Effects of dietary supplementation of trans-anethole on the intestinal antioxidant status, immune function, and liver lipid metabolism in broilers

Caiyun Yu, Tian Wang and Zaibin Yang

Nanjing Agricultural University, Weigang Campus, Nanjing, China; College of Animal Sciences and Technology, Shandong Agricultural University, Tai’an, China

ABSTRACT
This study was aimed to investigate the effects of trans-anethole (TA) on intestinal antioxidant status, immune function and liver lipid metabolism in broilers. A total of 256 1-day-old Arbour Acres broilers were allocated to 4 treatment groups with 8 birds per replicate. TA was added to diets at a concentration of 0 (control), 400, 600, and 800 mg/kg for 42 days. Dietary supplementation of TA increased the average daily feed intake (ADFI) of birds throughout the entire period. TA supplemented at 400 mg/kg of diet contributed to the lowest jejunal and ileal malondialdehyde (MDA) concentration on d 42, and the highest ileal total superoxide dismutase (T-SOD) activity on d 21 and d 42. TA supplementation at the concentration of 400 and 600 mg/kg of diet increased mRNA expression of ileal interleukin (IL)-4 on d 21, and intestinal secretory immunoglobulin A (sIgA) concentration on d 42. Supplementation of TA at the concentration up to 800 mg/kg of diet had adverse effects on intestinal antioxidant status, immune function and liver lipid metabolism compared with control group. Differently, TA supplemented at 400 mg/kg led to lower mRNA expression of liver ACC1, FASN, sterol regulatory element-binding protein 1c (SREBP-1), and higher CPT1 on d 42. In conclusion, TA inclusion improved intestinal antioxidant status and immune function, and enhanced liver lipid metabolism of broilers.

HIGHLIGHTS
- Supplementation of 400 mg/kg of TA increased intestinal antioxidant status and immune function, and promoted liver lipid metabolism of broilers compared with non-supplemented group.

Introduction

It was widely evidenced that poultry performance is directly related to gastrointestinal function and health (Attia et al. 2019; Paraskeuas and Mountzouris 2019). The intestine health not only plays a key role in nutrient absorption, but also in the development of immune status against pathogen invasion (Oakley et al. 2014). Immunoglobulin and cytokines concentration are important parameters that reflect poultry immune response to pathogens and antigens (Wang et al. 2009; Shehata et al. 2021). Antibiotics have been used in poultry diets for improving intestinal health and preventing pathogen infection for many years. However, the prohibition on antibiotics has accelerated the research on seeking suitable natural alternatives with similar beneficial effects (Gernat et al. 2021; Tellez-Isaias et al. 2021). The world population is increasing, and producing affordable, high quality, safe, and nutritious turkey meat is of utmost importance. Several options of feed additives were reported to be used in poultry production, but which product would be the best choice and the ideal dosage should be studied in detail (Tellez-Isaias et al. 2021).

Trans-anethole (TA) is a natural phenylpropanoid with an anise flavour and is present in many essential oils of medical aromatic plants of more than 20 species (e.g., fennel, anise, and star anise). TA has been generally recognised as safe by the United States Food and Drug Administration (Sheikh et al. 2015; Aprotosoaie et al. 2016). TA has been widely used as a flavour agent in foods, cakes, cosmetics, alcoholic beverage, and perfumes (Aprotosoaie et al. 2016). Its multiple activities in animal and cell line have been widely reported, such as antimicrobial (Hançer Aydemir et al. 2017).
2018; Wieczynska and Cavoski 2018), antioxidant (Sá et al. 2018, 2020), anti-inflammatory (Kim et al. 2017; Zhang et al. 2018), promoting digestion (Reyer et al. 2017), gastroprotective (Asano et al. 2016) properties. Accumulating studies also revealed the antiobesity effects of TA (Kang et al. 2018; Rhee et al. 2018; Song et al. 2020). However, to the best of our knowledge, the information on the effects of TA on intestinal antioxidant and immune function, and liver lipid metabolism in broilers is not available yet. An earlier study conducted by us revealed the effects of different concentration of star anise oil (SAO) on laying performance and antioxidant status in laying hens, the results indicated that supplementation of SAO at the level of 200 to 600 mg/kg of diet enhanced antioxidant status of laying hens (Yu et al. 2018). Previous study found that supplementation of 400 mg/kg of TA increased nutrient utilisation and intestinal barrier function of broilers (Yu et al. 2021). Despite these findings, there has been dearth of the research on the antioxidant and anti-inflammatory effects of TA in broilers. Therefore, the present study was further conducted to investigate the effects of TA on the intestinal antioxidant status and immune function, and liver lipid metabolism of broilers, and gain the ideal dosage of TA.

Materials and methods

Ethics approval

All procedures were performed in accordance of the ethical guidelines of the Animal Care and Welfare Committee of the Nanjing Agricultural University, Nanjing, China (approval ref no. SYXX-2017-0027).

Preparation of trans-anethole

TA was purchased from Nanjing Dilger Medical Technology Co., Ltd (purity, 98.35%). The TA was stored in glass bottles in the dark and stored at 4°C until use.

Experimental design, birds and management

A total of 256 1-day-old male Arbour Acres broiler chicks, with similar initial body weight (39.75 ± 0.47 g) were obtained from a commercial hatchery (Yantai Land Animal Husbandry Co., Ltd) and randomly distributed into 4 treatment groups each consisting of 8 replicates with 8 birds per pen. Birds in control group (non-supplemented TA) were fed a corn-expanded soybean meal which was formulated to meet nutritional requirements recommended by Feeding Standard of chicken of the People’s Republic of China (NY/T 33-2004). Birds in TA groups were fed a basal diet supplemented with 400, 600, and 800 mg of TA/kg of diet, respectively. The ingredients and nutrient composition of the basal diet were shown in Table 1. Broilers were fed starter diet from d1 to d 21 and grower diet from d 22 to d 42. The TA was firstly mixed with soybean oil and then mixed with other ingredients. The experimental diet was fed as mash and prepared every 14 d and kept in airtight containers prior to feeding.

All of the birds were kept in an environmentally controlled room with ad libitum feeding and water. The temperature was gradually reduced from 35°C on the first day to 22°C by 0.5°C per day until the end of the experiment. In addition, all birds were immunised with Newcastle disease virus vaccine via eye dropping and Avian Influenza virus vaccine via injection on d 7, plus infectious bursa of Fabricius virus vaccine via drinking water on d 14.

Sample collection

At 21 and 42 days of age, 8 birds (1 bird per replicate) per treatment were stunned and slaughtered by cervical dislocation. The small intestine was immediately removed and placed on ice. 3-cm segments of jejunum and ileum from each bird in the same
position were dissected and flushed with ice-cold sterile saline. The samples were subsequently collected in freezing tube and rapidly frozen in liquid nitrogen, later stored at −80°C for sIgA determination and RNA extraction.

**Growth performance parameters**

Data on the body weight (BW) and feed intake of birds of each replicate were recorded weekly and used for calculating the average daily feed intake (ADFI), average daily gain (ADG), and feed/gain (F/G). Mortalities and health status were visually recorded daily to correct feed consumption.

**Antioxidant enzyme activities**

Frozen jejunum and ileum were weighed, and homogenised (3 min) with ice-cold physiologic saline in the ratio of 1:9 (wt/vol). The homogenates were then centrifuged at 3,000 g for 10 min at 4°C, and the supernatants were collected in 1.5 mL centrifuge tubes for determination of total superoxide dismutase (T-SOD) activity and malondialdehyde (MDA) concentration by using commercial assay kit (Jiancheng Bioengineering Institute, Nanjing, China) following manufacturer’s instructions.

**SIgA concentration examination**

The concentration of secretory immunoglobulin A (sIgA) was quantified using the jejunum and ileum homogenates mentioned above by commercial chicken enzyme-linked immune sorbent assay (ELISA) kits (Cusabio Biotech Co., Ltd, Wuhan, China) according to the manufacturer’s instructions. The results were expressed as μg per mg of proteins in jejunum and ileum of broilers.

**RNA extraction and quantitative real-time PCR (qPCR)**

The extraction of total RNA from the jejunum and ileum segments was performed using Trizol reagent (Thermo Fisher Scientific, Nanjing, China) in accordance with the manufacturer's instructions. The purity and concentration of RNA was determined by microspectrophotometer (NanoDrop-1000, Thermo Fisher Scientific, Waltham, UK). The reverse transcription PCR was subsequently conducted using the PrimeScript RT reagent Kit (TaKaRa Biotechnology Co., Ltd, Dalian, China) in order to reverse transcribe the RNA into cDNA by two steps: 37°C for 15 min and 85°C for 5 s. After diluting the cDNA 5-fold, qPCR reactions were done based on Applied Biosystems 7500 Real-time PCR System, using ChamQ SYBR® qPCR Master Mix Kit (Vazyme Biotechnology Co., Ltd, Nanjing, China) according to the manufacturer’s instructions. The primers were commercially synthesised by Sangon Biotechnology Co., Ltd (Shanghai, China), which are shown in Table 2. The relative mRNA abundance of target genes was analysed using the 2−ΔΔCt method, with β-actin as the endogenous reference gene.

**Statistical analysis**

All data were analysed in a randomised design by one-way ANOVA using the GLM procedure of SAS (SAS Institute Inc., 2001, Cary, NC). The data on performance parameters was analysed on a pen basis, whereas data on intestinal antioxidant and immune function, and liver lipid metabolism was analysed on individual bird. Statistical differences among treatments were separated by Tukey’s HSD test. Values were presented as means with a standard error of the mean (SEM). Significant differences are declared at p < 0.05.

**Results**

**Growth performance**

All of the birds were healthy and no mortality appeared. As shown in Table 3, overall growth of birds

| Table 2. Primer sequences used for quantitative real-time PCR. |
|----------------------|----------------------|----------------------|
| Primer | Primer sequence (5′→3′) | Product size, bp | GeneBank accession no. |
| IL-1β | F:TGCCTGCAGAAGAAGGCTG | 137 | NM_205424.1 |
| IL-2 | R:TCCCGAGATGGTTCAT | 140 | AF006631.1 |
| IL-4 | R:CCGGTTGATGTTACGGG | 102 | NM_001007079.1 |
| TLR4 | R:TTCCAAGCTAATGCGGCT | 82 | NM_001030693.1 |
| IFN-γ | R:AGTGGCTGAGGGTAGGTA | 134 | NM_205149.1 |
| ACC1 | R:CACCGACCCGAGTTCATT | 268 | NM_205505.1 |
| FASN | R:ACCGGACCTTCAATGCGATTG | 103 | NM_204126.2 |
| SREBP-1 | R:AGGCCGACCCGAGTTCATT | 107 | NM_001012898.1 |
| CPT1 | R:AGTCTTTTCCCCAGAATGAT | 103 | NM_20282.1 |
| LPL | R:GGTCCCTGATACTGCGGCT | 116 | NM_205518.1 |

IL-1β, Interleukin-1β; IL-2, Interleukin-2; IL-4, Interleukin-4; TLR4, Toll-like receptor 4; IFN-γ, Interferon-γ; ACC1, Acetyl-CoA carboxylase; FASN, Fatty acid synthase; SREBP-1, Sterol regulatory element-binding protein 1c; CPT1, Carnitine palmitoyltransferase 1; LPL, Lipoprotein lipase.
was not significantly affected by the inclusion of TA. However, the ADFI of birds supplemented with TA was higher \((p < 0.05)\) than that of control birds during the grower phase (d 22 to d 42) and entire trial period (d 1 to d 42). All birds had similar ADG, F/G and BW in either phase or the entire period of the experiment.

**Intestinal antioxidant status**

As shown in Table 4, TA supplemented at 400 mg/kg of diet contributed to the lowest MDA content in the jejunum and ileum on d 42, and the highest T-SOD activity in the ileum \((p < 0.01)\) on d 21 and d 42 compared with the control group.

**Intestinal sIgA concentration**

As shown in Figure 1, TA inclusion at the highest level (800 mg/kg) resulted in marginal \((p < 0.05)\) effect on the ileal sIgA concentration of broilers whereas had no effect \((p > 0.05)\) on that in the jejunum on d 21. Compared with control, TA supplementation resulted in an increased \((p < 0.05)\) concentration of sIgA in the jejunum and ileum of broilers on d 42.

**Gene expression of intestinal cytokines**

The gene expression data for the cytokine in the jejunum and ileum are summarised in Table 5. Compared with control group, dietary supplementation of 800 mg/kg TA significantly down-regulated the ileal expression of IL-2, IL-4, Toll-like receptor 4 (TLR-4) and Interferon-\(\gamma\) (IFN-\(\gamma\)) on d 21, and jejunal expression of IL-1\(\beta\), IL-4, TLR-4 and IFN-\(\gamma\), and ileal expression of IL-4 in broilers on d 21 was up-regulated \((p < 0.01)\) by consuming diets containing 400 or 600 mg/kg TA. TA supplementation had no significant effect on the jejunal expression of cytokine on d 21.

**Gene expression of lipid metabolism**

As exhibited in Table 6, 800 mg/kg of TA administration significantly down-regulated \((p < 0.05)\) liver mRNA levels of acetyl-CoA carboxylase (ACC1), and fatty acid synthase (FASN), while 400 and 600 mg/kg of TA had...
no significant effects on that compared with control group on d 21. Differently, TA supplemented at 400 mg/kg led to lower (p < 0.05) mRNA expression of liver ACC1, FASN, sterol regulatory element-binding protein 1c (SREBP-1) and higher mRNA expression of carnitine palmitoyltransferase 1 (CPT1) on d 42. There was no significant difference on the mRNA expression of CPT1 and lipoprotein lipase (LPL) on d 21, and LPL on d 42 among groups.

Discussion

The effects of phytogenic feed additives (PFA) on broiler performance, nutrient utilisation, and intestinal health have been a high-profiled worldwide research hotspot. There is little information about the effects of TA on gut health in broilers. This study investigated the effects of different concentration of TA on growth performance of broilers and observed that TA inclusion had no distinct effect on growth performance, but significantly increased the ADFI when supplemented at 400 and 600 mg/kg. It is well known that SAO with aromatic anise flavour could stimulate appetite thus increase the ADFI of broilers (Wang et al. 2011). Similar results were found that laying hens consumed SAO containing diets tended to have a higher ADFI (Yu et al. 2018). A recent study also found that inclusion of TA had no significant effects on ADG, BW and F/G, whereas the ADFI was significantly increased (Yu et al. 2021). However, it was reported that the growth performance of broilers was not affected by inclusion of essential oils consisting of menthol and anethole (Hafeez et al. 2016). Additionally, the experimental conditions, hygiene and animal age could affect the effects of TA on the growth performance of broilers (Goel et al. 2008; Attia et al. 2019; Shehata et al. 2021). In addition, the final BW obtained in this study is lower than standard. This phenomenon may be attributed to the mash feed instead of pellet feed in the entire experimental period. Taken together, the effects of TA on growth performance of broilers require to be further characterised.

The present study investigated the effects of TA on the intestinal antioxidant status, intestinal immune function and liver lipid metabolism of broilers. As expected, we found that appropriate dose of TA supplemented in broiler diet enhanced intestinal antioxidant capability, increased slgA concentration and decreased mRNA abundance of inflammatory cytokines. In addition, 800 mg/kg of TA had an inferior effect on gut antioxidant and immune status, indicating that the intestinal health of broilers may response
to TA supplementation in a dose dependent manner. Apparently, these encouraging findings indicate that well-dosed TA administration had a beneficial effect on improving intestinal antioxidant and immune status of broilers. Previous studies showed that TA had good antioxidant activity (Så et al. 2018; Wieczyńska and Cavoski 2018). The recent study performed by Ding et al. (2020) demonstrated that SAO could enhance antioxidant capability of White Leghorn birds via activation of nuclear factor E2-related factor 2 (Nrf2) signalling pathway under Escherichia coli challenge. Our previous study also found that dietary supplementation of SAO enhanced antioxidant status of laying hens and extended the shelf life of eggs, which may be attributed to the presence of TA in SAO (Yu et al. 2018). These findings supported our results that TA could enhance intestinal antioxidant capability, indicating the underlying role of TA on preventing oxidative stress in broilers. On the other hand, the anti-inflammatory activities of TA were widely reported (Cho et al. 2013; Kang et al. 2013; Ritter et al. 2013; da Rocha et al. 2017; Kim et al. 2017). It was reported that Foeniculum vulgare essential oil could ameliorate acetic acid-induced colitis in rats, and this may be attributed to the presence of TA and D-limonene (Rezayat et al. 2018). Based on the results of increased jejunal and ileal slgA concentration, and decreased gene expression of inflammatory cytokines in the present work, we concluded that TA enhanced intestinal immune response of grower broilers. In addition, the anti-inflammatory activity of TA may be closely linked with its metabolism process (Freire et al. 2005). Further research on the mechanism of anti-inflammatory effects of TA in broilers requires to be conducted.

The expression of genes related to synthesis and oxidation of fatty acid including ACC1, FASN, SREBP-1, CPT1 and LPL could reflect liver lipid metabolism (Zhao et al. 2020). The results of this study also revealed that TA administration enhanced liver lipid metabolism of broilers associated with lower mRNA expression of fatty synthetase. Consistent with our results, Song et al. (2020) found that TA activated lipid metabolism in HepG2 cells, suggesting that TA may be used to prevent fatty liver disease. Moreover, it was reported that TA suppressed the adipogenic differentiation of human mesenchymal stem cells and reactive oxygen species (ROS) generation (Rhee et al. 2018). TA also exhibited antiobesity effect through induction of browning in white adipocytes and activation of brown adipocytes (Kang et al. 2018). These encouraging results validated our results that TA could modulate liver lipid metabolism in broilers. Excessive deposition of fat has become a serious problem in broiler production, and excessive abdominal fat has been an important factor that restricts the production efficiency of broilers (Lang et al. 2019). In addition, it is widely known that heart disease is directly related to increased levels of serum cholesterol. Therefore,

### Table 5. The mRNA expression of cytokines in the jejunum and ileum of broilers fed diets with different concentration of Trans-anethole (TA) supplementation.

| Item | Dietary TA concentration, mg/kg | 0 | 400 | 600 | 800 | SEM | p Value |
|------|---------------------------------|---|-----|-----|-----|-----|--------|
| 21 d Jejunum | | | | | | | |
| IL-1β | 1.00 | 0.83 | 0.89 | 1.05 | 0.063 | 0.817 |
| IL-2 | 1.00 | 0.81 | 0.95 | 1.09 | 0.050 | 0.489 |
| IL-4 | 1.00 | 0.65 | 0.60 | 0.78 | 0.028 | 0.102 |
| TLR4 | 1.00 | 0.75 | 0.77 | 0.76 | 0.050 | 0.427 |
| IFN-γ | 1.00 | 0.98 | 1.08 | 1.15 | 0.067 | 0.884 |
| 42 d Ileum | | | | | | | |
| IL-1β | 1.00 | 0.76 | 0.82 | 0.91 | 0.088 | 0.007 |
| IL-2 | 1.00 | 1.09 | 1.12 | 0.38 | 0.071 | 0.004 |
| IL-4 | 1.00 | 1.92 | 1.54 | 0.72 | 0.104 | 0.004 |
| TLR4 | 1.00 | 0.95 | 0.98 | 0.88 | 0.046 | <0.001 |
| IFN-γ | 1.00 | 1.32 | 1.32 | 0.74 | 0.085 | 0.004 |

### Table 6. The mRNA expression of liver lipid metabolism of broilers fed diets with different concentration of Trans-anethole (TA) supplementation.

| Item | Dietary TA concentration, mg/kg | 0 | 400 | 600 | 800 | SEM | p Value |
|------|---------------------------------|---|-----|-----|-----|-----|--------|
| 21 d | | | | | | | |
| ACC1 | 1.00 | 1.03 | 0.73 | 0.27 | 0.056 | 0.001 |
| FASN | 1.00 | 0.84 | 0.69 | 0.37 | 0.053 | 0.009 |
| SREBP-1 | 1.00 | 0.50 | 0.73 | 0.81 | 1.42 | 0.824 |
| CPT1 | 1.00 | 0.67 | 0.91 | 0.71 | 0.051 | 0.231 |
| LPL | 1.00 | 1.04 | 1.19 | 0.91 | 0.045 | 0.102 |
| 42 d | | | | | | | |
| ACC1 | 1.00 | 0.50 | 0.82 | 0.97 | 0.067 | 0.041 |
| FASN | 1.00 | 0.30 | 0.84 | 1.08 | 0.047 | 0.001 |
| SREBP-1 | 1.00 | 0.31 | 0.83 | 0.62 | 0.074 | 0.038 |
| CPT1 | 1.00 | 1.64 | 1.31 | 1.12 | 0.106 | 0.030 |
| LPL | 1.00 | 0.72 | 0.88 | 0.95 | 0.075 | 0.096 |

**a** *Means within a row with different letters differ significantly (p < 0.05).
**b** Data are means for 8 replicates of 8 birds per replicate.
**c** Standard error of the means.
chicken with lower fat and cholesterol content has become research hotspot and an important concern for people (Golzadeh et al. 2012). The results in this study revealed that TA may be a promising tool for lower abdominal fat percentage in broilers and lower fat and cholesterol content in chicken. Further research investigating the mechanism of action of TA on modulating liver lipid metabolism needs to be conducted.

The inferior effect of 800 mg/kg of TA on the intestinal antioxidant and immune status is evidenced by our previous findings detecting negative effects of that on the nutrient digestibility and intestinal barrier function (Yu et al. 2021). The study conducted by Ding et al. (2020) observed similar results that supplementation of SAO at the level of 600 mg/kg of diet resulted in an negative effect on growth performance and antioxidant status of White Leghorn birds, and the author pointed out that the strong anise flavour led to stress responses of birds, thereby decreased the performance as the SAO level increased beyond a certain point. Some literature reported that broiler response is closely related to the inclusion concentration of PFA (Çiftçi et al. 2005; Mountzouris et al. 2011; Reyer et al. 2017). It was also pointed out that the smell of essential oils could affect appetite, then influence performance of broilers (Ertas et al. 2005). On the basis of that, the results obtained in the present study may be due to the highly pungent anise flavour of TA. The strong pungent anise smell may influence the diet’s taste and broilers’ appetite, thereby lead to stress response of broilers when added to the diet at a high concentration. This phenomenon suggested that the efficacy of TA in broilers is dose-dependent.

Conclusions

In conclusion, the results indicated that supplementation of 400 mg/kg of TA in broiler diets could increase intestinal antioxidant and immune function and promote liver lipid metabolism.

Ethical approval

The birds were raised in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Besides, the sampling procedures complied with the ethical guidelines of the Nanjing Agricultural University, Nanjing, China (approval ref no. SYXXK-2017-0027).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Key Research and Development Program of China [No. 2018YFD0501101].

Data available statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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