Effects of dietary Enterococcus faecium NCIMB 11181 supplementation on growth performance and cellular and humoral immune responses in broiler chickens

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ABSTRACT This study evaluated the effects of dietary Enterococcus faecium NCIMB 11181 on growth performance and immune response in broiler chickens. A total of 360 1-day-old Arbor Acres male birds were randomly assigned to 4 treatments that administered different dosages of E. faecium (0, 5 × 10^7, 1 × 10^8, and 2 × 10^8 CFU E. faecium/kg diet). The results revealed that average daily gain (ADG) changed quadratically, while feed conversion rate (FCR) increased linearly from day 22 to 35 and day 1 to 35 (P < 0.05). Supplementation of E. faecium at 5 × 10^7 CFU/kg diet resulted in increased ADG (P < 0.05) compared with the other groups. Birds fed with 2 × 10^8 CFU/kg E. faecium exhibited increased peripheral blood lymphocyte proliferation in response to concanavalin A (Con A) (P < 0.05) at day 35 and enhanced skin responses following phytohemagglutinin (PHA) injection (P < 0.05) at 12 h. Serum lysozyme activity at day 21 increased linearly with dietary E. faecium concentration (P < 0.05), the highest activity was observed in the 1 × 10^8 and 2 × 10^8 CFU E. faecium groups (P < 0.01). Serum levels of proinflammatory cytokines IL-1β, IL-2, IL-6, IFN-γ, and anti-inflammatory IL-4, IL-10 changed linearly or quadratically both at the initial and final phases (P < 0.05). In addition, BSA antibody titers were significantly increased following both primary and secondary inoculation when birds were fed with 1 × 10^8 or 2 × 10^8 CFU/kg E. faecium (P < 0.05). In comparison with other groups, birds received 5 × 10^7 CFU E. faecium exhibited the highest levels of serum IgG (P < 0.05) at day 35. Together, our results revealed that broiler diet supplemented with 5 × 10^7 CFU/kg E. faecium NCIMB 11181 was appropriate in relation to growth performance under normal conditions. Upon administration with higher dosages of E. faecium NCIMB 11181, obvious immune-stimulatory effects were observed following both cell-mediated and humoral immunity.

Key words: broiler, Enterococcus faecium, growth performance, immune response

INTRODUCTION Broilers are often exposed to multiple challenges during poultry production practices. These challenges include but are not limited to inadequate housing conditions, dietary toxins, pathogen infection, and feed withdrawal prior to slaughter or transportation. Additional issues can often occur as a result of compromised immune systems, disease susceptibility, inadequate growth performance, increased frequency of administration of antibiotics, carcass contamination, and pathogen carriage (Gomes et al., 2014; Tsiouris et al., 2015a,b). Administration of antibiotics at the early stages of a chick’s life can severely disrupt intestinal microbiota composition, resulting in a delay in immune system development and compromised immune function. These occurrences can render the chickens more prone to pathogen infections later in life (Simon et al., 2016). Therefore, it is of the utmost importance that we attempt to elucidate effective alternatives to antibiotics. Future treatment strategies should focus on immune system development and maturity, and enhanced innate immunity even in the absence of Antibiotics growth promoter (Jarosz et al., 2017). It has previously been reported that the early presence of beneficial microorganisms in the gastrointestinal tract (GIT) of broiler chicks facilitates resistance against pathogens by improving the health and integrity of the GIT. These microorganisms also help to improve both immune system function and growth (Cox and Dalloul, 2015). Thus, probiotics are considered viable immune stimulators.

Probiotics are live, non-pathogenic microorganisms that benefit host health and physiology by stabilizing the intestinal ecosystem. Probiotics also stimulate the immune system, modulate the immune response (Lyte,
Probiotic strains differ regarding the properties and clinical effects that they elicit; these differences are even observed when the strains belong to the same bacterial species (Vieira et al., 2013). E. faecium strain 11181 (isolated from pigs) is currently authorized by the EFSA Panel on Additives and Products or Substances used in Animal Feed as a supplement for fattening animals (Becquet, 2003, 2005; EFSA, 2005; Pajarillo et al., 2015). This strain has been shown to effectively increase daily weight gain and improve feed conversion in weaning piglets (Herzig et al., 2011), and consolidate the mucosal barrier (Fuller, 1989; Vanderpool et al., 2008; Ng et al., 2009). Enterococcus faecium is one of the first probiotic species approved by the EU and the FDA for animal feed (Franz et al., 2011). Previous studies have demonstrated that feed supplementation with E. faecium facilitates systemic and intestinal local mucosal immune responses (Benyacoub et al., 2003, 2005; Pollmann et al., 2005; Scharek et al., 2005; Szabo et al., 2009; Kreuzer et al., 2012b; Siepert et al., 2014). E. faecium has also been shown to increase the absorptive and secretory capacity of jejunal mucosa, improves intestinal barrier function, enhances disease resistance to pathogenic infection, prevents or treats diarrhea, and increases growth performances in pigs (Broom et al., 2006; Lodemann et al., 2006; Taras et al., 2006; Zeyn and Boldt, 2006; Scharek-Tedin et al., 2009; Mafamane et al., 2011; Bednorz et al., 2013; Klingspor et al., 2013; Wang et al., 2014; Kreuzer-Redmer et al., 2016). Furthermore, the addition of E. faecium to pig has displayed to modify intestinal bacterial communities by increasing the prevalence of beneficial bacteria and reducing pathogenic bacteria load. (Taras et al., 2006; Vahjen et al., 2007; Starke et al., 2013). Results from poultry experiments have revealed that supplementation of the diet with E. faecium improves growth performance and modulates intestinal microflora composition (Vahjen et al., 2002; Chichlowska et al., 2007; Mountzouris et al., 2007; Samli et al., 2007, 2010; Awad et al., 2008; Capcarova et al., 2010b; Luo et al., 2013). This supplementation also results in the regulation of intestinal mucosal immune responses and enhanced chicken resistance to intestinal pathogen infection including infections caused by Salmonella and Escherichia coli (Carina Audisio et al., 2000; Luo et al., 2013; Bobikova et al., 2015; Karaffova et al., 2015). E. faecium has also been shown to alter anti-oxidant status by exerting anti-oxidant properties (Capcarova et al., 2010b; Luo et al., 2013), influencing the density of the intestinal mucin layer (Levkut et al., 2012), changing blood biochemical parameters (Capcarova et al., 2010b), and regulating intestinal and hepatic proteome profiles (Luo et al., 2013; Zheng et al., 2016).

Animal Ethics Statement

All study procedures were approved by the Animal Care and Use Committee of China Agricultural University and were in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). All efforts were made to minimize the suffering of the animals.

Birds, Housing and Experimental Design

A total of 360 male 1-day-old Arbor Acres broiler chicks were randomly assigned to 4 dietary treatment groups each receiving a different dosage of E. faecium (NCIMB 11181, viable count ≥ 1 × 10^9 CFU/g; manufactured by Probiotics International Ltd. Co., UK). Each treatment group consisted of 6 replicate cages with 15 birds per replicate pen. Dietary treatments consisted of a control basal diet without E. faecium supplementation, and basal diets containing 5 × 10^7, 1 × 10^8, or 2 × 10^8 CFU/kg E. faecium, respectively. An antibiotic-free commercial basic diet was prepared first according to the National Research Council (NRC, 1994) requirements for starter (day 1 to 21) and grower (day 22 to 42) periods. The composition of the basal diet and nutrient levels is presented in Table 1. The experimental feed was formulated by mixing the basal diet with 50, 100, or 200 g of E. faecium per ton to reach 5 × 10^5, 1 × 10^6, or 2 × 10^6 CFU E. faecium/kg of diet. To ensure the homogeneity of the additives, approximately 5 kg of the basal diet was thoroughly mixed with the additive using a plastic bucket. The feed samples were taken and the E. faecium number was counted by using plate counts method after maintaining cultures in Sodium azide-cysteine-violet-esculin agar (CM 1507, Beijing Land Bridge Technology Co., LTD) at 37°C for 24 h under an anaerobic environment to ensure the probiotic dosages were performed correctly. The chicks were reared on net floor cages in a closed and ventilated house. Each pen had a floor space of 11,200 (160 × 70) cm^2 and was equipped with a separate feeding trough. Water was supplied through nipple drinkers. Water and feed were provided ad libitum. In accordance with the AA Broiler Management Guide, all
Calculated nutrient levels

<table>
<thead>
<tr>
<th>Items</th>
<th>1 to 21 d</th>
<th>22 to 35 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition (%)</td>
<td>25.5</td>
<td>24.7</td>
</tr>
<tr>
<td>Corn (7.8% CP)</td>
<td>54.6</td>
<td>60.7</td>
</tr>
<tr>
<td>Soybean meal (46% CP)</td>
<td>37.4</td>
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<tr>
<td>Soybean oil</td>
<td>3.52</td>
<td>3.28</td>
</tr>
<tr>
<td>Limestone-calcium carbonate</td>
<td>1.11</td>
<td>1.15</td>
</tr>
<tr>
<td>Calcium hydrogen phosphate</td>
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<td>1.63</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>DL-Methionine (98%)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>L-Lysine HCL (98%)</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Vitamin premix 2</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Mineral premix 3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Choline chloride (50%)</td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Calculated nutrient levels 4

Metabolizable energy (Kcal/kg) 2.950 3.000
Crude protein(%) 21.0 19.1
Calcium (%) 1.0 0.9
Available phosphorus (%) 0.48 0.40
Lysine (%) 1.15 1.04
Methionine (%) 0.50 0.41

1 Diets were in mash form.
2 Vitamin premix provided per kg of complete diet: vitamin A (retinylacetate), 9,500 IU; vitamin D3 (cholecalciferol), 2,500 IU; vitamin E (DL-a-tocopherol acetate), 30 IU; vitamin K3 (menadione sodium bisulfate), 2.65 mg; vitamin B12 (cyanocobalamin), 0.025 mg; biotin, 0.30 mg; folic acid, 1.25 mg; nicotinic acid, 50 mg; d-pantothenic acid, 12 mg; pyridoxine hydrochloride, 6.0 mg; riboflavin, 6.5 mg; thiamine mononitrate, 3.0 mg.

3 Mineral premix provided per kg of complete diet: iron, 80 mg; copper, 8 mg; manganese, 100 mg; zinc, 80 mg; iodine, 0.35 mg; selenium, 0.15 mg.

4 Calculated value based on the analysis of experimental diets.

chicks received continuous light for the first 24 h, and were then maintained under a 23-h light/1-h dark cycle for the remainder of the study. The room temperature was maintained at 33 to 34°C on the first 3 d, and then gradually decreased by 2°C/wk until a final room temperature of 22 to 24°C reached. The relative humidity was kept at 60 to 70% during the first week and then 50 to 60% thereafter.

Sampling and Measurement

Growth Performance. At 21 and 35 d of age, feed and birds were weighed by pen to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR). All pens were checked on a daily basis for deaths.

Relative Lymphoid Organ Weights. At 21 and 35 d of age, 6 healthy chickens per treatment (1 per replicate) were randomly chosen from each treatment group. Heparinized blood samples were collected from the wing vein and then diluted 1:1 with sterile calcium and magnesium-free Hank’s balanced salt solution (CMF-HBSS, Sigma). The diluted samples were placed on ice and then carefully layered into a tube containing an equal volume of Ficoll lymphocyte separation medium (Histopaque-1077, Tianjin HaoYang Biological Manufacture Co., Ltd., China) to form a distinct layer above the Ficoll. Following centrifugation at 400 × g for 30 min at room temperature, the white flocculent material on the interface between the plasma and the lymphocyte separation medium was transferred to a clean tube using a sterile transfer pipette. The lymphocyte suspension was washed 3 times with RPMI 1640 (Invitrogen Corp., Grand Island, NY) incomplete culture medium and then resuspended in 2 mL of RPMI 1640 complete culture medium supplemented with 5% (vol/vol) fetal calf serum, 0.5% penicillin (final concentration, 100 U/mL), 0.5% streptomycin (final concentration, 100 mg/mL), and 1% N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid (HEPES, final concentration, 24 mM; Amresco 0511, Amresco Inc., Cleveland, OH). The live cells were detected using atrypan blue dye exclusion technique and a microscope (DM6000B, Leica Microsystems, Germany). Cell suspensions were diluted to a final concentration of 1 × 10^7 cells/mL in RPMI 1640 medium for subsequent analysis.

Peripheral Blood Lymphocyte Proliferation. A 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) assay was used to determine the peripheral blood lymphocyte proliferation response. Briefly, 100 μL of PBMC suspension and 100 μL of RPMI 1640 in the absence or presence of 90 μg/mL concanavalin A (Con A; C2613, Sigma Chemical, Co.) or 50 μg/mL lipopolysaccharide (L3129, Sigma Chemical, Co.) were added into a 96-well microtiter plate (Costar 3599, Corning, Inc., Corning, NY). The cultures were set up in triplicate. After a 68-h incubation in a 5% CO2 incubator (MCO-18AIC CO2 incubator, Sanyo Electric Biomedical Co. Ltd., Tokyo, Japan) at 39°C, MTT was added to each cell culture to give a final concentration of 5 mg/mL. The cells were incubated for a further 4 h, and then 100 μL of 10% sodium dodecyl sulfate dissolved in 0.04 mol/L HCl solution was added to each well to lyse the cells and solubilize the MTT crystals. Finally, the absorbance value of each sample was read using an automated ELISA reader (model 550 Microplate Reader, Bio-Rad Pacific Ltd., Hong Kong, China) at 570 nm. Stimulation index (SI) for each sample was calculated based on the following formula: SI = (absorbance value of mitogen-stimulated cells)/(absorbance value of media without mitogen).

T Cell Sub-Populations in PBMC. The percentage of CD3^+^, CD4^+^, and CD8^+^ in peripheral blood lymphocyte samples was analyzed by flow cytometry as previously described (Fair et al., 2008; Jarosz
et al., 2017). Briefly, the primary monoclonal antibodies, IgG1κ mouse anti-chicken-CD3-SPRD-labelled antibody (8200–13), IgG1κ mouse anti-chicken-CD4-FITC-labeled antibody (8210–02), and IgG1κ mouse anti-chicken-CD8-RPE-labeled antibody (8220–09) (Southern Biotechnology Associates Inc., Birmingham, AL), were diluted in PBS (pH 7.2). A volume of 100 μL of PBMC (2 × 10⁶ cells) was added into a 1.0 mL Eppendorf tube; the contents of the tube were stirred with 25 μL of diluted primary monoclonal antibody (1:100 dilution) as well as the negative isotype control IgG (mouse IgG1κ-SPRD, mouse IgG1κ-FITC, and mouse IgG1κ-R-PE). After incubation for 45 min at room temperature, cells were washed twice with cold PBS and centrifuged for 30 min at 1,800 g to remove unbound primary antibodies. A total of 300 μL of hemolysin solution diluted in PBS (1:25) was added to each tube. Finally, cells were washed twice and brought to a final volume of 500 μL. Three-color flow cytometric analysis was conducted using a Coulter XL (Beckman Coulter Corp., Fullerton, CA) at Xi-Yuan Traditional Chinese Medicine Hospital, Chinese Academy of Medicine Science, China. The percentages of CD3+ T, CD4+ T, and CD8+ T cells in PBMC were subsequently calculated.

**PHA Skin Test.** The PHA skin test for cell-mediated immune response was conducted in 35-day-old chickens following previous published procedures (Sullivan and Erf, 2017). In brief, 50 μL containing 100 μg PHA (PHA-P, Sigma-Aldrich L1668, diluted in sterile saline (0.9%) to a final concentration of 2,000 μg/mL PHA-P) were injected intradermally with a 30-G needle into the right wattle of each bird. Same volume of sterile saline (0.9%) was simultaneously administered into the left wattle. Thickness of the wattle was measured prior to injection, 12 and 24 h after injection, using a vernier caliper with 0.01-mm precision (Peacock, Ozaki MFG. CO. Ltd, Tokyo, Japan). A SI was calculated as the change in the thickness of the PHA-injected wattle minus the change in thickness of the saline-injected side. Twelve birds per group (2 birds from each replicate pen) were randomly chosen for the PHA skin test.

**Serum Cytokines and Lysozyme Activity Analysis.** Blood samples were collected via the wing vein for each treatment group on day 21 and 35, respectively. The concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) in serum samples were determined with commercially available chicken cytokine ELISA kits (Genorise Scientific Inc, Paoli) according to the manufacturer’s instructions. For each assay, a control recombinant chicken cytokine sample was diluted over the recommended detection range to generate a standard curve, and the linearity was calculated using Excel with R² = 0.99. Sample concentrations were interpolated from the standard curve. Serum lysozyme activity was measured with commercial kits according to the manufacturer’s instructions (Jian Cheng Bioengineering Institute, Nanjing, China).

**Serum NDV-Specific Antibody Titers.** Antibody titers against Newcastle disease virus (NDV) were determined by ELISA using a Newcastle Disease Virus Antibody Test Kit from IDEXX laboratories Inc. (NDV Ab, IDEXX, US). The birds were vaccinated against NDV and infectious bronchitis virus (IBV) via eye drops on day 8 (primary immunization) and day 21 (booster immunization), respectively. Blood samples were collected via the wing vein for each treatment group on day 21 and 35, respectively. Serum samples were prepared and frozen at −30°C until further required for the associated assays. The samples were diluted (1/500 dilutions) and the operation was performed in accordance with the specifications supplied in the test kit. The plates were read by an ELISA plate reader (SpectraMax i3x Platform, Molecular Devices, LLC, Australia) at 650 nm. The results were calculated and shown as S/P values.

**Serum Total IgG**

On day 21 and 35, 6 healthy chickens were randomly chosen from each treatment group. Blood samples were collected from the wing vein and centrifuged at 3,000 × g for 10 min at 4°C. The serum was stored at −30°C until required for the assay. Serum levels of total IgG were quantified using a Chicken IgG ELISA Quantitation Kit (Cat. No. E30–104, Lot No.E30–104–30, Bethyl Laboratories, Inc.). The serum samples were diluted 1:125,000. The plates were read via an ELISA plate reader (SpectraMax i3x Platform, Molecular Devices, LLC) at 450 nm, and serum antibody concentrations were calculated using Gen 5 software.

**Serum Antibody Titers to BSA**

Two birds were selected from each treatment group and injected in the thigh muscle with 1 mL of 1% BSA (Roche 738,328, Roche, Basel, Switzerland) at day 14 and 24. Blood samples were collected from the wing vein of immunized birds on the 5th, 10th, and 15th day after primary and secondary immunization. The samples were analyzed for anti-BSA antibody titers with indirect ELISA as previously described (Parmentier et al., 2004). Briefly, 96-well plates were coated with 2 mg/mL BSA and incubated overnight at 4°C. After 5 washes with 0.01 M PBS-Tween (pH 7.4, 0.05% Tween 20), the plates were incubated with serum (1:100 dilution) at 37°C for 1.5 h. The specific IgG used to bind BSA was diluted 1:120,000 according to the specification peroxidase-labeled polyclonal antibodies against chicken IgG (Bethyl Laboratories, Inc.). The plates were subsequently rinsed and 100 μL of secondary antibody was added and incubated for 30 min at 37°C. A total of 100 μL of 0.05% tetramethylbenzidine was added after washing and the plates were
incubated for 30 min at room temperature in the dark. The reaction was terminated with 2 M sulfuric acid. The resultant color was measured at 450 nm using an ELISA plate reader (SpectraMax i3x Platform, Molecular Devices, LLC) after comparison with the negative control.

Statistical Analysis

All data were analyzed by 1-way analysis of variance using the general linear model procedure of SPSS 17.0 software. Linear and quadratic effects were tested and considered significant at $P < 0.05$, and $P$ value between 0.05 and 0.10 was classified as a tendency. Differences among the 4 treatment groups were compared using Duncan’s multicomparison test. Results were considered significant at the $P < 0.05$ level, and $P$ value between 0.05 and 0.10 was classified as a tendency.

**RESULTS**

**Growth Performance**

As shown in Table 2, ADG changed quadratically, whereas FCR increased linearly with increasing dietary levels of *E. faecium* from day 22 to 35 and over the entire experimental period ($P < 0.05$). Supplementation of the diet with *E. faecium* at a dosage of $5 \times 10^7$ CFU/kg resulted in higher ADG levels ($P < 0.05$) from day 22 to 35. An increase in ADG levels ($P = 0.091 < 0.1$) was also observed over the whole testing period compared with the control; however, no significant difference was observed among the probiotics-treated groups. In addition, the same dosage significantly improved FCR ($P < 0.05$) compared with the highest dosage *E. faecium*-treated group; however, there was no apparent difference between the highest dosage *E. faecium*-treated, the untreated control, and the $1 \times 10^8$ CFU/kg groups. ADFI from day 22 to 35 and day 1 to 35 was quadratically affected ($P = 0.056 < 0.1$, $P = 0.057 < 0.1$) by *E. faecium* levels, and the highest ADFI values were observed in the lowest dosage *E. faecium*-treated group. There was no notable difference in the mortality rate among the different groups ($P > 0.05$).

**Lymphoid Organ Index**

The data presented in Figure 1 indicated that different supplemental levels of *E. faecium* did not affect spleen relative weight at either 21 d or 35 d ($P > 0.05$).

**Cellular Immune Responses**

As shown in Figure 2, on day 21, the peripheral blood lymphocyte responses to the mitogen Con A and lipopolysaccharide (LPS) were not affected ($P > 0.05$) by the concentration of *E. faecium* in the diet ($P > 0.05$). However, at 35 d of age, supplementation with $2 \times 10^8$ CFU/kg of *E. faecium* enhanced peripheral blood lymphocyte proliferation responses to Con A ($P < 0.05$) and LPS ($P = 0.069 < 0.1$) compared with control; however, there was no significant difference among the different *E. faecium*-treated groups.

Figure 3 revealed the percentage of lymphocytes expressing CD3$^+$, CD4$^+$, and CD8$^+$ in the different groups. Different supplemental levels of *E. faecium* did not affect the percentage of T lymphocytes (expressing CD3$^+$), helper T cells (expressing CD4$^+$), cytotoxic T cells (expressing CD8$^+$), and the average ratio of CD4$^+$ T cells to CD8$^+$ T cells in the peripheral blood both at day 21 and 35 ($P > 0.05$). However, an increasing tendency in the percentage of the CD8$^+$ T-cell subpopulation was observed on day 35 in the group that were

### Table 2. Effects of dietary *E. faecium* on growth performance of broiler chickens.

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatment, CFU/kg</th>
<th>Statistic</th>
<th>SEM²</th>
<th>P-value</th>
<th>Linear</th>
<th>Quadratic</th>
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<tbody>
<tr>
<td>ADG, g</td>
<td>0</td>
<td>5 $\times$ $10^7$</td>
<td>1 $\times$ $10^8$</td>
<td>2 $\times$ $10^9$</td>
<td></td>
<td></td>
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<tr>
<td>Day 1 to 21</td>
<td>26.9</td>
<td>28.4</td>
<td>27.8</td>
<td>27.2</td>
<td>0.433</td>
<td>0.642</td>
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<tr>
<td>Day 22 to 35</td>
<td>96.7$^{a}$</td>
<td>102.3$^{b}$</td>
<td>99.1$^{a,b}$</td>
<td>97.3$^{a,b}$</td>
<td>0.802</td>
<td>0.045</td>
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<td>Day 1 to 35</td>
<td>54.8</td>
<td>58.0</td>
<td>56.3</td>
<td>55.2</td>
<td>0.490</td>
<td>0.091</td>
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<td>ADFI, g</td>
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<td>5 $\times$ $10^7$</td>
<td>1 $\times$ $10^8$</td>
<td>2 $\times$ $10^9$</td>
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</tr>
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<td>Day 1 to 21</td>
<td>40.7</td>
<td>42.6</td>
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<td>41.3</td>
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<td>0.736</td>
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<td>Day 22 to 35</td>
<td>157.3</td>
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<td>162.5</td>
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<td>2 $\times$ $10^9$</td>
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<tr>
<td>Day 1 to 21</td>
<td>1.513</td>
<td>1.500</td>
<td>1.512</td>
<td>1.521</td>
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<tr>
<td>Day 22 to 35</td>
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<td>1.618$^{a}$</td>
<td>1.650$^{b}$</td>
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<td>1.583$^{a}$</td>
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<td>1.626$^{b}$</td>
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<td>Mortality, %</td>
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<td>2 $\times$ $10^9$</td>
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<td>1.111</td>
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<td>2.222</td>
<td>1.111</td>
<td>0.518</td>
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<td>Day 1 to 35</td>
<td>1.111</td>
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<td>2.222</td>
<td>1.111</td>
<td>0.518</td>
<td>0.540</td>
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</table>

$^{a,b}$Means in the same column without common superscripts differ significantly ($P < 0.05$).

ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio = g of feed intake/g of BW gain, g/g.
Figure 1. Effect of dietary *E. faecium* on spleen relative weight of broiler chickens. The spleen relative weight was represented as organ weight (g) to BW (kg) based on 6 birds per treatment. Individual data points are presented as box plots, showing median (horizontal lines), the lower and upper quartiles (lower and upper borders of the boxes), and minimum and maximum values (lower and upper whiskers).

Figure 2. Effect of dietary *E. faecium* on peripheral blood lymphocyte proliferation of broiler chickens. Peripheral blood lymphocyte obtained at 21 d (A) or 35 d (B) post hatch were stimulated with concanavalin A (ConA) or lipopolysaccharide (LPS), and the stimulation index (SI) was calculated as described in the Materials and Methods section. Individual data points (n = 6) per each treatment are presented as box plots, showing median (horizontal lines), the lower and upper quartiles (lower and upper borders of the boxes), and minimum and maximum values (lower and upper whiskers). Different small letters on the bars indicated significant difference (*P* < 0.05).

PHA skin test results showed that the wattle thickness index at 12 and 24 h post-stimulation linearly increased (*P* < 0.05) with increasing levels of *E. faecium* in the diet (Figure 4). The birds fed with $2 \times 10^8$ CFU *E. faecium*/kg feed showed the strongest SI compared to the control group (*P* < 0.05) at 12 h after injection and a tendency at 24 h injection (*P* = 0.081 < 0.1).
Figure 3. Effect of dietary E. faecium on T-cell subpopulations in peripheral blood. Peripheral blood lymphocyte obtained at 21 d (A) or 35 d (B), and the subpopulation of T cell was calculated as relative cell counts (%) as described in the Materials and Methods section. Individual data points (n = 6) per each treatment are presented as box plots, showing median (horizontal lines), the lower and upper quartiles (lower and upper borders of the boxes), and minimum and maximum values (lower and upper whiskers).

**Serum Lysozyme Activity**

As shown in Figure 5, serum lysozyme activity on day 21 was linearly increased when the dietary E. faecium level was elevated. Moreover, greater serum lysozyme activity was observed in the 1 × 10^8 and the 2 × 10^8 CFU/kg E. faecium groups compared with the control group (P < 0.001). On day 35, compared with the other groups, the 2 × 10^8 CFU E. faecium group exhibited increased serum lysozyme activity (P = 0.085 < 0.1).

**Concentration of Serum Cytokines**

The data are presented in Table 3. At day 21, the proinflammatory cytokines INF-γ, IL-1β, IL-2, IL-6 and the anti-inflammatory cytokine IL-10 increased quadratically with increasing doses of E. faecium (P < 0.05). The results revealed that the probiotic supplementation resulted in higher serum IL-1β compared with the untreated birds (P < 0.05). IFN-γ and IL-6 levels significantly increased in the birds supplemented with 5 × 10^7 and 1 × 10^8 CFU E. faecium/kg diet compared with the control group (P < 0.05). Moreover, E. faecium addition stimulated the anti-inflammatory cytokines IL-10 production compared with the control, the highest concentration was observed in birds fed with 5 × 10^7 CFU E. faecium/kg diet. At day 35, serum proinflammatory cytokine IL-β and anti-inflammatory cytokine IL-4 levels were affected linearly with the dosage of E. faecium (P < 0.05) whereas IL-2 and the Th1/Th2 immune response indicator INF-γ/IL-4 ratio exhibited a quadratic relationship with the E. faecium dosage (P < 0.05). About the proinflammatory cytokines, higher IL-1β was observed in the birds fed with 2 × 10^8 CFU/kg E. faecium compared with the other 3 groups (P < 0.05). The serum IL-2 level in the birds fed with 1 × 10^8 CFU E. faecium/kg diet was significantly higher than that in the low and high dosage groups but no notable difference was displayed when compared with the control group. The middle and high dosage groups exhibited significantly increased serum INF-γ and IL-6 levels compared to the control and 5 × 10^7 CFU/kg group (P < 0.05). About the anti-inflammatory cytokines, all birds treated with E. faecium showed a notable increase in the serum IL-4 and IL-10 concentrations compared with the control.
Moreover, these 2 cytokines production were further enhanced when the broilers supplemented with $2 \times 10^8$ CFU *E. faecium*/kg diet.

**Antibody Production**

Figures 6 and 7 revealed the influence of the different doses of *E. faecium* on the serum levels of total IgG and NDV antibodies in broiler chickens. The highest IgG concentration was observed in birds receiving $5 \times 10^7$ CFU *E. faecium*/kg of diet ($P < 0.05$). NDV antibody titers at day 35 exhibited a quadratic relationship with increasing concentrations of *E. faecium* ($P < 0.05$). Birds raised on a diet supplemented with $1 \times 10^8$ CFU *E. faecium* showed numerically ($P = 0.081 < 0.10$) higher antibody titers at 35 day of age compared with the other groups. Anti-BSA antibody titers (Table 4) in 5 d after primary immunization were significantly increased in birds fed $1 \times 10^8$ and $2 \times 10^8$ CFU/kg of *E. faecium* compared with birds fed on the control diet ($P < 0.01$). At 15 d after secondary immunization, we observed an increase in the anti-BSA antibody titer in the $1 \times 10^8$ CFU *E. faecium* group compared with the other groups ($P < 0.05$).

**DISCUSSION**

Probiotic supplementation represents an interesting option that is currently being explored as a means of improving performance, enhancing immune competence, and reducing the amount and severity of enteric diseases in the poultry industry (Franz et al., 2011). In this study, we investigated the effects of dietary *E. faecium* NCIMB 11181 levels on growth performance and immune responses in broiler chickens. The results showed that ADG levels exhibited a quadratic relationship, while FCR exhibited a linear relationship ($P < 0.05$) with increasing levels of *E. faecium* in the diet in both grower and whole phases. Supplementation of the diet with *E. faecium* at a dosage of $5 \times 10^7$ CFU/kg resulted in higher ADG levels and improved FCR compared with the other dosage groups. In a
Table 3. Effect of dietary E. faecium on serum cytokines of boiler chicken.

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatment, CFU/kg</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5 × 10^7</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>26.02a</td>
<td>34.04b</td>
</tr>
<tr>
<td>IL-2, pg/mL</td>
<td>89.54</td>
<td>100.86</td>
</tr>
<tr>
<td>IL-4, pg/mL</td>
<td>61.02</td>
<td>65.46</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>58.48b</td>
<td>70.61bc</td>
</tr>
<tr>
<td>IL-10, pg/mL</td>
<td>60.04a</td>
<td>84.28c</td>
</tr>
<tr>
<td>INF-γ, pg/mL</td>
<td>44.46a</td>
<td>56.58b</td>
</tr>
<tr>
<td>INF-γ/IL-4</td>
<td>0.73</td>
<td>0.87</td>
</tr>
<tr>
<td>Day 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>39.06a</td>
<td>42.86a</td>
</tr>
<tr>
<td>IL-2, pg/mL</td>
<td>110.20ab</td>
<td>102.51a</td>
</tr>
<tr>
<td>IL-4, pg/mL</td>
<td>55.72a</td>
<td>69.58b</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>64.18a</td>
<td>70.04bc</td>
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<td>IL-10, pg/mL</td>
<td>62.35a</td>
<td>81.15c</td>
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<tr>
<td>INF-γ, pg/mL</td>
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<td>69.58b</td>
</tr>
<tr>
<td>INF-γ/IL-4</td>
<td>0.80</td>
<td>0.66</td>
</tr>
</tbody>
</table>

*a–cMeans in the same column without common superscripts differ significantly (P < 0.05).

IL-1β = interleukin-1β; IL-2 = interleukin-2; IL-4 = interleukin-4; IL-6 = interleukin-6; IL-10 = interleukin-10; INF-γ = interferon-γ.

Figure 6. Effect of dietary E. faecium on serum total IgG in broiler chickens. Individual data points (n = 6) per each treatment are presented as box plots, showing median (horizontal lines), the lower and upper quartiles (lower and upper borders of the boxes), and minimum and maximum values (lower and upper whiskers). Different small letters on the bars indicated significant difference (P < 0.05).

previous study, Samli et al (2007) observed that inclusion of E. faecium positively affected growth performance by increasing weight gain and improving the FCR in broiler chickens. Other researchers have reported that feeding a mixture of probiotics or symbiotics containing E. faecium elicited beneficial effects on both body weight and body weight gain in broiler chickens (Mountzouris et al., 2007; Awad et al., 2008). However, other studies showed that there was no generally significant promotion in relation to growth performance in non-infected birds (Luo et al., 2013) and non-infected pigs (Broom et al., 2006; Taras et al., 2006). These inconsistent results might be attributable to differences in properties of E. faecium strain to be used, inclusion dosage and timing, feed composition and digestibility, and health status of birds. In this study, the improvement in ADG by E. faecium was possibly attributed to increases in the number of lactic acid bacteria or alternative of intestinal mucosal structure thereby enhancing nutrient absorption (Samli et al., 2007).

Serum lysozyme activity was one of the indicators to assess the nonspecific immunity (Kim et al., 2012). Our results showed that serum lysozyme levels increased linearly with increases in dietary E. faecium levels, suggesting that E. faecium is capable of boosting nonspecific immune-competence in broiler chickens. The spleen is a peripheral lymphoid organ full of different types of lymphocytes. This organ plays an important role in the systemic immune response. In this study, dietary supplementation with E. faecium did not appear to effect spleen organ weight in the broiler chickens. Similar results were reported by Samli et al. (2007). In contrast, Luo et al. (2013) found that E. faecium supplementation promoted the development of immune organs in birds. The difference in results in relation to immune
organ relative weight could be related to the probiotic dosage used, the properties of the *E. faecium* strain used, the duration of feeding with experimental diets, or the animal health status.

In the current study, cell-mediated immune competence in the birds was assessed by measuring the relative prevalence of peripheral blood T lymphocytes subsets, lymphocyte proliferation response to mitogen Con A or LPS, and PHA skin test levels. Our findings indicated that the addition of $5 \times 10^7$ CFU/kg *E. faecium* 11181 to the diet resulted in a slight increase in the proportion of CD8$^+$ T cells in broilers. Similarly, several previous studies reported that supplementation with *E. faecium* showed increasing of the relative prevalence of CD8$^+$ cytotoxic T cells in the peripheral blood or gut tissue in pigs or broilers that were infected (Levkut et al., 2012; Kreuzer et al., 2012b; Wang et al., 2014). CD8$^+$ T cells, the cytotoxic T cells, are essential in immune protection against intracellular pathogens (Erf, 2004; Zhang and Bevan, 2011). Thus, a slight increase in the percentage of blood CD8$^+$ T cells possibly implied that *E. faecium* 11181 could induce lymphocyte differentiation and enhance anti-infection capacity in broilers. However, other studies have indicated that *E. faecium* administration had no clear effect or showed a significant reduction on CD8$^+$ T cell levels in the gut and/or blood of both healthy piglets and piglets challenged with *Salmonella* (Scharek et al., 2005; Mafamane et al., 2011; Kreuzer et al., 2012a). The inconsistent results might be related to the properties of *E. faecium* strain to be used, different tissues, challenged or not. Further studies need to be conducted to clarify why *E. faecium* induce lymphocytes to differentiate into more cytotoxic T cells.

Continuous feeding with high levels of *E. faecium* enhanced Con A-induced peripheral blood lymphocyte proliferation in broiler chickens. This result is in accordance with data published by Levkut et al. (2012), which suggested that *E. faecium* supplementation augmented T-cell proliferation. We also observed that birds fed diets supplemented with $2 \times 10^8$ CFU/kg *E. faecium* exhibited an improved wattle response to PHA. Furthermore, an increase in skin thickness after PHA stimulation suggested a stronger local immune system and increased resistance to disease. Both of these studies suggest that *E. faecium* boosts lymphocyte activity.

Measuring cytokine production is an integral part of evaluating the cell-mediated immune response (Siebert 2015).
and Walker, 2010). Cytokines can generally be divided into 2 types: proinflammatory (IL-1β, IL-2, IL-6, IFN-γ, and TNF-α) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β). Proinflammatory cytokines are essential for the development and functioning of both the innate and adaptive immune response. These immune cells eliminate pathogens from the host, while their overexpression is associated with pathological conditions of the immune system and potentially deleterious effects on host health and growth (Smith and Humphries, 2009). Anti-inflammatory cytokines are involved in reduced inflammatory response, immune tolerance, antibodies synthesis, and disease susceptibility (O’Garra and Vieira, 2007). In our study, *E. faecium* supplementation significantly increased the levels of serum cytokines, including IL-1β, IL-6, IL-10, and IFN-γ, over the whole experimental period. Both IL-2 and IL-4 were found to be more prevalent at the finisher phase; however, the ratio of Th1-type (IFN-γ) to Th2-type (IL-4) remained unaltered over the duration of the experiment. Therefore, modulation of the expression of proinflammatory and anti-inflammatory cytokines and other immune mediators may be an important mechanism in relation to the immunomodulatory effects of the *E. faecium* strain NCIMB 11181. Similarly, previous studies have demonstrated that *E. faecium* AL41 is capable of stimulating the expression of multiple cytokines such as TGF-β and IL-17 in the intestine of chickens challenged with *Salmonella enteritidis* (Karaffova et al., 2015). Additionally, cytokine production was promoted following *E. faecium* supplementation in animals (Tarasova et al., 2010; Mansour et al., 2014) or macrophages (Choi et al., 2012; Rho et al., 2017). However, other researchers have reported that piglets fed with *E. faecium* NCIMB 10415 exhibited significant reductions in the relative mRNA expression levels of IFN-γ, IL-8/CXCL8, IL-10, and the co-stimulatory molecule CD86 (Siepert et al., 2014). It is still unclear whether *E. faecium* induces immune stimulation in broilers. Indeed, many factors including the strain of probiotic used, the dosage and administration strategy, and the challenge status of chickens may affect potential immune modulation. Therefore, further studies were needed to conclusively elucidate the mechanisms that underpin the immunomodulatory effects of cytokines following probiotic administration in poultry. Together, our findings suggest that oral administration of *E. faecium* NCIMB 11181 promotes cell-mediated immune competence by enhancing T-cell proliferation and cytokine secretion, but not affecting the balance between Th1 and Th2 immune responses.

In broilers, humoral immunity could be assessed by determining serum antibody titers in primary and secondary responses to NDV vaccine, intramuscular injection of 1% BSA, or intramuscular injection of 7% sheep red blood cells (Fairbrother et al., 2004). Numerous studies have shown that probiotic supplementation enhances broiler humoral immune response by increasing the levels of immunoglobulin (Haghighi et al., 2005, 2006; Brisbin et al., 2011; Yang et al., 2012; Salim et al., 2013). In this study, the biggest increase in total serum natural IgG was observed in birds that were fed 5 × 10⁷ CFU *E. faecium*/kg diet. This result reflected the immunomogenicity of probiotics and suggested that *E. faecium* was capable of elevating natural antibody synthesis in the sera of chickens. Based on the finding in other species that natural antibodies were important for defense against pathogens (Ochssein et al., 1999; Baumgarth et al., 2005), it was possible that the effects of *E. faecium* bacteria in reducing colonization of intestinal pathogens may be due partly to the stimulation of natural antibodies. At the same time, we also found that the antibody titers against NDV quadratically increased with concomitant increases in dietary *E. faecium*. Furthermore, birds fed with 1 × 10⁸ CFU *E. faecium*/kg diet exhibited the highest anti-NDV antibody titers compared with other groups. Higher specific antibody titers to NDV induced by *E. faecium* correlated with increased protection from NDV infection. In addition, results from the current study revealed that birds fed diets supplemented with 1 × 10⁸ and 2 × 10⁸ CFU/kg *E. faecium* had higher serum anti-BSA antibody levels after primary and secondary immunization compared with the control group. The increased antigen response to thymus-dependent antigen BSA suggested that administration of *E. faecium* was beneficial to immunocompetence. Similar findings in earlier studies had indicated that dietary supplementation with different strains of *E. faecium* resulted in the stimulation of nonspecific or specific antibody production in the blood or intestinal content of birds (Talebi et al., 2008; Levkut et al., 2012; Karaffova et al., 2015) and piglets (Szabo et al., 2009; Wang et al., 2014). In accordance with the alterations in cytokine levels, we speculated that increased humoral immunity in *E. faecium*-fed chickens could be attributed to the activation and maturation of resident intestinal dendritic cells. This phenomenon could theoretically stimulate the activation and differentiation of different subsets of immune system cells, leading to the production of Th2 cytokines, such as IL-4, IL-10, and transforming growth factor β. Importantly, all of these cytokines are important for antibody production and isotype switching (Di Giacinto et al., 2005).

In conclusion, based on our findings, *E. faecium* addition conferred obvious immunomodulatory effects on the chicken immune system by regulating both cell-mediated and humoral immune competence in broiler chickens. In general, considering the results of the growth and slightly immune response, we suggest that supplementing the broiler diet with 5 × 10⁷ CFU/kg *E. faecium* NCIMB 11181 under non-challenged rearing conditions gives rise to improved growth performance. We also recommend that the optimal dose for immune stimulation is 1 to 2 × 10⁸ CFU/kg of diet. However, the mechanisms that underlie the immunomodulatory and protective effects elicited by *E. faecium* NCINB 11181 in relation to chicken health remain to be elucidated.
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Authors’ contributions: ZW designed the research; YW, WZ, and YG performed the research and analyzed the data; YW wrote the manuscript; ZW and YG participated in the revision of the manuscript. All authors contributed to the data interpretation and approved the final version of the manuscript.

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