Ann. Anim. Sci., Vol. 15, No. 3 (2015) 681–697 *DOI: 10.1515/aoas-2015-0010*

DE GRUYTER
OPEN

EFFECTS OF CLINOPTILOLITE (ZEOLITE) ON ATTENUATION OF LIPOPOLYSACCHARIDE-INDUCED STRESS, GROWTH AND IMMUNE RESPONSE IN BROILER CHICKENS*

Qiu Jue Wu[1]♦, Qin Yu Wang[1], Tian Wang[2], Yan Min Zhou[2]

[1]College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, Henan, PR China
[2]College of Animal Science and Technology, Nanjing Agricultural University, No. 6, Tongwei Road, Xuanwu District, Nanjing 210095, Jiangsu, PR China
♦Corresponding author: wuqiujue@163.com

Abstract

The effects of natural clinoptilolite (NCLI) and modified clinoptilolite (MCLI) were evaluated in broilers challenged with lipopolysaccharide (LPS) in a 21-d feeding trial. A total of 288 one-day-old chickens were allocated into three treatment groups: control, NCLI (2%) and MCLI (2%). Half of the birds from each treatment group were challenged with either 0.9% NaCl solution or LPS (250 µg/kg body weight, orally administered) at 16, 18 and 21 d of age. Before the LPS challenge, no dietary effect on bird growth performance was found (P>0.05). When LPS was orally administered, no significant changes in growth performance of broilers was found (P>0.05). However, small intestinal morphology and development, malondialdehyde (MDA) content of the jejunual and ileal mucosa, and superoxide dismutase (SOD) activity of the ileal mucosa were significantly affected (P<0.05). Supplementation with NCLI and MCLI significantly decreased the MDA contents of the jejunual and ileal mucosa and improved the SOD activity of the ileal mucosa and the development of the small intestine compared with the control group (P<0.05). The results indicated that NCLI and MCLI additions in feed had protective effects on the gut health of broilers against LPS challenge.

Key words: zeolite (clinoptilolite), broiler, growth performance, gut health

Lipopolysaccharide (LPS), a known endotoxin, is a glycolipid of the outer membrane of gram-negative bacteria (e.g., *Escherichia coli* and *Salmonella*). Commercial broilers are chronically challenged with airborne gram-negative bacteria and LPS. The inhalation and the penetration of gram-negative bacteria and LPS to the gas exchange parenchyma trigger an inflammatory response and immunological stress that

*This research was supported by a project funded by the Henan University of Science and Technology (09001656).

affect physiological and pathological processes of broilers (Lorenzoni and Wideman, 2008). When stressed, feed consumption, body-weight gain and feed conversion efficiency of chickens decreased (Yang et al., 2008), and health status of broilers was impaired (Wu et al., 2013). The change partitioned nutrients away from growth and toward processes associated with inflammatory immune responses in chickens (Korver and Klasing, 1997). The redox homeostasis, a typical indicator of the response to these stresses, is maintained by the balance between the production of reactive oxygen species (ROS), reactive nitrogen species, and the antioxidant defense system (Wu et al., 2013). In chickens, one approach to modulate the immune system and to maintain the balance between antioxidants and oxidative stress in broilers is to use feed additives to alleviate decreased performance following immunological and oxidative stimulation.

Clinoptilolite (CLI) is the optimum type of feed additive and is suitable for large numbers of applications. Clinoptilolite (natural zeolite) has a cage-like structure consisting of SiO_4 and AlO_4 tetrahedrals that contain water molecules and alkaline and alkali earth metals in the structural framework joined by shared oxygen atoms (Mumpton, 1999). Natural clinoptilolite contains most of the major and trace minerals that are essential for the growth of chickens, livestock, and aquatic animals. These minerals are in an ionic state and can be used in animal diets for improving the health condition of animals (Mumpton, 1999). Additionally, in the process of animal digestion harmful organisms (e.g., *Escherichia coli*, dysentery bacillus and *Salmonella*) and toxic gases (e.g., ammonia and hydrogen sulfide) may be adsorbed at any time by clinoptilolite, which can be used to eliminate the harmful substances from the body, and thus decrease the harm to the intestine (Charlton et al., 1988). The natural clinoptilolite also has unique catalytic, ion exchange, and ion selective properties and is acidic with thermal stability and high biological activity against toxic compounds. Because of its particular structural and replacement properties, the natural clinoptilolite, the crystals of which have a lamellar structure, has proven repeatedly to be suitable for use in feed, chemicals, veterinary medicine, and feed additives.

Clinoptilolite (CLI), as a feed additive in the diets of animals, was used with enormous success in animal breeding for many purposes in the last few decades. Most of the *in vitro* and *in vivo* studies of synthetic or modified CLI demonstrated that it adsorbs toxins that are damaging and even fatal to the growth of animals (Oguz, 2011; Majid and Davood, 2011), reduces oxidative stress (Ivkovic and Zabcic, 2002 a, b), eliminates heavy metals (Zhou, 2008) and radioactive elements (Mitrovic et al., 2007), controls bactericides and fungicides (Morishita et al., 1998), prevents diabetes (Oschilewski et al., 1985), and averts or diminishes some late sequelae of diabetes (Charlton et al., 1988). According to the accumulated evidence, CLI significantly affects the regulation of the immune system. Studies with animals revealed that CLI relieves immune stress (Miazzo et al., 2000) and deodorizes animal litter (Leung et al., 2007), as well as improving performance in rats, lambs, pigs, broilers and laying hens (Olver, 1997; Papaioannou et al., 2002). Additionally, CLI decreases the rate of passage of feed in the digestive system, which leads to a decrease in feed intake (Fethiere et al., 1990). If natural clinoptilolite (NCLI) and synthetic or modi-

fied CLI (MCLI) have significant immunostimulatory and antioxidant properties, they may provide clinical benefits as an oral dietary supplement. The purposes of the present study were to determine whether the two types of CLI were inert and nontoxic for animals, and furthermore, to compare the preventive effectiveness of two types of CLI on the growth performance, the relative weight of immune organs and the small intestinal morphology of broilers under challenge from *Escherichia coli* lipopolysaccharide (LPS).

Material and methods

The NCLI used in this study was collected from the Center of China Geological Survey (Nanjing, China). From X-ray diffractometry of the powder, the NCLI was approximately 85% clinoptilolite, 8% mordenite, 5% montmorillonite, and 2% silica minerals. The NCLI grain size was within the range of 0.15 to 0.2 mm. For the modification of natural CLI, the NCLI was calcined in a muffle oven at 400°C for 4 h, followed by the addition of formic acid, which was stirred in to ensure even distribution. The mixture was repeatedly washed with deionized water. After stirring, the sample was allowed to settle. The sediments were collected and dried in an air oven at 65°C for 2 h. After drying, the sediment was ground and passed through a 100-mesh sieve.

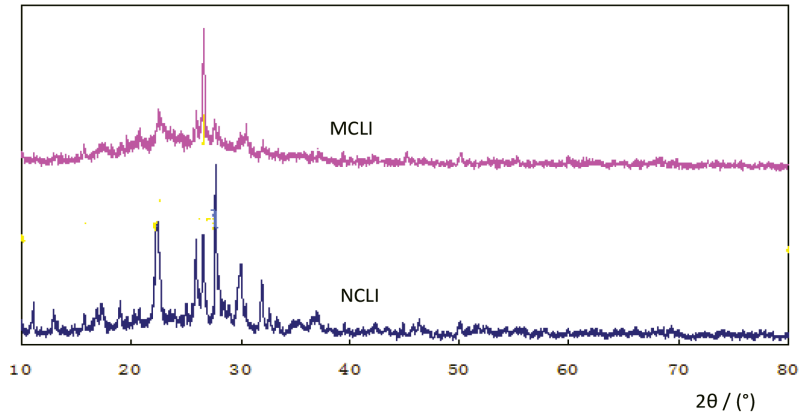

Figure 1. XRD patterns of natural clinoptilolite and formic acid modified clinoptilolite

The X-ray diffraction (XRD) graphs were obtained using an ASAP 2400 diffractometer with Cu Kα radiation (λ = 0.154 nm; 40 kV; 30 mA) at room temperature. The 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. The chemical composition determined by atomic absorption spectroscopy is shown in Table 1. The cation exchange capacity (CEC) was determined by leaching with 1 mol/L of ammonium acetate at pH 7, washing with 90% ethanol, displac-

ing the NH_4^+ with 1 mol/L of NaCl, and measuring the amount displaced with an autoanalyzer (Theng et al., 1997). The results are shown in Table 1. The Brunauere Emmette Teller (BET) specific surface area of the sample was measured by the multipoint BET method on an ASAP 2400 surface analyzer. The samples were outgassed at 133.322°K for 5 h at approximately 10^{-4} Torr. The results are shown in Table 1.

Table 1. The chemical composition, BET surface area and CEC of NCLI and MCLI

Item	SiO_2 (%)	Al_2O_3 (%)	CaO (%)	Fe_2O_3 (%)	K_2O (%)	MgO (%)	Na_2O (%)	TiO_2 (%)	LOS (%)	BET Surface Area (m^2/g^a)	CEC (mol(+)/kg[b])
NCLI	66.45	13.30	3.97	1.49	1.54	0.92	1.02	0.19	12.10	19.485	0.184
MCLI	69.52	11.96	3.80	1.12	1.32	1.03	0.55	0.08	6.85	24.993	0.232

[a]BET= Brunauere Emmette Teller.
[b]CEC= cation exchange capacity.

A total of 288 one-day-old commercial Arbor Acres broiler chicks in cages were randomly assigned to six dietary treatments based on the initial body weight ($P>0.05$). Six replicate cages containing eight chicks each were assigned to each treatment. All the birds were in wire cages with automatic watering, manual feeding, and with controlled light and temperature regimens. The broiler chicks were all fed mash form diets with the same component composition, and the only difference was the CLI supplementation. The basal diets were of the corn-soybean type. The diets were formulated based on the NRC standards (1998) to meet the nutrient requirements of the broilers (Table 2).

Table 2. Formulation and calculated composition of broiler diets, on as-fed basis

Ingredients (g/kg)	1–21d
Corn	578
Soybean meal (43%, crude protein)	325
Corn gluten meal	30
Soybean oil	27
Limestone	9.5
Dicalcium phosphate	17.5
Salt	3
Premix[a]	10
Total	1.000
Calculation of nutrients (g/kg)[b]:	
apparent metabolism energy (MJ/kg)	12.51
crude protein	211.5
Ca	9.7
available P	4.2
Lys	10.8
Met	4.8
Met+Cys	8.1

[a]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.
[b]The nutrient levels were on as-fed basis.

This experiment was designed as a 2 × 3 factorial with three dietary treatments: control group (birds fed with the basal diet), NCLI group and MCLI group (birds fed with the basal diet supplemented with 2% NCLI or 2% MCLI, respectively). Additionally, for each diet, birds received either a sham challenge or a challenge with orally administered LPS. The LPS (*E. coli* serotype O_{55}, B_5; Sigma Chemical, St Louis, MO, USA) was dissolved in a sterile 9 g/L (w/v) NaCl solution at 0.5 mg/mL to administer orally the desired dose of 0.5 mL/kg body weight. At 16, 18 and 21 d of age, half of the birds in each replicate were orally treated with LPS (250 μg/kg body weight) or an equivalent amount of sterile saline alone, which served as a control. Each replicate (8 birds) was the experimental unit for dietary treatment and challenge status (orally administered LPS or saline). The Institutional Animal Care and Use Committee of the Nanjing Agricultural University reviewed and approved all the experimental protocols.

During the 21-day experiment, the weight of chicks was measured weekly, and the mortality was recorded as it occurred. The feeds and feed residues were weighed on the same days as above to determine feed intake (FI) and feed/gain ratio (F/G). At the end of each experimental period (21 d), eight chicks from each treatment were randomly selected and slaughtered. The intestinal segments (duodenum, jejunum and ileum) were removed, emptied by gentle pressure and the length and weight were recorded. The pancreas, liver, heart, spleen, thymus and bursa of the broiler chicks were also removed and weighed. The relative organ weights (weight of organ/kg live body weight) were calculated. Three cross sections of the jejunum and ileum intestinal segments were fixed with formalin, prepared using standard paraffin embedding procedures, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. Blood samples were collected within 2 h after oral administration and were separated by centrifugation at 3,000 × g for 15 min at 4°C. The serum samples were frozen at –80°C until analysis. Approximately 0.3 g of the jejunual and ileal mucosa was used to prepare the mucosa homogenate. The mucosa was diluted 1:9 (wt/vol) with PBS solution and homogenized using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH, USA). The protein concentration, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) content of the mucosa homogenate were determined using a corresponding diagnostic kit (Nanjing Jiancheng Bioengineering Institute, China), according to the instructions of the manufacturer. Briefly, the MDA was measured with the barbiturate thiosulfate assay, and the SOD enzymes were measured with the xanthine oxidase method. The MDA concentrations were expressed as nmol per mg of protein of mucosa tissue, and the SOD activity was expressed as unit per mg of protein of mucosa.

The data were analyzed with the General Linear Model procedure of the statistical package for social sciences 18.0 (SPSS Inc., Chicago, IL, USA) as a 2 × 3 factorial design with dietary treatment and LPS challenge status as the main effects. The significant differences among different treatments were evaluated by least significant difference post hoc multiple comparisons tests, with the significance level set at 0.05.

Table 3. Effects of NCLI (2%) and MCLI (2%) on the growth performance of LPS (250 μg/kg)-induced immune stress broilers fed 1 to 21 d of age

Items	LPS(–)[d]			LPS(+)[d]			SEM[b]	P-values[f]		
	Control[e]	NCLI[e]	MCLI[e]	Control[e]	NCLI[e]	MCLI[e]		diet	stress	interaction
FI[c] (g/per bird)										
1–15 d	465	468	469	462	463	464	28	0.087	0.368	0.770
15–21 d	318	320	322	313	315	317	62	0.995	0.083	0.926
BWG[c] (g/per bird)										
1–15 d	543	535	537	549	545	542	15	0.580	0.848	0.983
15–21 d	504	502	501	508	507	506	32	0.281	0.171	0.661
F:G[c] (g/g)										
1–15 d	1.17	1.14	1.14	1.19	1.18	1.17	0.17	0.864	0.442	0.953
15–21 d	1.58	1.57	1.56	1.62	1.61	1.60	0.16	0.677	0.374	0.639

[a]Data represent means from 6 replicates per treatment, n=6.

[b]SEM = standard error of mean.

[c]BWG = body weight gain; FI = feed intake; F/G = feed intake / BW gain.

[d]LPS(–) = dietary treatment was orally administered sterile saline; LPS(+) = dietary treatment was orally administered lipopolysaccharide (LPS). Days of LPS injections = 16, 18 and 21 d of age.

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

[f]The P-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

Table 4. Effect of NCLI (2%) and MCLI (2%) on the relative organ weights (g/kg) of LPS (250 μg/kg)-induced immune stress broilers fed 1 to 21 d age

Items	LPS(–)[c]			LPS(+)[c]			SEM[b]	P-values[e]		
	control[d]	NCLI[d]	MCLI[d]	control[d]	NCLI[d]	MCLI[d]		diet	stress	interaction
Spleen	0.75 a	0.80 ab	0.84 ab	0.79 ac	0.97 b	0.92 bc	0.22	0.020	0.114	0.204
Thymus	1.97 a	2.81 b	2.93 b	3.12 c	3.24 c	3.68 d	0.16	0.005	0.007	0.259
Bursa of Fabricius	1.97 a	2.15 a	2.38 ab	2.52 b	2.44 b	2.33 b	0.65	0.806	0.005	0.104
Heart	7.57 a	7.29 a	6.39 b	7.85 a	7.54 a	7.79 a	0.20	0.034	0.129	0.002
Liver	25.68 a	24.53 a	26.71 a	26.82 a	26.06 a	27.16 a	0.46	0.146	0.537	0.241
Gizzard	31.46 a	28.15 b	31.02 ab	32.60 a	30.79 ab	30.98 a	0.63	0.056	0.194	0.468

[a]Data represent means from 6 replicates per treatment, n=6.

[b]SEM = standard error of mean.

[c]LPS(–) = dietary treatment was orally administered sterile saline; LPS(+) = dietary treatment was orally administered lipopolysaccharide (LPS). Days of LPS injections = 16, 18 and 21 d of age.

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

[e]The P-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

[f]Means with different letters in the same line differ significantly; lower cases represent $P<0.05$.

Table 5. Effects of NCLI (2%) and MCLI (2%) on the relative length (cm/kg) and relative weights (g/kg) of small intestine in the LPS (250 μg/kg)-induced immune stress broilers fed 1 to 21 d age

Items	LPS(–)[c]			LPS(+)[c]			SEM[b]	P-values[e]		
	control[d]	NCLI[d]	MCLI[d]	control[d]	NCLI[d]	MCLI[d]		diet	stress	interaction
Relative length										
duodenum	35.8 a	32.1 ab	31.4 ab	31.1 ab	29.6 b	31.2	0.53	0.028	0.005	0.184
jejunum	76.7 a	81.1	79.8 a	65.4 b	65.7	65.4	0.78	0.309	0.002	0.356
iIleum	71.7 a	70.4	71.3	65.1 b	65.7 b	66.9 b	1.37	0.745	0.004	0.985
Relative weights										
duodenum	9.4	9.2 a	8.9	8.4 b	8.5 ab	8.3	0.21	0.695	0.002	0.895
jejunum	14.71	15.85	16.31a	12.71 a	13.81 b	13.7 b	0.28	0.003	0.001	0.391
ileum	10.8 a	11.1	11.7 a	9.3 c	10.1	9.8 c	0.20	0.215	0.001	0.727

[a]Data represent means from 6 replicates per treatment, n=6.

[b]SEM = standard error of mean.

[c]LPS(–) = dietary treatment was orally administered sterile saline; LPS(+) = dietary treatment was orally administered lipopolysaccharide (LPS). Days of LPS injections = 16, 18 and 21 d of age.

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

[e]The P-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

[f]Least-square mean values (n=6) within a row with unlike letters were significantly different ($P<0.05$).

Table 6. Effects of NCLI (2%) and MCLI (2%) on the morphology (μm) of the intestinal mucosa in the LPS (250 μg/kg)-induced immune stress broilers fed 1 to 21 d age

Items	LPS(–)[c]			LPS(+)[c]			SEM[b]	P-values[e]		
	control[d]	NCLI[d]	MCLI[d]	control[d]	NCLI[d]	MCLI[d]		diet	stress	interaction
Jejunum										
villus height (μm)	816.2 a	910.1	971.9	779.7	819.1	913.9	32.72	0.002	0.009	0.001
crypt depth (μm)	121.0 a	112.4	106.7 c	138.2	128.5	106.0 c	21.24	0.005	0.005	0.005
villus height/crypt depth	6.75	8.10 b	9.11	5.64 d	6.37	8.62 f	5.44	0.006	0.002	0.008
muscle layer thickness of the submucosa (μm)	146.4	134.2	124.7	161.4 d	148.8 a	134.4 b	13.58	0.007	0.002	0.132
Ileum										
villus height (μm)	516.7 a	551.1 b	568.4	492.9 d	513.5	538.2	51.27	0.004	0.006	0.060
crypt depth (μm)	130.7	114.5 b	102.9	118.7 b	108.3 c	96.0 d	10.71	0.005	0.008	0.628
villus height/crypt depth	3.95 a	4.81	5.52 c	4.15	4.74 b	5.61 c	0.92	0.009	0.031	0.766
muscle layer thickness of the submucosa (μm)	152.6 a	139.5 b	120.1	132.2	118.0 c	101.6 d	9.86	0.009	0.009	0.762

[a]Data represent means from 6 replicates per treatment, n=6.

[b]SEM = standard error of mean.

[c]LPS(–) = dietary treatment was orally administered sterile saline; LPS(+) = dietary treatment was orally administered lipopolysaccharide (LPS). Days of LPS injections = 16, 18 and 21 d of age.

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

[e]The P-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

[f]Least-square mean values (n=6) within a row with unlike letters were significantly different ($P<0.05$).

Results

The effects of the treatments on the production performance as measured by body weight gain, feed intake and feed efficiency are shown in Table 3. Before the LPS challenge (0–15 d), no effect of dietary treatment on bird growth performance was found ($P>0.05$). With the oral administration of LPS, the body weight gain, feed intake and F/G of broilers were also not significantly different from those given saline ($P>0.05$). The natural clinoptilolite-LPS and modified clinoptilolite-LPS interactions did not affect the production performance of broilers.

The effects of treatments on the relative organ weights are presented in Table 4. In the groups not challenged with LPS, the relative weight of the thymus increased in the NCLI and MCLI groups compared with the control group ($P<0.05$). Compared with the control group, the inclusion of NCLI in the diets without LPS had no effect on the relative weights of the heart ($P>0.05$). However, the inclusion of MCLI in the diets without LPS significantly influenced the relative heart weight ($P<0.05$). No effect of dietary treatments on the relative weight of the spleen, bursa of Fabricius or liver was found ($P>0.05$) in the absence of LPS challenge. The oral administration of LPS significantly increased the relative weights of the thymus and bursa of broilers ($P<0.05$). The dietary inclusion of MCLI significantly increased the relative weights of the thymus of broilers in the presence of the LPS challenge. The dietary inclusion of NCLI and MCLI significantly influenced the relative weights of the spleen ($P<0.05$) in broilers with the LPS challenge. When LPS-challenged broilers were pretreated with NCLI or MCLI, no significant influence on the relative weights of the bursa of Fabricius, heart, liver or gizzard of broilers was found ($P>0.05$). The addition of NCLI and MCLI might attenuate LPS-induced immune stress, and significantly reduced the relative weight of the heart.

The effects of dietary NCLI and MCLI on the relative length and weight of the small intestines of LPS-induced immune stressed broilers are shown in Table 5. Before the LPS challenge, no dietary effect on the relative length and weight of the small intestine was observed ($P>0.05$). The oral administration of LPS significantly decreased the relative lengths of the jejunum and ileum of broilers, as well as the relative weights of the ileum ($P<0.05$). When LPS-challenged broilers were pretreated with NCLI or MCLI, there was no significant influence on the relative lengths of the duodenum, jejunum and ileum or on the relative weight of the duodenum ($P>0.05$). When LPS-challenged broilers were pretreated with NCLI, the relative weight of ileum increased ($P<0.05$). The relative lengths of the jejunum and ileum, as well as the relative weights of the ileum, in the unchallenged birds, were significantly higher than those of the challenged birds ($P<0.05$). The NCLI-LPS and MCLI-LPS interactions did not affect the development of the small intestine.

The effects of dietary NCLI and MCLI on the morphology of the small intestine of LPS-induced immune stressed broilers are shown in Table 6. Compared with LPS unchallenged broilers, the LPS decreased the villus height, crypt depth, villus height/crypt depth ratio and the thickness of the muscle layer of the submucosa of the jejunum and ileum (without the inclusion of the villus height/crypt depth of the ileum) ($P<0.05$). The addition of NCLI and MCLI to the diets of immune stressed

broilers minimized the deleterious effects of the LPS, and improved the morphology of the jejunum and ileum with or without orally administered LPS (P<0.05). Moreover, a significant difference in the morphology of the jejunum and ileum was found (P<0.05), and the MCLI was more effective at alleviating the LPS toxicity on the morphology of the jejunum and ileum of broilers. No significant (P>0.05) interaction between clinoptilolite and LPS was observed for the morphology of the ileum, but a significant (P<0.05) interaction effect on the villus height, crypt depth and villus height/crypt depth ratio was observed.

Table 7. Effects of NCLI (2%) and MCLI (2%) on SOD activity, MDA content of intestine mucosa and serum D-Lactic Acid and DAO levels of LPS (250 μg/kg)-induced immune stress broilers fed 1 to 21 d of age (unit / mg prot)

Items	LPS(–)[c]			LPS(+)[c]			SEM[b]	P-values[e]		
	control[d]	NCLI[d]	MCLI[d]	control[d]	NCLI[d]	MCLI[d]		diet	stress	inter-action
Jejunum										
SOD	185.32	189.78	190.26	143.56	147.23	155.97	7.06	0.107	0.051	0.103
MDA	0.36 b	0.25 b	0.19 b	0.97 a	0.49 b	0.32 b	0.07	0.004	0.000	0.001
Ileum										
SOD	125.67 a	131.42 a	130.27 a	80.65 b	93.06 ab	95.43 ab	6.98	0.007	0.001	0.119
MDA	0.32 b	0.20 b	0.17 b	0.63 a	0.32 b	0.29 b	0.03	0.007	0.001	0.119

[a]Data represent means from 6 replicates per treatment, n=6.

[b]SEM = standard error of mean.

[c]LPS(–) = dietary treatment was orally administered sterile saline; LPS(+) = dietary treatment was orally administered lipopolysaccharide (LPS). Days of LPS injections = 16, 18 and 21 d of age.

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

[e]The P-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

[f]Least-square mean values (n=6) within a row with unlike superscript letters were significantly different (P<0.05).

The effects of each treatment on the SOD activity in the intestinal mucosa are presented in Table 7. Among the groups not challenged with LPS, the SOD activity in the jejunal and ileal mucosa increased in the NCLI and MCLI groups compared with the control group (P>0.05). Moreover, dietary supplementation with NCLI and MCLI had no influence on the SOD activity in the jejunal and ileal mucosa of animals that were not orally administered LPS (P<0.05). The oral administration of LPS significantly decreased the SOD activity in the ileal mucosa of broilers (P<0.05) but had no influence on the activity in the jejunal mucosa of broilers (P>0.05). The dietary supplementation with NCLI or MCLI had no significant influence on the SOD activity in the jejunal and ileal mucosa of the LPS-treated broilers (P<0.05). The NCLI-LPS and MCLI-LPS interactions had no effect on the SOD activity in the jejunal and ileal mucosa.

The effects of each treatment on the MDA content in the intestinal mucosa are presented in Table 7. Among the LPS-unchallenged groups, the MDA content in the jejunal and ileal mucosa decreased in the NCLI and MCLI groups compared with the

control group (P>0.05). Moreover, dietary supplementation with NCLI and MCLI had no influence on the MDA content in the jejunal and ileal mucosa of animals that were not orally administered LPS (P<0.05). The dietary supplementation (with NCLI and MCLI) alone had no influence on the MDA content in the jejunal and ileal mucosa (P>0.05).

The oral administration of LPS significantly increased the MDA content in the intestinal mucosa of broilers (P<0.05). When LPS-challenged broilers were pretreated with NCLI and MCLI, the MDA contents in the jejunal and ileal mucosa decreased (P<0.05). The NCLI-LPS and MCLI-LPS interactions had no effect on the MDA content in the ileal mucosa, but the malondialdehyde content in the jejunal mucosa was altered.

Discussion

Bacterial lipopolysaccharide (LPS) is the major constituent of the outer membrane of gram-negative bacteria (*e.g*., *Salmonella typhimurium* and *Escherichia coli*) and is a potent bacterial endotoxin. When injected intravenously or orally administered to mammals, LPS leads to the acute phase response and the bacterial disease, with decreased growth and feed intake with the disruption of the immune system. Clinoptilolite, an adsorbent, can combine with LPS to create a stable complex and thereby decrease the availability of LPS for absorption from the gastrointestinal tract. Harvey et al. (1993) found no beneficial effect on growth performance of broiler chicks by adding clinoptilolite (5 g/kg diet) to an AF (3.5 ppm)-containing diet for 21 days. Ortatatli et al. (2005) reported that the addition of CLI (15 g/kg) to an AF-free diet did not cause any adverse effects on the parameters that were investigated. Our data agree with the results obtained by those researchers who found a protective effect of zeolite or clinoptilolite on BW gain. However, no significant differences were found for the feed:gain ratio. According to some research, it was suggested that clinoptilolite in diets contained mycotoxins, which might explain increased broiler performance (Oguz and Kurtoglu, 2000; Majid and Davood, 2011). The reasons for these differences might be due to the different type, dose, and physical characteristics of the clinoptilolite, the concentration of the toxin in the diet or the broiler strain used in the trials.

In the present study, no significant changes in poultry performance were found, but the morphology of the small intestine was significantly affected. Under immune stress conditions, it was speculated that the primary absorption sites for nutrients were in the jejunum and ileum because natural and modified clinoptilolite supplements led to greater absorptive surface area. This could have shifted the absorption site for nutrients to distal parts of the small intestine as a compensatory mechanism, and hence, no significant changes were observed on performance of the poultry (Awad et al., 2006 a). Moreover, results were also affected by the dose of orally administered LPS. The dose of orally administered LPS caused immune stress in broiler chickens; however, no internationally recognized recommended value was available that was studied during the beginning phase.

The liver and the immune system organs are the target organs for LPS and are primarily affected in bacterial lipopolysaccharide cases (Kuznetsova et al., 2011). It was confirmed that LPS affected the immune organs and hematopoietic organs first and then affected the other organs. In the present study, the toxic effects of LPS and the ameliorative efficacy of dietary adsorbents (NCLI and MCI) on the detrimental effects of LPS were investigated for pathological changes. The addition of natural and modified clinoptilolite to the diet partially reduced the severity of lesions in the organs examined. The beneficial effect of natural and modified clinoptilolite in this study might be related to the ability to adsorb the bacterial endotoxin. The accumulation of evidence indicates that an important mechanism of clinoptilolite action is the adsorption of active substances from the serum or intestine (Katic et al., 2006).

The natural clinoptilolite-LPS and modified clinoptilolite-LPS interactions involved the heart index, and the addition of natural and modified clinoptilolite attenuated the lipopolysaccharide-induced immune stress and significantly reduced the relative weight of hearts in broiler chicks. This was a clear demonstration that the addition of natural and modified clinoptilolite to the diet provided a moderate amelioration of LPS toxicity, and the modified clinoptilolite had a stronger effect than natural clinoptilolite.

The effects of dietary NCLI and MCLI on the relative length and weight of the small intestine of LPS-induced immune stressed broilers might be attributed to the LPS alteration of digestive and absorptive functions. When NCLI or MCLI was added to the diets of birds challenged with LPS, a significant increase in relative weight of the jejunum occurred ($P<0.05$). This result might be from the slower passage of food through the digestive tract, and the jejunum used the limited nutrients for its growth with higher priority over body weight increase during stress.

The addition of natural and modified clinoptilolite to the diet of immune stressed broiler chicks minimized the deleterious effects of LPS and improved the morphology of the jejunum and ileum, with or without orally administered LPS. These results might explain the improvement in the performance and intestine development of broilers supplemented with natural and modified clinoptilolite in the present study. Indeed, the NCLI and MCLI used in the present study was efficient in alleviating the effects of immune stress, which suggested that the effects of NCLI and MCLI were most likely because of its role as endotoxin adsorbent. However, the CLI perhaps, with increased digestibility of feeds, increased the passage rate of material from the gastrointestinal tract. Additionally, CLI can irritate the digestive tract and increase different parts of the muscle layer of the submucosa (Kubena et al., 1993). Moreover, 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, the NCLI reduced intestinal colonization and infectious processes, and ultimately decreased inflammatory processes of the intestinal mucosa, which increased villus heights and functions of secretion (Khambualai et al., 2009). Combined, the present results suggested that NCLI and MCLI might improve overall health status of the intestinal lumen due to the adsorptive function of the crystal structural cavities. These results demonstrated that clinoptilolite was effective as a toxin adsorbent and an immunostimulator (Pavelić et al., 2001).

The LPS caused adverse effects on the morphology of the small intestine in poultry. The adverse effects were attributed to irritant effects on the gastrointestinal tract (Awad et al., 2006 a, b). Toxins and endotoxins disrupt normal intestine cell function by inhibiting RNA, DNA, and protein synthesis, which inhibits cell division and stimulates a ribotoxic stress response (Rocha et al., 2005). Under the immune stress conditions, the morphological alterations in villus height, crypt depth, and muscle layer thickness of the submucosa of the broiler might contribute to reduced nutrient absorption in the jejunum and ileum. Moreover, the NCLI and MCLI prevented many of the adverse effects on the morphology of the small intestine caused by orally administered LPS. However, the mechanism by which this occurred needs further study.

Oxidative stress is a state of imbalance between the generation and the scavenging of reactive oxygen species, which results in excess free radicals, and thus impairment of intestinal barrier integrity (van Ampting et al., 2009). The LPS generates free radicals, and LPS is known to induce microglia activation, which causes the release of cytokines involved in the generation of free radicals species such as MDA in the intestinal cells. MDA is one of the most frequently used indicators of lipid peroxidation or as a biomarker for oxidative stress (Nielsen et al. 1997). An effective antioxidant defense system requires an increase in antioxidant enzyme activity, and SOD is one of the most important antioxidant enzymes (Zelko et al., 2002). Previous experiments suggested that some zeolites had antioxidant properties (Ivkovic et al., 2004).

In the current study, the LPS challenge affected the MDA content in the jejunual and ileal mucosa and the SOD activity of the ileal mucosa at 21 d ($P<0.05$). The serious adverse effects of oxidative damage on the intestinal mucosa that emerged might explain this phenomenon. The supplementation with NCLI and MCLI significantly decreased the contents of MDA in intestinal mucosa at 21 d and increased the SOD activity, particularly in the ileal mucosa. The increased antioxidant effects of NCLI and MCLI might be attributed to the adsorption activity, which reduces the oxidative stress of the LPS challenge. Additionally, NCLI and MCLI might adsorb free radicals that were generated and increase the protective effects of NCLI and MCLI on intestine health. This result is in agreement with the accumulated evidence that CLI played a role as an antioxidant. For example, Yarovan (2008) observed that the MDA concentration decreased after zeolite supplementation in dairy cows.

Moreover, we observed that the values of antioxidant indicators such as SOD activity in the intestinal mucosa increased with NCLI and MCLI supplementation ($P<0.05$), and these antioxidant enzymes decreased MDA content. Thus, the intake of NCLI and MCLI provided significant protection against oxidative stress by increasing the levels of important antioxidant enzymes. Ivkovic et al. (2002 b), Madhusudhan et al. (2009) and Wang et al. (2012) also found that antioxidant indicators increased significantly with zeolite or modified zeolite supplementation. These data suggested that NCLI and MCLI were able to increase physiological mechanisms against oxidative stress.

In conclusion, the dietary use of NCLI and MCLI had no obvious effect on reducing the toxic effect of LPS on the growth performance, but they did improve the development and morphology of the intestine, increase the liver and the immune sys-

tem organ weights in broilers, decrease the MDA contents in the intestinal mucosa at 21 d, and increase SOD activity. Thus, the addition of NCLI and MCLI to the diet of immune stressed broilers reduced the deleterious effects of LPS and did not provoke any adverse clinical effects on broilers, and NCLI and MCLI could be beneficial as feed additives in the broilers diet, with better performance with MCLI. Further studies on other aspects of their modes of action, including possible immunomodulatory and antioxidant actions of NCLI and MCLI, will be performed in the future. The combined results of this report provide data to support a role for NCLI and MCLI as feed additives to broilers.

Acknowledgements

The authors express the special thanks to Lingrui Kong, Li Dong and Jinke Tian for their skillful technical assistance for this research.

References

Awad W.A., Bohme J., Razzazi-Fazeli E., Ghareeb K., Zentek J. (2006 a). Effect of addition of a probiotic microorganism to broiler diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. Poultry Sci., 85: 974–979.

Awad W.A., Bohme J., Razzazi-Fazeli E., Ghareeb K., Zentek J. (2006 b). Effects of feeding deoxynivalenol contaminated wheat on growth performance, organ weights and histological parameters of the intestine of broiler chickens. J. Anim. Physiol. An. N., 90: 32–37.

Charlton B., Bacelj A., Mandel T.E. (1988). Administration of silica particles or anti-Lyt2 antibody prevents beta-cell destruction in NOD mice given cyclophosphamide. Diabetes, 37: 930–935.

Farr S.B., Kogoma T. (1991). Oxidative stress responses in *E. coli* and *S. typhimurium*. Microbiol. Res., 55: 561–585.

Fethiere R., Miles R.D., Harms R.H. (1990). Influence of synthetic sodium aluminosilicate on laying hens fed different phosphorus levels. Poultry Sci., 69: 2195–2198.

Harvey R.B., Kubena L.F., Ellisalde M.H., Phillips T.D. (1993). Efficacy of zeolitic ore compounds on the toxicity of aflatoxin to growing broiler chickens. Avian Dis., 37: 67–73.

Ivkovic S., Zabcic D. (2002 a). The effect of tribomechanically activated zeolite (TMAZ) on total antioxidant status of healthy individuals and patients with malignant disease. Free Radical Bio Med., 33 (suppl 1), p. 172.

Ivkovic S., Zabcic D. (2002 b). Antioxidative Therapy: nanotechnology product TMA-Zeolite reduces oxidative stress in cancer and diabetic patients. Free Radical Bio Med., 33 (suppl 2), p. 331.

Ivkovic S., Deutsch U., Silberbach A., Walraph E., Mannel M. (2004). Dietary supplementation with the tribomechanically activated zeolite clinoptilolite in immunodeficiency: effects on the immune system. Adv. Ther., 21: 135–147.

Khambualai O., Ruttanavut J., Kitabatake M., Goto H., Erikawa T., Yamauchi K. (2009). Effects of dietary natural zeolite including plant extract on growth performance and intestinal histology in Aigamo ducks. Brit. Poultry Sci., 50: 123–130.

Korver D.R., Klasing K.C. (1997). Dietary fish oil alters specific and inflammatory immune responses in chicks. J. Nutr., 127: 2039–2046.

Kubena L.F., Harvey R.B., Huff W.E., Elissalde M.H., Yersin A.G., Phillips T.D., Rottinghaus G.E. (1993). Efficacy of a hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and diacetoxyscirpenol. Poultry Sci., 72: 51–59.

Kuznetsova T.A., Somova L.M., Plekhova N.G., Drobot E.I. (2011). Pathogenetic role of *Yersinia pseudotuberculosis* endotoxin in hemostasis and microcirculation disturbance. B. Exp. Biol. Med., 150: 619–623.

Leung S., Barrington S., Wan Y., Zhao X., El-Husseini B. (2007). Zeolite (clinoptilolite) as feed additive to reduce manure mineral content. Bioresource Technol., 98: 3309–3316.

Lorenzoni A.G., Wideman Jr R.F. (2008). Intratracheal administration of bacterial lipopolysaccharide elicits pulmonary hypertension in broilers with primed airways. Poultry Sci., 87: 645–654.

Madhusudhan N., Basha P.M., Begum S., Ahmed F. (2009). Fluoride-induced neuronal oxidative stress and its amelioration by antioxidants in developing rats. Fluoride, 42: 179–187.

Majid G., Davood M. (2011). The use of clinoptilolite in broiler diet to decrease aflatoxin effects. International Conference on Applied Analysis and Algebra., 13: 38–43.

Miazzo R., Rosa C.A., De Queiroz C.E.C, Magnoli C., Chiacchiera S.M., Palacio G., Saenz M., Kikot A., Basaldella E., Dalcero A. (2000). Efficacy of synthetic zeolite to reduce the toxicity of aflatoxin in broiler chicks. Poultry Sci., 79: 1–6.

Mitrovic B., Vitorovic G., Vitorovic D., Dakovic A., Stojanovic M. (2007). AFCF and clinoptilolite use in reduction of (137)Cs deposition in several days' contaminated broiler chicks. J. Environ Radioactiv., 95: 171–177.

Morishita M., Miyagi M., Yamasaki Y., Tsuruda K., Kawahara K., Iwamoto Y. (1998). Pilot study on the effect of a mouthrinse containing silver zeolite on plaque formation. J. Clin. Hyp., 9: 94–96.

Mumpton F.A. (1999). La roca magica: Uses of natural zeolites in agriculture and industry. P. Natl. Acad. Sci. USA, 96: 3463–3470.

Nielsen F., Mikkelsen B.B., Nielsen J.B., Andersen H.R., Grandjean P. (1997). Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. Clin Chem., 43: 1209–1214.

Oguz H. (2011). A review from experimental trials on detoxification of aflatoxin in poultry feed. Eurasian J. Vet. Med. Sci., 27: 1–12.

Oguz H., Kurtoglu V. (2000). Effect of clinoptilolite on performance of broiler chickens during experimental aflatoxicosis. Brit. Poultry Sci., 41: 512–517.

Olver M.D. (1997). Effect of feeding clinoptilolite (zeolite) on the performance of three strains of laying hens. Brit. Poultry Sci., 38: 220–222.

Ortatatli M., Oguz H., Hatipoglu F., Karaman M. (2005). Evaluation of pathological changes in broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. Res. Vet. Sci., 78: 61–68

Oschilewski U., Kiesel U., Kolb H. (1985). Administration of silica prevents diabetes in BB-rats. Diabetes, 34: 197–199.

Papaioannou D.S., Kyriakis S.C., Papasteriadis A., Roumbies N., Yannakopoulos A., Alexopoulos C. (2002). Effect of in-feed inclusion of a natural zeolite (clinoptilolite) on certain vitamin, macro and trace element concentrations in the blood, liver and kidney tissues of sows. Res. Vet. Sci., 72: 61–68.

Pavelić K., Hadzija M., Pavelić L.B.J., Dikić I., Kralj M.K.M., Kapitanović M.H.B.S., Krizanac M.P.S., Stojković R., Subotić M.J.B., Colić M. (2001). Natural zeolite clinoptilolite: new adjuvant in anticancer therapy. J. Mol. Med., 78: 708–720.

Rocha O., Ansari K., Doohan F.M. (2005). Effects of trichothecene mycotoxins on eukaryotic cells: a review. Food Addit. Contam., 22: 369–378.

Theng B.K.G., Hayashi S., Soma M., Seyama H. (1997). Nuclear magnetic resonance and X-ray photoelectron spectroscopic investigation of lithium migration in montmorillonite. Clay Clay Miner., 45: 718–723.

van Ampting M.T., Schonewille A.J., Vink C., Brummer R.J.M., van der M.R., Bovee-Oudenhoven I.M.J. (2009). Intestinal barrier function in response to abundant or depleted mucosal glutathione in Salmonella-infected rats. BMC Physiology, 9, p. 6.

Wang L.C., Zhang T.T., Wen C., Jiang Z.Y., Wang T., Zhou Y.M. (2012). Protective effects of zinc-bearing clinoptilolite on broilers challenged with *Salmonella pullorum*. Poultry Sci., 91: 1838–1845.

Wu Y.N., Wu Q.J., Zhou Y.M., Hussain A., Wang T. (2013). Effects of clinoptilolite on growth performance and antioxidant status in broilers. Biol. Trace Elem. Res., 155: 228–235.

Yang X., Guo Y., He X., Yuan J., Yang Y., Wang Z. (2008). Growth performance and immune responses in chickens after challenge with lipopolysaccharide and modulation by dietary different oils. Anim., 2: 216–223.

Yarovan N.I. (2008). Effect of zeolites on adaptation processes in cows. Russ. Agri. Sci., 34: 120–122.

Zelko I.N., Mariani T.J., Folz J. (2002). Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Bio. Med., 33: 337–349.

Zhou Y.M. (2008). Mechanism of nutritional effect and harmful agent reduction of natural zeolite in broilers. Thesis PhD, Nanjing Agricultural University, Nanjing, China.

Received: 2 VIII 2014
Accepted: 12 XII 2014