

Effects of dietary sweeteners supplementation on growth performance, serum biochemicals, and jejunal physiological functions of broiler chickens

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ABSTRACT The objective of this study was to investigate the effects of dietary 3 kinds of sweeteners supplementation on growth performance, serum biochemicals, and jejunal physiological functions of broiler chickens for 21 D. A total of one hundred ninety-two 1-day-old male Ross 308 broiler chicks were randomly divided into 4 treatments with 6 replicates for each treatment. The treatments were basal diet (**CON**), a basal diet supplemented with 250 mg/kg stevioside (**STE**), a basal diet supplemented with 100 mg/kg sucralose (**SUC**), and a basal diet supplemented with 600 mg/kg saccharin sodium (**SAC**). All birds were housed in 3-level battery cages. The results showed that dietary STE supplementation increased ($P < 0.05$)

growth performance, serum total protein, serum albumin, and jejunal antioxidant capacity of broiler chickens. Both SUC and SAC supplementation decreased ($P < 0.05$) serum total protein and albumin. Dietary SAC supplementation impaired the intestinal integrity, permeability, and mucus layer of the jejunum in broiler chickens. In addition, SAC supplementation elevated ($P < 0.05$) the transcription expression level of jejunal bitter taste receptors and induced excessive jejunal apoptosis. Our data suggest that STE could be potentially applied as a growth-promoting and antioxidant feed additive in broiler chickens. Whereas, dietary supplementation with high level SAC has side-effects on the jejunal physiological functions of broiler chickens.

Key words: sweetener, broiler, stevioside, sucralose, saccharin sodium

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INTRODUCTION

Nowadays, high-potency sweeteners have been widely used in the animal feed for mammals, including pig and ruminants (Buerge et al., 2011; Moran et al., 2014; Ma et al., 2017). Owing to the high sweetness and low-calorie, some sweeteners are extensively used to improve the palatability of the feed (Figuroa et al., 2019). Sweet taste is also correlated with feed intake. A large number of studies have shown that dietary supplementation with sweeteners could promote the feed intake and thereby improve growth performance in livestock, including piglets (Sterk et al., 2008; Zhang et al., 2020), cattle (McMeniman et al., 2006), and goat (Han et al., 2019). Nevertheless, data are lacking about the impact of dietary supplementation with sweeteners on the feed intake

and body weight (**BW**) gain of broiler chickens. It is worthwhile to investigate the effects of sweeteners supplementation on the growth performance of broiler chickens.

Taste is mediated by taste receptors (Lee et al., 2017). It is well established that, in mammals, sweet substances could bind with the sweet taste receptors on the taste buds and induce the sweet signal transduction (Damak et al., 2003). Sweet taste receptors in mammals are consist of taste receptor family 1 member 2 (**T1R2**) and taste receptor family 1 member 3 (Chaudhari and Roper, 2010). However, T1R2 is missing in chickens, which could be the reason that chickens are insensitive to sweet substances (Ganchrow et al., 1990; Shi and Zhang, 2006; Cheled-Shoval et al., 2017). Unlike chickens, some bird, like the hummingbird, has evolved to adapt the umami taste receptors to respond to the sweetness (Baldwin et al., 2014). In addition, a study done by Milner (1969) showed that Japanese quail prefers sucrose solution rather than normal water because of palatability. Whether the umami taste receptors of broiler chickens could perceive sweetness has not been investigated yet.

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Interestingly, an increasing number of studies in mammals have suggested that sweeteners not only induce the sense of sweet taste but also exert additional biological functions in the gastrointestinal tract (Brown and Rother, 2012; Meyer-Gerspach et al., 2018; Hunter et al., 2019). Stevioside (STE) has been proven to exhibit anti-inflammatory activity in intestinal cells (Boonkaewwan et al., 2008). Sucralose (SUC) could reduce the beneficial bacteria in the gastrointestinal tract of rats (Schiffman and Rother, 2013). Consumption of saccharin sodium (SAC) has been suggested to be able to disrupt monolayer integrity using a human Caco-2 cell model in vitro (Santos et al., 2018). However, few studies have been focused on the physiological relationship between sweeteners and gastrointestinal tract in chickens (Kimmich et al., 1989). Understanding the biological functions of sweeteners on the gastrointestinal tract could be helpful for exploring new feed additives for broiler chickens.

Based on the findings above, we hypothesized that dietary supplementation with sweeteners might have physiological functions on the gastrointestinal tract of broiler chickens. Three sweeteners (STE, SUC, and SAC) that commonly used in our daily life were selected in this study. The present study was conducted to evaluate the effects of dietary sweeteners supplementation on growth performance, serum biochemicals, and jejunal physiological functions of broiler chickens.

MATERIALS AND METHODS

Animals and Treatment

The experiments were performed in accordance with the Animal Care and Use Committee of Nanjing Agricultural University, Nanjing, China (PZ2019088). A total of one hundred ninety-two 1-day-old male Ross 308 broiler chicks with similar original weights (41.45 ± 0.15 g) were purchased from a commercial hatchery. Broiler chicks were randomly assigned into 4 treatments with 6 replicates (cages) for each treatment and 8 birds per replicate. The whole experiment lasted for 21 D. The basal diet used in this study was according to the Nutrient Requirements of Poultry (Table 1) (Council, 1994). The 4 treatments were as follows: (1) broiler chickens fed a basal diet (CON); (2) broiler chickens fed a basal diet supplemented with 250 mg/kg STE; (3) broiler chickens fed a basal diet supplemented with 100 mg/kg SUC; (4) broiler chickens fed a basal diet supplemented with 600 mg/kg SAC. The supplemental level of STE was chosen according to our previous study (Jiang et al., 2019b). The supplemental levels of SUC and SAC were chosen according to a previous study to obtain equivalent sweetness with the amount of STE (Keast et al., 2004). Stevioside was purchased from Macklin (Shanghai, China). Sucralose and SAC were purchased from Aladdin (Shanghai, China). Any purity of these 3 sweeteners used in the present experiment is more than 98%. All birds were housed in 3-level battery cages (dimension of each cage:

Table 1. Ingredient composition and calculation of ingredients of the basal diet for broiler chickens.

Items	1 to 21 D
Ingredient (%)	
Corn	53.28
Soybean meal	38.57
Soybean oil	3.70
Dicalcium phosphate	1.98
Mineral premix	0.50
Vitamin premix	0.10
Limestone	1.05
Choline chloride (50%)	0.30
Salt	0.35
Methionine	0.17
Total	100
Calculation of nutrients	
Metabolizable energy, kcal/kg	2,953
Crude protein, %	21.57
Lysine, %	1.15
Methionine, %	0.49
Calcium, %	1.05
Available phosphorus, %	0.45

Provided per kilogram of diet: vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin E, 80 IU; vitamin K, 2.65 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; nicotinic acid, 50 mg; pantothenic acid, 20 mg; vitamin B6, 4 mg; folic acid, 1.25 mg; vitamin B12, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg; Fe, 80 mg; Zn, 75 mg; Mn, 100 mg; Cu, 8 mg; I, 0.35 mg; Co, 0.2 mg; and Se, 0.15 mg.

120 × 60 × 50 cm) in the animal house of the Nanjing Agricultural University with temperature control and continuous light. All broilers had ad libitum access to mash feed and water. The temperature of the room was maintained at 32 to 34°C for 7 D, and it was then gradually decreased by 1°C every 2 D until 26°C was reached. Furthermore, all broilers were inoculated with a Newcastle disease vaccine on seventh day and with an inactivated infectious bursal disease vaccine on 14th D. At day 14 and 21 of the experiment, all birds were weighed after fasting for 12 h to determine BW and average daily gain (ADG). The feed consumption by the broilers in a replicate (cage) was recorded to calculate average daily feed intake (ADFI). The spilled feed were collected and weighted to correct the final data of ADFI. Feed conversion ratio was defined as ADFI: ADG.

Sample Collection

At day 21 of the experiment, all birds were weighed, and 1 bird was selected from each replicate (6 broilers per treatment) with a BW close to the average BW in the respective cage. Blood samples were collected from the wing vein. Serum was then obtained after centrifugation at 3,000g for 15 min at 4°C, and it was stored at -20°C for biochemical assays. The birds were euthanized after blood collection, and the liver, thymus, bursa of fabricius, and breast muscle were carefully separated and weighed. The jejunum was then gingerly taken out. Section of approximately 2 cm in length was cut-off from the middle of each jejunum, and it was washed gently using phosphate buffer saline (pH 7.4) and promptly fixed in 4% paraformaldehyde. The jejunal mucosae were gently scraped by a glass microscope slide

from the rest of jejunum. The mucosae were stored at -80°C for the analysis of oxidative status and gene expression.

Measurement of Serum Biochemical Indexes and Diamine Oxidase Activity

Total protein (TP), albumin (ALB), total bilirubin, direct bilirubin, and uric acid were measured by using a commercial kit (NovaTech Co., Ltd., Shandong, China) and an automatic clinical biochemistry analyzer (NVAS6805, NovaTech Co., Ltd.). The activity of serum diamine oxidase (DAO) was measured using a commercial reagent kit (Jin Yibai Biological Technology, Nanjing, China). The whole experimental procedure was strictly performed according to the manufacturer's instructions.

Intestinal Morphology Analysis

After fixation in 4% paraformaldehyde for 24 h, the jejunal sections were soaked through a graded series of ethanol and xylene and embedded in paraffin. The jejunum were sectioned at 5 μm with a Leica RM2235 microtome (Leica Biosystems Inc., Buffalo Grove, IL). The sections were deparaffinized with xylene and rehydrated through a graded dilution of ethanol. Hematoxylin and eosin staining and Alcian blue-periodic acid Schiff staining were performed, respectively. The images of jejunum were acquired using an Olympus simon-01 microscope (Olympus Optical Co., Ltd., Beijing, China). The values of villus height (VH), crypt depth (CD), and the number of jejunal goblet cells were measured 5 times from different villus and crypts per section from each broiler using the Image-Pro Plus software 6.0 (Media Cybernetics, Inc., Rockville, MD).

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was used to determine the jejunal apoptosis. The whole experiment was performed using a commercial TUNEL BrightRed Apoptosis Detection Kit (A113, Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. First, the jejunal sections were deparaffinized, rehydrated, then incubated with Proteinase K (20 mg/ml) at room temperature for 20 min. Second, the sections were incubated with the terminal deoxynucleotidyl transferase enzyme with BrightRed Labeling Mix at 37°C for 60 min in the dark. Finally, the sections were stained with 4',6-diamidino-2-phenylindole staining solution (C1005, Beyotime Biotechnology, Shanghai, China) for 5 min in the dark. To ensure there was no nonspecific reaction, the negative control was performed without incubation of the TdT enzyme. The fluorescent images were acquired using a

LSM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Determination of Jejunal Oxidative Status

The amount of 0.2 g frozen jejunal mucosae was precisely weighed and homogenized in 2 mL of ice-cold saline. After being centrifuged at 12,000 g for 10 min at 4°C , the supernatants were separated to measure the oxidative status in the jejunal mucosae. A bicinchoninic acid protein assay kit (P0010, Beyotime Biotechnology) was used to measure the protein content. Catalase activity, superoxide dismutase activity, and glutathione peroxidase activity and malondialdehyde (MDA) content in the jejunal mucosae were assessed using commercial reagent kits (S0051, S0101, S0056, and S0131, Beyotime Biotechnology). All experimental procedures were performed according to the manufacturer's instructions. The final results were normalized to protein concentration in each sample.

Total RNA Extraction and mRNA Quantification

The total RNA of jejunal mucosae was extracted using the RNAiso Plus (9109, Takara Bio Inc., Dalian, China). The concentration and quality of total RNA was identified by a ND-2000 micro spectrophotometer (Thermo Scientific, Wilmington, USA). Afterward, the RNA was reverse-transcribed into complementary DNA using a HiScript II first Strand cDNA Synthesis Kit with gDNA wiper (R323-01, Vazyme Biotech). The gDNA wiper was added to remove the DNA, and a total of 1 μg of RNA was reverse-transcribed to complementary DNA. Complementary DNA was diluted 10 \times before real-time PCR. Real-time PCR was performed using the ChamQ SYBR qPCR Master Mix (Q311-02, Vazyme Biotech) on the QuantStudio 5 Real-Time PCR System (Thermo Scientific). The β -actin gene was selected to be the housekeeping gene to normalize the expression of the other target genes. The primers were synthesized by Sangon Biotech (Shanghai, China), and the primer sequences were shown in Table 2. All genes were assayed 3 times. The reaction program was set as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, followed by 60°C for 30 s. The amplification of a single product was verified by the melting curve. Relative gene expression levels were analyzed by the $2^{-\Delta\Delta\text{Ct}}$ method after normalization against β -actin.

Statistical Analysis

Data were statistically analyzed by one-way ANOVA with multiple comparisons among groups tested by Tukey's post hoc tests, using GraphPad Prism 7. The Shapiro-Wilk test was used to assess the normality distribution of the data. Data were presented as the mean and standard error of the mean. Differences were considered to be statistically significant at $P < 0.05$.

Table 2. Primer sequences used for RT-qPCR in this study.

Gene	Primer sequence (5' → 3')	Amplicon size (bp)	GeneBank accession number
<i>ggTAS1R1</i>	Forward: GTGTTCATCCCCACAACCAA Reverse: CACCACTGCCTCAAAGAAGG	137	XM_015297004.2
<i>ggTAS1R3</i>	Forward: CATTACCGTCTTCGCCACTC Reverse: CTCTGTTCAAATCGGGCTTC	143	XM_025142692.1
<i>ggTAS2R1</i>	Forward: TGCCAGTCTCATACCCTGGA Reverse: GAAGTTGCTGTGTGCGTTGT	100	AB249766.1
<i>ggTAS2R2</i>	Forward: TCAACGGGGAAGTGTGGAGA Reverse: GCATTGCATCTTCTTGGTGTGT	174	XM_004938927.3
<i>ggTAS2R7</i>	Forward: TGTGGCTGCGTCTTGTATGG Reverse: TGGCGCACAGATACCAAAAC	104	NM_001080719.1
<i>CLDN1</i>	Forward: CATACTCCTGGGTCTGGTTGGT Reverse: GACAGCCATCCGCATCTTCT	100	NM_001013611.2
<i>CLDN2</i>	Forward: CTGCTACCCCTCATTGGA Reverse: AACTCACTCTTGGGCTTCTG	140	NM_001277622.1
<i>ZO-1</i>	Forward: CTTCAAGTGTCTTCTTCTCCTCCTC Reverse: CTGTGGTTTCATGGCTGGATC	131	XM_015278975.2
<i>OCNL</i>	Forward: ACGGCAGCACCTACCTCAA Reverse: GGGCGAAGAAGCAGATGAG	123	NM_205128.1
<i>MUC2</i>	Forward: CCCTGGAAGTAGAGGTGACTG Reverse: TGACAAGCCATTGAAGGACA	143	XM_001234581.3
<i>β-actin</i>	Forward: TGTTACCAACACCCACACCC Reverse: TCCTGAGTCAAGCGCCAAAA	110	NM_205518.1

Abbreviations: *CLDN1*, claudin 1; *CLDN2*, claudin 2; *ggTAS1R1*, *Gallus gallus* taste receptor family 1 member 1; *ggTAS1R3*, *Gallus gallus* taste receptor family 1 member 3; *ggTAS2R1*, *Gallus gallus* taste receptor family 2 member 1; *ggTAS2R2*, *Gallus gallus* taste receptor family 2 member 2; *ggTAS2R7*, *Gallus gallus* taste receptor family 2 member 7; *MUC2*, mucin 2; *OCNL*, occludin; *ZO-1*, zonula occludens-1.

RESULTS

Growth Performance and Relative Organ Weight

The data of growth performance are shown in Table 3. At 14 D, the STE group had higher ($P < 0.05$) BW compared with the control and SUC groups. At 21 D, dietary supplementation with STE increased ($P < 0.05$) BW of broiler chickens compared with the control group. In addition, dietary supplementation with STE increased ADFI and significantly

differed from control only from 1 to 14 D ($P < 0.05$), while supplementation with STE significantly increased ($P < 0.05$) ADG compared with the control and SUC groups from 1 to 14 D. There were no differences ($P > 0.05$) in ADFI and ADG among any groups during 15 to 21 D. In general, from 1 to 21 D, dietary STE supplementation elevated ($P < 0.05$) ADFI and ADG of broiler chickens compared with the control group. There were no significant differences ($P > 0.05$) in feed conversion ratio among any groups during the whole experimental period. As shown in Table 4, there were no differences ($P > 0.05$) in the

Table 3. Effects of supplementation with sweeteners on the growth performance of broiler chickens.

Items ¹	Treatments				SEM	P-value
	CON	STE	SUC	SAC		
BW (g)						
14 D	360.15 ^b	405.70 ^a	360.33 ^b	381.31 ^{a,b}	10.48	0.027
21 D	638.06 ^b	717.12 ^a	653.30 ^{a,b}	668.38 ^{a,b}	17.84	0.045
ADFI (g/D)						
1–14 D	39.20 ^b	45.35 ^a	42.98 ^{a,b}	40.49 ^{a,b}	1.20	0.017
15–21 D	58.35	63.26	62.34	65.05	3.06	0.570
1–21 D	47.46 ^b	53.44 ^a	52.07 ^{a,b}	49.58 ^{a,b}	1.25	0.031
ADG (g/D)						
1–14 D	24.49 ^b	28.02 ^a	24.56 ^b	26.13 ^{a,b}	0.81	0.029
15–21 D	39.70	44.49	44.01	38.86	1.99	0.153
1–21 D	29.82 ^b	33.79 ^a	31.37 ^{a,b}	30.58 ^{a,b}	0.90	0.046
FCR (g:g)						
1–14 D	1.61	1.62	1.65	1.65	0.07	0.951
15–21 D	1.48	1.43	1.51	1.58	0.08	0.684
1–21 D	1.60	1.59	1.66	1.62	0.05	0.768

^{a,b}Means with different letters within a row are significantly different ($P < 0.05$).

n = 6.

Abbreviations: CON, broiler chickens fed a basal diet; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium; SEM, standard error of means; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose.

¹BW: body weight; ADFI: average daily feed intake; ADG: average daily gain; FCR: feed conversion ratio.

Table 4. Effects of supplementation with sweeteners on the relative organ weight of broiler chickens at 21 D.

Items	Treatments				SEM	P-value
	CON	STE	SUC	SAC		
Liver (g/kg)	16.99	18.56	17.79	17.65	0.58	0.324
Thymus (g/kg)	1.68	1.61	1.84	1.61	0.15	0.683
Bursa of Fabricius (g/kg)	1.57	1.45	1.68	1.75	0.11	0.517
Breast muscle (%)	20.14	19.30	19.28	18.38	0.40	0.054

n = 6.

Abbreviations: CON, broiler chickens fed a basal diet; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium; SEM, standard error of means; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose.

relative organ weight of liver, thymus, bursa of fabricius, and breast muscle among any groups.

Serum Biochemical Indexes

The effects of sweeteners supplementation on serum biochemical indexes of broilers at 21 D are shown in Table 5. Daily STE supplementation significantly increased ($P < 0.001$) the concentration of TP and ALB compared with the other 3 groups. Both SUC and SAC supplementation reduced ($P < 0.001$) the level of serum ALB compared with the control group. Moreover, the supplementation of sweeteners did not alter the concentration of total bilirubin, direct bilirubin, and uric acid in the serum ($P > 0.05$).

Intestinal Morphology and Permeability

To observe the effects of sweeteners supplementation on the jejunal morphology of broilers, hematoxylin and eosin staining was performed (Figure 1). The data of jejunal VH and CD are shown in Figure 2. Saccharin sodium supplementation markedly decreased ($P < 0.001$) the VH in the jejunum of broilers compared with the other 3 groups (Figure 2A). There were no differences ($P > 0.05$) in the CD and VH/CD of the jejunum among any groups (Figure 2B and 2C). Furthermore, to

Table 5. Effects of supplementation with sweeteners on the serum biochemical indexes of broiler chickens at 21 D.

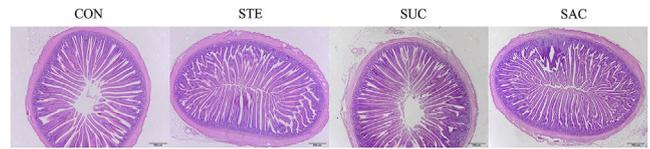
Items ¹	Treatments				SEM	P-value
	CON	STE	SUC	SAC		
TP (g/L)	18.00 ^b	25.11 ^a	13.70 ^b	15.44 ^b	1.32	<0.001
ALB (g/L)	14.50 ^b	18.38 ^a	10.75 ^c	11.25 ^c	0.66	<0.001
TBIL (μmol/L)	37.97	32.74	31.80	36.79	3.62	0.603
DBIL (μmol/L)	7.98	7.06	8.44	10.08	0.93	0.188
UA (μmol/L)	333.61	251.75	324.88	407.02	47.26	0.251

^{a-c}Means with different letters within a row are significantly different ($P < 0.05$).

n = 6.

Abbreviations: CON, broiler chickens fed a basal diet; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium; SEM, standard error of means; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose.

¹TP: Total protein; ALB: albumin; TBIL: total bilirubin; DBIL: direct bilirubin; UA: uric acid.

**Figure 1.** Representative images of H&E staining on the intestinal morphology of broiler chickens. Scale bar = 500 μm. Abbreviations: CON, broiler chickens fed a basal diet; H&E, hematoxylin and eosin staining; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium.

evaluate the jejunal permeability, the activity of DAO in the serum of broilers was assessed (Figure 2D). The data showed that dietary supplementation with SAC significantly elevated ($P < 0.05$) the serum DAO activity compared with the CON group. The jejunal goblet cells indicated by arrows were observed using Alcian blue-periodic acid Schiff staining (Figure 3). Statistical results showed that dietary supplementation with SAC notably reduced ($P < 0.001$) the number of jejunal goblet cells compared with other 3 groups.

TUNEL Assay

To estimate the effects of sweeteners supplementation on the jejunal apoptosis of broilers, TUNEL assay was performed (Figure 4). As shown by TUNEL assay, the apoptotic cells were distributed mainly in the apical region of the jejunal villus. Daily supplementation of SAC obviously increased the number of apoptotic cells in the jejunal villus. While STE and SUC supplementation had no influences on the jejunal apoptosis.

Oxidative Status of the Jejunal Mucosa

As shown in Figure 5, STE supplementation significantly increased ($P < 0.05$) the activities of catalase, superoxide dismutase, and glutathione peroxidase in the jejunal mucosa compared with the control group. There was no effect of either SUC or SAC supplementation on the oxidative status of the jejunal mucosa ($P > 0.05$). In addition, supplementation with sweeteners did not affect the content of MDA in the jejunal mucosa ($P > 0.05$).

Jejunal Gene Expression

Real-time PCR was used to determine the effects of sweeteners supplementation on the gene expression of taste receptors and tight-junction-related genes. There were no differences ($P > 0.05$) on the gene expression of *ggTAS1R1* and *ggTAS1R3* in the jejunum among any groups (Figure 6). Saccharin sodium supplementation significantly increased ($P < 0.05$) the gene expression of *ggTAS2R1*, *ggTAS2R2*, and *ggTAS2R7* compared with the control group (Figure 6). In addition, sweeteners supplementation had no effect ($P > 0.05$) on the transcription level of claudin 1, claudin 2, zonula occludens-1, and occludin in the jejunum (Figure 7).

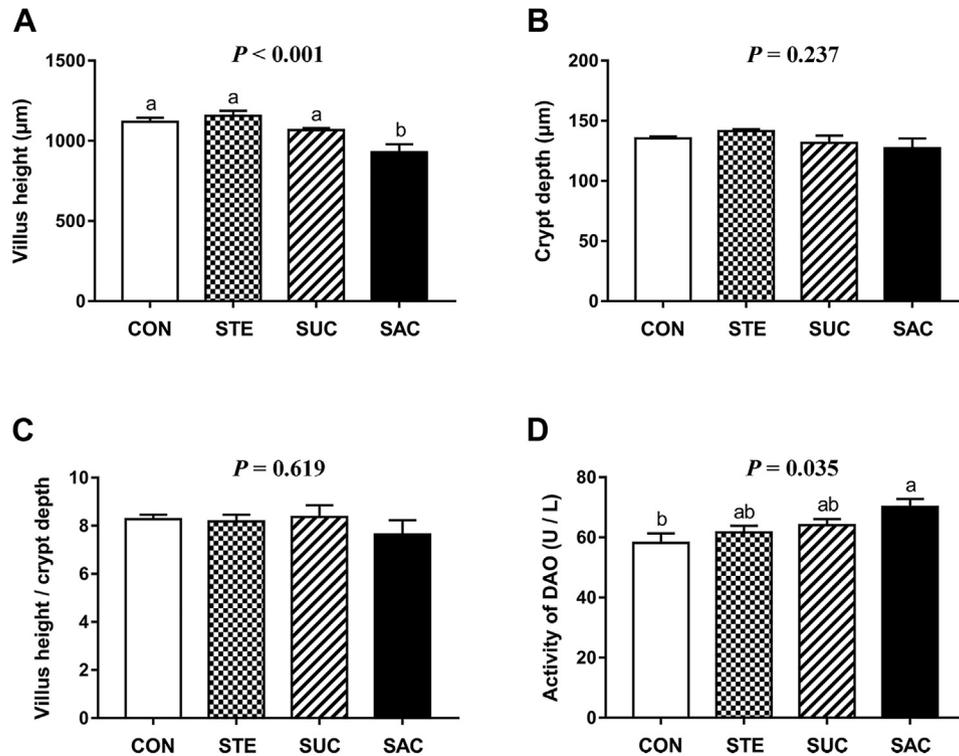


Figure 2. Effects of supplementation with sweeteners on the intestinal integrity and permeability of broiler chickens. (A) Villus height of the jejunum. (B) Crypt depth of the jejunum. (C) Villus height/crypt depth of the jejunum. (D) Activity of serum diamine oxidase (DAO). Data are presented as mean value \pm SEM ($n = 6$). Values without the same letter (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: CON, broiler chickens fed a basal diet; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium.

Dietary supplementation with SAC significantly decreased ($P < 0.05$) the mRNA expression level of jejunal mucin 2 (*MUC2*) (Figure 7).

DISCUSSION

Mammals are sensitive to sweet taste because of advanced evolution of sweet taste receptors on the taste buds (Ahn et al., 2016). Unlike mammals, chickens are lacking T1R2, one of the taste receptor family gene responding to sweeteners in mammals (Shi and Zhang, 2006). This leads to the insensitivity to sweet substances

of chickens (Shi and Zhang, 2006). Consistently, in the present study, broiler chickens did not show specific response to 2 artificial sweeteners (SUC and SAC). Dietary supplementation with SUC and SAC had no influence on the growth performance of broiler chickens. However, STE supplementation surprisingly promoted the feed intake, which, in turn, increased the growth performance of broiler chickens at an early age. In accordance with our results, a previous study has also shown that STE supplementation could increase the average BW gain of broiler chickens at 0–2 wks of age (Atteh et al., 2008). In addition, STE could be

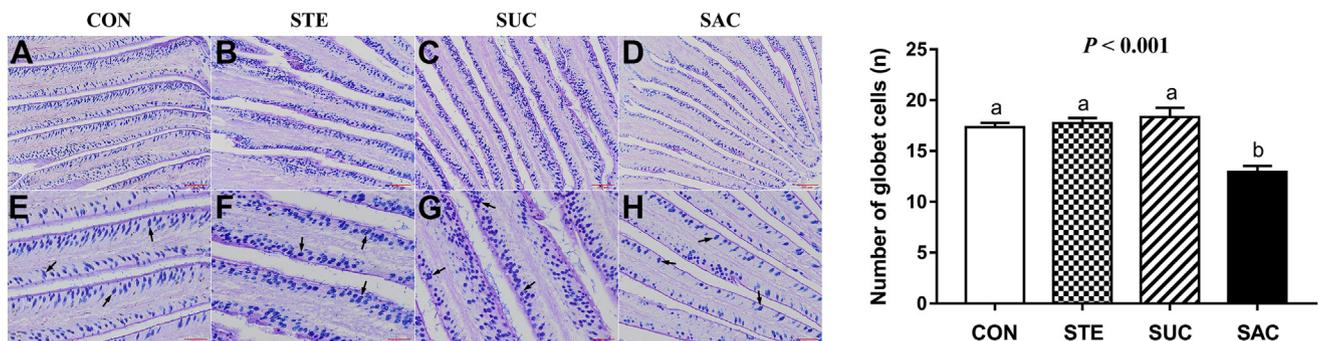


Figure 3. Representative images of AB-PAS staining on the jejunum of broiler chickens. (A–D) AB-PAS staining on the jejunum. Scale bar = 100 μ m. (E–H) Enlargement of AB-PAS staining on the jejunum. Scale bar = 50 μ m. The histogram represents the number of jejunum goblet cells in different treatment groups. Data are presented as mean value \pm SEM ($n = 6$). Values without the same letter (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: AB-PAS, Alcian blue-periodic acid Schiff staining; CON, broiler chickens fed a basal diet; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium.

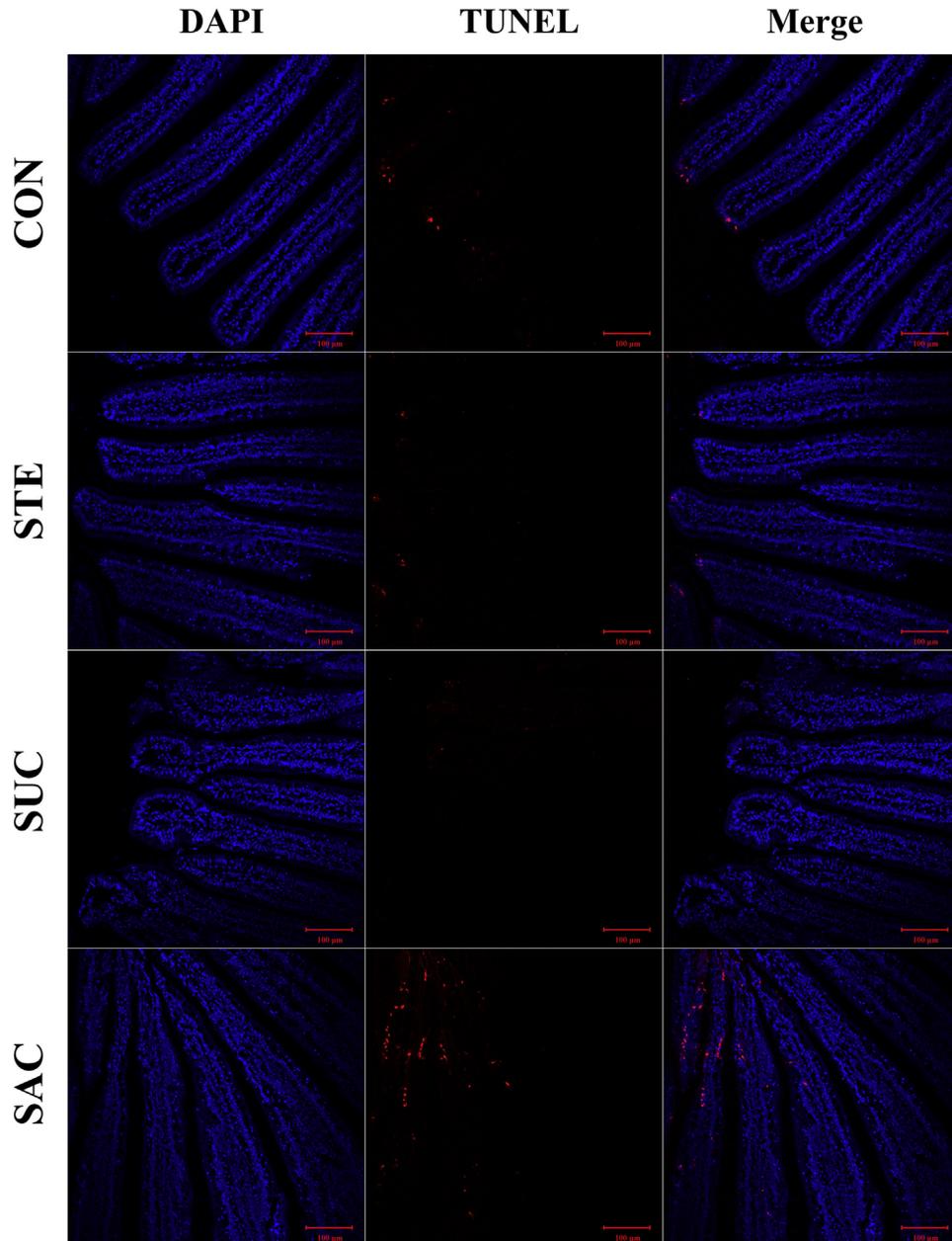


Figure 4. Representative images of TUNEL assay on the jejunal sections of broiler chickens by immunofluorescence. The blue color represents the total cell nuclei, and the red color represents the apoptotic cells in the jejunum. Scale bar = 100 μm . Abbreviations: CON, broiler chickens fed a basal diet; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium.

hydrolyzed to steviol by the intestinal microflora (Renwick and Tarka, 2008). A recent study has shown that dietary supplementation with STE could alter the microflora distribution in the cecum of broiler chickens (Wu et al., 2019). The gut microbiota plays an important role in producing short-chain fatty acids and neuropeptides, which could affect the feed intake of animals (Cryan et al., 2019; Metzler-Zebeli et al., 2019a; Metzler-Zebeli et al., 2019b). Because chickens are insensitive to sweet taste, promoted feed intake induced by STE supplementation might be associated with orexigenic neuropeptides secreted by the gut microbiota or the hypothalamus. This hypothesis requires further investigation and validation.

The serum biochemical parameters represent the physiological status of animals (Zhang et al., 2019). Stevioside has been proven to exert immunomodulatory activity in vitro (Boonkaewwan et al., 2006; Sehar et al., 2008). In chickens, it has been suggested that STE supplementation could increase the concentration level of IgA and IgG (Wu et al., 2019). Identically, elevated serum concentration levels of TP and ALB induced by STE supplementation suggested that STE could potentially enhance the immunity and protein synthesis of broiler chickens. This result was in agreement with the increase of growth performance. Moreover, the present data showed that dietary supplementation with SUC and SAC negatively altered the protein

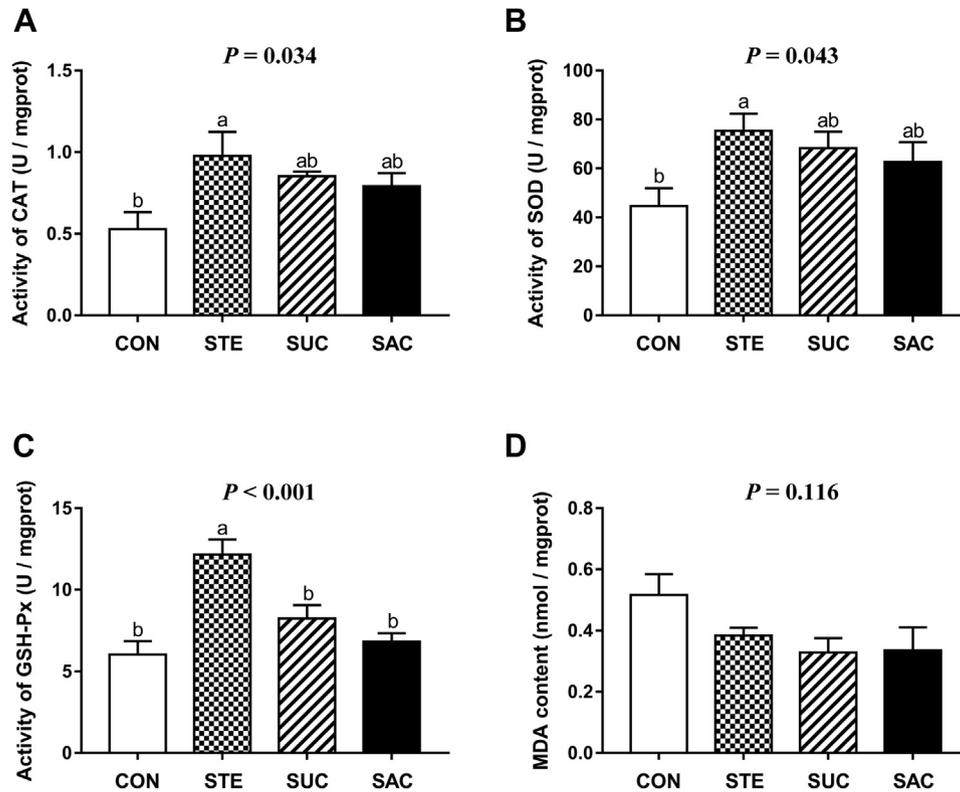


Figure 5. Effects of supplementation with sweeteners on the intestinal oxidative status of broiler chickens. (A) Activity of jejunal CAT. (B) Activity of jejunal SOD. (C) Activity of jejunal GSH-Px. (D) Content of jejunal MDA. Data are presented as mean value \pm SEM ($n = 6$). Values without the same letter (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: CAT, catalase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; CON, broiler chickens fed a basal diet; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium.

synthesis of broiler chickens. Consistent with our results, daily administration of SAC also decreased serum TP and ALB contents in rats (Abdelaziz and Ashour, 2011). Therefore, our data suggest that dietary STE supplementation could enhance the protein synthesis, whereas SUC and SAC supplementation could reduce it in broiler chickens.

Intestinal integrity plays some functionally significant roles in preventing pathogens invasion in broiler

chickens. Dietary supplementation with SAC impaired the intestinal morphology, as indicated by the decreased VH in the jejunum. Similarly, a previous study has also shown that SAC could disrupt the barrier function of intestinal epithelial cells in vitro (Santos et al., 2018). The structural damage of the jejunal villus suggested that the ability of nutrients absorption was reduced by SAC supplementation in broiler chickens. Decreased VH also represented a fragile physical barrier in the

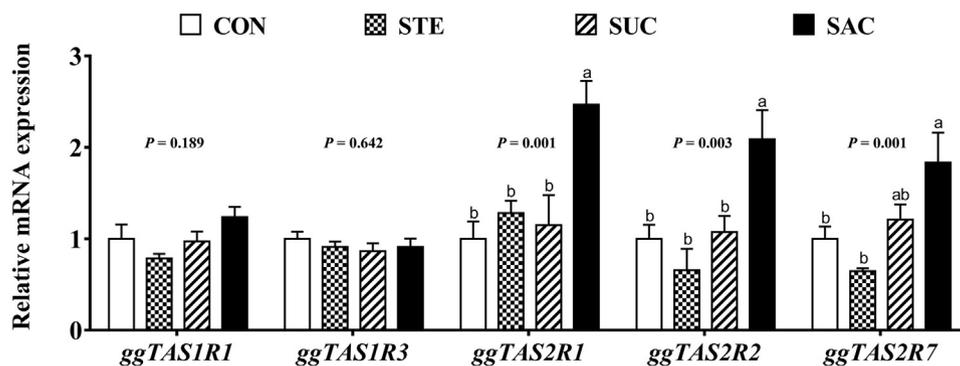


Figure 6. Effects of supplementation with sweeteners on jejunal mRNA expression of taste receptors in broiler chickens. Data are presented as mean value \pm SEM ($n = 6$). Values without the same letter (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: CON, broiler chickens fed a basal diet; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium; *ggTAS1R1*, *Gallus gallus* taste receptor family 1 member 1; *ggTAS1R3*, *G. gallus* taste receptor family 1 member 3; *ggTAS2R1*, *G. gallus* taste receptor family 2 member 1; *ggTAS2R2*, *G. gallus* taste receptor family 2 member 2; *ggTAS2R7*, *G. gallus* taste receptor family 2 member 7.

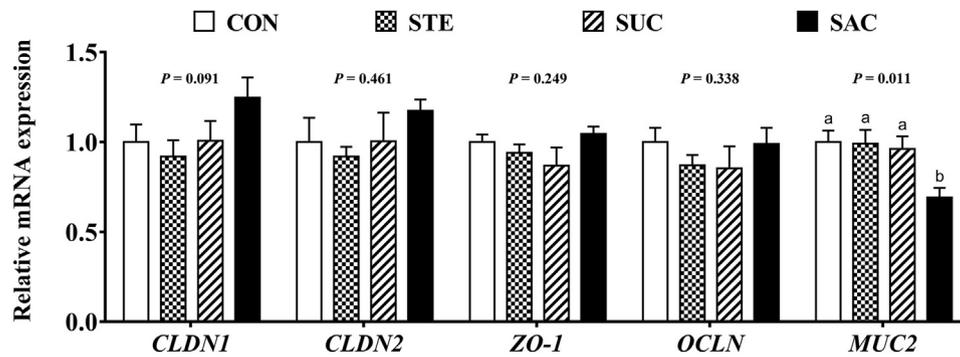


Figure 7. Effects of supplementation with sweeteners on jejunal mRNA expression of tight junction genes in broiler chickens. Data are presented as mean value \pm SEM ($n = 6$). Values without the same letter (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: CON, broiler chickens fed a basal diet; STE, broiler chickens fed a basal diet supplemented with 250 mg/kg stevioside; SUC, broiler chickens fed a basal diet supplemented with 100 mg/kg sucralose; SAC, broiler chickens fed a basal diet supplemented with 600 mg/kg saccharin sodium; *CLDN1*, claudin 1; *CLDN2*, claudin 2; *ZO-1*, zonula occludens-1; *OCLN*, occludin; *MUC2*, mucin 2.

jejunum, which might result in increased infection rate of pathogenic bacteria and thereby damage the gut health (Shao et al., 2013). In addition, DAO is mainly generated by the intestinal mucosae, and it can be released into the blood once the mucosal barrier is damaged. The activity of DAO in the serum reflects the intestinal permeability (Gilani et al., 2017). In the present study, increased serum DAO activity suggested that supplementation with SAC could impair the intestinal permeability, which was consistent with the damaged jejunal morphology. Furthermore, goblet cells are mainly responsible of secreting mucus in the gut. Lower goblet cell density was observed in the SAC supplemented group, and this could result in decreased secretion of intestinal mucus. Intestinal mucus is essential for efficient nutritional uptake, and it contains many immunomodulatory molecules (Johansson and Hansson, 2016). The intestinal mucus layer also has protective effect on the gut of broiler chickens (Hermans et al., 2010). The loss of production in intestinal mucus might lead to intestinal mucosal damage in broiler chickens. Moreover, *MUC2*, the main gel-forming mucin gene, is secreted from goblet cells and controls the formation of mucus layer (Johansson and Hansson, 2016). Several studies have indicated that lack of *MUC2* could result in less mucus, which increases the development of inflammation in mice (Velcich et al., 2002; Johansson et al., 2008). Our finding of decreased transcription level of jejunal *MUC2* also suggested that the mucus secretion of jejunum was damaged by supplementation with SAC. This result was in accordance with lower goblet cells density and impaired intestinal permeability in the jejunum. Collectively, our results have shown that dietary supplementation with SAC could impair intestinal integrity, permeability, and mucus layer of broiler chickens.

Emerging evidence has shown that high-potency sweeteners could induce oxidative stress in mammals (Simintzi et al., 2007; Erbaş et al., 2018; Iyaswamy et al., 2018). To evaluate whether sweeteners used in the present study could cause oxidative stress in the jejunum of broiler chickens, the antioxidant capacity

and MDA content in the jejunal mucosae were determined. The results suggested that those 3 sweeteners did not induce oxidative stress in the jejunal mucosae. Nevertheless, our recent study has demonstrated that dietary supplementation with STE is able to alleviate lipopolysaccharide-induced intestinal mucosal damage through antioxidant and anti-inflammatory effects in broiler chickens (Jiang et al., 2019b). In agreement, STE could also enhance the antioxidant capacity of jejunal mucosae in the present study. Thus, our data suggest that dietary supplementation with sweeteners had no harm to the oxidative status of jejunal mucosae.

Taste receptors have been indicated to exist in many other organs beyond taste buds in mammals, including the gastrointestinal tract (Behrens and Meyerhof, 2011). Similarly, a previous study has demonstrated that umami taste receptors and bitter taste receptors are expressed in the gut of chickens (Cheled-Shoval et al., 2015). Although hummingbird has adapted the umami taste receptors to sense the sweetness, our data showed that the jejunal umami taste receptors (*ggTAS1R1* and *ggTAS1R3*) had no response to the sweeteners in broiler chickens. Surprisingly, SAC supplementation remarkably increased the transcription expression level of all 3 bitter taste receptors (*ggTAS2R1*, *ggTAS2R2*, and *ggTAS2R7*). Several previous studies have reported that SAC is capable of binding the bitter taste receptors at high concentration (Behrens et al., 2017; Kuhn et al., 2004). A previous study has also shown that high-dose SAC treatment could damage testicular functions via activating testicular bitter taste receptors, whereas low-dose and middle-dose SAC treatments could not activate the bitter taste receptors and have no adverse effects on the testicular functions in mice (Gong et al., 2016). Therefore, in the present study, there is a possibility that the activation of jejunal bitter taste receptors was because of the high supplemented level of SAC in the feed.

In addition, our previous study has shown that bitter taste receptors exert biological functions in the jejunum

(Jiang et al., 2019a). The activation of bitter taste receptors could increase cytosolic Ca^{2+} concentration (Freund et al., 2018). Excessive Ca^{2+} concentration in the jejunum is possible to induce apoptosis via calpain/caspase-dependent mechanism. Despite the fact that cell apoptosis is vital for the turnover and homeostasis of intestinal epithelium, the intestinal mucosal damage of broiler chickens is likely associated with excessive cell apoptosis in the intestines (Gunther et al., 2013). In the present study, obviously amplified apoptotic rate was observed in the jejunal epithelial cells of SAC supplemented group. Similarly, a recent study has shown that daily administration of SAC increases ovarian apoptosis in female rats (Ngekure et al., 2019). Another study has also reported that treatment with a complex of SAC and acesulfame K increases the expression levels of proapoptotic proteins in human colonic cell line (Bua et al., 2019). Hence, the adverse effects on the jejunal function caused by supplementation with high level SAC might be associated with excessive apoptosis induced by the upregulation of bitter taste receptors.

In summary, dietary supplementation with STE increased the growth performance of broiler chickens and improved antioxidant capacity of jejunum mucosa. Dietary supplementation with SUC had no effects on either growth performance or jejunal physiological functions but decreased the serum protein synthesis of broiler chickens. Saccharin sodium supplementation impaired the intestinal integrity, permeability, and mucus layer of the jejunum in broiler chickens. In addition, SAC supplementation enhanced the jejunal apoptosis, which was associated with the activation of jejunal bitter taste receptors. Our results suggest that STE has the potential to be used as a growth-promoting and antioxidant feed additive in broiler chickens. Whereas, dietary supplementation with high level SAC could impair the intestinal physiological functions of broiler chickens.

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