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

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**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 (Boriss, 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; Karaoğlu and Durdağ, 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 sinensis*, *Alisma canaliculatum*, *Citrus junos* and *Punica granatum* are important. Previous studies on the effects of natural plant materials on growth performance showed varying

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results (Alcicek et al., 2004; Grashorn, 2010); fermented 
*Ailmos calaniculatum*, *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 
sinesis* 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, bitter 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 sinensis* 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 
sinesis-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 biloba* leaf powder, whereas fermented *Camelia 
sinesis* (FCS) contains 60% defatted rice bran, 30% 
pomegranate peel extract and 10% *Camelia sinensis* 
leaf powder. After mixing the ingredients to prepare FGB 
and FCS, samples were inoculated with 30% (v/v) 
*Lactobacillus plantarum* KCTC 3099 and *Lactobacillus 
adipophilus* KCTC 3111 and fermented for 2 days at 
37°C and 40% moisture in a commercial fermenter (W- 
1000; Wonsahlho 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 
centralization, 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 17.87% 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<sub>10</sub> CFU/g for FGB 
and FCS, respectively, while the *Saccharomyces* spp. 
population was 6.62 and 6.36 log<sub>10</sub> 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.
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% Camellia sinensis probiotic; (5) FCS2 = basal diet+0.4% Camellia 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<sup>2</sup>/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 recording 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

<table>
<thead>
<tr>
<th>Ingredients (g/kg as fed basis)</th>
<th>Starter diet (0 to 3 weeks)</th>
<th>Finisher diet (4 to 5 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn grain</td>
<td>575.8</td>
<td>606.4</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>268.0</td>
<td>249.0</td>
</tr>
<tr>
<td>Corn gluten</td>
<td>50.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>22.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Animal fats</td>
<td>45.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Common salt</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>21.4</td>
<td>20.0</td>
</tr>
<tr>
<td>Limestone</td>
<td>9.2</td>
<td>8.8</td>
</tr>
<tr>
<td>Vitamin-mineral premix</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Choline</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>L-lysine HCl (78%)</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>2.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**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                         |

1Vitamin-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, 90 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 Camellia sinensis-based probiotics

<table>
<thead>
<tr>
<th>Nutrient content (g/kg DM)</th>
<th>Fermented Ginkgo biloba probiotics (FGB)</th>
<th>Fermented Camellia sinensis probiotics (FCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>234.2</td>
<td>178.7</td>
</tr>
<tr>
<td>Crude protein</td>
<td>119.8</td>
<td>118.6</td>
</tr>
<tr>
<td>Crude fat</td>
<td>24.1</td>
<td>21.5</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>98.3</td>
<td>116.6</td>
</tr>
<tr>
<td>Crude ash</td>
<td>66.6</td>
<td>70.3</td>
</tr>
<tr>
<td>Nitrogen free extract</td>
<td>533.0</td>
<td>503.6</td>
</tr>
<tr>
<td><strong>Microbial population (log10 CFU/g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>2.2x10^8</td>
<td>2.2x10^8</td>
</tr>
<tr>
<td>Saccharomyces spp.</td>
<td>2.5x10^8</td>
<td>2.4x10^8</td>
</tr>
</tbody>
</table>

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, Lactobacillus 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 log10 CFU/ml.
Statistical analyses: All data were subjected to ANCOVA 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 elevated in the FGB and FCS supplemented groups relative to control (p<0.05), whereas, 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 Camellia 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 (Grashom, 2010). Natural plants and phyogenic 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 Lactobacillus 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 gastrointestinal 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 *Camellia sinensis*-based probiotics on growth performance of broilers

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>FGB1</th>
<th>FGB2</th>
<th>FCS1</th>
<th>FCS2</th>
<th>SEM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBW (g/bird)</td>
<td>46.95</td>
<td>46.90</td>
<td>47.04</td>
<td>47.03</td>
<td>46.99</td>
<td>0.15</td>
<td>0.99</td>
</tr>
<tr>
<td>FBW (g/bird)</td>
<td>1867.86</td>
<td>1954.48</td>
<td>2009.03</td>
<td>1875.27</td>
<td>1802.46</td>
<td>30.46</td>
<td>0.04</td>
</tr>
<tr>
<td>0-3 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG (g/bird)</td>
<td>40.76</td>
<td>42.05</td>
<td>43.09</td>
<td>39.69</td>
<td>39.68</td>
<td>0.78</td>
<td>0.05</td>
</tr>
<tr>
<td>ADFI (g/bird)</td>
<td>64.74</td>
<td>63.85</td>
<td>63.77</td>
<td>62.98</td>
<td>62.82</td>
<td>0.57</td>
<td>0.18</td>
</tr>
<tr>
<td>FCR</td>
<td>1.56b</td>
<td>1.52ab</td>
<td>1.48a</td>
<td>1.56a</td>
<td>1.58b</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>4-5 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG (g/bird)</td>
<td>68.93</td>
<td>73.18</td>
<td>75.51</td>
<td>70.75</td>
<td>72.30</td>
<td>2.00</td>
<td>0.38</td>
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<tr>
<td>ADFI (g/bird)</td>
<td>136.98</td>
<td>127.33</td>
<td>128.28</td>
<td>125.27</td>
<td>130.58</td>
<td>4.37</td>
<td>0.42</td>
</tr>
<tr>
<td>FCR</td>
<td>1.99a</td>
<td>1.74a</td>
<td>1.73a</td>
<td>1.76b</td>
<td>1.81a</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>0-5 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG (g/bird)</td>
<td>52.03</td>
<td>54.00</td>
<td>56.06</td>
<td>52.24</td>
<td>52.73</td>
<td>0.87</td>
<td>0.04</td>
</tr>
<tr>
<td>ADFI (g/bird)</td>
<td>93.63</td>
<td>89.30</td>
<td>89.57</td>
<td>87.90</td>
<td>89.92</td>
<td>1.88</td>
<td>0.34</td>
</tr>
<tr>
<td>FCR</td>
<td>1.60a</td>
<td>1.64a</td>
<td>1.60a</td>
<td>1.68b</td>
<td>1.71a</td>
<td>0.02</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

1-4Means 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 *Camellia sinensis*-based probiotic; FCS2 = basal diet+0.4% fermented *Camellia sinensis*-based probiotic.

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>FGB1</th>
<th>FGB2</th>
<th>FCS1</th>
<th>FCS2</th>
<th>SEM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>9.048</td>
<td>9.308</td>
<td>9.488</td>
<td>9.168</td>
<td>9.448</td>
<td>0.163</td>
<td>0.348</td>
</tr>
<tr>
<td>Yeast</td>
<td>8.925</td>
<td>9.573</td>
<td>9.780</td>
<td>9.700</td>
<td>9.815</td>
<td>0.264</td>
<td>0.220</td>
</tr>
<tr>
<td>Salmonella</td>
<td>9.710</td>
<td>9.388</td>
<td>9.570</td>
<td>9.408</td>
<td>9.586</td>
<td>0.233</td>
<td>0.084</td>
</tr>
<tr>
<td>E. coli</td>
<td>8.630</td>
<td>7.256</td>
<td>7.190</td>
<td>7.128</td>
<td>7.206</td>
<td>0.239</td>
<td>0.004</td>
</tr>
</tbody>
</table>

1-4Means 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 *Camellia sinensis*-based probiotic; FCS2 = basal diet+0.4% fermented *Camellia 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, punicalin 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 *Camellia 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 Carneilia sinensis-based probiotics on immunity of broilers.

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 Carneilia sinensis-based probiotic; FCS2 = basal diet+0.4% fermented Carneilia 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; Aattori et al., 2002) and combination of polyphenolic compounds (derived from Punica granatum, Ginkgo Biloba and Carneilia sinensis) (Al-Masad, 2012; Abuelosaad et al., 2013). Improvement in the immune status and lower mortality was reported after inclusion of Ginkgo biloba extract and Carneilia 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; Abuelosaad 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 downturn 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 Carneilia sinensis+Punica granatum) 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 Carneilia 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.

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