Original Research Article

Tolerance and safety evaluation of sodium sulfate: A subchronic study in laying hens

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ABSTRACT

Sodium sulfate (Na_2SO_4) is a readily available chlorine-free source of sodium, which could be used to reduce sodium chloride to maintain the ratio between chlorine and sodium in poultry diets. Dietary supplementation with excessive levels of Na_2SO_4 might be detrimental to bird's health and performance. A subchronic study was carried out to investigate the potential adverse effects of an accidental oversupply of Na_2SO_4 in the diets of laying hens. Four hundred and fifty 21-week-old Hy-Line White layers were randomly assigned to 5 treatments with 6 replicates. The birds were fed diets supplemented with 0 (control), 0.3%, 0.6%, 1.5%, and 3.0% Na_2SO_4 for 8 weeks. Laying performance, egg quality parameters, clinical blood parameters, histopathology, intestinal barrier functions, and intestinal microflora composition were measured. No clinical signs of intoxication or mortality were observed during the experimental period. The results of this study showed that the optimal levels of Na_2SO_4 (0.3% to 0.6%) significantly improved the laying rates, average daily egg mass, and eggshell quality of hens compared to the control ($P < 0.05$). However, 3.0% Na_2SO_4 produced negative effects on laying performance, eggshell quality, blood biochemistry, and particularly on liver and kidney pathology, and intestinal morphology and barrier functions compared with the controls. Although minor changes were observed in clinical blood parameters of hens receiving 1.5% Na_2SO_4, these were not considered to be of toxicological significance due to the absence of any organ pathological changes in hens. In conclusion, our results indicated that a Na_2SO_4 concentration of 1.5% was non-deleterious to laying hens after a daily administration for 56 d, namely that dietary supplementation of up to 5 times the maximum recommended dose is safely tolerated by laying hens.

1. Introduction

Sodium (Na) is involved in several physiological processes, such as regulation of acid-base balance, osmotic pressure, and cell permeability (Olanrewaju et al., 2007). Sodium is also required for the absorption of monosaccharides and amino acids, and a Na deficiency in body could decrease the utilization of protein and carbohydrates (Smith et al., 2000; Gal-Garber et al., 2003). An adequate amount of sodium intake has reported to have beneficial influences on the bird's growth performance (Watkins et al., 2005). Sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) are commonly used as the additional sodium sources in poultry diets. In commercial production, 0.3% NaCl is usually included in hen diets to satisfy the requirements of Na and Cl. However, an excess of dietary Cl may disturb the balance of Cl^- and carbonate ion (CO_3^{2-}) and adversely affect calcium carbonate synthesis, resulting in poor performance (Mushtaq et al., 2007) and eggshell quality (Jonchère et al., 2012).

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Sodium sulfate (Na$_2$SO$_4$), known as Glauber's salt, has been tested as an alternative and cost-effective chlorine (Cl)-free source of Na for birds (Ahmad and Sarwar, 2005; Wang et al., 2019). In addition to Na, the supplemental sulfur (S) found in Na$_2$SO$_4$ may be incorporated into some biological S-containing compounds with antioxidant properties such as methionine, cysteine, taurine, and glutathione (Battin and Brumaghim, 2009; Del-Vesco et al., 2014). The substitution of Na$_2$SO$_4$ for NaCl in hen diets has been found to improve the eggshell quality (Faria et al., 2000). In addition, previous studies indicated that Na$_2$SO$_4$ could moderately stimulate the digestive tract mucosa, increase the gastrointestinal motility, and regulate the Na$^+$/K$^+$-ATPase activity (Gal-Garber et al., 2003), thereby improving the digestive function of poultry.

High dietary Na$_2$SO$_4$ intake could cause osmotic alterations in the intestinal lumen, resulting in intestinal functional disorders accompanied by increased excreta (Smith et al., 2000; Jankowski et al., 2011; Mushtaq et al., 2013). These intestinal disorders may consequently increase the risk of viral and bacterial infection and other health issues in poultry (Nie et al., 2018). High sodium intake also affects dietary cation−anion balance (DCAB, defined as Na$^+$+K$^+$−Cl$^−$), which determines blood pH and therefore influences enzymatic efficiency and amino acid metabolism. Moreover, excess sulfur intake results in the destruction of vitamin D, loss of membrane permeability, fluid collection around the breast meat, and an increase in fecal production (Alam and Anjum, 2003).

Little is known regarding the tolerance of excessive Na$_2$SO$_4$ in hens. Therefore, this study was carried out to investigate the detrimental effects of Na$_2$SO$_4$ oversupply in hens with a commercial diet (containing 0.3% NaCl). Laying performance, egg quality, clinical blood parameters, histopathological changes of visceral organs, intestinal barrier functions, and intestinal microflora composition were taken as variables to estimate the tolerance of dietary Na$_2$SO$_4$ in laying hens.

2. Materials and methods

The experimental use of animals and related procedures were performed according to the Chinese Guidelines for Animal Welfare and approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

2.1. Experimental design and treatments

This tolerance evaluation study was conducted based on The Guidelines for Tolerance Evaluation of Feed Additives in Target Animals issued by the European Food Safety Authority (EFSA, Question No EFSA-Q-2016-00553, 2017). An announcement from the Ministry of Agriculture of China No. 2625 (2017) specifies that the optimal Na$_2$SO$_4$ dose range for chickens is from 0.1% to 0.3% of the diet. According to a previous study in laying hens, the maximum recommended dose (MRD) of Na$_2$SO$_4$ is 0.3% of the diet (Fu, 2019). Taken together, the MRD of Na$_2$SO$_4$ was set at 0.3% of the diet. In the present study, 3 tolerance treatments were designed to supplement 0.6% (2-fold MRD), 1.5% (5-fold MRD), and 3.0% (10-fold MRD) Na$_2$SO$_4$ in the diets of laying hens.

This study was conducted on 21-week-old (the start stage of egg production cycle) Hy-Line White laying hens for a 56-day period with a completely randomized design. Four hundred and fifty hens were randomly allocated to 5 treatment regimens with 6 replicates per treatment. Each replicate consisted of 15 birds housed in 5 adjacent cages with 3 birds per cage (45 cm × 45 cm × 50 cm). Birds received diets supplemented with 0 (control), 0.3%, 0.6%, 1.5%, and 3.0% Na$_2$SO$_4$ for 8 weeks. The analyzed purity of Na$_2$SO$_4$ (Baimei Chemical Industry Co., Ltd, Jiangsu, China) used in this study was 99.3%.

2.2. Diets and management

The basal diet was formulated based on the China Agricultural Standard (NY/T 33-2004) to meet the nutrient requirements of laying hens, and the experimental diets were formulated with Na$_2$SO$_4$ supplementation at the expense of zeolite powder (Table 1). The analyzed potassium, sodium, chloride, and sulfur contents and the cation−anion balances (calculated from the analyzed Na$^+$, K$^+$, and Cl$^−$ contents) of the experimental diets are shown in Table 2. Birds were kept with ad libitum access to feed and water (analyzed Na, K, Cl, and S concentrations were 9.5, 5.2, 3.0, and 83.2 mg/L, respectively), and maintained in an environmentally controlled room (temperature: 23 ± 2 °C; and relative humidity: 55% to 75%) with 16 h of light and 8 h of darkness per day.

2.3. Laying performance and egg quality measurement

Egg production and mass were monitored daily, and feed disappearance was recorded weekly. At the end of the feeding trial, the laying rates, egg mass, average daily feed intake (ADFI), and the feed efficiency (g feed/g egg mass) were calculated. Freshly laid eggs collected at the start (d 0) and the end (d 56) of the feeding trial were used for egg quality measurements. Haugh unit, albumen height, yolk color, and eggshell strength were determined using a digital egg tester (DET-6000, Nabel Co., Ltd, Kyoto, Japan) on 4 eggs per replicate. The albumen and yolk were separated and individually weighed. The ratios of each individual to egg weight were calculated. Eggshell thickness was measured (without shell membrane) at 3 points (air cell, equator, and sharp end) with an Egg Shell Thickness Gauge (Abou-Elkhair et al., 2018). Initial egg quality measurements prior to starting this trial (d 0) were taken for baseline purposes only and did not result in any treatment differences.

2.4. Clinical blood parameters

At d 56, blood samples were taken from 60 randomly selected birds (2 birds per replicate) through the wing vein. The blood samples were placed into 2 mL heparin lithium-containing tubes for routine analysis of blood, and another 4 mL was collected for clinical biochemistry analysis. Blood routine test parameters including red blood cell count (RBC), white blood cell count (WBC), hemoglobin (HGB), hematocrit (HCT), mean hemoglobin (MCH), and red cell distribution width (RCDW) were measured with an automated hematology analyzer (Sysmex XE2100, Kobe, Japan).

Clinical biochemistry measurements were obtained from the plasma of blood samples centrifuged at 3,000 × g at 4 °C for 20 min. Concentrations of calcium (Ca), phosphorus (P), potassium (K), sodium (Na), chlorine (Cl), total proteins (TP), albumin (ALB), glucose (GLU), uric acid (UA), creatinine (CR), total bilirubin (TBIL), and activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured using a Chemical Analyzer (Randox,

Table 1
Ingredients and nutrient levels of the experimental diets (%, air-dry basis).

Item	Na_2SO_4 supplemental level, %				
	0	0.3	0.6	1.5	3.0
Ingredients					
Corn	56.83	56.83	56.83	56.83	56.83
Soybean meal	27.67	27.67	27.67	27.67	27.67
Soybean oil	1.91	1.91	1.91	1.91	1.91
Zeolite powder	3.00	2.70	2.40	1.50	0
Na_2SO_4	0	0.30	0.60	1.50	3.00
CaHPO4	0.90	0.90	0.90	0.90	0.90
Limestone	8.90	8.90	8.90	8.90	8.90
NaCl [1]	0.30	0.30	0.30	0.30	0.30
Premix [2]	0.37	0.37	0.37	0.37	0.37
Methionine	0.12	0.12	0.12	0.12	0.12
Total	100.00	100.00	100.00	100.00	100.00
Nutrient levels[3]					
ME, Mcal/kg	2.71	2.71	2.71	2.71	2.71
CP	16.86 (16.84)	16.86 (16.85)	16.86 (16.88)	16.86 (16.84)	16.91 (16.85)
Calcium	3.56 (3.60)	3.56 (3.57)	3.56 (3.60)	3.56 (3.58)	3.56 (3.58)
Phosphorus	0.60	0.60	0.60	0.60	0.60
Available phosphorus	0.35	0.35	0.35	0.35	0.35
Lysine	0.95	0.95	0.95	0.95	0.95
Methionine	0.40	0.40	0.40	0.40	0.40

[1] Diets containing salt (NaCl), based on NRC (1994) with minimum Na^+ and Cl^- contents. Dietary cation−anion balance (DCAB, $K^+ + Na^+ - Cl -$) of the basal diet was 180.1 mEq/kg, and the analyzed sulfur content was 0.23%.

[2] Premix provided the following per kilogram of the diet: vitamin A, 6,250 IU; vitamin D_3, 3,125 IU; vitamin E, 15 IU; vitamin K, 2 mg; thiamine, 1 mg; riboflavin, 8.5 mg; calcium pantothenate, 50 mg; niacin acid, 32.5 mg; pyridoxine, 8 mg; folic acid, 5 mg; vitamin B_{12}, 5 mg; choline chloride, 500 mg; Fe, 60 mg; Cu, 8 mg; Mn, 60 mg; Zn, 60 mg; Se, 0.3 mg; and I, 1.0 mg.

[3] The values in parentheses are analyzed values. Others are calculated values calculated from tables of feed composition and nutritive values provided by Feed Database in China (2016).

Table 2
Analyzed potassium, sodium, and chloride contents and DCAB in the experimental diets (%, air-dry basis)[1].

Item	Na_2SO_4 supplemental level, %				
	0	0.3	0.6	1.5	3.0
Potassium	0.741	0.752	0.732	0.757	0.747
Sodium	0.138	0.229	0.320	0.634	1.160
Chloride	0.248	0.245	0.256	0.245	0.262
Sulfur	0.225	0.302	0.346	0.543	0.867
DCAB[2], mEq/kg	180.1	224.9	254.0	400.7	622.3

[1] Values are the means of 6 analyses per sample.

[2] DCAB, dietary cation−anion balance ($K^+ + Na^+ - Cl^-$).

London, UK). Diamine oxidase (DAO) activities in plasma were assayed using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). D-lactate levels in plasma were measured using commercial kits (Abcam, UK). These parameters cover wide ranges of potential toxicities including possible effects on electrolyte balance, metabolism, and damages to major organ systems (Lu et al., 2017).

2.5. Histopathology and intestinal morphology

After blood samples were taken, the selected birds (2 birds per replicate) were euthanized and subjected to postmortem examinations. Livers and kidneys were excised and weighed in situ (paired organs were weighed together), and the relative organ weights were calculated as follows: Relative organ weight (%) = Organ weight/Live body weight × 100. Portions of visceral organs and approximately 1.5 cm intestinal segments (jejunum and

ileum) were fixed in 4% formaldehyde for 24 h. The formalin-fixed samples were embedded in paraffin, and 5-μm-thick sections were cut and stained with hematoxylin-eosin using the method described by Jia et al. (2019). Histopathological changes and intestinal morphology were examined using a Nikon Eclipse 80i microscope (Nikon, Japan).

Blind histopathological observation of the stained liver and kidney sections were performed under a light microscope at a 400× final magnification. Hepatic injury was evaluated using the following parameters: bile-duct proliferation, nuclear condensation, hepatocyte necrosis, fatty degeneration, and inflammatory cell infiltration (Kraieski et al., 2017; Ma et al., 2012). The pathological changes in kidneys were assessed by renal tubular dilatation and atrophy, hyaline vacuolar degeneration, and tubular epithelial cell swelling or necrosis (Ma et al., 2018; Shi et al., 2018). Intestinal morphology analyses were carried out on 2 cross-sections per sample, 15 well-oriented villus per section (30 intact well-oriented villus per sample) were measured (Poloni et al., 2020). Villus height (VH) was determined from the tip of the villus to the villus crypt junction, and the crypt depth (CD) was measured as the depth of the invagination between adjacent villus.

2.6. Parameters of caeca and excreta

Approximately 1.0-g digesta and excreta samples were diluted in 9.0 mL of sterilized physiological saline for pH analysis with a digital pH meter (HI99161, Hanna, Italy). Digesta and excreta moisture contents were measured using a drying oven at 105 °C to a constant weight, and calculated from the weight difference.

2.7. Cecal microflora population

The viable counts of *Lactobacillus*, *Escherichia coli*, *Clostridium perfringens*, and *Bifidobacteria* in the cecal digesta were conducted according to the methods described by Zhao et al. (2013) with some modifications. In brief, cecal digesta contents (1.0 g) were taken and diluted 10 times with sterile ice-cold anoxic PBS (0.1 mol/L; pH 7.0) and homogenized. Each homogenate was serially diluted from 10^{-1} to 10^{-8} and the diluted samples (0.1 mL) were subsequently plated on selective agar media for enumeration of target bacterial groups. The *Lactobacillus*, *E. coli*, *C. perfringens,* and *Bifidobacterium* were enumerated using Lactobacilli MRS agar, MacConkey agar, Perfringens TSC agar, and Wilkins-Chalgren agar supplemented with glacial acetic acid (1 mL/L) and mupirocin (100 mg/L). Plates were incubated aerobically at 37 °C for 24 h (MacConkey agar and Perfringens TSC agar), for 72 h (Wilkins-Chalgren agar) or anaerobically at 37 °C for 24 h (Lactobacilli MRS agar). Results were expressed as $\log_{10}$ colony-forming units per gram of fresh cecal digesta.

2.8. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from jejunal and ileal mucosa (6 birds from each treatment) using the RNAiso Plus method (Code No. 9108, Takara). The first-strand cDNA was synthesized using a PrimeScript RT reagent kit with gDNA Eraser (Code No. RR047A, Takara). Transcriptional changes were then identified by quantitative PCR using ABI 7500 Fast Real-Time PCR system with SYBR Premix Ex Taq II (Code No. RR820A, Takara). Primer sequences are shown in Table 3. Relative expression abundance of target genes was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Data were subjected to ANOVA using the GLM procedure of the SPSS software (SPSS Inc., Chicago, IL, USA) followed by Tukey multiple comparisons. Linear and quadratic effects for the

Table 3
Sequences of real-time PCR primers.

Gene name	GenBank no.	Primers (5′-3′)	Annealing temperature, °C	Products
ZO-1	XM _413773	F: CCAAAGACAGCAGGAGGAGA R: TGGCTAGTTTCTCTCGTGCA	55	217
Claudin-1	NM_001013611.2	F: TGGCCACGTCATGGTATGG R: AACGGGTGTGAAAGGGTCATAG	55	62
Occludin	NM_205128.1	F: GAGCCCAGACTACCAAAGCAA R: GCTTGATGTGGAAGAGCTTGTTG	55	68
β-actin	NM_205518.1	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	54	110

ZO-1 = zonula occludens protein-1.

Table 4
Laying performance from a Na$_2$SO$_4$-tolerance evaluation study in the hens (21 to 28 weeks of age, n = 6).

Item	Na$_2$SO$_4$ supplemental level, %					SEM	*P*-value		
	0	0.3	0.6	1.5	3.0		Na$_2$SO$_4$	Linear	Quadratic
Laying rate, %	85.59[b]	88.40[a]	88.33[a]	86.43[b]	82.17[c]	0.44	<0.001	<0.001	0.001
ADFI, g/d per hen	104.50	104.03	104.40	103.83	104.00	0.90	0.981	0.674	0.921
Egg mass, g/d per hen	46.73[ab]	47.58[a]	47.74[a]	47.08[a]	44.74[b]	0.52	0.003	0.012	0.001
Feed efficiency[1], g/g	2.24[ab]	2.19[b]	2.19[b]	2.21[ab]	2.33[a]	0.03	0.016	0.052	0.004

ADFI = average daily feed intake.
[a–c] Means without a common superscript with a row differ significantly (*P* < 0.05).
[1] Feed efficiency is the ratio of feed to egg mass.

Table 5
Egg quality from a Na$_2$SO$_4$-tolerance evaluation study in the hens (28 weeks of age, n = 6).

Item	Na$_2$SO$_4$ supplemental level, %					SEM	*P*-value		
	0	0.3	0.6	1.5	3.0		Na$_2$SO$_4$	Linear	Quadratic
Albumen percentage, %	64.59	64.36	64.24	65.27	65.47	0.63	0.548	0.189	0.395
Yolk percentage, %	23.94	23.68	23.88	24.21	24.41	0.37	0.656	0.217	0.448
Eggshell percentage, %	10.83[a]	10.88[a]	10.96[a]	10.75[a]	9.16[b]	0.30	0.001	0.001	0.004
Albumen height, mm	7.72	7.90	7.95	7.78	7.70	0.18	0.815	0.792	0.271
Haugh unit	88.77	89.81	90.04	88.97	88.66	0.97	0.785	0.731	0.277
Yolk color (Roche scale)	6.17	7.33	6.83	6.83	7.33	0.34	0.123	0.099	0.516
Eggshell strength, kg/cm^2	3.98[bc]	4.50[ab]	4.79[a]	4.02[bc]	3.40[c]	0.16	<0.001	0.004	0.001
Eggshell thickness, mm	0.37[b]	0.41[ab]	0.42[a]	0.37[b]	0.34[c]	0.01	<0.001	<0.001	<0.001

[a–c] Means without a common superscript within a row differ significantly (*P* < 0.05).

Table 6
Blood routine parameters from a Na_2SO_4-tolerance evaluation study in the hens (28 weeks of age)[1].

Item	Na_2SO_4 supplemental level, %					SEM	P-value		
	0	0.3	0.6	1.5	3.0		Na_2SO_4	Linear	Quadratic
WBC, $\times 10^9$/L	291.70	290.10	297.78	291.58	288.04	7.12	0.900	0.797	0.511
RBC, $\times 10^{12}$/L	2.84^{ab}	2.91^a	2.86^{ab}	2.62^{bc}	2.46^c	0.07	<0.001	0.065	0.015
HGB, g/L	92.37^a	91.60^a	89.13^{ab}	84.20^{bc}	80.52^c	1.61	<0.001	<0.001	0.180
HCT, %	36.22	36.03	35.20	35.95	31.90	1.31	0.138	0.055	0.220
MCH, pg	32.55	31.61	31.28	32.24	32.77	1.00	0.812	0.735	0.272
RCDW, %	7.93	8.08	7.98	8.02	7.63	1.21	0.616	0.331	0.251

WBC = white blood cells; RBC = red blood cells; HGB = hemoglobin; HCT = hematocrit; MCH = mean hemoglobin; RCDW = red cell distribution width.
$^{a-c}$ Means without a common superscript within a row differ significantly ($P < 0.05$).
[1] Results are the means of 6 replicates of 2 hens each.

responses to sodium sulfate levels were performed using orthogonal contrasts. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Laying performance

No treatment-related clinical signs of intoxication or increased mortality were found throughout the feeding period. Mean laying rates, egg mass, and feed efficiency over the whole experiment period (21 to 28 weeks) showed significant linear or quadratic response to the increasing Na_2SO_4 supplement levels (Table 4, $P < 0.05$). Dietary supplementation with 0.3% and 0.6% Na_2SO_4 increased the laying rates of hens compared to the control ($P < 0.05$). Nevertheless, 3.0% Na_2SO_4 supplementation decreased hen's laying rates compared to the control and 0.3% Na_2SO_4 groups ($P < 0.05$). No significant differences were observed in the laying rates, egg mass or feed efficiency of hens receiving 1.5% Na_2SO_4 compared to the control ($P > 0.05$).

3.2. Egg quality

Eggshell percentage, strength, and thickness responded linearly and quadratically with the increasing supplemental levels of Na_2SO_4 (Table 5, $P < 0.05$). Dietary supplementation with 0.6% Na_2SO_4 showed the highest eggshell percentage, strength, and thickness ($P < 0.05$), whereas hens receiving 3.0% Na_2SO_4 showed the lowest eggshell percentage, strength, and thickness ($P < 0.05$). No statistical differences were found across treatments for albumen percentage, yolk percentage, albumen height, Haugh unit, or yolk color ($P > 0.05$).

3.3. Clinical blood parameters

As shown in Table 6, RBC and HGB declined linearly or quadratically with increasing Na_2SO_4 supplement levels ($P < 0.05$). Compared with the control and 0.3% Na_2SO_4 groups, blood RBC and HGB decreased significantly in hens receiving 3.0% Na_2SO_4 for 8 weeks ($P < 0.05$). No statistically significant changes were observed in WBC, HCT, MCH, or RCDW across treatments ($P > 0.05$). For biochemical parameters, activities of ALT, AST, and DAO along with the concentrations of TP, TBIL, UA, CRE, and D-lactate showed significant linear or quadratic responses to increased dietary Na_2SO_4 supplement levels (Table 7, $P < 0.05$). No statistically significant differences were found in all selected biochemical parameters of hens receiving 1.5% or less Na_2SO_4 compared with the control ($P > 0.05$). Dietary supplementation with 3.0% Na_2SO_4 significantly increased the activities of ALT ($P < 0.001$) and AST ($P = 0.044$), as well as the concentrations of TBIL ($P = 0.040$),

Table 7
Plasma biochemical parameters from a Na_2SO_4-tolerance evaluation study in the hens (28 weeks of age)[1].

Item	Na_2SO_4 supplemental level, %					SEM	P-value		
	0	0.3	0.6	1.5	3.0		Na_2SO_4	Linear	Quadratic
Liver function									
ALT, U/L	2.07^b	2.10^b	2.44^b	2.89^b	4.11^a	0.30	<0.001	0.001	0.016
AST, U/L	194.50^b	206.17^b	199.00^b	219.50^b	267.43^a	0.56	0.044	0.008	0.186
ALP, U/L	156.23	151.63	162.53	173.90	170.13	13.74	0.767	0.260	0.967
GLU, mmol/L	11.28	11.55	12.00	11.71	10.41	0.97	0.810	0.609	0.291
TP, g/L	50.89^a	47.30^{ab}	48.57^{ab}	46.72^{ab}	40.12^b	2.35	0.040	0.006	0.308
ALB, g/L	30.36	30.07	33.20	31.57	30.57	0.89	0.120	0.506	0.077
TBIL, μmol/L	2.46^b	2.12^b	2.63^b	2.43^b	3.65^a	0.18	<0.001	<0.001	0.002
Kidney									
UA, mg/L	170.00^b	155.00^b	204.17^{ab}	196.67^b	252.17^a	12.84	<0.001	<0.001	0.091
CRE, μmol/L	37.67^b	40.67^b	46.33^b	44.50^b	57.67^a	2.33	<0.001	<0.001	0.153
Intestinal permeability									
D-Lactate, μmol/mL	1.72^b	1.66^b	1.82^b	1.87^b	3.00^a	0.09	<0.001	<0.001	<0.001
DAO activity, U/L	7.40^b	7.51^b	7.63^b	8.63^{ab}	9.17^a	0.33	0.002	<0.001	0.171

ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GLU = glucose; TP = total protein; ALB albumin; TBIL = total bilirubin; UA = uric acid; CRE = creatinine; DAO = diamine oxidase.
$^{a-b}$ Means without a common superscript within a row differ significantly ($P < 0.05$).
[1] Results are the means of 6 replicates of 2 hens each.

Table 8
Plasma ion concentration (mmol/L) from a Na_2SO_4-tolerance evaluating study in the hens (28 weeks of age)[1].

Item	Na_2SO_4 supplemental level, %					SEM	*P*-value		
	0	0.3	0.6	1.5	3.0		Na_2SO_4	Linear	Quadratic
Calcium	7.44[b]	8.02[ab]	8.27[a]	7.44[b]	6.58[c]	0.18	<0.001	<0.001	<0.001
Phosphorus	2.72	2.89	2.91	2.80	2.76	0.09	0.530	0.986	0.124
Potassium	4.32	4.28	4.31	4.03	3.95	0.11	0.056	0.008	0.316
Sodium	133.78	131.10	137.08	135.19	134.55	3.04	0.906	0.709	0.616
Chloride	102.63[b]	102.36[b]	103.84[ab]	105.36[ab]	114.31[a]	2.77	0.029	0.006	0.086
Electrolyte balance[2]	35.46[a]	35.02[a]	37.55[a]	33.86[ab]	24.18[b]	1.74	<0.001	0.004	0.001

[a–c] Means without a common superscript within a row differ significantly at $P < 0.05$.
[1] Results are the means of 6 replicates of 2 hens each.
[2] Electrolyte balance, $Na^+ + K^+ - Cl^-$.

UA ($P < 0.001$), and CR ($P < 0.001$) compared with the control and 0.3% groups. Plasmic D-lactate levels and DAO activity in hens receiving 3.0% Na_2SO_4 significantly increased compared with the control and low dose (0.3% and 0.6%) groups ($P < 0.05$).

3.4. Plasma ion concentration

The concentrations of Ca and Cl, and the electrolyte balance ($Na^+ + K^+ - Cl^-$) showed significant linear or quadratic responses to the increasing Na_2SO_4 supplement levels (Table 8, $P < 0.05$). Compared with the control and 0.3% groups, plasma Cl concentration increased ($P < 0.05$) whereas Ca concentration and the electrolyte balance ($Na^+ + K^+ - Cl^-$) decreased ($P < 0.05$) in hens exposed to 3.0% Na_2SO_4. Dietary supplementation with 1.5% or less Na_2SO_4 did not significantly affect the Ca, P, K, Na, and Cl concentrations and the electrolyte balance in plasma as compared to the control ($P > 0.05$).

3.5. Relative organ weight and histological observations

The hepatic and renal microscopic results of hens receiving 0.3% to 1.5% Na_2SO_4 were generally in accordance with those of the control. The appearances of central vein, bile duct, and hepatocytes in the liver (Fig. 1A), glomerulus, and renal tubules in the kidney (Fig. 1B) were normal in the treated groups of hens. However, extensive pathological changes in these organs were observed in hens receiving 3.0% Na_2SO_4. Dietary supplementation with 3.0% Na_2SO_4 resulted in severe hepatic pathological changes, evidenced by minor fatty degeneration and inflammatory cell infiltration in the liver tissue. Microscopic examination also showed slight tubular dilatation, swelling of renal tubular epithelial cells, and hyaline degeneration in the kidney tissue of hens after receiving 3.0% Na_2SO_4 for 8 weeks. In comparison to the control, no differences ($P > 0.05$) were observed in the relative organ weights of the livers (Fig. 1C) and kidneys (Fig. 1D) of the treated hens.

3.6. Intestinal morphology and barrier functions

Compared with the control and 0.3% groups, dietary supplementation with 3.0% Na_2SO_4 decreased the VH as well as the VH-to-CD ratios in the jejunum and ileum (Fig. 2A to C, $P < 0.05$). No significant differences in VH, CD, or VH-to-CD ratios were observed between the 1.5% Na_2SO_4 and control groups ($P > 0.05$). As shown in Fig. 3A to B, 0.3% and 0.6% supplemental Na_2SO_4 upregulated *ZO-1* and occludin mRNA expression abundance in the jejunum ($P < 0.05$), and 0.6% Na_2SO_4 upregulated *ZO-1*,

claudin-1, and occludin mRNA expression in the ileum ($P < 0.05$) relative to controls. Supplementation with 3.0% Na_2SO_4 resulted in significant downregulation of occludin and *ZO*-1 mRNA expression in the jejunum and ileum ($P < 0.05$). In addition, compared to the 0.6% group, 1.5% Na_2SO_4 supplementation decreased *ZO-1* and occludin transcript levels in the jejunum, and *ZO-1* and claudin-1 transcript levels in the ileum ($P < 0.05$). No significant differences existed between the 1.5% Na_2SO_4 and control groups ($P > 0.05$).

3.7. Cecal microflora, pH values, and moisture

The moisture contents in cecal digesta and excreta, cecal *Lactobacillus*, *Bifidobacteria* and *E. coli* concentrations, and the pH of excreta responded linearly or quadratically with the increased Na_2SO_4 levels (Table 9, $P < 0.05$). Compared with the control, diets with 1.5% or more Na_2SO_4 increased the moisture contents in cecal digesta and excreta ($P < 0.05$). The pH values of the excreta from hens receiving 3.0% Na_2SO_4 increased compared to that from hens in the other groups ($P < 0.05$). Cecal *E. coli* concentrations in hens receiving 3.0% Na_2SO_4 increased ($P < 0.05$), whereas cecal *Lactobacillus*, and *Bifidobacteria* concentrations decreased ($P < 0.05$) compared with the controls. No significant differences existed in cecal *C. perfringens* concentrations among groups ($P > 0.05$).

4. Discussion

4.1. Laying performance

The current work provides evidence that Na_2SO_4 supplementation remarkably affects the laying rates of hens during the pre-peak laying period. Dietary supplementation with 0.3% or 0.6% Na_2SO_4 dramatically increased the laying rates of hens, supporting the findings of a previous study (Wei et al., 2015). Interestingly, short-term feeding (3 weeks post-treatment) of high-dose Na_2SO_4 (1.5% and 3.0%) also significantly increased the laying rates. This improvement in laying performance might be due to that Na_2SO_4 could increase the bird's appetite (Mushtaq et al., 2013) evidenced by the increased ADFI of hens in second and third weeks of receiving 1.5% and 3.0% Na_2SO_4 (Appendix Fig. 1A). Nevertheless, long-term continuous exposure to 3.0% Na_2SO_4 caused significant reductions in laying rates, egg mass, and feed efficiency after 4 to 8 weeks of treatments (Appendix Fig. 1 B, 1C, and 1D) in the current study, indicating that Na_2SO_4 supplementation might have cumulative toxic effects on hens. The observed reductions might be attributed to the detrimental or adverse effects of Na_2SO_4 on blood
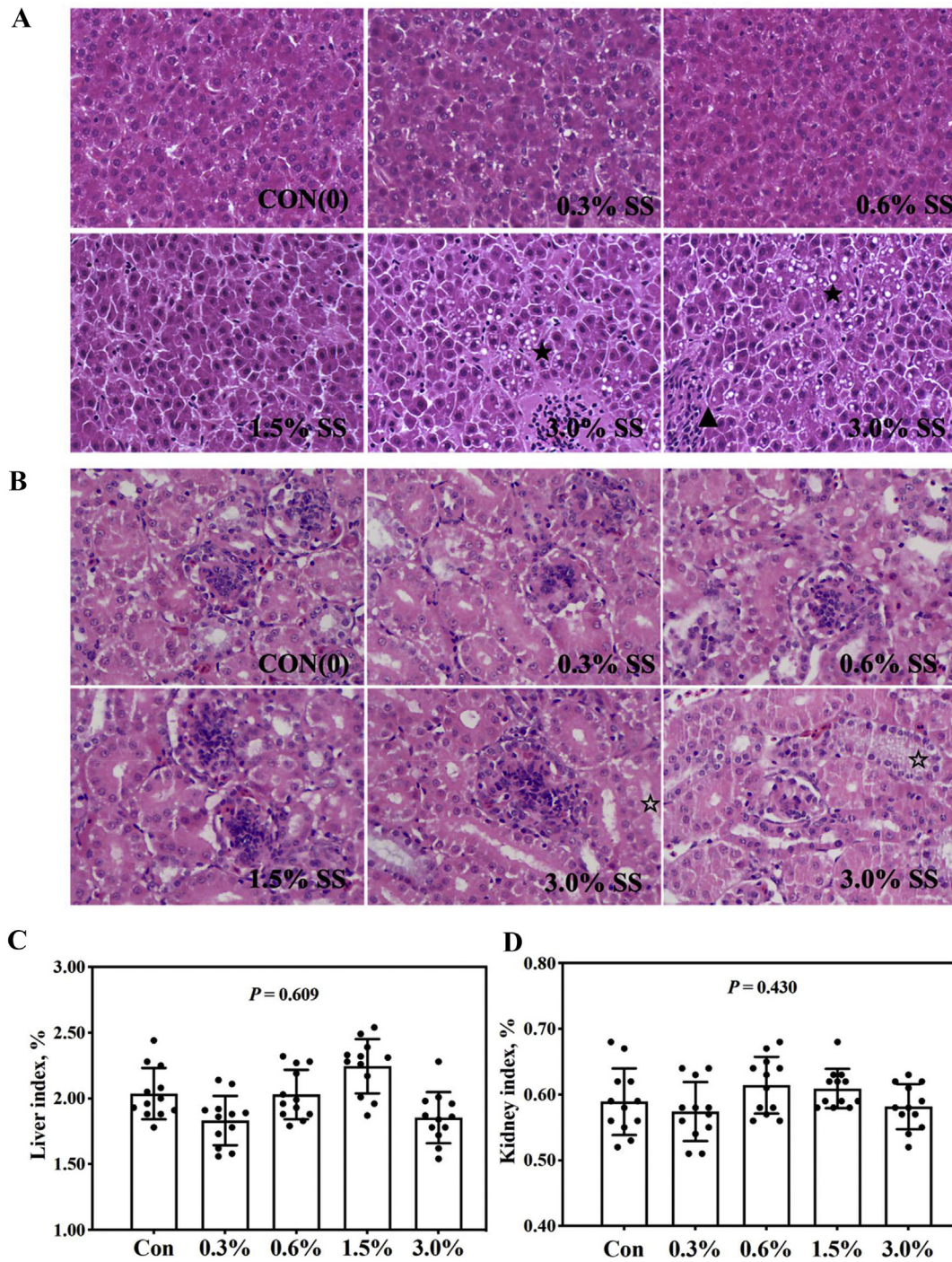
Fig. 1. Hematoxylin-eosin staining (HE, 400× magnification) of the liver (A) and kidney (B), and relative organ weight of the liver and kidney (C and D) of hens supplemented with 0 (control), 0.3%, 0.6%, 1.5%, and 3.0% of sodium sulfate (SS), respectively, for 8 weeks. ▲ indicates inflammatory cell infiltration and accumulation in the viscera of laying hens. ★ indicates fatty degeneration in the liver. ☆ indicates hydropic degeneration in the kidney.
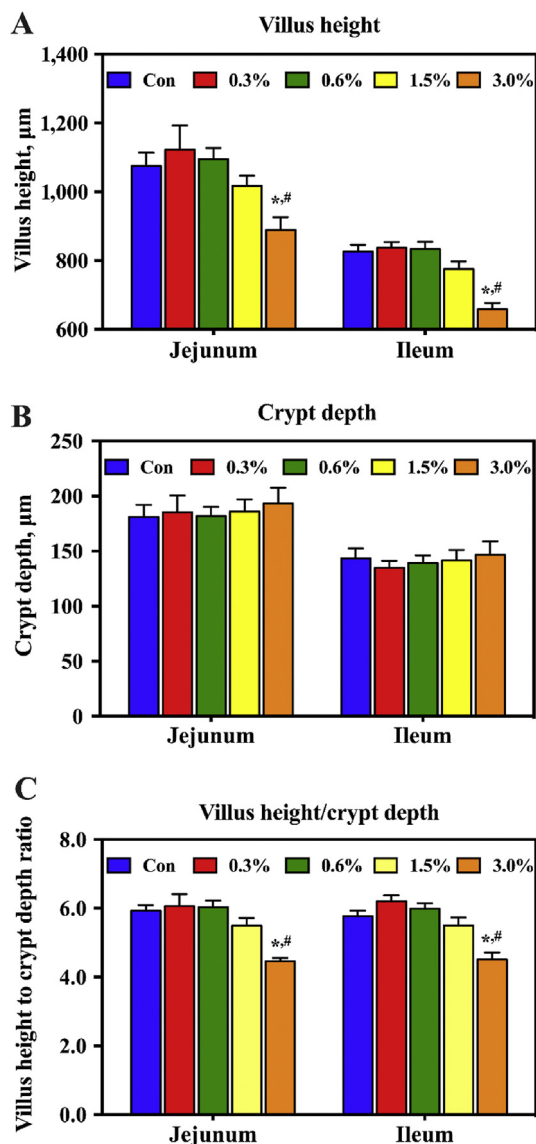
Fig. 2. Villus height (A), crypt depth (B), and villus height-to-crypt depth ratio (C) in the jejunum and ileum sections of hens supplemented with 0 (control), 0.3%, 0.6%, 1.5%, and 3.0% of sodium sulfate for 8 weeks. Data are presented as means ± standard error ($n = 6$). *, Significantly different from control (0) at $P < 0.05$, and #, significantly different from the recommended dose (0.3%) at $P < 0.05$.

electrolyte balance, histopathological changes, and gut health indices when an overdose of Na_2SO_4 was included in the feed.

4.2. Egg quality

Dietary supplementation with 0.3% to 0.6% Na_2SO_4 showed the higher eggshell percentages, thickness, and strength than any other treatment, which is consistent with the findings of Wei et al. (2015). The improvements in eggshell quality indices are likely attributed to the increased Ca absorption and retention associated with Na_2SO_4, since Ca is vital in eggshell formation (Gu et al., 2013). However, hens receiving 3.0% Na_2SO_4 experienced a notable reduction in eggshell quality,

corresponding with the declined Ca concentrations in plasma. High dietary Na and S intakes have been reported to interfere with the absorption of dietary Ca from the gut (Lichtorowicz et al., 2012). Na^+ has been reported to mediate Ca^{2+} uptake via the membrane-localized Na^+/Ca^{2+} exchange pump (Blaustein and Lederer, 1999), which has the capacity to translocate Na^+ and Ca^{2+} in opposite directions determined by the prevailing Na^+ gradient. Elevated Na levels in diets may disturb the balance of Na^+ and Ca^{2+} and thus decrease Ca availability for eggshell formation. In addition, the analyzed Cl^- ion levels in plasma were significantly elevated (from 102.6 to 114.3 mmol/L), which might also account for the notable reduction in eggshell quality. This adverse response is associated with reduced carbonic anhydrase activity in the shell gland caused by excess Cl intake. Since excess Cl^- intake limits the HCO_3^- supply to the shell gland, which in turn decreases the Ca uptake to the shell gland and consequently results in increased urinary Ca (Chen and Balnave, 2001).

4.3. Clinical blood parameters

More recent sulfur toxicity data (Alam and Anjum, 2003) derived from blood profiling of chickens indicate significantly reduced HGB concentrations and RBC in birds supplemented with 2% to 4% sulfur versus controls. In this study, compared with the control, RBC counts and HGB concentrations in hens decreased as a result of receiving 1.5% or 3.0% Na_2SO_4 for 8 weeks, supporting the findings of Alam and Anjum (2003). Although RBC declined in 1.5% and 3.0% groups, it was within the normal range (reference standard: 1.3 to 3.2 $\times 10^{12}$/L; current result: 2.46 to 2.91 $\times 10^{12}$/L) (Averbeck, 1992). However, the HGB concentrations in hens treated with 3.0% Na_2SO_4 were below the normal range for birds (reference standard: 85.2 to 175 g/L; current result in 3.0% group: 80.52 g/L) (Averbeck, 1992), indicating that serious pathological changes had occurred. Contrarily, previous studies showed that RBC counts were significantly increased in laying hens exposed to 1.8% or more Na_2SO_4 (Wei et al., 2015) or in broilers exposed to 0.245% NaCl (Fu et al., 2018). Therefore, further studies are needed to better elucidate this phenomenon.

4.4. Liver and kidney histopathology

The relative organ weight reflects the status of organ development in birds. Our results revealed that supplementation with up to 3.0% Na_2SO_4 had no observed detrimental effects on organ development. The hepatic and renal microscopic results of hens receiving 1.5% or less Na_2SO_4 were generally in accordance with the control hens. Histopathological lesion and detrimental changes in hematological parameters associated with the long-term 3.0% Na_2SO_4 exposure occurred mainly in the liver, kidney, and intestinal tract, corroborating the findings of Wei et al. (2015). Plasma ALT and AST activity and TBIL levels are biomarkers of hepatic damage and impaired hepatobiliary function in clinical examination (Chen et al., 2018). In this study, plasma AST and ALT activity, and TBIL levels were remarkably elevated in hens receiving 3.0% Na_2SO_4, indicating that an excess of Na_2SO_4 in the diet resulted in liver injury and pathological changes, which were confirmed by microscopic examination of the liver. Hens receiving 3.0% Na_2SO_4 had notable hepatic pathological changes characterized by obvious minor fatty degeneration and inflammatory cell infiltration in the liver tissue. The levels of plasma CRE and UA are considered
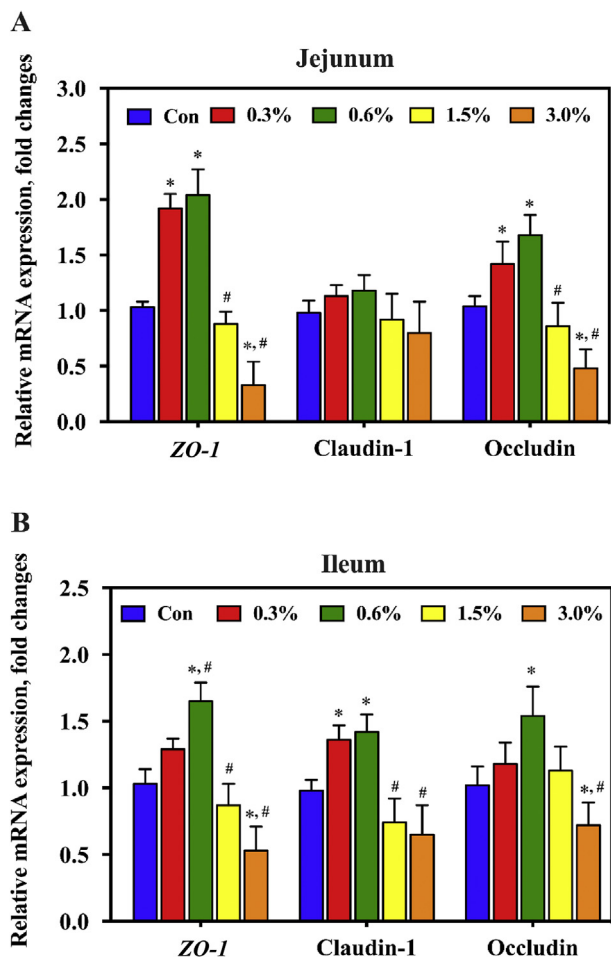
Fig. 3. Changes in the relative expression of zonula occludens protein-1 (*ZO-1*), claudin-1, and occludin in the jejunum (A) and ileum (B) sections of hens supplemented with 0 (control), 0.3%, 0.6%, 1.5%, and 3.0% of sodium sulfate for 8 weeks. Data are presented as means ± standard error ($n = 6$). *, Significantly different from control (0) at $P < 0.05$. #, Significantly different from the recommended dose (0.3%) at $P < 0.05$.

excretion through the kidney. The renal dysfunction might therefore be ascribed to long-term exposure to excessive levels of Na_2SO_4 leading to an increase in the absorption of sodium, which aggravates the bird's burden of renal excretion over time and consequently triggers renal dysfunction.

4.5. Intestinal morphology and barrier functions

Few studies have investigated the effects of dietary Na_2SO_4 on intestinal morphology and barrier functions (Jankowski et al., 2011; Lichtorowicz et al., 2012). In this study, improvements in intestinal villi-crypts absorptive areas and barrier functions were, to some extent, observed in low-level Na_2SO_4-supplemented hens. The transcript levels of all the tested tight junctions were upregulated to varying degrees at low (0.3% and 0.6%) Na_2SO_4 supplementation levels, with this effect being most obvious at 0.6% Na_2SO_4. The improvement in intestinal mucosal morphology and barrier functions in Na_2SO_4-supplemented hens might be associated with the antioxidant properties of S-containing compounds synthetized from Na_2SO_4 (Battin and Brumaghim, 2009). These results indicated that an appropriate amount of Na_2SO_4 supplementation may have partially improved intestinal barrier functions, which may be beneficial to intestinal health and then improve egg production finally.

Furthermore, intestinal permeability was evaluated using blood D-lactate levels and DAO activity. In this study, D-lactate levels and DAO activity in plasma increased notably in the 3.0% group compared with the control and low dose (0.3% and 0.6%) groups, indicating damages to the intestinal physical barrier induced by excessive exposure to Na_2SO_4. This finding was confirmed by the decreased relative abundances of jejunal or ileal *ZO-1*, claudin-1, and occludin transcript levels observed in hens receiving 3.0% Na_2SO_4. In addition, shortened VH and lower VH-to-CD ratios were observed in hens receiving 3.0% Na_2SO_4. Taken together, long-term exposure to high-dose (3.0%) Na_2SO_4 will damage the barrier functions of intestinal mucosa and increase intestinal permeability to some degree.

4.6. Cecal digesta parameters and microflora composition

Na_2SO_4-supplemented diets linearly increased the moisture in digesta and excreta in the current study. This phenomenon is likely attributed to the large osmotic changes in the intestinal lumen induced by excessive Na intake and the limited ability of kidney to reabsorb water from the distal renal tubules (Mushtaq et al., 2013). Combined with the findings of previous studies (Jankowski et al., 2011; Oviedo-Rondon et al., 2001), these

biochemical markers of renal damage (Chen et al., 2018), and these levels increased in hens receiving 3.0% Na_2SO_4, corresponding with the pathological changes (slight swelling of the renal tubular epithelial cells) in the kidney tissue. These results are consistent with a previous study (Adekunmisi and Robbins, 1987), which revealed that sodium intake might stimulate UA

Table 9
Parameters of caeca and excreta from a Na_2SO_4-tolerance evaluation study in the hens (28 weeks of age)[1].

Item	Na_2SO_4 supplemental level, %					SEM	*P*-value		
	0	0.3	0.6	1.5	3.0		Na_2SO_4	Linear	Quadratic
Caeca parameters									
pH of digesta	7.63	7.46	7.54	8.07	8.02	0.41	0.215	0.291	0.172
The moisture of digesta, %	79.49[b]	80.76[b]	80.99[b]	82.10[a]	83.31[a]	1.34	0.008	0.002	0.247
Cecal microflora, log_{10} CFU/g									
Lactobacillus	8.34[a]	8.80[a]	8.67[a]	8.39[a]	8.16[b]	0.06	<0.001	<0.001	<0.001
Bifidobacteria	8.45[ab]	8.75[a]	8.72[a]	8.36[ab]	8.24[b]	0.10	0.003	0.016	0.003
Escherichia coli	6.69[c]	6.50[c]	6.53[c]	6.93[b]	7.21[a]	0.06	<0.001	<0.001	<0.001
Clostridium perfringens	7.33	7.21	7.24	7.35	7.40	0.05	0.111	0.124	0.054
Excreta parameters									
pH of excreta	6.43[b]	6.57[b]	6.72[b]	6.92[ab]	7.43[a]	0.13	<0.001	<0.001	0.098
The moisture of excreta, %	74.29[c]	75.07[c]	75.59[c]	78.28[b]	82.58[a]	1.41	0.001	0.001	0.112

[a–c] Means without a common superscript within a row differ significantly ($P < 0.05$).
[1] Results are the means of 6 replicates of 2 hens each.

results indicate that excessive Na$_2$SO$_4$ intake (3.0% of the diet) will cause severe intestinal dysfunction. High moisture content in digesta has been reported to increase the rate of intestinal microflora proliferation and consequently result in disorders of the intestinal microflora (Smith et al., 2000). In this study, hens receiving 3.0% Na$_2$SO$_4$ decreased *Bifidobacteria* and *Lactobacillus* and increased *E. coli* colonization in the cecum. Interestingly, low levels of Na$_2$SO$_4$ supplementation to hens promoted the growth and activity of *Bifidobacteria* and *Lactobacillus* to some extent, and decreased *E. coli* colonization in the cecum. These results indicate that Na$_2$SO$_4$ supplementation has the potential to modulate the cecal microbiota, and these modifications might be linked to the moisture changes in the cecal content (Smith et al., 2000). However, little information is available about the positive effects of Na$_2$SO$_4$ on intestinal microflora in hens, further studies are needed to confirm and expand on these findings.

5. Conclusions

In summary, our results indicate that dietary supplementation with 0.3% to 0.6% Na$_2$SO$_4$ is of great benefit to improve the laying performance and eggshell quality of laying hens. Although minor changes of clinical blood parameters were observed in hens receiving 1.5% Na$_2$SO$_4$, these are not considered toxic due to the absence of any significant organ changes in these hens. However, long-term high dose (3.0% of the diet) of Na$_2$SO$_4$ had adverse effects on laying performance and eggshell quality caused by the adverse alterations in electrolyte balance, histological lesions, and disturbed hepatic, renal, and intestinal functions. Hence, a Na$_2$SO$_4$ concentration of 1.5% was non-deleterious to laying hens after a daily administration for 56 d, namely that dietary supplementation of up to 5 times the maximum recommended dose is safely tolerated by laying hens.

Author contributions

Dongyou Yu designed and supervised the experiments. **Bing Liu** and **Jiaming Zhu** carried out the animal trials. **Bing Liu**, **Qin Zhou**, and **Jiaming Zhu** collected and analyzed samples, as well as performing statistical analysis. **Bing Liu** prepared the draft manuscript. **Dongyou Yu**, **Jiaming Zhu** and **Qin Zhou** revised and finalized the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

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