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Supplementation of Lactic Acid and Citric Acid in Diets Replacing Antibiotic and its Influence on Broiler Performance, Meat Yield and Immune Response up to 42 Days of Age

K. Vijaya Lakshmi[1], G. Shyam Sunder[2]

[1]Department of Poultry Science, College of Veterinary Science, Rajendranagar, Hyderabad-30 and [2]Project Directorate on Poultry, Rajendranagar, Hyderabad-30

Abstract: ***The present study aimed at evaluating two organic acids, Lactic acid (LA) and Citric acid (CA), each of which at 1.0 and 2.0% levels for replacing antibiotic (AB-Virginiamycin 11mg/kg) from diets. In a feeding trial with 270 broiler chicks (Vencobb) LA and CA were tested in 2 x 2 factorial design, and compared with AB and control diets from day-one to 42 d of age in cages. Each of the 6 test diets was offered to 9 replicates of 5 chicks, and their growth performance, carcass attributes, bone morphology and immune response were recorded. Results on broiler growth revealed that supplemental LA or CA at 2% level in diets significantly ($p \leq 0.01$) enhanced body weight (2109-2195g) compared to AB and Control (1960-1995g). The improvement in weight gain with organic acids was 9.6-10.9% over the control and 7.75- 8.9% over AB diets. Both organic acids reduced feed intake, which coupled with higher weight gain produced better FCR compared to AB diet. LA inclusion in diets recorded higher meat yields (>3.1%) and breast meat yield (>9.7%) with desirable decline in abdominal fat. LA even at 1% level supported higher tibia weight (5.96 g), length (8.11cm) and low leg scores (1.08), indicating better bone condition. LA and CA at 2% level improved immune competence in broilers measured as humoral and cell mediated response, compared to the AB and control diets. Conclusively, the results of present study revealed significant advantages of LA and CA supplementation at 2% level on the broiler performance, meat yields, bone conformation and immune response over AB and control diets, implying that organic acids could replace antibiotic from broiler diets effectively and beneficially.***

Keywords: Organic acid, Growth, carcass yield, Immune response

1. Introduction

Antibiotics are supplemented in broiler diets as feed additive on regular basis to protect the chicken from commonly occurring diseases. Although, antibiotics promote growth by sanitizing the digestive system from different microbial influences, there exists a strong public concern to move away from the use of antibiotics fearing their residual effects on consumers. Therefore, short chain fatty acids (organic acids) are used as alternatives to antibiotics to promote better performance in broilers. Dibner and Butin (2002) suggested that organic acids enhanced protein and energy digestibility by reducing microbial competition with the host nutrients, endogenous nitrogen losses, production of ammonia and other growth depressing microbial metabolites. They also minimized the incidence of sub-clinical infections and secretion of immune mediators leading to better performance in the birds.

Although most of the literature on organic acids indicated positive influence on weight gain in broiler chicken, wide variability existed among the organic acids and their levels of supplementation. Some of the acidifiers like the formic acid (Islam et al., 2008), propionic acid (Gorniowicz and Dziadek, 2002; Alikhosravi et al., 2008) and butyric acid (Leeson et al., 2005) were required at levels less than 1% in broiler diets, and higher levels had no additional advantage. In contrast, lactic acid (Versteegh and Jongbloed 1999), citric acid (Andrys et al., 2003), acetic acid (Abdel –Fattah et al., 2008), fumaric acid (Islam et al., 2008; Sheikh Adil et al., 2010), malic acid, tartaric acid etc., (Sheikh Adil et al., 2010) needed inclusion levels higher than 1% in diet, preferably between 1-3%. Organic acids with longer chain such as citric, lactic or benzoic acids had progressively less acidifying power and hence, required at higher inclusion rates, particularly when growth rate or survival of *coli* form bacteria were considered *in-vitro* (Murray, 2010). Therefore, in the current study emphasized on the need to evaluate the response of broiler chickens to lower levels of LA and CA, and their effectiveness in replacing antibiotic from diets.

2. Materials and Methods

Stock, husbandry and experimental diets

A feeding trial was undertaken with 270 day-old commercial male broiler chicks of Vencobb brand. They were individually wing banded, weighed and randomly distributed to 6 dietary groups with 9 replicates of 5 chicks each, and housed in stainless steel battery brooders in open sided house. Each battery brooder had three tiers with two cells per tier (60 cm x 75 cm x 45 cm - for 5 chicks). Incandescent lights were used to brood chicks at 34 ± 1^0C up to 7 d of age, and the temperature was gradually reduced to 26 ± 1^0C by 21 d of age after which no supportive heat was provided. They were vaccinated against Marek's disease, Newcastle's disease and Infectious Bursal disease following the standard schedule. Mortality was recorded throughout experimental period for calculating the percent livability up to 42 d of age.

A control diet was formulated with maize and soybean meal (Table 1) to which Lactic acid (LA) or Citric acid (CA) was added at two levels, 1.0 and 2.0% in a factorial pattern and compared with a diet supplemented with antibiotic (AB), Virginiamycin at 11mg/kg. The control diet was not supplemented with LA, CA or AB. Thus, 6 experimental diets were constituted that were individually offered to the dietary groups at *ad libitum* from day one to 42 d of age. During the 42-d period, a pre-starter diet (0-10d) was formulated to contained 22% protein and 2950 kcal ME/kg, and in continuity for the starter diet (11-21d), the protein was 21% protein and ME was 3050 kcal/kg. Further, in the finisher diet (22-42 d) the dietary protein was reduced to 20.2% and the ME increased to 3150 kcal/kg.

Carcass parameters and leg scores

On the 43rd day, 6 birds from each dietary group were randomly selected, starved over night, weighed and sacrificed by cervical dislocation for measuring the eviscerated meat yield; breast meat, abdominal fat, spleen and bursal weights as percentage of pre-slaughter weight.

The soundness of hock joints was physically examined in all birds, and leg abnormalities were recorded on the 42nd day following the score card described by Watson et al., (1970). The birds that appeared normal were scored 1; slight swelling of tibia metatarsal joint as 2; marked swelling of the joint as 3; swelling of the joint with symptoms of slipped achilles tendon as 4 and swelling with marked degree of slipped tendon as 5.

Immune Response

The humoral immune response was evaluated by inoculating a non-pathogenic antigen, the Sheep Red Blood Cells (SRBC). Sheep blood was collected by juglar puncture in EDTA tubes and washed thrice with 0.85% saline solution. The broiler chickens (6 no) from each dietary group were injected 0.1 ml of 0.5% SRBC suspension into brachial vein on the 36th d and the blood samples were collected after 5 d post-inoculation on the 41st d. Subsequently, the micro-haemagglutination activity of serum was estimated following the procedure of Wegmann and Smithies (1966). Antibody titres were expressed as log2 of the reciprocal of the highest dilution in which agglutination occurred (Brugh, 1978).

The cell mediated immune response was assessed by conducting the cutaneous basophilic hypersensitivity test *in-vivo* by using PHA-P (Phytohaemagglutinin – plant origin). Broiler chicken (6 no) from each test groups were selected on the 36th d and the toe thickness between the 2nd and 3rd inter-digital space of both feet, left and right was measured by micrometer. Immediately after measurements, 100 μg of PHA-P suspended in 0.1 ml of phosphate buffer saline (PBS) and 0.1 ml of PBS alone, were respectively injected into right and left foot (control). The web swelling of both feet was measured after 24 hours of initial injection. The response was determined by subtracting the skin thickness of first measurement from the second and the values of left foot (control) from the right foot (Corrier and Deloach, 1990).

On the 43rd d, broiler chicken were sacrificed for meat traits from which the bursa and spleen were collected, weighed individually and expressed as the percent pre-slaughter weight.

Statistical analysis

The experiment was one with of completely randomized design with 6 dietary treatments. Since, two organic acids were tested at similar levels of inclusion, two-way ANOVA was conducted on the organic acids and the same was compared with AB and control groups using the General Linear Models Procedure of SAS (1991). The means were compared by Duncan's Multiple Rang Test (Duncan 1955). A level of ($p \leq 0.05$ or 0.01) was used as the criterion for statistical significance.

3. Results and Discussion

Growth, Feed intake and FCR in broilers

The average body weights were significantly ($P< 0.01$) higher with the inclusion of LA and CA in diets compared to antibiotic and control diets at 42 d of age (Table 2). There was no significant difference between the two acids in influencing the broiler growth, but their inclusion at 2% level was better than 1%. However, inclusion of LA and CA in diets was significantly ($p \leq 0.01$) better than *Virginiamycin* (11mg/kg diet) in the diet. The feed consumed per unit weight gain indicated that the FCR was significantly better in groups fed organic acids at both levels of inclusion, followed by AB and control diets. The interaction also revealed the advantage of organic acids at 2% level for FCR followed by 1% level, AB and control diets.

Several studies concluded that the dietary organic acids increased the nutrient utilization and improved the digestible coefficient of protein (El-kerdawy, 1996; Abdel-Azeem *et al.*, 2000 and Abdo, 2004). The decreased gastric emptying rate was perhaps the possible mechanism to improve the protein digestion in GI-tract. The data on uric acid, which is the major end product of protein metabolism in poultry (Sturkie, 1986) revealed that dietary addition of organic acid slightly reduced serum concentration of uric acid, contributing to better utilization of protein and amino acid digestibility, culminating in better growth. Another important aspect of our study was the addition of antibiotic (AB), *Virginiamycin* (11mg/kg) in diet, which showed no significant improvement in body weight and remained equivalent to the control diet, and inferior to the organic acids because AB did not have the ability to stimulate the pancreatic secretions for higher nutrient availability and improved performance (Gauthier, 2002).

Further, the broilers fed CA showed significantly higher feed consumption, but with no change in FCR. It was hypothesized by Atapattu and Nelligaswatta (2005) that inclusion of CA in diets at 2% level reduced the pH of the feed and digesta of the crop and gizzard. Appetite control nerve endings are located in the crop of the birds, which were perhaps stimulated in the low pH environment created by the CA. Perhaps, LA was not as effective as CA, and hence less feed intake. Nevertheless, the reduction in feed intake due to the presence of LA and CA coupled with higher body weight resulted in better FCR followed by AB and control groups.

Eviscerated meat yield, breast meat and abdominal fat

The percent eviscerated meat yield and breast meat were significantly ($p \leq 0.01$) higher and the abdominal fat was lower in the broilers fed organic acids up to 42 d of age compared to AB and control diets (Table 3). The concentration of organic acids at 1 and 2% levels in diets did not show any variation between the levels on meat parameters, but were better than AB and control diets. The interaction between organic acids and their levels also confirmed the trend of results mentioned above.

The significant features of the present study were those of higher breast meat yield and lower abdominal fat ($p \leq 0.01$) due to organic acids in diets. Organic acids used in our experimental diets for 42d perhaps maintained sufficient undissociated acid molecules, which appeared to produce bacteriostatic or bactericidal effect leading to better breast meat yield than diets without organic acids (Leeson *et al.* 2005). Similar to our findings, Izat *et al.* (1990) reported significant reduction in abdominal fat content in male broiler chickens with diets supplemented, with propionic acid. The reduction in abdominal fat might be due to elevated serum T_3 concentration caused by dietary acidification. The hyperthyroidism and peripheral conversion of T_4 to T_3 signified superior metabolic rate and growth rate, coupled with lower fat deposition (Abdel-Fattah *et al*., 2008). This effect was observed to be more pronounced with LA supplemented at 3% level compared to CA or Acetic acid at 1.5% and 3%, respectively in diets. However, in our study the same effects were realized even with lower levels (1-2%) of LA and CA supplementation in diets.

Bone measures

The average tibia weight (5.31-6.12g) and length (6.16-8.40cm) was significantly ($p \leq 0.01$) higher and leg score values (1.08-1.50) were lower in groups fed diets supplemented with LA and CA compared to the control diet. No significant difference was observed in leg scores between the two organic acids and AB diets (Table 2 & 3). However, organic acids supplemented at 2% in diets promoted higher tibia weight and tibia length with optimum leg score compared to 1% level and AB. Significant ($p \leq 0.01$) decrease in tibia weight and length was recorded in control group, which were offered the diet with no antibiotic and no organic acid in the diet. The interaction between acids and their levels of inclusion showed increased tibia weight, tibia length and low leg scores when both acids were used at 2% levels, indicating the possible improvement in the bone measures could be due to improved mineralization. Further, the diets supplemented with AB were better than control diet, indicating that dietary supplementation of AB had definite advantage on tibia compared to control. This was also confirmed by low leg scores with organic acids and AB supplementation.

The influence of organic acids can be probably attributed to improved availability of Ca and P by virtue of decreased pH in the upper GI tract and their stimulating effect on intestinal villi, (Senkoylu *et al.* 2007). It was also observed that CA improved Ca availability by chelating Ca and reducing the formation of insoluble Ca-phytate-complexes (Boling *et al*. 2000). Even in aged laying hens (64 wk), Nezhad *et al.* (2008) found increased tibia mineralization (ash content) with addition of CA to diets. It was possible that the organic acids in our study produced similar effects with significant increases in tibia weight and length, and consequently low leg scores in broiler chicken.

Immune response

The antibody titers against sheep red blood cells (SRBC) antigen were significantly ($p \leq 0.01$) higher in broilers fed diets supplemented with LA and CA over control diets (Table 4). Diets with AB were intermediate to treatments with organic acids and control. Organic acids supplemented at 2% level were more effective than 1% in stimulating humoral immune response. However, both diets with organic acids maintained higher ($p \leq 0.01$) antibody titers than AB followed by control diets. The interaction revealed that 2% level in both the acids maintained significantly ($p \leq 0.01$) higher immune response compared to other treatments, suggesting it to be the ideal level for optimal humoral response. The cell mediated immunity recorded as cutaneous basophil hypersensitivity (CBH) was higher in broilers that were fed LA and CA diets compared to the control group. The interaction between the acids and their levels showed a similar trend suggesting the advantage of organic acid supplementation for better CBH response. The control group recorded significantly ($p \leq 0.01$) lower weights of spleen and bursa compared to the groups fed organic acid supplemented diet, suggesting that inclusion of organic acids had better lymphoid organ weights in turn indicating the better immune response.

Broilers offered LA and CA in diets at 2% responded positively to the humoral and cell mediated immunity with increased weights of lymphoid organs at 42 d of age compared to AB supplemented and control diets (Table 4). Enhanced immune response in broilers to acidifiers was also witnessed by Abdel- Fattah *et al*. (2008) and Rama Rao *et al* (2004). The relative increase in the weight of spleen and bursa, the two primary lymphoid organs was considered as an indication of the immunological advances. The establishment of immune response associated with dietary acidification could be on account for their inhibitory effects against the pathogenic microorganisms throughout the GI tract providing scope for improved nutrient absorption. The higher immunity due to organic acids can also be attributed to higher nutrient efficiency, which might have triggered immunogenic cells to record higher immune response (Rama Rao *et al* 2004). It was therefore, obvious that LA and CA inclusion in broiler diets at 2% level effectively enhanced immune response, which perhaps had positive effect on broiler performance and meat yield.

Evidently, the results of this study demonstrated that organic acids, LA or CA supplemented at 2% level in diets significantly enhanced growth, FCR meat yield, breast meat, tibia bones and reduced abdominal fat and leg scores in broilers up to 42 d of age, indicating a clear advantage over the antibiotic supplemented diet. Organic acids effectively replaced antibiotic from diets.

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Table 1: Ingredient composition of basal diet (g/kg)

Ingredient	*Pre-starter (0-10d)*	*Starter (11-21d)*	*Finisher (22-42d)*
Maize	560.600	569.370	574.009
Soya bean meal	376.840	354.350	335.894
Common salt	4.500	4.500	4.200
Di-cal Phosphate	19.020	19.200	17.684
Shell grit powder	7.230	7.340	8.616
DL methionine	2.370	2.090	1.993
L lysine HCl	0.560	0.060	0.000
AB2D3K*	0.150	0.150	0.150
B complex *	0.150	0.150	0.150
Choline Chloride	0.600	0.600	0.600
Toxin binder	2.000	2.000	2.000
Trace minerals**	1.200	1.200	1.200
Anti-oxidant	0.200	0.200	0.200
Coccidiostat	0.500	0.500	0.500
Vegetable oil	23.780	37.950	52.354
Nutrient composition			
^{1}ME (kcal/kg)	2950	3050	3150
^{2}Crude protein (%)	22	21	20.2
^{2}Lysine (%)	1.2600	1.15	1.09
^{2}Methionine (%)	0.56	0.52	0.50
^{1}Calcium (%)	0.90	0.90	0.90
^{1}Avail. P (%)	0.45	0.45	0.42

* Vitamin premix provided per kg diet: Vitamin A, 12375 IU; vitamin D3, 1800 IU; vitamin E 6 mg; vitamin K 1.5 mg; riboflavin 7.5 mg; vitamin B1 0.6 mg; B6 1.2 mg; B12 6 mcg; Niacin 9 mg; Calcium pantothenate 6 mg.
** Trace mineral provided per kg diet: Mn 60 mg, Zinc 80 mg, Iron 25 mg, Copper 10 mg, Iodine 1mg and Selenium 0.1mg.
^{1} calculated nutrient
^{2} Estimated nutrient

Table 2: Influence of LA and CA inclusion in diets at different concentrations on the performance of broiler chicken at 42 d of age and its comparison with AB and control diets

Org acids and levels	B. wt (g)	FCR	Livability (%)	Leg score
LA 1.0%	2109^{b}	1.71^{cd}	97.2	1.08^{b}
LA 2.0%	2188^{a}	1.66^{d}	96.2	1.08^{b}
CA 1.0%	2148^{ab}	1.73^{c}	93.7	1.00^{b}
CA 2.0%	2195^{a}	1.65^{d}	95.0	1.08^{b}
AB	1995^{c}	1.83^{b}	92.5	1.25^{ab}
Control	1960^{c}	1.91^{a}	90.7	1.50^{a}
Source of variation	^{2}Significance of treatment effect			
Org acids x levels	^{1}**	**	NS	**
Organic acids	**	**	NS	**
Levels	**	**	NS	**

Means within a column having different superscripts are statistically different (^{2}p ≤ **0.01 or *0.05)
LA- Lactic acid: CA – Citric acid: AB-Antibiotic

Table 3: Influence of LA and CA at different levels of inclusion in diets on the eviscerated meat yield, breast meat, abdominal fat (%), tibia weight (g) and tibia length (cm) of broiler chicken at 42 days of age and its comparison with AB and control diets

Org acids and levels	*Eviscerated yield*	*Breast meat yield*	*Abdominal fat*	*Tibia weight*	*Tibia length*
LA 1.0%	75.27^{a}	15.35^{a}	1.12^{b}	5.96^{ab}	8.11^{ab}
LA 2.0%	75.37^{a}	15.08^{ab}	1.16^{b}	6.12^{a}	8.33^{a}
CA 1.0%	74.85^{a}	15.36^{a}	1.11^{b}	5.92^{b}	7.98^{bc}
CA 2.0%	74.91^{a}	14.82^{b}	1.09^{b}	6.07^{ab}	8.40^{a}
AB	72.91^{b}	13.87^{c}	1.24^{b}	5.67^{c}	7.75^{c}
Control	73.04^{b}	13.68^{c}	1.60^{a}	5.31^{d}	6.16^{d}
Source of variation	^{2}Significance of treatment effect				
Org acids x levels	**	**	**	**	**
Organic acids	**	**	**	**	**
Levels	**	**	**	**	**

Means within a column having different superscripts are statistically different (^{2}p ≤ **0.01 or *0.05)
LA- Lactic acid: CA – Citric acid: AB-Antibiotic

Table 4: Effect of organic acids and antibiotic inclusion in diets on the humoral immune response (antibody titers), cell mediated response and weight of lymphoid organs in broiler chickens at 42 d of age

Org acids and levels	SRBC (log_2)	CBH	Spleen	Bursa
LA 1.0%	6.33^{c}	236^{a}	0.180^{a}	0.184^{a}
LA 2.0%	6.69^{a}	239^{a}	0.179^{a}	0.183^{a}
CA 1.0%	6.50^{b}	237^{a}	0.173^{a}	0.179^{a}
CA 2.0%	6.63^{ab}	247^{a}	0.174^{a}	0.175^{a}
AB	5.57^{d}	222^{b}	0.157^{b}	0.141^{b}
Control	5.35^{e}	207^{c}	0.143^{c}	0.127^{b}
Source of variation	^{2}Significance of treatment effect			
Org acids x levels	**	**	**	**
Organic acids	**	**	**	**
Levels	**	**	**	**

Means within a column having different superscripts are statistically different (^{2}p ≤ **0.01 or *0.05)
LA- Lactic acid: CA – Citric acid: AB-Antibiotic
SRBC: sheep red blood cell; CBH: Cutaneous Basophil hyper-sensitivity

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