ABSTRACT: To evaluate and compare the effects of Bacillus coagulans-fermented Ginkgo biloba (FG) and nonfermented Ginkgo biloba (NFG) on the immunity status of broiler chickens, 180 1-d-old female Arbor Acres chicks were divided into 3 groups and fed either a basal diet, a basal diet supplemented with 0.3% NFG, or a basal diet supplemented with 0.3% FG. Blood samples were taken on the seventh (before vaccination), 14th, 21st, 28th and 35th day for the assessment of serum IL-18 and interferon-γ (IFN-γ) levels by ELISA. In addition, Newcastle disease antibody titer analysis was made via hemagglutination and hemagglutination inhibition test methods. On d 35, 6 chickens from each group were sacrificed and the thymus, liver, spleen, small intestine (jejunum segment), cecum, and bursa of Fabricius from each chicken were removed for analysis. RNA was isolated for defensin expression detection by real-time PCR (q-PCR). The results showed that serum IL-18 and IFN-γ levels decreased after treatment with NFG and FG compared with untreated control chickens. The ND antibody titers did not differ significantly between the 3 groups on the seventh, 14th, 21st and 28th day; however, on the 35th day, the ND antibody titers of the NFG and FG chickens were both significantly higher than those of control group chickens. Defensin RNA expression levels were inhibited by NFG; however, they were induced by FG. In conclusion, fermentation of Ginkgo biloba with Bacillus coagulans can promote the beneficial effect of Ginkgo biloba on the immunity status of broiler chickens.

Key words: Bacillus coagulans, broiler chicken, defensin, Ginkgo biloba, interleukin-18

INTRODUCTION

During the last decade, bacterial resistance to antibiotics has become a widespread concern. In 1999, the European Union banned the use of most antibiotic feed additives in Europe, and in 2006, a complete ban was enforced, with discussions regarding their restricted use remaining ongoing (Windisch et al., 2008). As a consequence, phytogenic feed additives have gained much attention recently (van Beek, 2002; Guo, 2003; Lin et al., 2008; van Beek and Montoro, 2009; Amin et al., 2012) as herbs and their extracts are known for their beneficial properties, such as antimicrobial (Özer et al., 2007) and antioxidant activities (Wei and Shibamoto, 2007), as well as beneficial effects on gut function (Windisch et al., 2008). Ginkgo biloba L. (Boonkaew and Camper, 2005) contains a number of biologically active compounds, including flavonol glycosides, terpene lactones, and proanthocyanidins, which have shown efficacy in the treatment of vascular and cardiac diseases, as well as some cancers (Brinkley et al., 2010; Sochocka et al., 2010; Chen et al., 2011; Amieva et al., 2013; Barton et al., 2013). Although some studies have shown that flavonoids can have some beneficial effects in ani-
mals (Feng et al., 2011), most research has focused on their antimicrobial, neuroprotective, antimitochondrial, anticancer, and cardioprotective effects (Yoshikawa et al., 1999; Bent et al., 2005; Biggs et al., 2010; Vardy et al., 2013). However, other studies have shown that *G. biloba* also possesses an immunostimulatory activity (Villaseñor-García et al., 2004; Sochocka et al., 2010).

Fermentation has been used for the manufacture of biological materials with health-promoting properties and to eliminate undesirable flavors (Dei et al., 2008; Ng et al., 2011). It has mostly been used in the processing of various fruits, feeds, and vegetables (Kim et al., 2012; Swain et al., 2014) but has also been utilized as a practical way to ameliorate the overproduction of herbs. In the present study, we developed a fermentation process with *Bacillus coagulans* that could not only preserve the bioactive substances of *G. biloba* leaves, but may also enhance their functionality. Thus, we investigated the effects of fermented and nonfermented *G. biloba* on the immune status of broiler chickens.

**MATERIALS AND METHODS**

**Animals**

All animal-based procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of Southern China Agricultural University.

**Culturing of Bacillus Coagulans**

The *Bacillus coagulans* used in this study was a laboratory strain obtained from Wens Research Institution (Guangdong Province, China). It was cultivated using glucose broth liquid medium at 30°C for 14–18 h before inoculation (1 × 10^7 cfu/mL).

**Preparation of Fermented G. Biloba Leaves**

*G. biloba* leaves were purchased from a qualified local traditional Chinese medicine (TCM) supplier. All leaves used in this study were from the same batch. Leaves were powdered by an electric grinder and passed through a 20-mesh sieve. The powder was then mixed with corn starch, soybean meal powder, and 2% nutritive salt (MnSO₄·H₂O: MgSO₄·7H₂O: K₂HPO₄·NaCl: glucose = 0.2: 1: 4: 5: 9.8) so that the final mixture contained 73% *G. biloba* leaves, 15% corn starch, 10% soybean meal, and 2% nutritive salt. The *G. biloba* mixture was then divided into 2 portions after autoclave sterilization. One lot was untreated (i.e., it was not fermented), and the other lot was first wetted with sterilized tap water until the humidity reached 75% under a sterile environment and then fermented using *Bacillus coagulans* at 30°C for 48 h by packing the mixture into stainless steel containers. The mixture was then gently pressed and the containers sealed with adhesive film to create a relatively anaerobic environment. The inoculation quantity was controlled at 10%. *Bacillus coagulans* is a Gram-positive, endospore-forming, heat-resistant, facultatively anaerobic bacterium (Nakamura et al., 1988). The fermented product was spread onto stainless steel pans and dried in an oven overnight at 50°C. The dry matter was then collected and passed through a 20-mesh sieve.

**Detection of Total Flavonoid and Short-chain Fatty Acid Contents**

The detection of the total flavonoid and terpene lactone contents as well as the content of the short-chain fatty acids (SCFA) in the fermented and nonfermented *G. biloba* samples was performed. The total flavonoid content was measured by HPLC with an evaporative light scattering detector (ELSD) according to the method described by van Beek and Montoro (2009). Ginkgolic acid and SCFA contents were measured by HPLC/UV.

The changes of the constituents before and after fermentation are shown in Table 1. All chemicals were purchased from Sigma–Aldrich (St Louis, MO), and all solvents were of chromatographic grade.

### Table 1. Changes in the composition of *G. biloba* leaves before and after sterilization and fermentation

<table>
<thead>
<tr>
<th></th>
<th>Sterilization</th>
<th>Fermentation</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before After</td>
<td>Before After</td>
<td></td>
</tr>
<tr>
<td>Total flavonoids (mg/g)</td>
<td>22.43 19.89</td>
<td>19.89 19.01</td>
<td>−0.88</td>
</tr>
<tr>
<td>Bilobalide (mg/g)</td>
<td>8.45 7.67</td>
<td>7.67 7.34</td>
<td>−0.33</td>
</tr>
<tr>
<td>Ginkgolide A (mg/g)</td>
<td>6.63 6.03</td>
<td>6.03 5.79</td>
<td>−0.24</td>
</tr>
<tr>
<td>Ginkgolide B (mg/g)</td>
<td>5.45 4.98</td>
<td>4.98 4.47</td>
<td>−0.51</td>
</tr>
<tr>
<td>Ginkgolide C (mg/g)</td>
<td>6.16 5.77</td>
<td>5.77 5.33</td>
<td>−0.44</td>
</tr>
<tr>
<td>Ginkgolic acid (mg/g)</td>
<td>7.44 1.16</td>
<td>1.16 0.24</td>
<td>−0.92</td>
</tr>
<tr>
<td>Acetate (mg/g)</td>
<td>0.23 0.23</td>
<td>0.23 15.32</td>
<td>15.09</td>
</tr>
<tr>
<td>Propionate (mg/g)</td>
<td>0.14 0.14</td>
<td>0.14 4.12</td>
<td>3.98</td>
</tr>
<tr>
<td>Butyrate (mg/g)</td>
<td>0.00 0.00</td>
<td>0.00 2.28</td>
<td>2.28</td>
</tr>
<tr>
<td>L-Lactate (mg/g)</td>
<td>0.00 0.00</td>
<td>0.00 32.34</td>
<td>32.34</td>
</tr>
</tbody>
</table>
Table 2. Nutritional composition of basal dry feed diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>1 to 21 d</th>
<th>22 to 35 d</th>
<th>Nutrient level</th>
<th>1 to 21 d</th>
<th>22 to 35 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>59.20</td>
<td>64.22</td>
<td>ME (MJ/kg)</td>
<td>12.30</td>
<td>12.60</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>31.36</td>
<td>24.69</td>
<td>Crude protein</td>
<td>20.07</td>
<td>20.30</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>0.50</td>
<td>0.40</td>
<td>Calcium</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>2.00</td>
<td>4.00</td>
<td>Available phosphorus (%)</td>
<td>0.45</td>
<td>0.40</td>
</tr>
<tr>
<td>Lard</td>
<td>2.03</td>
<td>2.83</td>
<td>Lysine (%)</td>
<td>1.13</td>
<td>1.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.10</td>
<td>1.27</td>
<td>Methionine (%)</td>
<td>0.50</td>
<td>0.44</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.39</td>
<td>1.27</td>
<td>Methionine + cysteine (%)</td>
<td>0.82</td>
<td>0.74</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.20</td>
<td>0.25</td>
<td>Threonine (%)</td>
<td>0.97</td>
<td>0.81</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.07</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Methionine</td>
<td>0.15</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin premix¹</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
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</tr>
<tr>
<td>Trace mineral premix²</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Provided per kilogram of diet: 24 mg vitamin A, 4,600 IU vitamin D3, 37.5 mg vitamin E, 6.0 mg vitamin K3, 1.3 mg thiamin, 7.5 mg riboflavin, 4.5 mg vitamin B6, 24 μg vitamin B12, 51 mg niacin, 400 mg choline chloride, 1.5 mg folic acid, 0.2 mg biotin, and 13.5 mg pantothenic acid.

²Provided per kilogram of diet: 37.5 mg Zn (as ZnSO₄), 37.5 mg Mn (as MnO₂), 37.5 mg Fe (as FeSO₄·7H₂O), 3.75 mg Cu (as CuSO₄·5H₂O), 0.83 mg I (as KI), and 0.3 mg Se (as Na₂SeO₃·5H₂O).

³The metabolic energy value of the diet was calculated according to NRC (1994) guidelines.

Experimental Design and Animal Husbandry

A total of 180 1-d-old healthy female commercial Arbor Acres chicks (body weight 42 ± 2 g) were purchased from a local commercial hatchery (Wens Food Groups, Guangdong, China). Chicks were randomly allocated into 3 treatment groups (60 chicks per group) with 3 replicates for each group: (1) control group (Cont.), fed a basal diet; (2) nonfermented group (NFG), fed a basal diet + 0.3% G. biloba mixture; (3) fermented group (FG), fed a basal diet + 0.3% fermented G. biloba mixture. The chemical composition of the basal feed is shown in Table 2. The experimental diets were formulated to meet National Research Council guidelines (NRC, 1994). Nutrient requirements are also shown in Table 2. All birds were placed into rearing isolators designed for chickens and housed in a room with the temperature maintained at 34°C–35°C for 3 d. The environmental temperature was then gradually reduced by 1°C every day until a final temperature of 25°C was reached. A 12-h light–dark cycle (0600 to 1800 h light) was used throughout the trial, and all broiler chickens had ad libitum access to feed and water. On the seventh day, Newcastle disease (ND) vaccination (live LaSota strain, period of validity: 4 wk) was conducted, and no other vaccine was used during the remainder of the trial period. The experimental design and procedures were approved by the Animal Care and Use of Committee of Southern China Agriculture University following the requirements of the Regulations for Administration of Affairs Concerning Experimental Animals of China. The rearing period was 5 wk.

Serum Sampling Procedure

Nine chicks per group were randomly selected for blood sampling on the seventh (before vaccination), 14th, 21st, 28th, and 35th day. Chicks were fasted for 12 h before blood sampling. Blood samples were kept at room temperature for 2 h before refrigeration at 4°C overnight. Then, the serum was separated by centrifugation at 3,500 rpm for 10 min at 4°C. After that, serum samples were collected into 1.5-mL Eppendorf tubes and frozen at −20°C for IL-18, IFN-γ, and antibody titer analysis.

IL and IFN ELISA Analysis and ND Antibody Titer Detection

The IL-18 and IFN-γ levels of all 3 groups were determined using a chicken IL-18 ELISA kit (TSZ ELISA, Waltham, MA) and a chicken IFN-γ ELISA kit (TSZ ELISA) according to the manufacturer’s instructions. ND antibody titer analysis was made using hemagglutination and hemagglutination inhibition test methods (Allan and Gough, 1974).

Defensin-related Gene Expression

After sacrificing the birds, the thymus, liver, spleen, small intestine (jejunum segment), cecum, and bursa of Fabricius (approximately 100 mg of each) were removed and washed with 0.1% diethylpyrocarbonate (DEPC)-treated water. All organ tissues were then immersed in 1 mL of RNA store reagent (Tiangen Inc., Beijing, China), kept at 4°C overnight, and then transferred to a −80°C deep freeze until required. Tissue collection was performed in 10 min. Total RNA isolation of the samples was conducted using an RNA isolation kit (Takara, Dalian, China) according to the manufacturer’s instructions. The concentrations and purities of the RNA preparations were determined spectrophotometrically by taking absorbance at 260 and 280 nm in a NanoDrop instrument. It was ensured that the 260/280 ratios of the samples were > 1.8. The concentration of each RNA sample was computed and then adjusted with RNase-free water. PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China) was employed for reverse transcription. RNA (2.0 μg each) isolated from each bird sample was added to a 25.0-μL reaction system. Cycle parameters...
for the reverse-transcription procedure were 1 cycle of 42°C, 2 min; 1 cycle of 4°C, 5 min; 1 cycle of 37°C, 15 min; 1 cycle of 85°C, 5 s. First-strand cDNA was synthesized and used as a template for real-time PCR or stored at −20°C for further use.

Oligonucleotide primers (Table 3) were designed, synthesized, and standardized for PCR conditions. The mRNA expressions of galectin (Gal)-1, Gal-5, Gal-6, Gal-9, Gal-10, and β-actin were measured in triplicate using the TaqMan real-time PCR system (ABI 7500FAST Sequence Detection System; Applied Biosystems, Carlsbad, CA) with specific TaqMan primers/probes (Applied Biosystems). Quantitative analysis of the PCR was performed according to the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The q-PCR was conducted using a SuperReal PreMix kit (Tiangen Inc., Beijing, China) with specific TaqMan primers/probes (Applied Biosystems). Quantitative analysis of the PCR was performed according to the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The q-PCR was conducted using a SuperReal PreMix kit (Tiangen Inc., Beijing, China). The reaction system (20 µL) contained 10 µL of SuperReal PreMix, 0.2 µL of the reference dye, 0.6 µL of primers (10 µmol/L of the sense primer and 10 µmol/L of the antisense primer), 0.3 µL of the probe, and 1 µL of the cDNA template. For the PCR reaction, the following experimental run protocol was used: denaturation program, 95°C for 1 min; amplification and quantification program, 95°C for 5 s, 60°C for 15 s, repeated 40 times. Amplification was performed using standard conditions, and the amount of target transcript was presented as a relative expression (2^{−ΔCT}) or fold induction (2^{−ΔΔCT}) in comparison to unstimulated controls after being normalized to the expression of β-actin as an endogenous reference.

Statistical Analysis

Data were analyzed by the one-way ANOVA procedure with SPSS 18.0 software (SPSS Inc., Chicago, IL). If a significant effect was observed, the significance between the treatments was identified by a least significant difference posthoc multiple comparisons test. Results were expressed as the treatment mean with their respective pooled SE. Relative gene expression data were analyzed by Student’s t test to measure significance. A probability value of p < 0.05 was taken to be statistically significant.

RESULTS

Composition of Fermented G. Biloba

The methods described by van Beek and Montoro (2009) were used to measure the total flavonoid contents in G. biloba samples before and after sterilization and fermentation, as well as the levels of the 3 main terpene lactones and ginkgolic acids. SCFA were also quantified by HPLC/UV, and the results are shown in Table 1. The sterilization and fermentation processes caused small reductions in the levels of flavonoids, bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C in the G. biloba leaves. In addition, the levels of acetate and L-lactate increased sharply after fermentation.
To evaluate the effect of nonfermented *G. biloba* leaves and fermented *G. biloba* on immune-related cytokines, serum IL-18 and IFN-γ levels were analyzed, and the results are shown in Table 4. After 7 d of treatment with nonfermented *G. biloba* leaves, the concentrations of serum IL-18 (\(p = 0.171\)) and IFN-γ (\(p = 0.06\)) were slightly lower compared to control group chickens, but the differences were not significant. In addition, the IL-18 (\(p = 0.018\)) and IFN-γ (\(p = 0.012\)) levels for the FG group were lower than the control group after 7 d. By the 14th day, the cytokines of all 3 groups had increased markedly, which may be a normal response to the ND vaccination. However, the concentrations of serum IL-18 and IFN-γ in both the *G. biloba*-treated groups were lower than those of the control group, and the cytokine levels of the treated groups were comparable.

Statistical analysis showed that there were significant differences between the NFG group and the control group and between the FG group and the control group with regards to the serum IL-18 and IFN-γ levels (\(p < 0.05\)); however, no significant differences were found between the NFG and FG groups. After 21, 28, and 35 d of treatment, the cytokines levels were similar to those of d 14 for the *G. biloba*-treated chickens (with no significant differences between the NFG and FG groups), and the serum IL-18 and IFN-γ levels remained lower than the control group (\(p < 0.05\)).

### ND Antibody Titer

To explore whether nonfermented and fermented *G. biloba* have an additive effect on immunity resulting from the ND vaccine, we compared the antibody titers of the 3 groups using hemagglutination and hemagglutination inhibition test methods. The results are shown in Fig. 1. At d 7 (the day of the ND vaccination), the antibody titers of the 3 groups were comparable, and no significant differences were observed. At d 14 (7 d after ND vaccination), the antibody titers of the 3 groups began to increase and reached a peak at d 21. The antibody titers for d 28 and 35 decreased in all 3 groups, and the decreases were most evident in the untreated control group. The antibody titers for d 21 and 28 in the 3 groups were comparable with no significant differences found between any 2 groups, although the antibody titers of both the NFG and FG group were slightly higher than the control group. However, the antibody titers of the NFG and FG groups at d 35 were comparable, and both were significantly higher than that of the control group.

### In Vivo Defensin Gene Expression

To find out whether or not *G. biloba* can increase the expression of defensins in vivo, we treated chickens with fermented and nonfermented *G. biloba* for 35 d and then extracted RNA from the thymus, liver, spleen, small intestine (jejunum segment), cecum, and bursa of Fabricius for real-time PCR analysis. The defensin expressions are shown in Fig. 2.
In thymus tissue, the expression levels of Gal-6 ($p = 0.032$) and Gal-9 ($p = 0.012$) for the NFG group were decreased compared to the control group. However, the expression levels of Gal-1, Gal-5, and Gal-10 showed no significant differences ($p > 0.05$) compared to the control group. On the other hand, the expression levels of Gal-6 ($p = 0.018$) and Gal-9 ($p = 0.022$) in the FG group were increased compared to the control group. Similarly, the expression levels of Gal-1, Gal-5, and Gal-10 for the FG group were slightly higher than those of the control group, although no significant differences were observed. The results also suggested that there were differences between the NFG and FG groups with regards to the expression of Gal-6 and Gal-9 (Fig. 2), with the NFG group showing decreased expression compared to the FG group.

In spleen tissue, the RNA expression levels of Gal-6 ($p = 0.043$), Gal-9 ($p = 0.031$), and Gal-10 ($p = 0.036$) for the NFG group were reduced compared to the control group. However, the expression level of Gal-1 ($p < 0.01$) for the NFG group was higher than that of the control group, while the levels of Gal-5 were comparable. Meanwhile, the expression levels of Gal-1 ($p = 0.037$), Gal-5 ($p = 0.003$), Gal-9 ($p = 0.017$), and Gal-10 ($p = 0.024$) of the FG group were higher than those of the control group; however, the RNA expression level of Gal-6 for the FG group was lower than that of the control group, albeit with no significant difference. Comparing the RNA expression levels of the NFG and FG groups, we found that the expression levels of all 5 defensins for the FG group were higher; however, significant differences were found only for Gal-5 ($p = 0.010$), Gal-9 ($p = 0.041$), and Gal-10 ($p = 0.021$; Fig. 3).

In the bursa of Fabricius, only the expression of Gal-1 ($p < 0.01$) for the NFG group was lower than that of the control group, and no significant differences were found between the NFG and control group for the other 4 defensins. The RNA expression levels of Gal-1 ($p = 0.009$), Gal-9 ($p = 0.004$), and Gal-10 ($p = 0.045$) for the FG group were higher than those of the control group, while the expression levels of Gal-5 and Gal-6 for the FG and control groups were comparable with no significant differences between them. As to the differences between the NFG and FG groups, the expression levels of Gal-1 ($p = 0.001$), Gal-9 ($p = 0.004$), and Gal-10 ($p = 0.031$) for the FG group were
higher than those of the NFG group, while the levels of Gal-5 and Gal-6 were comparable (Fig. 4).

In liver tissue, although the expression levels of all 5 defensins for the NFG group were not identical to those of the control group, no significant differences were observed. However, the expression level of Gal-9 ($p = 0.049$) was significantly different when the FG and control groups were compared. Similarly, the expression level of Gal-9 ($p = 0.023$) for the FG group was significantly higher than that of the NFG group (Fig. 5).

In small intestine tissue, the expression levels of all the assessed defensins for the NFG group showed no significant differences compared to the control group, with the exception of Gal-1 ($p = 0.037$), which were higher than the control group. On the contrary, the expression levels of Gal-1 ($p = 0.042$), Gal-5 ($p = 0.002$), Gal-9 ($p = 0.008$), and Gal-10 ($p = 0.029$) for the FG group were increased compared to the control group. Similarly, the expression levels of Gal-1 ($p = 0.042$), Gal-5 ($p = 0.002$), Gal-9 ($p = 0.003$), and Gal-10 ($p = 0.029$) for the FG group were also higher than those of the NFG group (Fig. 6).

In cecum tissue, the expression levels of all the assessed defensins for the NFG group showed no significant differences compared to the control group, with the exception of Gal-9 ($p = 0.018$), which was lower than the control group. Meanwhile, the expression levels of Gal-6 ($p = 0.028$) and Gal-9 ($p = 0.031$) for the FG group were higher than those of the control group. Furthermore, Gal-6 ($p = 0.024$) and Gal-9 ($p = 0.045$) expression levels were higher for the FG group than for the NFG group (Fig. 7).

DISCUSSION

According to an industry analysis report in 2013, over 4,000 tons of $G. bilo$ba leaves are produced in China each year. While some are used to manufacture the commercial $G. bilo$ba extract EGB 761, much is wasted. Thus, it is of interest to investigate additional uses for $G. bilo$ba to avoid its overproduction. Recent studies have suggested that $G. bilo$ba may exhibit immunostimulatory effects in vitro and in vivo. For example, Lee et al. (2011) found that an extract of $G. bilo$ba could decrease the sensitivity of monocytes to lipopolysaccharide, which suggested it may have potential applications in the clinical treatment of immune diseases. Meanwhile, Sochocka et al. (2010) indicated...
that G. biloba has beneficial effects on innate immunity, suggesting that G. biloba may be considered as a substitute for antibiotics in the animal healthcare industry.

Fermentation has been used as a traditional process to produce biological materials with health-promoting properties. Bacillus coagulans is generally recognized as a safe lactic acid bacterium, which is approved for use by the Food and Drugs Administration (FDA). It is characterized by its strong resistance to heat, acid, and bile salts, and it can also produce beneficial SCFA and lactic acid (Benson et al., 2012), which help regulate metabolism and the inflammatory response (Mortensen and Clausen, 1996; den Besten et al., 2013). Therefore, in this study, we investigated the effects of fermented and nonfermented G. biloba on broiler chickens by assessing defensin expression and serum IL-18 and interferon γ (IFN-γ) levels. We additionally evaluated the influence of G. biloba on antibody titers after Newcastle disease (ND) vaccination.

In the present study, SCFA (for example, acetate, propionate, and butyrate) and L-lactate increased after fermentation to different degrees. Furthermore, the ginkgolic acid content in G. biloba leaves dropped by 79.3% after fermentation. Such changes in fermented G. biloba leaves are favorable for the health of animals because SCFA can exert multiple beneficial effects on energy metabolism (den Besten et al. 2013, 2014) and innate immunity (Bentley-Hewitt et al., 2012), and ginkgolic acid is the main cause of adverse reactions to G. biloba leaves and may be linked to cytotoxicity, sensitization, mutagenic activity, carcinogenesis, liver toxicity, and kidney toxicity (Ahlemeyer et al., 2001; Hecker et al., 2002; Ahlemeyer and Kriegstein, 2003). Although the total flavonoids and terpene lactones of G. biloba leaves were decreased slightly after fermentation, the degree of this reduction was small. While previous studies have revealed that the biological effects of G. biloba leaves are mainly due to the flavonoids and terpene lactones, most studies have focused on the antitumor and anticancer effects of G. biloba, as well as its effects in angiocardioopathy and Alzheimer’s disease (McKenna et al., 2000; Christen and Maixent, 2002). However, the present study mainly focused on the effect of nonfermented and fermented G. biloba on the immunity of boiler chickens to evaluate its potential as a feed additive.

To this effect, serum IL-18 and IFN-γ levels were assessed and found to be comparable at d 7 (before ND vaccination) for all 3 groups. However, on d 14, the serum IL-18 and IFN-γ levels increased rapidly in all groups, and this was presumed to be due to the ND vaccination. It should be noted that, at d 14, the IL-18 and IFN-γ levels of the G. biloba-treated groups began to decrease and were lower compared to those of the control group. At d 28 and 35, the IL-18 and IFN-γ levels for the G. biloba-treated groups were also lower than those of the control group. IL-18 is known to induce IFN-γ production, which activates macrophages (He et al., 2011). Furthermore, He et al. (2011) demonstrated that IL-18 may fulfill its function through IFN-γ. The reason for this decline may be due to flavonoids and polysaccharides that can modulate and normalize the expression of IFN-γ and IL-18 (Hämäläinen et al., 2007; Sochocka et al., 2010). Some reports have already shown that G. biloba can inhibit the production of cytokines, including tumor necrosis factor (TNF-α), IL-2, IL-4, IL-6, IFN-α, and IFN-γ (Chandrasekaran et al., 2001; Cheng et al., 2003; Chen et al., 2012). In addition, some researchers have found that the activation of TNF-α, IL-6, and IL-18, and the activation of the nuclear factor (NF)-κB pathway can lead to inflammation cascade effects and tissue damage (Morriss et al., 1989; Neurath et al., 1997). The downregulation of these cytokines seems to be important for the prevention of cell apoptosis (Serrano-Garcia et al., 2013). The reason for the decreases seen in the levels of these cytokines following treatment with G. biloba may be due to a homeostatic mechanism involving the flavonoids and polysaccharides of fermented and nonfermented G. biloba for the maintenance of the Th1/Th2 balance in response to extracellular pathogens. Villaseñor-García et al. (2004) found that G. biloba extract can decrease elevated phagocytic functions through deactivation of IL-6 and NF-κB and delayed-type hypersensitivity reactions resulting in the production of antibodies. The result of the present study is consistent with previously reported results and suggest that G. biloba may produce its anti-inflammatory and beneficial effects on the immune system through the downregulation of IL-18 and IFN-γ expression.

The results of the ND antibody titer showed that the antibody levels of the control group and both treatment groups were comparable at d 7, 14, 21, and 28, which suggested that G. biloba does not have an effect on increased antibody levels after vaccination. However, the ND antibody titers for the NFG and FG groups were significantly higher than that of the control group after 35 d, which implied that G. biloba may enhance the effect of the ND vaccine. We presumed that this extending effect may involve the anti-apoptosis property of G. biloba as Ergun et al. (2005) previously showed that the apoptosis of human lymphocytes was inhibited by G. biloba extract. Similar findings have demonstrated that G. biloba-pretreated thymus cells can exhibit resistance to apoptosis (Tian et al., 2003). Furthermore, lymphocytes isolated from the spleens of aged mice showed decreased apoptosis...
after treatment with *G. biloba* for 2 wk (Schindowski et al., 2001). In addition, Zhang et al. (2006) found that T lymphocytes (CD3+) and T helper cells (CD4+), together with the CD4/CD8 ratio, in humans increased significantly after treatment with *G. biloba* for 12 wk.

Defensin is an important antimicrobial substance involved in the first line of host defense and can act on a broad spectrum of microorganisms by destroying their membrane integrity, resulting in lysis (Lazarev and Govorun, 2010). In the current study, the RNA expression levels of Gal-5, Gal-6, Gal-9, and Gal-10 for the NFG group showed tissue-dependent decreases compared to the control group. However, it seems that the expression level of Gal-1 was not affected by the use of nonfermented *G. biloba*. These results suggested that nonfermented *G. biloba* may prime certain tissues and downregulate levels of some antimicrobial defensins. The expression and regulation of defensins differ considerably in different tissues (van Dijk et al., 2008; Mukhopadhyaya et al., 2010), and the exact regulative mechanism is still not fully understood, especially in chickens. There are 2 known pathways that regulate the expression of defensins: the immune deficiency (IMD) pathway and the Toll-like receptor and NF-κB pathway. Some studies have shown that NF-κB regulates defensin expression at the transcriptional level and Sochocka et al. (2010) found that *G. biloba* extract can modulate the innate immunity of leukocytes by inhibiting NF-κB activation, which may partially help to explain how *G. biloba* suppresses the expression of defensins. In the present study, we also evaluated the effect of fermented *G. biloba* leaves on defensin expression, and the results showed that the expression levels of 5 defensins were upregulated to different degrees in a tissue-dependent manner, which suggested that treatment with fermented *G. biloba* could stimulate tissues with higher levels of antimicrobial defensins, potentially producing additional protection against infection by pathogenic bacteria. Probiotic metabolites, such as SCFA (e.g., acetate, propionate, and butyrate) and lactate, produced by *Bacillus coagulans* may bring beneficial effects to animals and humans. Benson et al. (2012) found that the probiotic metabolites of *Bacillus coagulans* may support the maturation of antigen-presenting cells, while other studies have shown that probiotics can induce the expression of defensins (Wehkamp et al., 2004; Shi, 2007). Furthermore, Bentley-Hewitt et al. (2012) found that SCFA (e.g., acetate, propionate, and butyrate) can induce human intestinal HT29 cells to increase human β-defensin 1 and 2 expression. Butyrate has been demonstrated to activate extracellular-signal-regulated kinases (ERK) and p38 mitogen-activated protein kinases (MAPK), which play a key role in activating defensin transcription (Ding et al., 2001; Yang et al., 2001). More recently, butyrate and propionate processes have shown the ability to induce antimicrobial peptide production (McBain et al., 1997; Schauber et al., 2004; Schwab et al., 2007). We suggest that the suppression effect of nonfermented *G. biloba* on defensin expression is attenuated by fermentation with *Bacillus coagulans* and, due to the SCFA (i.e., acetate, propionate, and butyrate) that were synthesized in *G. biloba* during the fermentation, fermented *G. biloba* may even promote the expression of defensins. The upregulated expression of defensins by fermented *G. biloba* in some tissues can directly improve innate immunity against invading pathogens, which is especially important in the gastrointestinal tract due to the constant exposure to a variety of microbial species, including commensals and pathogens (Wehkamp et al., 2007).

**Conclusions**

The results of the current study have implied that the fermentation of *G. biloba* with *Bacillus coagulans* may reverse its effect on the expression of defensins in chickens, which may result in enhanced protection against pathogenic infection. Furthermore, dietary *G. biloba* may exhibit an immune-regulation effect and antiapoptosis property in chickens due to the flavonoids and polysaccharides in *G. biloba* leaves, regardless of fermentation. Therefore, we suggest *G. biloba* as a potential feed additive that could have beneficial effects on the immunity status of broiler chickens. However, the regulation and control mechanism of *G. biloba* on innate and adaptive immunity have not yet been fully elucidated and require further study.

**LITERATURE CITED**


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