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Full Length Research Paper

The effects of supplementing an organic acid blend and/or microbial phytase to a corn-soybean based diet fed to broiler chickens

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This experiment was designed to investigate the effects of supplementation with phytase, either alone or in combination with an organic acid blend on growth performance, some internal organs measurements and tibia development, intestinal and cecal microbiology. One-day-old male Ross 308 strain broiler chickens (n=140) were allocated to 5 dietary treatments in a randomized complete block design. The treatments were as follows: (1) Positive control (PC); (2) Negative control (NC) that was the same as diet PC except that available phosphorus was reduced by 0.25 percentage units; (3) NC plus microbial phytase (MP) (NC + MP; 500 FTU/kg); (4) NC plus organic acid blend (OA) (NC + OA), and (5) NC + MP + OA. Body weight gain (BWG) was not improved by supplementation of OA to the NC diet. In contrast, BWG was increased by MP, and MP+OA interaction (P<0.001) compared to NC group. Tibia ash content was lowest (P<0.05) for birds fed the all NC diets compared to the PC and NC+P diet. The addition of phytase slightly increased tibia phosphorus according to NC+OA and NC+P+OA diets (P<0.05). NC diet resulted in increased weight of gizzard, heart, liver and bursa of Fabricious and length of duodenum, jejunum and ileum. In ileal digesta, lactic acid bacteria (LAB) counts were significantly increased for birds fed OA, whereas *Escherichia coli* were significantly decreased compared to PC group (P<0.001).

Key words: Broiler, phytase, organic acid, performance, bone development, intestinal measurements, intestinal microbiota.

INTRODUCTION

Phosphorus is essential for the growth of poultry, particularly for bone development. Monogastric animals consume diets composed mostly of oilseed and cereal grains that contain high level of P present in the form of phytic acid or phytate. The P in this form is generally unavailable to poultry due to low phytase activity found in the digestive tract (Nelson, 1976; Cromwell, 1992).

There are many factors which affect efficient utilization and phytate bound phosphorus in poultry diets. Genotype, age of bird, dietary calcium and phosphorus level, dietary vitamin D_3, dietary fibre content, type of feed ingredients and supplementation of diets with exogenous microbial phytase have found to be effective in increasing phytate hydrolisis (Singh, 2008). In addition, more recent studies showed that organic acids can increase phytate P utilisation by poultry (Bolling et al., 1998; Boling et al., 2000; Boling-Frankenbach et al., 2001; Brenes et al., 2003).

Studies examining the effects of microbial phytase in the digestive tract of monogastric animals revealed that its maximum activity could be reached at lower pH values. In this connection, some authors evaluated potential beneficial effects of citric acid on the efficacy of microbial phytase preparations in pigs and broiler chickens (Li et al., 1998; Radcliffe et al., 1998; Brenes et al., 2003).

Organic acids have been evaluated numerous times

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for their efficacy in improving growth performance in pigs and chickens (Giesting and Easter, 1985; Patten and Waldroup, 1988; Skinner et al., 1991; Risley et al., 1992; Jongbloed et al., 2000). However, little work with organic acids, other than citric acid, has investigated their efficacy for improving phytate P utilization.

In poultry production, organic acids have been studied as a tool to reduce unwanted bacteria (Griggs and Jacob, 2005), and formic acid in particular has been shown to be particularly effective against *Escherichia coli* (Ratcliff, 2000). The beneficial effects of organic acids on the productive traits of pigs have been demonstrated in many studies, but consistent data have not been obtained for poultry (Langhout, 2000).

On the other hand, various organic acids, especially citric and ascorbic acids have been investigated on the phosphorus digestibility (Zobac et al., 2004; Rice et al., 2002; Omogbenigun et al., 2003; Rafacz-Livingston et al., 2005; Smulikowska et al., 2010). The results of organic acids on the phosphorus digestibility of broilers are controversial.

The aim of our experiment was to investigate the effects of supplementation with phytase, either alone or in combination with an organic acid blend on performance, some internal organs evolution, bone development and intestinal microbiota of broilers.

MATERIALS AND METHODS

Animals and housing

Male Ross 308 broilers were obtained from a local parent stock supplier and randomly transferred to compact-type three-tier cage, four chicks per cage. Battery cages were equipped with wire mesh, nipple drinkers and trough feeders. The experiment consisted of five dietary treatments and was set up in completely randomized design in which 28 chicks were randomly assigned to each of the five treatments, seven replicates each. The experiment lasted for 21 days and the chicks were fed the experimental diets throughout the experimental period. Feed and water provided *ad libitum*. The lighting regime was 23 h/day. The birds were weighed individually at 1, 7, 14 and 21 days of age.

Diets

Five corn-soybean meal-based diets consisting of a positive control (PC) formulated according to NRC (1994); a negative control (NC) that was the same as diet PC except that available phosphorus was reduced by 0.25 percentage units; NC plus microbial phytase (MP) (NC+MP; 500 FTU/kg); NC plus organic acid (OA) (Salgard®; NC+OA); and NC+MP and OA (NC+MP+OA) (Table 1).

Microbial phytase was provided by BASF Group, Ludwigshafen, Germany and Salgard® was provided by Optivite LTD., Notts, UK. Salgard is a feed treatments that helps protect against bacterial and fungal challenge composed of propionic acid (20,000 mg/kg), ammonium propionat (85,000 mg/kg), ammonium format (160,000 mg/kg) and formic acid (35,000 mg/kg).

Experimental diets were formulated using ration-formulation software (UFFDA) to be isocaloric and isonitrogenous following National Research Council recommendations (1994). Diets were formulated to contain 23% crude protein and 3152 kcal/kg, and other essential nutrients (Table 1). Birds were fed the experimental diets *ad libitum* in mash form. Feed intake was recorded weekly. The feed conversion ratio (FCR) was calculated as grams of feed consumed per chick divided by grams of weight gain per chick.

Gastrointestinal tract and some internal organs measurements

At 21 days of age, two birds taken randomly from each cage (14 birds per treatment) were starved overnight and killed by cervical dislocation. The birds were then weight and their organs harvested. Organ analyses included proventriculus, gizzard, abdominal fat, hearth, pancreas, liver, duodenum, jejenum, ileum, cecum, thymus, bursa of Fabricious and spleen weights and duodenum, jejenum, ileum and cecum lengths. The gastrointestinal tract was portioned into proventriculus, gizzard, pancreas, duodenum, jejenum, ileum and ceca. The ileum, defined as the region from Meckel's diverticulum to a point 40 mm proximal to the ileocecal junction. The jejenum was defined as the portion of intestine extending from the bile duct enterance to Meckel's diverticulum. All organs weights and lengths were expressed as a percent of body weight.

Ileal and cecal contents of two chicks from each replicate pooled and homogenized, then one gram sample diluted with 4 ml deionised water and pH was measured immediately by pH meter (Inolab level 1, WTW GmbH, Weilheim, Germany).

Bone development measurements

At the end of experiment, chicks were killed by cervical dislocation and right tibias were collected for bone ash, Ca and P determination. Tibias were pooled by replicate pens, autoclaved, and all adhering tissue was removed. Bones were dried for 24 h at 100°C, weighed, and then dry ashed for 24 h in a muffle furnace at 600°C. Ash weight of the tibia was expressed as a percentage of dry bone weight (Chung and Baker, 1990). The tibia length and width were measured.

The total phosphorus was measured spectrophotometrically at a wavelength of 420 nm after mineralising the sample by breaking down and converting with vanadomolybdate. The calcium was determined by the method of titration with 0.1 M potassium permanganate ($KMnO_4$) volumetric standard (AOAC, 2000).

Intestinal microbiota

Samples of the ileal and cecal contents were immediately collected. The entire contents were transferred under aseptic conditions into sterile glass tubes in which they kept on ice until subsequent inoculation into agar. Accordingly, as the incubation medium; MRS agar (MERCK, 1.10660) was used for lactic acid bacteria (LAB) and malt extract agar (MERCK, 1.05398) was used for yeast. LAB and yeast counts of the ileum and cecum contents were obtained at 30°C following a 3-day incubation period. Coliform bacteria were grown aerobically on VRB agar (MERCK, 1.01406) at 37°C for 24 to 48 h. The LAB, yeast and coliform bacteria counts, and the average number of live bacteria determined per gram of original ileal and cecal contents. Bacterial colonies were counted, and the average number of live bacteria was calculated based on per gram of original ileal and cecal contents. All quantitative data were converted into logaritmic colony forming.

Statistical analysis

Collected data were recorded on a weekly basis and statistically

Table 1. The ingredients and chemical composition of experimental basal diets (as-fed basis).

Ingredients (g/kg diet)	Positive control (PC)	Negative control (NC)
Corn	455.01	463.65
Soybean meal (44% CP)	313.02	311.00
Full-fat soybean (37.5% CP)	142.13	142.53
Soybean oil	51.51	48.60
Limestone	14.41	24.11
DCP	16.91	3.11
Salt	2.65	2.65
DL-Methionine	1.86	1.85
Vitamin-mineral Premix[1]	2.50	2.50
Total	1000.00	1000.00
Calculated nutrient content[2]		
ME (kcal/kg)	3152	3152
Crude protein (%)	23.00	23.00
Crude fiber (%)	3.97	3.98
Ether extract (%)	9.69	9.43
Crude ash (%)	5.92	5.65
Methionine+Cystine (%)	0.90	0.90
Lysine (%)	1.28	1.28
Methionine (%)	0.53	0.53
Tryptophan (%)	0.33	0.33
Calcium (%)	1.00	1.00
Total Phosphorus (%)	0.72	0.47
Nonphytate phosphorus (%)	0.45	0.20
Linoleic acid (%)	5.00	4.87

[1]Provided per kilogram of diet: vitamin A, 8,000 IU (as retinyl acetate); vitamin D_3, 2,500 IU (as cholecalciferol); vitamin E, 30 mg (as -tocopheryl acetate); vitamin K_3, 2.5 mg (as menadione sodium bisulfite); vitamin B_1 2 mg (thiamine); vitamin B_2, 5 mg (as riboflavin); vitamin B_6, 2 mg (as pridoxamine); vitamin B_{12}, 0.01 mg (as cyanocobalamin); niacin, 30 mg; calcium-D-pantothenate, 8 mg; folic acid, 0.5 mg; D biotin, 0.045 mg; choline chloride, 300 mg; vitamin C, 50 mg; MnO_2, 70 mg; $FeSO_4\_7H_2O$, 35 mg; ZnO, 70 mg; $CuSO_4\_5H_2O$, 8 mg; $Ca(IO_3)_2$.. [2]Based on NRC (1994) values for feed ingredients.

subjected to ANOVA using the PASW Statistics18 packed program for windows (PASW Statistics18, 2010). The differences between group means were evaluated by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Performance

The result of growth performance is shown in Table 2. Birds fed the PC diet exhibited significantly higher BWG and feed efficiency (P<0.001). In our experiment, BWG was not improved by supplementation of OA to the NC diet. In contrast, BWG was increased by MP and MP+OA interaction (P<0.001) compared to NC group. Microbial phytase and MP+OA significantly improved the BWG by 17.67 and 12.21% respectively in NC diets (P<0.001). FCR was significantly improved by the addition of MP plus OA preparation. Further improvements occurred with MP alone (P<0.001). As expected, decreasing available phosphorus (AP) in diet caused a negative effect on the performance of chicks. These results were in agreement with those reported by Qian et al. (1996) and Punna and Roland (1999). Similarly, the positive effect of phytase on performance in broiler chicken diets has already been reported (Rama et al., 1999; Ahmad et al., 2000) . Qian et al. (1996) furthermore indicated that microbial phytase seems to be more efficient in diets with none or low levels of inorganic P supplementation in both pigs and poultry.

In the literature, positive effects of different organic acids on feed utilization or dressing percentage have been reported (Dibner and Buttin, 2002), but Rafacz-Livingston et al. (2005) did not find any positive effects on performance in response to supplementation of different organic acid blends.

In this current study, the addition of phytase to the low-AP diet, exhibited improved feed intake (FI). Broilers fed the NC diet and the addition of OA tended to have lower FI than those fed the other diets. Decreased feed intake resulting from low levels of available phosphorus in diets

Table 2. Effects of experimental diets on broiler performance (0 to 21days).

Treatment	Weight gain (g/bird)	Feed intake (g/bird)	FCR
PC	677.23[a]	940.32[a]	1.388[c]
NC	476.38[d]	780.36[b]	1.638[a]
NC+P	560.58[b]	845.53[ab]	1.508[b]
NC+OA	499.70[cd]	766.24[b]	1.533[ab]
NC+P+OA	534.56[bc]	805.34[b]	1.507[b]
SEM	14.565	17.723	0.020
P level	<0.001	0.006	<0.001

Values with different letters in the same column are statistically significantly different (P<0.05).

Table 3. Effects of experimental diets on organ weights (g/ 100 g body weight).

Organ	PC	NC	NC+P	NC+OA	NC+P+OA	SEM	P level
Proventriculus	0.598	0.585	0.535	0.514	0.540	0.0131	0.205
Gizzard	2.388[b]	2.729[a]	2.487[ab]	2.562[ab]	2.453[ab]	0.0447	0.128
Abdominal fat	0.751[a]	0.509[b]	0.653[ab]	0.577[b]	0.534[b]	0.0261	0.011
Heart	0.684[b]	0.753[a]	0.755[a]	0.722[ab]	0.775[a]	0.0096	0.014
Pankreas	0.381	0.368	0.384	0.381	0.385	0.0068	0.945
Liver	2.595b	2.834[a]	2.879[a]	2.834[a]	2.808[a]	0.0352	0.075

Values with different letters in the same row are statistically significantly different (P<0.05).

Table 4. Effects of experimental diets on small intestine weight (g/ 100 g body weight) and length (cm/ 100 g body weight).

Organ	PC	NC	NC+P	NC+OA	NC+P+OA	SEM	P level
Duodenum weight	1.568	1.696	1.626	1.724	1.659	0.0228	0.214
Duodenum length	3.607[c]	4.488[a]	3.895[bc]	4.169[ab]	4.148[ab]	0.0716	<0.001
Jejenum weight	3.820	3.699	3.661	3.684	3.622	0.0945	0.977
Jejenum length	8.958[c]	11.615[a]	10.062[b]	10.578[b]	10.327[b]	0.1898	<0.001
Ileum weight	2.339	2.209	2.291	2.268	2.235	0.0569	0.965
Ileum length	6.647[b]	8.600[a]	7.857[a]	8.091[a]	8.259[a]	0.1649	<0.001
Cecum weight	1.301	1.512	1.219	1.224	1.336	0.0568	0.494
Cecum length	2.096[c]	2.874[a]	2.524[b]	2.630[ab]	2.515[b]	0.0604	<0.001

Values with different letters in the same row are statistically significantly different (P<0.05).

fed to poultry is a well-known phenomenon (Kiiskinen et al., 1994). In the current study, however, this effect was partly compensated for the low-P diets supplemented with phytase.

This results were similar to those reported by Radcliffe et al. (1998), who indicated that no synergistic effects with the combination of phytase and citric acid on pigs performance in low-AP diets. In contrast, other authors reported that citric acid (20 to 60 g/kg) only had a positive effect on performance in low-AP diets (Boling et al., 2001).

Effects of dietary treatments on some internal organs

Effect of phytase and OA, individually or in combination on the relative weight and length of different sections of intestinal organs are presented in Table 3 to 5. The relative weights of proventriculus, pancreas, thymus and spleen were not influenced by dietary additions (P>0.05), but relative weights of gizzard, heart, liver and bursa of Fabricious, and relative length of small intestine segments differed among treatments (P<0.05). Generally, NC diet resulted in increased weight of gizzard, heart,

Table 5. Effects of experimental diets on lymphoid organs weights (g/ 100 g body weight).

Organ	PC	NC	NC+P	NC+OA	NC+P+OA	SEM	P level
Thymus, g	0.484	0.525	0.439	0.497	0.489	0.01478	0.521
bursa of Fabricious, g	0.268^{b}	0.327^{a}	0.344^{a}	0.326^{a}	0.302^{ab}	0.00749	0.007
Spleen, g	0.080	0.086	0.086	0.099	0.083	0.00322	0.360

Values with different letters in the same row are statistically significantly different (P<0.05).

Table 6. Effects of experimental diets on ileal and cecal pH.

Treatment	Ileum pH	Cecum pH
PC	6.862^{bc}	5.650^{c}
NC	7.212^{a}	5.569^{c}
NC+P	7.171^{ab}	5.813^{ab}
NC+O	6.631^{c}	5.879^{a}
NC+P+OA	7.409^{a}	5.495^{c}
SEM	0.0862	0.0472
P level	0.002	0.006

Values with different letters in the same column are statistically significantly different (P<0.05).

Table 7. Effects of experimental diets on feed microbiota (log cfu/g feed contents).

Treatment	LAB	Yeast	*E. coli*	Mould
PC	NF	2.934^{a}	3.104^{a}	NF
NC	NF	2.462^{ab}	2.113^{b}	NF
NC+P	NF	0.000^{b}	1.239^{c}	NF
NC+OA	NF	2.159^{b}	1.651^{c}	NF
NC+P+OA	NF	2.462^{ab}	2.723^{a}	NF
SEM	-	0.348	0.230	-
P level	-	<0.001	<0.001	-

NF: Not found. Values with different letters in the same column are statistically significantly different (P<0.05).

Table 8. Effects of experimental diets on ileum microbiota (log cfu/g ileal content).

Treatment	LAB	Yeast	*E. coli*
PC	4.474^{b}	4.963^{b}	4.184^{c}
NC	3.653^{d}	5.420^{a}	3.602^{e}
NC+P	3.997^{c}	4.703^{c}	5.313^{a}
NC+OA	4.785^{a}	4.415^{d}	3.784^{d}
NC+P+OA	4.832^{a}	3.860^{e}	4.282^{b}
SEM	0.086	0.099	0.111
P level	<0.001	<0.001	<0.001

Values with different letters in the same column are statistically significantly different (P<0.05).

Table 9. Effects of experimental diets on cecum microbiota (log cfu/g cecum content).

Treatment	LAB	Yeast	*E. coli*
PC	3.817^{b}	4.645	4.485^{d}
NC	2.915^{a}	4.706	4.474^{d}
NC+P	4.047^{c}	4.811	5.342^{a}
NC+OA	4.310^{b}	4.780	4.886^{a}
NC+P+OA	4.848^{a}	4.845	5.279^{b}
SEM	0.119	0.036	0.070
P level	<0.001	0.411	<0.001

Values with different letters in the same column are statistically significantly different (P<0.05).

liver and bursa of Fabricious and length of duodenum, jejunum and ileum. The relative weight of gizzard, hearth, liver and bursa of Fabricious were lower in PC groups when compared to the all NC groups.

There are several studies which indicate that microbial phytase supplementation increases body weight gain, feed intake and feed efficiency in broiler chickens (Simons et al., 1990; Broz et al., 1994; Denbow et al., 1995; Mitchell and Edwards, 1996; Sebastian et al., 1996a, b). These conclusions are in agreement with our results; we also noticed that NC diet may result a increased duodenum, jejunum and ileum length because of decreased feed efficiency. The beneficial effect of NC diets supplemented with phytase may be decreased length of duodenum, jejunum and ileum.

Intestinal microbiology

The effects of dietary treatments on feeds, ileum and cecum microbiota (log cfu/g contents) are shown in Tables 7 to 9. LAB and moulds did not seem to be influenced in feed. In NC diets, yeast and *E. coli* numbers were consistently decreased compared to PC groups.

Table 8 reveals the effects of dietary treatments on ileal microbiota (log cfu/g ileal content). In ileal digesta, LAB counts were significantly increased for birds fed OA (P<0.001), whereas *E. coli* were significantly decreased compared to PC group (P<0.001). Intestinal pH was reduced (P<0.05) by supplementation of OA (Table 6). We can assume that OA inhibit pathogen bacteria growth.A decrease in the population of yeast in ileal digesta was observed in NC+P+OA groups (P<0.001).

The results of present study demonstrate that the

Table 10. Effects of experimental diets on tibia development.

Treatment	Weight[A] (%)	Length (cm)	Width (cm)	Ash[B] (%)	Ca (%)	P (%)
PC	0.265[a]	6.233[a]	0.572[a]	48.243[a]	30.661	18.366[ab]
NC	0.240[b]	5.640[b]	0.480[c]	43.021[ab]	29.164	17.834[ab]
NC+P	0.247[b]	5.752[b]	0.524[b]	42.224[ab]	31.172	18.928[a]
NC+OA	0.234[b]	5.681[b]	0.507[bc]	41.224[b]	31.401	16.593[c]
NC+P+OA	0.243[b]	5.693[b]	0.517[bc]	43.344[ab]	31.714	17.216[bc]
SEM	0.003	0.046	0.008	0.975	0.629	0.217
P level	0.002	<0.001	<0.001	0.173	0.744	0.002

[a]Percentage of the live body weight. [b]Percentage of dry bone weight. Values with different letters in the same row are statistically significantly different (P<0.05).

addition of the organic acid mixture that contains propionic acid (20,000 mg/kg), ammonium propionate (85,000 mg/kg), ammonium format (160,000 mg/kg) and formic acid (35,000 mg/kg) had a limited effect on feed and intestinal microbiology. Organic acid activity would reduce the total microbial population but would be particularly effective against *E. coli* and other acid-intolerant microorganisms (Dibner and Buttin, 2002).

Organic acids are one of the most efficient feed additives for mould prevention. For example, propionic acid inhibits the growth of fungi and prevents production of mycotoxins (Paster et al., 1988; Marin et al., 1999). Usage of some acids, such as formic, propionic, and HMB have broader antimicrobial activities and can be effective against bacteria and fungi, including yeast (Partanen and Mroz, 1999; Doerr et al., 1995; Enthoven et al., 2002).

We notice that addition of organic acid mixture may result in a reduced mould, yeast and *E. coli* growth in feed and ileal and cecal digesta.

Effects of dietary treatments on tibia characteristics

The effects of dietary treatments on tibia characteristics are shown in Table 10. Tibia ash content was lowest (P<0.05) for birds fed the all NC diets compared to the PC and NC+P diet. Tibia ash is considered to be the most sensitive criterion for assessing response to P availability in poultry (Onyango et al., 2003, 2005). Supplementing the NC diet with phytase in this study significantly improved tibia ash (P<0.05). Chicks fed diets supplemented with phytase had a 7.20% increase in tibia ash (From 43.021 to 46.120%) compared to chicks consuming a diet with no added phytase in NC diets. In contrast, in our experiment, dramatic decreases in BWG and tibia ash were observed when OA was supplemented to a P-deficient corn-soybean meal diet. Results showed that adding organic acid to the low available phosphorus diets did not increase the percentage of tibia ash.

From previous studies, we deduced that organic acids may increase the utilities of phosphorus. Adding the mixture of citric acid and sodium citrate in (1:1 ratio) to rat diets with lack of calcium and phosphorus inhibit from rickets diseases (Shohl, 1937). In contrast, Boling et al. (2000) reported that in laying, hens fed with corn-soybean meal, the citric acid did not affect utility of phosphorus.

Tibia Ca did not differ any of the groups. But the addition of phytase slightly increased tibia phosphorus according to NC+OA and NC+P+OA diets (P<0.05).

Keshavarz (2000) observed in pullets and Rama et al. (1999) in chickens that tibia ash was not influenced by phytase in diets. However, our experiment phytase supplementation to low-P diets significantly increase tibia ash (P<0.05). The increase in tibia ash has been reported by several authors in chickens and considered to be a good indicator of bone mineralization (Ahmad et al., 2000; Leeson et al., 2000). This improvement in ash percentage in tibia can be related to increase in mineral retention.

In this study, the tibia ash content and tibia Ca and P were the lowest in group fed diet NC with supplement of OA but without phytase, and addition of phytase to these diet increased tibia ash content and tibia phosphorus most effectively. Boling et al. (2000) hypothesized that citric acid may competitively chelate Ca and reduce the binding of Ca to phytate, thereby preventing the formation of insoluble Ca-phytate complexes. This may result in the dietary phytate being more susceptible to phytase. This theory may also be true for the blend of organic acids used in this study, which contained, among others, citric acid. Better understanding of interactions between organic acids, phytic acid and phytase may allow improvement in P utilization from different plant feedstuffs by chickens.

The present study clearly indicated that supplementation of broiler diets with P is not necessary with adequate activities of phytase enzyme.

Consequently, the results of the present study clearly demonstrate that the addition of microbial phytase significantly improved performance and bone development of broilers; however, no improvement in performance and bone development was observed with supplementation of organic acid blend. However, addition of the blend of organic acid to broiler diets decreases

E. coli in the diets and intestine.

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