Dietary L-theanine alleviated lipopolysaccharide-induced immunological stress in yellow-feathered broilers

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Abstract
L-theanine, a natural nonprotein amino acid with a high biological activity, is reported to exert anti-stress properties. An experiment with a 3 × 2 factorial arrangement was conducted to investigate the effects of dietary L-theanine on growth performance and immune function in lipopolysaccharide (LPS)-challenged broilers. A total of 432 one-day-old male yellow-feathered broilers were randomly assigned to 3 dietary treatments (control, antibiotic and L-theanine diets) with 2 subgroups of each (6 replicate cages; 12 birds/cage). Birds from each subgroup of the 3 dietary treatments were intra-abdominally injected with the same amount of LPS or saline at 24, 25, 26 d of age. Both dietary L-theanine and antibiotic improved (P < 0.05) the growth performance of birds before LPS injection (d 1 to 21). The effect of dietary L-theanine was better (P < 0.05) than that of antibiotic. Lipopolysaccharide decreased feed intake (FI) and body weight gain (BWG) from d 22 to 28 (P < 0.05), BWG and feed to gain ratio (F:G) from d 29 to 56 (P < 0.05), increased mortality in different growth periods (P < 0.05), elevated the levels of serum cortisol, α1-acid glycoprotein (α1-AGP), interleukin-6 (IL-6) on d 24 and 25 (P < 0.05), reduced immune organ indexes and contents of jejunal mucosal secretory immunoglobulin A (sIgA) on d 28 (P < 0.05). The decreased FI and BWG, as well as increased F:G and mortality in LPS-challenged birds, were alleviated by dietary L-theanine or antibiotic from d 29 to 56. Dietary L-theanine mitigated the elevated serum α1-AGP level on d 25, serum IL-6 concentration on d 24 and 26, and the decreased jejunal mucosal sIgA content on d 28 of the LPS-challenged birds. The results indicated that L-theanine had potential to alleviate LPS-induced immune stress in broilers.

1. Introduction
The modern broiler industry has been greatly expanding to meet increasing requirement of growing population (Niu et al., 2015). However, various factors, such as pathogenic microorganisms, heavy vaccinations, drug abuse and other external elements in the intensive production system will attack birds to induce the loss of immune homeostasis and trigger immune stress response directly or indirectly (Liu et al., 2015). Under stress, chickens may be vulnerable to suffer from enteric diseases, which will affect their intestine health and growth performance, inflicting huge financial losses to poultry production (Yang et al., 2011; Liu et al., 2014).

Traditionally, utilization of in-feed antibiotics can enhance the animal growth via maintaining intestinal structure and function (Broom, 2018), regulating intestinal flora and controlling gut pathogens (Looft et al., 2014), and direct modulating immune system (Pomorskamol & Pejsak, 2012). Nevertheless, the European Union and some other countries have forbidden the use of antibiotics in livestock feed according to consideration of antibiotics.
resistance causing negative effects on human health (Huyhebaert et al., 2011). It is reported that regulation of immune system in birds by certain nutrients or antibiotic-free substances appears to be a way to avoid or lower use of antibiotics (Takahashi, 2012). Therefore, development of safe and effective nutritional additives to modulate the immune function is of great significance to protect chickens from immune stress (Liu et al., 2015).

L-theanine, first isolated from green tea leaves in the late 1940s, is a unique natural nonprotein amino acid accounting for more than 50% of the total free amino acids in green tea leaves (Sakato, 1949; Vuong et al., 2011; Mu et al., 2015). Now it is commonly thought that L-theanine is only detected in Camellia sasanqua, camellia japonica, Camellia oleifera and Xerocomus badius (Wen et al., 2012). L-theanine exists in nature with a relatively high biological activity, while synthetic theanine is normally prepared as a racemic mixture of L- and D-forms (Desai et al., 2005). L-theanine is usually used as a therapeutic or medicinal agent and additive in consumer products. Most of studies in the bio-medical field demonstrated that L-theanine had nervous regulation and protection effect (Sumathi et al., 2015), antioxidative properties (Wang et al., 2014), anti-stress response (Kimura et al., 2007), and immune regulation (Kurihara et al., 2013; Li et al., 2016). However, there are limited reports on application of L-theanine in livestock animals.

In the present study, we used lipopolysaccharide (LPS), a potent activator of innate immune response from the outer membrane of Gram-negative bacteria (Li et al., 2015a), to induce immunological stress in broilers. Then, we investigated the growth performance, blood parameters, immune organ indexes and jejunal mucous secretory immunoglobulin A (sIgA) in broilers supplemented with or without L-theanine to clarify the effect of L-theanine on regulation of immunological stress response and growth performance of broilers.

2. Materials and methods

2.1. Animals, management and experimental design

All animal protocols for this study were approved by the Hunan Agricultural University Animal Ethics Committee (Changsha, China). A total of 432 one-day-old male WENS yellow-feathered broilers purchased from a commercial hatchery (WENS Co., Ltd., Guangdong, China) were randomly allotted to 3 dietary groups and each group consisted of 2 subgroups. Each subgroup included 6 replicate cages with 12 birds per cage. There is no difference for the weight of broilers among all cages. Birds in 3 groups received a corn-soybean meal basal diet in mash form or a basal diet supplemented with antibiotics (200 mg/kg colistin sulfa for d 1 to 21, and 150 mg/kg for d 22 to 56) or 800 mg/kg L-theanine. At 24, 25 and 26 d of age, birds from each subgroup of the 3 dietary treatments were intra-abdominally injected with 0.2 mL sterile saline or LPS (Escherichia coli) serotype O127:88; Sigma–Aldrich Inc., St. Louis, MO, USA) dissolved in saline at adequate doses of 600 μg/kg BW. Therefore, birds were divided into 1 of 6 treatments (birds fed control, antibiotic, or L-theanine diets; LPS-challenged birds fed basal, antibiotic, or L-theanine diets) (Fig. 1). The corn-soybean basal diet was formulated to satisfy or exceed the NRC (1994) recommendations for the birds during the starter (d 1 to 21) and grower-finisher (d 22 to 56) period (Table 1). L-theanine (>90% purity measured by HPLC) was gifted from National Research Center of Engineering Technology for Utilization of Functional Ingredients from Botanicals (Changsha, China). The feeding trial was carried out in experimental chicken house affiliated to College of Animal Science and Technology (Changsha, China) for 56 d. All birds were reared in an environmentally controlled with triple-layer wire battery cages (150 cm × 145 cm × 70 cm) and had ad libitum access to feed and fresh water. The room temperature was maintained about 34 °C for the first week and then gradually reduced by 2 °C per consecutive week until it reached 24 °C. Continuous light system was provided in the house and the room relative humidity was kept between 50% and 65%. Bird per cage was weighed at 08:00 on d 1, 22, 29 and 57. Feed intake (FI) on a cage basis was recorded at 21, 28 and 56 d of age to calculate body weight gain (BWG), FI and feed:gain ratio (F:G). Mortality was recorded daily and expressed as percentage.

2.2. Sample collection

One bird per replicate was randomly selected to collect about 5 mL of blood from brachial vein using 5-mL vacuum tubes on d 24, 25 and 26 at 2 h after LPS or saline injection. Serum samples were detached through centrifugation at 2, 000 × g for 10 min at 4 °C, and then the supernatant was dispensed into 1.5-mL Eppendorf tubes and stored at −20 °C until analyzed. At 28 and 56 d of age, one chick per cage was randomly chosen to collect serum samples according to the above methods, and then slaughtered to isolate the mid-sections of jejunum. The jejunal contents were flushed with saline to scrape the mucosa, which was placed in 1.5-mL Eppendorf tubes and stored at −80 °C for further detection. Besides, the bursa, thymus and spleen of each bird were excised and weighed to calculate the indexes of immune organ with the following formula: Relative organ weight (%) = (Organ weight/Body weight) × 100.

2.3. Measurements of serum index and jejunal mucous sIgA contents

The concentrations of cortisol (CORT), α1-acid glycoprotein (α1-AGP) and interleukin-1β (IL-1β) in serum were measured with ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, Hubei, China) according to the manufacturer’s manual. In brief, standards or sera were added to the appropriate microtiter plate wells with horseradish peroxidase (HRP) conjugated antibody specific to CORT, α1-AGP or IL-1β. The competitive inhibition reaction was launched between pre-coated CORT, α1-AGP or IL-1β and these in serum. The substrate 3,3,5,5-tetramethyl benzidine solution (TMB) was added to the wells and the color was developed. Samples were arranged in triplicates and the average value of each sample was used for analysis. The absorbance was read at a wavelength of 450 nm by a multiskan spectrum microplate spectrophotometer (Thermo Fisher Scientific [China], Co., Ltd, Shanghai, China) within 5 min after adding the stop solution.

The serum concentration of interleukin-6 (IL-6) was detected with a chicken sandwich ELISA kit. Briefly, IL-6 existed in serum or standards was captured by chicken antibody specific for IL-6 pre-coated onto a microplate. Then, the captured IL-6 was bound to biotin-conjugated antibody labeled with HRP. The addition of substrate TMB triggered a chromogenic enzymatic reaction catalyzed by HRP and the color developed in proportion to the amount of IL-6 in the serum. The OD values were read at 450 nm with a multiskan spectrum microplate spectrophotometer within 5 min after adding the stop solution. The detection range of IL-6 is between 15.6 and 1,000 pg/mL.

The content of jejunal mucous sIgA were measured in accordance with instructions introduced by a competitive inhibition chicken ELISA kit. The principle of the assay is similar with that of CORT, α1-AGP or IL-1β. Mucous samples were vortexed and centrifuged at 4,000 r/min for 10 min. Then, the supernatant was collected and diluted 1:1,000 before the assay. On the basis of the...
manufacturer, the detection range of sIgA is between 0.234 and 60 μg/mL.

2.4. Statistical analysis

Data were analyzed using the GLM procedure of SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for a 3 × 2 factorial arrangement: 3 diets (control, antibiotics, L-theanine diets) × 2 immune stress (saline and LPS). The main effects of dietary treatments and immune stress, as well as their interactions were determined. Differences among the treatment mean values were assessed by ANOVA using Duncan’s multiple comparisons test. Statistical significance was determined at a probability level of 0.05.

Table 1

| Item                      | d 1 to 21 | d 22 to 56 |
|---------------------------|-----------|------------|
| Ingredients, %            |           |            |
| Corn                      | 59        | 61         |
| Wheat middling and reddog | 3.9       | 4          |
| Soybean meal              | 25.4      | 22         |
| Fish meal                 | 2         | –          |
| Soybean oil               | 1.8       | 1.4        |
| Cottonseed protein        | 4         | 7.8        |
| Dicalcium phosphate       | 1.2       | 1.1        |
| Limestone                 | 1.4       | 1.4        |
| Sodium chloride           | 0.3       | 0.3        |
| Premix 1                  | 1         | 1          |
| Total                     | 100       | 100        |

Nutrient levels

Metabolizable energy, MJ/kg 12.42 12.31
Crude protein              21.2 20.45
Lysine                     1.22 1.11
Methionine                 0.49 0.43
Methionine + Cystine       0.86 0.81
Calcium                    0.95 0.81
Total phosphorus           0.64 0.58

1 Premix provided per kilogram of complete feed: vitamin A 1,200 IU; vitamin D3 2,500 IU; vitamin E 20 mg; vitamin K3 3.0 mg; vitamin B6 3.0 mg; vitamin B12 0.03 mg; pantothenic acid 20.0 mg; niacin 50.0 mg; biotin 0.1 mg; folic acid 1.5 mg; Cu 8.0 mg; Fe 100 mg; Mn 100 mg; Zn 75.0 mg; I 0.7 mg; Se 0.4 mg.
2 Nutrient levels were calculated values.

3. Results

3.1. Growth performance

As shown in Table 2, birds in L-theanine group had higher FI, BW, and lower F:G than those in control and antibiotic groups (P < 0.05) before LPS challenge (d 1 to 21). Birds injected with LPS appeared a lower FI and BW on d 22 to 28 (P < 0.05), a decline in BW and F:G on d 29 to 56 (P < 0.05), and more mortality in each recorded stage (P < 0.05), when compared with the saline-treated broilers. Dietary L-theanine or antibiotic alleviated the decreased FI and BWG, as well as the increased F:G and mortality in LPS-challenged birds on d 29 to 56 and d 1 to 56. Furthermore, there was an LPS × diet interaction for FI on d 29 to 56 and d 1 to 56 (P < 0.05).

3.2. Serum parameters

As shown in Table 3, LPS increased the serum CORT, α1-AGP, IL-6 concentrations on d 24 and 25 (P < 0.05). There was a LPS × diet interaction for serum α1-AGP content (P < 0.05), as evidenced by a mitigation of the elevated serum α1-AGP level in birds injected with LPS on d 25 by L-theanine addition. Dietary antibiotic and L-theanine alleviated the increased serum IL-6 concentration on d 24 and 26 (P < 0.05).

3.3. Immune organ index

Lipopolysaccharide reduced (P < 0.05) the relative weights of bursa, thymus and spleen in birds on d 28 (Fig. 2A). Dietary antibiotic or L-theanine addition exerted no influence (P > 0.05) on relative organ weights.

3.4. Jejunal mucous secretory immunoglobulin A content

Jejunal mucosal sIgA content in the LPS-challenged birds decreased (P < 0.05), but L-theanine addition could retard the downtrend on d 28 (Fig. 3A). Lipopolysaccharide did not make a difference in jejunal mucosal sIgA content (P > 0.05), but dietary...
Table 2
Effect of dietary L-theanine on growth performance, mortality of LPS-challenged broilers.

| Item | LPS | FL, g | BWG, g | F:G | Mortality, % |
|------|-----|-------|--------|-----|--------------|
|      |     | d 1 to 21 | d 22 to 28 | d 29 to 56 | d 1 to 56 |
|      |     | d 1 to 21 | d 22 to 28 | d 29 to 56 | d 1 to 56 |
| Control | − | 532.3a | 259.5 | 854.3b | 1,632.4c |
|         | + | 528.8b | 225.4 | 819.1a | 1,473.8b |
| Antibiotic | − | 566.2b | 300.2 | 1,221.3d | 2,087.6a |
|         | + | 555.5b | 229.6 | 1,175.5e | 1,920.6b |
| L-theanine | − | 589.1b | 274.3 | 708.0c | 1,564.7cd |
|         | + | 589.9b | 250.4 | 701.3c | 1,548.3cd |
| SEM |    | 2.561 | 6.852 | 17.518 | 19.598 |
|      |    | 2.514 | 6.282 | 16.598 | 18.578 |
| P-value | LPS | 0.495 | 0.004 | 0.382 | 0.834 |
|       | Diet | <0.001 | 0.356 | <0.001 | 0.017 |
|       | Interaction | 0.057 | 0.356 | <0.001 | 0.017 |

LPS — lipopolysaccharide; FI — feed intake; BWG — body weight gain; F:G — feed to gain ratio.

Within a column, different superscript letters mean significant difference (P < 0.05).

1 Control, basal diet; antibiotic, basal diet supplemented with antibiotics; L-theanine, basal diet supplemented with L-theanine.

Table 3
Effect of dietary L-theanine on serum indexes of LPS-challenged broilers.

| Item | LPS | CORT, ng/mL | α1-AGP, ng/mL | IL-1β, pg/mL | IL-6, pg/mL |
|------|-----|-------------|---------------|--------------|-------------|
|      |     | d 24 | d 25 | d 26 | d 24 | d 25 | d 26 | d 24 | d 25 | d 26 | d 24 | d 25 | d 26 | d 24 | d 25 | d 26 |
| Control | − | 5.51 | 8.114 | 9.78 | 1,735.894 | 1,577.359 | 2,297.164 | 47.4 | 55.8 | 54.02 | 48.78 | 53.89 | 30.96 | 29.32 | 28.8b | 26.01 | 27.58d |
|         | + | 6.538 | 11.276 | 14.139 | 1,864.182 | 2,014.143 | 1,769.866 | 60.54 | 61.04 | 65.4 | 51.2 | 63.71 | 53.76b | 33.94 | 30.37a | 25.51 | 25.92c |
| Antibiotic | − | 3.538 | 8.584 | 11.649 | 1,618.703 | 1,547.024 | 1,779.886 | 45.8 | 53.43 | 57.84 | 44.22 | 48.14 | 25.57b | 32.21 | 31.65a | 25.62 | 30.26b |
|         | + | 9.163 | 10.325 | 5.177 | 2,124.647 | 2,328.470 | 2,101.373 | 71.97 | 59.49 | 64.47 | 49.86 | 55.58 | 44.3b | 37.02 | 30.3a | 25.95 | 31.91b |
| L-theanine | − | 3.237 | 4.612 | 5.758 | 1,665.433 | 1,439.782 | 1,862.147 | 41.78 | 56.43 | 69.73 | 50.42 | 46.55 | 25.71d | 32.51 | 28.42b | 24.95 | 35.77a |
|         | + | 4.895 | 8.986 | 5.602 | 1,656.783 | 1,615.188 | 1,834.951 | 51.03 | 53.94 | 70.34 | 59.43 | 68.55 | 25.87ab | 37.99 | 27.14b | 26.39 | 32.53ab |
| SEM |    | 0.433 | 0.559 | 1.137 | 41.237 | 41.301 | 72.823 | 3.553 | 2.909 | 4.064 | 3.008 | 3.471 | 2.06 | 0.701 | 0.278 | 0.34 | 1.088 |
| P-value | LPS | 0.005 | 0.013 | 0.742 | 0.017 | <0.001 | 0.6 | 0.032 | 0.619 | 0.452 | 0.354 | 0.072 | 0.003 | 0.002 | 0.529 | 0.54 | 0.632 |
|       | Diet | 0.09 | 0.09 | 0.09 | 0.117 | 0.002 | 0.593 | 0.368 | 0.901 | 0.541 | 0.565 | 0.689 | 0.011 | 0.1 | <0.001 | 0.589 | 0.031 |
|       | Interaction | 0.094 | 0.636 | 0.17 | 0.051 | 0.022 | 0.083 | 0.602 | 0.804 | 0.864 | 0.905 | 0.661 | 0.077 | 0.966 | 0.07 | 0.513 | 0.649 |

LPS — lipopolysaccharide; CORT — cortisol; α1-AGP — α1-acid glycoprotein; IL-1β — interleukin-1β; IL-6 — interleukin-6.

Within a column, different superscript letters mean significant difference (P < 0.05).

1 Control, basal diet; antibiotic, basal diet supplemented with antibiotics; L-theanine, basal diet supplemented with L-theanine.

< < < means broilers treated with saline, while > > > means broilers injected with LPS.
antibiotic and L-theanine greatly increased \((P < 0.05)\) the level of jejunal mucosal sIgA (Fig. 3B) on d 56.

4. Discussion

Immune stress induced by LPS resulted in compromised growth performance of broilers (Yang et al., 2011; Haziak et al., 2014; Tan et al., 2014; Fowler et al., 2015; Zhang et al., 2017), which was mainly attributed to reallocation of nutrients. To be specific, diet nutrients were away from growth, but toward processes associated with inflammatory immune response and synthesis of various mediators such as cytokines (Liu et al., 2015; Li et al., 2015b). Besides, immunological stress easily caused intestinal structure injury (Hu et al., 2011; Li et al., 2015a), intestinal digestion and absorption disorder (Feng et al., 2012; Liu et al., 2015) and body metabolic disturbance of animal (Klasing, 2007; Liu et al., 2014). In this study, dietary L-theanine significantly improved growth performance of broilers before LPS challenge, which was similar to the results described previously (Hwang et al., 2008; Wen et al., 2012). Lipopolysaccharide decreased the BWG on d 22 to 28 and on d 29 to 56, which was possibly due to simultaneously compromised FI. Dietary L-theanine alleviated the decreased FI and BWG, as well as increased F:G and mortality in LPS-challenged birds on d 29 to 56 and d 1 to 56, indicating that L-theanine might play a protective role on the growth of infected broilers. These benefits were possibly due to anti-stress and antioxidant characteristics of L-theanine. Maintenance and improvement of anti-stress and antioxidant status in the gastrointestinal tract were good for digestion and absorption of nutrients to promote animal growth. Besides, LPS reduced the immune organ indexes of broilers on d 28 and dietary
L-theanine addition had no influence on it, which indicated that the severe immunosuppressive model of broilers was mimicked (Wu et al., 2015; Alizadeh et al., 2016).

The fluctuation of serum CORT and α1-AGP levels could reflect physiological stress of animals. Regulation of stress was closely related to the hypothalamus-pituitary-adrenal axis (HPA) (Koolhaas et al., 1999; Dickerson and Kemeny, 2004). Cortisol is a glucocorticoid hormone secreted by the adrenal cortex, which plays a vital role in relieving stress. Once animals were exposed to external stimulation, the level of serum CORT would rise and nutrients metabolism was strengthened to meet the need of normal physiological function (Nakamura et al., 1998; Liu et al., 2015). As one of acute phase proteins (APP), α1-AGP is mainly synthesized by the liver and particularly participates in immune defensive function. It was reported that the level of serum α1-AGP would increase under acute inflammation (Nakamura et al., 1998). In the present study, the serum CORT and α1-AGP concentrations elevated after LPS challenge, suggesting that broilers suffered from stress and acute inflammation, which provided an evidence for growth inhibition in LPS-challenged broilers. Mitigation the elevated serum α1-AGP level in birds injected with LPS by L-theanine addition on d 25 might be correlated with its anti-stress property. Some investigations showed that L-theanine could pass through the blood–brain barrier to participate in neuromodulation and reduce psychological and physiological stress responses (Juneja et al., 1999; Kimura et al., 2007; Cho et al., 2008; Haskell et al., 2008; Yoto et al., 2012).

Immune stress is often associated with the inflammation response, and cytokines released in the inflammation response could promote metabolism and suppress anabolism of carbohydrate, protein and fat to inhibit growth of animals (Klasing, 1988; Johnson, 1997; Webel et al., 1997; Sijben et al., 2001; Hwang et al., 2008). Pro-inflammatory cytokines such as IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) originated from macrophages were the major cellular messengers initiating metabolic cascade alterations following the innate immune response (Jiang et al., 2015). Lipopolysaccharide was demonstrated to increase pro-inflammatory
cytokines secretion and suppress animal growth (Takahashi, 2012; Tan et al., 2014; Li et al., 2015b). Results of the present study results showed that serum IL-1β, IL-6 levels of broilers significantly increased after LPS injection, and then gradually back to the previous level, which indicated that broilers began to adapt immunological stress and metabolic function gradually recovered to normal. The increased serum IL-6 concentration on d 24 and 26 were alleviated by L-theanine suggested that dietary L-theanine could mit inflammation response.

Gastrointestinal tract of broilers is sensitive to various external antigens, and the intestinal mucosa plays an important role in gut barrier function (Yang et al., 2011). Secretory immunoglobulin A is the main antibody present at mucosal surfaces, which have long been considered as a first line of defense via providing passive immune protection against invading antigens and can protect the intestinal epithelium from enteric pathogens and toxins (Mantis et al., 2011). Immune stress could cause intestinal injury and affect intestinal immune function (Glaser and Kiecolt-Glaser, 2005). Results of the present study research showed that L-theanine addition could retard the decreased jejunal mucosal slaG contents in the LPS-challenged broilers on d 28 and elevated the level of jejunal mucosal slaG on d 56. This might be connected with a report by Wen et al. (2012) that L-theanine improved intestinal immune function of broilers via γδT cell-mediated humoral immune regulation.

5. Conclusion

Results from present study indicate that L-theanine could exert a protective role in the growth and immune function of LPS-challenged broilers.

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