ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 15 (2): 62-71, 2016
ISSN 1682-8356

Effect of Fermented *Ginkgo biloba* and *Camelia sinensis*-Based Probiotics on Growth Performance, Immunity and Caecal Microbiology in Broilers

Ye-Jin Kim[1], A.B.M. Rubayet Bostami[1], M.M. Islam[1], Hong Seok Mun[1], S.Y. Ko[2] and Chul-Ju Yang[1]
[1]Department of Animal Science and Technology, Sunchon National University,
255 Jungangno, Suncheon, Jeonnam 540-950, Republic of Korea
[1]En-Biotech. Co. Ltd., 276-19, Juseok-ro, Juam-myeon, Suncheon-si, Jeollanam-do, Korea

Abstract: Present study was conducted to evaluate the effects of dietary supplementation with fermented *Ginkgo biloba* and *Camelia sinensis*-based probiotics on growth performance, immunity and caecal microbiology in broilers. A total of three hundred twenty day old Ross broilers were randomly allocated based on completely randomized design into five treatments with eight replications (eight birds per replicate). Dietary treatments included: (1) Control (basal diet); (2) FGB1 = basal diet+0.2% fermented *Ginkgo biloba* probiotics; (3) FGB2 = basal diet+0.4% fermented *Ginkgo biloba* probiotics; (4) FCS1 = basal diet+0.2% fermented *Camelia sinensis* probiotics, (5) FCS2 = basal diet+0.4% fermented *Camelia sinensis* probiotics. Results of the present study elucidated that average daily gain was higher in FGB2 than FCS1 and FCS2 ($p<0.05$) during starter period; where feed intake was unaffected after dietary supplementation during starter, finisher and overall period ($p>0.05$). However, feed conversion ratio was improved in FGB2 during starter period ($p<0.05$), as well as in FGB1, FGB2, FCS1 and FCS2 during finisher and overall period relative to control ($p<0.05$). In addition, serum immunoglobulin was elevated in the FGB and FCS supplemented group compared to control ($p<0.05$). Moreover, dietary supplementation of FGB and FCS significantly suppressed caecal pathogenic *E. coli* ($p<0.05$). To sum up, dietary FGB and FCS can be utilized as potential feed additives in broiler nutrition with significant improvement in the growth performance, immunity and suppression of pathogenic caecal *E. coli*. Further detailed study is required on mechanism and meat quality analysis in broilers.

Key words: Fermented probiotics, growth performance, immunity, caecal microbiology, broiler

INTRODUCTION

After banning of antibiotic growth promoters, alternative sources of feed additives are the continuous research interest of the animal nutritionist. Since antibiotic was banned with the main concern of possible negative impact on the human health through microbial resistance via food chain. Therefore, alternative feed strategies concentrated by the researchers on the medicinal plants and their derivatives, probiotics, prebiotics, symbiotics, organic acids and oligosaccharides (Fulton *et al*., 2002). According to scientific information, there are around 250,000 to 500,000 species of plants in the world (Borris, 1996); among them small numbers (1 to 10%) are used as food for man and animals (Cowan, 1999). Compared to the synthetic products and chemicals, plants and their derivatives are considered natural and safe, less toxic, residue free and have growth promoting efficacy (Hashemi *et al*., 2008). Probiotics are beneficial microorganisms and having gut microbial modulating capacity thereby promote growth and feed efficiency in animals (Kyriakis *et al*., 1999). Among different probiotic microorganisms, *Lactobacillus* and *Saccharomyces* are the most effective for broiler nutrition because of their antimicrobial, immunomodulatory and brush bordering activities (Ehrmann *et al*., 2002; Karaoglu and Durdag, 2005). Utilizing probiotics to preserve feedstuffs or feed fermentation (such as liquid fermenting feed or silage for ruminants and pigs) has been practiced for many years in industries where fermented feed has the potential to improve growth performance, feed efficiency, nutrient digestibility and immunity in livestock (Feng *et al*., 2007; Cho *et al*., 2013). Although fermented feed research is ongoing, there is lack of wide knowledge and information regarding utilization of fermented feeds and there is also large variety of fermented feed to simulate different natural resources for poultry nutrition. Medicinal plants are abundant in different geographical positions all over the world including Korea, japan and china. Korean medicinal plants and natural sources including *Ginkgo biloba*, *Camelia sinesnis*, *Alisma canaliculatum*, *Citrus junos* and *Punica granatum* are important. Previous studies on the effects of natural plant materials on growth performance showed varying

Corresponding Author: Chul-Ju Yang, Department of Animal Science and Technology, Sunchon National University, 255 Jungangno, Suncheon, Jeonnam 540-950, Republic of Korea

results (Alcicek *et al*., 2004; Grashorn, 2010); fermented *Alisma canaliculatum, Citrus junos* and *Punica granatum* effectively improve weight gain in broilers when they supplemented separately (Hossain *et al*., 2012; Ahmed *et al*., 2014; Bostami *et al*., 2015); whereas, fermented *Ginkgo biloba* and *Camelia sinensis* have no effect on weight gain of birds during individual supplementation (Cao *et al*., 2012; Sarker *et al*., 2010). Although the actual mode of action and combination of different plant derived constituents and probiotics is difficult to determine due to the wide composition of phytobiotics and functions of the gastro intestinal tract (Grashorn, 2010; Erdogan *et al*., 2010); herbal oil mixture was found to lead significant improvement in body weight gain and feed intake (Alcicek *et al*., 2004).

To develop functional feed additives, considerable effort has been devoted to identification of effective combinations of medicinal plant byproducts; because most byproducts are considered waste products that resulted environmental pollution, even though they contain utilitarian plant secondary metabolites. It is generally accepted that, utilization of plant byproducts can reduce the feed cost, where health promotion is the bonus for animal production. Natural plant materials are composed of primary (carbohydrates, proteins and fats) and secondary (essential oils, bitterns and phenolic compounds) metabolites (Wald, 2003); therefore, it was expected that combinations of plant materials and fermentation with probiotics would have synergistic effects on broilers in the present study. Considering utilization of minimal cost of byproduct along with health promoting efficacy (due to the phytochemicals); combinations of plant materials and fermentation with probiotics for the development of potential feed additives for broilers was the main purpose of the current study. However, to the best of our knowledge no studies have investigated the effects of a combination of *Punica granatum* with *Ginkgo biloba* and *Camelia sinesnis* after fermentation with *Lactobacillus* spp. and *Saccharomyces* spp. on broiler nutrition to date. Therefore, the present study was conducted to investigate the effects of *Ginkgo biloba+Punica granatum* and *Camelia sinensis+Punica granatum* that had been fermented with *Lactobacilli* and *Saccharomyces* spp. on the growth performance, immunity and caecal microbiology in broilers.

MATERIALS AND METHODS

Preparation of fermented *Ginkgo biloba* and *Camelia sinensis*-based probiotics: *Ginkgo biloba* leaf and *Camelia sinensis* was obtained from Boseong, Republic of Korea. *Punica granatum* byproduct, which is a Goheung-gun cultivar, was collected from a juice manufacturing company. The byproduct was composed of about 80% peels and rinds and 20% seed. *Ginkgo biloba* leaf, *Camelia sinensis* leaf and *Punica granatum* were then dried in a forced air oven (Doori TEC, Doori TEC, FA, Co., Ltd.) at 80°C for 3 d and subsequently ground into powder that could pass through a 0.15 mm sieve using a milling machine. Samples were then tightly packed in polythene plastic bags, after which they were sealed and kept at room temperature until needed. *Ginkgo biloba* leaf, *Camelia sinensis* leaf and *Punica granatum* samples were analyzed in triplicate for crude protein (CP), ether extract (EE), moisture and ash as described by the Association of Official Analytical Chemists (AOAC, 2000). The fatty acid composition was determined by a direct method for fatty acid methyl ester (FAME) synthesis using a gas chromatograph (GC). The pH was measured using a digital pH meter (Docu-pH + meter, Sartorius, USA).

Fermented *Ginkgo biloba* (FGB) contains 60% defatted rice bran, 30% pomegranate peel extract and 10% *Ginkgo bloba* leaf powder, whereas fermented *Camelia sinessis* (FCS) contains 60% defatted rice bran, 30% pomegranate peel extract and 10% *Camelia sinessis* leaf powder. After mixing the ingredients to prepare FGB and FCS, samples were inoculated with 30% (v/w) *Lactobacillus plantarum* KCTC 3099 and *Lactobacillus acidophilus* KCTC 3111 and fermented for 2 days at 37°C and 40% moisture in a commercial fermenter (W-1000; Wonbalhyo Industry Co., Incheon, South Korea). Fermented medium was again inoculated with 30% (w/v) *Saccharomyces cerevisiae* KCTC 7904 and fermented for 3 days at 37°C. Fermentation with microbial inoculum was conducted using a cycle of 5 hours standing and 3 hours shaking to ensure proper mixing and fermentation. Subsequently, the fermented sample was dried in a forced air oven (Doori TEC, Doori TEC, FA, Co., Ltd.) at 32°C for 2 days to reduce the moisture levels. During fermentation with microbial inoculum, there was a cycle of 5 h standing and 3 h shaking to obtain the proper mixing and fermentation. Finally, FGB and FCS were stored in an air-tight plastic bag until being mixed with basal diet. The microbial concentration, proximate composition, trace minerals, fatty acids and pH of FGB and FCS were analyzed in triplicate and presented in Table 2. The experimental FGB and FCS contained 11.98 and 11.86% CP, 23.42 and 17.67% moisture, 2.41 and 2.15% crude fat, 9.83 and 11.66% crude fiber, 6.66 and 7.03% crude ash and 53.30 and 50.36% NFE, respectively. The *Lactobacillus* spp. population was 7.67 and 7.37 $\log_{10}$ cfu/g for FGB and FCS, respectively, while the *Saccharomyces* spp. population was 6.62 and 6.36 $\log_{10}$ cfu/g in FGB and FCS, respectively. The fatty acid composition of FGB and FCS is presented as supplementary Table 1. The pH of the FGB was 3.20-3.25, whereas in FCS it was 3.34-3.38.

Supplementary Table 1: Fatty acid composition of the Fermented *Ginkgo biloba* probiotics (FGB) and Fermented *Camelia sinensis* probiotics (FCS)

Parameters		Fermented *Ginkgo biloba*-based probiotics (FGB)		Fermented *Camelia sinensis*-based probiotics (FCS)	
		Mean	SD	Mean	SD
Feed fatty acid composition (g/100g of fatty acids)					
Lauric acid	(C12:0)	0.917	0.031	0.967	0.051
Palmitic acid	(C16:0)	14.503	0.095	14.833	0.097
Margaric acid	(C17:0)	3.147	0.035	3.127	0.047
Stearic acid	(C18:0)	8.743	0.123	8.863	0.116
Eicosanoic acid/Arachidic acid	(C20:0)	3.453	0.070	3.620	0.085
Heneicosylic acid	(C21:0)	0.583	0.035	0.643	0.181
Tricosylic acid	(C23:0)	27.173	0.152	27.807	0.186
[1]SFA		58.520	0.515	59.860	0.282
Palmitoleic acid	(C16:1n7)	3.048	0.059	3.050	0.085
Oleic acid	(C18:1n9)	14.573	0.530	14.360	0.363
Eicosaenoic acid	(C20:1n9)	0.983	0.055	0.947	0.075
Tetracosaenoic acid	(C24:1n9)	0.630	0.075	0.613	0.085
[2]MUFA		19.235	0.342	18.970	0.153
a-Linolenic acid	(C18:3n3)	1.410	0.046	1.213	0.085
Eicosapentanoic acid	(C20:5n3)	3.143	0.176	2.877	0.100
Docosahexaenoic acid	(C22:6n3)	3.223	0.197	3.200	0.317
[3]n-3		7.777	0.369	7.290	0.187
Linoleic acid	(C18:2n6)	12.797	0.050	12.543	0.085
DGLA	(C20:3n6)	1.170	0.050	1.033	0.080
Arachidonic acid	(C20:4n6)	2.127	0.021	2.093	0.055
[4]n-6		16.093	0.100	15.670	0.195
[5]PUFA		23.870	0.401	22.960	0.111

[1]∑SFA = saturated fatty acid; [2]∑MUFA = mono-unsaturated fatty acid; [3]∑n-3 = total omega 3 fatty acid; ∑n-6 = total omega 6 fatty acid; [5]∑PUFA = polyunsaturated fatty acid

Experimental design, dietary treatments and bird's husbandry: Experimental birds were reared in the Sunchon National University experimental farm, Suncheon, Republic of Korea. A total of three hundred twenty day old Ross broiler chicks were randomly allocated into five treatments with eight replications (eight birds per replicate) in a completely randomized design. Dietary treatments were: (1) Control (basal diet); (2) FGB1 = basal diet+0.2% *Ginkgo biloba* probiotic; (3) FGB2 = basal diet+0.4% *Ginkgo biloba* probiotic; (4) FCS1 = basal diet+0.2% *Camelia sinensis* probiotic, 5) FCS2 = basal diet+0.4% *Camelia sinensis* probiotic. The basal diet was formulated to meet the Nutrient Requirements of Poultry (National Research Council, NRC, 1994, Washington DC, USA) and applied for a total of 5 weeks in two stages: starter (0 to 3 weeks) and finisher (4 to 5 weeks). All diets were in mashed form. The ingredients, chemical composition and vitamin and mineral content of the basal diets are shown in Table 1. To conduct the present experiment, all guidelines for the care and use of animals in research were followed based on the Korean Ministry for Food, Agriculture, Forestry and Fisheries (2008). Broilers were reared in a closed, ventilated, wire-floor caged broiler house (100 cm long x 90 cm wide x 40 cm high/cage) with a floor space of 1,125 cm^2/bird. The cages had a linear feeder in the front and a nipple drinker in the back to provide *ad libitum* feed intake and free access to water. The internal temperature of the broiler house was set and maintained at 34°C for the first week, after which it was gradually reduced to 23 at 3°C per week and then maintained at this temperature until the end of the total experimental period. The internal relative humidity was maintained at around 50% throughout the experimental period.

Measurement of growth performance: Continuous lighting was provided for the entire experimental period and there was no vaccination or medication program. Chicks were inspected daily and dead birds were removed following recoding of the mortality (pen, date and body weight). Feed intake and body weight (BW) were recorded weekly by replicate and the average daily feed intake (ADFI), average daily gain (ADG) and FCR (feed to gain ratio) per cage were then calculated by period and for the total experimental period.

Collection and analyses of blood and caecal samples: At the termination of the feeding trial, 2 birds close to the mean body weight were randomly selected from each pen for blood sample collection. Blood samples were collected (10 mL) from the wing veins of the selected birds into a 10-mL anticoagulant-free vacutainer tube (Greiner Bio-One GmbH, Kremsmunster, Austria). The samples were subsequently stored on ice during the period of collection and then immediately centrifuged to

Table 1: Feed ingredients and chemical compositions of the basal diets

Items	Starter diet (0 to 3 weeks)	Finisher diet (4 to 5 weeks)
Ingredients (g/kg as fed basis)		
Corn grain	575.8	606.4
Soybean meal	268.0	249.0
Corn gluten	50.0	35.0
Soybean oil	22.0	22.0
Animal fats	45.0	50.0
Common salt	2.5	2.5
Dicalcium phosphate	21.4	20.0
Limestone	9.2	8.8
Vitamin-mineral premix[1]	3.0	3.0
Choline	0.8	0.7
L-lysine HCl (78%)	2.4	1.6
DL-Methionine	2.0	1.0
Calculated composition (g/kg DM)		
ME (MJ/kg)	130.3	132.7
Moisture	120.7	130.8
Crude protein	208.9	191.2
Ether extract	46.5	24.3
Crude fiber	44.2	37.1
Crude ash	56.3	56.1
Calcium	10.5	8.1
Available phosphorus	5.5	4.5
Lysine	14.2	11.0
Methionine	4.9	4.5

[1]Vitamin-mineral mixture provided the following nutrients per kilogram of diet: vitamin A, 15,000 IU; vitamin D3, 1,500 IU; vitamin E, 20.0 mg; vitamin K3, 0.70 mg; vitamin B12, 0.02 mg; niacin, 22.5 mg; thiamine, 5.0 mg; folic acid, 0.70 mg; pyridoxine, 1.3 mg; riboflavin, 5 mg; pantothenic acid, 25 mg; choline chloride, 175 mg; Mn, 60 mg; Zn, 45 mg; I, 1.25 mg; Se, 0.4 mg; Cu, 10.0 mg; Fe, 72 mg; Co, 2.5 mg (Bayer Korea Ltd., Dongjak-Ku, Seoul, Korea)

Table 2: Chemical composition of fermented *Ginkgo biloba* and *Camelia sinensis*-based probiotics

Nutrient content (g/kg DM)	Fermented *Ginkgo biloba* probiotics (FGB)	Fermented *Camelia sinensis* probiotics (FCS)
Moisture	234.2	176.7
Crude protein	119.8	118.6
Crude fat	24.1	21.5
Crude fiber	98.3	116.6
Crude ash	66.6	70.3
Nitrogen free extract	533.0	503.6
Microbial population (log_{10} CFU/g)		
Lactobacillus spp.	$2.2x10^9$	$2.2x10^9$
Saccharomyces spp.	$2.5x10^8$	$2.4x10^8$

separate the serum (centrifugation for 15 min at 1,610 x g at 4°C). Then, the serum samples were carefully transferred to plastic vials and stored at -20°C until immunoglobulin analysis was performed. The concentrations of serum IgG, IgA and IgM were assayed using appropriately diluted samples by a sandwich ELISA with chicken-specific IgG (Cat. No. E30-104), IgA (Cat. No. E30-103) and IgM (Cat. No. E10-101) ELISA quantitation kits (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer's instructions. Each experiment was run in duplicate and the results represent the means of triplicate experiments. The absorbance of each well at 450 nm was measured within 30 min using a microplate autoreader (Thermo Lab Systems, Helsinki, Finland). The concentrations of IgG, IgA and IgM were determined using standard curves constructed from the respective immunoglobulin standards and the results were expressed as mg/ml of serum.

Selected chickens were slaughtered at the end of 5th week of experimental period to measure the microflora concentration of caeca, where caecal contents were collected carefully from each bird. Feed withdrawal period of 12 h were maintained. The collected caecal contents were serially diluted in sterile saline in the 1:10 dilution and then cultured on agar media (duplicate for each). The culture media for *E. coli*, *Salmonella*, *Lactobacillus* and yeast were MacConkey Sorbitol Agar; Salmonella Shigella Agar; Lactobacilli MRS (Mann, Rogosa and Sharpe) Agar and Potato Dextrose Agar, respectively. Incubation in the anaerobic condition at 37°C for 24 h (*E. coli* and *Salmonella)* and 48 h (*Lactobacillus* and yeast) were done followed by the smearing of supernatant of 100 µl onto the agar plate. Following enumeration of microbial colonies in the duplicate incubated agar plates, microbial counts were expressed as log_{10} CFU/ml.

Statistical analyses: All data were subjected to ANOVA using the General Linear Models (GLM) function of the Statistical Analysis System (SAS, 2003). Each cage was considered as the experimental unit for growth performance parameters (BW, ADG, ADFI and FCR), whereas an individual bird served as the experimental unit for immunity and caecal microbiology. A probability level of $p<0.05$ was considered as statistically significant and a level of $p<0.10$ was considered as statistical tendency.

RESULTS

Broiler growth performance: Growth performance of broilers is shown in Table 3. Dietary supplementation of FGB2 induced a significant increase in ADG during the starter period compared to FCS1 and FCS2 ($p<0.05$) and during the overall period (0 to 5 weeks) in comparison to the control ($p<0.05$). In addition, the ADFI of broilers during the starter, finisher and overall period did not differ significantly after dietary supplementation ($p>0.05$). However, the FCR was significantly improved during starter period only in FGB2 inoculated birds and during the finisher and the overall period in all birds that received basal diets supplemented with FGB1, FGB2, FCS1 and FCS2 relative to the control diet ($p<0.05$). Moreover, the FCR was significantly improved in the FGB2 relative to that of the FCS1 and FCS2 supplemented groups ($p<0.05$) during the overall period.

Broiler's immunity: The serum immunoglobulin status was shown in Fig. 1. It was observed that, serum IgM was significantly substantially elevated in the FGB and FCS supplemented groups relative to control ($p<0.05$); where, serum IgA was tended to be higher in the FGB1 and FGB2 supplemented group compared to control ($p<0.10$). However, there was found no significant differences on serum IgG after dietary supplementation.

Caecal microbiology of broilers: Dietary supplementation of FGB and FCS on caecal microbiology was presented in Table 4. It was observed that, the caecal *E. coli* was significantly repressed after FGB and FCS supplementation than that of unsupplemented group ($p<0.05$); however, although the *Lactobacillus* spp. and yeast was found higher in FGB and FCS supplementation, it was not significant ($p>0.05$). In addition, *Salmonella* content was also unaffected in both the FGB and FCS supplemented group ($p>0.05$). There was no significant differences between FGB and FCS group based on caecal microbial content ($p<0.05$). Overall, both higher and lower level of FGB and FCS was effective on suppression of pathogenic *E. coli* concentration ($p<0.05$).

DISCUSSION

Natural plants are composed of bioactive compounds; where the active chemical constituents of the *Ginkgo biloba* leaf are flavonoids (flavone glycosides, primarily composed of quercetin), polysaccharides and terpenoids (ginkolides and bilobalides) (van Beek and Montoro, 2009), while the natural derivatives of *Camelia sinensis* are phytochemicals (polyphenols, flavonols and caffeine), polyphenols (epigallocatechin gallate, epigallocatechin, epicatechin gallate and epicatechin) and flavonols (kaempferol, quercetin and myricetin), enzymes, amino acids, carbohydrates, lipids, sterols, related compounds and dietary minerals (Khan and Mukhtar, 2013). In addition, the bioactive compounds present in *Punica granatum* are ellagitannin, punicalagin, pedunculagin and punicalin, flavonoids, anthocyanins and some other phenolic compounds (Kanatt *et al*., 2010). Natural plants or their parts containing different primary and secondary metabolites are considered phytobiotics (where secondary metabolites are of primary interest) and are utilized in animal feed alone or in combination (Grashorn, 2010). Natural plants and phytogenic products can control and limit the growth and colonization of numerous pathogenic and nonpathogenic species of bacteria in the gastrointestinal tract of chickens, resulting in increased efficiency in the utilization of feed and enhanced growth of animals (Bedford, 2000). Probiotics have been used for the past few decades to improve growth performance by enhancing the efficiency of feed utilization in poultry (Mountzouris *et al*., 2007). In addition, polysaccharides and oligosaccharides that are not effectively digested, but regarded as beneficial in animals after fermentation by anaerobic and colonic bacteria are commonly known as prebiotics (Zhang *et al*., 2003). These compounds stimulate the growth of beneficial organisms such as *Lactobacilli* and *Bifidobacteria* while controlling pathogenic organisms such as *E. coli* and *Salmonella*, resulting in improved health and performance of animals (Zhang *et al*., 2003). The combination of probiotics and prebiotics that include both beneficial microorganisms and substrates exerts synergistic effects on the gastro-intestinal tract and consequently promotes the growth of animals (Patterson and Burkholder, 2003). In addition, microbial fermentation of medicinal plants as well as herbs and spices has long been practiced, resulting in products enriched with vitamins, enzymes and growth factors (Ng *et al*., 2011); accepted by animals with conferring nutrient availability and considered useful tool for producing biological materials with health-promoting properties (Hong *et al*., 2004). The results of the present study revealed significant improvement of the ADG and FCR, indicating that symbiotic effect indeed occurred, which was also consistent with a study of plant derived essential oil mixture in broilers (Alcicek *et al*., 2004). Additionally, the lack of significant changes in feed intake were concordant with the supplementation of broiler diets with combined quercetin and alfa-tocopherol

Table 3: Effect of fermented *Ginkgo biloba* and *Camelia sinensis*-based probiotics on growth performance of broilers

	Dietary treatments						
Parameters	Control	FGB1	FGB2	FCS1	FCS2	SEM	p-value
IBW (g/bird)	46.95	46.96	47.04	47.03	46.99	0.15	0.99
FBW (g/bird)	1867.86^{b}	1954.48^{ab}	2009.03^{a}	1875.27^{b}	1892.46^{b}	30.46	0.04
0-3 weeks							
ADG (g/bird)	40.76^{ab}	42.05^{ab}	43.09^{a}	39.89^{b}	39.68^{b}	0.76	0.05
ADFI (g/bird)	64.74	63.95	63.77	62.98	62.82	0.57	0.18
FCR	1.59^{a}	1.52^{ab}	1.48^{b}	1.58^{a}	1.59^{a}	0.02	0.03
4-5 weeks							
ADG (g/bird)	68.93	73.18	75.51	70.75	72.30	2.00	0.38
ADFI (g/bird)	136.96	127.33	128.28	125.27	130.58	4.37	0.42
FCR	1.99^{a}	1.74^{b}	1.70^{b}	1.78^{b}	1.81^{b}	0.05	0.02
0-5 weeks							
ADG (g/bird)	52.03^{b}	54.50^{ab}	56.06^{a}	52.24^{b}	52.73^{b}	0.87	0.04
ADFI (g/bird)	93.63	89.30	89.57	87.90	89.92	1.88	0.34
FCR	1.80^{a}	1.64^{bc}	1.60^{c}	1.68^{b}	1.71^{b}	0.02	0.0001

$^{a, b}$Means with different superscripts within the same row are significantly different ($p<0.05$). S

EM: Standard error of mean, IBW: Initial body weight, FBW: Final body weight, ADG: Average daily gain, ADFI: Average daily feed intake, FCR: Feed conversion ratio (feed: gain ratio); Control (corn-soybean based basal diet); FGB1 = basal diet+0.2% fermented *Ginkgo biloba*-based probiotic; FGB2 = basal diet+0.4% fermented *Ginkgo biloba*-based probiotic; FCS1 = basal diet+0.2% fermented *Camelia sinensis*-based probiotic, FCS2 = basal diet+0.4% fermented *Camelia sinensis*-based probiotic

Table 4: Effect of fermented *Ginkgo biloba* and *Camelia sinensis*-based probiotics on caecal microbiology of broilers (log_{10} CFU/g)

	Dietary treatments						
Parameters	Control	FGB1	FGB2	FCS1	FCS2	SEM	p-value
Lactobacillus	9.048	9.308	9.488	9.188	9.448	0.163	0.346
Yeast	8.925	9.578	9.780	9.700	9.815	0.284	0.220
Salmonella	9.710	9.368	9.570	9.408	9.598	0.233	0.864
E. coli	8.630^{a}	7.255^{b}	7.190^{b}	7.128^{b}	7.205^{b}	0.239	0.004

$^{a, b}$Means with different superscripts within the same row are significantly different ($p<0.05$).

SEM = Standard error of mean. Control (corn-soybean based basal diet); FGB1 = basal diet+0.2% fermented *Ginkgo biloba*-based probiotic; FGB2 = basal diet+0.4% fermented *Ginkgo biloba*-based probiotic; FCS1 = basal diet+0.2% fermented *Camelia sinensis*-based probiotic, FCS2 = basal diet+0.4% fermented *Camelia sinensis*-based probiotic

(Sohaib *et al*., 2015). Zhang *et al*. (2012) reported that *Aspergillus*-fermented *Ginkgo biloba* was not effective at improving weight gain in broilers; however, we found a significant increase in ADG after FGB supplementation during the starter and overall period. The additional impact and variation in the current observation might be attributed to the addition of *Punica granatum* with *Ginkgo biloba* and fermentation with *Lactobacilli* and *Saccharomyces* spp. Because the combination of different phytochemicals such as flavonoids (flavonol and flavone glycosides), terpenoids (ginkolides and bilobalides), tannins (ellagitannins, punicalagin, punicallin and pedunculagin), anthocyanins and the organic acids from *Ginkgo biloba* and *Punica granatum* might have contributed to the antimicrobial (Hara-Kudo *et al*., 2005) and immunomodulatory properties (Nishida *et al*., 2006), as well as the anti-carcinogenic (Mukhtar and Ahmad, 1999), antioxidative and free-radical scavenging activities (Le Bars *et al*., 1997; Rajan *et al*., 2011), which consequently help to improve body weight gain and feed efficiency of broilers (Cao *et al*., 2005). Zhang *et al*. (2012) evaluated *Aspergillus*-fermented *Ginkgo biloba* and found that FCR was significantly improved during days 22 to 42 and 1 to 42. Consistent with these findings, FCR was found to be improved in the FGB supplemented group in the present study ($p<0.05$). In the case of FCS supplementation, we observed improved FCR, but Sarker *et al*. (2010) reported no significant improvement in FCR following treatment with fermented *Camelia sinensis* probiotics. This inconsistency might have been due to variations in the probiotic preparation, (in the present study, 30% *Punica granatum* was added by replacing wheat bran) and the probiotic microorganisms (*Lactobacilli* and *Saccharomyces* spp.), thus successful benefit of combination of plant material is proved, although required further detailed mode of action study.

In the present study, the significantly higher ADG during the starter (0 to 3 weeks) and overall period (0 to 5 weeks) and better FCR relative to FCS was reflected in the FGB supplemented birds. The significant difference in FCR between FGB and FCS indicated a higher benefit of FGB due to combination with *Punica granatum* relative to FCS with similar probiotics mixtures (*Lactobacilli* and *Saccharomyces* spp.). It has been reported that flavonoids are more easily and rapidly absorbed in the intestines after fermentation (Izumi *et al*., 2000). Total polysaccharides, CP and total amino acids can be increased in fermented *Ginkgo biloba* relative to non-fermented and total flavonoid contents decreased

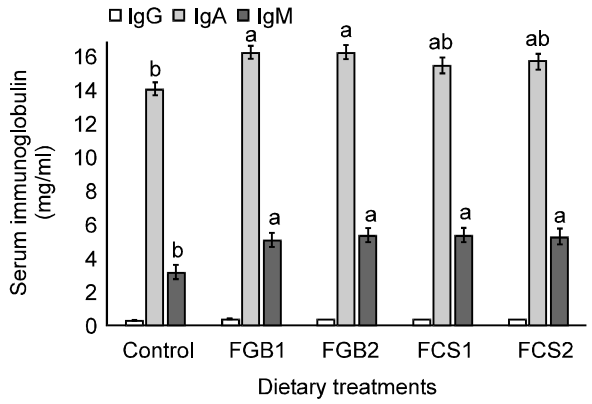

Fig. 1: Effect of fermented *Ginkgo biloba* and *Camelia sinensis*-based probiotics on immunity of broilers.
[a,b]Means with different superscript letters within the similar bars are significantly different ($p<0.05$). Error bar indicated standard error. Control (corn-soybean based basal diet); FGB1 = basal diet+0.2% fermented *Ginkgo biloba*-based probiotic; FGB2 = basal diet+0.4% fermented *Ginkgo biloba*-based probiotic; FCS1 = basal diet+0.2% fermented *Camelia sinensis*-based probiotic, FCS2 = basal diet+0.4% fermented *Camelia sinensis*-based probiotic

slightly due to microbial synthesis of the enzyme (Cao *et al*., 2012). Where, microbial enzymes act on the conversion of flavonoids to aglycones (bioactive components) and helps to exert beneficial impact on broiler nutrition (Hsu and Chiang, 2009).

Probiotics secrete useful enzymes, organic acids, vitamins and nontoxic antibacterial substances after ingestion, thereby improving the gut microflora and influencing the local and systemic immune systems (Jun *et al*., 2002). Where, plant derived flavones and terpenes have immune promoting activities which helps to enhance the lymphocyte synthesis, phagocytosis activity and cytokin release (Zhao *et al*., 2011). Elevation of serum immunoglobulins (IgM, predominant isotype of natural antibodies) in the current study might be attributable to enlargement of the splenic lymphocyte due to probiotic effect (Shimizu *et al*., 1981; Aattouri *et al*., 2002) and combination of polyphenolic compounds (derived from *Punica granatum*, *Ginkgo Biloba* and *Camelia sinensis*) (Al-Masad, 2012; Abuelsaad *et al*., 2013). Improvement in the immune status and lower mortality was reported after inclusion of *Ginkgo biloba extract* and *Camelia sinensis* in broilers (Yang *et al*., 2003; Yang *et al*., 2008; Sarker *et al*., 2010; Khalaji *et al*., 2011). Zhou *et al*. (2015) reported that, 10% inclusion of fermented *Ginkgo biloba* was beneficial to improve the immune function in case of weaned piglets which support our study of combined fermented medicinal plant supplementation. Hossain *et al*. (2012) and Kim *et al*. (2010) studied on medicinal plants with probiotics while they suggested that, *A. canaliculatum*, *Viscum album* and *Cornus officinalis* with probiotics could be used as alternative to antibiotics for the improvement of growth performance and immunity of growing pigs and broilers. Research on the modes of action of combined phytobiotic substances and probiotics also implicated that, there is a possibility of synergistic effects between these classes of compounds, which might help to improve the immunity, health and performance (Kim *et al*. 2007; Sarker *et al*., 2010).

The significant suppression of the *E. coli* in the caecal microbiology in the present study indicated the symbiotic effect on successful microbial balance (although the *Lactobacillus* and yeast content was non-significantly higher; which was consistent with Hara-Kudo *et al*. (2005) report of antimicrobial effects without affecting the lactic acid bacteria). Presence of flavonoids (Mandalari *et al*., 2007; Abuelsaad *et al*., 2013) and generation of different metabolites (lactic acids, organic acids) (having antimicrobial properties) after microbial fermentation might be act as lethal action to pathogenic bacteria (Zheng *et al*., 1999; Heres *et al*., 2003), which might consequently be acted on suppression of *E. coli* in the present study. Polyphenols has been reported in increment of *Lactobacilli* populations and downtrend of *Bacteroidaceae* in the caecal content of chicken (Terada *et al*., 1993). Therefore, combination of natural materials along with microbial fermentation helps to modulate the gut microbial population (suppressing pathogenic *E. coli*) and helps to improve the performance and immunity of birds in the present study which was concurred with Table 4 and Fig. 1.

Conclusion: Natural plant-based probiotic development through combination of natural plant resources (*Ginkgo biloba+Punica granatum* and *Camelia sinensis+Punica grantum*) and fermentation with probiotic microorganisms (*Lactobacilli+Saccharomyces* spp.) was found effective on growth performance, immunity and caecal microbiology. In the current study, supplementation of the diet with fermented *Ginkgo biloba* (FGB) and *Camelia sinensis* (FCS)-based probiotics significantly improved the average daily gain and feed conversion ratio compared to control ($p<0.05$). Where feed intake among the dietary treatments did not differ significantly. In addition, dietary FGB and FCS supplementation was effective on improvement of the bird's immunity and favored the gut microbial modulation through suppression of caecal pathogenic *E. coli* ($p<0.05$). Therefore, dietary FGB and FCS can be utilized as potential feed additives in broiler diets for positive impact on the performance, immunity and

caecal microbiology. Further detailed study is required on mechanism and meat quality analysis in broilers.

Conflict of interest: We are confirming that, there is no any conflict of interest associated with this research and publication. In addition, all the authors are agreed to publish this article and approved thereby.

ACKNOWLEDGEMENT

This study (Grants No. C0193546) was supported by Business for Academic-industrial Cooperative establishments funded Korea Small and Medium Business Administration in 2014.

REFERENCES

Aattouri, N., M. Bouras, D. Tome, A. Marcos and D. Lemonnier, 2002. Oral Ingestion of Lactic Acid Bacteria by Rats Increases Lymphocyte Proliferation and Interferon Production. Br. J. Nutr., 87: 367-373.

Abuelsaad, A.S., I. Mohamed, G. Allam and A.A. Al-Solumani, 2013. Antimicrobial and Immunomodulating Activities of Hesperidin and Ellagic Acid Against Diarrheic Aeromonas Hydrophila in a Murine Model. Life Sci., 93: 714-722.

Ahmed, S., H. Mun, M. Islam, S. Kim, J. Hwang, Y. Kim and C. Yang, 2014. Effects of *Citrus junos* by-Products Fermented with Multistrain Probiotics on Growth Performance, Immunity, Caecal Microbiology and Meat Oxidative Stability in Broilers. Br. Poult. Sci., 55: 540-547.

Alçiçek, A., M. Bozkurt and M. Cabuk, 2004. The Effect of a Mixture of Herbal Essential Oils, an Organic Acid Or a Probiotic on Broiler Performance. S. Afr. J. Anim. Sci., 34: 217-222.

Al-Masad, M., 2012. Effect of Vitamin C and Zinc on Broilers Performance of Immunocompetence Under Heat Stress. Asian. J. Anim. Sci., 6: 76-84.

AOAC, 2000. Official Methods of Analysis of AOAC International, 17th Edn., AOAC International, Gaitherburg, MD, USA.

Bedford, M., 2000. Removal of Antibiotic Growth Promoters from Poultry Diets: Implications and Strategies to Minimise Subsequent Problems. Worlds Poult. Sci. J., 56: 347-365.

Borris, R.P., 1996. Natural Products Research: Perspectives from a Major Pharmaceutical Company. J. Ethnopharmacol., 51: 29-38.

Bostami, A., S. Ahmed, M. Islam, H. Mun, S. Ko, S. Kim and C. Yang, 2015. Growth Performance, Fecal Noxious Gas Emission and Economic Efficacy in Broilers Fed Fermented Pomegranate Byproducts as Residue of Fruit Industry. Int. J. Adv. Res., 3: 102-114.

Cao, B., Y. Karasawa and Y. Guo, 2005. Effects of Green Tea Polyphenols and Fructo-Oligosaccharides in Semi-Purified Diets on Broilers' Performance and Caecal Microflora and their Metabolites. Asian-Aust. J. Anim. Sci., 18: 85-89.

Cao, F.L., X.H. Zhang, W.W. Yu, L.G. Zhao and T. Wang, 2012. Effect of Feeding Fermented Ginkgo Biloba Leaves on Growth Performance, Meat Quality and Lipid Metabolism in Broilers. Poult. Sci., 91: 1210-1221.

Cho, J., Z. Zhang and I. Kim, 2013. Effects of Fermented Grains as Raw Cereal Substitutes on Growth Performance, Nutrient Digestibility, Blood Profiles and Fecal Noxious Gas Emission in Growing Pigs. Livest. Sci., 154: 131-136.

Cowan, M.M., 1999. Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev., 12: 564-582.

Ehrmann, M., P. Kurzak, J. Bauer and R. Vogel, 2002. Characterization of Lactobacilli Towards their use as Probiotic Adjuncts in Poultry. J. Appl. Microbiol., 92: 966-975.

Erdogan, Z., S. Erdogan, O. Aslantaþ and S. Celik, 2010. Effects of Dietary Supplementation of Synbiotics and Phytobiotics on Performance, Caecal Coliform Population and some oxidant/antioxidant Parameters of Broilers. J. Anim. Physiol. Anim. Nutr., 94: 40-48.

Feng, J., X. Liu, Z. Xu, Y. Lu and Y. Liu, 2007. The Effect of Aspergillus Oryzae Fermented Soybean Meal on Growth Performance, Digestibility of Dietary Components and Activities of Intestinal Enzymes in Weaned Piglets. Anim. Feed Sci. Technol., 134: 295-303.

Fulton, R.M., B.N. Nersessian and W.M. Reed, 2002. Prevention of Salmonella Enteritidis Infection in Commercial Ducklings by Oral Chicken Egg-Derived Antibody Alone Or in Combination with Probiotics. Poult. Sci., 81: 34-40.

Grashorn, M., 2010. Use of Phytobiotics in Broiler nutrition-an Alternative to Infeed Antibiotics. J. Anim. Feed Sci., 19: 338-347.

Hara-Kudo, Y., A. Yamasaki, M. Sasaki, T. Okubo, Y. Minai, M. Haga, K. Kondo and Y. Sugita-Konishi, 2005. Antibacterial Action on Pathogenic Bacterial Spore by Green Tea Catechins. J. Sci. Food Agric., 85: 2354-2361.

Hashemi, S.R., Z. Idrus, M.H. Bejo, F. Abas and M.N. Somchit, 2008. Acute Toxicity Study and Phytochemical Screening of Selected Herbal Aqueous Extract in Broiler Chickens. Int. J. Pharmacol., 4: 352-360.

Heres, L., B. Engel, F. Van Knapen, J.A. Wagenaar and B.A. Urlings, 2003. Effect of Fermented Feed on the Susceptibility for Campylobacter Jejuni Colonisation in Broiler Chickens with and without Concurrent Inoculation of *Salmonella Enteritidis*. Int. J. Food Microbiol., 87: 75-86.

Hong, K., C. Lee and S.W. Kim, 2004. Aspergillus Oryzae GB-107 Fermentation Improves Nutritional Quality of Food Soybeans and Feed Soybean Meals. J. Med. Food, 7: 430-435.

Hossain, M.E., G.M. Kim, S.K. Lee and C.J. Yang, 2012. Growth Performance, Meat Yield, Oxidative Stability and Fatty Acid Composition of Meat from Broilers Fed Diets Supplemented with a Medicinal Plant and Probiotics. Asian-Aust. J. Anim. Sci., 25: 1159.

Hsu, M. and B. Chiang, 2009. Effect of Bacillus Subtilis natto-fermented Radix Astragali on Collagen Production in Human Skin Fibroblasts. Process Biochem., 44: 83-90.

Izumi, T., M.K. Piskula, S. Osawa, A. Obata, K. Tobe, M. Saito, S. Kataoka, Y. Kubota and M. Kikuchi, 2000. Soy Isoflavone Aglycones are Absorbed Faster and in Higher Amounts than their Glucosides in Humans. J. Nutr., 130: 1695-1699.

Jun, K., H. Kim, K. Lee, H. Paik and J. Kang, 2002. Characterization of Bacillus Polyfermenticus SCD as a Probiotic. Korean J. Microbiol. Biotech., 30: 359-366.

Kanatt, S.R., R. Chander and A. Sharma, 2010. Antioxidant and Antimicrobial Activity of Pomegranate Peel Extract Improves the Shelf Life of Chicken Products. Int. J. Food Sci. Tech., 45: 216-222.

Karaoglu, M. and H. Durdag, 2005. The Influence of Dietary Probiotic (*Saccharomyces cerevisiae*) Supplementation and Different Slaughter Age on the Performance, Slaughter and Carcass Properties of Broilers. Int. J. Poult. Sci., 4: 309-316.

Khalaji, S., M. Zaghari, K. Hatami, S. Hedari-Dastjerdi, L. Lotfi and H. Nazarian, 2011. Black Cumin Seeds, Artemisia Leaves (*Artemisia sieberi*) and Camellia L. Plant Extract as Phytogenic Products in Broiler Diets and their Effects on Performance, Blood Constituents, Immunity and Cecal Microbial Population. Poult. Sci., 90: 2500-2510.

Khan, N. and H. Mukhtar, 2013. Tea and Health: Studies in Humans. Curr. Pharm. Des., 19: 6141-6147.

Kim, D., S. Kim, D. Yu, G. Kang, J. Kim, H. Kang, B. Jang, J. Na, O. Suh and I. Jang, 2007. Effects of Single or Mixed Supplements of Plant Extract, Fermented Medicinal Plants and Lactobacillus on Growth Performance in Broilers. Kor. J. Poult. Sci., 34: 187-196.

Kim, K., G. Kim, M. Hossain, S. Park and C. Yang, 2010. Effect of *Alisma canaliculatum*, *Viscum albumas* and *Cornus officinalis* probiotics feed additives on growth performance and immunity in growing pigs. In: Proceeding of the Annual Congress of Korean Society of Animal Sciences and Technology, Jinju. South Korea, pp: 217.

Kyriakis, S., V. Tsiloyiannis, J. Vlemmas, K. Sarris, A. Tsinas, C. Alexopoulos and L. Jansegers, 1999. The Effect of Probiotic LSP 122 on the Control of Post-Weaning Diarrhoea Syndrome of Piglets. Res. Vet. Sci., 67: 223-228.

Le Bars, P.L., M.M. Katz, N. Berman, T.M. Itil, A.M. Freedman and A.F. Schatzberg, 1997. A Placebo-Controlled, Double-Blind, Randomized Trial of an Extract of Ginkgo Biloba for Dementia. JAMA, 278: 1327-1332.

Mandalari, G., R. Bennett, G. Bisignano, D. Trombetta, A. Saija, C. Faulds, M. Gasson and A. Narbad, 2007. Antimicrobial Activity of Flavonoids Extracted from Bergamot (Citrus Bergamia Risso) Peel, a Byproduct of the Essential Oil Industry. J. Appl. Microbiol., 103: 2056-2064.

Mountzouris, K.C., P. Tsirtsikos, E. Kalamara, S. Nitsch, G. Schatzmayr and K. Fegeros, 2007. Evaluation of the Efficacy of a Probiotic Containing Lactobacillus, Bifidobacterium, Enterococcus and Pediococcus Strains in Promoting Broiler Performance and Modulating Cecal Microflora Composition and Metabolic Activities. Poult. Sci., 86: 309-317.

Mukhtar, H. and N. Ahmad, 1999. Mechanism of Cancer Chemopreventive Activity of Green Tea. Proc. Soc. for Exp. Biol. and Med., 220: 234-238.

Ng, C., C. Wang, Y. Wang, W. Tzeng and Y. Shyu, 2011. Lactic Acid Bacterial Fermentation on the Production of Functional Antioxidant Herbal Anoectochilus Formosanus Hayata. J. Biosci. Bioengin, 111: 289-293.

Nishida, T., B. Eruden, K. Hosoda, H. Matsuyama, K. Nakagawa, T. Miyazawa and S. Shioya, 2006. Effects of Green Tea (*Camellia sinensis*) Waste Silage and Polyethylene Glycol on Ruminal Fermentation and Blood Components in Cattle. Asian-Aust. J. Anim. Sci., 19: 1728.

Patterson, J.A. and K.M. Burkholder, 2003. Application of Prebiotics and Probiotics in Poultry Production. Poult. Sci., 82: 627-631.

Rajan, S., S. Mahalakshmi, V. Deepa, K. Sathya, S. Shajitha and T. Thirunalasundari, 2011. Antioxidant Potentials of Punica Granatum Fruit Rind Extracts. Int. J. Pharm. Pharm. Sci., 3: 82-88.

Sarker, M.S.K., S. Ko, G. Kim and C. Yang, 2010. Effects of Camellia Sinensis and Mixed Probiotics on the Growth Performance and Body Composition in Broiler. J. Med. Plant Res., 4: 546-550.

SAS, 2003, Version 9.1, SAS Institute, Cary, NC, USA

Shimizu, T., I. Mifuchi and T. Yokokura, 1981. Mitogenic effect of lactobacilli on murine lymphocytes. Chem. Pharmac. Bull., 29: 3731-3734.

Sohaib, M., M.S. Butt, M.A. Shabbir and M. Shahid, 2015. Lipid Stability, Antioxidant Potential and Fatty Acid Composition of Broilers Breast Meat as Influenced by Quercetin in Combination with α-Tocopherol Enriched Diets. Lipids in Health and Dis., 14: 1.

Terada, A., H. Hara, S. Nakajyo, H. Ichikawa, Y. Hara, K. Fukai, Y. Kobayashi and T. Mitsuoka, 1993. Effect of Supplements of Tea Polyphenols on the Caeeal Flora and Caeeal Metabolites of Chicks. Microb. Ecol. Health Dis., 6: 3-9.

van Beek, T.A. and P. Montoro, 2009. Chemical Analysis and Quality Control of Ginkgo Biloba Leaves, Extracts and Phytopharmaceuticals. J. Chromatography A., 1216: 2002-2032.

Wald, C., 2003. Gewurze and Co.-eine Ubersicht. Lohmann Information, 3: 7-11.

Yang, C., I. Yang, D. Oh, I. Bae, S. Cho, I. Kong, D. Uuganbayar, I. Nou and K. Choi, 2003. Effect of Green Tea by-Product on Performance and Body Composition in Broiler Chicks. Asian-Aust. J. Anim. Sci., 16: 867-872.

Yang, X., Y. Lin and Y. Li, 2008. Effect of Ginkgo Biloba Extract on Growth Performance, Slaughter Performance and Immune Index in Broilers [J]. J. Fujian Agric. Forestry Univ. (Natural Science Edition), 3: 016.

Zhang, X., F. Cao, Z. Sun, W. Yu, L. Zhao, G. Wang and T. Wang, 2012. Effect of Feeding Aspergillus Niger-Fermented Ginkgo Biloba-Leaves on Growth, Small Intestinal Structure and Function of Broiler Chicks. Livest. Sci., 147: 170-180.

Zhang, W.F., D.F. Li, W.Q. Lu and G.F. Yi, 2003. Effects of Isomalto-Oligosaccharides on Broiler Performance and Intestinal Microflora. Poult. Sci., 82: 657-663.

Zhao, J., Y. Su, A. Chen, H. Yuan, L. Liu and W. Wu, 2011. Effect of Ginkgo Leaf Parenteral Solution on Blood and Cochlea Antioxidant and Immunity Indexes in OM Rats. Molecules, 16: 10433-10442.

Zheng, G., L.Z. Yan, J.C. Vederas and P. Zuber, 1999. Genes of the sbo-alb Locus of Bacillus subtilis Are Required for Production of the Antilisterial Bacteriocin Subtilosin. J. Bacteriol., 181: 7346-7355.

Zhou, H., C. Wang, J. Ye, H. Chen and R. Tao, 2015. Effects of Dietary Supplementation of Fermented *Ginkgo biloba* L. Residues on Growth Performance, Nutrient Digestibility, Serum Biochemical Parameters and Immune Function in Weaned Piglets. Anim. Sci. J., 86: 790-799.