm, respectively) in comparison with control and PFA150 groups (178.43 and 179.38 µm, respectively; $p<0.05$). Similarly, supplementation of PFAs in PFA100 group (11.59) increased the goblet cell number as compared to other dietary treatments at d 42 ($p<0.05$).

DISCUSSION

The availability of a vast variety of phytogenic products comprising of various bioactive molecules and active ingredients in the market makes the comparison of the results of the scientific investigations very difficult. In this study, a blend of different spices, and essential oils was used which made it even harder to interpret the role of each component in the blend for the results obtained. Therefore, the interpretation of results requires attention to these facts.

The present study showed that BWG, FI, and FCR of broilers were not statistically different among the treatments. These results are no different than those reported by Botsoglu *et al.* (2002) and Lee *et al.* (2004) who found that dietary supplementation of oregano essential oil (100 and 50 mg/kg, respectively) did not affect the broilers' performance. Fukayama *et al.* (2005) reported that growth performance of broilers remained unaffected following the dietary supplementation of oregano extract at 0, 25, 50, 75, and 100 mg/kg. Similarly, dietary addition of 250 and 500 ppm phytogenic feed additive (based on essential oils of oregano (carvacrol), cinnamon (cinnamaldehyde), eucalyptus (cineole/eucalyptol), artemisia (artemisinin), and clover (trifoline)) had no effect on growth performance of broilers (Toledo *et al.*, 2007). Dietary supplementation of 250 ppm garlic based PFAs (organopolysulphide compounds) did not improve the growth performance of broilers (Kumar *et al.*, 2010). Rizzo *et al.* (2010) concluded that broilers fed diets supplemented with 1000 ppm of PFA (based on 20% active ingredients as essential oils of clove, thyme, cinnamon, and capsicum) or 100 ppm of PFA (based on essential oils of oregano and cinnamon, and capsicum oleoresin) or 500 ppm of PFA (containing eucalyptus oil, Chinese cinnamon oil, Chilean boldo leaves, and fenugreek seeds) had no difference in growth performance. Likewise, supplemental PFA containing turmeric extract, citrus extract, grape seed extract, Chinese cinnamon essential oil, Chilean boldo leaves, and fenugreek seeds had no effect on growth performance of broilers (Fascina *et al.*, 2012). Similar findings were reported by Abudabos & Alyemni (2013) in broilers fed 0.01% of a blend of essential oils including 29% active ingredients (piperine, curcumin, thymol, and eugenol). Hafeez *et al.* (2016) reported that growth performance was not different in broilers fed diets supplemented with 100 mg/kg PFA (based on carvacrol, thymol, limonene) or 150 mg/kg PFA (based on menthol and anethole), or without supplementation. Abildgaard *et al.* (2010) reported that broilers fed diets without the blend of essential oils (0 mg/kg) had better growth performance than those fed 100, and 200 mg/kg of a blend of essential oils containing thymol, eugenol, piperine, and curcumin. In contrast, some other studies showed that dietary PFAs supplementation improved the performance of broilers. Jamroz *et al.* (2005) reported that the inclusion of 100 mg PFAs (containing carvacrol, cinnamaldehyde, and capsicum oleoresin) per kg of the diet improved the performance of broilers. Spernakova *et al.* (2007) reported similar findings in broilers fed rosemary powder at 500 mg/kg of the diet. Fortification of broiler diet with a blend of phytogenic extracts at 1 g/kg also increased the BW gain and enhanced the performance (Lippens *et al.*, 2005). Similarly, the dietary supplementation of 1 g/kg thyme essential oil (Cross *et al.*, 2007) and 5 g/kg thyme herb (Toghyani *et al.*, 2010) improved the performance of broilers. The actual mechanism of action by which PFAs improve

the performance of broilers is not known, however, it may be due to the stabilization of feed components, improvement of the gut environment, concentration of enviable microflora or by the continuous stimulation of pancreatic and the digestives enzymes (Windisch *et al.*, 2008). Apparently, the variation in the results of the present study compared to previous studies seems to occur due to the differences in a number of factors. The variation in the efficacy of PFAs may arise due to the difference in the selection of plants that possess various bioactive biomolecules. In addition, the use of different parts of the plants may influence the results as well as the variations in the effectiveness of PFAs supplemented to the poultry diets. Most studies do not mention the concentration of active ingredients in PFA rather they list the active ingredients or the name of the components only. Therefore, it is very hard to make a comparison on the basis of the concentration of the active ingredient. However, the difference in the composition of PFAs suggests the difference in the results. Moreover, it is more likely that the concentration of the bioactive molecules would vary greatly among the PFAs in addition to the extracts or essential oils of the plants used that lead towards an inconsistency in the concentration of the bioactive molecules in PFAs. Additionally, the inclusion level of PFAs may play an important role. Most of the studies have used the mixture of different plant extracts, essential oil, herbs, and spices. Only fewer studies have emphasized the use of individual plant or its extracts. The quantity of the bioactive molecules in an individual plant has been an unsolved question. This implies that the exact inclusion level cannot be reached until the quantification of bioactive molecules in the individual plant is not accomplished in order to determine the effective level of application in poultry diets. It is important that the composition of the diet as well as the genetic potential of the broilers determine the efficacy of PFAs. Another possibility is that the components of PFAs used in the present study might have behaved differently resulting in failure to improve the growth performance of broilers. It is speculated that the diets used in the present study would have been successful to fully express the genetic potential for growth performance of broilers not leaving any room for further dietary supplementation of PFAs to improve the growth performance of broilers.

Most studies have reported a decrease in the pathogenic bacteria such as coliforms, *E. coli*, *C. perfringens*, and *Salmonella* spp. and improvement in lactobacilli populations in the intestines of broilers (Jamroz *et al.*, 2003; 2005; Mitsch *et al.*, 2004; Jang *et al.*, 2007; Siragusa *et al.*, 2008; McReynolds *et al.*, 2009; Murugesan *et al.*, 2015; Wati *et al.*, 2015; Manafi *et al.*, 2016). Only a few studies reported that dietary PFAs supplementation were unable to reduce the total aerobic and anaerobic bacteria, *E. coli*, *Clostridium* spp., *C. jejuni*, and improve the lactobacilli, and bifidobacteria populations in the broilers (Peric *et al.*, 2010; Hermans *et al.*, 2011; Vukic-Vrajnes *et al.*, 2013; Mountzouris *et al.*, 2014). The present study showed that dietary PFAs supplementation were ineffective to the selected caecal microbial populations. Usually, the antimicrobial activity of PFAs is attributed to the presence of the volume of essential oils in it. According to Hammer *et al.* (1999), most essentials oils showed their *in vitro* antimicrobial activity at an inclusion level ranging from 0.03 to 2.0% (v/v). However, the essential oils are supplemented in feed at lower levels than those required for their *in vitro* antimicrobial activity (Hafeez *et al.*, 2016). This might have occurred in the present study. The gut microbiota of broilers becomes more stable against changes as the age progresses compared to the younger chicks (Torok *et al.*, 2009). This might be another reason for the failure of dietary PFAs to improve the caecal microbial population of broilers in the present study. The molecular study on gut microbiome of broilers might present the true picture of dynamics of gut microbiota of broilers following dietary supplementation of PFAs.

In this study, dietary supplementation of PFAs significantly affected the villus height and diameter, crypt depth, muscularis thickness, and goblet cell number per villus in the jejunal sections of broilers in comparison with the control group on 21 and 42 days of age. Overall, villus height, diameter, and goblet cell number increased whereas crypt depth and muscularis thickness decreased in response to the dietary PFAs. This shows a promising effect of PFAs on gut health of broilers. The increased villus height and villus diameter are linked with increased surface area which, in turn, leads to better digestion and absorption (Murugesan *et al.*, 2014). Similarly, a higher number of goblet cell per villus indicates higher production of mucins and glycoprotein compounds that bind with the pathogenic bacteria thus preventing their attachment with the intestinal mucosa (Chacher *et al.*, 2017). The intestinal crypts act as a reservoir of epithelial cells so they are indicative of epithelial cell turnover or renewal rate. The cell turnover is a nutrient consuming process that uses vital nutrients that would otherwise be consumed for growth (Markovic *et al.*, 2009). Muscularis thickness reveals the germ load in broiler's intestine.

Shallow crypts and thin muscularis show that the bird has lower germ load indicating that it might spare nutrients for growth. The results of gut morphology in the present study suggest that dietary PFAs would have improved performance and gut microbiota in broilers that actually did not occur. It might be suggestive of low levels of essential oils in the phytogenic product used in this study. It is speculated that the inclusion levels used in the present study might be sufficient to improve the gut morphometry of broilers and higher inclusion levels might be needed for improvement in growth performance and caecal microbial populations.

CONCLUSIONS

The present study has shown the results quite different from those previously reported by different researchers on the effect of PFAs on growth performance, gut morphology, and gut microbiota of broilers. The study revealed that the different levels of dietary PFAs supplementation in broilers improved the gut morphology only despite most studies reporting the improvement of growth performance and gut microbiota, and only a few studies showing improvement in the gut morphology of broilers. Since the *in vitro* studies have shown that antimicrobial activity of PFAs requires a higher concentration of essential oils that is not in practice while applying in the feeds. Therefore, higher inclusion levels of the phytogenic product may be helpful in enhancing the growth performance and gut microbiota of broilers. Furthermore, under the conditions of the present study, the inclusion rate of dietary PFAs may be different for different criteria e.g. enhancing the growth performance, and improving the gut microbiota and gut morphology.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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