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Intestinal Absorption Function of Broiler Chicks Supplemented with *Ginkgo* Leaves Fermented with *Bacillus* Species

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Abstract.- This study was carried out to evaluate the effect of supplementing diets with *Bacillus subtilis* var. *natto (Bac natto)* or *Bac licheniformis*-fermented *Ginkgo biloba* leaves (FG) on growth performance, intestinal morphology, absorption function and apoptosis in chicks. A total of 240 one-day-old Arbor Acress broilers, were randomly allocated to 3 dietary treatments with 8 replications of 10 birds each. Birds were fed basal diets (control), basal diets supplemented with 0.35% FG with *Bac natto* (FBN group), or basal diets supplemented with 0.35% FG with *Bac licheniform* (FBL group), respectively, for 42 d. The results showed ($P<0.05$) an improvement with low mortality due to F/G in FBN and FBL groups during the period of 22-42 d and 0-42 d, respectively compared to the control group. Compared with the control group, birds fed with FG diets had higher ($P<0.05$) activities of pancreatic, duodenal and jejunal protease as well as pancreatic and duodenal amylase. Duodenal villus height and VH/CD ratio, as well as jejunal VH/CD ratio was improved ($P<0.05$) in FBN or FBL groups. In addition, decreased ($P<0.05$) duodenal apoptosis index (AI) was observed in FG groups compared with the control group. Birds in both FG groups exerted an increased ($P<0.05$) plasma D-xylose, whereas broilers fed FBN diets led to lower ($P<0.05$) serum urea nitrogen (SUN) levels compared with the control group. The present study may indicate that application of FG in the diet proved to have positive influence on feed efficiency and intestinal absorption functions.

Key words: Broilers, fermented *ginkgo* leaves, digestive enzyme activities, intestinal absorption.

INTRODUCTION

Over the last decade, the importance of gastrointestinal tract health in broiler chicks has been increasingly recognized due to its contribution to their overall health and performance (Mountzouris *et al*., 2007). The use of antibiotics at subtherapeutic levels has been a cornerstone of the poultry industry for the control of subclinical diseases, maintenance of gut health and growth promotion for decades. However, antibiotic use tends to produce antibiotic resistance and have led to an increase in intestinal stress, which may further lead to decreased absorptive function and enable colonization by pathogens. This may pose a severe health hazard for birds and consumers of poultry products. Therefore, an urgent need exists to find alternative strategies that can effectively control intestinal health and retain growth-promoting properties similar to antibiotics.

With regards to this, the combined use of microbials and natural phytogenic resource has earned attention as viable alternative means for enhancing growth performance and improving intestinal health and function common to poultry (Steiner, 2006). *Bacillus* are aerobic, endospore-forming bacteria that are well defined and have recently shown tremendous promise because of their inherent capacity to form spores that can withstand harsh environmental stress and transitions during storage and handling (Cartman *et al*., 2008). *Bacillus* spores are extremely suitable strains candidates used for fermentation because of their longevity and stability.

Ginkgo biloba L. (Family: Ginkgoaceae) is a traditional herb in China, which has been used in some areas of the world, showing high physiological activities in therapies for inflammations, vascular and cardiac diseases and cancers (Puebla-Pérez *et al*., 2003; Ye *et al*., 2007; Sochocka *et al*., 2010; Chen *et al*., 2011). Leaves of *Ginkgo biloba* are well

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known for its high content of flavonoids. Chemically, the active components of *Ginkgo biloba* leaf are flavonoids (flavone glycosides, primarily composed of quercetin), polysaccharides (polymers of glucose, rhamnose, arabinose, mannose, galactose and xylose) and terpenoids (ginkolides and bilobalides) (Li *et al*., 2012; van Beek and Montoro, 2009). There were numerous reports in animals indicated that *Ginkgo* flavonoids posses many beneficial effects, including antioxidant and anti-inflammatory (Jenkins and Atwal, 1995; Cao *et al*., 2002; Ding *et al*., 2009).

In recent years, fermentation is a useful tool for producing biological materials with health promoting properties and is an optimal way to solve overproduction and stimulate processing use of various fruits and vegetables (Dei *et al*., 2008; Ng *et al*., 2011). Studies have demonstrated that fermentation not only alters the original bioactivities of Chinese herbs, resulting in new treatment effects, but also enhances the original treatment efficacy (Miyake *et al*., 2005; Lin and Chiang, 2008). China has a large scale production of ginkgo leaves for years, about forty thousand tons every year, and mainly distributes in Jiangsu, Shandong, Sichuan, Zhejiang provinces. In the last few years, large-scale cultivation of ginkgo has been initiated. Therefore, it is important to find out a way to utilize this herbal resource as feed ingredient and unveil its potential economic value in feed industry. Our previous researches confirmed that the use of 0.5% *Aspergillus niger* fermented-*Ginkgo biloba* leaves products minimized the deleterious effects of endotoxin, improved the intestinal development, absorption and immunity in immune-stressed chickens (Zhang *et al*., 2013). Dietary total flavonoids and polysaccharides were most likely the key compounds responsible for the health-improving effect of the fermentation products (Cao *et al*., 2012). Despite these findings, there has been a dearth of information on the possible intestinal protective effect of FG.

Bacillus spp. can be found in the normal intestinal flora of poultry and are capable of germinating and resporulating in the gastrointestinal tract of chickens (Barbosa *et al*., 2005; Tam *et al*., 2006; Cartman *et al*., 2008). Spores are known to withstand the process of pelletizing feed and, once ingested, germinate in the gastrointestinal tract because of the influence of pH, nutrients, and other relevant factors (Shivaramaiah *et al*., 2011). In their vegetative form, *Bacillus* spp. produce extracellular enzymes that may enhance digestibility and absorption of nutrients in addition to overall immune function of the gut (Samanya and Yamauchi, 2002; Chen *et al*., 2009). To promote processing of ginkgo leaves and improve the quality of pelletized feed, we have developed a process for *Bacillus* species fermentation wherein the functionality of this resources is preserved and enhanced. The aim in this study was to investigate the effect of supplementing diets with *Bacillus subtilis* var. *natto (Bac natto)* or *Bac licheniformis* - fermented *Ginkgo biloba* leaves on growth performance, intestinal morphology and absorption function of chicks.

MATERIALS AND METHODS

Preparation of starter cultures

The *Bac natto* and *Bac licheniform* used in this study was a laboratory strain obtained from the College of Chemical Engineering, Nanjing Forest University, Nanjing, Jiangsu, China. Both *Bacillus* species were cultured by an agar plating technique using Sabouraud dextrose agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37°C for 24 h.

Preparation of fermented-ginkgo leaves sample

Comminuted (2.0-mm sieve) dried ginkgo leaves picked during the last third of September (ginkgo garden for leaf use, Nanjing Forestry University, Jiangsu Province, China) were used for this study. They were divided into 2 lots after autoclave sterilization. One lot was untreated (no fermentation), and the other lot was fermented using *Bac natto* and *Bac licheniform*, respectively. The fermentation medium contained 10 g solid medium (ginkgo leaves : corncob: wheat bran = 14 : 3 : 3) and 12 mL nutritive salt (glucose : urea: $MgSO_4 \cdot 7H_2O$: KH_2PO_4 = 30 : 30 : 1 : 6) and was inoculated with 10% of the *Bac natto* and *Bac licheniform* seed, respectively and then cultivated at 37°C, for a 48 h aerobic fermentation with the initial pH7.0. The fermented sample was spread on a polythene sheet in a room at 30-40°C, dried for 6 d

Table I.- Changes of main nutritional ingredients and amino acids contents before and after fermentation

***Ginkgo* leaves**	**Fermented with *Bac natto***		**Increment %**	**Fermented with *Bac licheniform***	**Increment %**
	Before	**After**		**After**	
Total flavonoids /mg of quercetin equivalents/g	6.6	7.5	13.64	7.7	16.67
Polysaccharides/g/kg	4.37	6.42	46.91	6.48	48.28
Protein/g/kg	21.06	33.43	58.74	30.29	43.83
Total amino acid/g/kg	15.653	20.513	31.05	18.348	17.22
Total ginkgolic acid g/kg	1.664	0.054	-96.75	0.049	-97.06
Protease activity (Unit/g)	100	260	160.00	252	152.00
β-glucosidase activity (Unit/g)	0.4	3.5	775.00	3.46	765.00
Concentration of Indispensable amino acid /g/kg					
Lysine	7.7	8.48	10.13	7.85	1.95
Threonine	8.75	10.07	15.09	10.49	19.89
Leucine	12.34	13.86	12.32	14.05	13.86
Isoleucine	5.93	6.25	5.40	6.61	11.47
Methionine	2.18	4.63	112.39	3.07	40.83
Tryptophan	5.29	5.26	-0.57	5.51	4.16
Phenylalanine	7.66	8.88	15.93	8.76	14.36
Tyrosine	5.29	5.26	-0.57	5.51	4.16
Histidine	4.55	5.45	19.78	5.65	24.18
Glycine	9.91	22.86	130.68	31.28	215.64
Arginine	9.46	13.74	45.24	14.05	48.52

upto about 900 g/kg of the dry matter, and ground to pass through a 0.15-mm sieve. The changes of the ingredients before and after the fermentation are shown in Table I. Repetitious examination showed that the proportion of components in polysavone was constant within a minute range.

Experimental design and animal husbandry

A total of 240 one-day-old healthy commercial Arbor Acres chicks with the initial body weight (BW) 48.85 g were purchased from a local commercial hatchery. Birds were randomly placed in 40 wire cages (200 cm×155 cm×80 cm) with 10 chickens (0.15 m^2 per chick) each in a 3-level battery and housed in a room maintained at a brooding temperature of 34°C to 35°C for 5 d, and then the environmental temperature was gradually reduced by 1°C every 2 days until a final temperature of 22°C was reached in keeping with normal brooding practice. The light regimen was a 12-h light–dark cycle (06:00-18:00 h light) throughout the trial. Meanwhile, all broiler chickens were *ad libitum* access to the feed and water. The experimental design and procedures were approved by the Animal Care and Use of Committee of Nanjing Forestry University following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals of China (The State Science and Technology Commission, 1988). The rearing period was 6 weeks.

During the entire rearing period, all chickens were randomly allocated to 3 treatment groups with 8 replicates 10 animals each (male and female were half-and-half). A cage was treated as one replicate. The 3 treatment groups were allocated to the control group (basal diet), FBN and FBL groups (basal diets supplemented with 0.35% FG with *Bac natto* or 0.35% FG with *Bac licheniform*, respectively, at the expense of wheat bran). The percentage of all other major ingredients remained the same across treatments. The composition of the corn-soybean basal diets and nutrient levels for the starter (1 to 21 d) and grower phase (22 to 42 d) formulated to meet NRC (1994) nutrient requirements are shown in Table II. The dietary total flavonoids and polysaccharides contents for the broilers are shown in Table III.

Table II.- Ingredients and nutrient composition of broiler diets, on fed basis.

Ingredients (%)	Starter	Grower	Nutrient levels[2]	1-21 d	22-42 d
Maize	60.34	64.68	Metabolizable energy, MJ/kg	12.30	12.60
Soybean meal	30.22	24.23	Crude protein, %	20.07	20.30
Wheat bran	0.50	0.40	Calcium, %	1.00	0.90
Corn gluten meal	2.00	4.00	Available phosphorus, %	0.45	0.40
Lard	2.03	2.83	Lysine, %	1.13	1.00
Limestone	1.10	1.27	Methionine, %	0.50	0.44
Dicalcium phosphate	1.39	1.27	Methionine + cystine, %	0.82	0.74
Sodium chloride	0.20	0.25	Threonine, %	0.97	0.81
L-Lysine	0.07	0.16			
DL-Methionine	0.15	0.11			
Premix[1]	1.00	1.00			
Total	100	100			

[a]Premix provided per kilogram of diet: transretinyl acetate, 24mg; cholecalciferol, 6mg; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyidoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 7.5 mg; Mn (from manganese sulfate), 110 mg; Zn (from zinc oxide), 65 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg; Bacitracin Zinc, 30 mg.

Table III.- Total flavonoids and polysaccharides contents in the diets of broilers.

Dietary treatments[1]	Total flavonoids/mg of quercetin equivalent/kg		Total polysaccharides/mg/kg	
	1-21 d	22-42 d	1-21 d	22-42 d
Cont.	0.092	0.071	0.059	0.056
FBN	0.519	0.531	0.353	0.364
FBL	0.521	0.533	0.356	0.365

[1]Cont., basal diet; FBN, basal diet with 0.35% *Bac natto* fermented *ginkgo* leaves; FBL, basal diet with 0.35% *Bac licheniform* fermented *ginkgo* leaves;

Chickens were weighed individually at the age of 1, 21, and 42 d to determine average daily body weight gain (BWG). Feed consumption on a cage/replicate basis was recorded daily, the uneaten feed was discarded, and the feeders were replenished with fresh feed. Average daily feed intake (FI) and feed-to-gain ratio (F/G) were calculated.

Sampling procedure

At the termination of the study, 8 chicks per treatment (one bird per replicate) were selected 12 hrs after feed deprivation. Individual blood samples were taken and serum were separated by centrifugation at 3500 rpm for 15 min, at 4°C. Serum samples were frozen at -20°C for serum urea nitrogen (SUN) analysis. After that, all birds were euthanized by exsanguination. A 1-cm segment of the duodenum, jejunum and ileum was taken from the middle part of each intestinal section, and immediately rinsed with cold physiological saline and fixed in 4% paraffin. The tissue samples were serially dehydrated in graded ethanol solutions (50, 70, 80, 96, and 100%), cleared with xylene and embedded in paraffin wax. Tissue samples were then sectioned using a microtome at a thickness of 5 μm (3 cross sections from each sample) for further determining the terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick-end labelling (TUNEL) and histomorphometry. Next, pancreas and intestinal digesta of duodenum and jejunum were collected. The sampling of small intestinal digesta and the pancreas tissue was conducted according to the procedures described by Jin *et al*. (2000).

Measurement of the main nutritional ingredients in FGL

The aluminum chloride colorimetric method described by Chang *et al*. (2002) and Verzelloni *et al*. (2007) was used to determine the total flavonoids content with some modifications. Briefly, aliquots of 1.0 g fermented samples or diets were dissolved and extracted with 200 mL of 80% alcohol for 6 to 8

h at 90°C. The extraction was made up to 200 mL with distilled water after filtration. One milliliter solution or standard solution was mixed with 1.5 mL methanol, 1 mL of 1% aluminum chloride (substituted with distilled water in blank probe), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After 30 min incubation, absorbance at 415 nm was determined against a distilled water blank on a UV-1206 spectrophotometer (Shimadzu, Kyoto, Japan). All samples were made in triplicate, and mean values of total flavonoids contents are expressed as milligrams of quercetin equivalents per gram of product calculated according to the standard calibration curve. Quercetin standard solutions were prepared by dissolving catechin in water at a concentration ranging from 2.5 to 25 mg in 100 mL.

The concentration of total polysaccharides was determined by colorimetric method of phenol-suluric acid assay (Dubois *et al*., 1956) taking D-glucose as standard control. The protein content was determined by the method described by Bradford (1976). Content of the crude protein was determined by Kjeldahl determination assay, nitrogen was determined using the Kjeltec Analyzer Unit (2300, Sweden).

The amino acid composition of the unfermented and fermented products was determined using an automatic amino acid analyzer L-8500 (Hitachi, Tokyo, Japan) after the samples were hydrolyzed in 6M HCl for 22 h at 110°C, as described in Zhang *et al*. (2013).

The content of total ginkgolic acids in *Ginkgo biloba* leaves was determined by HPLC. The FG was milled, and extracted with 4.8 mL methanol, shake 3 min, then mixed with 1.2 mL water. The mixture was sonicated for 20 min, and centrifuged at 14000 rpm for 10 min. After that, the supernatant was filtrated through 0.45 μm. A Alltima C18 (4.6 mm × 250 mm, 5 μm) and the mobile phase of methanol and 1% acetic acid (90:10) were used, the flow rate was 1.0 mL × min ($^{-1}$), and the wavelength was 310 nm. The content was calculated with external standard method.

One gram of each sample was taken and mixed with 15 mL of 100 mM citrate buffer having pH 4.8. The samples were incubated for 30 min in shaker at 200 rpm followed by centrifugation at 9000 × *g* and 4°C for 15 min and supernatant was analyzed for enzyme activities.

β-glucosidase activity was determined by modified method of Peralta *et al*. (1997). The reaction was carried out by incubating 0.2 ml *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (9 mM) with 0.2 ml crude enzyme and 1.6 ml sodium acetate buffer (200 mM, pH 4.6) at 50 ± 1 C for 15 min. The reaction was stopped by adding 2 ml of sodium carbonate (100 mM). The release of *p*-nitrophenol was measured at 405 nm from a *p*-nitrophenol standard curve. One international unit of β-glucosidase activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min under given assay conditions.

Protease activity was analyzed using the modified method of Lynn and Clevette-Radford (1984), and were determined using a corresponding diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Protease activity unit was defined as milligrams of azocasein degraded during 2 h of incubation at 38°C per milligram of fermented product. Crude protein from fermented product was determined by the method of Lowry *et al*. (1951). Ovine serum albumin was used as a standard.

Digestive enzyme activities in pancreatic tissue and small intestinal contents

Activities of amylase, lipase and protease in the homogenate supernatants of pancreatic tissue and small intestinal contents were determined with commercial kits (Jiancheng Biochemical Reagent Co., Nanjing, People's Republic of China) according to the manufacturer's instructions.

Intestinal histomorphometry

Three cross-sections for each sample were stained with hematoxylin and eosin. Histological sections were examined using an image analyzer (Nikon NIS-Elements BR, Nikon Co., Tokyo, Japan) to measure villus height and crypt depth. Villus height was measured from the tip of the villus to the villus–crypt junction, and the crypt depth was defined as the depth of the invagination between adjacent villi (Zhang *et al*., 2013). A total of 10 intact, well-oriented crypt villus units were selected per bird for each intestinal cross section, and the average of these values was used to express the

mean values of villus height (VH) and crypt depth (CD) for each bird.

Assay of plasma D-xylose concentration and serum urea nitrogen (SUN)

For the D-xylose absorptive test, another six birds (one bird per pen) of each treatment were given a dose of 0.5 g kg^{-1} BW D-xylose at a concentration of 5% (wt/vol distilled water) via oral gavage. One hour later, plasma was obtained from wing vein and serum D-xylose concentration was determined by the method described by Doerfler *et al*. (2000). SUN concentration was determined using commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

TUNEL assay

Apoptosis of tissue samples was evaluated by *in situ* TUNEL labelling. This method detects extensive DNA degradation, a characteristic event that often occurs in the early stages of apoptosis (Schwartzman and Cidlowski, 1993). The TUNEL assay was carried out on paraffin-embedded sections of intestinal samples. Briefly, paraffin was removed from the sections using graded concentrations of xylene and ethanol and then they were dehydrated. The sections were digested with 20 mg/mL proteinase K for 15 min at room temperature. The sections were then washed and incubated with the TUNEL reaction mixture (enzyme solution and labelling solution) for 60 min at 37°C in a humidified atmosphere. The slides were counterstained with hematoxylin and examined under a fluorescence inversion microscope (Olympus I × 51, Tokyo, Japan). The apoptotic index (AI) was calculated by percentage, as follows: AI = (the number of apoptotic cells/the total number of cells) × 100%.

Statistical analysis

The design for this experiment was a completely randomized design with 8 replications. Unless otherwise stated, all data are presented as means and pooled S.E.M. The statistical significance of differences among the treatments (Cont., FBN or FBL) was evaluated using the mixed procedure of SAS® version 9.1 (SAS Institute, Inc., Cary, NC). The statistical differences between treatments were determined by one-way ANOVA. Statements of significance were $P<0.05$ unless noted otherwise.

RESULTS

Changes of main nutritional ingredients before and after fermentation

The results of the changes of main nutritional ingredients before and after fermentation in ginkgo leaves, presented in Table I, reveal that ginkgo leaves fermented with Bacillus *subtilis* var. *natto (Bac natto)* or *Bac licheniformis* improves nutritional value, especially total flavonoids (approximately 13.64%, 16.67%, respectively), polysaccharides (46.91%, 48.28%), protein (58.74%, 43.83%), and total amino acid (31.05%, 17.22%) contents, respectively. While the content of ginkgolic acid, which has been recognized as hazardous compounds with suspected cytotoxic, allergenic properties decreased sharply (96.75% and 97.06%, respectively) after fermentation.

Growth performance

As shown in Table IV, no significant differences ($P>0.05$) occurred in BWG and FI among treatments in the period of 1-21 d, 22-42 d and the overall period. With regard to F/G, there was no significant difference ($P>0.05$) among treatment groups in the period of 1-21 d, however, improved ($P<0.05$) F/G in FBN and FBL groups was observed than that of the control group in the period of 22-42 d and the overall period. Meanwhile, lower ($P<0.05$) mortalities were observed ($P<0.05$) in both the dietary supplemented groups compared with the control.

Digestive enzyme activities and intestinal histomorphometry

Birds fed with FBN and FBL diets had higher ($P<0.05$) activities of pancreatic, duodenal and jejunal protease as well as pancreatic and duodenal amylase, compared with the control group (Table V). Feeding FBN or FBL supplemented diets increased duodenal VH and VH/CD ratio, as well as jejunal VH/CD ratio compared with the control; FBL group showed the highest ($P<0.05$) increase in jejunal VH compared with the control group (Table VI).

Table IV.- Growth performance of broilers fed diets supplemented with fermented *Ginkgo biloba* leaves.

Items	Dietary treatments[1]			SEM[2]	P-value
	Cont.	FBN	FBL		
BWG, g/bird/d[3]					
d 0-21	28.80	31.25	31.23	0.21	0.086
d 22-42	80.39	84.20	83.31	0.88	0.064
d 0-42	54.60	57.73	57.27	0.63	0.073
FI, g/bird/d [3]					
d 0-21	44.71	46.88	47.09	0.56	0.069
d 22-42	139.70	138.82	133.79	1.98	0.078
d 0-42	92.20	92.35	91.58	0.89	0.071
F/G[3]					
d 0-21	1.55	1.50	1.51	0.09	0.064
d 22-42	1.74[a]	1.65[b]	1.61[b]	0.07	0.043
d 0-42	1.69[a]	1.60[b]	1.60[b]	0.06	0.048
Mortality rate (%)	2.43[a]	1.78[b]	1.89[b]	0.10	0.036

[a-c]Means within the same row that do not share a common superscript are significantly different (P<0.05) . n=8.
[1]Cont., Basal diet; FBN, basal diet with 0.35% *Bac natto* fermented *ginkgo* leaves; FBL, basal diet with 0.35% *Bac licheniform* fermented *ginkgo* leaves;
[2] Standard error of the mean based on pooled estimate of variation.
[3] BWG, body weight gain; FI, Feed intake; F/G, Feed gain ratio.

Table V.- Digestive enzyme activities of broilers fed diets supplemented with fermented *Ginkgo biloba* leaves.

Items	Dietary treatments[1]			SEM[2]	*P*-value
	Cont.	FBN	FBL		
Pancreas					
Protease, unit[3]	146.21[b]	154.19[a]	158.88[a]	3.46	0.025
Lipase, Sigma-Tietz unit[4]	34.46	39.52	40.70	0.92	0.067
Amylase, Somogyi unit[5]	33.16[b]	37.41[a]	38.87[a]	1.37	0.082
Duodenum					
Protease, unit	82.62[b]	90.46[a]	92.98[a]	0.89	0.038
Lipase, Sigma-Tietz unit[4]	20.36	20.73	20.68	0.56	0.085
Amylase, Somogyi unit[5]	26.43[b]	34.08[a]	35.49[a]	0.36	0.042
Jejunum					
Protease, unit	68.88[b]	75.46[a]	74.13[a]	2.10	0.041
Lipase, Sigma-Tietz unit[4]	18.27	18.82	17.99	0.59	0.064
Amylase, Somogyi unit[5]	15.14	16.39	16.83	0.84	0.072

[a-b]Means within the same row that do not share a common superscript are significantly different (P<0.05). n=8.
[1]Cont., Basal diet; FBN, basal diet with 0.35% *Bac natto* fermented *ginkgo* leaves; FBL, basal diet with 0.35% *Bac licheniform* fermented *ginkgo* leaves;
[2]Standard error of the mean based on pooled estimate of variation.
[3]Protease activity unit was defined as milligrams of azocasein degraded during 2 h of incubation at 38°C per milligram of intestinal digesta protein or pancreas.
[4] Amylase activity unit (1 Somogyi unit) was defined as the amount of amylase that will cause formation of reducing power equivalent to 1 mg of glucose in 30 min at 38°C per milligram of intestinal digesta protein or pancreas.
[5] Lipase activity unit (1 Sigma-Tietz unit) was equal to the volume (milliliter) of 0.05 M NaOH required to neutralize the fatty acid liberated during 6 h incubation with 3 mL of lipase substrate at 38°C per milligram of intestinal digesta protein or pancreas.

Table VI.- Small intestine morphology of broilers fed diets supplemented with fermented *Ginkgo biloba* leaves.

Items	Dietary treatments[1]			SEM[2]	*P*-value
	Cont.	FBN	FBL		
Duodenum					
Villous height (µm)	1215.08[b]	1358.23[a]	1383.78[a]	58.46	0.036
Crypt depth (µm)	204.61	193.93	190.70	17.45	0.061
Villous height to crypt depth ratio	5.94[b]	7.00[a]	7.26[a]	0.54	0.082
Jejunum					
Villous height (µm)	784.89[b]	859.3 [ab]	883.31[a]	68.70	0.047
Crypt depth (µm)	168.84	163.77	163.37	9.26	0.068
Villous height to crypt depth ratio	4.65[b]	5.25[a]	5.41	0.45	0.04
Ileum					
Villous height (µm)	225.24	236.75	242.41	14.90	0.061
Crypt depth (µm)	109.62	112.92	107.94	7.30	0.073
Villous height to crypt depth ratio	2.05	2.10	2.25	0.18	0.062

[a-b]Means within the same row that do not share a common superscript are significantly different (P<0.05). n=8.
[1] Cont., basal diet; FBN, basal diet with 0.35% *Bac natto* fermented *ginkgo* leaves; FBL, basal diet with 0.35% *Bac licheniform* fermented *ginkgo* leaves;
[2] Standard error of the mean based on pooled estimate of variation.

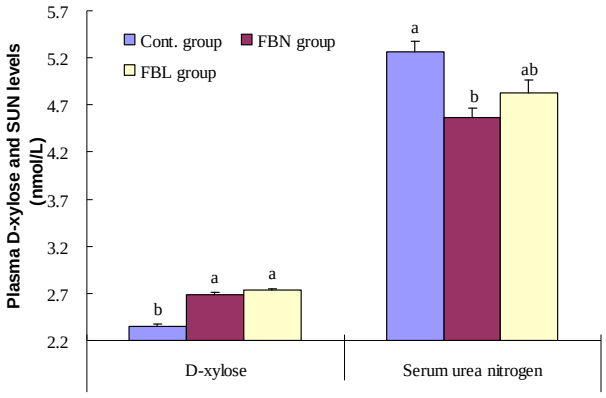

Fig. 1. Plasma D-xylose and SUN levels of broilers fed diets supplemented with fermented *Ginkgo biloba* leaves
[a-b]Means within the same column that do not share a common superscript are significantly different (P<0.05). n=8.
[1]Cont., basal diet; FBN, basal diet with 0.35% *Bac natto* fermented *ginkgo* leaves; FBL, basal diet with 0.35% *Bac licheniform* fermented *ginkgo* leaves.
SUN, serum urea nitrogen

Plasma D-xylose and serum urea nitrogen (SUN) level

Based on the results presented in Figure 1, birds in both groups FBN and FBL exerted a significant increased (P<0.05) plasma D-xylose as compared to the control group as shown in Figure 2. Whereas broilers fed FBN diets led to lower (P<0.05) SUN levels compared with the control group (Fig. 2).

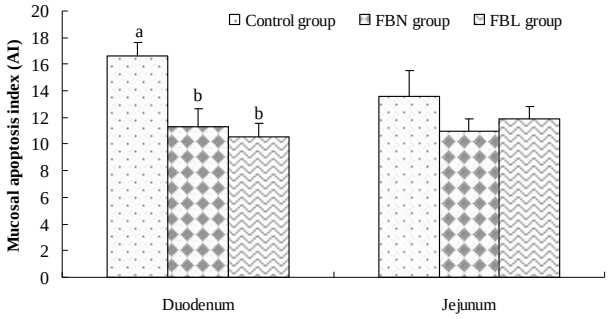

Fig. 2. Mucosal apoptosis index (AI) of broilers fed diets supplemented with fermented *Ginkgo biloba* leaves
[a-c]Means within the same column that do not share a common superscript are significantly different (P<0.05). n=8.
[1]Cont, basal diet; FBN, basal diet with 0.35% *Bac natto* fermented *ginkgo* leaves; FBL, basal diet with 0.35% *Bac licheniform* fermented *ginkgo* leaves

Apoptosis detection of duodenal mucosa

Apoptosis of intestinal tissue was evaluated by *in situ* TUNEL labeling. As shown in Figure 2,

dietary treatment had no effect on mucosal apoptosis index (AI) in jejunum. Significant decreased duodenal AI ($P<0.05$) was observed in FBN and FBL groups compared with the control group.

DISCUSSION

Flavonoids are a large group of polyphenolic compounds, numerous studies have revealed that the flavonoids stem from their aglycones (Hendrich 2002; Kawakami *et al*., 2005), and flavonoids aglycones are more easily and rapidly absorbed in the intestines after fermentation (Izumi *et al*., 2000). The present study was designed to evaluate the effects of fermented *Ginkgo biloba*-leaves, with the view of its potential inclusion in chicken diets as an alternative feed resource for feeding poultry. This study used oriental *Ginkgo biloba*-leaves that are most common cultivated in many parts of the world and are grown on commercial scale in parts of China. The question addressed in this paper was whether the intake of fermented *Ginkgo biloba* leaves could beneficially affect the host, by selectively stimulating the growth or intestinal absorption function or cell proliferation of the small intestines, thus improving gut's functions through maintaining intestinal mucosal structure.

In the present study, the improved F/G in FG groups may be attributed to the improvement in intestinal health status of birds fed diets supplemented with FG. The structure of the intestinal mucosa can reveal some information on gut health. Stressors that are present in the digesta can lead to relatively rapid changes in the intestinal mucosa because of the close proximity of the intestinal contents to the mucosal surface. One possible hypothesis about changes in intestinal morphology, such as shorter villi and deeper crypts, has been associated with the presence of toxins (Xu *et al*., 2002). Shortening of the villus decreases the surface area for nutrient absorption. The crypt can be regarded as a villus factory, and a large crypt indicates fast tissue turnover and a high demand for new tissue (Yason *et al*., 1987). Demand for energy and protein for gut maintenance is higher compared with other organs. A fast-growing broiler devotes about 12% of the newly synthesized protein to the digestive tract (Yason *et al*., 1987). Any additional tissue turnover will increase nutrient requirements for maintenance and will therefore lower the efficiency of the bird. A shortening of the villus and a large crypt can lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, diarrhea, reduced disease resistance, and lower overall performance. In the present study, the VH of the duodenum and jejunum were affected as dietary supplementation of FG. These results are in accordance with the results for performance. The crypt is the area where stem cells divide to permit renewal of the villus; a large crypt indicates fast tissue turnover and a high demand for new tissue. This may be explained by the results of decreased mucosal apoptosis index in duodenum and jejunu.

Pancreatic and intestinal digestive enzymes play a crucial role in the digestion and absorption of ingested macromolecules. From the present results, it may be postulated that the supplementation of fermented *Ginkgo biloba* leave would trigger the secretion of digestive enzymes under certain circumstances (*e.g*., age of birds, dose of phytogenic, bird species, type and quality of basal diet, bird health, and environmental and management conditions), which could enhance digestion of nutrients in the intestine. Furthermore, the increased intestinal protease and amylase is in accordance with the lower urea nitrogen excretion (Chah *et al*., 1975), and the reason is likely to be the better dietary nitrogen utilization in FBN and FBL group than the control group. Lower urea nitrogen excretion and the improvement of enzyme activities in small intestinal contents of broilers may be mostly due to the inactivation of anti-nutritional factor. Fermentation may decrease or eliminate anti-nutritional constituents (Chen *et al*., 2009). Hong *et al*. (2004) showed that large-size peptides, such as antigenic proteins, could be degraded to small-size peptides. In the present study, reduction in antinutritional factors–protein complexes might have spared more protein and starch for digestion (Viveros *et al*., 2001; Mansoori *et al*., 2007), and the reduction in anti-nutritional factors–enzyme complexes may have spared digestive enzymes to enhance the digestibility of the nutrients (Mahmood *et al*., 2006, 2008; Farran *et al*., 2001), ultimately resulting in more efficient utilization of the diets and

improved F/G of the broilers. Ginkgolic acid has been recognized as hazardous compounds with suspected cytotoxic and allergenic properties. In the present study, the content of ginkgolic acid was decreased sharply after fermentation (Liu and Zeng, 2009). Therefore, the improvement of activities of intestinal enzymes in broilers fed with FG presented here may be associated with degradation of protein by enzymes and ginkgolic acid in FG (Table 1), and this needs further research (Hong *et al*., 2004). Furthermore,

D-xylose is a poorly metabolized pentose, which is absorbed from the upper small intestine, primarily by passive diffusion and, to a lesser extent, by the same active transport system responsible for absorbing glucose (Hu *et al*., 2011). The D-xylose absorption test has been used in poultry to provide a simple, specific, and sensitive test for intestinal absorption and malabsorption (Doerfler *et al*., 2000). The decreased plasma D-xylose as an indicator of malabsorption that has been demonstrated in several cases of viral-induced intestinal dysfunction in poults. Histologically, the malabsorptive condition was reflected as villus atrophy and crypt hypertrophy (Perry *et al*., 1991). As expected, a marked elevation (P=0.023) of plasma D-xylose levels was observed after FG administration, which may result from the improved intestinal morphology and digestive enzymes activity after FG administration.

In conclusion, the results indicated that supplementation of 0.35% FG in the diets, improved feed efficiency, intestinal morphology, digestion and absorption function of broilers. Further studies are needed to assess fermented *Ginkgo biloba* leaves as a medicinal herb feed additive in broilers for enhanced growth performance and its potential use in practical diets.

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