Bacillus amyloliquefaciens supplementation alleviates immunological stress in lipopolysaccharide-challenged broilers at early age

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ABSTRACT This study was conducted to investigate the effect of *Bacillus amyloliquefaciens* (**BA**) on the immune function of broilers challenged with lipopolysaccharide (**LPS**). 192 one-day-old male Arbor Acre broiler chickens were randomly distributed into four treatments: 1) broilers fed a basal diet; 2) broilers fed a basal diet supplemented with BA; 3) LPS-challenged broilers fed a basal diet; and 4) LPS-challenged broilers fed a basal diet supplemented with BA. Each treatment consisted of six replicates with eight broilers per replicate. Broilers were intraperitoneally injected with either 500 μg LPS per kg body weight or sterile saline at 16, 18 and 20 d of age. LPS decreased the average daily gain (**ADG**, $P = 0.001$) and average daily feed intake ($P = 0.001$). The decreased ADG ($P = 0.009$) and increased feed conversion ratio ($P = 0.047$) in LPS-challenged broilers were alleviated by BA. LPS increased the relative spleen weight ($P = 0.001$). Relative spleen ($P = 0.014$) and bursa ($P = 0.024$) weights in the LPS-challenged broilers were reduced by BA. LPS increased white blood cell (**WBC**) numbers ($P = 0.001$). However, the WBC numbers ($P = 0.042$) and the ratio of lymphocytes to WBC ($P = 0.020$) in LPS-challenged broilers were decreased with BA treatment. LPS decreased plasma lysozyme activity ($P = 0.001$), but increased concentrations of plasma corticosterone ($P = 0.012$) and IL-2 ($P = 0.020$). In contrast, BA increased lysozyme activity in plasma ($P = 0.040$). LPS increased mRNA abundances of splenic toll-like receptor 4 ($P = 0.046$), interferon γ ($P = 0.008$), *IL-1β* ($P = 0.045$) and *IL-6*, ($P = 0.006$). *IL-2* ($P = 0.014$) and *IL-6* ($P = 0.074$) mRNA abundances in LPS-challenged broilers were reduced by BA, although BA had an opposite effect for *IL-10* mRNA expression in those broilers ($P = 0.004$). In conclusion, BA supplementation could partially alleviate the compromised growth performance and immune status of broilers under immune stress induced by LPS challenge at early age.

Key words: *Bacillus amyloliquefaciens*, immunological stress, lipopolysaccharide, spleen, broiler

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INTRODUCTION

Probiotics are defied as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Sanders, 2008; Hill et al., 2014). Recent studies have confirmed that probiotic bacteria could induce regulatory T cells or Th2 cells that could produce anti-inffiammatory cytokines, IL-10 and IL-4 and reduce auto-reactive T-cells, which produce pro-inffiammatory cytokines as interferon γ (**IFN-γ**) (Matsuzaki et al., 1997; Calcinaro et al., 2005; von Boehmer, 2005; Kwon et al., 2010). *Bacillus amyloliquefaciens* (**BA**) is a major workhorse for the production of a variety of extracellular enzymes including phytase, α-amylases, cellulase, metalloproteases and proteases that could enhance digestibility and absorption of nutrients (Gould et al., 1975; Gracia et al., 2003; Lee et al., 2008). Meanwhile, BA secretes bacteriocins including subtilin and barnase that can inhibit the growth of pathogenic bacteria such as *Clostridium perfringens* and *Escherichia coli* (Lisboa et al., 2006; Ulyanova et al., 2011). BA isolated from the soils of North East Himalayas has been shown to reduce the protein and mRNA levels of pro-inffiammatory cytokines such as tumor necrosis factor and IL-1β in the colon of colitis mice induced by dextran sulfate sodium (Hairul Islam et al., 2011). Additionally, the surfactin lipopeptides produced by *Bacillus subtilis* have been verified as anti-inffiammatory agents (Eun et al., 2008). An in vivo trial has also shown that, in rats, surfactin from *Bacillus subtilis* can reduce the acute inffiammation of septic shock induced by lipopolysaccharide (**LPS**) from *Escherichia coli* (Hwang et al., 2007). Recently, Gao et al. (2014) found that oral administration of a surfactin lipopeptide puriffied from a BA culture could down-regulate the secretion of tumor necrosis factor and stimulate the production of IL-10 in the splenocytes of NOD mice.

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LPS, the primary component of the outer membrane of gram-negative bacteria, serves as a potent activator of innate immune responses that results in the production of pro- and anti-inflammatory mediators (Ulevitch and Tobias, 1995). LPS is widely used to induce immunological stress in broilers, which is characterized by compromised growth performance and enhanced production of pro-inffiammatory cytokines (Takahashia et al., 1997; Xie et al., 2000; Mireles et al., 2005; Yang et al., 2011). For broilers, recent studies have shown that dietary BA inclusion can enhance growth performance and nutrient digestibility, improve intestinal integrity, reduce fecal noxious gas emissions and inhibit the proliferation of pathogenic bacteria (Ahmed et al., 2014; Lei et al., 2014). However, little is known about the protective effect of BA on broilers under immune stress. The current study was therefore conducted to investigate the effect of BA inclusion on broilers during immunological stress induced by LPS originating from *Escherichia coli*.

MATERIALS AND METHODS

Experimental Design, Diets and Management

The animal care and use protocol was approved by Nanjing Agricultural University Institutional Animal Care and Use Committee. One hundred and ninety two one-day-old male Arbor Acre broiler chickens were randomly divided into two groups and each group included two subgroups. Each subgroup consisted of six replicates (one replicate per cage) with eight birds per replicate. Birds in the two groups then received a maize-soybean meal basal diet in mash form or a basal diet supplemented with 1.08×10^{10} colony-forming units (CFU) BA per kg feed for 21 d. At 16, 18 and 20 d of age, birds from each subgroup of the two groups were then intraperitoneally injected with 0.5 ml sterile saline (8.6 g/l) or LPS (*Escherichia coli* serotype O55.B5, Sigma Chemical, St Louis, MO, USA) dissolved in 8.6 g/l sterile saline (1 mg/ml) at the appropriate dose of 500 μg/kg body weight. The doses and routes of LPS administration referred to the previous studies (Rajput et al., 2013; Zhang et al., 2010). Thus, birds were subjected to one of four treatments: 1) broilers fed a basal diet; 2) broilers fed a basal diet supplemented with BA; 3) LPS-challenged broilers fed a basal diet; and 4) LPS-challenged broilers fed a basal diet supplemented with BA. The basal diet was formulated according to the National Research Council (1994) to meet the nutrient requirements of the broiler. The BA was provided by Prof. Lu from the College of Food Science and Technology at Nanjing Agricultural University. All birds were placed in three-level wired battery cages and housed in an environmentally controlled room maintained between 32°C to 34°C from 1 to 7 d, which was then gradually reduced to 26°C at the rate of 3°C to 4°C per week and then kept constant thereafter. Continuous light was provided for the entire period of experiment. Feed and fresh water were available *ad libitum*. Body weight of broilers were measured at 1, 15 and 21 d of the experiment, and feed intake on a cage basis was recorded at 15 and 21 d of age to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**).

Sample Collection

At 20 d of the experiment, six chickens from each treatment (1 bird per pen) were randomly selected and weighed, EDTA or heparinized blood samples were collected within 2 h post-injection for further analysis. After decapitation, thymus, spleen and bursa were removed to determine relative organ weight and were expressed relative to body weight (g per kg of body weight). After that, spleen samples were immediately collected and stored in liquid nitrogen for further measurement.

Blood Parameters Determination

The blood sample collected in EDTA tube was to determine blood cell counts. The numbers of red blood cells (**RBC**) and white blood cells (**WBC**), and the ratio of lymphocytes to WBC were determined using a blood counter (Coulter STKS model, Coulter electronics, Ltd, Luton, UK) with adapted dilutions.

The blood sample collected in heparinized tube was taken by centrifugation at 2,000 $\times$ *g* for 15 min at 4°C to obtain plasma and stored at −80°C until analysis. Plasma samples were measured for lysozyme activity with a lysozyme assay kit (Nanjing Jiancheng Bioengeering Institute, Nanjing, China). The methodology used in the kit was described by Kreukniet et al. (1995) using *Micrococcus lysodeikticus cells* as substrate. In detail, a series of concentrations of crystalline lysozyme dissolved in phosphate buffer were adopted to build the standard curve. The standard dilution series of crystalline lysozyme and plasma samples were then analyzed for their lysozyme actively during the lysis of *Micrococcus lysodeikticus*. The content of corticosterone (**COR**) and IL-2 in plasma was determined using a commercial available ^{125}I-RIA kit (Beijing Research institute of Biotechnology, Beijing, China) according to manufacturer's guideline.

Messenger RNA Quantification

Messenger RNA abundance was determined according to method described by Zhang et al. (2014). RNA was isolated using TRIzol Reagent (TaKaRa Biotechnology, Dalian, Liaoning, China) from snap-frozen spleen samples using the manufacturer's protocol. RNA integrity was checked on 1% agarose gel with ethidium bromide staining. The RNA concentration and purity were determined from OD260/280 readings (ratio>1.8) using a NanoDrop ND-1000 UV

Table 1. Sequences for real-time PCR primers.

Gene[1]	Gene Bank ID	Primer sequence, sense/antisense	Length
CD14	NM˙001139478.1	TGGACGACTCCACCATTGAC CCATCTCCTGCACCTGAGTG	132
TLR4	NM˙001030693.1	AGGCACCTGAGCTTTTCCTC TACCAACGTGAGGTTGAGCC	96
MyD88	NM˙001030962.1	ATCCGGACACTAGAGGGAGG GGCAGAGCTCAGTGTCCATT	115
TIRAP	NM˙001024829.1	CCTGGTGCAGGTACCAGATG AGCTCCCTGGGGTAATCCTT	109
IFN-γ	NM˙205149.1	CACTGACAAGTCAAAGCCGC ACCTTCTTCACGCCATCAGG	87
IL-1β	NM˙204524.1	GTACCGAGTACAACCCCTGC AGCAACGGGACGGTAATGAA	112
IL-2	NM˙204153.1	GTCCATTCTGGGACCACTGT CCAACGTACATTTTGAGCCCG	107
IL-4	NM˙001007079.1	GTGCCCACGCTGTGCTTAC AGGAAACCTCTCCCTGGATGTC	82
IL-6	NM˙204628.1	AGGGCCGTTCGCTATTTGAA CAGAGGATTGTGCCCGAACT	72
IL-7	NM˙001037833.1	TTTTCAGGTTTGCACGCAGG ATTCCAGCAAGATCGAAGGCT	133
IL-10	NM˙001004414.2	GGAGCTGAGGGTGAAGTTTGA GACACAGACTGGCAGCCAAA	129
β-actin	NM˙205518.1	TTGGTTTGTCAAGCAAGCGG CCCCCACATACTGGCACTTT	100

[1]CD14, cluster of differentiation 14; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; TIRAP, toll interleukin 1 receptor adaptor protein; IFN-γ, interferon γ; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-7, interleukin 7; IL-10, interleukin 10.

spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After determining the RNA concentration, 1 μg of total RNA was reverse-transcribed into complementary DNA using the PrimeScript^{TM} RT Reagent Kit (TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer's guidelines. Real-time PCR was carried out on an ABI StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. The primer sequences for the target and reference genes (cluster of differentiation 14 (***CD14***), toll-like receptor 4 (***TLR4***), myeloid differentiation factor 88 (***MyD88***), toll interleukin 1 receptor adaptor protein (***TIRAP***), *IFN-γ*, *IL-1β*, *IL-2*, *IL-4*, *IL-6*, *IL-7* and *IL-10*) are given in Table 1. Brieffiy, the reaction mixture was prepared using 2 μl of complementary DNA, 0.4 μl of forward primer, 0.4 μl of reverse primer, 10 μl of SYBR Premix Ex Taq^{TM} (TaKaRa Biotechnology, Dalian, Liaoning, China), 0.4 μl of ROX Reference Dye (TaKaRa Biotechnology, Dalian, Liaoning, China) and 6.8 μl of double-distilled water. Each sample was tested in duplicate. PCR consisted of a prerun at 95°C for 30 s and 40 cycles of denaturation at 95°C for 5 s, followed by a 60°C annealing step for 30 s. The conditions of the melting curve analysis were as follows: one cycle of denaturation at 95°C for 10 s, followed by an increase in temperature from 65 to 95°C at a rate of 0.5°C/s. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) after normalization against the reference gene β-actin. The values of saline-treated broilers fed the basal diet were used as a calibrator.

Statistical Analysis

Two-way ANOVA was employed to determine the main effects of diet and LPS and their interaction using the general linear model procedure of SPSS software (version 16.0; SPSS, Inc.). When P values of interaction of main effects less than 0.10, differences among the treatments were examined by one-way ANOVA using Duncan's multiple range test, which were considered signifficant at $P < 0.05$, and P values between 0.05 and 0.10 were considered as a trend. Data are presented as means with their pooled standard errors.

RESULTS

Growth Performance

No significant difference (Table 2) was observed in the growth performance of broilers before LPS challenge ($P > 0.05$). LPS challenge induced decreased ADG ($P = 0.001$) and ADFI ($P = 0.001$) of broilers. In this experiment, BA inclusion ameliorated the compromised ADG ($P = 0.009$) and FCR ($P = 0.047$) in LPS-challenged broilers compared with those given a basal diet.

The Relative Weights of the Thymus, Bursa and Spleen

LPS challenge resulted in (Table 3) an increased relative weight of spleen ($P = 0.001$), with the same tendency also noted for bursa ($P = 0.052$). The increased relative weight of spleen ($P = 0.014$) and bursa

Table 2. Effect of *bacillus amyloliquefaciens* supplementation on the growth performance of broilers challenged with lipopolysaccharide[1].

		Saline		LPS			*P*-value		
Item[2]		Con	BA	Con	BA	SEM	Stress	Diet	Stress×Diet
1-15 d	ADG (g/d)	20.31	20.11	20.11	20.86	0.44	–	0.757	–
	ADFI (g/d)	28.23	27.24	27.31	27.52	0.24	–	0.422	–
	FCR (g:g)	1.40	1.37	1.37	1.34	0.03	–	0.671	–
16-21 d	ADG (g/d)	71.38^{a}	68.03^{a,b}	52.95^{c}	61.88^{b}	1.07	0.001	0.206	0.009
	ADFI (g/d)	105.83	105.76	91.86	93.38	1.11	0.001	0.749	0.727
	FCR (g:g)	1.49^{b}	1.56^{a,b}	1.76^{a}	1.52^{b}	0.04	0.142	0.265	0.047

$^{a–c}$Means within a row with different superscripts are different at $P < 0.05$.

[1]Con, basal diet; BA, basal diet supplemented with *bacillus amyloliquefaciens*; LPS, lipopolysaccharide.

[2]ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Table 3. Effect of *Bacillus amyloliquefaciens* supplementation on relative immune organ weight of broilers challenged with lipopolysaccharide at 20 d of age (g/kg of body weight)[1].

	Saline		LPS			*P*-value		
Item	Con	BA	Con	BA	SEM	Stress	Diet	Stress×Diet
Thymus	3.34	3.53	2.88	3.63	0.21	0.677	0.270	0.506
Bursa	1.88^{b}	2.34^{b}	2.74^{a}	2.27^{b}	0.10	0.052	1.000	0.024
Spleen	0.93^{b}	0.92^{b}	1.50^{a}	1.04^{b}	0.04	0.001	0.011	0.014

$^{a–b}$Means within a row with different superscripts are different at $P < 0.05$.

[1]Con, basal diet; BA, basal diet supplemented with *Bacillus amyloliquefaciens*; LPS, lipopolysaccharide.

Table 4. Effect of *Bacillus amyloliquefaciens* supplementation on blood characteristics of broilers challenged with lipopolysaccharide[1].

	Saline		LPS			*P*-value		
Item[2]	Con	BA	Con	BA	SEM	Stress	Diet	Stress×Diet
RBC (10^6/mm^3)	2.62	2.41	2.60	2.57	0.07	0.623	0.411	0.505
WBC (10^3/mm^3)	206.55^{c}	209.25^{c}	465.75^{a}	329.95^{b}	15.23	0.001	0.049	0.042
Lymphocyte[3] (%)	75.68^{a,b}	77.33^{a,b}	81.98^{a}	71.50^{b}	1.13	0.918	0.074	0.020

$^{a–c}$Means within a row with different superscripts are different at $P < 0.05$.

[1]Con, basal diet; BA, basal diet supplemented with *Bacillus amyloliquefaciens*; LPS, lipopolysaccharide.

[2]RBC, red blood cells; WBC, white blood cells.

[3]Value presented as percentage of total white blood count.

($P = 0.024$) induced by LPS was counteracted by BA supplementation. However, broilers exhibited a similar relative thymus weight among groups ($P > 0.05$).

Whole Blood Cells

LPS injection (Table 4) increased the WBC numbers ($P = 0.001$). However, BA diet decreased the numbers of WBC ($P = 0.049$) regardless of LPS challenge. In addition, a tendency towards a decreased ratio of lymphocyte to WBC ($P = 0.074$) was observed in BA diet-fed broilers compared with their basal diet-fed counterparts. The increased levels of WBC numbers ($P = 0.042$) and lymphocyte to WBC ratio ($P = 0.020$) induced by LPS challenge were alleviated by BA supplementation.

Plasma Parameters

LPS challenge (Table 5) decreased lysozyme activity ($P = 0.001$), but increased concentrations of COR ($P = 0.012$) and IL-2 ($P = 0.020$). Irrespective of LPS challenge, the BA diet resulted in decreased plasma IL-2 content ($P = 0.056$) and increased lysozyme activity ($P = 0.040$). Additionally, LPS and BA had no interaction effect on these parameters ($P > 0.05$).

Splenic Gene Expression

As indicated in the Table 6, LPS challenge increased mRNA abundances of *TLR4* ($P = 0.046$), *IFN-γ* ($P = 0.008$), *IL-1β* ($P = 0.045$) and *IL-6* ($P = 0.006$). There was however, a tendency for decreased mRNA expressions of *CD14* ($P = 0.062$), *IL-1β* ($P = 0.051$) and *IL-6* ($P = 0.065$) observed in the broilers fed diets supplemented with BA compared with the basal

Table 5. Effect of *Bacillus amyloliquefaciens* supplementation on plasma parameters of broilers challenged with lipopolysaccharide[1].

	Saline		LPS			*P*-value		
Item[2]	Con	BA	Con	BA	SEM	Stress	Diet	Stress×Diet
COR (ng/ml)	8.24	8.08	12.28	9.63	0.52	0.012	0.188	0.242
IL-2 (ng/ml)	3.96	3.11	5.98	4.27	0.31	0.020	0.056	0.504
Lysozyme (U/ml)	143.28	181.53	72.74	103.10	7.82	0.001	0.040	0.804

[1]Con, basal diet; BA, basal diet supplemented with *Bacillus amyloliquefaciens*; LPS, lipopolysaccharide.

[2]COR, corticosterone; IL-2, interleukin 2.

Table 6. Effect of *Bacillus amyloliquefaciens* supplementation on splenic gene expression of broilers challenged with lipopolysaccharide[1].

	Saline		LPS			*P*-value		
Item[2,3]	Con	BA	Con	BA	SEM	Stress	Diet	Stress×Diet
CD14	1.00	0.80	0.87	0.64	0.05	0.211	0.062	0.908
TLR4	1.00	0.91	1.33	1.05	0.05	0.046	0.110	0.389
MyD88	1.00	0.94	1.09	0.78	0.05	0.737	0.101	0.262
IFN-γ	1.00	1.01	1.36	1.18	0.04	0.008	0.334	0.288
IL-1β	1.00	0.88	1.23	1.01	0.04	0.045	0.051	0.520
IL-2	1.00^{b}	1.13^{b}	1.50^{a}	0.96^{b}	0.06	0.187	0.106	0.014
IL-6	1.00^{b}	0.99^{b}	1.53^{a}	1.12^{b}	0.05	0.006	0.065	0.074
IL-10	1.00^{a}	0.64^{a,b}	0.35^{b}	1.11^{a}	0.08	0.590	0.237	0.004

[a–b]Means within a row with different superscripts are different at $P < 0.05$.

[1]Con, basal diet; BA, basal diet supplemented with *Bacillus amyloliquefaciens*; LPS, lipopolysaccharide.

[2]CD14, cluster of differentiation 14; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; TIRAP, toll interleukin 1 receptor adaptor protein; IFN-γ, interferon γ; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-7, interleukin 7; IL-10, interleukin 10.

[3]Expressed in arbitrary units. The mRNA level of each target gene for the Saline-Con group was assigned a value of 1 and normalized against β-actin.

diet control. The increased *IL-2* ($P = 0.014$) and *IL-6* ($P = 0.074$) mRNA expression in broilers challenged with LPS was reduced by BA inclusion. However, BA had an opposite effect for *IL-10* mRNA expression ($P = 0.004$) in those broilers. Additionally, there were no significant differences in the expressions of *MyD88*, *TIRAP*, *IL-4*, and *IL-7* between treatments or interactions ($P > 0.05$, data not shown).

DISCUSSION

Immune stress induced by LPS challenge results in compromised growth performance of broilers (Xie et al., 2000; Yang et al., 2008; Shen et al., 2010; Yang et al., 2011), mainly due to the diversion of available nutrients away from growth to support immune-related processes and synthesis of various mediators such as cytokines (Xie et al., 2000; Brzek and Konarzewski, 2007; Yang et al., 2008; Shen et al., 2010; Yang et al., 2011). In the present study, LPS decreased the weight gain of broilers during 6 d post-challenge, which was possibly due to simultaneously compromised feed intake (Table 2). The compromised weight gain and feed conversion ratio of broilers suffering from LPS challenge were alleviated by BA supplementation (Table 2), indicating that BA might exert a protective effect on the growth of the broilers under immunological stress. An in vivo study has also found that BA inclusion increased the body weight of dextran sulfate induced colitis mice model by suppressing the production of pro-inffiammatory cytokines (Hairul Islam et al., 2011).

The bursa is a vital lymphoid organ and is the main site for the development and maturation of B-lymphocytes and the development of a diverse repertoire of antibody specificities (Paramithiotis and Ratcliffe, 1994; Glick, 1995). The spleen functions as a secondary lymphoid tissue where lymphocytes are activated with antigens derived from an infection (Pozo et al., 2009). The increased spleen size in the LPS-challenge broilers was associated with the simultaneously increased pro-inffiammatory cytokine production, either in the spleen or plasma, suggesting that the increase in spleen weight was possibly due to the greater activity of this organ (Liu et al., 2015) and emergency need for cytokine synthesis during the systemic inffiammatory response. A similar result was also observed by Koutsos et al. (2006) and Liu et al. (2015). The decreased bursa and spleen weight seen in LPS-challenged broilers fed a BA diet (Table 3) indicated that BA supplementation could exert a beneficial effect on the homeostatic mechanisms within these immune organs and we suggest that the beneficial effect could be attributed to the anti-inflammatory effect of BA (Eun et al., 2008; Hairul Islam et al., 2011; Gao et al., 2014).

WBC and lymphocytes in the blood are considered a late response to LPS stimulation (Xie et al., 2000; Shen et al., 2010). In this study, consistent with the results by Xie et al. (2000) and Shen et al. (2010),

we also found that LPS increased WBC numbers in whole blood (Table 4). COR as a glucocorticoid has a potential immune-suppressive and pro-inffiammatory cytokine regulatory effect (Chen et al., 2009). Baert et al. (2005) have shown that LPS injection elevated plasma COR in broiler chickens, which is consistent with our study. IL-2 is a Th-1-associated cytokine that plays a central role in adaptive immunity and is classified as a pro-inflammatory cytokine (van Roon et al., 1995; Tang et al., 1997). In the current study, the increased pro-inffiammatory cytokine in the plasma, IL-2, was positively associated with the simultaneously increased COR (Table 5). The lysozyme present in the external secretions, and in polymorphonuclear leukocytes and macrophages, is highly active against gram-positive bacteria. The lysozyme found in the blood can degrade the glucosidic bonds in the cell wall of *Escherichia coli* and *Staphylococcus* (Zhang et al., 2005). However, LPS challenge could reduce the antimicrobial capacity in the blood as demonstrated by the decreased lysozyme activity observed in the plasma. The pro-inflammatory response induced by LPS challenge, an increased WBC, lymphocytes to WBC ratio and IL-2 concentration, were ameliorated by BA supplementation (Tables 4 and 5), which further confirmed the anti-inflammatory effect of BA. In addition, the same effect exerted by BA was also found for plasma lysozyme activity (Table 5), which in turn indicated that BA could compensate for the antimicrobial ability in those broilers under immunological stress.

The TLR family can recognize structural components unique to bacteria, fungi and viruses, and transmit signals to activate inflammatory responses (Miller et al., 2005). The ligands that are recognized by TLRs mainly include lipopeptides (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5) and CpG DNA (TLR9) (Poltorak et al., 1998; Aliprantis et al., 1999; Hayashi et al., 2001). Activation of LPS-responsive cells, such as monocytes and macrophages, occurs rapidly after LPS interacts with circulating LPS-binding protein and CD14, a glycosylphosphatidylinositol-linked cell surface glycoprotein present in soluble form in blood or as a membrane-bound form in myeloid lineage cells that is necessary for sensitive responses to LPS (Ulevitch and Tobias, 1995; Schletter et al., 1995; da Silva Correia et al., 2001). Stimulation of the extracellular domain of a TLR triggers the intracellular association of MyD88 with its cytosolic domain for the activation of NF-kappaB (Miller et al., 2005), which ultimately leads to the synthesis and release of a number of pro-inflammatory mediators, including IL-1, IL-6 and tumor necrosis factor (Chow et al., 1999). In our study, we found that LPS challenge increased mRNA abundances of *TLR4*, *IFN-γ*, *IL-1β* and *IL-6* in the spleen (Table 6). Our result was in accordance with the report by Liu et al. (2015) in which LPS increased mRNA expression of *TLR4*, *IL-1β* and *IL-6* in jejunal mucosa of chickens. We suggest that enhanced pro-inflammatory cytokine mRNA could be due to the increases in *TLR4* mRNA, rather than *MyD88* and *CD14*. This conclusion is based on observations by others (Liu et al., 2015), in which the increased *TLR4* and pro-inflammatory cytokine mRNA in the jejunum of chickens induced by LPS was not accompanied by elevated *MyD88* mRNA.

The BA isolated from the soils of North East Himalayas has been shown to reduce the protein and mRNA levels of pro-inffiammatory cytokines such as tumor necrosis factor and *IL-1β* in the colon of dextran sulfate induced colitis mouse model (Hairul Islam et al., 2011). Similarly, a decreased *IL-1β* mRNA expression was observed in the spleen of broilers fed a BA diet (Table 6). It has been shown that surfactin lipopeptides produced by BA have anti-inffiammatory properties as demonstrated by reducing *CD 40*, *54* and *80* and major histocompatibility class II expression on macrophages, inhibiting nitric oxide production in antigen-presenting cells and suppressing activation of $CD4^+$ T cells (Eun et al., 2008). Gao et al. (2014) recently showed that oral administration of a surfactin lipopeptide originating from a BA culture could promote the production of *IL-10* in the splenocytes of NOD mice. In the current study, we found that BA treatment also promoted the synthesis of *IL-10* mRNA in the spleen of LPS-challenged broilers (Table 6). The *IL-10* is an anti-inflammatory cytokine, which inhibits many pro-inflammatory cytokines including *IL-2* (de Waal Malefyt et al., 1993) and *IL-6* (Kambayashi et al., 1995). Thus, the decrease in splenic *IL-2* and *IL-6* mRNA in the LPS-challenged broilers fed a BA diet may result from elevated *IL-10* mRNA.

In conclusion, the results obtained in this study show that BA supplementation could exert protective effects on the growth performance and immune function of broilers under immunological stress induced by LPS challenge from *Escherichia coli*.

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