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# Dietary Supplementation with Medium-Chain Fatty Acids and Sodium Butyrate Improves Antioxidant and Anti-Inflammatory Capacity in Laying Hens

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## Simple Summary

Laying hens, due to their extended rearing periods and demanding production requirements, are highly susceptible to various stressors, which not only compromise their health but also adversely affect their productive performance. Notably, as hens age, they often experience increased inflammation alongside a decline in antioxidant capacity. This study aimed to investigate whether the supplementation of medium-fatty acids and sodium butyrate in the diet could ameliorate oxidative stress and suppress inflammatory responses in Hy-Line Gray laying hens. The results demonstrated that dietary inclusion of these fatty acids enhanced the antioxidant defense system and significantly reduced inflammation levels in the hens. These findings suggest that medium-chain fatty acids and sodium butyrate improve antioxidant capacity and modulate inflammatory responses in laying hens, offering a nutritional strategy to support the health of laying hens.

## Abstract

Oxidative stress and inflammatory responses adversely affect the health and reproductive functions of laying hens. This study aimed to investigate the effects of dietary supplementation with medium-fatty acids (MCFAs) and sodium butyrate (SB) on the antioxidant capacity and inflammatory status of laying hens. A total of 240 Hy-Line Gray laying hens were randomly assigned to three groups: control group fed a basal diet, an MCFA group fed the basal diet supplemented with 1000 mg/kg MCFAs, and an MS group fed the basal diet supplemented with a 50:50 mixture of MCFAs and SB at 1000 mg/kg. After an 8-week feeding trial, antioxidant function and inflammatory responses were assessed in serum and tissue samples. The results demonstrated that dietary inclusion of MCFAs and SB enhanced antioxidant enzyme activities in the liver and spleen, upregulated the expression of antioxidant-related genes, and concurrently downregulated pro-inflammatory cytokine mRNA expression. Furthermore, the composite of MCFAs and SB ameliorated oxidative stress and inflammation in reproductive tissues. These findings suggest that supplementation with MCFAs and SB in the diet effectively enhances antioxidant defenses and suppresses inflammatory responses in laying hens, presenting a viable nutritional approach to promote the health of laying hens.

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Academic Editor: Kun Li

Received: 9 November 2026
Revised: 25 November 2026
Accepted: 25 November 2026
Published: 27 November 2026

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**Keywords:** medium-chain fatty acids; sodium butyrate; antioxidant capacity; inflammatory; laying hens

## 1. Introduction

Poultry reared under intensive breeding conditions are influenced by multiple factors, such as management, ambient temperature, stocking density, and nutrition [1]. These variables can induce stress, which subsequently result in reduced production performance and metabolic disorders in poultry. Generally, the production and clearance of free radicals in the body remain in a relatively balanced state. Free radicals, such as reactive oxygen species (ROS), serve as cell signaling molecules and participate in various physiological regulatory processes, while the body maintains redox homeostasis through its antioxidant system. However, poultry are highly susceptible to harsh environmental factors and are therefore more prone to oxidative stress, which leads to decreased antioxidant enzyme activity, a gradual decline in free radical scavenging capacity, and a disruption of the oxidative and antioxidative balance [2,3]. Research has shown that oxidative stress adversely affects laying hens by diminishing production performance, lowering egg yield, reducing feed intake, and increasing the feed conversion ratio [4]. Oxidative stress also resulted in oxidative damage and inflammatory responses in the liver of laying hens by pathological liver lesions, increased levels of ROS, and the upregulation of inflammatory cytokine expression [5].

It is important to recognize that, beyond diminishing the poultry antioxidant capacity, elevated generation of ROS and lipid peroxidation products can disrupt hepatic lipid metabolism and lead to abnormal accumulation of lipids within the liver. Furthermore, lipid peroxidation products have the potential to activate inflammatory signaling pathways, including nuclear factor-κB (NF-κB), thereby promoting hepatocellular inflammation and injury [5]. Oxidative stress exerts an impact on the reproductive performance of laying hens, which is closely associated with their egg-laying performance. Specifically, oxidative stress can trigger apoptosis in ovarian granulosa cells, resulting in follicular atresia [6]. Granulosa cells serve as the primary functional cells responsible for steroid hormone synthesis. Under the influence of follicle-stimulating hormone, they synthesize progesterone and, in coordination with the theca cells, contribute to the production of estradiol. This collaborative process is essential for follicular development and ovulation [7]. Oxidative stress can lead to ovarian damage, which is one of the reasons that restricts fertility. In light of these adverse effects, nutritional strategies aimed at enhancing antioxidant capacity have attracted widespread attention as a method to alleviate oxidative stress in laying hens.

Medium-chain fatty acids (MCFAs) and sodium butyrate (SB) possess extensive potential applications in poultry production, attributable to their regulation on metabolism. MCFAs are relatively water-soluble. Their emulsification, digestion and absorption processes do not rely on the emulsification of bile salts and the hydrolysis of pancreatic lipase. Upon entry into intestinal epithelial cells, MCFAs are transported directly to the liver via the portal vein. Furthermore, due to their short carbon chain length, MCFAs do not require the carnitine palmitoyltransferase (CPT1/CPT2) shuttle system to access the mitochondria for β-oxidation. Instead, they can traverse the mitochondrial membrane directly to enter the matrix for oxidation, thereby facilitating a rapid energy supply. Furthermore, MCFAs have the capacity to modulate the host's glycolipid metabolism [8]. Coconut oil is rich in MCFAs, mainly caprylic, capric and lauric acids. Research indicates that the inclusion of coconut oil in poultry feed does not significantly affect the growth performance of broiler chickens, although it does enhance lipid metabolism. Conversely, another study demonstrates that

supplementation with MCFAs can improve both the growth performance and antioxidant capacity of broilers [9,10]. In comparison to MCFAs, short-chain fatty acids (SCFAs) are more efficient as energy substrates. SCFAs are primarily produced by intestinal microbiota through the fermentation of carbohydrates such as dietary fiber and resistant starch. Among these, acetic acid plays a crucial role in maintaining the host's energy homeostasis, whereas butyric acid serves as the principal energy source for colonocytes, promoting the proliferation of intestinal epithelial cells and the expression of tight junction proteins, thereby reinforcing the integrity of the intestinal barrier. It is important to highlight that the SB utilized in this study was administered exogenously and represents the sodium salt derivative of butyric acid. Sodium butyrate has been demonstrated to confer health benefits through its antioxidant properties and its capacity to suppress inflammatory processes [11–13].

The beneficial effects of single supplementation of MCFAs or SCFAs on poultry growth performance have been demonstrated. Nevertheless, the majority of current research predominantly addresses the supplementation of individual fatty acids, with comparatively limited investigations into the combined administration of MCFAs and SCFAs. Research has demonstrated that the combined application of these two factors can enhance the intestinal health of broiler chickens [14]. However, it remains unclear whether the combined supplementation of MCFAs and SB can enhance the antioxidant capacity of laying hens and alleviate oxidative stress. Therefore, this study aims to investigate the effects of combined supplementation of MCFAs and SB on the antioxidant capacity of laying hens, with the objective of providing a scientific basis for the application of such combined supplementation in laying hen production.

## 2. Materials and Methods

### 2.1. Experimental Materials

The MCFA supplement comprised hexanoic acid (≥1.5%), caprylic acid (≥20%), capric acid (≥13%), and lauric acid (≥5.5%), formulated with silicon dioxide as the carrier. The SCFA supplement used was sodium butyrate (≥98%). Both supplements were provided by Jiangxi Kangbo Agriculture and Animal Husbandry Co., Ltd. (Fuzhou, China).

### 2.2. Experimental Design, Diets, and Management

A total of 240 healthy Hy-Line Gray laying hens at 49 weeks of age, with similar body weights and laying rates, were randomly allocated into 3 treatment groups, with 10 replicates per group and 8 hens per replicate. The experimental treatments consisted of three groups: the control group (CON), which received the basal diet; the MCFA group, which was fed the basal diet supplemented with 1000 mg/kg of a commercial MCFAs product; and the MS group, which received the basal diet supplemented with a total of 1000 mg/kg of a 1:1 mixture of MCFAs and SB. The MCFA product utilized in this study is a commercially available complex of medium-chain fatty acids, and its inclusion level was determined based on the manufacturer's recommendations, previous dosages reported in broiler feed studies, and preliminary experiments conducted by the authors [15–17]. The 1:1 mixture (50% MCFA and 50% SB) for the MS group was determined in our group's prior work (unpublished). The basal diet was formulated according to the Agricultural Industry Standard of the People's Republic of China, Chicken Feeding Standard (NY/T 33—2004) [18]. The dietary ingr edients and nutrient levels are presented in Table 1. A one-week adaptation period was conducted prior to the formal 8-week experimental period to ensure the uniformity of the initial conditions among all hens. During the trial period, the hens were housed in battery cages with 8 birds per cage. The environment was consistently maintained in a clean and well-ventilated condition, ensuring adequate illumination with a lighting schedule of 16 h of light followed by 8 h of darkness. Feed and

water were provided ad libitum, and routine immunization and disinfection procedures were strictly followed. The ambient temperature was regulated at approximately 23 °C, while relative humidity was maintained at about 65%. Each cage was equipped with one feeder and two nipple drinkers for water supply. A routine flock inspection was performed each night at 21:00 prior to the lights being extinguished. Feed troughs were cleaned weekly, coinciding with the recording of feed intake, and the aisles were cleaned every two days. All experimental protocols were conducted in accordance with the ethical guidelines set forth by the Laboratory Animal Welfare and Ethics Committee of Jiangxi Agricultural University (Approval No. JXAULL-2021-036).

**Table 1.** Composition and nutritional levels of the basal diet (air-dry basis).

| Ingredients | Content (%) | Nutrient Levels | Content (%) |
|---|---|---|---|
| Corn | 57.50 | Metabolic energy, MJ·Kg$^{-1}$ | 10.73 |
| Soybean meal | 20.00 | Crude protein | 16.20 |
| DDGS | 6.00 | Crude fat | 3.59 |
| Corn germ meal | 3.00 | Methionine | 0.28 |
| Soybean oil | 0.50 | Lysine | 0.72 |
| Limestone | 8.00 | Threonine | 0.62 |
| Premix [1] | 5.00 | Calcium | 3.71 |
| Total | 100.00 | Available Phosphorus | 0.25 |

[1] The premix provides the following mineral per kilogram of diet: Cu 12.50 mg, Fe 80.00 mg, Zn 85.00 mg, Mn 80.00 mg, I 1.10 mg, and Se 0.30 mg. The premix provides the following vitamins per kilogram of diet: vitamin A 9250.00 IU, vitamin D3 3500.00 IU, vitamin E 19.00 IU, vitamin K3 1.23 mg, vitamin B1 2.00 mg, vitamin B2 6.50 mg, vitamin B6 3.25 mg, and vitamin B12 0.02 mg. DGSS, distillers' dried grain with solubles.

### 2.3. Serum Antioxidant Parameters

At the end of the experiment, one hen was randomly selected from each replicate of every treatment group for blood sampling. Blood samples were collected from the wing vein of each hen, and 6 mL of blood was withdrawn per hen. After clotting at room temperature for 30 min, samples were centrifuged at 3000× *g* for 10 min to isolate serum. The pale yellow supernatant was harvested, aliquoted, and stored at −80 °C for subsequent analyses. The activities of total superoxide dismutase (T-SOD, Cat. No. A001-3-2), catalase (CAT, Cat. No. A007-1-2), and total antioxidant capacity (T-AOC, Cat. No. A015-3-1), as well as the malondialdehyde (MDA, Cat. No. A003-1-2) content in the serum, were determined using commercial assay kits according to the manufacturer's instructions. All kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### 2.4. Determination of Tissue Antioxidant Parameters

At the end of the experiment, one hen was randomly sampled from each of the 10 replicates per treatment group, yielding 10 tissue samples per treatment group. Following blood collection, each hen was sacrificed, and tissue samples of the liver, jejunal mucosa, spleen, uterus, ovary and magnum were collected and stored at −80 °C. For analysis, the tissue samples were thawed on ice. Approximately 0.15 g of each tissue was accurately weighed and placed in a homogenization tube with pre-cooled physiological saline at a ratio of 1:9 (*w*/*v*). Grinding beads were added, and the samples were homogenized using a tissue homogenizer at 4 °C. The homogenates were then centrifuged at 3000× *g* for 10 min at 4 °C, and the supernatants were collected for subsequent analysis. The activities of T-SOD, CAT, and T-AOC, as well as MDA content, were determined using commercial assay kits according to the manufacturer's instructions. The total protein concentration of the supernatant was determined using a BCA protein assay. All kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### 2.5. RT-PCR Analysis

Approximately 0.15 g of tissue samples (liver, spleen, jejunal mucosa, uterus, ovary, and magnum) was placed in an RNase-free centrifuge tube with 1 mL of Trizol reagent (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China) and lysed on ice for 5 min. After adding 200 µL of chloroform, the mixture was thoroughly mixed and incubated on ice for 10 min. The samples were then centrifuged at 12,000× *g* for 20 min at 4 °C. Subsequently, 400 µL of the upper aqueous phase was carefully transferred to a new 1.5 mL RNase-free tube, mixed with an equal volume of isopropanol, and incubated for 10 min. After centrifugation at 12,000× *g* for 10 min at 4 °C, the supernatant was discarded. The RNA pellet was washed three times with 75% ethanol (prepared with RNase-free water) and air-dried at room temperature. The RNA was dissolved in 30–50 µL of DEPC-treated deionized water. The concentration and purity of the RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA) and diluted with DEPC water to a uniform concentration. Genomic DNA removal and reverse transcription were performed using a HiFiScript All-in-one RT Master Mix for qPCR kit (Vazyme, China) to synthesize cDNA for subsequent quantitative analysis. Real-time quantitative PCR reactions were performed using SYBR Green and total volume of the reaction system is 10 µL. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the reference gene *β-actin*. The specific primer sequences used for RT-qPCR are listed in Table 2.

**Table 2.** Nucleotide sequences of the primers used for real-time quantitative PCR [1].

| Gene | Primer Sequence (5′-3′) | Accession Number | Fragment Size (bp) |
|---|---|---|---|
| *β-actin* | F: GATTTCGAGCAGGAGATGGC<br>R: GCCAATGGTGATGACCTGAC | NM_205518 | 118 |
| *CAT* | F: TGGCGGTAGGAGTCTGGTCT<br>R: GTCCCGTCCGTCAGCCATTT | NM_001031215.2 | 139 |
| *SOD* | F: ATGTGACTGCAAAGGGAGGA<br>R: AGCTAAACGAGGTCCAGCAT | NM_205064.1 | 98 |
| *HO-1* | F: AGGGTTGGCTTTCTTCACCT<br>R: TTCTGCAGCGCCTCAAATAC | NM_205344.2 | 125 |
| *Nrf2* | F: ACAGTTCTCCTGGGAGTGTG<br>R: CGGCTCCACAGAAGGAAGTA | MN416129.1 | 198 |
| *GSH-Px* | F: AACTGCAGGACGACATCGAG<br>R: TTGTATGACAGAGGTGCGGG | NM_001277853.3 | 86 |
| *Keap1* | F: ATGTACCAGATCGACAGCGT<br>R: AACTCCTCCTGCTTGGAGAC | MN416132.1 | 186 |
| *IL-1β* | F: TCTGCCTGCAGAAGAAGCC<br>R: CTCCGCAGCAGTTTGGTCAT | NM_204524.2 | 139 |
| *IL-6* | F: GGCTTCGACGAGGAGAAATG<br>R: ACTCAGCCATGATGCTTCTTC | HM179640.1 | 124 |
| *TNF-α* | F: GGACAGCCTATGCCAACAAG<br>R: CGCTCCTGACTCATAGCAGA | XM_046927265.1 | 150 |
| *IL-10* | F: CTCTGAGCACAGTCGTTTGG<br>R: TCGTCTGGTGTTTGCAGTTG | NM_001004414.4 | 133 |

[1] *β-actin*, beta-actin; *CAT*, catalase; *SOD*, superoxide dismutase; *HO-1*, heme oxygenase-1; *Nrf2*, nu-clear factor erythroid 2-related factor 2; *GSH-Px*, glutathione peroxidase; *Keap1*, kelch-like ECH-associated protein 1; *IL-1β*, interleukin-1 beta; *IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor-alpha; *IL-10*, interleu-kin-10.

### 2.6. Statistical Analysis

All data were statistically analyzed using one-way analysis of variance via SAS 9.4 software (SAS Institute Inc., Cary, NC, USA), followed by Tuk ey's multiple comparison test to evaluate the differences among treatment means. The results are presented as means and SEM. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Serum Antioxidant Biomarkers

Serum antioxidant biomarkers are presented in Table 3. Serum CAT activity in the MCFA and MS group were significantly higher than that in the control group ($p < 0.05$). Dietary supplementation with MCFA and SB did not significantly affect T-SOD, T-AOC and MDA compared with the control ($p > 0.05$).

**Table 3.** Effects of dietary MCFAs and SB supplementation on serum antioxidant biomarkers of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| CAT (U/mL) | 0.95 [b] | 2.41 [a] | 2.14 [a] | 0.135 | 0.003 |
| T-SOD (U/mL) | 446.51 | 480.51 | 477.90 | 10.545 | 0.064 |
| T-AOC (U/mL) | 0.60 | 0.61 | 0.60 | 0.037 | 0.984 |
| MDA (nmol/mL) | 3.20 | 4.36 | 4.31 | 0.589 | 0.312 |

[1] CAT, catalase; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity; MDA, malondialdehyde. [a,b] Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB.

### 3.2. Liver, Spleen and Jejunal Mucosa Antioxidant Biomarkers

As shown in Table 4, both MCFA and MS group showed significantly elevated hepatic CAT and T-SOD activities relative to the control ($p < 0.05$). Hepatic CAT and T-SOD activities were significantly higher in the MS group than in the CON and MCFA groups ($p < 0.05$). No statistically significant differences were observed in hepatic T-AOC and MDA concentrations across the three groups ($p > 0.05$). In the spleen, T-AOC was significantly higher in the MCFA and MS groups than in the CON group ($p < 0.05$). Furthermore, the MCFA group exhibited a significant decrease in MDA levels ($p < 0.05$). Similarly, compared with the control group, the jejunal mucosal activities of CAT and T-SOD were significantly elevated in both the MCFA and MS groups ($p < 0.05$). Furthermore, the jejunal mucosal MDA content markedly decreased in the MCFA group ($p < 0.05$).

**Table 4.** Effects of dietary MCFAs and SB supplementation on antioxidant biomarkers of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| liver | | | | | |
| CAT (U/g prot) | 227.94 [c] | 274.64 [b] | 354.23 [a] | 14.024 | <0.001 |
| T-SOD (U/mg prot) | 445.09 [c] | 513.83 [b] | 626.96 [a] | 9.234 | <0.001 |
| T-AOC (mmol/g prot) | 0.12 | 0.12 | 0.10 | 0.006 | 0.087 |
| MDA (nmol/mg prot) | 0.51 | 0.56 | 0.53 | 0.022 | 0.281 |
| spleen | | | | | |
| CAT (U/g prot) | 19.63 | 19.41 | 20.76 | 0.898 | 0.541 |
| T-SOD (U/mg prot) | 55.14 | 53.91 | 57.57 | 1.126 | 0.099 |
| T-AOC (mmol/g prot) | 0.080 [c] | 0.087 [b] | 0.094 [a] | 0.002 | <0.001 |
| MDA (nmol/mg prot) | 1.10 [a] | 0.97 [b] | 0.98 [ab] | 0.033 | 0.029 |
| jejunal mucosa | | | | | |
| CAT (U/g prot) | 37.51 [b] | 40.86 [a] | 43.51 [a] | 0.894 | 0.001 |
| T-SOD (U/mg prot) | 366.05 [b] | 488.83 [a] | 497.10 [a] | 11.975 | <0.001 |
| T-AOC (mmol/g prot) | 0.05 | 0.06 | 0.06 | 0.001 | 0.524 |
| MDA (nmol/mg prot) | 0.20 [a] | 0.15 [b] | 0.18 [ab] | 0.013 | 0.049 |

[1] CAT, catalase activity; T-SOD, total superoxide dismutase activity; T-AOC, total antioxidant capacity; MDA, malondialdehyde. [a,b,c] Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB. Prot, protein.

### 3.3. Effects on Liver Antioxidant and Inflammatory Gene Expression

As shown in Table 5, dietary supplementation significantly altered hepatic mRNA expression of antioxidant genes. Specifically, the MS group exhibited a markedly higher level of catalase (*CAT*) expression compared to the CON group ($p < 0.05$). Nuclear factor erythroid 2-related factor 2 (*Nrf2*) expression was significantly upregulated in both the MCFA and MS groups, accompanied by a significant downregulation of its negative regulator kelch-like ECH-associated protein 1 (*Keap1*) compared with CON group ($p < 0.05$). Furthermore, the expression levels of heme oxygenase-1 (*HO-1*) were markedly increased and glutathione peroxidase (*GSH-Px*) expression was significantly increased only in the MS group compared with the control group, while the MCFA group did not differ significantly from either group. Regarding inflammatory genes, the mRNA expression of interleukin-1 beta (*IL-1β*) and interleukin-6 (*IL-6*) in the MCFA group was significantly lower than that in the CON group ($p < 0.05$). The anti-inflammatory cytokine interleukin-10 (*IL-10*) and inflammatory cytokine tumor necrosis factor-alpha (*TNF-α*) expression did not differ significantly among groups ($p > 0.05$).

**Table 5.** Effects of dietary MCFAs and SB supplementation on liver antioxidant and inflammatory gene expression of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| *CAT* | 1.00 $^{b}$ | 1.29 $^{ab}$ | 1.41 $^{a}$ | 0.071 | 0.037 |
| *SOD* | 1.00 | 1.60 | 1.32 | 0.119 | 0.113 |
| *Keap1* | 1.00 $^{a}$ | 0.63 $^{b}$ | 0.55 $^{b}$ | 0.078 | 0.026 |
| *Nrf2* | 1.00 $^{b}$ | 1.49 $^{a}$ | 1.31 $^{a}$ | 0.072 | 0.006 |
| *HO-1* | 1.00 $^{b}$ | 1.76 $^{a}$ | 1.68 $^{a}$ | 0.118 | 0.004 |
| *GSH-Px* | 1.00 $^{b}$ | 1.25 $^{ab}$ | 1.45 $^{a}$ | 0.101 | 0.023 |
| *IL-1β* | 1.00 $^{a}$ | 0.45 $^{b}$ | 0.62 $^{ab}$ | 0.089 | 0.027 |
| *IL-6* | 1.00 $^{a}$ | 0.46 $^{b}$ | 0.90 $^{a}$ | 0.091 | 0.026 |
| *TNF-α* | 1.00 | 0.63 | 0.68 | 0.090 | 0.192 |
| *IL-10* | 1.00 | 2.03 | 2.47 | 0.331 | 0.065 |

[1] *CAT*, catalase; *SOD*, superoxide dismutase; *Keap1*, kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase-1; *GSH-Px*, glutathione peroxidase; *IL-1β*, interleukin-1 beta; *IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor-alpha; *IL-10*, interleukin-10. $^{a,b}$ Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB.

### 3.4. Effects on Spleen Antioxidant and Inflammatory Gene Expression

The expression of splenic *SOD* was significantly upregulated in the MS group compared with CON and MCFA groups (Table 6, $p < 0.05$). Meanwhile, the expression of *Nrf2* and *GSH-Px* were significantly upregulated in the MCFA group compared with the CON group, while the expression of *Keap1* was significantly downregulated in both the MCFA and MS groups ($p < 0.05$). However, no significant differences were observed among the three groups regarding the expression of *CAT* and *HO-1* ($p > 0.05$). Data of inflammatory genes show that the expression of *IL-1β* was significantly downregulated in the MS group compared with the CON and MCFA group ($p < 0.05$). The expression of inflammatory cytokines *TNF-α*, *IL-6*, and *IL-10* did not differ significantly among the groups ($p > 0.05$).

**Table 6.** Effects of dietary MCFAs and SB supplementation on spleen antioxidant and inflammatory gene expression of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| *CAT* | 1.00 | 1.25 | 1.34 | 0.146 | 0.288 |
| *SOD* | 1.00 $^{b}$ | 1.03 $^{b}$ | 1.94 $^{a}$ | 0.174 | 0.005 |
| *Keap1* | 1.00 $^{a}$ | 0.64 $^{b}$ | 0.56 $^{b}$ | 0.051 | <0.001 |
| *Nrf2* | 1.00 $^{b}$ | 1.42 $^{a}$ | 1.29 $^{ab}$ | 0.101 | 0.029 |
| *HO-1* | 1.00 | 1.20 | 1.42 | 0.191 | 0.322 |
| *GSH-Px* | 1.00 $^{b}$ | 1.43 $^{a}$ | 1.26 $^{ab}$ | 0.073 | 0.009 |
| *IL-1β* | 1.00 $^{a}$ | 0.81 $^{ab}$ | 0.46 $^{b}$ | 0.098 | 0.009 |
| *TNF-α* | 1.00 | 0.73 | 1.09 | 0.134 | 0.186 |
| *IL-6* | 1.00 | 0.83 | 0.80 | 0.204 | 0.758 |
| *IL-10* | 1.00 | 1.18 | 1.49 | 0.166 | 0.140 |

[1] *CAT*, catalase; *SOD*, superoxide dismutase; *Keap1*, kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase-1; *GSH-Px*, glutathione peroxidase; *IL-1β*, interleukin-1 beta; *IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor-alpha; *IL-10*, interleukin-10. $^{a,b}$ Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB.

### *3.5. Effects on Jejunal Mucosa Antioxidant Gene Expression*

As presented in Table 7, relative to the CON group, the expression of the *CAT* and *GSH-Px* in the jejunal mucosa of laying hens were markedly elevated in the MCFA group ($p < 0.05$) and the expression of *CAT* and *GSH-Px* in the MS group were no significant difference compared with the CON group ($p > 0.05$). Furthermore, both the MCFAs and MS groups demonstrated a significant downregulation of *Keap1* expression ($p < 0.05$).

**Table 7.** Effects of dietary MCFAs and SB supplementation on jejunal mucosa antioxidant gene expression of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| *CAT* | 1.00 $^{b}$ | 1.69 $^{a}$ | 1.11 $^{ab}$ | 0.176 | 0.033 |
| *SOD* | 1.00 | 1.12 | 1.00 | 0.161 | 0.848 |
| *Keap1* | 1.00 $^{a}$ | 0.65 $^{b}$ | 0.68 $^{b}$ | 0.104 | 0.003 |
| *Nrf2* | 1.00 | 1.04 | 1.26 | 0.072 | 0.048 |
| *HO-1* | 1.00 | 1.17 | 1.22 | 0.146 | 0.560 |
| *GSH-Px* | 1.00 $^{b}$ | 2.20 $^{a}$ | 1.29 $^{b}$ | 0.143 | 0.003 |

[1] *CAT*, catalase; *SOD*, superoxide dismutase; *Keap1*, kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase-1; *GSH-Px*, glutathione peroxidase. $^{a,b}$ Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB.

### *3.6. Dietary Supplementation with MCFAs and SB Mitigates Oxidative Stress and Inflammation in the Reproductive Tissues of Laying Hens*

Considering the effects of dietary MCFAs and SB on the antioxidant properties and inflammation of reproductive tissues (uterus, ovary and magnum). MCFAs and SB administration enhanced the antioxidant properties. As depicted in Table 8, the laying hens in MS group had higher mRNA expression of *Nrf2* in the uterus than CON group and *SOD* expression was significantly higher only in the MS group compared with the control group ($p < 0.05$), while the MCFA group did not differ significantly from either group. Laying hens in the MCFA group demonstrated significantly elevated expression levels of *Nrf2* and *HO-1* compared to those in the CON group ($p < 0.05$). *Keap1* expression was

markedly reduced in both the MCFA and MS groups compared to the CON group ($p < 0.05$). In the ovary, CAT expression was significantly increased only in the MS group, whereas SOD expression was significantly increased in both supplemented groups compared with the control group ($p < 0.05$). The mRNA expression of *Keap1*, *Nrf2*, *HO-1*, and *GSH-Px* did not differ significantly between the MS and CON groups ($p > 0.05$). In the magnum, upregulations in *CAT*, *SOD*, and *GSH-Px* expression were also observed in the MS group when compared to the CON group, and *Keap1* was significantly downregulated in both supplemented groups ($p < 0.05$).

**Table 8.** Effects of dietary MCFAs and SB supplementation on reproductive tissues antioxidant gene expression of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| uterus | | | | | |
| *CAT* | 1.00 | 1.18 | 1.09 | 0.153 | 0.718 |
| *SOD* | 1.00 $^{b}$ | 1.68 $^{ab}$ | 2.23 $^{a}$ | 0.221 | 0.005 |
| *Keap1* | 1.00 $^{a}$ | 0.65 $^{b}$ | 0.72 $^{b}$ | 0.060 | 0.007 |
| *Nrf2* | 1.00 $^{b}$ | 1.90 $^{a}$ | 1.59 $^{a}$ | 0.145 | 0.003 |
| *HO-1* | 1.00 $^{b}$ | 1.65 $^{a}$ | 1.02 $^{b}$ | 0.130 | 0.005 |
| *GSH-Px* | 1.00 | 1.26 | 1.62 | 0.206 | 0.142 |
| ovary | | | | | |
| *CAT* | 1.00 $^{b}$ | 1.61 $^{ab}$ | 1.87 $^{a}$ | 0.171 | 0.011 |
| *SOD* | 1.00 $^{c}$ | 1.76 $^{a}$ | 1.38 $^{b}$ | 0.088 | 0.001 |
| *Keap1* | 1.00 | 0.98 | 0.97 | 0.072 | 0.965 |
| *Nrf2* | 1.00 $^{b}$ | 1.51 $^{a}$ | 1.09 $^{b}$ | 0.010 | 0.007 |
| *HO-1* | 1.00 | 1.17 | 1.24 | 0.107 | 0.306 |
| *GSH-Px* | 1.00 | 1.10 | 1.21 | 0.103 | 0.410 |
| magnum | | | | | |
| *CAT* | 1.00 $^{b}$ | 1.61 $^{ab}$ | 1.94 $^{a}$ | 0.150 | 0.006 |
| *SOD* | 1.00 $^{b}$ | 1.54 $^{ab}$ | 2.32 $^{a}$ | 0.196 | 0.009 |
| *Keap1* | 1.00 $^{a}$ | 0.68 $^{b}$ | 0.62 $^{b}$ | 0.101 | 0.011 |
| *Nrf2* | 1.00 | 1.33 | 1.32 | 0.122 | 0.149 |
| *HO-1* | 1.00 | 1.20 | 1.56 | 0.197 | 0.130 |
| *GSH-Px* | 1.00 $^{b}$ | 1.59 $^{a}$ | 1.48 $^{a}$ | 0.090 | 0.001 |

[1] *CAT*, catalase; *SOD*, superoxide dismutase; *Keap1*, kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase-1; *GSH-Px*, glutathione peroxidase. $^{a,b}$ Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB.

Regarding the inflammatory status of reproductive tissues, the MCFA and MS group exhibited a reduced mRNA expression level of pro-inflammatory cytokine *IL-1β* in the uterus compared to the CON laying hens (Table 9, $p < 0.05$). The expression of the anti-inflammatory cytokine *IL-10* was significantly elevated in the MCFA group compared with the control group, while the MS group did not differ significantly from either group. The mRNA expression of *TNF-α* and *IL-16* did not differ significantly among the groups ($p > 0.05$). In the ovary, the MS group exhibited a significantly lower *TNF-α* expression compared with the CON group ($p < 0.05$). Furthermore, the *IL-6* expression was significantly downregulated in the MCFA group compared with both the CON and MS groups ($p < 0.05$). No significant differences in the mRNA expression of *IL-1β*, *TNF-α*, *IL-6* and *IL-10* were observed in the magnum among the CON, MCFA and MS groups ($p > 0.05$).

**Table 9.** Effects of dietary MCFAs and SB supplementation on reproductive tissues inflammatory gene expression of laying hens [1].

| Items | Treatments | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | CON | MCFA | MS | | |
| uterus | | | | | |
| *IL-1β* | 1.00 [a] | 0.36 [b] | 0.52 [b] | 0.075 | <0.001 |
| *TNF-α* | 1.00 | 0.58 | 0.94 | 0.154 | 0.180 |
| *IL-6* | 1.00 | 0.95 | 0.63 | 0.181 | 0.328 |
| *IL-10* | 1.00 [b] | 2.61 [a] | 1.68 [ab] | 0.322 | 0.013 |
| ovary | | | | | |
| *IL-1β* | 1.00 | 0.77 | 0.74 | 0.154 | 0.460 |
| *TNF-α* | 1.00 [a] | 0.69 [ab] | 0.32 [b] | 0.116 | 0.006 |
| *IL-6* | 1.00 [a] | 0.41 [b] | 0.94 [a] | 0.142 | 0.013 |
| *IL-10* | 1.00 | 1.07 | 1.28 | 0.158 | 0.453 |
| magnum | | | | | |
| *IL-1β* | 1.00 | 0.67 | 0.96 | 0.224 | 0.560 |
| *TNF-α* | 1.00 | 0.71 | 0.74 | 0.111 | 0.189 |
| *IL-6* | 1.00 | 0.78 | 0.84 | 0.150 | 0.389 |
| *IL-10* | 1.00 | 1.01 | 1.03 | 0.210 | 0.998 |

[1] *IL-1β*, interleukin-1 beta; *IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor-alpha; *IL-10*, interleukin-10. [a,b] Means within a row lacking a common superscript differ ($p < 0.05$). MCFA, diet with 1000 mg/kg commercial MCFAs; MS, diet with 1000 mg/kg 1:1 MCFAs and SB.

## 4. Discussion

Stress is a prevalent factor in poultry production and constitutes a significant adverse influence on poultry health and productivity. It frequently results in diminished production performance, impaired intestinal health, and reduced immune function, thereby causing substantial detrimental effects on poultry production. Laying hens are highly susceptible to oxidative stress due to prolonged feeding cycles and intense laying pressure, which can be triggered by a variety of factors [3]. Existing studies have demonstrated that oxidative stress reduces egg production, weakens nutrient absorption, and compromises ovarian function in laying hens [19,20]. Moreover, hens at the late laying phase are more sensitive to stress and suffer from greater oxidative damage compared with younger hens. This is mainly attributed to the progressive decline in the hens' antioxidant defense capacity with increasing age. In laying hens, the activities of antioxidant enzymes decrease and the ability to scavenge ROS is consequently weakened, leading to the accumulation of excessive free radicals in the body and exacerbating oxidative damage [21]. Meanwhile, laying hens commonly experience chronic inflammation during this period, which further disrupts the redox homeostasis of the organism. MCFAs and SB not only serve as essential energy substrates but also play crucial roles in modulating immune and antioxidant defenses. Our previous studies have demonstrated that MCFAs can enhance the laying hen antioxidant capacity [15]. And SCFA significantly promotes intestinal health in broiler chickens [22]. In comparison, SCFA (calcium butyrate), is more cost-effective and economically feasible for practical production applications. Accordingly, this study aims to substitute MCFAs with selected SB in feed formulations to harness the synergistic effects of both types of fatty acids, thereby mitigating oxidative stress and inflammation in laying hens.

Serum antioxidant biomarkers serve as indicators of the overall redox status in laying hens. The findings of the present study demonstrated that dietary supplementation with MCFAs and SB significantly enhanced CAT activity in laying hens. As a critical enzyme involved in the scavenging of reactive oxygen species, elevated CAT activity improves the organism's capacity to decompose hydrogen peroxide ($H_2O_2$), thereby mitigating the accumulation of free radicals and providing a protective effect against oxidative damage induced by $H_2O_2$ accumulation [23]. Regarding serum antioxidant parameters, MS group did not

exhibit a significant alleviation of oxidative stress in laying hens. Although the difference was not statistically significant, the serum MDA levels in this group were marginally higher compared to the control group. These findings suggest that fatty acid supplementation may not have elicited a pronounced redox response at the serum level, potentially due to the relatively brief duration of treatment and the influence of experimental stress.

It is well established that the liver, as the primary organ responsible for metabolic processes, has a high propensity to generate substantial quantities of reactive oxygen species. Concurrently, the spleen, serving as a critical immune organ, plays a vital role in sustaining immune function and modulating inflammatory responses. Antioxidant enzymes play a critical role in neutralizing free radicals within the organism, and their activity serves as a direct indicator of the tissue's oxidative defense capacity. In the present study, the activities of T-SOD and CAT in the liver were significantly elevated in hens receiving MCFA and MS supplementation compared to the control group. Notably, the MS group exhibited significantly higher enzyme activities than the MCFA-only group, indicating that partial substitution of MCFAs with SB can further enhance hepatic antioxidant enzyme activity, surpassing the effects observed with MCFA supplementation alone. This result is consistent with previous studies on the antioxidant function of fatty acids [24,25]. Meanwhile, the mRNA expression of *CAT*, *Nrf2*, *HO-1* and *GSH-Px* was significantly upregulated in the liver of laying hens in the MS group relative to the CON group, whereas *Keap1* mRNA expression was significantly downregulated. In addition, similar trends were observed in the spleen, with T-AOC activity significantly increased in the MS and MCFA groups, accompanied by significant downregulation of *Keap1* expression. And Nrf2 expression in the MCFA group were significantly elevated. Because long-term egg-laying imposes a heavy metabolic burden that triggers oxidative stress and subsequent immune damage in the spleen of laying hens, enhancing the spleen's antioxidant capacity helped alleviate the oxidative stress-induced immune damage. The antioxidant capacity of fatty acids may be related to the Nrf2/Keap1 signaling pathway. Numerous studies have demonstrated that the beneficial effects of antioxidants on poultry are mediated via this pathway [26–28]. Nrf2 is a pivotal transcription factor responsible for maintaining cellular redox homeostasis, with its activity negatively controlled by Keap1. Upon oxidative stress, Nrf2 dissociates from Keap1 and escapes Keap1-mediated ubiquitination and proteasomal degradation. Stabilized Nrf2 accumulates in the cytoplasm and subsequently translocates into the nucleus, where it heterodimerizes with small Maf proteins. This complex recognizes and binds to antioxidant response elements (AREs) within the promoters of target genes, triggering the transcription of an array of downstream cytoprotective genes that collectively strengthen the cellular capacity to scavenge ROS and attenuate oxidative injury [29]. In alignment with prior research [30], this study observed a downregulation of *Keap1* expression alongside an upregulation of *Nrf2* and its downstream target genes. These findings suggest that MCFAs and SB may mediate antioxidant effects through the activation of the Nrf2/Keap1 signaling pathway.

It is noteworthy that Nrf2 is involved not only in the regulation of the host's antioxidant defense mechanisms but also in modulating inflammatory responses. In addition to activating downstream antioxidant enzyme genes, Nrf2 exerts anti-inflammatory effects by interacting with the NF-κB signaling pathway, with a complex bidirectional regulatory relationship existing between the two [29]. On one hand, the activation of Nrf2 initiates the transcription of the *HO-1* gene, which not only facilitates the clearance of free radicals but also inhibits the NF-κB signaling pathway, thereby attenuating the release of pro-inflammatory cytokines such as *TNF-α*, *IL-1β*, and *IL-6*. On the other hand, NF-κB can reciprocally regulate Nrf2. NF-κB binds to the κB response elements within the promoter region of the *Nrf2* gene, inducing its expression at the transcriptional level [31]. Addi-

tionally, Rac1, functioning as an upstream activator, can trigger NF-κB activation, which subsequently upregulates *Nrf2* expression. The elevated Nrf2 further promotes the transcription of downstream HO-1, which in turn inhibits NF-κB activation, thereby reducing its inflammatory activity [32,33]. The results of this study showed that fatty acid supplementation activated the expression of *Nrf2* and its downstream antioxidant genes in the liver and spleen of laying hens, while inhibiting tissue inflammatory responses. Compared with the control group, the expression of pro-inflammatory factor *IL-1β* in the spleen of the MS group were significantly downregulated. Fatty acids can reduce inflammation by regulating redox homeostasis. In addition, the study found that NF-κB can also regulate the transcription of Nrf2 by activating histone deacetylase 3 (HDAC3), thereby affecting signaling ability [34]. It is worth noting that the antioxidant and anti-inflammatory effects of nbutyric acid are also closely related to HDAC. Studies have shown that nbutyric acid can exert antioxidant effects by inhibiting HDACs; however, it should be noted that unlike the pathway that regulates Nrf2 transcription, nbutyric acid mainly regulates the production of pro-inflammatory mediators by acting on macrophages [35]. This mechanism is different from the regulation of the Nrf2/Keap1 pathway.

In addition to the liver and spleen, MCFAs and SB can also act on the jejunal mucosa to improve its oxidative damage state. In this study, the gene expression of *CAT* and *GSH-Px* in the jejunal mucosa of laying hens in the MCFA group was significantly upregulated, while the expression of *Keap1* was significantly downregulated. Studies have confirmed that MCFAs can enhance intestinal health by improving intestinal morphology and promoting the proliferation of beneficial bacteria [36]. SCFAs (metabolites of gut microorganisms) have a more direct effect. A study of an organ bath system based on laying hens found that SCFAs can regulate jejunal motility and barrier function [37]. Dietary supplementation with SCFAs was also shown to enhance the antioxidant capacity of the jejunum of laying hens and improve the structure of the intestinal flora [22]. It should be noted that the difference between the MS group and the control group in this study did not reach a significant level, but its antioxidant index was numerically better than that of the control group. This phenomenon is speculated to be related to the complex ratio of medium and SB. When the two are compounded in different proportions, they may affect each other within a certain range, resulting in the synergistic effect not being fully realized. The specific mechanism needs further research and exploration.

Oxidative stress exerts detrimental effects on the reproductive tissues of laying hens, impairing both reproductive function and egg production performance. Numerous studies have demonstrated that oxidative stress disrupts steroid hormone synthesis, accelerates follicular apoptosis, and induces ovarian follicular atrophy as well as mitochondrial dysfunction, thereby compromising normal ovarian function [19,38,39]. MCFAs and SB act as antioxidants, helping to alleviate oxidative stress in the reproductive tissues of laying hens. This study measured that the expression of antioxidant genes *CAT* and *SOD* in the ovaries of laying hens in the MS group was significantly upregulated and the *TNF-α* inflammatory factor was significantly downregulated. Fatty acids may enhance the antioxidant defense of the ovaries and inhibit inflammation by activating the Nrf2 pathway. The expanded region of the oviduct in laying hens, responsible for protein secretion, constitutes the site of albumen formation. The glandular activity within this segment directly influences the quality of the egg white; damage to these glands results in a reduction in the consistency of the albumen [40]. The uterus serves as the central site for eggshell mineralization. Oxidative stress and inflammation can impair its secretory and mineralization functions, leading to a decline in eggshell quality [41]. With increasing laying age, the antioxidant activity in the magnum and uterus of the oviduct significantly declines, leading to a deterioration of tissue antioxidant function. This results in the accumulation of oxidative damage products

such as MDA, ultimately manifesting as reductions in eggshell strength, albumen height, and Haugh units [42]. The findings of this study indicate that supplementation with fatty acids can upregulate the expression of *CAT*, *SOD*, and *GSH-Px* in the magnum, thereby preserving glandular secretory function. Concurrently, MCFAs and SB supplementation downregulates the expression of the uterine negative regulatory factor *Keap1* and upregulates antioxidant-related genes, collectively enhancing the uterine antioxidant capacity. These molecular changes are conducive to the improvement of both albumen and eggshell quality. Furthermore, existing studies have demonstrated that FoxO1 serves as a critical regulatory factor in oxidative stress-induced apoptosis of follicular granulosa cells, with its expression markedly upregulated under oxidative stress conditions [20,39]. There is a potential synergistic regulatory relationship between the Nrf2 pathway and FoxO1 in modulating oxidative stress within reproductive tissues. However, the present study did not assess the expression levels of FoxO1 or related signaling molecules, leaving the role of FoxO1 in fatty-acid-mediated regulation of oxidative stress in the reproductive tissues of laying hens unclear. This aspect warrants further investigation in future research. A limitation of this study is the absence of a group treated with sodium butyrate alone, which precludes the separate evaluation of the sole effect of SB or any potential MCFA–SB interaction. Nevertheless, the observed improvement in the MS group supports the practical value of the combined formulation. And the expression of antioxidant and inflammation-related genes was assessed solely at the mRNA level across various tissues, without determining whether HDCA is involved in their regulation. Secondly, although the regulatory effects of fatty acids on antioxidant and inflammation-related gene expression in reproductive tissues were evaluated, reproductive function indicators such as ovarian reproductive capacity were not measured. These parameters would provide a more robust reflection of the impact of fatty acids on the reproductive system.

## 5. Conclusions

In the present study, dietary supplementation with MCFAs alone or combined with SB modulated the antioxidant capacity and inflammatory responses in laying hens. The combined supplement altered the activity of antioxidant enzymes and the mRNA abundance of antioxidant-related genes. Supplementation with MS may confer beneficial effects on the antioxidant and inflammatory status of laying hens. Consequently, complexes represent a promising nutritional intervention strategy to mitigate oxidative stress and inflammatory responses in laying hens.

**Author Contributions:** Conceptualization, H.Z.; methodology, J.L. (Jiyun Long); validation, W.L. and J.L. (Jin Liu); formal analysis, W.L.; investigation, W.L., Y.Z., C.Z. and Y.B.; resources, H.Z.; data curation, C.Z.; writing—original draft preparation, W.L.; writing—review and editing, J.L. (Jin Liu); visualization, Q.L. and G.L.; supervision, J.L.(Jin Liu); project administration, H.Z.; funding acquisition, H.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by the China Postdoctoral Science Foundation project (2023M731435), Project of Jiangxi Province Natural Science Foundation (Nos. 20252BAC240656, 20242BAB20306), and National Natural Science Foundation of China (32202707).

**Institutional Review Board Statement:** The animals used for this research were approved by the Animal Care and Use Committee of the Animal Nutrition Institute, Jiangxi Agriculture University on 12 March 2021(Ethics Approval Code: No. JXAULL-2021-036).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

**Conflicts of Interest:** Author Jiyun Long was employed by Jiangxi Kangbo Agriculture and Animal Husbandry Co., Ltd. which also provided the MCFAs and SCFAs supplements. The remaining authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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