Allium hookeri supplementation improves intestinal immune response against necrotic enteritis in young broiler chickens

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ABSTRACT Three hundred birds (1 day old) were randomly assigned to 6 groups (n = 50 birds/treatment) and fed a basal diet (control) or basal diet supplemented with *Allium hookeri* (AH) root (1 or 3%). At day 14, half of the birds in each group were orally challenged with *E. maxima* 41A (1×10^4 cells/chicken), followed by *C. perfringens* infection (1×10^9 cfu/chicken) on day 18. Necrotic enteritis (NE)-associated infections and intestinal immune response were assessed by average body weight gain, lesion score, and oocyst shedding. The effect of dietary supplementation, AH, on transcript levels of pro-inflammatory cytokines, and tight junction proteins and mucin protein in the jejunum, were quantified by quantitative real-time (qRT)-PCR. At day 20, birds fed with diet supplementation (3% of AH) significantly weighted more than the control group. Although the NE-challenged had significantly reduced average body weight gain, there was no significance in the effect between diet × NE-challenge interactions on the average body weight gain. Among the NE-challenged groups, gut lesion score and oocyst shedding were significantly decreased in birds given AH (1 or 3%) compared to the control group. There was a correlation between diet and NE infection with regards to interleukin (IL)-17A, and inducible nitric oxide synthase (iNOS). The up-regulated transcript levels of cytokines IL-8, IL-17A, iNOS, and LITAF by NE challenged groups were significantly reduced by AH (1 or 3%) supplementation. Down-regulated expression levels of tight junction (TJ) proteins: junctional adhesion molecule 2 (JAM2), occluding, and intestinal mucin 2 (MUC2) by NE challenge, was up-regulated by the addition of AH (1 or 3%) supplementation. All TJ proteins (JAM2, ZO1, Ocluddin and MUC2) in the jejunum had a significant diet × NE-challenge interaction. These findings demonstrate that dietary supplementation of AH in chicken feed could be beneficially used to improve chicken health against NE.

Key words: *Allium hookeri*, necrotic enteritis, tight junction protein

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INTRODUCTION

Antibiotics are commonly used in the animal industry to promote growth and prevent disease and pathogen, as well as to treat sick animals. However, concerns about increasing antibiotic resistance led to bans on antibiotics for growth promotion (**AGPs**). Restrictions on the use of AGP has resulted in poorer feed conversion efficiencies and increased mortality rate (Cervantes, 2015) resulting from intestinal diseases such as necrotic enteritis (**NE**) (Dahiya et al., 2006).

NE is a widespread disease in broilers, with considerable financial relevance, because it costs the US poultry industry over six billion dollars annually (Wade and Keyburn, 2015). The significant risk factor for the development of NE is an intestinal environment that promotes the disease's progression. Cooper and Songer (2009) reported that the chickens fed with a high energy and protein-rich feed, for example wheat- or barley-based diets, experienced NE rates of up to ten times greater than those on maize-based diets. NE can easily occur in modern, high-intensity poultry farming where feeding environments require high nutrition intake in a short amount of time. So far, many studies have reported reduced incidences of NE using antibiotic alternative feed additive (Jackson et al., 2003; Geier et al., 2010; Timbermont et al., 2010).

Modulation of innate immunity using natural foods, including extracts from herb species and medicinal plants, offers another opportunity to enhance poultry

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health and reduce the negative effects of pathogen infection (Lillehoj and Lee, 2012). For example, dietary capsicum and *Curcuma longa* oleoresins improved body weight and reduced lesions in NE challenged chickens (Kim et al., 2015). In an in vitro study conducted by Lee et al. (2010a), milk thistle (*Silybum marianum*), turmeric (*Curcuma longa*) and mushroom extracts showed enhanced innate immunity in chickens. Also, in an in vivo test, dietary supplementation of chickens with dried mushroom and broccoli extracts showed growth promoting effect and antioxidant properties (Giannenas et al., 2010; Mueller et al., 2012). One of these medicinal plants is *Allium hookeri* (**AH**) which belongs to the family Lilaceae and is found in China (Yunnan Province), India, Bhutan, Sri Lanka, and Myanmar. AH has been used to treat coughs, colds, fatigue, and to recover immunity (Sharma et al., 2011). According to the medical dictionary of Myanmar, AH can be used to treat cancer or inflammation because it contains large amounts of methyl sulfonyl methane (Bae and Bae, 2012). Recently, Hwang et al. (2015) reported that AH contains abundant flavonoids and phenols and the leaf showed remarkable free radical scavenging activity. Several other studies have also reported the beneficial effects of AH bioactive substances (Song et al., 2014; Kim et al., 2017).

NE can be prevented by boosting the innate immune response and managing the inflammation caused by this infection. In this present study, the effect of AH on the immune enhancement and intestinal integrity was investigated in NE challenged chickens. In addition, differences according to contents of AH were compared and the efficacy value was evaluated as a feed additive.

MATERIALS AND METHODS

Preparation of A. Hookeri Supplement

AH root powder used in this study was verified based on morphological assessment, and the powder was 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. 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.

Bird Husbandry and Diets

Experimental protocol and procedures were approved by the Small Animal Care Committee of the Beltsville Agricultural Research Center (Beltsville, MD). Three hundred male Ross broiler chickens (1 day old) were purchased from Longenecker's Hatchery (Elizabethtown, PA) and assigned randomly to six groups, with each group containing 50 birds (n = 50/group). Each group was divided into seven cages (7 cages/treatment).

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

Ingredients (g)	Low protein	High protein
Corn	69.01	55.78
Soybean meal	23.99	37.03
Soybean oil	2.75	2.97
Dicalcium phosphate	2	1.8
Calcium carbonate	1.4	1.51
Salt	0.35	0.38
Poultry Vit Mix[1)]	0.2	0.22
Poultry Mineral Mix[2)]	0.15	0.15
DL-Methionine	0.1	0.1
Choline-chloride, 60%	0.05	0.06
Total	100	100
Calculated values (DM basis, %)		
CP, %	18	24.00
Ca, %	1.19	1.20
Avail. P, %	0.54	0.51
Lys, %	1	1.40
Met, %	0.42	0.49
Cys + Met, %	0.65	0.80
TMEn, kcal/kg	3585	3450

[1]Vitamin mix provided the following nutrients per kg of diet: vitamin A, 2,000 IU; vitamin D3, 22 IU; vitamin E, 16 mg; vitamin K, 0.1 mg; vitamin B1, 3.4 mg; vitamin B2, 1.8 mg, 3.4 mg; vitamin B2, 1.8 mg; vitamin B6, 6.4 mg; vitamin B12 0.013 mg; biotin, 0.17 mg; pantothenic acid, 8.7 mg; folic acid, 0.8 mg; niacin, 23.8 mg.

[2]Mineral mix provided the following nutrients per kg of diet: Fe, 400 mg Zn, 220 mg; Mn, 180 mg; Co, 1.3 mg; Cu, 21 mg; Se, 0.2 mg.

The birds were housed in Petersime starter brooder unit with an automatically controlled temperature environment and were continuously fed from the hatch with an antibiotic-free, certified organic starter diet (Table 1) or an antibiotic-free, certified organic starter diet with AH root (1 or 3%) ad libitum for 20 days.

Experimental NE Disease Model

Experimental NE model was designed based on a previous reported study (Jang et al., 2013). The chickens were infected with *E. maxima* 41A (1.0×10^4 oocyst/bird) by oral gavage on day 14 post-hatch followed by co-infection with *C. perfringens* strain Del-1 (1.0×10^9 colony forming units [cfu]/bird) by oral gavage on day 18. To facilitate the development of NE, birds were fed a starter diet containing 17% crude protein (dry matter basis), between day 1 and 18 post-hatch, followed by a standard grower diet containing 24% crude protein (dry matter basis), between day 18 and 20 post-hatch. Diagram of experimental design is presented in Figure 1.

Lesion Scores

Birds were humanely sacrificed using cervical dislocation by well-trained personnel. On day 20 post-hatch, six birds from each group were randomly selected for lesion score. Two equal sections of 10 cm jejunum were removed and necrotic enteritis lesion were scored on scale from 0 (none) to 4 (high) as described by Lee et al. (2013). Lesion scores were evaluated blindly by 4 independent observers.

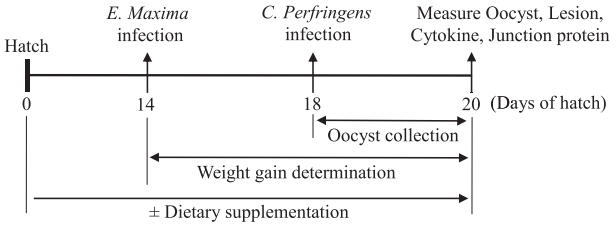

Figure 1. Diagram of classical experimental design. Chickens were fed with basal diet supplemented with 1% or 3% of AH and co-infected with *E. maxima* and C. perfringens at 14 and 18 days post-hatch, respectively. Intestinal tissues were collected at 20 days post-hatch and body weight was individually measured between 0 and 6 dpi with *E. maxima*.

Body Weight Gain

Body weight was measured individually on day 14 (before infection with *E. maxima*) and day 20 post hatch (6 days post-infection (**dpi**) with *E. maxima,* and day 2 post-infection with *C. perfringens*). Later on, average body weight gain of each group was calculated per cage.

Oocyst Shedding

Oocyst production and shedding was assessed as described by Lee et al. (2016). Feces were collected from 20 birds in 5 cages (4 birds/cage) for 4 days, starting on 6 dpi, and water was added up to 3 liters. The fecal materials were ground and homogenized, and two samples were taken and diluted. The oocysts were counted microscopically using a McMaster counting chamber. The total number of oocysts was calculated using the following formula:

$$\begin{aligned}\text{Total oocysts/bird} = {} & \text{oocyst count} \times \text{dilution factor} \\ & \times (\text{fecal sample volume/counting} \\ & \times \text{chamber volume})/ \\ & \text{number of birds per cage}\end{aligned}$$

Total RNA Extraction and Reverse Transcription

At 2 dpi with *C. perfringens*, a segment of intestinal jejunum tissues was collected and immediately placed in RNAlater. The samples were cut and opened longitudinally, washed three times with ice-cold Hank's balanced salt solution (Sigma, St. Louis, MO) to remove gut contents, and the samples were placed in RNAlater. Mucosa layer was carefully scraped away using a surgical scalpel and total RNA was isolated using TRIzol (Invitrogen). 5 μg of total RNA was reverse transcribed to cDNA using QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Oligonucleotide primers sequences used for chicken pro-inflammatory cytokines, including interleukin (IL)-1β, IL-2, IL-8, IL-17A, inducible nitric oxide synthase (iNOS), lipoplysaccharide (**LPS**)-induced tumor necrosis factor-α factor (LITAF), and intestinal tight junction (TJ) proteins junctional adhesion molecule 2 (JAM2), occludin, Zonula occludens-1 (ZO1), and MUC2 are shown in Table 2. The primer sequences of tight junction proteins JAM2, ZO1, and MUC2 genes were adapted from Chen et al. (2015). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Polymerase chain reaction (PCR) amplification and detection was carried out using the Agilent Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA) and the Brilliant SYBR Green qRT-PCR Master Mix (Stratagene, La Jolla, CA). 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 using the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize mRNA expression levels between samples within individual experiments, the mean threshold cycle value (Ct) for the corresponding target and GAPDH products were calculated by pooling values from all samples in that experiment.

Statistical Analysis

Statistical analysis was carried out using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL), and all data were expressed as means ± SEM for each treatment. The data were analyzed by two-way ANOVA to examine the interactive effect of diet and NE challenge (main effects) on dependent variables. When the main effects were significant, differences among treatments were separated using Duncans multiple range test. Probability (P) value < 0.05 was considered statistically significant.

RESULTS

Effect of Dietary Supplementation with AH on Body Weight

No chickens showed any clinical abnormalities during the experimental period. Figure 2 shows the average body weight gain comparison at different dosage of AH-based diets in NE challenged model. NE challenge showed significantly reduced ($P < 0.001$) body weight gain at 48 h post-NE challenge (20 days post-hatch) compared to the uninfected group. Among the NE challenged group, chickens fed the diet supplemented with 3% AH showed significantly increased ($P < 0.05$) body weight gain compared to NE challenged birds fed with basal diet. However, there was no significant effect on growth in the uninfected treatment groups for any of

Table 2. Sequence of oligonucleotide primers used in qRT-PCR.

RNA target	Primer sequences* (5–3)	Accession No.
GAPDH	F: 5′ -GGTGGTGCTAAGCGTGTTAT-3′ R: 5′ -ACCTCTGTCATCTCTCCACA-3′	NM_204,305
IL-1β	F: 5′ -TGGGCATCAAGGGCTACA-3′ R: 5′ -TCGGGTTGGTTGGTGATG-3′	NM_204,524
IL-2	F: 5′ -TCTGGGACCACTGTATGCTCT-3′ R: 5′ -ACACCAGTGGGAAACAGTATCA-3′	NM_204,153.1
IL-6	F: 5′ -CAAGGTGACGGAGGAGGAC-3′ R: 5′ -TGGCGAGGAGGGATTTCT-3′	NM_204,628
IL-8	F: 5′ -GGCTTGCTAGGGGAAATGA-3′ R: 5′ -AGCTGACTCTGACTAGGAAACTGT-3′	NM_205,498.1
IL-17A	F: 5′ -CTCCGATCCCTTATTCTCCTC-3′ R: 5′ -AAGCGGTTGTGGTCCTCAT-3′	NM_204,460.1
LITAF	F: 5′ -TGTGTATGTGCAGCAACCCGTAGT-3′ R: 5′ -GGCATTGCAATTTGGACAGAAGT-3′	NM_204,267
TNFSF15	F: 5′ -CCTGAGTATTCCAGCAACGCA-3′ R: 5′ -ATCCACCAGCTTGATGTCACTAAC-3′	NM_0,010,24578.1
Occludin	F: 5′ -GAGCCCAGACTACCAAAGCAA R: 5′ -GCTTGATGTGGAAGAGCTTGTTG	NM_205,128.1
ZO1	F: 5′ -CCGCAGTCGTTCACGATCT R: 5′ -GGAGAATGTCTGGAATGGTCTGA	XM_01,527,8981.1
JAM2	F: 5′ -AGCCTCAAATGGGATTGGATT R: 5′ -CATCAACTTGCATTCGCTTCA	NM_0,010,06257.1
MUC2	F: 5′ -GCCTGCCCAGGAAATCAAG R: 5′ -CGACAAGTTTGCTGGCACAT	NM_0,013,18434.1

*F = Forward primer; R = Reverse primer.

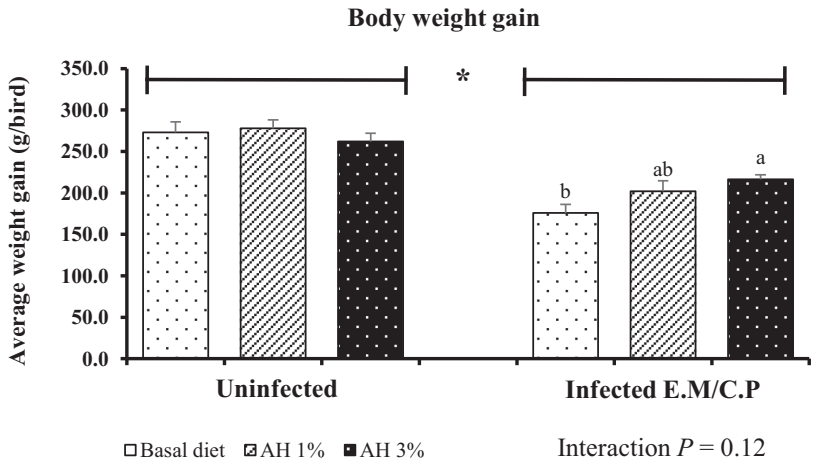

Figure 2. Average body weight gain comparison at different doses of AH-based diets in *E. Maxima*/*C. perfringens* infection. Chickens were fed from hatch with a basal diet or a diet supplemented with either 1 or 3% AH. At 14 days post-hatch, chickens were either uninfected (control) or orally infected with E. 1×10^4 oocyst of *E. maxima*. At 18 days post-hatch, *E. maxima* infected chickens were orally infected with 1×10^9 colony-forming units of *C. Perfringens*. The data were analyzed by two-way ANOVA and each bar presents the mean ± SEM (n = 6) and ∗ represent significant main effect of NE challenge ($P < 0.05$). Diet × NE challenge interaction presented P-value.

the diets. The effect of diet × NE challenge interaction on the body weight gain was not significant.

Effect of Dietary Supplementation with AH on Lesion Score and Oocyst Shedding

At 48 h post-NE challenge (20 days post-hatch), NE challenged birds fed basal diet showed markedly thickened mucosa, hemorrhages, and intestinal lesion (Figure 3A). However, birds fed diets supplemented with AH (1, 3%) had significantly reduced ($P < 0.05$) lesion scores compared to birds fed basal diet. The mean of oocyst shedding number per birds is presented in Figure 3B. Birds in uninfected group excreted no oocyst and the birds fed diets supplemented with AH 3% significantly shed less ($P < 0.05$) oocyst per bird compared to birds with fed basal diet.

Effect of Dietary Supplementation with AH on Cytokine Expression

The expression level of cytokines in the jejunum is presented in Figure 4. At 48 h post-NE challenge (20 days post-hatch), there was significant effect of diet × NE challenge interaction in iNOS ($P < 0.001$) and IL-17A ($P < 0.01$), and statistically significant NE challenge effect were also found ($P < 0.05$) in IL-1β, IL-17A, iNOS and LITAF. NE challenge significantly

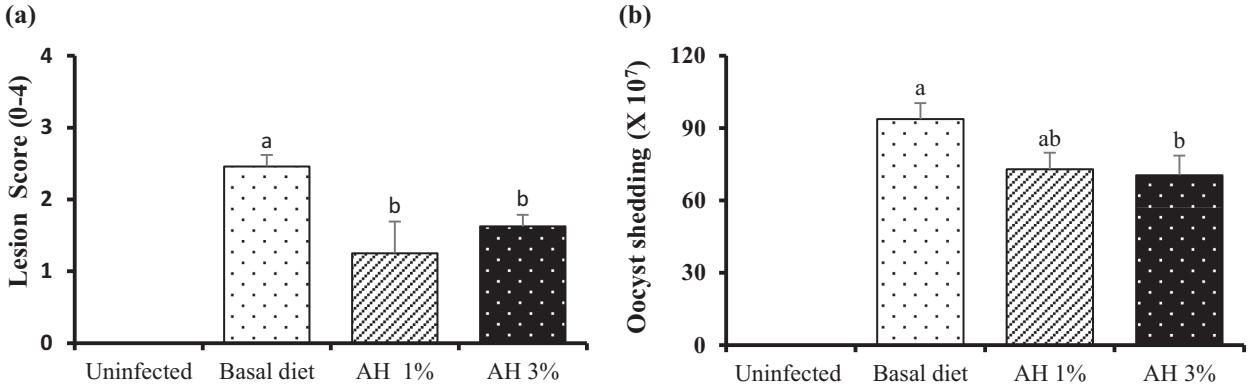

Figure 3. Effect of AH root on lesion score (A) and fecal oocyst output (B). Chickens were fed from hatch with a basal diet or a diet supplemented with 1 or 3% of AH respectively. At 14 day post-hatch, chicken were either uninfected or orally infected with E. 1×10^4 Oocyst of *E. maxima*. At 18 day post-hatch, *E. maxima* infected birds were orally infected with 1×10^9 colony-forming units of *C. Perfringens*. (A) Gut lesion scores were determined at 20 days post-hatch. (B) Fecal oocyst shedding was collected between 6 and 8 days from *E. maxima* infected groups and counted. The data were analyzed by one-way ANOVA and means were separated using Duncan's multiple range test. Each bar presents the mean ± SEM (n = 6) and a, b, c mean values with unlike letters were significantly different ($P < 0.05$).

induced increased expression level of cytokines compared to uninfected group. Among the birds challenged with NE, cytokines expression levels including IL-8, IL-17A, iNOS, and LITAF were significantly lower in the birds fed diets supplemented with 1 or 3% AH compared to birds fed basal diet. The birds in unchallenged groups showed significant difference ($P < 0.05$) between treatment groups in IL-1β and IL-8. However, pattern of those transcription levels were not consistent. IL-2 showed no significant difference between NE challenge and uninfected control, nor was there a correlation between diet and NE challenge.

Effect of Dietary Supplementation with AH on Intestinal Tight Junction Protein and Mucin Expression

The effect of diet × NE challenge interaction were shown in JAM2 ($P < 0.05$), ZO1 ($P < 0.001$), Occludin ($P < 0.001$), and MUC2 ($P < 0.05$) and is presented in Figure 5. At 48 h post-NE challenge (20 days post-hatch), significant NE challenge effects were found ($P < 0.05$) in ZO1 and Occludin. NE challenge intensely reduced expression level of TJ (JAM2 and ZO1) and MUC2 in birds fed with basal diet compared to birds in unchallenged groups. Among the birds challenged with NE, the expression level of TJ proteins (JAM2, ZO1, Occludin) and MUC2 in birds fed diets supplemented with 1 or 3% AH were significantly up-regulated ($P < 0.05$) compared to birds fed basal diet. The birds fed diet supplemented with 1% AH showed consistently high expression levels in TJ proteins. ZO1 and occludin in unchallenged group showed significantly down-regulated ($P < 0.05$) expression level in birds fed with AH (1 or 3%) compared to basal diet group. There was no significant difference of MUC2 in the unchallenged group.

DISCUSSION

Intestinal health has recently been the subject of intense studies in poultry production (Yang et al., 2017; Gadde et al., 2017a). The general function of gut is not limited to only digestion and absorption of nutrients, but it also contributes in the activation of both innate defense mechanisms and adaptive immune responses (Brisbin et al., 2008). When gut health is compromised, digestion and nutrient absorption are affected which, in turn, can have a detrimental effect on feed conversion and feed efficiency, leading to a greater susceptibility to disease and economic loss. Of the intestinal diseases in poultry, NE may be most economically important, because it has been shown to impair feed conversion and reduce growth in broilers (Caly et al., 2015). The causative agent of necrotic enteritis is *Clostridium perfringens*, a Gram-positive spore-forming anaerobe, and the best-known predisposing factor for necrotic enteritis is mucosal damage caused by coccidial pathogens. *C. perfringens* and *Eimeria* spp. perform synergistically in inducing necrotic enteritis lesions (Timbermont et al., 2011).

The previous studies conducted by our lab have demonstrated that ethanol extracts of AH leaf and root increased chicken spleen lymphocytes proliferation and suppressed chicken tumor cell growth in a dose-dependent fashion in vitro. It also induced nitric oxide secretion by chicken macrophages, and potent free radical scavenging capacity of AH was also shown by the 2, 2-diphenyl-1-picrylhydrazyl assay (Lee et al., 2017b). Another in vivo study has demonstrated the anti-inflammatory and tight junction protein recovery effect of dietary AH in LPS-challenged chickens. LPS-challenge induced up-regulation of pro-inflammatory cytokines (IL-1β, IL-8, tumor necrosis factor superfamily member 15 (TNFSF15), and LITAF) in duodenum and serum α -1-acid glycoprotein (α-1-AGP) level. It also induced down-regulation of expression level in

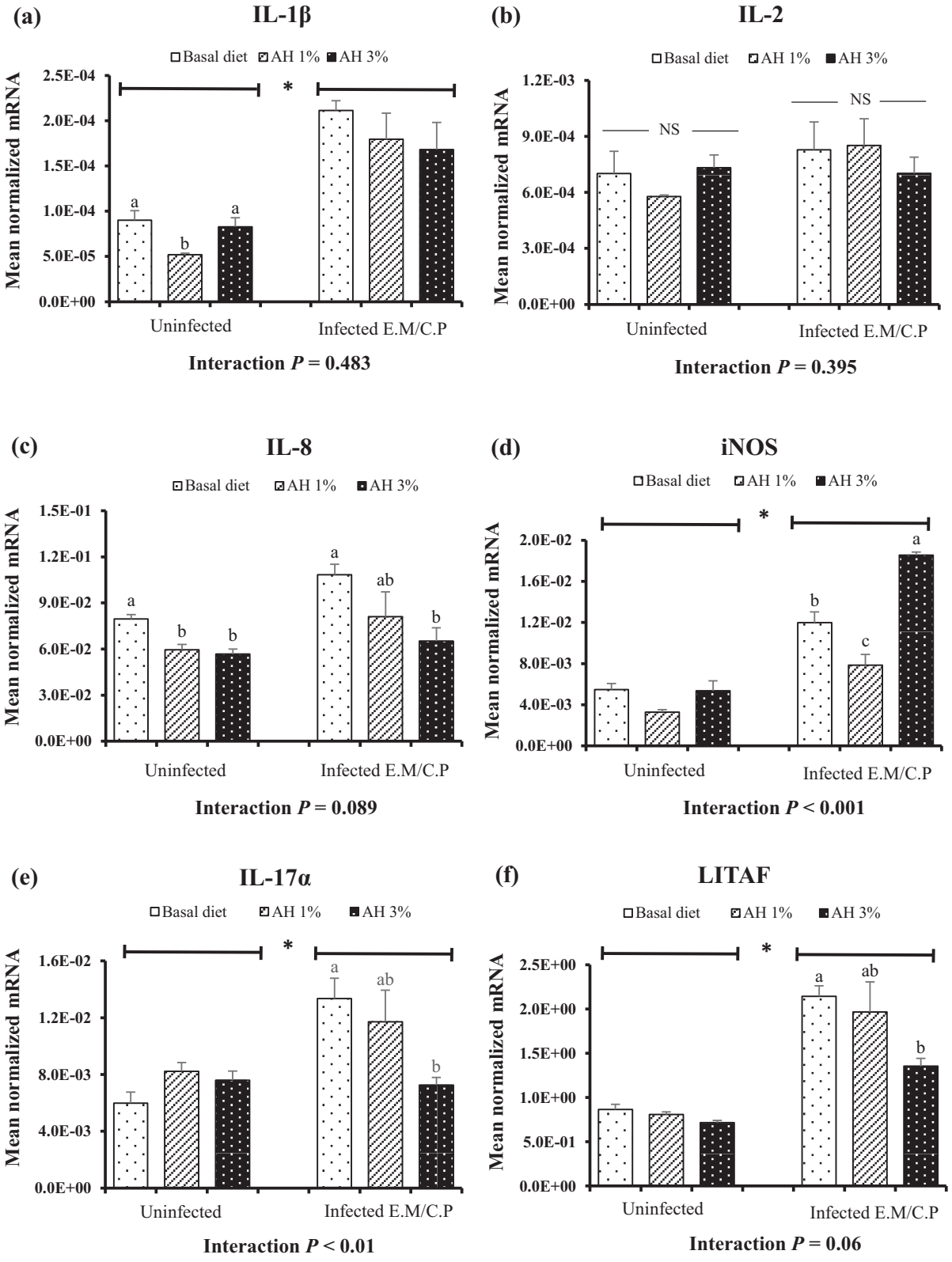

Figure 4. Levels of transcripts of pro-inflammatory cytokines comparison at different dosage of AH-based diets in *E. Maxima*/*C. perfringens* infection. Chickens were fed from hatch with a basal diet or a diet supplemented with 1 or 3% of AH respectively. At 14 day post- hatch, chicken were uninfected or orally infected with E. 1×10^4 Oocyst of *E. maxima*. At 18 day post-hatch, *E. maxima* infected birds were orally infected with 1×10^9 colony-forming units of *C. Perfringens*. Transcript levels of cytokines (IL-1, IL-2, IL-8, IL-17a, iNOS, and LITAF) in jejunum were measured by qRT-PCR and normalized to GAPDH transcript levels. The data were analyzed by two-way ANOVA and each bar presents the mean ± SEM (n = 6).^{a,b,c} mean values with unlike letters were significantly different, and ∗ represent significant main effect of NE challenge ($P < 0.05$). Diet × NE challenge interaction presented P-value.

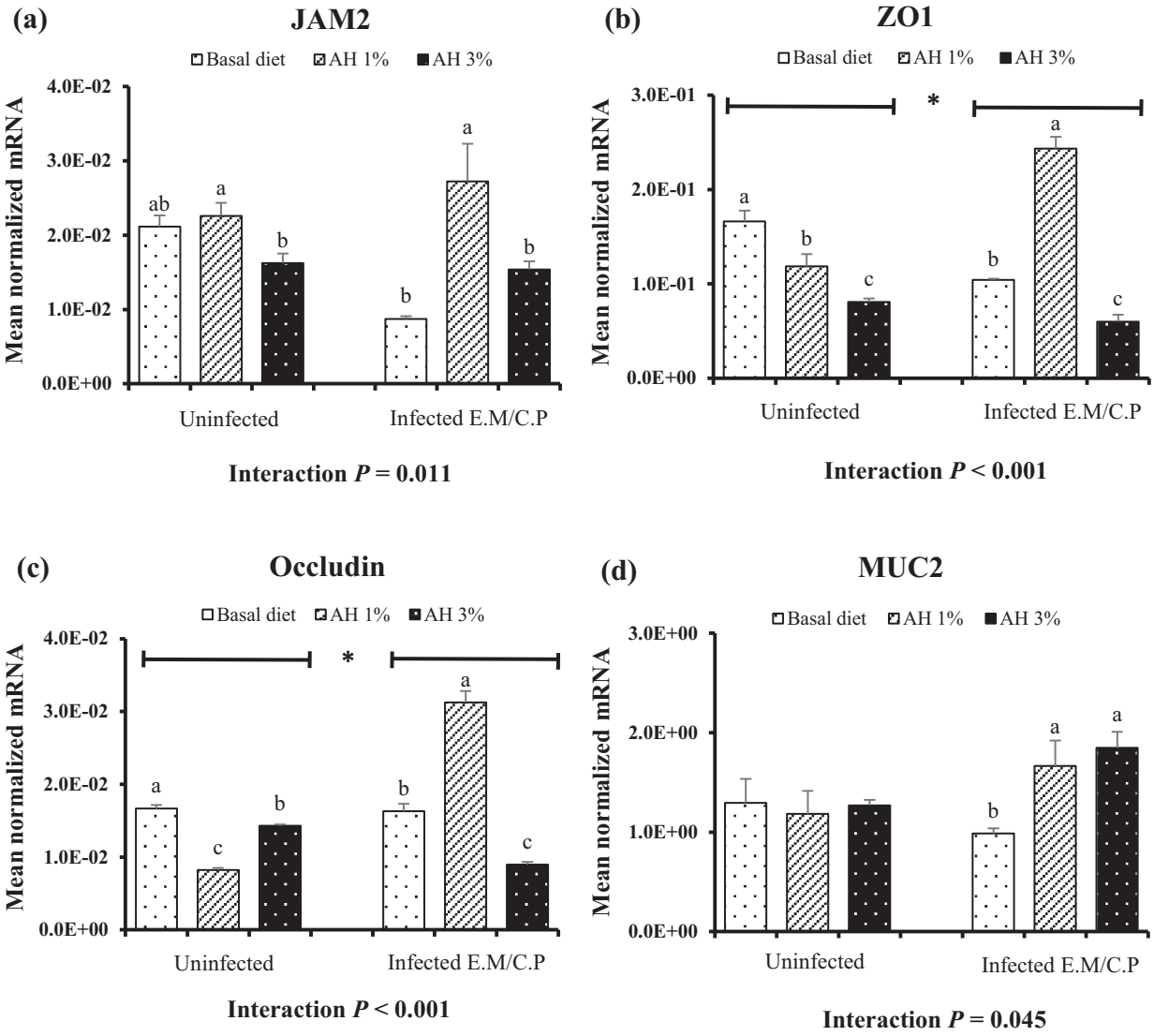

Figure 5. Levels of transcripts of intestinal tight junction proteins and mucin comparison at different dosage of AH-based diets in *E. Maxima*/*C. perfringens* infection. Chickens were fed from hatch with a basal diet or a diet supplemented with 1 or 3% of AH respectively. At 14 day post-hatch, chicken were uninfected or orally infected with E. 1×10^4 Oocyst of *E. maxima*. At 18 day post-hatch, *E. maxima* infected birds were orally infected with 1×10^9 colony-forming units of *C. Perfringens*. Transcript levels of junction proteins and mucin (JAM2, ZO1, Occludin, and MUC2) in jejunum were measured by qRT-PCR and normalized to GAPDH transcript levels. The data were analyzed by two-way ANOVA and each bar presents the mean ± SEM (n = 6).[a,b,c] mean values with unlike letters were significantly different, and ∗represent significant main effect of NE challenge ($P < 0.05$). Diet × NE challenge interaction presented P-value.

TJ protein and mucin gene. However, up-regulated cytokines and serum α-1-AGP level were reversed by AH supplemented diet. Besides, down-regulated expression levels of tight junction and mucin gene induced by LPS-challenge were up-regulated by dietary supplementation of AH (Lee et al. 2017a). Based on the previous studies, the present study was undertaken to investigate whether dietary supplementation of AH would influence growth performance, inflammatory immune activities in the jejunum, and intestinal barrier function during the immunological stress induced by *Clostridium/Eimeria* co-infected commercial broilers.

From the results presented in Figure 2, supplementation of broiler diet with AH did not have a significant influence on the chicken growth performance in uninfected groups. However, following *Clostridium/Eimeria* co-infection, the average body weight gain in infected groups rapidly decreased compared to the uninfected group. The infected birds fed with supplement 3% AH showed significantly higher body weight gain compared to NE-challenged birds fed basal diet. These results are similar to our previous in vivo study which revealed that chickens fed supplement with 1% AH showed higher body weight gain among the LPS-injected groups at 24 h post-LPS injection.

In this study, bloody diarrhea was prolonged and severe in the *Clostridium/Eimeria* co-infected groups, especially on day 20 post-hatch. However, no chickens in this study died as a result of *CP/Eimeria* infection. At day 20 post-hatch, total gross intestinal NE lesion scores showed significantly decreased levels in the birds fed diets supplemented with AH (1 and 3%) compared

to birds given basal diet. Average fecal oocyst shedding was also significantly reduced in the birds fed diet supplemented with 3% AH compared to birds fed basal diet. Historically, the severity of experimental *Eimeria* infection in chickens have been assessed by loss of body weight gain, presence of intestinal lesion, and excretion of fecal oocyst (Lee et al., 2010b). These are the disease parameters that reflect host immunity status in avian coccidiosis (Lillehoj et al., 2007). In the present study, showing less loss of body weight gain, decreased lesion score, and oocyst shedding by AH supplementation in NE-challenge could be attributed to improved host immune response. Some medicinal plants contain phytochemical compounds that can mediate in multiple disease-related signaling pathways (Chang et al., 2013). And many studies have reported that natural products and herbal remedies are emerging as attractive and alternative methods to resist coccidiosis (Wang et al., 2008; Lillehoj et al., 2011; Kim et al., 2013; Peek et al., 2013).

Cytokines are essential effector molecules of innate and adaptive immunity against pathogenic microorganisms. Previous studies demonstrated that several pro-inflammatory cytokines or chemokine such as IL-1β, IL-8, IL-17, and LITAF were produced in response to experimental *Eimeria* infections (Hong et al., 2006b; Lee et al., 2011). From the results illustrated in Figure 4, *Clostridium*/*Eimeria* co-infection stimulated increased production of pro-inflammatory cytokines (IL-1β, IL-8, IL-17A, iNOS, and LITAF). However, expression levels of IL-8, IL-17A, iNOS, and LITAF showed decrease in the chickens fed diet supplemented with 1 or 3% AH. Our current observation corroborates with previous in vitro studies conducted by other groups. Kim et al. (2012) reported that methanol extract of AH mediated production of pro-inflammatory cytokines, IL-6, and tumor necrosis factor-α, in LPS-induced RAW264.7 cells. According to a study by Jang et al. (2017), methanol extract of AH inhibited gene expression level of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-induced RAW264.7 cells. IL-1β is a representative powerful pro-inflammatory cytokine mainly produced by stimulated macrophages, monocytes and other cells (Laurent et al., 2001). Generally, IL-1β is not expressed in healthy cells or tissue but acts as a mediator of inflammation in mammals and avians (Fasina et al., 2008). IL-17 is also involved in a pro-inflammatory cytokine produced by T helper 17 (Th17) CD4+ T cells, a subpopulation of cells phenotypically and functionally distinct from the classical Th1 and Th2 lymphocyte lineages (Iwakura et al., 2011). IL-8 is a CXC chemokine that attracts leukocytes to sites of inflammation on mucosal surfaces (Park et al., 2008). High concentration of NO synthesized by iNOS, can mediate inflammation and cause cell death by inducing apoptosis (Wink and Mitchell, 1998). LITAF (predominantly expressed in spleen and intestinal intraepithelial lymphocytes) expression is up-regulated following stimulation of macrophages with bacterial endotoxin or *Eimeria* species infection (Hong et al., 2006a). These pro-inflammation cytokines (IL-1β, IL-8, IL-17A, iNOS, and LITAF) are directly or indirectly related with inflammation and have been used as markers to detect inflammation. Therefore, showing decreased expression levels of pro-inflammatory cytokines in NE challenged group is an indication that either host innate immune was improved or the inflammation was mediated by AH. IL-2 is an important cytokine which controls several immune mechanisms, including stimulation of natural killer T-cells (Min et al., 2005). Several studies have shown that IL-2 is up regulated followed by infection with *Eimeria* species (Choi and Lillehoj, 2000; Miyamoto et al., 2002). It was expected that NE challenge in this study would induce IL-2 expression. However, in contrast with other studies, there was no significant difference between NE-challenge effect as well as diet $\times$ NE challenge correlation.

Maintaining tight junctions of intestinal epithelial cells is important to improve health. It regulates major immune functions, increases absorption rate of nutrients, maintains homeostasis, and protects against invading pathogens (Rajput et al., 2013). From the results presented in Figure 5, in contrast with pro-inflammatory cytokines, NE challenge significantly reduced expression levels of JAM2 and ZO1 in jejunum compared to birds in the uninfected groups. However, the expression levels in birds given 1% AH showed 2-fold greater increase when compared to NE- challenged birds fed a basal diet. This increased expression of JAM2 and ZO1 translates to improved barrier function in intestine, especially during invasion of pathogenic microorganisms. Mucin 2 is one of the major secreted mucins expressed by intestinal goblet cells and acts as a protective barrier for the intestine (Dharmani et al., 2009, Gadde et al., 2017b). Elevated mucus secretion as a consequence of *Eimeria* inoculation has shown to cause increased incidences of *C. perfringens* colonization in chickens, implying that the mucus layer provides a nutritional substrate favoring growth of the bacterium (Collier et al., 2008). Several studies have demonstrated that this necrotic pathogen induces decrease expression of MUC2 in chickens (Forder et al., 2012; Awad et al., 2017). In agreement with previous studies mentioned, decreased expression of MUC2 was observed in the NE challenged group compared to the uninfected group. However, dietary supplementation of AH (1, 3%) dramatically led to increased regulation of MUC2. Interestingly, NE challenge increased the expression levels of cytokines and consequently down-regulated the expression of junction proteins. Ethanol/methanol extracted AH might give similar beneficial results as the dry powder form, similar to results obtained from other studies using extracts of garlic and other herbs (Cross et al., 2007; Naidoo et al., 2008; Hashemi and Davoodi, 2011; Abudabos et al., 2018). Using the extract fraction of AH might require less to be added to the feed, however, this would require further in vivo studies.

In conclusion, it has been demonstrated that supplementation of broiler diet with AH led to less loss of body weight gain and decreased lesion and oocyst shedding in NE-challenged birds. Additionally, up-regulated pro-inflammatory cytokines during inflammation induced by NE were down-regulated by dietary supplementation of AH (1 or 3%). In contrast, NE challenge caused down-regulation of tight gut junction proteins and mucin gene expression. However, infected chickens fed a diet supplemented with AH showed reversed expression levels in tight gut junction proteins and mucin. These results provide scientific evidence that dietary supplementation of AH enhances innate immunity in broilers. Further studies will be valuable in understanding both the molecular signaling mechanism and how immune responses are initiated. Moreover, supplementation of broiler diet with AH could be used to improve host immune response in chickens.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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