Trans-anethole exerts protective effects on lipopolysaccharide-induced acute jejunal inflammation of broilers via repressing NF-κB signaling pathway

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ABSTRACT This study aimed to explore the effects of *trans*-anethole (**TA**) on lipopolysaccharide (**LPS**)-induced acute jejunal inflammation model of broilers. A total of 160 one-day-old broilers (male; Arbor Acres) were randomly allocated into four treatment groups with 8 replicates of 5 birds each. On d 20, the dose of 5 mg/kg body weight LPS solution and the equal amount of sterile saline were intraperitoneally injected into LPS-challenged and unchallenged broilers, respectively. Compared with the control group, LPS decreased ($P < 0.05$) the villus height (**VH**) and the ratio of villus height to crypt depth (**VCR**) but increased ($P < 0.05$) the crypt depth (**CD**), meanwhile, enhanced ($P < 0.01$) the levels of interleukin-6 (**IL-6**), interleukin-1beta (**IL-1β**) and tumor necrosis factor-alpha (**TNF-α**) but decreased ($P < 0.01$) the level of interleukin-10 (**IL-10**). The group supplemented with 600 mg/kg of TA had lower ($P < 0.01$) CD and higher ($P < 0.01$) VCR than the LPS group. TA increased ($P < 0.01$) the level of IL-10 and decreased ($P < 0.01$) the level of IL-1β. The mRNA expression levels of *IL-6*, nuclear factor kappa B (***NF-κB***), *TNF-α* were up-regulated ($P < 0.05$) and the levels of *IL-10* and inhibitor of NF-κB alpha (***IκBα***) were down-regulated ($P < 0.05$) by LPS as compared with the control group. TA down-regulated ($P < 0.05$) the increased mRNA expression levels of genes caused by LPS, as well as up-regulated ($P < 0.05$) the levels of *IL-10* and *IκBα*. Furthermore, LPS down-regulated ($P < 0.05$) and up-regulated ($P < 0.05$) the protein expression levels of IκBα and NF-κB p65, respectively. TA up-regulated ($P < 0.05$) the level of IκBα and down-regulated ($P < 0.05$) the level of NF-κB p65. The conclusion of this study is that TA could exert protective effect on the LPS-induced acute jejunal inflammation of broilers via repressing the activation of NF-κB and the 600 mg/kg is the optimal dose against LPS-induced acute jejunal inflammation of broilers.

Key words: *trans*-anethole, acute inflammation, intestine, lipopolysaccharide, broiler, NF-κB signaling pathway

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INTRODUCTION

A former publication has elucidated a summary of the intestinal inflammation phenotypes of poultry that are categorized as physiological inflammation, pathological inflammation, metabolic inflammation and sterile inflammation (Kogut et al., 2018). The physiological inflammation is a primal and acute phase reaction to the disturbed balance between intestinal microbes and intestinal tolerance characterized by the increased intestine permeability, which occurs specifically following the release of virulence factors from dying bacteria (Abraham and Medzhitov, 2011). However, once the virulence factors such as lipopolysaccharide (**LPS**) are out of control, the physiological inflammation could be transformed to pathological inflammation and chronic inflammation, which ultimately leads to tissue damage and even organ failure (Barton, 2008). The LPS, an endotoxin existing in the gut, emerges from the gram-negative bacteria and consists of O-antigen, core oligosaccharide domain and lipid A. The lipid A is a conserved region responsible for the LPS recognition by the pattern recognition receptors (**PRRs**) including toll-like receptors (**TLRs**) (Bidne et al., 2018). Numerous studies have confirmed that the LPS is mainly recognized by toll-like receptor 4 (**TLR4**), which can induce the production of proinflammatory cytokines such as interleukin-6 (**IL-6**) through activating the NF-κB signaling pathway (Sanjabi et al., 2000; Lai et al., 2017; Tang et al., 2021).

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Trans-anethole (**TA**), as a major active component of essential oil that extracted from various herbal plants such as *Illicium verum*, has been confirmed to possess multiple functions including antioxidant, antifungal, anti-inflammatory and gastroprotective (Domiciano et al., 2013; Luís et al., 2019; Sharafan et al., 2022). In addition, the preparations of TA have been used in cosmetic, food and medical industries for TA is identified as a safe compound by the Expert Panel of the Flavour and Extract Manufacturer Association (**FEMA**) (Newberne et al., 1999). TA is reported to have an aromatic odor and a sweet taste thus contributing to being the constitute of perfume and spice. However, the visible light and high temperature can transform TA to its isomer *cis*-anethole which is toxic, but the cis-form has a negligible amount in essential oil (Aprotosoaie et al., 2016). Although the majority of previous studies have shown the potent anti-inflammatory effect of TA and its mechanisms on mammals (Ponte et al., 2012; Kim et al., 2017; Samadi-Noshahr et al., 2021a), few studies have elucidated the anti-inflammatory effect of TA and its mechanism on poultry. Further, the NF-κB signaling pathway has attracted the interest of most researchers for decades, which is also confirmed to be repressed by the administration of TA in the inflammatory models of animals (Feng et al., 2019). Hence, we hypothesized that TA could show protective effects on the LPS-induced acute jejunal inflammation of broilers via repressing the NF-κB signaling pathway.

MATERIALS AND METHODS

Preparation of Materials

TA was purchased from Nanjing Dilger Medical Technology Co., Ltd (Nanjing, China), whose purity was 98.35%. The storage method of TA was keeping in glass bottles avoiding light at 4°C. The doses of TA in the experiment were referred to the earlier study (Yu et al., 2021). LPS (O55:B5) was purchased from Sigma-Aldrich Chemical Inc. (#L2880, St. Louis, MO), dissolved with equal proportion in 0.86% (w/v) sterile saline when used for administration, the dosage of which was referred to previous studies (Zhang et al., 2020; Chen and Yu, 2021).

Animals and Experimental Design

The experiment was performed by raising 160 one-day-old broilers (male; Arbor Acres) following the protocols approved by the Institution of Animal Care Committee of Nanjing Agriculture University. The healthy birds with similar weight were obtained from Yantai Land Animal Husbandry Co., Ltd (Yantai, China) and randomly allocated into 4 treatment groups with 8 replicates of 5 birds each. Four treatment groups were classified as control group (**CON**), lipopolysaccharide group (**LPS**), lipopolysaccharide group plus 400 mg/ kg *trans*-anethole (LPS + TA400) and lipopolysaccharide group plus 600 mg/kg *trans*-anethole (LPS + TA600). The CON and LPS groups were fed with basal diet which was formulated to meet the nutrient requirements of broilers following the Feeding Standard of chicken of the People's Republic of China (NY/T 33-2004), the other groups were fed basal diet supplemented with 400 or 600 mg/kg TA, respectively. The ingredients and nutrient composition of the basal diet are shown in Table 1. On d 20, the dose of 5 mg/kg body weight (**BW**) LPS solution and the equal amount of sterile saline were intraperitoneally injected into LPS-challenged broilers and unchallenged broilers, respectively. Birds were kept in a house with controllable temperature and humidity, and had free access to water and feed. The duration of the experiment was 21 d. The growth performance before LPS administration has shown in our previous study (Tong et al., 2022), which exhibited that TA at the dose of 600 mg/kg had higher average daily feed intake (**ADFI**) of broilers compared with other groups.

Table 1. Ingredients and nutrient composition of the basal diet[1] (%, as fed-basis).

Ingredient	%	Nutrient levels[1]	%
Corn	55.60	Metabolizable energy, Mcal/kg	2.87
Expanded soybean meal	29.00	Crude protein	20.95
Cottonseed meal	2.50	Total calcium	0.96
Wheat flour	4.00	Total phosphorus	0.66
Hydrolyzed feather meal	1.50	Total lysine	1.11
Soybean oil	2.00	Total methionine	0.35
Dicalcium phosphate	0.90	Total threonine	0.82
Limestone	1.50		
Bentonite	1.00		
Premix[2]	2.00		
Total	100.00		

[1]All nutrient levels were analyzed values, except metabolizable energy.
[2]Supplied per kilogram of diet: vitamin A, 11,500 IU; cholecalciferol, 3,500 IU; vitamin E, 30 mg; vitamin K_3, 5 mg; thiamin, 3.38 mg; riboflavin, 9.0 mg; pyridoxine, 8.96 mg; vitamin B_{12}, 0.025 mg; choline chloride, 800 mg; calcium pantothenate, 13 mg; niacin, 45 mg; biotin, 0.15 mg; folic acid, 1.20 mg; Mn, 60 mg; Fe, 66.5 mg; Zn, 88 mg; Cu, 8.8 mg; I, 0.70 mg; Se, 0.288 mg.

Sample Collection

On d 21, eight broilers per group were chosen randomly to sacrifice with cervical dislocation. The segments about 1.5 cm cut from the middle of jejunum were fixed in 4% paraformaldehyde for morphological measurement. After that, the segments about 3 cm of jejunum were dissected and the remaining digesta of the segments were rinsed by precooling sterile saline. The segments were collected into sterile tubes and then stored in liquid nitrogen for further analyses.

Morphological Measurement of Jejunum

Paraformaldehyde-fixed segments of jejunum were dehydrated in xylene, embedded in paraffin and sectioned (5 μm) with a microtome for hematoxylin-eosin (**H&E**) staining. The villus height (**VH**) and crypt depth (**CD**)

Table 2. Gene-specific primers sequences for quantitative real-time PCR.

Gene name[1]	GenBank[2]	Primer sequence[3] (5′→3′)	Length
IL-2	AY510091.1	TGCAGTGTTACCTGGGAGAAG TTGCATTCACTTCCGGTGTG	148
IL-4	AJ621249.1	AGCCTCCACAATTGTTTGGG TGAAGTAGTGTTGCCTGCTG	139
IL-6	AB302327.1	AACAACCTCAACCTGCCCAA AGGTCTGAAAGGCGAACAGG	112
IL-8	DQ393272.2	CCTCCTCCTGGTTTCAGCTG TGGCGTCAGCTTCACATCTT	136
IL-10	NM_012854.2	CAGACCAGCACCAGTCATCA TCCCGTTCTCATCCATCTTCTC	96
IL-1β	NM_204524.2	TTTTTGAGCCCGTCACCTTC AGCACTTCTGGTTGATGTCG	111
NF-κB	NM_001012887.2	AAGATCTGGTGGTGTGCCTG AGTGGAACCTTTCGCGGATT	137
IκBα	NM_001001472.2	CAGCACTACACTTGGCCGTA GGAGTAGCCCTGGTAGGTCA	101
TNF-α	HQ739087.1	GAACCCTCCGCAGTACTCAG AACTCATCTGAACTGGGCGG	116
TLR4	KP410249.1	CGGCTCCGCATCTTGGATAT GGGCTTGGAGTGGCTTGTAT	148
IFN-γ	NM_205149.1	TGTAGCTGACGGTGGACCTA GCGGCTTTGACTTGTCAGTG	134
β-Actin	NM_205518.1	ACCGGACTGTTACCAACACC CCTGAGTCAAGCGCCAAAAG	116

[1]IL, interleukin; *NF-κB*, nuclear factor kappa B; *IκBα*, inhibitor of NF-κB alpha; *TNF-α*, tumor necrosis factor-α; *TLR4*, toll-like receptor 4; *IFN-γ*, interferon-gamma.
[2]GenBank Accession Number.
[3]Shown as the forward primer then the reverse primer.

were observed using light microscope (Olympus CX31, Tokyo, Japan), and then the length of ten villi and crypts in each section were measured by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD). The results of measurement were used to calculate the ratio of villus height to crypt depth (**VCR**).

Determination of Inflammatory Cytokines

The levels of IL-6, interleukin-10 (**IL-10**), interleukin-1beta (**IL-1β**), and tumor necrosis factor-alpha (**TNF-α**) in the jejunum were determined by ELISA kits (Jiangsu Meimian Industry Co., Ltd, Yancheng, China) according to the manufacturer's instructions.

Extraction of RNA and Fluorescence Quantitative Real-Time PCR Analysis

The total RNA was extracted from jejunum samples using Trizol reagent (Nanjing Vazyme Biotech Co., Ltd, China) following the manufacturer's instructions. The extractive RNA was used to determine the purity and concentration using a spectrophotometer (NanoDrop Products, Wilmington, DE) and the concentration of RNA was unified using DNase/RNase-free ddH_2O (Beyotime Biotechnology, Shanghai, China) to 500 ng/mL for reverse transcription. The reverse transcription reaction of RNA was performed with the EasyScript All-in-one First-Strand cDNA Synthesis SuperMix for quantitative real-time qPCR products (TransGen Biotech, Beijing, China). The fluorescence quantitative real-time PCR of cDNA was performed with PerfectStart Green qPCR SuperMix (TransGen Biotech, Beijing, China) based on Applied Biosystems QuantStudio 7 Flex apparatus. The mRNA expression levels of target genes relative to *β-actin* were calculated using $2^{-\triangle\triangle CT}$ method. The primer sequences of target genes and housekeeping gene are shown in Table 2.

Western Blot Analysis

Four replicates per group were randomly selected to perform western blot analysis in order to determine the protein expression level related to NF-κB signal pathway in the jejunum. Nuclear proteins were extracted from the whole proteins using the Nuclear Protein Extraction Kit (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) following the manufacturer's instructions. The concentrations of nuclear and cytoplasmic proteins were detected using the Bicinchoninic Acid (**BCA**) Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions. After unifying the concentration of protein to 5 ng/mL, adding the SDS-PAGE Sample Loading Buffer (5X; Beyotime Biotechnology, Shanghai, China) into the protein and then denaturalizing the protein at 95°C for 5 min. The protein gel was prepared using the 12.5% PAGE Gel FAST Preparation Kit (Epizyme Biomedical Technology Co., Ltd, Shanghai, China) following the manufacturer's instructions, on which the protein samples and protein marker were loaded for protein electrophoresis. After that, the target gels were separated from the whole protein gel and transferred to the polyvinylidene fluoride (**PVDF**) membranes at 4°C. The membranes of target proteins were closed in blocking buffer for 1.5 h, then incubated overnight at 4°C with the primary antibodies against nuclear

Table 3. Effects of *trans*-anethole on jejunal morphology of LPS-challenged broiler.[1]

Items[2]	CON	LPS	LPS + TA400	LPS + TA600	*P*-value
VH (μm)	1031.34 ± 35.03[a]	936.35 ± 30.11[b]	938.81 ± 29.85[b]	883.25 ± 25.80[b]	0.016
CD (μm)	214.98 ± 10.60[b]	252.36 ± 8.42[a]	240.28 ± 11.56[a,b]	184.01 ± 9.92[c]	<0.001
VCR (μm/μm)	4.87 ± 0.27[a]	3.73 ± 0.15[b]	3.99 ± 0.27[b]	4.88 ± 0.24[a]	0.002

[a-c]The values in the same row with different superscripts means significantly different ($P < 0.05$).

[1]The values are represented as mean ± SEM, n = 8.

[2]VH, villus height; CD, crypt depth; VCR, villus height to crypt depth ratio. CON, control group fed with basal diet and treated with saline; LPS, group fed with basal diet and treated with lipopolysaccharide; LPS+TA400, group fed with 400 mg/kg *trans*-anethole and treated with lipopolysaccharide; LPS + TA600, group fed with 600 mg/kg *trans*-anethole and treated with lipopolysaccharide.

factor kappa B p65 (**NF-κB p65**; Proteintech Group, Inc., Wuhan, China), inhibitor of NF-κB alpha (**IκBα**; Proteintech Group, Inc., Wuhan, China), β-Actin (Affinity Bioscicences Co., Ltd, Jiangsu, China), Lamin B1 (Proteintech Group, Inc., Wuhan, China). Subsequently, the membranes were washed using Tris-buffered saline containing 0.1% Tween 20 (**TBST**) buffer for 3 times and incubated with the secondary antibody for 1.5 h. At last, putting the bands in the enhanced chemiluminescence (**ECL**) reagent (Nanjing Vazyme Biotech Co., Ltd, Nanjing, China) for the detection of protein expression level using the ChemiDoc MP Imaging System apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). β-Actin and Lamin B1 were used as an internal reference for cytoplasmic protein and nuclear protein, respectively.

Statistical Analysis

The data were analyzed using one-way ANOVA of SPSS software (ver. 21.0; IBM-SPSS, Inc., Chicago, IL) and represented as the mean ± SEM. The significant differences between groups were analyzed by the Tukey's HSD test of SPSS software. $P < 0.05$ and $P < 0.01$ means significant and highly significant differences, respectively. $P > 0.05$ means no significant differences.

RESULTS

Jejunal Morphology

As shown in Table 3, compared with the control group, LPS challenge significantly decreased ($P < 0.05$) the VH and VCR of jejunum but significantly increased ($P < 0.05$) the CD of jejunum. The group supplemented with 600 mg/kg of TA had lower ($P < 0.01$) CD and higher ($P < 0.01$) VCR than the LPS group. As shown in Figure 1, the LPS group exhibited structural deteriorations including the VH and CD, which were attenuated by dietary 600 mg/kg of TA.

Inflammatory Cytokines Levels in the Jejunum

As shown in Table 4, LPS challenge enhanced ($P < 0.01$) the levels of IL-6, IL-1β and TNF-α, meanwhile, decreased ($P < 0.01$) the level of IL-10 in the jejunum compared with the control group. However, compared with the LPS group, dietary supplementation of TA increased ($P < 0.01$) the level of IL-10 and decreased ($P < 0.01$) the level of IL-1β, but made no effect ($P > 0.05$) on the levels of IL-6 and TNF-α.

Expression Levels of Inflammation-related Genes in the Jejunum

The relative expression levels of inflammation-related genes in the jejunum of broilers are shown in Figure 2. LPS challenge up-regulated ($P < 0.05$) the jejunum mRNA expression levels of interleukin-2 (***IL-2***), *IL-6, NF-κB, TNF-α, TLR4*, and interferon-gamma (***IFN-γ***) but down-regulated ($P < 0.05$) the levels of interleukin-4 (***IL-4***), *IL-10* and *IκBα* as compared with unchallenged birds. However, there were no significant differences ($P > 0.05$) in the levels of interleukin-8 (***IL-8***) and *IL-1β* between the control group and LPS group. Compared with the LPS group, dietary supplementation of TA down-regulated ($P < 0.05$) the mRNA expression levels of *IL-2, IL-6, NF-κB, TNF-α, TLR4*, and *IFN-γ*, also up-regulated ($P < 0.05$) the mRNA expression levels of *IL-10* and *IκBα*. Furthermore, birds supplemented with 600 mg/kg TA had lower ($P < 0.05$) levels of *NF-κB, TNF-α* and *IFN-γ* than the birds supplemented with 400 mg/kg TA.

Relative Protein Expression Levels in the Jejunum

As shown in Figure 3, compared with the control group, LPS challenge down-regulated ($P < 0.05$) and up-regulated ($P < 0.05$) the relative protein expression levels of IκBα and NF-κB p65, respectively. However, compared with the LPS group, dietary supplementation of TA up-regulated ($P < 0.05$) the level of IκBα and down-regulated ($P < 0.05$) the level of NF-κB p65.

DISCUSSION

The intestinal tract has a complex immune system, despite that, a variety of commensal bacteria and fungi attach to the mucosal cells in the gut. Thus, the risks of

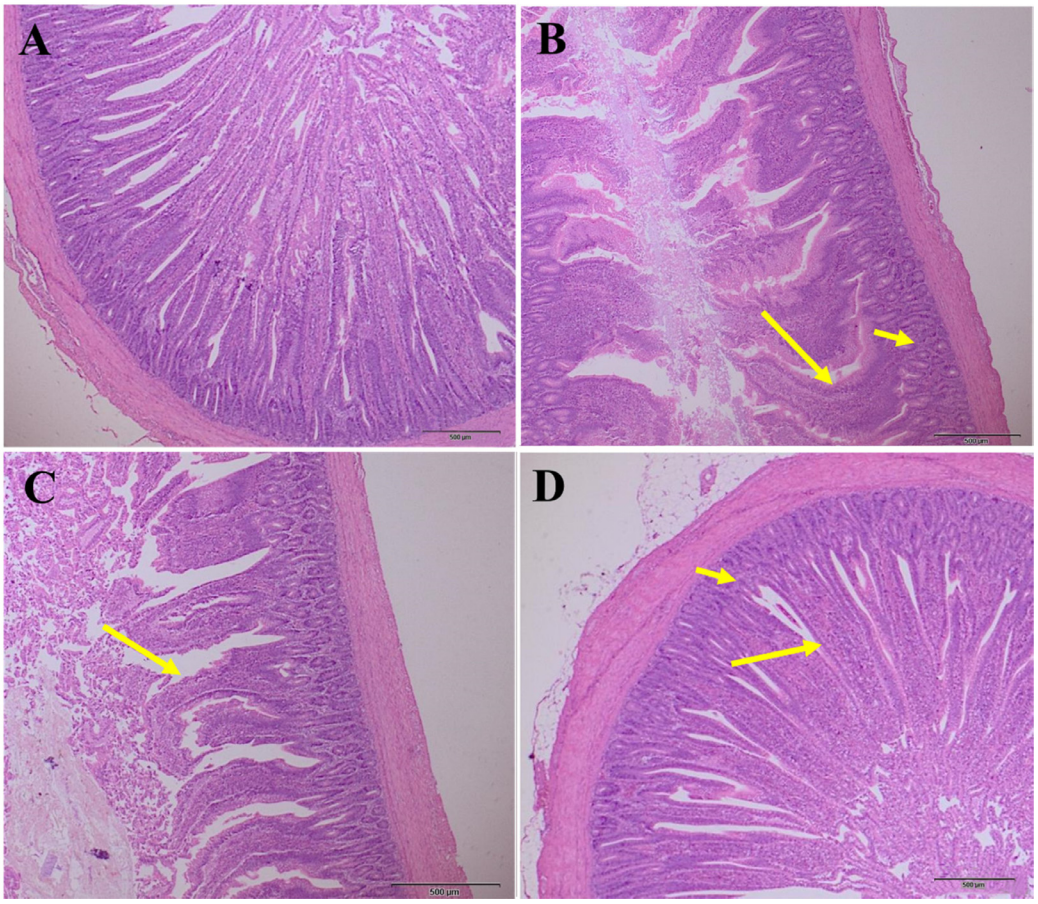

Figure 1. Effects of *trans*-anethole on jejunal morphology of LPS-challenged broilers. (A) control group fed with basal diet and treated with saline, (B) group fed with basal diet and treated with lipopolysaccharide, (C) group fed with 400 mg/kg *trans*-anethole and treated with lipopolysaccharide, (D) group fed with 600 mg/kg *trans*-anethole and treated with lipopolysaccharide.

bacterial infection increase concomitant with the death and dissolution of detrimental bacteria, such as *Escherichia coli* (Kelly et al., 2012). The LPS is located in the outermost membrane of gram-negative bacteria, which can be released into circulation when the permeability of intestine gets increased (Xing et al., 2021). The circulating LPS is commonly identified as an endotoxin that can trigger acute inflammatory response, at this time, the host is more vulnerable of the environmental stimuli and stressors, which can in turn result in chronic inflammatory diseases, endotoxemia and death (Cavaillon, 2018). In addition, Han (2002) demonstrated that the intestinal endotoxemia can further aggravate acute liver injury and even induce acute liver failure, which suggested that there is an interplay between liver and intestine on the pathogenesis of liver injury. Therefore, more and more research on quelling the LPS-induced detriments were carried out upon the animal models of mimicking endogenous LPS stimulation. Numerous studies have concluded that it is feasible to embark on suppressing the acute inflammation responded to LPS (Donovan and Grundy, 2012; Hsiang et al., 2015; Matos et al., 2021). To date, studies concerning the anti-inflammatory effect of TA have focused on the experiments of rats and cells, but few on the acute inflammation model of poultry (Kang et al., 2013; Estevao-Silva et al., 2014; Samadi-Noshahr et al., 2021b). The small intestine of chicken is short and simple consisting of duodenum, jejunum and

Table 4. Effects of *trans*-anethole on the jejunal inflammatory cytokine levels of LPS-challenged broilers.[1]

Items[2]	CON	LPS	LPS+TA400	LPS+TA600	*P*-value
IL-6, ng/g protein	29.60 ± 0.85^{b}	37.46 ± 2.65^{a}	25.00 ± 1.33^{b}	27.18 ± 1.72^{b}	<0.001
IL-10, ng/g protein	56.79 ± 1.61^{b}	50.17 ± 1.14^{c}	57.97 ± 1.25^{b}	67.97 ± 2.01^{a}	<0.001
IL-1β, ng/g protein	85.80 ± 2.65^{b}	115.70 ± 4.68^{a}	67.97 ± 4.05^{b}	84.43 ± 6.81^{b}	<0.001
TNF-α, ng/g protein	54.80 ± 1.56^{b}	86.82 ± 3.93^{a}	74.63 ± 3.71^{a}	76.52 ± 5.04^{a}	<0.001

[a-c]The values in the same row with different superscripts means significantly different ($P < 0.05$).

[1]The values are represented as mean ± SEM, n = 8.

[2]IL-6, interleukin-6; IL-10, interleukin-10; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; CON, control group fed with basal diet and treated with saline; LPS, group fed with basal diet and treated with lipopolysaccharide; LPS+TA400, group fed with 400 mg/kg *trans*-anethole and treated with lipopolysaccharide; LPS + TA600, group fed with 600 mg/kg *trans*-anethole and treated with lipopolysaccharide.

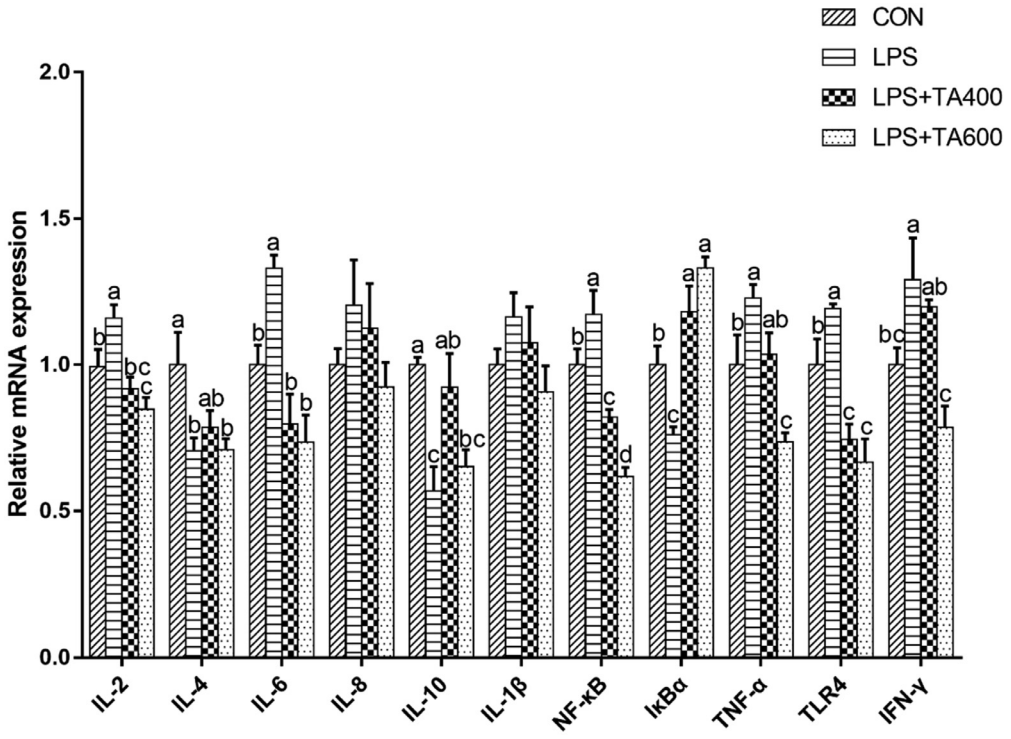

Figure 2. Effects of *trans*-anethole on the relative mRNA expression of genes related to inflammation in the jejunum of LPS-challenged broilers. The values are represented as mean (n = 8) with their standard errors. Bars with unlike letters means significantly different. Abbreviation: *IL-2*, interleukin-2; *IL-4*, interleukin-4; *IL-6*, interleukin-6; *IL-8*, interleukin-8; *IL-10*, interleukin-10; *IL-1β*, interleukin-1beta; *NF-κB*, nuclear factor kappa B; *IκBα*, inhibitor of NF-κB alpha; *TNF-α*, tumor necrosis factor-alpha; *TLR4*, toll-like receptor 4; *IFN-γ*, interferon-gamma; CON, control group fed with basal diet and treated with saline; LPS, group fed with basal diet and treated with lipopolysaccharide; LPS + TA400, group fed with 400 mg/kg *trans*-anethole and treated with lipopolysaccharide; LPS + TA600, group fed with 600 mg/kg *trans*-anethole and treated with lipopolysaccharide.

ileum. Moreover, the jejunal epithelium is an effective barrier that can prevent endotoxin from constantly enhancing the inflammatory response (Rodrigues and Choct, 2018). Our recent study has reported that TA had protective effect on acute liver inflammation of broilers (Tong et al., 2022). Similarly, the aim of this study was to investigate the influence of TA on LPS-induced acute jejunal inflammation of broilers through observing the jejunal morphology, determining the inflammatory cytokines levels and detecting the expression levels of genes and proteins related to NF-κB signaling pathway in the jejunum.

The jejunal morphology is dynamic due to its specific villus-crypt tissue structure, which plays an important role in intestinal health. The continuous turnover of villus-crypt tissue renders the gut epithelium to be a barrier (Gehart and Clevers, 2019). Thereby, decreased villus height and increased crypt depth are hallmarks of intestinal injury. Many researchers have proven that the LPS administration has negative effects on the VH and CD (Yang et al., 2014; Zheng et al., 2020; Zhang et al., 2021). In line with that, in this study, LPS-challenged broilers had not only lower VH and VCR but also higher CD than unchallenged broilers. However, dietary supplemented with 600 mg/kg of TA attenuated the variation of CD and VCR, which is in accordance with the results of Yu et al. (2022a) that TA improved the VH and VH/CD in the model of necrotic enteritis-induced broilers. It may attribute to the improved effect of TA on feed intake because of the association between the villus-crypt structure and nutrient absorption (Yu et al., 2021). It is worthwhile to mention that the CD of group supplemented with 600 mg/kg TA was much lower than that of control group, which may be due to the rate of crypt cells proliferation (Tappenden, 2014). Furthermore, we also observed that the dose of 600 mg/kg of TA had more apparent effects on CD and VCR than the dose of 400 mg/kg of TA, but there is no significant difference between broilers supplemented with 600 mg/kg and 400 mg/kg of TA on VH. It may be influenced by the sustained replenishment from crypt to villus (Gehart and Clevers, 2019).

When LPS exists in the blood circulation, the signal recognition mechanism of LPS initiates rapidly. The LPS is primarily recognized by and binds with the lipopolysaccharide-binding protein (**LBP**) secreted from liver, then, the LPS-LBP dimer binds with the cluster of differentiation 14 (**CD14**) in order to transferring the LPS to the myeloid differentiation factor 2 (**MD2**). The LPS is finally recognized by TLR4 with the help of MD2 (Fenton and Golenbock, 1998). The recognition procedure above occurs outside the cells. The TLR4 can transduce the signal into the intracellular effectors, which ultimately leads to the activation of NF-κB signaling pathway. NF-κB signaling pathway have long been researched for its complexity and multifunctional effects on inflammation and diseases such as cancer (Yu et al., 2020; Liu et al., 2022). The activation of NF-κB

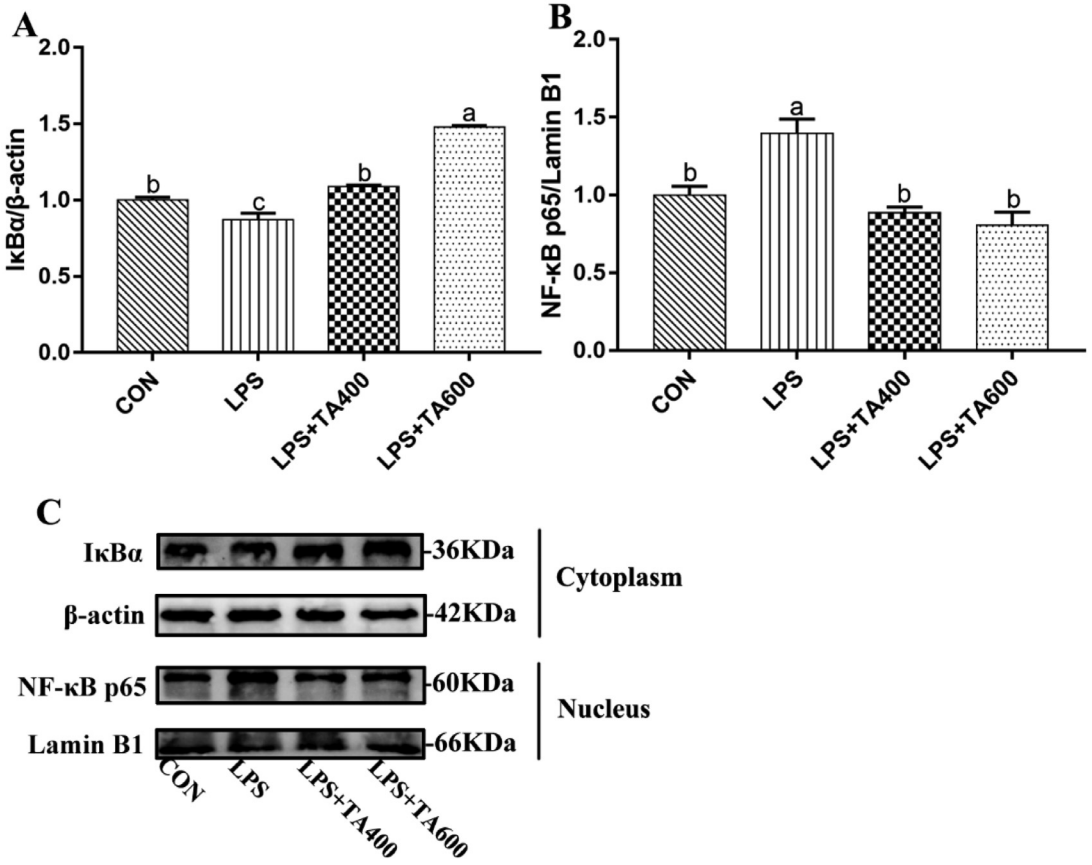

Figure 3. Effects of *trans*-anethole on the protein expression of NF-κB pathway in the jejunum of LPS-challenged broilers. The values are represented as mean (n = 4) with their standard errors. Bars with unlike letters means significantly different. Abbreviations: NF-κB p65, nuclear factor kappa B p65; IκBα, inhibitor of NF-κB alpha; CON, control group fed with basal diet and treated with saline; LPS, group fed with basal diet and treated with lipopolysaccharide; LPS + TA400, group fed with 400 mg/kg *trans*-anethole and treated with lipopolysaccharide; LPS + TA600, group fed with 600 mg/kg *trans*-anethole and treated with lipopolysaccharide.

pathway can elicit the transcription of proinflammatory genes, which is critical for inflammatory response (Jing et al., 2022). It is well-established that IL-1β, IL-6 and TNF-α are the prototypical proinflammatory cytokines rapidly released from immune cells contributing to the acute phase reaction, whereas, IL-10 and IL-4 are the cytokines against the inflammatory response by suppressing the production of proinflammatory cytokines (Wojdasiewicz et al., 2014). Furthermore, an earlier study has illustrated that the excessive IFN-γ can cause tissue damage and inflammation (Kak et al., 2018). Accordingly, the results in the current study exhibited that LPS challenge increased the levels of IL-6, IL-1β, and TNF-α, decreased the level of IL-10 in the jejunum. This is in accordance with the results of Han et al. (2020), who showed that LPS provoked the increased levels of IL-6, IL-1β, and TNF-α in the liver of broiler chickens. A former study has reported that anethole reduced the increased expression levels of IL-1β, IL-6, and TNF-α in the model of enterotoxigenic Escherichia coli-challenged piglets (Yi et al., 2021). In line with that, in this study, dietary with TA improved the decreased level of IL-10 and attenuated the increased level of IL-1β in the jejunum, but made no effect on the levels of IL-6 and TNF-α. Additionally, TA also down-regulated the mRNA expression levels of *IL-2, TLR4* and *IFN-γ* and up-regulated the level of *IL-10*. However, we noted that TA down-regulated the mRNA expression levels of *IL-6* and *TNF-α* from the results of qPCR analysis. The discrepancy between the expression level of mRNA and protein may be explained by the post-transcriptional modification of mRNA and the post-translational modification of protein (Courtney, 2021; Leutert et al., 2021).

The central components of NF-κB signaling pathway are the transcription factor NF-κB and its inhibitor protein IκB (Hayden and Ghosh, 2008). The NF-κB family is consisted of five subunits, p50, p52, p65, c-Rel and RelB. Two of them bind to form a NF-κB dimer, which is generally composed by p50 or p52 and p65, c-Rel or RelB as p65, c-Rel and RelB have the transcription activation domain responsible for the positive regulation of proinflammatory genes expression (Magnani et al., 2000). The most canonical one in the NF-κB dimers is the combination of p50 and p65, which generally binds with IκBα protein keeping inactive state in the cytoplasm. A diversity of stimuli such as LPS are capable of induing the ubiquitination of IκBα, which contributes to the release and translocation of NF-κB into the nucleus and ultimately initiating the inflammatory reaction (Lawrence, 2009; Liu et al., 2021a,b). The results of this study showed that LPS challenge down-regulated and up-regulated the mRNA expression level of *IκBα* and *NF-κB*, respectively. Similarly, the protein expression

levels of IκBα in the cytoplasm as well as NF-κB p65 in the nucleus were down- and up-regulated by LPS, respectively. These results are in agreement with the results of Ju et al. (2019), who revealed that LPS enhanced the NF-κB expression and decreased the IκBα expression in the model of acute lung injury rats. A previous study has reported that anethole decreased the level of NF-κB in myocardial infarction rats (Younis and Mohamed, 2022). In addition, another earlier study has demonstrated that anethole suppressed nuclear localization of NF-κB protein in human prostate cancer cells (Elkady, 2018). In line with them, in this study, birds dietary of TA had lower mRNA expression level of *NF-κB* and higher level of *IκBα*, simultaneously lower protein expression level of NF-κB p65 in the nucleus and higher level of IκBα in the cytoplasm of jejunum, which implied that TA could suppress the activation of NF-κB signaling pathway in the jejunum of broilers.

Taken together, TA could improve the broken jejunal morphology, reduce the production of proinflammatory cytokines via suppressing the activation of NF-κB signaling pathway in the broilers. Furthermore, the dose of 600 mg/kg is more efficient than the dose of 400 mg/kg from the observation of jejunal morphology and qPCR analysis, which may exhibit that the 600 mg/kg of TA is the optimal dose in inhibiting the inflammation of broilers. This is in agreement with the conclusion of Yu et al. (2022b), who revealed that TA administration at 600 mg/kg ameliorated subclinical NE infection of broilers more efficiently. However, much scientific work remains to be done to investigate the anti-inflammatory effect of TA on LPS-induced acute inflammation in broilers more specifically, for example, to narrow down the concentration gradient of TA.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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