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Intestinal Development and Function of Broiler Chickens on Diets Supplemented with Clinoptilolite

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ABSTRACT: The purpose of this study was to evaluate the effect of natural clinoptilolite (NCLI) and modified clinoptilolite (MCLI) on broiler performance, gut morphology, intestinal length and weight, and gut digestive enzyme activity. A total of 240 d-old male chicks were randomly assigned to 3 treatments, each of which comprised 8 pens of 10 chicks per pen. Birds in the control group were fed the basal diet, while those in the experimental groups were fed diets supplemented with NCLI at 2% (NCLI group), or MCLI at 2% (MCLI group), respectively, for 42 d. Compared with the control, supplementation with NCLI or MCLI had no significant ($p>0.05$) effects on productive parameters from d 1 to 42. Supplementation with NCLI or MCLI had no influence on the relative length and weight of small intestine at d 1 to 21. But supplementation with NCLI or MCLI significantly reduced the relative weight of duodenum. Supplementation with MCLI and NCLI was associated with greater ($p<0.05$) villus height in the jejunal and ileal mucosa compared with those areas in the controls from d 1 to 42. However, supplementation with NCLI and MCLI had no significant ($p>0.05$) influence on the crypt depth in the jejunal and ileal mucosa compared with those in the controls. The addition of either NCLI or MCLI to the diet improved the activities of total protease, and amylase in the small intestinal contents. In conclusion, supplementation with NCLI or MCLI in diets improved intestinal morphology, increased the intestinal length and weigh and gut digestive enzyme activity. (**Key Words:** Broiler, Clinoptilolite, Histology, Gut, Digestive Enzyme)

INTRODUCTION

Natural clinoptilolite (NCLI) is a natural zeolite, which is among the aluminosilicate materials. The aluminosilicate structure is negatively charged and attracts cations that come to reside inside the pores and channels (Mumpton and Fishman, 1977). Zeolites have large empty spaces, or cages, which can accommodate large cations, molecules and cationic groups. The basic structure of zeolites is biologically neutral, so this kind of zeolites have found diverse applications as adsorbents, ion exchangers and catalysts in industry, agriculture, veterinary medicine, sanitation and environmental protection. They are also used as a feed additive (Mumpton and Fishman, 1977; Mumpton, 1999; Martin-Kleiner et al., 2001).

Data support a favorable situation for potential applications in animal feeding. Numerous studies were shown that added CLI as a dietary supplement to the rations of cattle, pigs and poultry frequently were resulted in beneficial weight gains and less subject to disease, and show regular digestions, as well as an increase in appetite of animal. Because zeolites can slows the passage rate of digesta through the digestive tract and controls the release of nutrients in the gut (Evans, 1993; Olver, 1997; Papaioannou et al., 2002). On the other hand, zeolite's primary values are as growth promoters and carriers of a number of macro- and microchemical elements which are necessary for the vital activity of living organisms (such as vitamins; minerals, antibiotics and other active compounds). As growth promotoers zeolites appear to act as a buffer in the animals digestive system, storing nitrogen in the form of ammonium and releasing it gradually by ion exchange with zeolite (Maeda and Nosé, 1999). And the microelements present in zeolite, like calcium, potassium, sodium, as well as the majority of microelements can enhanced mineral metabolism, increased the content of macro- (Ca, K, Na) and microelements in the tissues and the organs. Due to the presence of these elements, which are capable of getting involved in the exchange, the ion composition of the chyme

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changes, which normalizes the pH and optimizes the activity of digestive enzymes, favorable effect on feed components hydrolysis over a wider range of pH, improved energy and protein retention (Cabezas et al., 1991; Shadrin, 1998; Parisini et al., 1999; Teimuraz et al., 2009). It is also possible that CLI remove toxins and create changes in enzymology and immunological responses (Oguz, 2011).

Similar effects were observed with some synthetic zeolites. Zeolites NaX, NaY, NaA, and CaA were evaluated *in vitro* for their ability to protect animal against the effects caused by bacteria or toxins, and further improving animal growth performance by enhancing nutrient absorption in cows, lambs, pigs and laying hens (Pond, 1995; Olver, 1997; Miazzo et al., 2000; Heather et al., 2009). However, further studies are needed to investigate the effect of CLI specifically on gut morphology, gut development and gut digestive enzyme activity in broilers.

It is well known that NCLI and modified CLI (MCLI) can affect the nutrient absorption and animal growth performance. Enterocyte enzymatic activity, structure and development are the most important features of the intestinal mucosal physiology. However, no information is available regarding the effect of adding NCLI and MCLI to broiler diets on the intestinal morphology. Based on this concept, the aim of the study is to evaluate the impact of dietary supplementation with these compounds, to test whether they also have an overall beneficial effect on broiler growth performance gut morphology, gut development and gut digestive enzyme activity of broiler chickens.

MATERIALS AND METHODS

Birds, housing and diets

A total of 240 d-old Arbor Acres male broiler chicks were allocated to three dietary treatments in a randomized complete block design for 42 d, each of which was replicated three times with 10 broilers per replicate. The dietary treatments were: i) basal diet, ii) basal diet+2% NCLI, iii) basal diet+2% MCLI. All birds were housed in wire cages in a 3-level battery, and housed in pens of identical size (1.75×6 m) in a deep litter system.

All the procedures were approved by the Institutional Animal Care and Use Committee of the Nanjing Agricultural University. Birds were housed in an environmentally controlled room. The initial temperature of 32°C was gradually reduced according to the age of the birds, reaching 20°C at the end of the experiment. The lighting cycle was 24 h from 1 to 3 d of age, 18 h from 4 to 20 d of age, 21 h from 21 to 35 d of age, and 23 h from 35 to 42 d of age.

The basal diets were of the maize-soya bean type. Broilers were fed a starter diet from d 1 to 21 and a grower diet from d 22 to 42 (Table 1). The diets were formulated in accordance with the NRC (1994) guidelines to meet the nutrient requirements of broilers. Diet compositions are shown in Table 3. Fresh diets were prepared once a week and were stored in sealed bags at 4°C.

The NCLI used in this study was collected from the Center of China Geological Survey (Nanjing). The grain-size distributions for the samples studied were 0.15 to 0.2 mm. For modification of natural CLI, the starting material was calcined in a muffle oven at 400°C for 4 h, and formic acid was gently stirred in to ensure good dispersion. The mixture was repeatedly washed with deionized water. After stirring, the sample was allowed to settle. The sediment was oven-dried at 65°C for 2 h, then ground in an agate mortar and sieved through a 100-mesh.

Performance and the length and weight of intestinal segments

During the overall experimental period, weights of chicks were measured weekly. Feed supplied and feed leftover were weighed on the same days as above, to calculate the feed intake (FI) and feed/gain ratio (F/G).

Table 1. Formulation and calculated composition of broiler diets (on fed basis)

Item	1-21 d	22-42 d
Ingredients (%)		
Maize	59.1	64.3
Soybean meal	30.6	24.3
Corn gluten meal	3.8	4.5
Lard	1.7	2.5
Limestone	1.31	1.23
Dicalcium phosphate	1.77	1.58
Sodium chloride	0.42	0.33
L-lysine	0.15	0.16
DL-methionine	0.15	0.1
Premix*	1	1
Calculation of nutrients		
Apparent metabolism energy (MJ/kg)	12.27	12.77
Crude protein (%)	21.2	19.3
Ca (%)	1.0	0.91
Available P (%)	0.43	0.38
Lysine (%)	1.08	0.95
Methionine (%)	0.50	0.43
Methionine+cystine (%)	0.82	0.73

* Premix provided per kg of diet: limestone, 3.3 g; L-lysine·HCl, 1.5 g; Dl-methionine, 1.3 g; VA 10,000 IU, VD_3 3,000 IU, VE 30 IU, menadione, 1.3 mg, thiamine 2.2 mg, riboflavin, 8 mg, nicotinamide 40 mg, choline chloride 600 mg, calcium pantothenate 10 mg, pyridoxine·HCl, 4 mg, biotin 0.04 mg, folic acid 1 mg; vitamin B_{12} (cobalamine) 0.013 mg, Fe (from ferrous sulphate) 80 mg, Cu (from copper sulphate) 8 mg, Mn (from manganese sulphate) 110 mg, Zn (Bacitracin Zn), 65 mg, iodine (from calcium iodate) 1.1 mg, Se (from sodium selenite), 0.3 mg.

Mortalities were recorded daily and were used to adjust the total number of birds by the end of 42 d, to determine the feed intake and F/G of the broilers. At the end of each experimental period (21 or 42 d), 8 broilers per group (one bird per replicate) from each treatment were randomly selected and weighed after feed deprivation for 12 h, were slaughtered. Then, the intestinal segments were excised. The small intestine was divided into 3 segments: duodenum (from gizzard outlet to the end of the pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the cecum junction). The contents of the duodenum, jejunum, and ileum (aseptically) were emptied by gentle pressure, then the length and weight were recorded.

Morpholoical measurement of the jejunal and ileal mucosa

Three cross-sections for each intestinal segment (jejunum and ileum) were fixed with formalin solution and were prepared using standard paraffin embedding procedures by sectioning at 5 μm thickness, and staining with hematoxylin and eosin. A total of 15 intact, well-oriented crypt-villus units were measured in each type of tissue from each broiler. Villus height and crypt depth were determined using an image processing and analyzing system (version 6.0, Image-Pro Plus), and were expressed as micrometers (μm).

Digestive enzyme activities of intestinal contents

The samples of duodenum, jejunum, and ileum contents (0.2 g) were homogenized with 4 ml icecold saline (0.9% NaCl). The digesta sample were stored immediately at -70°C until it be used. The small intestinal digesta samples were diluted 10×, based on the sample weight, with ice-cold PBS (pH 7.0), homogenized for 60 s, and sonicated for 1 min with three cycles at 30 s intervals. The sample was then centrifuged at 6,000 g for 15 min at 4°C. The supernatants were divided into small portions and stored at -70°C for assay of enzyme assays. Protease, trypsin, chymotrypsin and amylase were measured according to the methods described by Lhoste et al. (1993).

Statistical analysis

Analyses of variance were performed using the General Linear Model procedure of statistical package for social sciences 18.0 (SPSS Inc., Chicago, IL, USA) as a completely randomized design. Results are presented as mean±standard error of the mean (SEM). The significant differences among different treatment means were investigated using Duncan's new multiple range test. Effects were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Effects of NCLI (2%) and MCLI (2%) on the growth performance of broilers

The effects of the dietary treatments on broiler chicks' feed intake, average body weight gain (BWG), and F/G data in the periods of starter, grower, and the whole trial are presented in Table 2. It can be seen that no significant differences were observed between treatments from 1 to 42 d.

In the present study, the results presented in Table 2 show that adding NCLI and MCLI to the diets of broilers from 1 to 42 d of age produced no significant differences in terms of BWG, F/G. These findings are in agreement with

Table 2. Effects of NCLI (2%) and MCLI (2%) on the growth performance of broilers

Item[1]	Diet treatments			SEM	p value
	Control[3]	NCLI[3]	MCLI[3]		
BWG[2] (kg)					
1 to 21 d	0.542	0.551	0.564	0.011	0.976
22 to 42 d	1.392	1.421	1.435	0.017	0.432
1 to 42 d	1.934	1.972	1.999	0.015	0.831
FI[2] (g/bird/d)					
1 to 21 d	41.12	40.97	40.83	0.786	0.765
22 to 42 d	130.85	129.87	128.01	1.235	0.798
1 to 42 d	171.97	170.84	168.84	2.034	0.874
F:G[2]					
1 to 21 d	1.593	1.560	1.519	0.034	0.792
22 to 42 d	1.974	1.918	1.873	0.036	0.823
1 to 42 d	1.867	1.819	1.774	0.021	0.715

[1] Data represent means from 8 replicates per treatment, SEM = Standard error of mean.
[2] BWG = Body weight gain; FI = Feed intake; F/G = Feed intake/BW gain.
[3] Control = Basal diet; NCLI = Basal diet supplemented with 2% natural Clinoptilolite; MCLI = Basal diet supplemented with 2% formic acid modified clinoptilolite.

those of Evans (1989), who concluded from several experiments that CLI had no consistent beneficial effects. Similarly, the lack of response exhibited by BWG with CLI supplementation concurs with previous reports by Olver (1989), Elliot and Edwards (1991) and Zhou (2008). But some researchers reported that the supplementation of CLI to the diet improves the health status and body weight gain as well as feed efficiency of the animals (Ly et al., 2007). The expected effects of zeolites (CLI) may exhibit variation due to such factors as nature, purity, concentration, particle distribution, the CLI content of the zeolite and formulation composition in the diet. Moreover, Shariatmadari (2008) consider as this phenomenadepending on the aims and objectives of the experimental programme.

No effects of diet on mortality were detected in the present study. These findings suggested that further research regarding CLI as a feed additive is required. Numerous reports indicated that CLI is harmless, by including CLI into mixed feed; it is well tolerated by the animals and improves the production characteristics of broilers (Elliot and Edwards, 1991; Trckova et al., 2004).

Effects of NCLI and MCLI on the relative length, weights of intestine and morphology (μm) of intestinal mucosa in broilers

The effects of dietary NCLI and MCLI on the relative length and weight of small intestine in broilers are shown in Table 3. Broilers were pretreated with NCLI or MCLI, there was no dietary effect on the relative length and weight of duodenum, jejunum and ileum ($p>0.05$) in the period of 1 to 21. Moreover, there was no significant influence on the relative length of duodenum and ileum ($p>0.05$), and the relative weights of ileum ($p>0.05$) in the period of 22 to 42. When broilers were treated with MCLI, the relative length and weight of jejunum were significant increased ($p<0.05$). However, the relative weight of duodenum in the NCLI group were significantly lower than the control group and the MCLI group ($p<0.05$), and those of the MCLI group were significantly lower than the control group ($p<0.05$).

Morphological measurements of jejunal and ileal mucosae are presented in Table 4. During the overall experimental period the villus height in the jejunal mucosa in the MCLI group were higher ($p<0.05$) than those of the control group and NCLI group. The villus heights in the jejunal mucosa in the NCLI group were greater ($p<0.05$) than those in the control group, but they were lower ($p<0.05$) than in the MCLI group. The villus height in the ileal mucosae of chicks receiving the NCLI and MCLI in the feed was significantly greater than in the control group ($p<0.05$), but there were no significant differences between the two groups ($p>0.05$) during the overall experimental period. Supplementation with NCLI and MCLI had no significant ($p>0.05$) influence on the crypt depth in the jejunal and ileal mucosa compared with the controls during the overall experimental period. During the overall experimental period, the MCLI-supplemented group

Table 3. Effects of NCLI (2%) and MCLI (2%) on the relative length (cm/kg) and relative weights (g/kg) of small intestine in broilers

Items[1]	Diet treatments			SEM	p value
	Control[2]	NCLI[2]	MCLI[2]		
1 to 21 d					
Relative length					
Duodenum	34.47	31.55	31.05	0.84	0.206
Jejunum	73.50	74.26	73.60	0.69	0.980
Ileum	67.71	69.63	70.74	0.63	0.606
Relative weights					
Duodenum	9.52	8.62	8.59	0.29	0.347
Jejunum	14.67	15.35	15.87	0.37	0.433
Ileum	10.34	10.73	11.10	0.22	0.384
22 to 42 d					
Relative length					
Duodenum	11.27	10.71	10.59	0.39	0.769
Jejunum	25.04[a]	24.44[a]	29.54[b]	0.94	0.042
Ileum	28.70	28.17	31.97	0.85	0.140
Relative weights					
Duodenum	7.61[a]	3.95[b]	5.34[c]	0.43	0.002
Jejunum	8.44[a]	8.37[a]	10.56[b]	0.41	0.036
Ileum	8.05	7.93	9.03	0.22	0.079

[1] Data represent means from 8 replicates per treatment, SEM = Standard error of mean.
[2] Control = Basal diet; NCLI = Basal diet supplemented with 2% natural Clinoptilolite; MCLI = Basal diet supplemented with 2% formic acid modified clinoptilolite.
[3] Means with different superscript letters in the same line differ significantly; Lowercases represent $p<0.05$.

Table 4. Effects of NCLI (2%) and MCLI (2%) on the morphology (μm) of the intestinal mucosa in broilers

Items	Diet treatments			SEM	p value
	Control[2]	NCLI[2]	MCLI[2]		
1 to 21 d					
Jejunum					
Villus height (μm)	818.78[a]	904.96[b]	969.35[c]	12.62	0.002
Crypt depth (μm)	123.00	114.17	106.03	3.80	0.194
Villus height:crypt depth	6.66[a]	7.93[ab]	9.16[b]	0.31	0.003
Ileum					
Villus height (μm)	517.83[a]	549.32[b]	570.76[b]	6.21	0.001
Crypt depth (μm)	131.49	118.74	105.06	5.68	0.166
Villus height:crypt depth	3.95[a]	4.66[ab]	5.45[b]	0.23	0.012
22 to 42 d					
Jejunum					
Villus height (μm)	1,054.94[a]	1,193.63[b]	1,344.99[c]	14.69	0.001
Crypt depth (μm)	146.44	134.53	121.22	4.75	0.091
Villus height:crypt depth	7.21[a]	8.88[ab]	10.13[b]	0.38	0.001
Ileum					
Villus height (μm)	789.49[a]	844.64[b]	902.55[b]	9.27	0.002
Crypt depth (μm)	148.44	128.55	125.13	4.27	0.051
Villus height:crypt depth	5.32[a]	6.57[ab]	7.21[b]	0.23	0.001

[1] Data represent means from 8 replicates per treatment, SEM = Standard error of mean.
[2] Control = Basal diet; NCLI = Basal diet supplemented with 2% natural Clinoptilolite; MCLI = Basal diet supplemented with 2% formic acid modified clinoptilolite.
[3] Means with different superscript letters in the same line differ significantly; Lowercases represent $p<0.05$.

differed significantly from the control group in terms of the villus height to crypt depth ratio in the jejunal and ileal mucosa ($p<0.05$).The villus height to crypt depth ratio in the NCLI group was not significantly different from either the control group or the MCLI group ($p>0.05$).

The present study showed change in the relative weight of the jejunum in birds fed with NCLI or MCLI supplemented diet ($p<0.05$). These may be associated with slower passage of ingest through the digestive tract, and the jejunum utilized the limited nutrients for its growth with higher priority over body weight increase. Greater villus heights in the jejunal and ileal mucosa indicate that the function of the intestinal villi was increased (Ruttanavut and Yamauchi, 2010). In the present study, increases were observed in villus height and villus height to crypt depth ratio in the small intestinal mucosa of the broiler chicks supplemented with NCLI and MCLI. These results are in agreement with the findings of Tatar et al. (2008), who suggest that zeolite can stimulate villi of the small intestine. Such improvement in the morphology of the intestinal mucosa may be explained by the lower numbers of *E. coli* and *Salmonella*. It is reported that NCLI, a mucus stabilizer, effectively acts by attaching to the mucus to reinforce the intestinal mucosal barrier, and helps in the regeneration of the epithelium, therefore reducing intestinal colonization and infectious processes. This ultimately decreases inflammatory processes at the intestinal mucosa, thus increasing villus height and secretory activity (Loddi et al., 2004). Furthermore, increased villus size was also associated with activated cell proliferation in the crypt (Lauronen et al., 1998). In conclusion, the present results and related literature suggested that these intestine morphological changes might be induced by improved jejumum and ileum lumen due to adsorptive function of the crystal structural cavities of CLI (Khambualai et al., 2009), because a crystal structure of CLI is thought to induce epithelial cell generation in broilers (Mumpton and Fishman, 1977).

Effects of NCLI (2%) and MCLI (2%) on the activities of digestive enzymes of the intestinal contents in broilers (U/g)

As shown in Table 5, NCLI and MCLI had an effect on digestive enzyme activities of duodenum content. Markedly increased activities of digestive enzyme including protease, chymotrypsin, trypsin and amylase in the small intestinal contents were observed in the NCLI and MCLI-treated groups during the overall experimental period ($p<0.05$).

In the present experiment, supplementation with NCLI and MCLI could significant improved the activities of the digestive enzymes in the small intestinal contents ($p<0.05$). Our results were consistent with the previous studies of the clay minerals. It has been reported that the addition of clay to the feedstuffs improved the nutrient digestibility and the

Table 5. Effects of NCLI (2%) and MCLI (2%) on the activities of digestive enzymes of the intestinal contents in broilers (U/g)

Items[1]	Diet treatments			SEM	p value
	Control[2]	NCLI[2]	MCLI[2]		
1 to 21 d					
Duodenum					
Amylase	446.09[a]	632.27[b]	645.23[b]	20.03	0.002
Trypsin	4.38[a]	5.47[b]	5.83[b]	0.14	0.001
Chymotrypsin	5.23[a]	6.23[b]	6.50[b]	0.64	0.000
Protease	6.84[a]	8.21[b]	8.62[b]	0.89	0.001
Jejumum					
Amylase	468.35[a]	576.88[b]	620.75[b]	15.20	0.002
Trypsin	5.11[a]	6.53[b]	6.88[b]	0.18	0.001
Chymotrypsin	5.93[a]	6.85[b]	7.28[b]	0.15	0.003
Protease	7.07[a]	8.59[b]	9.08[b]	0.21	0.001
Ileum					
Amylase	462.78[a]	546.48[b]	552.61[b]	11.48	0.001
Trypsin	4.89[a]	6.19[b]	6.31[b]	0.18	0.002
Chymotrypsin	5.73[a]	6.91[b]	7.14[b]	0.15	0.001
Protease	6.88[a]	8.37[b]	8.49[b]	0.22	
22 to 42 d					
Duodenum					
Amylase	422.82[a]	465.15[b]	479.48[b]	7.37	0.001
Trypsin	5.35[a]	6.12[b]	6.26[b]	0.11	0.001
Chymotrypsin	5.47[a]	7.16[b]	6.83[b]	0.17	0.002
Protease	7.02[a]	8.35[b]	8.94[b]	0.19	0.002
Jejumum					
Amylase	439.59[a]	586.26[b]	601.60[b]	16.52	0.001
Trypsin	5.31[a]	6.23[b]	6.28[b]	0.12	0.001
Chymotrypsin	6.12[a]	7.18[b]	7.19[b]	0.13	0.012
Protease	7.14[a]	8.29[b]	8.53[b]	0.23	0.002
Ileum					
Amylase	436.74[a]	535.62[b]	554.78[b]	13.13	0.007
Trypsin	5.30[a]	6.73[b]	6.96[b]	0.18	0.001
Chymotrypsin	6.09[a]	7.01[b]	7.22[b]	0.14	0.002
Protease	7.25[a]	8.86[b]	8.99[b]	0.21	0.006

[1] Data represent means from 8 replicates per treatment, SEM = Standard error of mean.
[2] Control = Basal diet; NCLI = Basal diet supplemented with 2% natural Clinoptilolite; MCLI = Basal diet supplemented with 2% formic acid modified clinoptilolite.
[3] Means with different superscript letters in the same line differ significantly; Lowercases represent $p<0.05$.

enzymatic activity of gastrointestinal secretions (Cabezas et al., 1991; Ouhida et al., 2000; Alzueta et al., 2002; Hu et al., 2004). Because, the ion-exchange properties of the zeolite could alter the pH and increase the content of macro- (Ca, K, Na) and microelements in the gastrointestinal fluids (Teimuraz et al., 2009), thereby changing the enzymatic activity of gastrointestinal secretions (Martin-Kleiner et al., 2001). Moreover, some reports indicate that the villi and microvilli of intestinal mucosa can affect the secretion of digestive enzymes (Gao, 1998). In the present study, increases in villus height and villus height:crypt depth ratio were observed in the small intestinal mucosa of chicks supplemented with NCLI and MCLI. Such improved intestinal mucosal morphology may be explained by the higher enzymatic activity of gastrointestinal contents.

From this study, the following conclusion can be drawn. The supplementation of NCLI and MCLI into the diets of broiler chicks can exert beneficial effect in the gut morphology, gut development and gut digestive enzyme activity. But the mechanism(s) of CLI on the gastrointestinal tract has not yet been studied. Thus, NCLI and MCLI can be beneficial as a feed additive in the broilers diet, and there is a need for further research to understand and clarify the mechanism(s) involved.

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