Effects of clinoptilolite and modified clinoptilolite on the growth performance, intestinal microflora, and gut parameters of broilers[1]

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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, and its relation to gut circumstances. A total of two hundred forty 1-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, whereas those in the experimental groups were fed diets supplemented with NCLI at 2% (NCLI group) or MCLI at 2% (MCLI group) for 42 d. The results showed that 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 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. Total viable counts of *Escherichia coli* were significantly ($P < 0.05$) decreased by MCLI and NCLI from d 1 to 21. The NCLI and MCLI significantly increased the total viable counts of *Lactobacillus acidophilus* from d 22 to 42. Small intestine and cecal pH values in the MCLI group were found to be lower ($P < 0.05$) than those in other groups. Total volatile fatty acid concentrations were significantly ($P < 0.05$) decreased in both experimental groups from d 22 to 42. This study showed that NCLI or MCLI, as feed additives for broilers, had a positive effect on gut parameters by acting on microbial populations of the digestive tract.

Key words: clinoptilolite, growth performance, gut, broiler

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INTRODUCTION

Natural clinoptilolite (**NCLI**) is a natural zeolite that has a 3-dimensional tetrahedral network of SiO_4 and AlO_4 (Mumpton and Fishman, 1977). Zeolites are further characterized by the presence of channels and cavities, which can selectively adsorb water and exchange cations. Zeolites have 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).

Studies have revealed that clinoptilolite (**CLI**) is able to adsorb toxins that are damaging and even fatal to the growth of animals (Majid and Davood, 2011; Oguz, 2011), to eliminate heavy metals (Zhou, 2008) and radionuclides (Branislava and Gordana, 2004), and to improve animal performance (Papaioannou et al., 2002), decrease rate of passage of feed passing in the digestive system, and as a result, lead to a decrease in feed intake (Fethiere et al., 1990). Olver (1983) reported that NCLI reduced the viable counts of *Salmonella* Enteritidis and *Escherichia coli* in the proximal and distal gut, and reduced mortality in broiler chickens. Moreover, CLI also attracts and buffers excess protons that cause acidity. Clinoptilolite can thus exert a stabilizing effect on the intestinal barrier, and improve many conditions including acid reflux, *Candida* infections, and arthritis (Katsoulos et al., 2005; Szajewska et al., 2006). However, Cabuk et al. (2004) reported that growth performance of broilers fed CLI was not affected. Therefore, further studies are needed to determine whether CLI has positive effects on broiler production performance and gut parameters.

An in vitro study showed that NCLI adsorbs *E. coli* and *Salmonella typhimurium*, but showed no bactericidal effect (Mavilia et al., 1999). It has been shown

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that chemical modification of clinoptilolites with organic cations can increase hydrophobicity of the mineral surface, thus providing a high affinity and showing bactericidal effect against *E. coli* and its toxins (Uchida et al., 1992; Aleksandra et al., 2005). Both in vivo studies have demonstrated that different synthetic or modified CLI prevent the effects of bacteria or toxins, or prevent colonization, or bind toxins, further improving animal growth performance by enhancing nutrient absorption in rats, lambs, pigs, and laying hens (Pond and Yen, 1983; Olver, 1997; Richter et al., 2009). In accordance with these findings, synthetic or modified CLI (**MCLI**) may provide application benefits as a feed additive.

In recent years, CLI-based acidifying complexes have been prepared by a series of processes between the CLI and inorganic acidic substances. These complexes exhibited high adsorption activity and an antibacterial effect. However, the causticity of these inorganic acidifiers has caused some environmental concerns. Organic acids are weak acids, and their antibacterial properties have received much attention recently. They have thus been used largely as feed additives for poultry and swine. Formic acid, an organic acidifier with high causticity and antibacterial effect, has been used as a feed additive (acidifier) in the livestock industry. Studies are needed to determine whether formic acid-modified CLI has positive effects on adsorption and antibacterial properties in vitro, and on the growth performance and other parameters of broiler growth. To our knowledge, there are no data on the effects of feeding diets containing organic acid-modified CLI to broilers. Therefore, studies are also needed to determine whether MCLI has positive effects on in vitro properties and on growth performance and other parameters of broiler growth. 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 and gut parameters.

MATERIALS AND METHODS

Chemicals

The NCLI used in this study was collected from the Center of China Geological Survey (Nanjing). From x-ray diffractometry of the powder, the sample was shown to consist of about 85% CLI, 8% mordenite, 5% montmorillonite, and 2% silica minerals. 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.

The x-ray diffraction (**XRD**) graphs were obtained using an ASAP 2400 diffractometer with Cu Kα radiation ($\lambda = 0.154$ nm; 40 kV, 30 mA) at room temperature. Diffractograms were scanned from 10° to 80° in the 2θ range in 0.02° steps at a scanning rate of 5° min^{-1}, as shown in Figure 1. The samples were studied as powders. Chemical composition, determined by atomic absorption spectroscopy, is shown in Table 1. The cation exchange capacity was determined by leaching with 1 mol/L of ammonium acetate at pH 7, washing with 90% ethanol, displacing the $NH4^+$ with 1 mol/L of NaCl, and measuring the amount displaced with an autoanalyzer (Theng et al., 1997). Results are shown in Table 2. The Brunauere-Emmette-Teller (**BET**) specific surface area of the sample was measured by the multipoint BET method on an ASAP 2400 surface analyzer. Samples were outgassed at 133.322 K for 5 h at about 10^{-4} Torr (Rouquerol et al., 1999). Results are shown in Table 2.

Experimental Design, Birds, and Management

A total of two hundred forty 1-d-old Arbor Acres male broiler chicks were allocated to 3 dietary treatments in a randomized complete block design for 42 d, each of which was replicated 3 times with 10 broilers per replicate. The dietary treatments were 1) basal diet, 2) basal diet + 2% NCLI, 3) basal diet + 2% MCLI. All birds were housed in wire cages in a 3-level battery. The basal diets were of the maize-soybean type. Broilers were fed a starter diet from d 1 to 21 and a grower diet from d 22 to 42. 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.

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.

Table 1. The chemical composition of natural clinoptilolite (NCLI) and modified clinoptilolite (MCLI)

Sample	SiO_2 (%)	Al_2O_3 (%)	CaO (%)	Fe_2O_3 (%)	K_2O (%)	MgO (%)	Na_2O (%)	TiO_2 (%)	LOST (%)
NCLI	66.45	13.30	3.97	1.49	1.54	0.92	1.02	0.19	12.10
MCLI	69.52	11.96	3.80	1.12	1.32	1.03	0.55	0.08	6.85

Table 2. The Brunauere-Emmette-Teller (BET) surface area and cation exchange capacity (CEC) of natural clinoptilolite (NCLI, 2%) and modified clinoptilolite (MCLI, 2%)

Item	Sample	
	NCLI	MCLI
BET surface area (m^2/g)	19.485	24.993
CEC [mol(+)/kg]	0.184	0.232

Table 3. Formulation and calculated composition of broiler diets, as-fed basis

Item	1 to 21 d	22 to 42 d
Ingredient (g/kg)		
Corn	578	625
Soybean meal (43%, CP)	325	265
Corn gluten meal	30	35
Soybean oil	27	35
Limestone	9.5	10.5
Dicalcium phosphate	17.5	16.5
Salt	3	3
Premix[1]	10	10
Total	1,000	1,000
Calculation of nutrients (g/kg)		
AME (MJ/kg)	12.51	12.93
CP	211.5	192.5
Ca	9.7	9.0
Available P	4.2	4.0
Lys	10.8	9.5
Met	4.8	4.3
Met + Cys	8.1	7.1

[1]Premix provided per kilogram of diet: limestone, 3.3 g; L-Lysine·HCl, 1.5 g; DL-methionine, 1.3 g; vitamin A (transretinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-*rac*-α-tocopherol acetate), 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 sulfate), 80 mg; Cu (from copper sulfate), 8 mg; Mn (from manganese sulfate), 110 mg; Zn (Bacitracin Zn), 65 mg; iodine (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

Sample Collection and Procedures

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 and feed/gain ratio (**F/G**). 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 (1 bird per replicate) from each treatment were randomly selected and sent to the Veterinary Laboratory at Onderstepoort for bacterial analysis of the cecal contents. After slaughtering, the birds' 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 end of pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the cecum junction). The contents of the duodenum, jejunum, ileum, and cecum were collected aseptically for gut pH measurement. For assessing *Salmonella typhimurium*, *E. coli*, and *Lactobacillus acidophilus* colonization of the cecum, approximately 1 g of cecal content was aseptically collected from each bird. The sedimented cecal contents were frozen in liquid N until volatile fatty acid (**VFA**) analysis was performed. Approximately 2-cm lengths of the proximal jejunum and proximal ileum were removed and flushed with ice-cold PBS at pH 7.4 and immediately placed in 10% formalin solution for gut morphology measurements (Yang et al., 2007).

Intestinal Microbial Populations

One gram of mixed cecal contents was determined by serial dilution (10^{-1} to 10^{-7}) in PBS. Triplicate plates were then inoculated with 0.1-mL samples and incu-

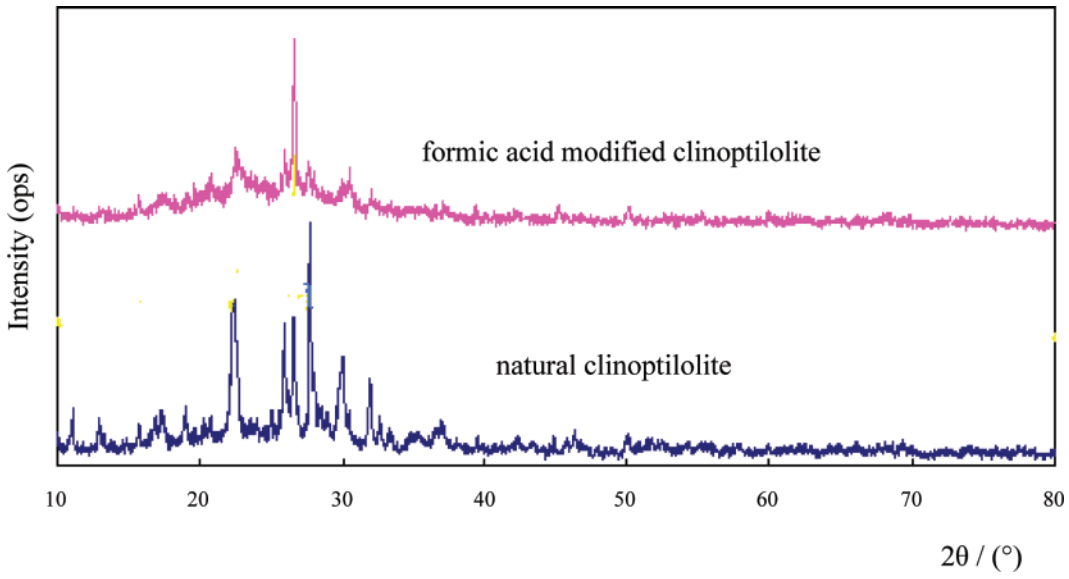

Figure 1. X-ray diffraction patterns of natural clinoptilolite and modified clinoptilolite.

bated at 37°C aerobically or anaerobically as appropriate. The dilutions were plated on the culture medium. *Lactobacilli acidophilus* were enumerated on de Man, Rogosa, and Sharpe agar (Merck, Frankfurter Straße, Darmstadt, Germany) after 48 h at 37°C; *E. coli* were counted on MacConkey agar (Merck) and incubated aerobically at 37°C for 24 h as red colonies. The population of *Salmonella typhimurium* was determined on bismuth sulfite agar incubated at 37°C for 24 h.

Morphological 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; Olympus CX31-32 Co2 optical microscope, Olympus Optical Co. Ltd., Tokyo, Japan) and were expressed as micrometers.

pH and VFA Analyses

Values of pH in different parts of the gastrointestinal tract were measured immediately by using a digital pH meter. To determine the pH, 1 g samples of gut content from duodenum, jejunum, ileum and cecum were collected aseptically in 9 mL of sterilized physiological saline (1:10 dilution) and pH values were determined (Al-Natour and Alshawabkeh, 2005).

Frozen digesta samples were held at 4°C in the refrigerator overnight before analysis. The VFA concentrations of cecal contents were determined by gas chromatography. Briefly, approximately 2 to 3 g of thawed digesta was suspended in 3 mL of 0.1 *M* phosphoric acid (H_3PO_4) and centrifuged (15 min at 12,000 × g) at 4°C. One-tenth of a milliliter of sodium hydrogen sulfate (62.2% wt/vol $NaHSO_4$) was added to 1 mL of the supernatant and transferred into a Thunberg tube. After the sample was frozen in liquid N and air was eliminated, it was bathed in liquid N in an insulated flask overnight. Subsequently, the gas chromatography was equipped with a flame ionization detector and a polyethylene glycol packed column (1.5 m long, 5.6 mm). The column was operated at 120°C with high purity helium, at 20 mL/min, as the carrier gas (Yang et al., 2007).

Statistical Analysis

Analyses of variance were performed using the GLM procedure of Statistical Package for the Social Sciences 18.0 (SPSS Inc., Chicago, IL) as a completely randomized design. Results are presented as 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

Growth Performance

The effects of the dietary treatments on broiler chicks' feed intake, average BW gain (**BWG**), and F/G data in the periods of starter, grower, and the whole trial are presented in Table 4. It can be seen that no significant differences were observed between treatments from 1 to 42 d. The general health status of broiler chickens was excellent for all treatments throughout the entire experimental period, showing livability more than 97.50%. There was no significant difference between the groups with respect to livability.

Intestinal Morphology

Morphological measurements of jejunal and ileal mucosae are presented in Table 5. During the overall experimental period, the villus height in the jejunal mucosa in the MCLI group was 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 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 2 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 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 the control group or the MCLI group ($P > 0.05$).

pH Value of Small Intestinal and Cecal Contents

The effects of NCLI and MCLI addition to the diets on the pH value of the small intestinal and cecal contents were evaluated (Table 6). From 1 to 21 d, compared with the controls, the pH of ileal and cecal contents was significantly decreased in the MCLI group ($P < 0.05$), and the pH of ileal and cecal contents in the NCLI group was not significantly different from the control group or the MCLI group ($P > 0.05$). The pH of the jejunal and duodenal contents of chicks receiving the NCLI and MCLI in the feed was not significantly different from those of the controls ($P > 0.05$), and

Table 4. Effects of NCLI (2%) and MCLI (2%) on the growth performance of broilers[1]

Item	Diet treatment[2]			SEM
	Control	NCLI	MCLI	
1 to 21 d				
FI[3] (g/bird per day)	61.7	61.4	61.3	0.79
BWG[3] (g/bird)	791	774	760	23.2
Feed/gain ratio (g/g)	1.64	1.67	1.69	0.034
22 to 42 d				
FI (g/bird per day)	164	164	164	1.2
BWG (g/bird)	1,680	1,694	1,710	42.5
Feed/gain ratio (g/g)	2.05	2.03	2.02	0.073
1 to 42 d				
FI (g/bird per day)	225	225	225	2.0
BWG (g/bird)	2,471	2,468	2,470	23.7
Feed/gain ratio (g/g)	1.92	1.92	1.91	0.023
Liveability (%)				
1 to 21 d	97.5	98.8	98.8	0.65
1 to 42 d	97.5	98.8	98.8	0.73

[a,b]Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]Data represent means from 8 replicates of 10 chickens per treatment.

[2]Control = basal diet; NCLI = basal diet + 2% natural clinoptilolite; MCLI = basal diet + 2% formic acid modified clinoptilolite.

[3]BWG = BW gain; FI = feed intake.

there was no significant difference between the 2 experimental groups ($P > 0.05$). From d 22 to 41, compared with the controls, the pH of duodenal and ileal contents was significantly decreased in the MCLI group ($P < 0.05$), and the pH of duodenal and ileal contents in the NCLI group was not significantly different from the control group and the MCLI group ($P > 0.05$). The pH of the cecal content of chicks receiving NCLI was lower ($P < 0.05$) than that in the control, but higher than that in the MCLI group ($P < 0.05$). The cecal pH of the MCLI group was significantly higher than that in the controls ($P < 0.05$). The pH of the jejunal contents of chicks receiving the NCLI and MCLI in the feed was not significantly different from that in the controls ($P > 0.05$), and there was no significant difference between the 2 experimental groups ($P > 0.05$).

Cecal Microbial Populations

Cecal microflora of chicks is presented in Table 7. Supplementation with NCLI and MCLI reduced ($P < 0.05$) the total viable counts of *E. coli* in the cecal contents of broiler chicks compared with those of the controls during the total experimental period. Supplementation with NCLI reduced ($P < 0.05$) the total viable counts of *Salmonella typhimurium* in the cecal contents

Table 5. Effects of NCLI (2%) and MCLI (2%) on the morphology (μm) of the intestinal mucosa in broilers[1]

Item	Diet treatment[2]			SEM
	Control	NCLI	MCLI	
1 to 3 wk				
Jejunum				
Villus height (μm)	819[c]	905[b]	969[a]	12.6
Crypt depth (μm)	123	114	106	3.8
Villus height:crypt depth	6.66[b]	7.94[ab]	9.14[a]	0.314
Ileum				
Villus height (μm)	518[b]	549[a]	571[a]	6.2
Crypt depth (μm)	132	119	105	5.7
Villus height:crypt depth	3.94[b]	4.61[ab]	5.44[a]	0.235
4 to 6 wk				
Jejunum				
Villus height (μm)	1,055[c]	1,194[b]	1,345[a]	14.7
Crypt depth (μm)	145	135	121	4.8
Villus height:crypt depth	7.28[b]	8.84[ab]	11.11[a]	0.384
Ileum				
Villus height (μm)	789[b]	845[a]	902[a]	9.3
Crypt depth (μm)	148	129	125	4.3
Villus height:crypt depth	5.33[b]	6.55[ab]	7.22[a]	0.233

[a–c]Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]Data represent means from 8 replicates of 10 chickens per treatment.

[2]Control = basal diet; NCLI = basal diet + 2% natural clinoptilolite; MCLI = basal diet + 2% formic acid modified clinoptilolite.

Table 6. Effects of NCLI (2%) and MCLI (2%) on the pH value in the small intestinal and cecal contents of the experimental broiler chicks[1]

	Diet treatment[2]			
Item	Control	NCLI	MCLI	SEM
1 to 3 wk				
Duodenum	6.27	6.25	6.23	0.041
Jejunum	6.33	6.30	6.29	0.032
Ileum	6.83[a]	6.66[ab]	6.52[b]	0.052
Cecum	6.71[a]	6.48[ab]	6.28[b]	0.062
4 to 6 wk				
Duodenum	5.94[a]	6.23[ab]	6.21[b]	0.043
Jejunum	6.23	6.22	6.26	0.024
Ileum	6.56[a]	6.48[ab]	6.30[b]	0.043
Cecum	6.63[a]	6.42[b]	6.25[b]	0.052

[a,b]Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]Data represent means from 8 replicates of 10 chickens per treatment.

[2]Control = basal diet; NCLI = basal diet + 2% natural clinoptilolite; MCLI = basal diet + 2% formic acid modified clinoptilolite.

of chicks compared with values for the controls in the starter period, but had no ($P > 0.05$) effect in the growing period. Chicks fed with MCLI had lower ($P < 0.05$) viable counts of *Salmonella typhimurium* in the cecal contents than did the NCLI and control groups during wk 1 to 3 and wk 4 to 6. *Lactobacillus acidophilus* in the cecal content was not affected by NCLI in the starter period, but supplementation with NCLI increased ($P < 0.05$) the total viable counts of *Lactobacillus acidophilus* in the cecal contents of chicks compared with values for the controls in the growing period. Dietary inclusion of MCLI increased the viable counts of *Lactobacillus acidophilus* ($P < 0.05$) during wk 1 to 3 and wk 4 to 6.

VFA

The concentration of volatile fatty acids in the cecal contents is presented in Table 8. From d 1 to 21, compared with the control group, the molar proportion of butyric acid was significantly increased ($P < 0.05$) in chicks fed the NCLI, but the molar proportion of butyric acid in those receiving MCLI was not significantly different from the control group or the NCLI group ($P > 0.05$). Supplementation with NCLI and MCLI had no ($P > 0.05$) effect on the molar proportions of acetic acid and propionic acid. Total VFA concentrations in both experimental groups were also not significantly different from those of the controls. From d 22 to 42, compared with the controls, the molar proportion of acetic acid and total VFA concentration were significantly decreased in both the MCLI and NCLI groups ($P < 0.05$), but there were no significant differences between the 2 groups ($P > 0.05$). Supplementation with NCLI and MCLI had no ($P > 0.05$) effect on the molar proportions of propionic acid in the experimental chicks compared with those in the control. The molar proportion of propionic acid in chicks fed NCLI was significantly higher than in the control ($P < 0.05$), but was not significantly different from corresponding values in the MCLI group ($P > 0.05$), and there were no significant differences in proportions of propionic acid between the control group and the MCLI group ($P > 0.05$).

DISCUSSION

The results presented in Table 4 show that adding NCLI and MCLI to the diets of broilers from d 1 to 42 produced no significant differences in terms of BWG. These findings are in agreement with those of Evans

Table 7. Effects of NCLI (2%) and MCLI (2%) on cecal microflora of broiler chicks[1]

	Diet treatment[2]			
Item	Control	NCLI	MCLI	SEM
1 to 3 wk				
Escherichia coli	7.93[a]	7.77[b]	7.64[c]	0.031
Salmonella	3.89[a]	3.82[a]	3.43[b]	0.052
Lactobacillus	8.80[b]	8.88[b]	9.30[a]	0.062
4 to 6 wk				
Escherichia coli	7.82[a]	7.70[b]	7.35[c]	0.055
Salmonella	3.52[a]	3.49[a]	3.14[b]	0.044
Lactobacillus	8.49[c]	8.99[b]	9.12[a]	0.073

[a–c]Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]Data represent means from 8 replicates of 10 chickens per treatment.

[2]Control = basal diet; NCLI = basal diet + 2% natural clinoptilolite; MCLI = basal diet + 2% formic acid modified clinoptilolite.

Table 8. Effects of NCLI (2%) and MCLI (2%) on the concentration of volatile fatty acids (VFA) in the cecal contents (mmol/L)[1]

	Diet treatment[2]			
Item	Control	NCLI	MCLI	SEM
1 to 3 wk				
Acetic acid	6.20	7.28	5.97	0.181
Propionic acid	0.67	0.68	0.95	0.291
Butyrate acid	0.50[b]	1.17[a]	0.72[ab]	0.114
Total VFA	7.37	9.14	7.63	0.232
4 to 6 wk				
Acetic acid	12.5[a]	5.79[b]	4.46[b]	0.875
Propionic acid	1.35	1.05	1.28	0.124
Butyrate acid	1.86[b]	2.46[a]	2.13[ab]	0.103
Total VFA	15.7[a]	9.31[b]	7.29[b]	0.942

[a,b]Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]Data represent means from 8 replicates of 10 chickens per treatment.

[2]Control = basal diet; NCLI = basal diet + 2% natural clinoptilolite; MCLI = basal diet + 2% formic acid modified clinoptilolite.

(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). Some researchers have however reported that dietary supplementation with CLI improves the health status and BW gain as well as feed efficiency of the animals (Papaioannou et al., 2004).

In the present investigation, supplementation with NCLI and MCLI did not appear to influence chicken feed intake. Livability, which ranged from 97.5 to 98.8%, was unaffected by treatments in the present study ($P > 0.05$). Indeed, the mortality rates determined for all treatments in the present study were much better than those observed in the commercial field. The low mortality may be related to the environment conditions of the experimental house in comparison with commercial farms. These findings suggest that further research into CLI as a feed additive is required. Numerous reports indicate that CLI is harmless as a component of mixed feed. It is well tolerated by the animals and improves the production characteristics of broilers (Elliot and Edwards, 1991; Trckova et al., 2004). The expected effects of zeolites (CLI) may vary because of such factors as nature, purity, concentration, particle distribution, the CLI content of the zeolite and composition of the formulation in the diet.

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 (Cik et al., 2001). 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). Narrow and long villi indicate a faster multiplication of the base of the crypt, with cells migrating faster to the tips of the villi. The turnover of the epithelial cells would, therefore, be shorter (Nordstrom and Dahlqvist, 1973). Greater villus height and numerous mitotic cells in the intestine are indicators that the function of the intestinal villi is activated (Langhout et al., 1999; Yasar and Forbes, 1999; Incharoen et al., 2009). Numerous, hypertrophied epithelial cells protrude into the intestinal lumen (Shamoto and Yamauchi, 2000; Tarachai and Yamauchi, 2000). These reports suggest that the intestinal villi and epithelial cells of chicks used in the preset study can be considered hypertrophied. Related literature suggests that CLI has cavities in its crystal structure (Coombs et al., 1997) and has the properties of a cation exchanger (Mumpton, 1999). These functions of zeolite might induce hypertrophied intestinal villi and epithelial cells (Mumpton and Fishman, 1977; Incharoen et al., 2009; Khambualai et al., 2009).

Table 6 presents pH values of intestine and ileum contents. Values of pH in the NCLI- and MCLI- supplemented groups were lower than those in the control group. These results are in agreement with the findings of Ismail et al. (2011), who found that ileum pH values in the zeolite group were lower than those in the control group. This could be a result of the high affinity of zeolites. It has been reported that the high affinity of CLI for water and osmotically active cations may facilitate fermentation in the gut, and osmotic activity may regulate gut pH by buffering hydrogen ions of organic acids (Szajewska et al., 2006; Dschaak et al., 2010). Moreover, it has been reported that there is a

relationship between pH, viscosity, and the density of ingesta. An increase in viscosity reduced the density of ingesta, causing an increase in the pH of intestinal content (Taylor and Jones, 2004; Ismail et al., 2011). Similar to our results, Evans (1989) found that natural zeolite can reduce the rate of passage of digesta through the digestive tract and decrease the pH of the intestinal contents.

It is well known that NCLI, as aluminosilicate compounds, could be much more selective than other clay minerals as adsorbates, and are used in animal nutrition for several beneficial effects. They adsorb bacteria selectively and bind certain toxins. This led to speculation that they might reduce *E. coli* and *Salmonella* (Olver, 1989). Adsorption was the main interaction between NCLI and bacteria. The adsorption effect was related to the high specific area and sorption capacity of CLI. In animal medicine, NCLI has been used as an antidiarrheal drug to treat the diseases of gram^{positive} and gram^{negative} bacteria (Vrzgula et al., 1988; Bartko et al., 1995; Rodriguez-Fuentes et al., 1997). These researchers proposed that the ameliorative effect on the diarrhea syndrome of calves and pigs might result from the increased sequestration of the enteropathogenic *E. coli*. The CLI is able to adsorb and partially inactivate the thermolabile *E. coli* enterotoxin in vitro, thus restricting its attachment to the intestinal cell-membrane receptors (Ramu et al., 1997). In the present study, the total viable counts of *E. coli* and *Salmonella typhimurium* of MCLI and NCLI groups significantly decreased relative to the control group. This result is consistent with the finding of Afaf et al. (2011), who reported that adding zeolite to the broiler diet significantly reduced *Salmonella* contamination in the ceca. The lower counts of *E. coli* and *Salmonella typhimurium* may be explained by the cecum pH. It is reported that decreasing cecum pH could reduce *E. coli* and *Salmonella* occurrence, and promote *Lactobacillus* proliferation (Boranic, 2000; Trckova et al., 2004). Moreover, this phenomenon could possibly mean that the broiler chicks in the NCLI and MCLI groups were less susceptible to disease than those broiler chicks not fed CLI. Mumpton and Fishman (1977) suggest that the presence of zeolite in the diet of broiler chicks could effectively prevent mortality.

The greater number of bacteria adsorbed by CLI was also attributed to the rough surface of CLI particles, which therefore provides a better microenvironment for the adsorption of bacteria (Hrenovic et al., 2005). Using scanning electron microscopy, we found that modified CLI exhibits unusual characteristics compared with the classic curves obtained from formic acid-prepared material. The surface of modified CLI was rough (Xu et al., 2008). Therefore, CLI could sequestrate harmful bacteria in the gut and form stable sorption complexes, then alter the fermentation of existing bacteria and control the concentration and molar proportions of VFA, ameliorating their adverse effect on the broiler chicks.

From the results, the following conclusions can be drawn. The growth performance of broiler chicks was not affected, but gut morphology was affected in the sense of increased villus height and villus to crypt depth, decrease in pH, and lower *Salmonellla* and *E. coli* counts, which suggest that the health of the intestine was improved by the NCLI and MCLI. Therefore, the use of NCLI or MCLI as a beneficial feed additive in broiler chicken diets is recommended. Further research is need to understand and clarify the mechanism(s) involved.

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