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Dietary *Allium hookeri* reduces inflammatory response and increases expression of intestinal tight junction proteins in LPS-induced young broiler chicken

Youngsub Lee[a,1], Sung-hyen Lee[b,1], Ujvala Deepthi Gadde[a], Sung-taek Oh[a], Sung-jin Lee[c], Hyun S. Lillehoj[a,*]

[a] *Animal Bioscience and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD, USA*
[b] *National Institute of Agricultural Sciences, Rural Department Administration, 166, Nongsaengmyeong-ro, Isoe-Myeon, Wanju-Gun, Jeollabuk-do, South Korea*
[c] *College of Animal Life Sciences, Kangwon National University, Chuncheon 200-701, South Korea*

1. Introduction

There is increasing interest in optimizing feed efficiency and growth rate during commercial poultry production. Indeed, poultry meat production has been greatly increased with the introduction of antibiotic growth promoters (AGP). However, the appearance of antibiotic-resistant bacteria and the associated increasing concern for food safety have demonstrated that there is a need to develop viable alternatives that can ensure optimal animal health and performance. The exact mechanism of action of antibiotic growth promoters is currently unknown (Lillehoj and Lee, 2012). Therefore, in depth research to define antibiotics mechanism of action, and finding safe alternative additives are required. Regarding poultry, most efforts has been focused on plant derived nutrients and organic acids (Hassan et al., 2010; Bravo et al., 2014).

Allium hookeri (AH), a member of the Alliaceae family, subgenera *Amerallium*, is found in Greece, southern China (Yunnan Province), Bhutan, Sri Lanka, and India, and it has been used by locals to treat coughs and colds and to heal burn and wounds (Sharma et al., 2011). Other members of this family include green onion, garlic, and onion, which contain abundant organo-sulfur compounds that have been reported to have anti-oxidant, anti-cancer, anti-coagulation, anti-cholesterol, and antibacterial activities (Welch et al., 1992; Keusgen, 2002; Banerjee and Maulik, 2002; Hsu et al., 2004; Vazquez-Prieto and Miatello, 2010; K.H. Kim et al., 2012). *In vitro* studies conducted previously in our laboratory (Lee et al., 2016) showed that an ethanol extract of AH activated poultry innate immunity. Briefly, AH increased the proliferation of chicken spleen lymphocytes, and it inhibited the growth of tumor cells in a dose-dependent fashion; it was also shown to induce NO secretion by chicken macrophages.

Chickens challenged with lipopolysaccharide (LPS) are a good model to study inflammation and stress, and these models have been characterized widely (De Boever et al., 2009; Kaiser et al., 2012). Indeed, LPS present in the cell wall of Gram-negative bacteria induces the activation of nuclear factor κB (NF-κB), a transcription factor present in macrophages and mononuclear cells that induces the expression of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), as well as the production of inflammatory mediators (Guzik et al., 2003). LPS also affects gut tight junctions (TJ) proteins. In contrast, intestinal permeability and barrier function are regulated by TJ proteins, and they are down-regulated by LPS and tumor necrosis factor-α (Park et al., 2010). Thus far, few studies have assessed the effects of AH on the early phase of the immune response and intestinal integrity maintenance in chickens. The present study was conducted with the aim of evaluating the inflammatory response modulation and TJ gene expression induced by LPS in chickens fed a diet supplemented with different dosages of AH root or fermented root, compared with non-supplemented controls. At 24 h post LPS-challenge, body weight gain was monitored and α-1-acid glycoprotein (α-1-AGP) levels in sera were detected by an enzyme-linked immunosorbent assay (ELISA). The levels of pro-inflammatory cytokines (interleukin (IL)-1b, IL-6, IL-8, tumor necrosis factor superfamily member 15 (TNFST15), and LPS-induced tumor necrosis factor-α factor (LITAF), as well as tight junction proteins (junctional adhesion molecule 2 (JAM2), Zonula occludens-1 ZO1, and occludin), and intestinal mucin 2 (MUC2) were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) to demonstrate the anti-inflammatory effect of AH as a feed additive.

2. Materials and methods

2.1. Preparation of A. hookeri supplement

All samples (AH root and fermented root) used in this study were

* Corresponding author at: Animal Bioscience and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, Beltsville, MD 20705, USA.
E-mail address: hyun.lillehoj@ars.usda.gov (H.S. Lillehoj).
[1] Equal contribution.

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verified based on a morphological assessment, and they were obtained from the Agricultural Development & Technology Center (Sunchang, South Korea). A voucher specimen (RDAAH15) was preserved at the National Institute of Agricultural Sciences (Jeonju, South Korea). Fermented AH root was provided by the Center for Healthcare Technology Development, Chonbuk National University (Chonbuk, South Korea). Briefly, *Saccharomyses cerevisiae* strain MG111 was used in the fermentation process. Dried AH root was soaked in cultured enzyme at room temperature for 24 h. Later on, first fermentation was carried out in a fermenter for 3 days at 45 °C. The fermentation process was repeated three times.

All verified samples were freeze dried (PVTFD 10R; Ilsin Lab, Yangju, South Korea), pulverized in a 40-mesh grinder (FM909T; Hanil Precision Ind., Co., Ltd., Wonju, South Korea), and stored at − 75 °C until use.

2.2. Experimental design and LPS challenge

A total of 150 male Ross 708 broiler chickens (1-day-old) were purchased from Longenecker's Hatchery, Elizabethtown, PA, USA, and assigned randomly to six groups, each containing 25 birds. Each group was divided into five cages (n = 25/group, five cages/treatment). Birds were housed in a Petersime starter brooder unit with an automatically temperature controlled environment. Feed and water were provided *ad libitum*. One group was fed an antibiotic-free, starter diet (Table 1) containing 23.5% (dry matter basis) crude protein (control). Other groups were fed a basal starter diet supplemented with either AH roots or fermented root powders at concentrations of 1% or 5% between d 1 and 8. In this randomized design experiment, cages were used as replicate units. Experimental protocol and procedures were approved by the Small Animal Care Committee of the Beltsville Agricultural Research Center (BACUC, ARS, BARC-East).

Experimental design diagram was shown in Fig. 1. One hundred and twenty-five chickens in each dietary treatment (n = 5; 25 birds/cage) were injected intraperitoneally with *Escherichia coli* O55:B5 LPS (Sigma–Aldrich, St. Louis, MO, USA) at 1 mg/kg body weight, and 25 birds were injected with sterile phosphate-buffered saline (PBS) as a negative control. LPS dosage determination was based on previous reports (Leshchinsky and Klasing, 2001; Matson et al., 2005). Body weight was measured individually on days 7 and 8 (0 and 24 h post-LPS injection, respectively) and later on, average body weight gain was calculated per cage.

Table 1
The ingredient and nutrient composition of the basal diet.

Ingredients (g)	Basal
Corn	557.8
Soybean meal	370.3
Soybean oil	29.7
Dicalcium phosphate	18.0
Calcium carbonate	15.1
Salt	3.8
Poultry vit mix[a]	2.2
Poultry mineral mix[b]	1.5
DL-Methionine	1.0
Choline-chloride, 60%	0.6
Total	1000
Calculated values (DM basis, %)	
CP (g/kg)	235
Ca (g/kg)	12.0
Avail. P (g/kg)	50.1
Lys (g/kg)	14.0
Met (g/kg)	4.90
Cys + Met (g/kg)	8.00
TMEn, kcal/kg	3450

[a] Vitamin mix provided the following (per kg of diet): thiamin·mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; DCa pantothenate, 12 mg; vitamin B_{12} (cobalamin), 12.0 μg; pyridoxine·HCL, 4.7 mg; D-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 μg; trans-retinyl acetate, 1892 μg; allrac α tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.

[b] Trace mineral mix provided the following (per kg of diet): manganese ($MnSO_4 \cdot H_2O$), 60 mg; iron ($FeSO_4 \cdot 7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 0.15 mg; selenium (NaSe03), 0.3 mg.

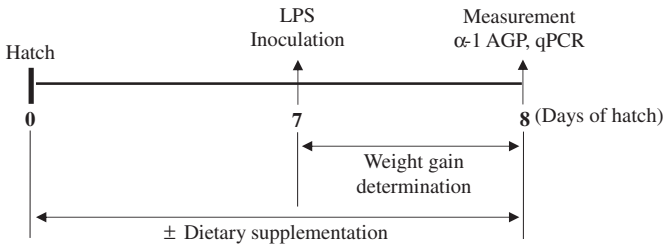

Fig. 1. Experimental design diagram. Chickens were fed diets non-supplemented or supplemented with AH root (1, 5%) or fermented root (1, 5%) and challenged with LPS or PBS at 7 days. At 24 h post-LPS challenge, α-1 AGP level in serum was measured by ELISA and gene expression level in intestine were measured by qRT-PCR. Body weight were measured individually at 7 day and 8 day (0, 24 h post LPS-challenge) as shown in the diagram. And later on, average body weight gain was calculated per cage.

2.3. Sample collection

Four chickens were selected randomly from each group at 24 h post-LPS injection, and used for collecting blood and small intestine samples. Blood samples (four birds/treatments) without anticoagulants were collected by cardiac puncture immediately following euthanasia, and sera were separated by centrifugation at 12,000 × *g* for 20 min at 4 °C. Intestinal (ileum) sections were collected and stored in RNA preservation buffer (RNAlater®, Applied Biosystems, Foster City, CA, USA) at − 20 °C until further use for the isolation of RNA and a gene expression analysis.

2.4. Serum α-1-AGP ELISA

Acute phase protein (α-1-AGP) levels in serum were measured using an ELISA kit (Life Diagnostics Inc., West Chester, PA, USA) according to the manufacturer's instructions. Briefly, 10,000-fold diluted samples were placed in the wells of a microtiter plate with immobilized anti-chicken α-1-AGP antibodies and incubated for 45 min. Following incubation, the plates were washed and treated with horseradish peroxidase-conjugated anti-chicken α-1-AGP antibodies and incubated for 45 min, which was followed by color development with a substrate. The optical density (OD) was determined at 450 nm using an automated microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). OD values were converted to concentrations (ng/mL) based on a standard curve generated using known quantities of recombinant α-1-AGP standard protein.

2.5. RNA extraction and qRT-PCR

Total RNA was isolated from the jejunum samples in RNAlater® using TRIzol (Invitrogen, Carlsbad, CA, USA). Briefly, jejunum samples were washed using ice-cold Hank's Balanced Salt Solution (Sigma–Aldrich) to remove the gut contents and the RNAlater®. Approximately 50 mg of the tissue was homogenized in 1 mL of TRIzol reagent using a handheld rotor-stator homogenizer (TissueRuptor; Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA pellets were washed once with 75% ethanol, air dried, and dissolved in RNase-free water. The RNA concentration was determined using a NanoDrop (ND-1000) spectrophotometer (NanoDrop Products, Wilmington, DE, USA) by measuring the absorbance at 260 nm, and the purity was checked by measuring the OD_{260}/

Table 2
Oligonucleotide primer used for quantitative RT-PCR.

Type	Target gene	Primer sequence[a] (5′-3′)	PCR product size (Kb)
Reference	GAPDH	F-GGTGGTGCTAAGCGTGTTAT R-ACCTCTGCCATCTCTCCACA	264
Proinflammatory	IL1β	F-TGGGCATCAAGGGCTACA R-TCGGGTTGGTTGGTGATG	244
	IL6	F-CAAGGTGACGGAGGAGGAC R-TGGCGAGGAGGGATTTCT	254
	IL8	F-GGCTTGCTAGGGGAAATGA R-AGCTGACTCTGACTAGGAAACTGT	200
	IL17F	F-TGAAGACTGCCTGAACCA R-AGAGACCGATTCCTGATGT	117
	TNFSF15	F-CCTGAGTATTCCAGCAACGCA R-ATCCACCAGCTTGATGTCACTAAC	292
TJ proteins	Occludin	F-GAGCCCAGACTACCAAAGCAA R-GCTTGATGTGGAAGAGCTTGTTG	68
	ZO1	F-CCGCAGTCGTTCACGATCT R-GGAGAATGTCTGGAATGGTCTGA	63
	JAM2	F-AGCCTCAAATGGGATTGGATT R-CATCAACTTGCATTCGCTTCA	59
Mucin	MUC2	F-GCCTGCCCAGGAAATCAAG R-CGACAAGTTTGCTGGCACAT	59

[a] F = forward primer; R = reverse primer.

OD_{280} ratio. Total RNA (1 μg) was reverse transcribed using the StrataScript first-strand synthesis system kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. Oligonucleotide primers for chicken pro-inflammatory cytokines, including IL-1b, IL-6 IL-8, TNFSF, LITAF, and intestinal tight junction proteins, including JAM2, occludin, ZO1, and MUC2, as well as glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as a housekeeping control, are listed in Table 2. The primer information for the TJ- and MUC2-enoding genes was adapted from Chen et al., 2015. Amplification and detection were conducted using the Agilent Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA) and the Brilliant SYBR Green qRT-PCR Master Mix (Stratagene). Each sample was analyzed in triplicate under the following PCR conditions: denaturation at 95 °C for 10 min, followed by amplification at 72 °C for 1 min for 40 cycles. Standard curves were generated using log10 diluted standard RNA to calculate the amplification efficiency, and the levels of individual transcripts were normalized to those of GAPDH by the Q-gene program (Muller et al., 2002). To normalize individual replicates, the logarithmic-scaled cycle threshold (Ct) values were transformed to linear units of normalized expression before calculating the means and standard errors of the mean (SEM) for the references and individual targets, followed by determination of mean normalized expression using the Q-gene program.

2.6. Statistical analysis

A statistical analysis was calculated using SPSS software ver. 22.0 for Windows (IBM-SPSS, Inc., Chicago, IL, USA). All data were expressed as the mean ± SEM for each treatment, and significant differences between the control and dietary treatment were determined by one-way analysis of variance with a Duncan multiple test. A probability (P) value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of dietary supplementation with AH on average body weight gain

Body weight gain was measured at d 7 and at 24 h post-LPS injection (8 d of age). No chickens showed any clinical abnormalities during the experimental period, and the effects of dietary AH treatment on broiler body weight gain are presented in Fig. 2. LPS challenge significantly reduced average body weight gain at 24 h post-injection, compared with chickens injected with PBS. Among the chickens injected with LPS, birds fed diets supplemented with 1% AH fermented root showed significantly increased ($P < 0.05$) body weight gains compared with LPS-injected chickens fed basal diets.

3.2. Effect of dietary supplementation with AH on serum α-1-AGP levels

Chickens serum α-1-AGP concentrations at 24 h post-LPS injection are shown in Fig. 3. LPS injection substantially increased the levels of α-1-AGP in serum, and within the LPS-injected chickens, all chickens fed diets supplemented with root and fermented root exhibited lower α-1-AGP levels, except chickens fed a diet supplemented with 1% AH root, compared with the LPS-injected chickens fed a basal diet. However, those decreased levels were higher than that of the PBS control.

3.3. Effect of dietary supplementation with AH on cytokine transcript levels

The IL-1b, IL-8, TNFSF15, and LITAF transcript levels in the intestine were higher in LPS-injected chickens fed the non-supplemented diet, compared with chickens that were given a PBS injection (Fig. 4). Among the LPS-injected groups, IL-1b and LITAF expression levels were significantly lower in chickens fed diets supplemented with AH root or fermented root. Especially, chickens fed diets supplemented with 1% AH root and 5% fermented root exhibited lower IL-1b expression levels than chickens that received PBS. TNFSF15 expression was lower in chickens fed a diet supplemented with fermented root (1% and 5%), and only the chickens that were fed 1% fermented root showed lower expression levels of IL-8. The IL-6 level did not differ significantly between groups that were challenged with LPS or PBS.

3.4. Effect of dietary supplementation with AH on intestinal tight junction proteins and mucin expression in jejunum

The expression profiles of tight junction proteins (JAM, occludin, and ZO1) and mucin (MUC2) in the jejunum are shown in Fig. 5. LPS challenge significantly reduced the expression levels of junction proteins (JAM, occludin, and ZO1) and mucin (MUC2) in chickens fed the non-supplemented diet, compared with chickens that received a PBS injection. Among the LPS-challenged groups, the occludin expression level was significantly higher in the chickens fed diets supplemented with all the AH treatments (root and fermented roots), and the occludin level of chickens that were fed 5% fermented root was as high

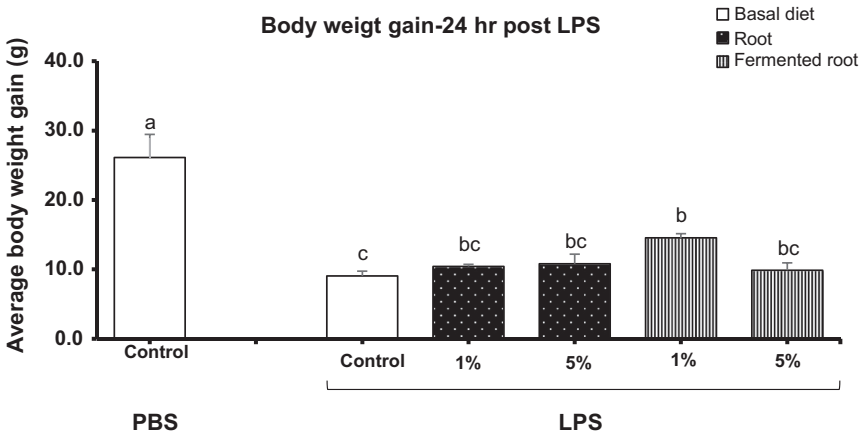

Fig. 2. Effects of dietary *A. hookeri* root or fermented root on body weight gain in chicken at 24 hrs-post LPS injection. Average body weight gain of broilers (24 h after LPS challenge) fed diets non-supplemented with *A. hookeri* root (1, 5%) or fermented root (1, 5%) and challenged with LPS or PBS at 7 days of age. The data were analyzed by one-way ANOVA and mean were separated using Duncan's multiple range test. Each bar represents the mean ± SEM (n = 4) and ^{a,b,c} mean values with unlike letters were significantly different ($P < 0.05$).

as that of the PBS control group. Only chickens fed a diet supplemented with 1% fermented root exhibited increased JAM2 expression, and no difference was found in the ZO1 levels. Interestingly, MUC2 expression was also significantly higher in chickens fed a diet supplemented with 1% fermented root.

4. Discussion

The present study was conducted to investigate whether dietary supplementation of AH would influence growth performance, inflammatory immune activities in the intestine, and intestinal barrier function during the immunological stress induced by LPS injection.

The results presented here showed significantly greater body weight gain in the chickens fed a diet supplemented with 1% fermented AH, compared with LPS-injected chickens fed a basal diet, at 24 h post-LPS-challenge. In a previous study, Roh et al. (2016) reported the beneficial effect of AH on the body weight of diabetic rats. According to the report, streptozotocin-induced diabetic rats showed a marked decrease in body weight, which was compensated significantly by dietary AH feeding. However, unlike in the rats, AH did not significantly increase body weight in type II diabetic mice (Kim et al., 2015). The differences between our results and those of previous studies could be attributable to the differences in animal species or experiment methods. Therefore, further research is needed to validate the effect of AH.

α-1-AGP is an acute phase protein, which means that its concentration is elevated during inflammation. In chickens, several studies have used α-1-AGP as a marker for systemic non-specific inflammation or gut barrier health (Adler et al., 2001; Peebles et al., 2014). In the present study, the serum α-1-AGP concentration was > 5-fold higher following LPS injection, compared with that of the PBS-injected group. Compared with the PBS-injected group, chickens fed diets supplemented with 5% AH root and fermented root (1% and 5%) exhibited significantly lower serum α-1-AGP levels. Both 5% AH root and 5% fermented AH root showed similar effects.

Several studies have demonstrated the inhibitory effect of AH on LPS-induced pro-inflammatory cytokines in mice. C.H. Kim et al. (2012) reported that a methanol extract of AH root significantly inhibited LPS-induced nitric oxide (NO) formation in a dose-dependent manner, and it decreased cytokine production (TNF-α and IL-6) in LPS-stimulated RAW264.7 macrophages. In another experiment, a 70% AH root ethanol extract was used to treat LPS-stimulated RAW264.7 cells, and similar results were obtained. The 70% AH root ethanol extract inhibited the production of IL-1β and NO, and it induced the production of heme oxygenase-1 (HO-1), the expression of which is known to prevent cell damage and apoptosis (Bae and Bae, 2012). Similar reductions in the levels of pro-inflammatory cytokines were observed in our study. LPS injection substantially increased the IL-1β, IL-8, TNFSF15, and LITAF transcript levels, compared with those of chickens that were given PBS, and among the LPS challenge groups, chickens fed diets supplemented with AH root or fermented root exhibited lower

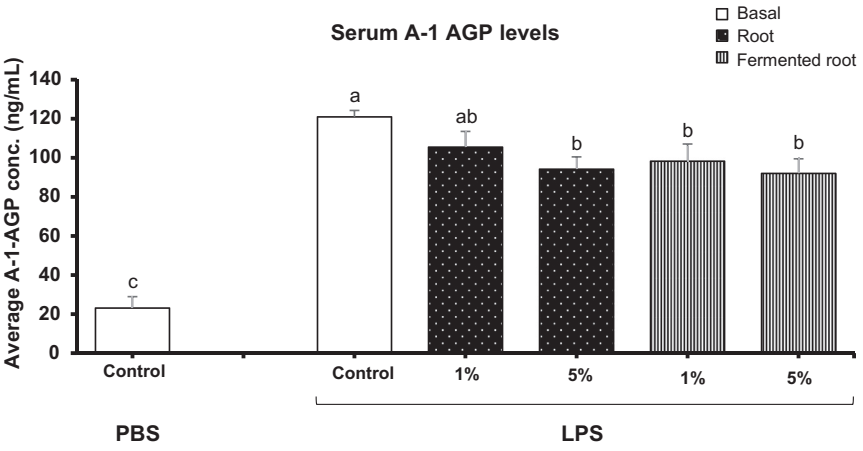

Fig. 3. Effects of dietary *A. hookeri* root or fermented root on the α-1-AGP levels in chicken sera at 24 h-post LPS injection. α-1-AGP concentration in the serum of chickens (n = 4) fed diets non-supplemented or supplemented with *A. hookeri* root (1, 5%) or fermented root (1, 5%) and challenged with LPS or PBS at 7 days of age. Serum samples were collected 24 h post-LPS injection. The data were analyzed by one-way ANOVA and mean were separated using Duncan's multiple range test. Each bar represents the mean ± SEM (n = 4) and ^{a,b,c} mean values with unlike letters were significantly different ($P < 0.05$).

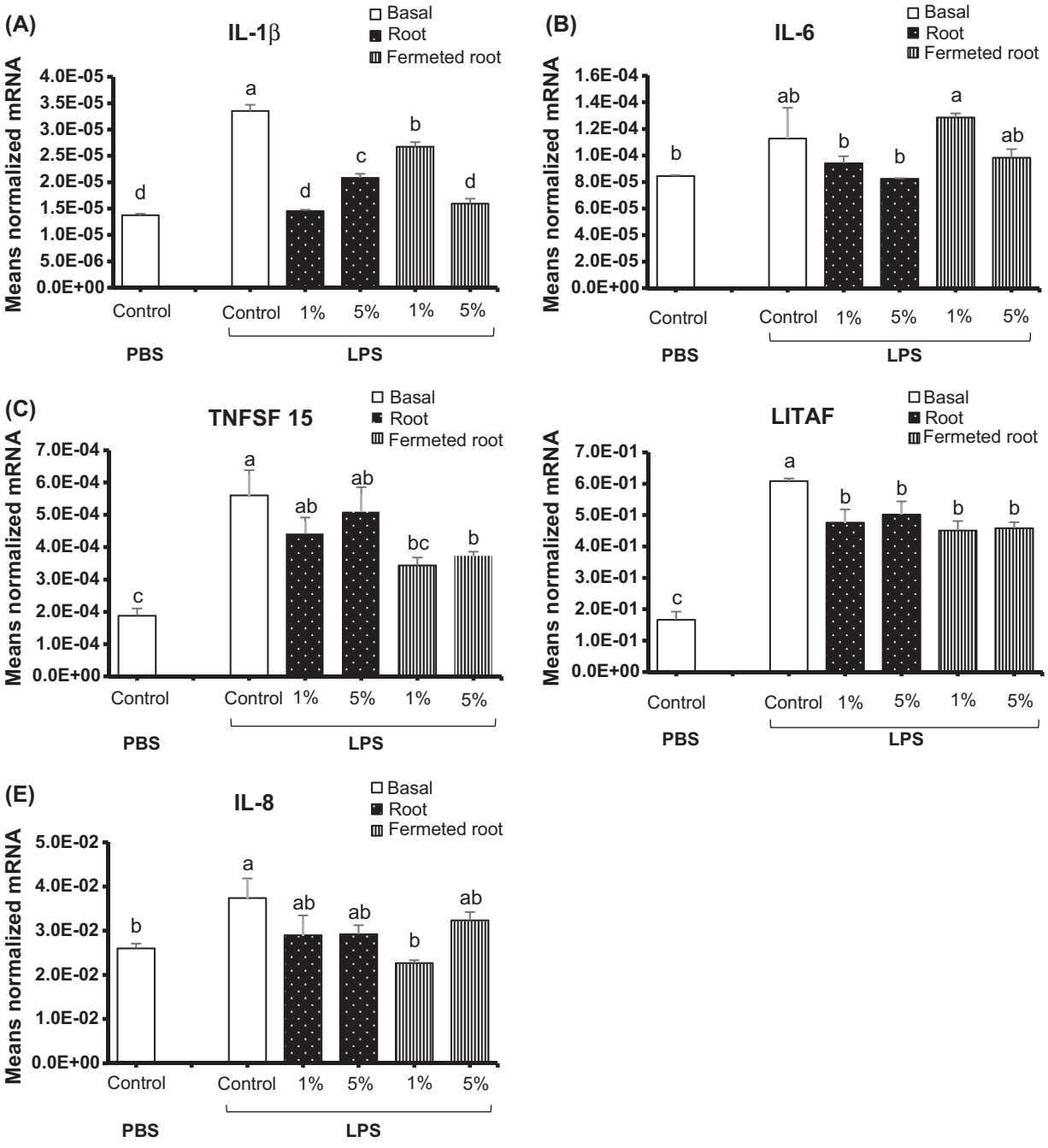

Fig. 4. Effects of dietary *A. hookeri* root or fermented root on the levels of transcripts of intestinal tight junction proteins and mucin. Chickens were fed diets non-supplemented or supplemented with *A. hookeri* root (1, 5%) or fermented root (1, 5%) and challenged with LPS or PBS at 7 days of age. Transcript levels of IL-1b, IL-6, IL-8, TNFSF15 and LITAF in the jejunum were measured by quantitative RT-PCR and normalized to GAPDH transcript levels. The data were analyzed by one-way ANOVA and mean were separated using Duncan's multiple range test. Each bar represents the mean ± SEM (n = 4) and ^{a,b,c} mean values with unlike letters were significantly different ($P < 0.05$).

transcript levels compared with those of chickens fed the non-supplemented diet. Especially for TNFSF15 and LITAF, fermented root more effectively down-regulated gene expression levels than non-fermented root.

The major structural and functional components of TJs include occludin, claudin, JAM2, JAM3, and ZO1 (Anderson, 2001; Tsukita et al., 2001). These proteins play important roles in the regulation of intestinal permeability and barrier function. Gastrointestinal tract is mostly covered by a mucosal surface and organized in two layers. The inner layer is stratified, dense, and firmly adherent to the epithelial cells and does not allow bacteria to penetrate. While the outer layer, is the first line of defense of the host innate immune system (Johansson et al., 2011). MUC2 gene expression is fundamental in maintaining gel layer architecture on the intestinal surface and has been used as a marker for gut health in other species (Forder et al., 2012; Dkhil et al., 2013; Li et al., 2015).

In the present study, LPS treatment significantly reduced the expression levels of JAM, ZO1, and MUC2 in the jejunum at 24 h post-injection. Feeding chickens the diet supplemented with 1% fermented AH root upregulated the expression of JAM and MUC2. Improvement of epithelial and endothelial barrier function could be explained by the increased expression of JAM and MUC2. Interestingly, LPS challenge increased the expression levels of cytokines and consequently down-regulated the expression of junction proteins. Dietary supplementation of AH clearly proved to be beneficial to growing poultry because it down-regulated cytokine levels and increased the

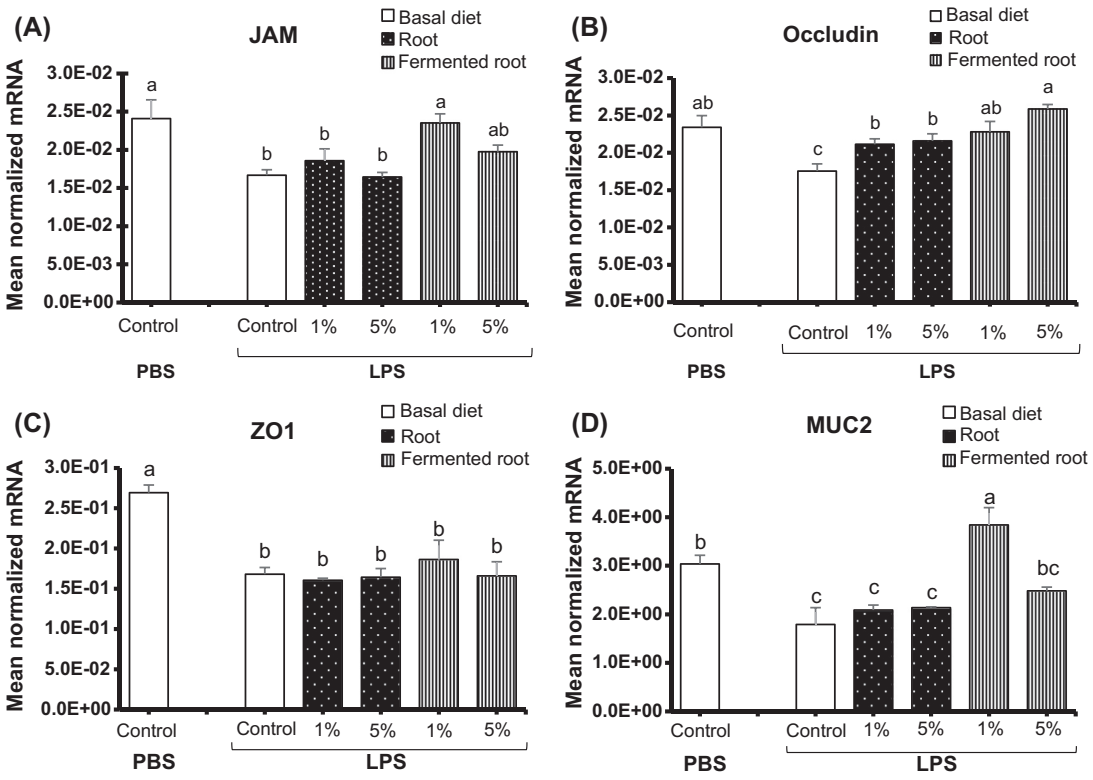

Fig. 5. Effects of dietary *A. hookeri* root or fermented root on the levels of transcripts of intestinal tight junction proteins and mucin. Chickens were fed diets non-supplemented or supplemented with *A. hookeri* root (1, 5%) or fermented root (1, 5%) and challenged with LPS or PBS at 7 days of age. Transcript levels of JAM2, Occludin, ZO1, and MUC2 in the ileum were measured by quantitative RT-PCR and normalized to GAPDH transcript levels. The data were analyzed by one-way ANOVA and mean were separated using Duncan's multiple range test. Each bar represents the mean ± SEM (n = 4) and ^{a,b,c} mean values with unlike letters were significantly different ($P < 0.05$).

expression of TJ proteins.

5. Conclusion

In this study, we demonstrated that different amounts of AH root and fermented root increased the body weight of chickens and the expression levels of TJ proteins and mucin. In contrast, they decreased serum α-1-AGP levels and pro-inflammatory cytokines during the inflammatory response. Therefore, these findings provide scientific evidence that dietary AH is beneficial to poultry production because it promotes gut integrity and enhances innate immunity. Further research is needed to corroborate our findings and to define the poultry immune response.

Acknowledgment

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