Effects of coated sodium butyrate on performance, egg quality, nutrient digestibility, and intestinal health of laying hens

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ABSTRACT This study determined the effects of coated sodium butyrate (**CSB**) on production performance, egg quality, nutrient digestibility, and intestinal health of laying hens. We divided a total of 800 Lohmann laying hens, aged 51 wk, into 4 treatment groups: 0 (**CON**), 300 (**CSB1**), 500 (**CSB2**), and 800 (**CSB3**) mg/kg of CSB. Each group comprised 20 birds, with 10 replicates set. A 12-wk monitoring process was conducted for each laying hen. Compared to CON, dietary supplementation of CSB did not affect the average daily feed intake or the egg weight. The CSB3 group demonstrated a linear increase in the production performance ($P < 0.05$), with decreased feed conversion ratio ($P < 0.05$). CSB2 and CSB3 exhibited markedly elevated egg mass ($P < 0.05$). The CSB supplementation markedly enhanced the yolk color ($P < 0.05$). CSB1 improved the digestibility of dry matter ($P = 0.029$). No significant differences were observed among dietary treatments in the duodenal morphology ($P > 0.05$). The three dosages of CSB reduced the crypt depth ($P < 0.05$) in the jejunum, whereas CSB3 exhibited an increase in the villus height (**VH**; $P = 0.048$). The CSB3 group showed a markedly elevated ileal VH ($P = 0.011$). CSB supplementation significantly increased the butyric acid content in the cecum ($P = 0.009$). The hens fed on the 800 mg/kg CSB diet showed a significant increase ($P = 0.029$) in butyric acid content in the ileum. The CSB3 group showed an elevation in microbial diversity ($P < 0.05$). Additionally, at the phylum level, the CSB3 increased the enrichment of *Bacteroidetes*, the CSB2 increased *Firmicutes*, and the abundance of *Deferribacteres* was increased in CSB2 and CSB3 groups ($P < 0.05$). An enrichment of *Muribaculaceae* (family) was observed in the CSB3 group. In conclusion, dietary supplementation of CSB improved production, yolk color, intestinal morphology, butyrate content, and microbial composition in laying hens.

Key words: coated sodium butyrate, laying hens, production performance, egg quality, intestinal health

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INTRODUCTION

The use of antibiotics for nontherapeutic purposes in the diets of poultry is currently forbidden in numerous countries. Antibiotic substitutes with disease-resistance and growth-promoting effects, like phytochemicals, prebiotics, probiotics, acidifiers, and enzymes (Pearlin et al., 2020). Butyrate is one of these substitutes which can be used as an antibacterial (Jerzsele et al., 2012) and as the prime source of enterocyte energy (Friedman and Bar-Shira, 2005). Sodium butyrate, being most studied, is an acidifier that can be converted to butyric acid within the avian alimentary canal, and has attracted widespread attention recently. As an easily available energy source for poultry, it exerts certain nutritional functions in intestinal mucosal growth and structure. Sodium butyrate has bactericidal and bacteriostasis effects when used as a supplement and can reduce the pathogenic microbiota in the intestine (Ahsan et al., 2016), improve the feed conversion rate, and stimulate the immune system (Herrera et al., 2009; Lakshmi et al., 2011). Dietary supplementation with sodium butyrate positively affects avian intestinal health and physiological activities (Elnesr et al., 2019).

However, the offensive odor of sodium butyrate (Bedford and Gong, 2018) has an adverse effect on feed intake (Lin et al., 2020). It is, therefore, prepared in diverse forms, like butyrate glycerides or sodium butyrate. Generally, 2 forms of sodium butyrate are adopted for animal feed-coated sodium butyrate (**CSB**) and uncoated sodium butyrate (**UCSB**). UCSB is absorbed

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immediately in the anterior section of the alimentary canal before it can reach the distal intestine (Hume et al., 1993; Claus et al., 2007), thus, limiting the efficiency of sodium butyrate throughout the gastrointestinal tract (Piva et al., 2007). The application of sodium butyrate on poultry is thus restricted in practice. CSB comprises a secondary coating of sodium butyrate of different purity through intelligent microencapsulation technology, overcoming the defect of ordinary sodium butyrate. CSB ensures the delivery of sodium butyrate in the entire alimentary canal (Roda et al., 2007), where the active ingredients reach the intestinal tract and play their role (Immerseel et al., 2004). It was found that hens supplemented with CSB could enhance intestinal morphology and performance (Chamba et al., 2014; Kaczmarek et al., 2016).

There have been several experimental studies with sodium butyrate on livestock and poultry in recent years. However, studies on CSB to the intestinal health of laying hens are limited. The effects of CSB on laying hens have mainly been investigated for production performance. This study, therefore, aimed to investigate the effect of dietary supplementation of CSB at diverse doses on intestinal health, nutrient digestibility, egg quality, and production performance in laying hens and explored the optimal supplemental level. Sobczak and Kozlowski (2016) studied the effects of dietary 700 mg/kg 70% CSB on laying rate and physiological indicators of laying hens. Pires et al. (2020) studied the effects of 30% CSB supplementation (350 mg/kg, 700 mg/kg, 1,000 mg/kg) on the performance and egg quality of laying hens. According to previous studies and actual applications in production, we chose 300 mg/kg, 500 mg/kg, and 800 mg/kg as the supplemental level of CSB.

MATERIALS AND METHODS

Experimental Design and Management

The approval for the experimental protocols used in the study was provided by the Animal Care and Use Committee of Sichuan Agricultural University, China. In total, 800 Lohmann laying hens were pre-fed adaptively with a control diet for 2 wk. The hens were then assigned similar egg production rates, aged 51 to 62 wk into four treatments-0 (**CON**), 300 (**CSB1**), 500 (**CSB2**), and 800 (**CSB3**) mg/kg of **CSB** into their basal diet. The CSB used in this study was obtained from Adisseo Life Sciences Products (Shanghai) Co., Ltd. 10 replicates were set for every treatment, where 2 cages were set for each replicate. Each cage (100 cm length × 64 cm width × 42 cm height) housed 10 hens and was equipped with nipple-type drinkers and a feed trough along the length of the cage. The birds were raised under environmentally controlled conditions of 24°C, 50 to 65% humidity and 16-h/8-h light-dark cycle. All treatments were equally assigned into layer houses to minimize the impacts on the environment. The birds had free access to water and experimental diets throughout the 12-wk experimental period. The mental state of the hens was observed daily, and the mortality was recorded promptly. A corn-soybean-type diet was used as the basal diet, where the nutrient levels and composition were determined based on NRC (1994) and Chinese Chicken Breeding Standard (2004) (Table 1).

Productive Performance and Sample Collection

The eggs were collected daily, and the total egg weight, (broken) egg numbers, soft, and dirty eggs of each replicate were recorded. The mortality was recorded daily throughout the experimental period. The feed intake was recorded weekly, along with the determined average egg weight, daily egg production rate, feed conversion ratio (**FCR**), and broken eggs rate. The feed intake was calculated as the difference between feed offered and leftovers to determine the amount of feed consumed/hen/day, in grams. Meanwhile, the FCR was determined as the ratio of total feed intake (g) to the total egg weight (g). The egg mass was calculated by multiplying the laying rate (%) by the average weight of eggs (g) and divided by 100.

After the experiments, 40 hens were randomly selected (1 from every replicate) to sacrifice using cervical dislocation. The duodenal, jejunal, and ileal segments of about 2-cm long were then harvested and fixed

Table 1. Composition and nutrient level of the basal diet (as-fed basis).

Ingredients,%	Contents, %
Corn(7.8% of CP)	62.39
Soybean meal(43% of CP)	19.48
Soybean oil	1.00
Corn protein meal(55% of CP)	3.60
Limestone(fine)	6.20
Limestone(coarse)	3.10
Dicalcium phosphate·$2H_2O$	1.56
Lysine·H_2SO_4(70%)	0.08
DL-Methionine (99%)	0.08
Unite bran	1.46
Sodium chloride	0.30
Choline chloride	0.10
Vitamin premix[1]	0.50
Mineral premix[2]	0.15
Total	100.00
Nutrient level, %	
ME, kcal/kg	2680.00
Crude protein	15.38
Calcium	4.00
Total phosphorus	0.57
Available phosphorus	0.37
D-Lysine	0.73
D-Methionine	0.34
D-Threonine	0.58
D-Tryptophan	0.16
D-Methionine + D-Cysteine	0.58

[1]Vitamin premix provided the contents below in diet (/kg): VA, 9,950 IU, VB_1, 37.7 mg, VB_2, 12 mg, D-pantothenate, 18.2 mg, VB_6, 7.55 mg, VB_{12}, 0.5 mg, VD_3 5,000 IU, VE:70 IU, VK_3, 4.47 mg, Biotin, 4 mg, VC, 195 mg, niacin acid, 70.35 mg.

[2]Mineral Premix offered the contents below in diet (/kg): Cu (as copper sulfate), 9.6 mg; Fe (as ferrous sulfate), 64 mg; Mn (as manganese sulfate), 121.5 mg; Zn (as zinc sulfate), 57 mg; I (as potassium iodide), 0.60 mg; Se (as sodium selenite), 0.36 mg.

with 4% neutral formaldehyde for histological analysis (Xiong et al., 2018). Fresh cecal and ileal contents were further obtained, transferred to a sterile microtube, and preserved under −80°C to explore the intestinal microbial populations (cecal contents) and short-chain fatty acids (**SCFAs**; Wang et al., 2019).

Determination of Egg quality

A total of 80 eggs, that is, 20 eggs under every treatment (2 from each replicate), were harvested and adopted to determine the egg quality after 12 wk. The eggshell breaking strength was assessed by using the eggshell force gauge (model II, Robotmation Co., Ltd., Tokyo, Japan). Additionally, the eggshell thickness gauge (Robotmation Co., Ltd.) was utilized to measure egg thicknesses in three varied regions of the egg (equatorial region, small end, large end). Further, the egg multitester (EMT-7300, Robotmation Co., Ltd.) was used to assess the albumen height, egg weight, yolk color, and Haugh units. The egg quality was measured as reported by Ebeid et al. (2012).

Determination of Nutrient Digestibility

This study additionally performed a metabolic digestibility assay using the indicator approach for determining nutrient digestibility. Specifically, a diet containing 0.5% chromic oxide was provided to the laying hens after the feeding experiment as the indigestible indicator. A total of 40 hens (10 under every treatment) were placed individually in separate cages. After acclimatization for 4 d, excreta samples from each bird (nearly 50 g/d) were gathered for a further 48 h of the trial, followed by immediate preservation under −20°C for further analyses (Sales and Janssens, 2003). The potential contamination of the excreta samples by foreign materials, feed, or feathers was avoided during collection. A forced-air drying oven was utilized to dry the excreta samples under 65°C.

Samples of the diets and feces were explored for moisture by oven drying (930.15), crude protein (**CP**) by Kjeldahl (990.03), calcium and total phosphorus (985.01) as well as ash by incineration (942.05) according to the description of the AOAC International (2007). Moreover, adiabatic bomb calorimetry was conducted following specific protocols (Parr Instrument Company, IL) to determine the gross energy (GE). The data on the composition of diets and feces were used to calculate the digestibility coefficients of dry matter (**DM**), CP, GE, total phosphorus (**TP**), ash, and calcium (Song et al., 2012), where digestibility (%) = 100% × (nutrient ingested-nutrient excreted)/nutrient ingested.

Intestinal Morphology Analysis

The intestinal segments soaked in 4% paraformaldehyde were removed, which were dehydrated with ethanol, cleared based on xylene, embedded in paraffin wax as well as sectioned with a Leica CM1860 microtome; the tissues were then cut into 5-μm thin sections and transferred on glass slides with the sections being subsequently subject to hematoxylin-eosin staining (Liu et al., 2020), followed by determination of crypt depth (**CD**) and villus height (**VH**). Ten straight and intact villi were selected from every sample to observe their morphology using the Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD). The CD refers to the invagination depth between neighboring villi, whereas VH indicates the distance between the villus top to the crypt-villus junction. The VH to CD ratio was defined as VH/CD.

Determination of SCFA Concentrations

The concentration of acetate, propionate, and butyric acid in the cecal chyme was determined using a gas chromatograph (VARIAN CP-3800). Approximately 0.7 g of the sample (mass was precisely recorded) was taken into a 2 mL centrifuge tube, followed by dilution using ultra-pure water (1.5 mL), 30 min standing as well as 15 min centrifugation at 20,000 g (sample concentration in the extract is M). Thereafter, 1 mL supernatant was transported into the novel tube to blend with 210 mmol/L crotonic acid (23.3 μL) and 25% metaphosphoric acid (0.2 mL). Once the mixture was incubated for 30 min at 4°C, it was subject to 10 min centrifugation at 20,000 × g, followed by filtration into a 1.5 mL tube. Methanol (0.9 mL) was subsequently added, and the mixture was subject to 5 min centrifugation at 10,000 × g, followed by filtration of the supernatant with the 0.22 μm membrane as well as collection in the 1.5 mL tubes for further analysis (Li et al., 2021).

Cecal Microbial Diversity

The study adopted the QIAamp DNA stool Mini Kit (QIA-192 Gen, GmbH Hilden, Germany) to extract cecal chyme DNA. The isolation was confirmed by 2% agarose gel electrophoresis, and the concentration of extracted DNA was determined by using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, EUA). Before sequencing, the above 16S rDNA V3- V4 region of each sample was amplified with a set of primers targeting the 16S rRNA gene region. Sequencing libraries were generated using NEB Next Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) following manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and Agilent Bioanalyzer 2100 system. A library was constructed by pyrosequencing of amplicon using the Illumina MiSeq platform, which was verified by Qubit and Q-PCR. After the library was qualified, the library was sequenced using HiSeq2500 PE250. Sequencing and bioinformatics analysis were performed by Novo Genomics Bioinformatics Technology Co., Ltd. (Beijing, China).

Table 2. Effect of CSB supplementation in the diet on laying hen production performance[1].

Item	CSB level(mg/kg)				SEM	*P*-value		
	0	300	500	800		ANOVA	Linear	Quadratic
Egg production, %	87.41	87.77	89.01	90.52	0.44	0.051	0.007	0.021
Egg weight, g	62.09	61.69	61.84	61.91	0.11	0.642	0.658	0.519
Egg mass (g/d/hen)	54.27[b]	54.15[b]	55.05[ab]	56.04[a]	0.30	0.081	0.018	0.039
FCR	2.00[b]	2.02[b]	2.00[b]	1.96[a]	0.01	0.025	0.028	0.009
ADFI, g	108.70	109.40	110.20	109.60	0.40	0.637	0.355	0.462

Abbreviations: ADFI, average daily feed intake; FCR, feed conversion ratio; CSB, coated sodium butyrate; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate.

Egg mass = egg production × egg weight/100.

[a,b]Averages with diverse superscripts in the column showed a significant difference ($P < 0.05$).

[1]Average from 10 replicates.

Operational taxonomic units (**OTUs**) were clustered, which were later adopted to investigate the alpha-diversity (Simpson, Shannon) and richness (Chao) at the threshold of 97%. Non-metric multidimensional scaling (**NMDS**) analysis was performed based on the Bray-Curtis distance matrix calculated by OTU information to show the beta diversity using principal component analysis (**PCA**) and Mothur method. Meanwhile, Silva linear discriminant analysis (**LDA**) effect size (**LEfSe**) method was used to explore the differences among different treatments.

Statistical Analysis

The statistical analysis was performed through one-way ANOVA using the SAS 9.2 general linear model (**GLM**) package (version 9.2, SAS Institute Inc., Cary, NC). When the significance of the therapeutic effect was detected among several comparisons, the averages of the diverse treatments were compared using Duncan's test. The CONTRAST statement was utilized for linear and quadratic trend analysis to assess the effects of the CSB supplementation dose on the different parameters. Furthermore, broken-line, asymptotic, and quadratic model regression was carried out using the nonlinear (**NLIN**) procedure of SAS, and the best-fitted model was chosen according to the coefficient of determination and *P*-value (Bai et al., 2022). The results were displayed in the form of the means and standard error of means, where $P < 0.05$ indicates the statistical significance and $0.05 \leq P < 0.1$, the statistical trend.

RESULTS

Production Performance and Egg Quality

Supplementing 800 mg/kg CSB in the diet markedly reduced the FCR ($P = 0.025$) and elevated egg production (linear, $P = 0.006$) from 1 to 12 wk in comparison to the control group, as presented in Table 2. In addition, the hens fed with 500 mg/kg and 800 mg/kg of the CSB supplemented diets demonstrated greater egg mass ($P < 0.05$). However, the supplementation did not affect the ADFI or the egg weight. The yolk color was higher ($P < 0.01$, Table 3) in the CSB1, CSB2, and CSB3 groups when compared with that in the control group. However, there existed no obvious difference in the Haugh unit, albumen height, eggshell thickness, eggshell weight, or eggshell strength among the groups ($P > 0.05$).

Nutrient Digestibility

A marked elevation was observed in the digestibility of DM in the CSB1 group when compared with the control group ($P < 0.05$, Table 4). Additionally, an increasing trend was observed in the digestibility of GE ($P = 0.051$) and TP ($P = 0.076$), whereas the digestibility of CP, calcium, and ash remained largely unchanged after CSB supplementation ($P > 0.05$).

Intestinal Morphology

Dietary CSB supplementation did not result in a change in the duodenal morphology ($P > 0.05$, Table 5).

Table 3. Effect of diverse CSB supplementation doses in laying hen egg quality[1].

Item	CSB level(mg/kg)				SEM	*P*-value		
	0	300	500	800		ANOVA	Linear	Quadratic
Albumen height, mm	7.57	7.50	7.58	7.11	0.09	0.192	0.091	0.125
Yolk color	9.28[b]	10.20[a]	10.56[a]	10.32[a]	0.13	<0.01	<0.01	<0.01
Haugh unit	85.94	85.68	85.84	83.61	0.56	0.408	0.162	0.257
Eggshell weight, g	6.69	6.48	6.70	6.65	0.06	0.500	0.958	0.796
Eggshell strength, kg/cm^2	4.60	4.35	4.47	4.62	0.08	0.614	0.831	0.458
Eggshell thickness, mm	0.41	0.44	0.42	0.41	0.01	0.571	0.753	0.645

Abbreviations: CSB, coated sodium butyrate; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate; SEM, standard errors of mean.

[a,b]Average with diverse superscripts in the column shows significant difference ($P < 0.05$).

[1]Average from 10 replicates, and 2 eggs were selected from every replicate.

Table 4. Effects of supplementation of CSB on nutrient retention (%) in laying hens[1].

Item	CSB level (mg/kg)				SEM	*P*-value		
	0	300	500	800		ANOVA	Linear	Quadratic
DM, %	69.09[b]	73.26[a]	68.42[b]	70.88[ab]	0.64	0.029	0.928	0.806
CP, %	46.81	47.10	48.10	49.28	0.89	0.780	0.304	0.576
GE, %	77.00	79.96	76.52	78.56	0.49	0.051	0.759	0.821
TP, %	38.34	42.54	34.95	33.03	1.40	0.076	0.059	0.091
Ash, %	36.39	43.13	46.74	42.45	1.75	0.214	0.167	0.109
Ca, %	57.46	56.82	56.13	54.80	1.47	0.933	0.531	0.803

Abbreviations: Ash, total ash; Ca, calcium; CP, crude protein; CSB, coated sodium butyrate; DM, dry matter; GE, gross energy; TP, total phosphorus; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate.

[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

[1]Average from 10 replicates.

The CSB3 group demonstrated elevated VH of jejunum and ileum when compared with the control group ($P < 0.05$). Meanwhile, a significant decrease was observed in the CD ($P = 0.036$) of the CSB groups, with the high dosage (800 mg/kg) showing the greatest effect. Relative to the control group, the VH/CD of hens remained largely unchanged after CSB supplementation ($P > 0.05$).

SCFA Concentrations

The laying hens in the CSB3 group showed an increased ($P < 0.05$, Table 6) content of butyrate in the ileum compared to the hens in the other CSB groups and the CON group. The addition of CSB also remarkably elevated ($P < 0.05$) the butyrate level in the cecum. No marked differences were observed among the treatments with isobutyric acid, propionic acid, acetic acid, valeric acid, and isovaleric acid ($P > 0.05$). CSB supplementation elevated the contents of total short chain fatty acids in cecal chyme.

Cecal Microbial Composition

According to the rarefaction curves (Figure 1A), each sample approached the saturation plateau, indicating that the sequencing data was reasonable and covered all the species in the sample. There were 946 common OTUs in cecal microbiota among the 4 groups, with each group possessing diverse specific OTUs. According to the Venn diagram (Figure 1B), the control group and CSB groups, respectively, had 211, 201, 312, and 231 unique OTUs. This study detected a total of 10 phyla by bacterial microbial analysis, out of which 4 phyla were predominant in the CSB groups and the control groups, including *Bacteroidetes, Proteobacteria*, and *Firmicutes*, which occupied over 90% of the overall sequences (Figure 1C). As shown in Table 7, the addition of 800 mg/kg CSB increased *Bacteroidota* ($P < 0.05$). The 500 mg/kg CSB had higher *Firmicutes* ($P < 0.05$) enrichment than that in CON layers. Moreover, in comparison to the CON group, *Deferribacteres* was higher in CSB2 and CSB3 groups ($P < 0.05$). With the increase in CSB level, *Fusobacteriota* was decreased linearly ($P < 0.05$).

Alpha and Beta Diversity of Cecum Microbiota

Chao1, ACE, Simpson, and Shannon indexes indicate the microbial diversity and richness separately. This study conducted NMDS and PCA for the intuitive measurement of the degree of similarity across diverse microbial communities under the four treatments. The fecal

Table 5. Effect of diverse CSB supplementation doses in laying hen intestinal morphology[1].

Item	CSB level (mg/kg)				SEM	*P*-value		
	0	300	500	800		ANOVA	Linear	Quadratic
Duodenum								
Villus height (μm)	1,400.80	1,374.50	1,384.80	1,230.46	35.15	0.310	0.124	0.203
Crypt depth (μm)	213.66	212.38	210.74	195.26	5.56	0.647	0.270	0.449
VH:CD	6.55	6.60	6.72	6.40	0.18	0.938	0.845	0.860
Jejunum								
Villus height (μm)	1,003.74[b]	1,186.48[ab]	1,133.54[ab]	1,259.86[a]	33.83	0.048	0.481	0.278
Crypt depth (μm)	181.91[a]	157.06[b]	155.60[b]	155.11[b]	4.06	0.036	0.018	0.016
VH:CD	6.78	5.75	6.99	7.09	0.21	0.085	0.271	0.202
Ileum								
Villus height (μm)	816.92[b]	800.29[b]	797.18[b]	1,011.82[a]	28.68	0.011	0.019	0.005
Crypt depth (μm)	133.80	145.03	131.36	162.72	5.32	0.129	0.121	0.180
VH:CD	6.20	5.54	6.07	6.54	0.19	0.302	0.341	0.210

Abbreviations: CSB, coated sodium butyrate; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate.

[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

[1]Averages from 10 replicates.

Table 6. Effect of dietary feed with different concentrations of CSB on SCFA in ileum and Cecum (mmol/L)[1].

Item	CSB level (mg/kg)				SEM	*P*-value		
	0	300	500	800		ANOVA	Linear	Quadratic
Ileum								
Acetic acid	2.52	1.92	1.34	2.31	0.34	0.933	0.867	0.817
Propionic acid	0.16	0.20	0.13	0.18	0.02	0.771	0.963	0.970
Isobutyric acid	0.08	0.02	0.04	0.05	0.01	0.270	0.366	0.181
Butyrate	0.05[b]	0.05[b]	0.01[b]	0.33[a]	0.04	0.009	0.046	0.011
Isovaleric acid	0.06	0.03	0.03	0.05	<0.01	0.144	0.479	0.076
Valeric acid	0.04	0.03	0.02	0.03	0.01	0.701	0.479	0.551
T-SCFAs	2.56	2.21	2.26	2.69	0.37	0.964	0.907	0.868
Cecum								
Acetic acid	40.22	42.66	46.76	49.46	2.67	0.641	0.191	0.430
Propionic acid	12.71	15.33	14.95	15.15	0.87	0.689	0.361	0.527
Isobutyric acid	1.01	0.85	0.96	1.03	0.07	0.816	0.822	0.698
Butyrate	4.95[b]	8.38[ab]	9.30[a]	10.20[a]	0.67	0.029	0.005	0.011
Isovaleric acid	1.05	1.01	1.01	1.01	0.05	0.995	0.836	0.967
Valeric acid	0.92	0.92	0.99	0.99	0.07	0.969	0.639	0.897
T-SCFAs	63.02	73.31	73.15	76.58	4.01	0.666	0.257	0.486

Abbreviations: CSB, coated sodium butyrate; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate.

[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

[1]Average from 10 replicates.

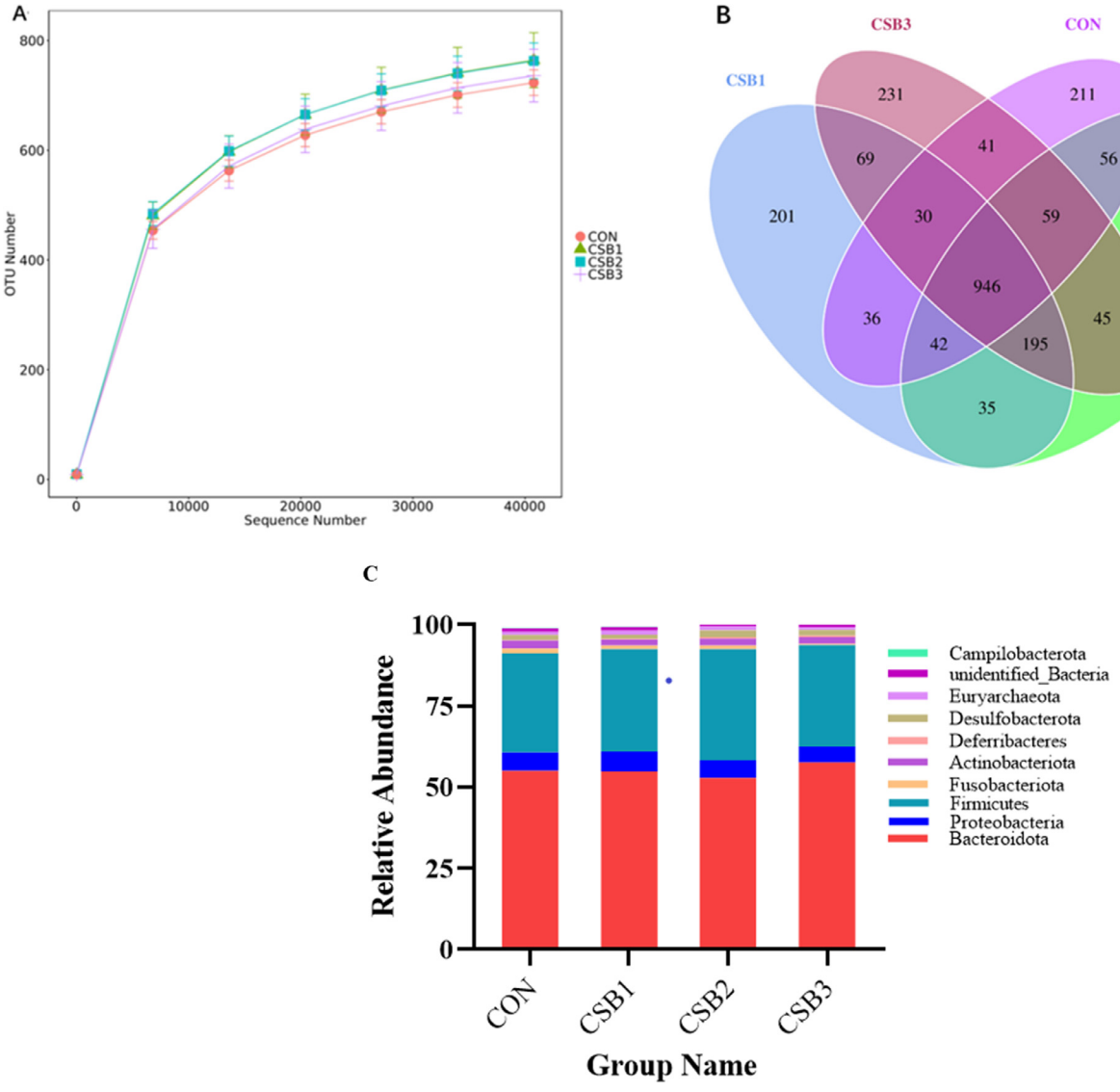

Figure 1. Rank abundance curve showing microbial OTUs of respective samples (A). Venn diagram showing cecal microorganisms in the samples (B). Effects of coated sodium butyrate coating on the relative abundances of those 10 most significant phyla within cecal microbiota. Phylum level classifications for observed features (C). Abbreviations: CON, control group; CSB1-CSB3, 300, 500, and 800 mg/kg CSB, respectively; OTUs, Operational taxonomic units.

Table 7. Effect of different CSB supplementation doses on the relative abundances of cecal microbiota at the phylum level (%)[1].

Item	CSB level (mg/kg)				SEM	*P*-value		
	0	300	500	800		ANOVA	Linear	Quadratic
Bacteroidota	51.26[b]	51.64[b]	51.24[b]	56.75[a]	0.72	0.005	0.007	0.003
Proteobacteria	5.27	5.92	5.39	4.81	0.21	0.378	0.351	0.247
Firmicutes	28.33[b]	29.82[ab]	32.81[a]	30.20[ab]	0.59	0.049	0.105	0.060
Fusobacteriota	1.31	1.09	1.12	0.38	0.15	0.091	0.021	0.049
Actinobacteriota	2.33	1.68	2.03	2.11	0.12	0.220	0.664	0.239
Deferribacteres	0.11[b]	0.17[b]	0.49[a]	0.44[a]	0.04	<0.001	<0.001	<0.001
Desulfobacterota	1.46	1.31	2.01	1.71	0.11	0.119	0.148	0.336
Euryarchaeota	0.89	1.18	1.18	0.78	0.11	0.514	0.784	0.315
Unidentified_Bacteria	1.00	0.97	1.08	0.94	0.03	0.209	0.964	0.567
Campilobacterota	0.06	0.06	0.09	0.06	0.01	0.509	0.730	0.710

Abbreviations: ADFI, average daily feed intake; CSB, coated sodium butyrate; FCR, feed conversion ratio; Tre, ANONA; Lin, Linear; Quad, Quadratic; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate.

[a,b]Average with diverse superscripts in the column shows significant difference ($P < 0.05$).

[1]Average from 10 replicates.

microbiota of CON and CSB groups were classified as 4 intersected clusters (Figure 2). The PCA plots for the groups revealed the different cecal microbial communities in the control group compared with the CSB2 group. The stress value was less than 0.2 ($P = 0.08$; Figure 2B), which indicates the feasibility of using NMDS in precisely reflecting different levels across diverse samples. According to Figure 3 (LEfSe), the CON group showed an increased abundance in *Clostridia* (class), while the CSB3 group had markedly enriched Muribaculaceae (family) abundance. Dietary CSB supplementation had no effect on ($P > 0.05$, Table 8) alpha diversity index of cecal microbiota except for Shannon index (linear effect, $P = 0.049$).

DISCUSSION

Performance is the most efficient indicator of the condition of laying hens. As an additive, CSB eventually decomposes to produce SCFAs, while short-chain fatty acids are beneficial to improve animal performance. The study revealed that dietary CSB supplementation does not significantly affect ADFI or egg weight among groups, which is consistent with the report by Sobczak and Kozlowski (2016). In addition, the study demonstrated that supplementation with 800 mg/kg of CSB increased egg production and had a positive impact on FCR throughout the trial period. A similar result was obtained by Miao et al. (2021), who reported that diet supplemented with CSB markedly elevated egg production and reduced FCR. The increase in egg production and feed efficiency may be attributed to the improved intestinal morphology and digestive capacity that induce the intestinal availability of other nutrients for the benefit of the intestinal health. However, Pires et al. (2020) reported that supplementing the diet with CSB did not affect FCR or egg production in laying hens. These inconsistencies may be related to the physiological stage of laying hens, the dosage of CSB, environmental

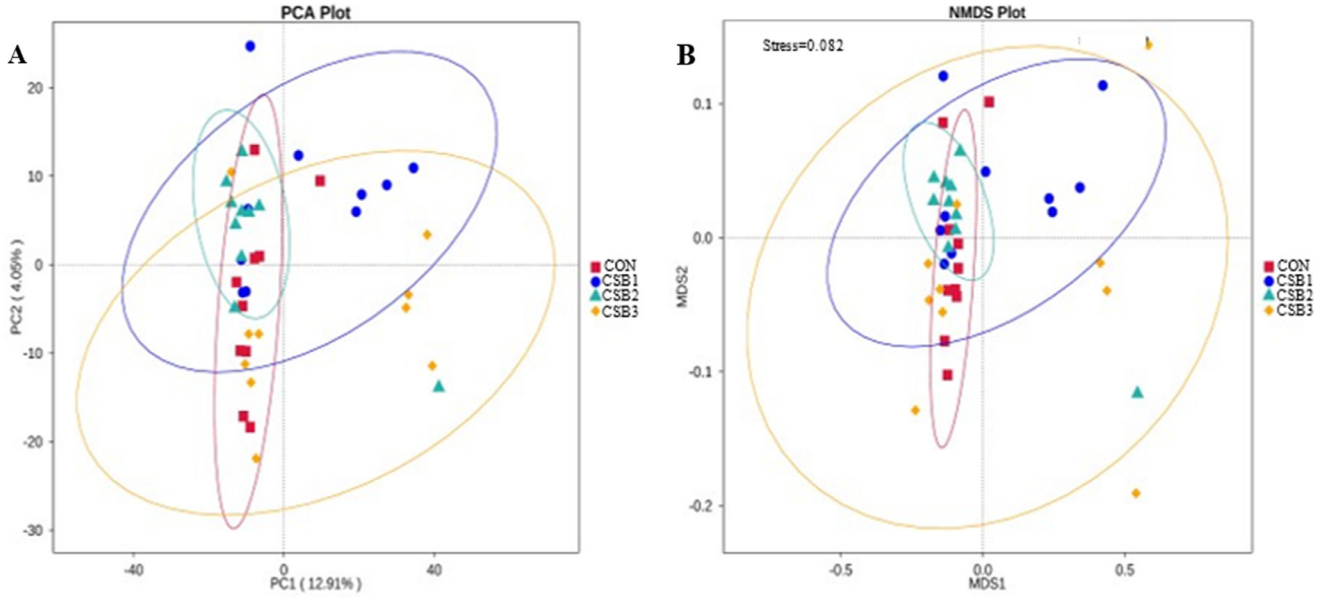

Figure 2. PCA plot according to weighted UniFrac (A) together with NMDS according to beta-diversity distance matrix via Qiime (B). Abbreviations: CON, control group; CSB1, 300 mg/kg coated with sodium butyrate; CSB2, 500 mg/kg coated with sodium butyrate; CSB3, 800 mg/kg coated with sodium butyrate.

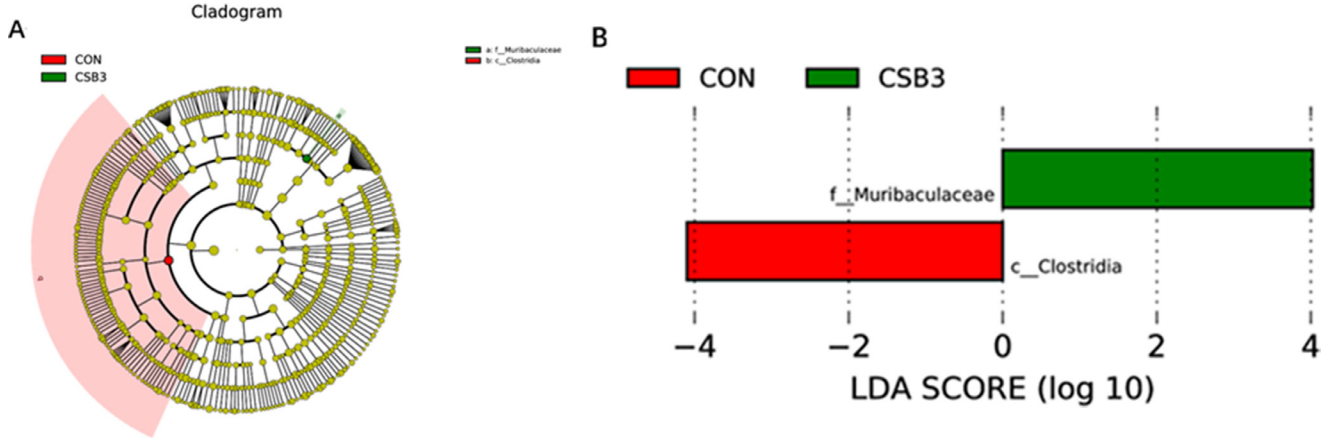

Figure 3. LEfSe analysis for the taxa with differential abundance among cecal microbiota. In a cladistic diagram, circles radiating from the inside out represent the classification level from phylum to genus (or species) (A). The histogram of distribution shows species whose LDA Scores > 4 (default value), species with statistically different biomarkers between the groups (B). Abbreviations: CON, control group; CSB1, 300 mg/kg coated with sodium butyrate; CSB2, 500 mg/kg coated with sodium butyrate; CSB3, 800 mg/kg coated with sodium butyrate; LEfSe, linear discriminant analysis effect size.

hygiene conditions, and the species of laying hens (Khong et al., 2014).

The color of the yolk is an important attribute for producers and, above all, consumers. Consumers are known to prefer darker, especially golden-orange yolks (Hasin et al., 2006). The deep yellow and golden-orange color of the yolk is suggested to indicate the health of an egg. By contrast, a lighter color may be associated with poor production or poor health of the hen (Englmaierová et al., 2014). Previous studies have shown no significant effect of CSB supplementation on the color of the yolk (Sobczak and Kozlowski, 2016). In this study, the CSB groups demonstrated remarkably enhanced yolk color in comparison with the control group. The color of the yolk is associated with pigment substances in the feed, like carotenoids (Lessire et al., 2017). Acidulants can promote the absorption of nutrients in the intestine (Freitag and Lückstädt, 2007), while CSB is an organic acid, which can retain its lipid-soluble component (Wang et al., 2019). The yolk color is related to dietary lutein content (Sun et al., 2013), lutein deposition will deepen the yolk color, and lutein is a fat-soluble substance (Johnson, 2004). BA is an effective component of CSB, which is lipophilic, so dietary CSB supplementation can promote intestinal absorption of lutein, thus deepening the yolk color. Heavy metal ions and unsaturated fatty acids in the feed will make the feed easy to be oxidized, easy to oxidize lutein, and lose its coloring ability so that the color of egg yolk becomes lighter. Yuan et al. (2016) showed that adding antioxidants in diets can increase yolk color, while CSB has an antioxidant effect (Miao et al., 2022). Therefore, the increase in yolk color of eggs in CSB group in this study may be due to the protective effect of CSB on carotenoids as an antioxidant.

Nutrient digestibility is an important index for measuring the status of animal health, feed nutritive value, digestive capacity, and additives on animal production efficiency (Zhao et al., 2008). Supplementing a broiler diet with sodium butyrate at 300 mg/kg did not notably affect the DM digestibility in previous studies (Smulikowska et al., 2009). However, this study demonstrated an increase in the digestibility of DM with dietary supplementation of 300 mg/kg of CSB. This is consistent with a study by Yang et al. (2010), who reported that supplementing the diets of laying hens with CSB can significantly increase the digestibility of DM and enhance nutrient metabolism in broilers (Riboty et al., 2016). Similarly, as suggested by Upadhaya et al. (2020), dietary supplementation of CBS in weaned piglets enhanced DM digestibility. Nutrient digestibility is firmly associated with avian absorption ability, while intestinal villous morphology aids nutrient absorption into the gut. Increasing the intestinal mucosal surface area in birds facilitates the transfer of nutrients from the intestine to the circulatory system (DeSesso and Jacobson, 2001), with shallow crypts and long villi support

Table 8. Effect of dietary feed different concentrations of CSB on Alpha diversity index of cecal microorganisms[1].

	CSB level (mg/kg)					*P*-value		
Item	0	300	500	800	SEM	ANOVA	Linear	Quadratic
Shannon	6.69	6.86	6.99	6.90	0.04	0.077	0.049	0.036
Simpson	0.97	0.98	0.98	0.98	<0.01	0.448	0.174	0.284
chao1	795.26	836.74	829.57	825.38	8.43	0.315	0.270	0.216
ACE	810.65	843.40	840.40	841.55	8.33	0.446	0.240	0.321

Abbreviations: CSB, coated sodium butyrate; 300, 300 mg/kg coated sodium butyrate; 500, 500 mg/kg coated sodium butyrate; 800, 800 mg/kg coated sodium butyrate.

[1]Average from 10 replicates.

Table 9. Estimations of dietary CSB requirements of laying hens based on the best quadratic models.

Dependent variable	Quadratic regression equation	*P*	R^2	Dietary CSB requirements (mg/kg)
Yolk color	$Y = -6.67E\text{-}06X^2 + 0.007X + 8.912$	<0.01	0.868	509.75
Crypt depth (Jejunum)	$Y = 8.00054E\text{-}005X^2 - 0.096 + 181.324$	0.016	0.969	600.38
Butyrate	$Y = -6.66667E\text{-}006X^2 + 0.012X + 5.029$	0.011	0.998	863.23

Abbreviations: CSB, coated sodium butyrate.
Regression equations according to dietary CSB supplementary doses.

the increased surface area to achieve an increased absorption capacity and normal intestinal development (Zhang et al., 2005; Yang et al., 2009). It has been reported that CSB decreased CD and increased VH:CD in the ileum (Xiong et al., 2018). In the case of poultry, due to the particularity of the digestive system, their duodenum is relatively short, and the absorption of nutrients is mainly concentrated in the jejunum and ileum (Chen et al., 2019). In this study, CSB treatment demonstrated positive effects on intestinal morphology, that is, shallow crypts were observed within the jejunum, and the ileal and jejunal villous heights were markedly elevated. However, a few studies have, on the other hand, also demonstrated no significant change in the VH and CD with the addition of sodium butyrate or CSB (Leeson et al., 2005; Czerwiński et al., 2012; Morel et al., 2019). This study confirmed that CSB improves intestinal morphology, indicating that dietary CSB supplementation is beneficial to intestinal health. The effect of CSB on nutrient digestibility may be attributed to CSB improving the intestinal environment and increasing the villus height and the absorption area. Meanwhile, assisting in gut development may be a key contributing factor to an acidifier's favorable impact on feed and growth efficiency (Guo et al., 2021), which could explain why adding CSB improves the performance of laying hens in the present study.

The conversion of ethyl acid to butyric acid by butyryl CoA and acetyl CoA transferases (Louis et al., 2004) or by sodium butyrate releases butyric acid in the intestine. This explains why sodium butyrate could potentially increase the intestinal SCFA content in broiler chickens, especially butyrate content (Guo and Cao, 2009). However, as confirmed in a previous study, sodium butyrate does not affect the content of SCFAs within the jejunum of broiler (Hu and Guo, 2007), which may be why sodium butyrate was absorbed into the front intestinal section. However, when sodium butyrate is coated using encapsulation technology, it assists in early butyric acid production; as a result, butyric acid can reach the distal GIT (Mallo et al., 2012). Wu et al. (2018) stated that broilers fed with additional CSB (800 mg/kg) reported greater butyric acid levels within the ileal chyme compared with the control group at 21 d. Zou et al. (2010) also reported that CSB significantly increased the butyric acid content in the ileum of broilers. Sobczak and Kozlowski (2016) reported that dietary supplementation of 700 g/t CSB could increase butyric acid content in the cecal content of laying hens compared with the control group. In line with previous reports, this study implies that supplementing the diet of laying hens with CSB could increase the content of butyric acid in the ileum and cecum, with the CSB3 group (800 mg/kg) showing the greatest improvement in the butyric acid content in the ileum.

It has been extensively suggested that intestinal flora has a vital effect on maintaining intestinal physiology and related activities (Sekirov et al., 2010). Moreover, the gut microbial diversity shows resistance to colonization of invasive pathogens—the higher the diversity, the more immune to the invasion of foreign microorganisms (Kühn et al., 1993). The diversity and composition of gastrointestinal microflora are closely related to the health of poultry. Sodium butyrate has been previously suggested to preserve gut microbial balance (Bortoluzzi et al., 2017). However, there are few reports about CSB on cecal microflora of poultry. As observed in this study, supplementing the diet in laying hens with 500 or 800 mg/kg CSB significantly enhances the diversity of microorganisms in the cecum. Conversely, some studies have also shown that sodium butyrate or butyric acid has no significant effect on the diversity of intestinal flora (Biagi et al., 2007; Yang et al., 2018; Zou et al., 2019). A primary reason for this could be that this study used CSB, which is mainly released in the posterior segment of the intestine, thereby directly affecting intestinal microbes. This study, moreover, demonstrated significant differences between CSB groups and CON group of cecal microbiota phylum in the abundance of *Firmicutes, Bacteroidota,* and *Deferribacteres. Bactleroidota*, and *Firmicutes* were the dominant phyla in the intestinal microbiotas of laying hens (Zou et al., 2019), which belong to beneficial bacteria. Yang et al. (2013) reported that *Firmicutes* and *Bacteroidota* are the dominant flora in the cecum of laying hens aged over 28 wk, mainly responsible for food fermentation in the gut (Ley et al., 2006). Besides, an increase in the *Muribaculaceae* (family), a dominant family of *Bacteroidetes,* was observed in laying hens supplemented with 800 mg/kg CSB in their diet (Chung et al., 2020). Many bacteria in *Firmicutes* can encode carbohydrate active enzymes, so *Firmicutes* have advantages in the hydrolysis and utilization of carbohydrates (Kaoutari et al., 2013) and also participate in the absorption and utilization of nutrients and energy metabolism; thus, they are conducive to the maintenance of body health (Videnska et al., 2014). Therefore, providing laying hens with appropriate supplementation of CSB in the diet is beneficial to the cecal microbes.

To conclude, dietary inclusion of CSB may improve the production performance, yolk color, intestinal morphology, and the digestibility of DM and could increase

the intestinal butyric acid content and microbial diversity in laying hens. It may also be deduced that dietary supplementation of 500 mg/kg to 800 mg/kg CSB is ideal for laying hens (Table 9).

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DISCLOSURES

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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