Influence of *Trans*-anethole on the nutrient digestibility and intestinal barrier function in broilers

Caiyun Yu,[*] Jingfei Zhang,[*] Hao Zhang,[*] Yanan Chen,[*] Chao Wang,[*] Lili Zhang,[*] Liren Ding,[†] Tian Wang,[*,1] and Zaibin Yang[‡]

[*]*College of Animal Sciences and Technology, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, P. R. China;* [†]*College of Animal Sciences and Technology, National Experimental Teaching Demonstration Centre of Animal Science, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, P. R. China; and* [‡]*College of Animal Sciences and Technology, Shandong Agricultural University, Tai'an, Shandong, 271018, P. R. China*

ABSTRACT This experiment was undertaken to investigate the effects of dietary *trans*-anethole (**TA**) at 5 levels (0, 200, 400, 600, and 800 mg/kg of diet) on the growth performance, apparent nutrient digestibility and intestinal barrier function in broilers. Three hundred twenty 1-day-old Arbor Acres broilers were randomly divided into the 5 dietary treatments with 8 replicates each for 42 d. Dietary TA supplementation increased ($P < 0.05$) average daily feed intake (**ADFI**), but had no effects ($P > 0.05$) on average daily gain (**ADG**), feed/gain (**F/G**), and body weight (**BW**) of broilers throughout the entire experimental period. The apparent metabolizable energy (**AME**) and nitrogen-corrected apparent metabolizable energy (**AMEn**), the apparent total tract digestibility of dry matter (**DM**), crude protein (**CP**), organic matter (**OM**), and gross energy (**GE**) showed a quadratic increase ($P < 0.05$) with the increasing TA concentration in the diet. The apparent ileal digestibility of Lys, Met, Leu, Thr, Ala, Tyr, and Pro were higher ($P < 0.05$) in birds fed TA diets compared with control group. Dietary supplementation of 400 mg/kg of TA increased ($P < 0.05$) mRNA levels of jejunal and ileal Na+/glucose co-transporter (SGLT1) on d 21 and d 42, oligopeptide transporter 1 (PepT1) on d 42, and ileal mRNA expressions of occludin (**OCLN**), claudin-1 (**CLDN-1**), and mucin 2 (**MUC2**), villus height (**VH**), crypt depth (**CD**), and VH:CD on d 21, as well as jejunal zonula-occludens-1 (**ZO-1**) and ileal mucin 2 on d 42. Linear or quadratic responses of the jejunal CD and villus VH:CD ratio occurred ($P < 0.01$) with increasing dietary TA concentration on d 42. The inclusion of 400 mg/kg TA decreased ($P < 0.05$) cecal *Escherichia coli* population on d 21 and d 42, but increased ($P < 0.05$) *Bifidobacterium* population on d 21 and ileal *Bifidobacterium* on d 42. In conclusion, 400 mg/kg of TA is the optimum concentration for increasing nutrient utilization and intestinal barrier function of broilers.

Key words: broiler, *trans*-anethole, growth performance, nutrient digestibility, intestinal barrier function

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INTRODUCTION

A favorable intestinal environment plays a key role in nutrient absorption and development of intestinal immune status (Clemente et al., 2012). It is widely confirmed that poultry performance is directly related to gastrointestinal function and health (Paraskeuas and Mountzouris, 2019). Intestinal health depends on the continuous interaction between diet and intestinal integrity, morphology, microbiota, and immunity (Du et al., 2016). Antibiotics were used in poultry diets for improving intestinal health and curing pathogen infection for many years. However, the prohibition on antibiotics has accelerated the research on seeking suitable natural alternatives with similar beneficial effects. Phytogenic feed additives (**PFA**) are reported to modulate gut health by positively affecting 4 interacting points as mentioned above, and then enhance livestock performance (Paraskeuas and Mountzouris, 2019; Pu et al., 2020; Xu et al., 2020).

Trans-anethole (**TA**), a main constituent of many essential oils of medicinal aromatic plants of more than 20 species (e.g., fennel, anise, and star anise), is a volatile terpenoid with anise flavor and easy to be deteriorated when exposed to light and high temperature. TA has been generally recognized as safe by the United States Food and Drug Administration (**FDA**) and widely used

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[1]Corresponding author: tianwangnjau@163.com

as an odorant in foods, cosmetics, alcoholic beverage, and perfumes (Sheikh et al., 2015; Aprotosoaie et al., 2016). Some data recorded in animal and cell line suggested that TA possess beneficial effects as a sensory additive in animal feed, including antimicrobial (Hançer Aydemir et al., 2018; Wieczyńska and Cavoski, 2018), antioxidant (Sá et al., 2018; Sá et al., 2020), anti-inflammatory (Kim et al., 2017; Zhang et al., 2018), ameliorating obesity (Kang et al., 2018; Rhee et al., 2018), and ameliorating hyperglycemia (Sheikh et al., 2015). In addition, some experimental data reported that TA had beneficial effects on cardiovascular and chronic diseases. The data recorded by Seo et al. (2018) firstly showed that TA could prevent hypertension induced by chronic exposure to both restraint stress and nicotine in rats. In vitro study showed that TA treatment suppressed the adipogenic differentiation of human mesenchymal stem cells (**hMSCs**), induced white adipocytes browning, and promoted lipid catabolism (Kang et al., 2018; Rhee et al., 2018), indicating its beneficial effects on obesity diseases. Based on its many biological activities, we hypothesized that TA may be used for growth promoters by improving the nutrient digestibility and intestinal barrier function in broilers. Therefore, the present study is first to investigate the effects of TA on the growth performance, nutrient digestibility, and intestinal barrier integrity in broilers.

Table 1. Ingredients and nutrient composition of the basal diet[1] (%).

Item	Starter (1−21 d)	Grower (22−42 d)
Ingredients		
Corn	55.60	54.40
Expanded soybean meal (46% CP)	29.00	24.15
Cottonseed meal	2.50	3.00
Wheat flour	4.00	4.00
Hydrolyzed feather meal	1.50	1.50
Soybean oil	2.00	7.25
Dicalcium phosphate	0.90	0.80
Limestone	1.50	1.50
Bentonite	1.00	1.00
Premix[2]	2.00	2.00
Titanium dioxide	0.00	0.40
Chemical composition, analyzed		
ME, calculated (Kcal/kg)	2,894	3,234
CP	21.50	19.51
Calcium	0.96	0.84
Total phosphorus	0.66	0.55
Lys	1.45	1.40
Met	0.54	0.50
Thr	0.91	0.80

[1]The experimental diet was the same basal diet supplemented with 0, 200, 400, 600, 800 mg of *trans*-anethole/kg of the basal diet.

[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.

MATERIALS AND METHODS

Preparation of Trans-*anethole*

The TA was kindly provided by Nanjing Dilger Medical Technology Co., Ltd (D105737, Nanjing, China). The analyzed purity of TA was 98.35%. The TA was stored in glass bottles in the dark and stored at 4°C until use.

Experimental Design, Birds and Management

This trial was carried out using a total of 320 one-day-old Arbor Acres broiler chicks (mixed sex) with similar initial body weight (39.75 ± 0.47 g) following the protocols of Animal Care approved by the Nanjing Agricultural University Animal Nutrition Research Institute (No. SYXK-2017-0027). The birds provided by a commercial hatchery (Yantai Land Animal Husbandry Co., Ltd, Yantai, China) were randomly allocated to 5 dietary treatments with 8 replicates of 8 birds each. Birds were fed mash corn-soybean meal based diets supplemented with 0, 200, 400, 600, and 800 mg/kg of TA respectively for 42 d in two phases (1−21 d and 22−42 d). The experimental diets were formulated to meet nutrient requirements recommended by Feeding Standard of chicken of the People's Republic of China (NY/T 33-2004). The ingredients and chemical composition of the basal diet are shown in Table 1. Four g/kg of Titanium dioxide (Zhejiang Jinghai New Material Co., Ltd, Quzhou, China) was externally added to the grower diet as an indicator for the ileal apparent amino acid digestibility measurement. TA was firstly mixed with soybean oil and then mixed with other ingredients. The experimental diet was prepared every 14 d and was kept in airtight containers prior to feeding.

All of the diets were fed as mash and the birds had free access to feed and water. All of the birds were kept in wire cages in a temperature-controlled environment and 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.

Growth Performance Parameters

Data on the body weight (**BW**) and feed intake of birds of each cage 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.

Sample Collection

Three birds per pen were collected for excreta on d 35 for the measurement of nutrients apparent total tract digestibility (**ATTD**). Total excreta from each individual were collected for 3 d after 7 d of adaption period. Afterward, the daily excreta of each replicate (3 birds) were mixed, weighed and placed in excreta collection trays in the same time during collection period and stored at 4°C until nutrients analysis. Feathers and shredded dry skin

were removed carefully from the excreta. At 39 d of age, the above birds were slaughtered by cervical dislocation for ileum digesta sampling 12 h after fasting in order to determine the apparent ileal digestibility (**AID**) of amino acids. The digesta from the Meckel's diverticulum to the ileocecal junction were immediately collected. The collected digesta was immediately freeze-dried, ground through 0.25-mm mesh, and mixed thoroughly until analysis of titanium and amino acid concentration.

At 21 and 42 d of age, 8 birds per group with average BW of its replicate were selected, stunned and subsequently sacrificed by cervical dislocation. Approximately 1.5 cm of middle jejunal and ileal segments were fixed in 4% paraformaldehyde for histomorphological analysis. Digesta in the ileum and cecum were collected into sterile 1.5 mL freezing tube for microbiota analysis. The another 3-cm jejunum and ileum segments were dissected and flushed with ice-cold sterile saline, then kept into 2 mL freezing tube until later analysis. The samples of intestinal segments and digesta were rapidly frozen in liquid nitrogen, later stored at −80°C for further analysis.

Chemical Analysis for Nutrient Digestibility

The samples of feed and excreta were analyzed according to the procedures of Association of Official Analytical Chemists (AOAC, 2000). The content of crude protein (**CP**) in the fresh samples was determined as 6.25 × Kjeldahl nitrogen. The contents of dry matter (**DM**), ether extract (**EE**), organic matter (**OM**) and gross energy (**GE**) were assayed by freeze-dried samples which were smashed to 40 meshes by grinder. The GE in the feed and excreta was determined using adiabatic bomb calorimeter (WHR-15, Changxing High Grade Educational Equipment Development Co. Ltd, Changxing, China). The apparent metabolizable energy (**AME**) and nitrogen-corrected apparent metabolizable energy (**AMEn**) value of the experimental diets was calculated according to Sibbald (1976). The ileal digesta samples were analyzed for amino acids using automatic amino acid analyzer (LA8080, Hitachi high-technologies Co. Ltd, Tokyo, Japan). Titanium concentration in the diet and ileal digesta samples was determined according to Short et al. (1996).

Morphological Measurements of the Jejunum and Ileum

The paraformaldehyde-fixed samples were dehydrated in graded ethanol, transparentized with xylol, and embedded in paraffin. The cross sections (5 μm) of intestinal segments were prepared with a microtome and stained with hematoxylin-eosin (**H&E**). A total of 10 well-oriented villus and crypts for each H&E-stained sections were randomly selected for measuring the villus height (**VH**), crypt depth (**CD**) with light microscope (Olympus CX31, Tokyo, Japan) and Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD). The mean of each cross section was used for statistical analysis.

RNA Extraction and Reverse Transcription-PCR Analysis

Extraction of total RNA was performed using the Trizol reagent (9108, TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer's instructions. The purity and concentration of RNA was determined by microspectrophotometer (NanoDrop-1000, Thermo Fisher Scientific, Waltham, UK). Subsequently, 500 ng of total RNA from each sample was reversely transcribed to cDNA using PrimeScript RT reagent Kit with gDNA Eraser (RR036A, TaKaRa Biotechnology). The synthetic cDNA was stored at −20°C until Real-time fluorescent quantitative PCR analysis and the amplified products were distinguished on 1% agarose gels.

Real-Time Fluorescent Quantitative PCR

Real-time PCR was performed in 96 well microplates using ChamQ SYBR qPCR Master Mix Kit (Q311-02, Vazyme-innovation in enzyme technology, Nanjing, China) based on Applied Biosystems 7500 Real-time PCR System. The primers which are listed on Table 2 were commercially synthesized by Sangon Biotechnology Co., Ltd (Shanghai, China). The relative mRNA abundance of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method with endogenous reference gene (β-actin).

Bacterial DNA Extraction and Quantification

The extraction of total bacterial genomic DNA from digesta samples of ileum and cecum was performed by TIANamp Stool DNA Kit (DP328, Tiangen Biotechnology Co., Ltd, Beijing, China) following manufacturer's instruction. Subsequently, RT-qPCR procedure was conducted to estimate the amount of *Escherichia coli, Lactobacillu* and *Bifidobacterium* using ChamQ SYBR qPCR Master Mix Kit (Q311-02, Vazyme Biotechnology Co., Ltd, Nanjing, China), and Applied Biosystems 7500 Real-time PCR System. As shown in Table 3, the primers targeting the 16S rRNA gene were found from the relevant literature (Walter et al., 2001; Bartosch et al., 2004; Rinttilä et al., 2004) and commercially synthesized via Sangon Biotechnology Co., Ltd. Specific standard curves were established for the quantification of above bacteria referred to Jiao et al. (2013). The bacteria copies were expressed as $\log_{10}$ cells/g of digesta for statistical analysis (Chen et al., 2013).

Statistical Analysis

All data were analyzed by one-way ANOVA using the GLM procedure of SAS (SAS Institute, 2001) and checked normality and homogeneity of variances before statistical analysis. The data on performance parameters and nutrient digestibility was analyzed on a pen basis, whereas data on intestinal barrier integrity was analyzed on individual bird. Values are presented as means with a standard error (**SEM**). Significant effects were analyzed

Table 2. Gene-specific primers and GenBank numbers of chickens.

Gene[1]	GeneBank ID	Primer sequences (5'→3')	Product size (bp)
SGLT1	NM_001293240.1	F:TGTGGGCATAGCAGGAACAG R:TACTCCGGCATTGTCACCAC	141
PepT1	NM_204365.1	F:TTCCCATGGAGTCAACAGGC R:GGCTGCTGCATTCTTGATGG	146
OCLN	NM_205128.1	F:ATGCACCCACTGAGTGTTGG R:GAGGTGTGGGCCTTACACAG	93
ZO-1	XM_015278981.2	F:AGCCCCTTGGTAATGTGTGG R:TTGGGCGTGACGTATAGCTG	87
CLDN1	NM_001013611.2	F:GGTATGGCAACAGAGTGGCT R:CAGCCAATGAAGAGGGCTGA	91
MUC2	JX284122.1	F:TGTGGTCTGTGTGGCAACTT R:GTGACATCAGGGCACACAGA	128
β-Actin	NM_205518.1	F:ACCGGACTGTTACCAACACC R:CCTGAGTCAAGCGCCAAAAG	116

Abbreviations: F, forward; R, reverse.
[1]SGLT1, Na^+/glucose co-transporter; PepT1, Oligopeptide transporter 1; OCLN, Occludin; ZO-1, Zonula occludens-1; CLDN-1, Claudin-1; MUC2, Mucin-2.

using Tukey's HSD test and declared at $P < 0.05$. In addition, orthogonal polynomial contrasts were performed to investigate the linear and quadratic effects of dietary TA supplementation level, and the significance was declared at $P < 0.05$.

RESULTS

Growth Performance

All of the birds were healthy and no mortality appeared. As shown in Table 4, overall growth of birds was not affected ($P > 0.05$) 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−d 42). All birds had similar ADG, F/G, and BW in either phase or the entire period of the experiment. Furthermore, TA inclusion level had no linear ($P > 0.05$) or quadratic ($P > 0.05$) effects on ADFI, ADG, and F/G, whereas linearly increased ($P = 0.022$) or tended ($P = 0.072$) to quadratically increase ADG of d 22 to d 42.

Nutrient Digestibility

The addition of TA had positive effects ($P < 0.05$) on AME and AMEn, ATTD of DM, CP, and GE (Table 5). Furthermore, the AME and AMEn, digestibility of DM, CP, OM, and GE were quadratically increased ($P < 0.05$) with increasing TA concentration. No significant effects of TA inclusion on the ATTD of EE and OM were seen ($P > 0.05$).

As shown in Table 6, means of the AID of Lys, Met, Leu, and Thr were higher ($P < 0.05$) in the TA supplemented groups compared with control group. Additionally, there was a quadratic effect ($P < 0.05$) of the supplemented TA on the AID of Lys, and Leu. The TA inclusion level had a linear ($P < 0.05$) or quadratic ($P < 0.05$) effect on AID of Met, Ile, Thr, and Val.

Gene Expression of Jejunal and Ileal Glucose and Amino Acid Transporters

Compared with the control group, the expression of Na^+/glucose co-transporter (**SGLT1**) and Oligopeptide transporter 1 (**PepT1**) in the jejunum and ileum of broilers was higher ($P < 0.05$) with 400 mg/kg of TA administration (Figure 1). TA inclusion had no effect ($P > 0.05$) on PepT1 expression in the jejunum of broilers on d 21. Additionally, TA inclusion level significantly affected jejunal and ileal expression levels of SGLT1 and PepT1. The inclusion of 800 mg/kg TA showed lowest SGLT1 and PepT1 expression compared with all other groups.

Barrier Integrity Related Gene Expression in Jejunum and Ileum

As shown in Table 7, compared with control group, TA supplemented at 400 mg/kg increased ($P < 0.05$) the mRNA abundance of occludin (**OCLN**), claudin-1

Table 3. Primer and probe sequences used for quantitative real-time PCR.

Item	Primer sequence (5'→3')	References
Escherichia coli	F:CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	Bartosch et al. (2004)
Lactobacillus	F:AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Walter et al. (2001)
Bifidobacterium	F:TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	Rinttilä et al. (2004)

Abbreviations: F, forward; R, reverse.

Table 4. The growth performance parameters of broilers fed diets with different concentration of TA supplementation[1].

	Dietary TA concentration, mg/kg						Effects (*P*-value)		
Item[2]	0	200	400	600	800	SEM	ANOVA	Linear	Quadratic
1−21 d									
ADFI, g	37.51	38.67	38.08	37.68	37.92	0.219	0.511	0.919	0.667
ADG, g	28.49	29.48	29.45	28.61	29.54	0.183	0.194	0.358	0.554
F/G, g/g	1.32	1.31	1.29	1.32	1.29	0.008	0.601	0.292	0.575
22−42 d									
ADFI, g	115.37[b]	115.67[b]	123.75[ab]	124.36[a]	122.13[a,b]	0.992	0.013	0.235	0.459
ADG, g	73.56	73.97	73.87	74.48	74.44	0.367	0.263	0.022	0.072
F/G, g/g	1.58	1.56	1.66	1.66	1.64	0.007	0.602	0.775	0.597
1−42 d									
ADFI, g	78.30[b]	81.10[ab]	83.60[a]	80.25[ab]	81.67[ab]	0.673	0.029	0.637	0.623
ADG, g	51.27	51.91	52.32	51.56	53.25	0.302	0.289	0.098	0.243
F/G, g/g	1.53	1.56	1.59	1.56	1.53	0.007	0.579	0.508	0.458
BW, g									
0 d	39.86	39.67	39.44	40.33	39.47	0.108	0.108	0.888	0.970
21 d	646.88	654.38	653.75	651.88	662.38	2.356	0.370	0.091	0.239
42 d	2191.69	2207.78	2205.06	2215.88	2225.61	9.222	0.267	0.229	0.491

[a-b]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates of 8 birds per replicate.
[2]Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; F/G, feed/gain.

Table 5. The total tract apparent digestibility of DM, CP, EE, OM, GE, AME, and AMEn of broilers fed diets with different concentration of TA supplementation[1].

	Dietary TA concentration, mg/kg						Effects (*P*-value)		
Item	0	200	400	600	800	SEM	ANOVA	Linear	Quadratic
DM, %	72.65[ab]	73.31[ab]	75.43[a]	74.21[ab]	71.28[b]	0.491	0.045	0.578	0.017
CP, %	54.69[b]	62.25[a]	61.13[a]	61.31[a]	59.19[ab]	0.766	0.007	0.137	0.004
EE, %	78.75	78.97	81.31	77.02	74.74	1.345	0.485	0.228	0.237
OM, %	77.22	79.69	78.86	77.85	76.57	0.431	0.184	0.268	0.032
GE, %	73.55[b]	76.83[ab]	79.12[a]	78.76[a]	75.85[ab]	0.635	0.018	0.134	0.003
AME, Kcal/kg	3,295[b]	3,442[ab]	3,544[a]	3,530[a]	3,398[ab]	0.119	0.018	0.134	0.003
AMEn, Kcal/kg	3,143[b]	3,253[ab]	3,341[a]	3,344[a]	3,229[ab]	0.112	0.048	0.142	0.009

Abbreviations: AME_n, nitrogen-corrected apparent metabolizable energy; CP, crude protein; DM, dry matter; GE, gross energy; OM, organic matter.
[a-b]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates of 3 birds per replicate.

Table 6. The apparent ileal digestibility of essential amino acids of broilers fed diets with different concentration of TA supplementation[1].

	Dietary TA concentration, mg/kg						Effects (*P*-value)		
Item,%	0	200	400	600	800	SEM	ANOVA	Linear	Quadratic
Lys	85.73[b]	86.10[b]	87.65[a]	85.73[b]	85.53[b]	0.118	<0.001	0.519	0.002
Met	78.10[b]	81.56[a]	82.95[a]	82.33[a]	81.36[a]	0.345	0.001	0.013	<0.001
Arg	93.30	93.25	93.12	93.64	92.78	0.147	0.477	0.537	0.611
His	82.37	83.89	83.70	83.89	83.53	0.232	0.221	0.168	0.088
Leu	82.43[b]	82.69[b]	84.07[a]	83.65[ab]	83.05[ab]	0.190	0.036	0.130	0.033
Ile	77.29[b]	78.46[ab]	78.27[ab]	79.02[ab]	80.14[a]	0.304	0.071	0.005	0.020
Phe	83.81	84.05	84.28	84.20	83.80	0.242	0.956	0.940	0.725
Thr	75.27[b]	75.84[ab]	76.06[ab]	76.95[a]	76.63[ab]	0.217	0.041	0.015	0.044
Val	75.78[b]	76.32[ab]	76.63[ab]	77.39[ab]	77.97[a]	0.266	0.078	0.005	0.018

[a-b]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates of 3 birds per replicate.

(**CLDN-1**), and mucin 2 (**MUC2**) in ileum of broilers on d 21, and jejunal zonula occludens-1 (**ZO-1**) and ileal MUC2 on d 42. Strangely, TA supplementation decreased ($P < 0.05$) ileal mRNA level of CLDN-1 on d 42. In addition, linear or quadratic responses of jejunal expression of CLDN-1 and MUC2, and ileal expression of OCLN on d 21, and ileal TJ protein expression except ZO-1 on d 42 occurred ($P < 0.05$) with increasing dietary TA concentration. There was also a quadratic ($P < 0.05$) effect of TA on ileal expression of CLDN-1 on d 21, and jejunal expression of MUC2 on d 42.

Morphological Measurements of Jejunum and Ileum

Broilers consuming TA-containing diets had considerably higher ($P < 0.05$) ileal VH and VH:CD ratio than

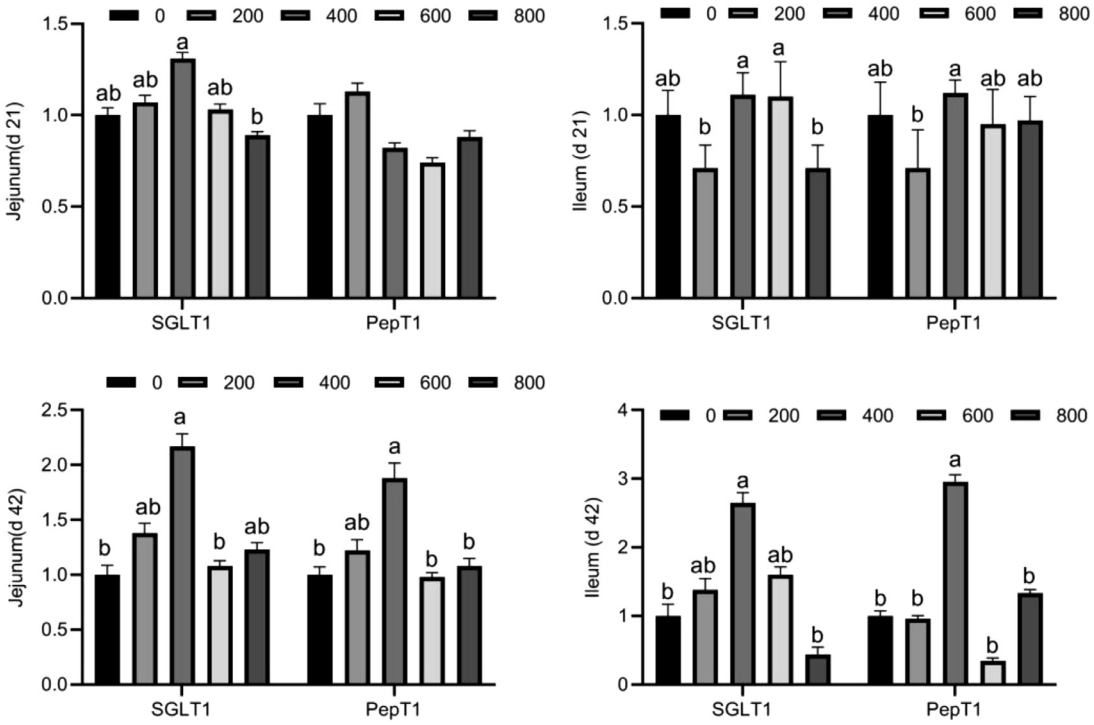

Figure 1. The gene expression of glucose and amino acid transporters in the jejunum and ileum of broilers. Values are means (n = 8), with their standard errors represented by vertical bars. [a-b]Means within a row with different letters differ significantly ($P < 0.05$). SGLT1, Na^+/glucose co-transporter; PepT1, Oligopeptide transporter 1.

control group, whereas had no differences ($P > 0.05$) on jejunal VH, CD, and VH:CD ratio on d 21 (Table 8). The lower CD and higher VH:CD ratio in the jejunum of broilers on d 42 was observed ($P < 0.05$) in TA group compared with the control group. In addition, the TA showed a linear ($P < 0.05$) or quadratic ($P < 0.05$) effect on the ileal VH and VH:CD ratio on d 21, and jejunal CD and VH:CD ratio ($P < 0.01$) on d 42.

Table 7. The mRNA expression of TJ protein in the jejunum and ileum of broilers fed diets with different concentration of TA supplementation[1].

	Dietary TA concentration, mg/kg						Effects (*P*-value)		
Item[2]	0	200	400	600	800	SEM	ANOVA	Linear	Quadratic
21 d									
Jejunum									
OCLN	1.00	0.67	1.33	0.47	0.74	0.096	0.072	0.186	0.223
ZO-1	1.00	0.97	0.95	0.99	1.21	0.034	0.141	0.440	0.096
CLDN-1	1.00^{ab}	0.91^{ab}	1.18^{a}	0.64^{b}	0.69^{b}	0.046	0.007	0.014	0.023
MUC2	1.00^{a}	1.09^{a}	0.68^{ab}	0.57^{b}	0.53^{b}	0.071	0.043	0.006	0.022
Ileum									
OCLN	1.00^{b}	1.19^{ab}	1.46^{a}	1.17^{b}	1.16^{b}	0.136	0.019	0.008	0.013
ZO-1	1.00	0.97	1.02	0.87	0.99	0.026	0.396	0.485	0.714
CLDN-1	1.00^{b}	0.92^{b}	1.47^{a}	1.43^{a}	0.14^{c}	0.042	<0.001	0.105	<0.001
MUC2	1.00^{b}	0.78^{b}	1.52^{a}	1.07^{ab}	0.82^{b}	0.060	0.007	0.743	0.717
42 d									
Jejunum									
OCLN	1.00	0.87	1.15	0.85	1.33	0.185	0.916	0.615	0.807
ZO-1	1.00^{b}	1.15^{ab}	1.36^{a}	1.13^{ab}	1.06^{b}	0.062	0.028	0.844	0.229
CLDN-1	1.00	0.30	0.31	0.48	0.68	0.104	0.207	0.542	0.076
MUC2	1.00	0.85	1.02	0.79	0.58	0.058	0.132	0.958	0.046
Ileum									
OCLN	1.00^{a}	1.14^{a}	1.01^{a}	0.51^{ab}	0.18^{b}	0.106	0.033	0.015	0.042
ZO-1	1.00^{ab}	0.73^{ab}	1.31^{a}	0.86^{ab}	0.41^{b}	0.078	0.014	0.352	0.652
CLDN-1	1.00^{a}	0.39^{b}	0.19^{b}	0.15^{b}	0.16^{b}	0.065	0.007	<0.001	<0.001
MUC2	1.00^{ab}	0.95^{ab}	1.90^{a}	1.38^{ab}	0.52^{b}	0.124	0.017	0.747	0.866

[a-c]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates of 1 bird per treatment.
[2]Abbreviations: OCLN, occludin; ZO-1, zonula occludens-1; CLDN-1, claudin-1; MUC2, mucin 2.

Table 8. Intestinal morphology of broilers fed diets with different concentration of TA supplementation[1].

Item[2]	Dietary TA concentration, mg/kg					SEM	Effects (*P*-value)		
	0	200	400	600	800		ANOVA	Linear	Quadratic
21 d									
Jejunum									
VH, μm	360.37	363.81	360.48	337.05	367.96	9.382	0.855	0.859	0.890
CD, μm	126.77	135.96	135.43	133.4	126.34	2.239	0.488	0.831	0.187
VH:CD	2.86	2.68	2.68	2.53	2.93	0.072	0.421	0.984	0.246
Ileum									
VH, μm	236.82^{b}	274.24^{ab}	300.18^{a}	281.93^{ab}	299.06^{a}	5.421	0.005	0.002	0.002
CD, μm	94.49^{b}	109.25^{a}	100.92^{ab}	92.22^{b}	94.13^{b}	1.601	0.011	0.169	0.086
VH:CD	2.50^{b}	2.52^{b}	2.99^{ab}	3.08^{a}	3.19^{a}	0.061	0.001	<0.001	0.003
42 d									
Jejunum									
VH, μm	384.41	362.9	420.45	445.23	364.95	14.849	0.370	0.099	0.136
CD, μm	115.33^{a}	113.90^{ab}	100.79^{b}	101.07^{b}	100.97^{b}	1.833	0.028	0.004	0.009
VH:CD	3.33^{b}	3.19^{b}	4.17^{a}	4.41^{a}	3.61^{ab}	0.133	0.035	0.002	0.007
Ileum									
VH, μm	279.26	322.91	307.69	290.93	284.81	7.590	0.424	0.678	0.316
CD, μm	87.66	89.51	83.90	86.40	80.69	1.255	0.254	0.067	0.152
VH:CD	3.19	3.61	3.67	3.37	3.53	0.098	0.541	0.538	0.489

[a-b]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates of 1 bird per treatment.
[2]CD, crypt depth; VH, villus height.

Microflora Populations in the Ileal and Cecal Digesta

As shown in Table 9, dietary supplementation of TA resulted in lower ($P < 0.05$) amount of cecal *E. coli* and higher *Bifidobacterium*, whereas had no effect ($P > 0.05$) on ileal microflora population compared with the control group on d 21. Additionally, birds fed TA diets tended ($P = 0.058$) to have higher amount of *Lactobacillu* in the cecal digesta on d 21. On d 42, the inclusion of TA increased ($P < 0.05$) the amount of ileal and cecal *Lactobacillu*, and ileal *Bifidobacterium*, but decreased ($P < 0.05$) the *E. coli* compared with the control group. Furthermore, linear ($P < 0.01$) or quadratic ($P < 0.01$) responses of *E. coli* quantity, and quadratic ($P < 0.01$) response of *Bifidobacterium* quantity in the cecal digesta occurred with increasing dietary TA concentration on d 21. At 42 d of age, the amount of *E. coli* in the ileum was quadratically ($P = 0.006$) decreased, and the *Lactobacillu* in the cecum was linearly ($P = 0.030$) increased.

DISCUSSION

The positive effects of TA in gastrointestinal health of broilers, such as enhancing nutrient utilization (Jamroz and Kamel, 2002), delaying gastric emptying (Asano et al., 2016), gastroprotector activity (Freire et al., 2005), antimicrobial (Senatore et al., 2013; Wieczyńska and Cavoski, 2018; Kwiatkowski et al., 2019), and anti-inflammatory (Paraskeuas et al., 2017; Paraskeuas and Mountzouris, 2019) properties, had

Table 9. Microflora population in the ileum and cecum content of broilers fed diets with different concentration of TA supplementation[1].

Item, $\log_{10}$ cells/g digesta	Dietary TA concentration, mg/kg					SEM	Effects (*P*-value)		
	0	200	400	600	800		ANOVA	Linear	Quadratic
21 d									
Ileum									
Escherichia coli	9.72	9.94	9.69	9.83	9.82	0.175	0.992	0.940	0.996
Lactobacillu	11.18	11.54	11.97	11.6	11.8	0.127	0.370	0.156	0.208
Bifidobacterium	7.07	6.76	7.01	7.2	7.28	0.119	0.693	0.303	0.453
Cecum									
Escherichia coli	12.18^{a}	10.66^{b}	9.42^{c}	9.36^{c}	11.16^{ab}	0.175	<0.001	0.001	0.003
Lactobacillu	9.02^{b}	9.42^{ab}	9.56^{ab}	10.61^{a}	8.83^{b}	0.195	0.058	0.585	0.123
Bifidobacterium	8.75^{b}	8.53^{b}	10.41^{a}	10.88^{a}	9.00^{b}	0.165	<0.001	0.162	0.004
42 d									
Ileum									
Escherichia coli	9.09^{a}	7.72^{b}	7.80^{b}	8.06^{b}	8.40^{ab}	0.129	0.014	0.329	0.006
Lactobacillu	9.03^{ab}	8.37^{bc}	9.67^{a}	8.55^{bc}	7.77^{c}	0.179	0.027	0.103	0.070
Bifidobacterium	6.81^{ab}	6.89^{ab}	7.28^{a}	6.87^{ab}	6.67^{b}	0.074	0.032	0.363	0.649
Cecum									
Escherichia coli	10.08^{a}	10.12^{a}	9.27^{b}	9.72^{ab}	9.89^{ab}	0.135	0.028	0.425	0.295
Lactobacillu	12.02^{b}	11.92^{b}	12.86^{ab}	13.47^{a}	12.11^{b}	0.190	0.042	0.030	0.073
Bifidobacterium	8.12	8.54	8.64	8.27	7.90	0.092	0.094	0.622	0.456

[a-c]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 8 replicates of 1 bird per treatment.

been widely reported. In this study, the results revealed that the inclusion of TA had no significant effects on ADG, BW and F/G, whereas the ADFI was significantly increased. Similar with our results, the growth performance of broilers was not affected by inclusion of plant essential oils containing TA (Amad et al., 2011; Hafeez et al., 2016). However, Ding et al. (2020) observed that the ADFI in White Leghorn broilers was quadratically increased with increasing dietary star anise oil (**SAO**) levels. Prior to that, the results found by us indicated that inclusion of SAO tended to quadratically increased ADFI of laying hens (Yu et al., 2018). This may be due to the aromatic anise flavor of SAO which could stimulate appetite, thereby increasing the ADFI (Ertas et al., 2005; Wang et al., 2011). Moreover, the experimental conditions, hygiene, animal age, diet type, and altered microbiota may also affect the performance response of broilers to TA (Goel et al., 2008).

Previously, we found that SAO may have nutrient releasing effects of broiler diets (Yu et al., 2019). Similarily, it was detected that the essential oil compound consisting of thymol and anethole significantly increased the ileal nutrient digestibility in broilers (Amad et al., 2011). The results of this study demonstrated that the inclusion of TA significantly increased the apparent digestibility of protein, energy and amino acids especially the AID of lysine and methionine as well as the transcript abundance of SGLT1 and PepT1 compared with control group. In accordance with that, Reyer et al. (2017) indicated that compound essential oils containing star anise induced a dose-dependent membrane recruitment of SGLT1 and PepT1 in Caco-2 cells. Kreydiyyeh et al. (2003) demonstrated that 0.05% SAO significantly increased jejunal glucose absorption, and TA is responsible for its biological effects. Moreover, a positive impact of TA on transcriptional level of lipid and carbohydrate metabolism was well demonstrated (Sheikh et al., 2015; Kang et al., 2018; Song et al., 2020), which provide evidences for the possible molecular mechanism of TA increasing nutrient digestibility of broilers. Taken together, the increase in nutrient digestibility in the present study can be attributed to the interaction of TA with the nutrient transport absorption function of intestinal epithelium. Additionally, the digestive stimulant functions of TA may contribute to a reduction of the protein, energy and amino acids concentration in the diets of broilers. The underlying mechanisms of promoting digestion of TA require more in-depth characterization.

Our results also found that dietary TA administration enhanced gene expression of TJ protein, improved intestinal morphology, and modulated ileal and cecal microbiota populations in broilers. The intestinal barrier integrity is performed by a layer of epithelial cells and TJ protein. TJ protein is mainly composed of transmembrane protein compounds such as OCLN, ZO cytosolic proteins, and the CLDN family (Paraskeuas and Mountzouris, 2019). Furthermore, MUC2 secreted by goblet cells has beneficial effects on intestinal barrier protection, thus playing a key role in intervening intestinal inflammation (Moughan et al., 2013). In the current study, the inclusion of TA led to higher levels of OCLN, CLDN-1, ZO-1, and MUC2 in the jejunum and ileum compared with control group. The results indicated that TA may enhance intestinal barrier function of broilers via increasing the mRNA levels of TJ protein and mucins. Similarly, previous studies reported the gastroprotector and mucous protective properties of TA (Schmeda-Hirschmann et al., 2002; Freire et al., 2005). This indicated that TA may protect the gastrointestinal mucous against aggressive factors. We also noted that TA supplementation decreased ileal mRNA level of CLDN-1. As we know, there is discrepancy between mRNA and protein levels of TJ protein due to post-translational modification (Zhang et al., 2020). Hence, this unreasonable result is inexplicable and requires to be confirmed by determination of protein level of CLDN-1. Some data showed that SAO containing 90% of TA increased the secretions of intestinal mucous and absorption surface area in the intestine (Jang et al., 2007; Amad et al., 2011). This is consistent with our results that VH and VH: CD was significantly increased by TA inclusion, which revealed a higher absorption surface area in the intestine. On the other hand, the antimicrobial activities of TA were widely reported, such as *Staphylococcus aureus* strains (Kwiatkowski et al., 2019), *Pseudomonas aeruginosa* (Hançer Aydemir et al., 2018), *E. coli* (Yi et al., 2021), *Klebsiella pneumonia* (Senatore et al., 2013) and so on. The antimicrobial properties of TA may be beneficial for gut microflora composition and favorable in intestinal health. Similar with that, the results of this study demonstrated that the cecal *E. coli* amount was significantly decreased whereas the *Lactobacillu* and *Bifidobacterium* were clearly increased. Some literature reported that the influence of PFA on intestinal microbiota population is directly linked with phytogenic composition and supplementation concentration (Cross et al., 2007; Mountzouris et al., 2011). We have concluded that the antimicrobial mechanisms of TA can be attributed to the degradation of cell wall, destruction of cytoplasmic membrane, or denaturing of membrane proteins, which result in ions, DNA, protein, and glucose leakage, lipid damage and finally cell death (Yu et al., 2020). Obviously, the increased nutrient digestibility in this study may also be attributed to the enhanced intestinal barrier function by TA in broilers.

Apparently, our findings indicated that the inclusion of 400 mg/kg TA appeared better nutrient digestibility and intestinal barrier function of broilers. However, 800 mg/kg TA had adverse effects on that, which is consistent with our previous finding that high concentration of SAO decreased the nutrient digestibility of laying hens (Yu et al., 2019). Previous studies reported that dietary phytogenic compounds concentration could affect broiler response (Ciftci et al., 2005; Soltan et al., 2008). The results conducted by Ding et al. (2020) also observed a dose-dependent efficacy of SAO on enhancing growth performance and antioxidant status of broilers. It was also reported that the addition concentration of PFA could affect the efficacy (Mountzouris et al., 2011; Reyer et al., 2017). As mentioned earlier, TA is the main

active constituent of SAO which is faint yellow with a highly anise flavor. 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. On the basis of that, an inferior effect of high concentration of TA on nutrient utilization and intestinal barrier integrity was detected. This phenomenon suggested that the nutrient digestibility and intestinal barrier integrity might response to TA inclusion in a dose dependent manner.

CONCLUSIONS

Inclusion of 400 mg/kg TA increased intestinal transcription abundance of SGLT1 and PepT1 transporters and intestinal barrier integrity, which may be the underlying mechanisms of TA exerting its promoting digestion functions.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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