# Long-term chemically protected sodium butyrate supplementation in broilers as an antibiotic alternative to dynamically modulate gut microbiota

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**ABSTRACT** Chemically protected sodium butyrate (**CSB**) is a new kind of sodium butyrate. Our previous study found that 1,000 mg/kg of CSB had the potential capacity of improving growth performance and promoting early development of small intestine in broilers. This study aimed to investigate the effect of long-term antibiotics or CSB supplementation for intestinal microflora dynamical regulation in broilers. One hundred ninety-two 1-day-old Arbor Acres male broilers were randomly allocated into 3 dietary treatment (8 replicates per treatment) and fed with a basal diet (**CON**), a diet supplemented with the antibiotics (enramycin, 8 mg/kg and aureomycin, 100 mg/kg) (**ANT**), or a diet supplemented with 1,000 mg/kg of CSB, respectively. Results showed that dietary supplementation of CSB or ANT treatment elevated the weight gain and feed conversion ratio (FCR; $P < 0.05$), as compared with control (CON) group. Additionally, CON, CSB, or ANT administration dynamically altered the gut microbiota composition as time goes on. The increased presence of potential pathogens, such as *Romboutsia* and *Shuttleworthia*, and decreased beneficial bacteria such as *Alistipes, Akkermansia*, and *Bacteroides* were verified in new gut homeostasis reshaped by long-term antibiotics treatment, which has adverse effects on intestinal development and health of broilers. Conversely, CSB supplementation could dynamically enhance the relative abundance of *Bacteroides*, and decrease *Romboutsia* and *Shuttleworthia* in new microflora, which has positive effects on intestinal bacteria of broilers compared with CON group. Meanwhile, CSB supplementation was significantly increased the concentration of propionic acid and total short chain fatty acids (**total SCFA**; $P < 0.05$) in comparison with CON and ANT groups. Moreover, CSB treatment significantly increased anti-inflammatory and antioxidative capacities ($P < 0.05$) of broilers compared with ANT group. Taken together, we revealed characteristic structural changes of gut microbiota throughout long-term CSB or ANT supplementation in broilers, which provided a basic data for evaluating the mechanism of action affecting intestinal health by CSB or ANT administration and CSB as an alternative to antibiotics in the broilers industry.

**Key words:** chemically protected sodium butyrate, gut microbiota, antibiotics, intestinal health, broiler

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## INTRODUCTION

The use of antibiotics, against pathogenic bacteria, have made numerous contributions to animal health and low dose supplement of antibiotics can also improve growth performance and reduce costs of animal husbandry in livestock production over the past few decades. However, the exposure of antibiotics is one of the major factors that cause environmental disruption, increase of drug-resistance bacteria and antibiotic residues in animals, which are the 3 hidden safety concerns (Bacanli and Basaran, 2019; Huang et al., 2020). In addition, increasing evidence has indicated that long-term antibiotics exposure has been documented to injure intestinal morphology and suppress gut barrier function, which cause dysfunction of intestinal immunity and increase the oxidative stress of intestine to threaten animal health (Busch and Binder, 2017; Karakan et al., 2021; Zhao et al., 2022). All these information reminds people to reconsider and prohibit the application of antibiotics in animal feed, and find some effective substitutes for antibiotics to promote animal production.



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The function of gut included digestion and absorption of nutrients, partly because of the role of intestinal microbiota (Adak and Khan, 2019). Emerging evidence has observed that the antibiotic exposure affected disorder of intestinal flora (Mu and Zhu, 2019; Hu et al., 2020; Ramirez et al., 2020) and failed to repress subsequent bacterial infection (Karam et al., 2016). A previous study by our team indicated that short-lincomycin administration could decrease beneficial bacteria and increase potential pathogens to affect intestinal health of young pigs (Tang et al., 2021). SCFA is one of the metabolic products of intestinal microbiota, which could enhance gut immunity and promote the absorption of nutrition (Tan et al., 2021). Nevertheless, there are risks effects of antibiotics. For example, lincomycin exposure decreased the levels of short chain fatty acids (**SCFA**), disrupted the intestinal morphology and immunosuppression in a previous research (Zhang et al., 2020). Some active promoting growth functional substance such as sodium butyrate, one of the supplements of butyrate, can be beneficial to intestinal microbiota and increase SCFA levels, which guarantee supplying the safety of livestock products (Zhao et al., 2022). Therefore, sodium butyrate is used as a substitute of antibiotics to promote animal growth and gut health in livestock production.

Recent research observed that sodium butyrate could enhance gut barrier and improve intestinal bacteria after entering into the intestines (Zou et al., 2019; Wang et al., 2020b). Chemically protected sodium butyrate (**CSB**) is a special form of sodium butyrate, which is protected by a physical and chemical modification technology of buffer salts to ensure enough butyrate into small intestine (Zhao et al., 2022). In previous study, CSB supplementation into diet had the potential capacity of improving growth performance and carcass composition in broilers (Yang et al., 2022; Zhao et al., 2022). However, there are also studies indicating that sodium butyrate supplementation did not influence the growth performance of broilers (Wu et al., 2018). Whereas, above studies also demonstrated that sodium butyrate supplementation could promote dominant bacteria of intestine by enhancing the abundance of *Bacteroidetes* and *Firmicutes* in broilers (Wu et al., 2018; Zhao et al., 2022). Further, the dynamic variation of gut microbiota after CSB supplementation or long-term antibiotics exposure in broilers is not clarified. Importantly, the dynamic variation of gut microbiota has been verified after long-term exposure of antibiotics, and these changes in the structure and function of microbiota could affect human health and animal production (Schwartz et al., 2020). Therefore, our purpose was to investigate the effects of CSB or antibiotics long-term administration on the dynamic variations of gut microbiota in broilers production, and provide a theoretical basis for CSB as one of effective substitutes for in-feed antibiotics in the broiler industry.

## MATERIALS AND METHODS

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Chinese Academy of Agriculture Sciences and experiments were approved by the Animal Ethics Committee of Experimental Animal Welfare and Ethical of Institute of Animal Science, Chinese Academy of Agriculture Sciences (IAS2021-105).

### Materials and Reagents

Chemically protected sodium butyrate (**CSB**) contains 54% sodium butyrate and was provided by Beijing Shengtaiyuan Bio-Technology Co., Ltd. (Beijing, China). Assay kits, including tumor necrosis factor (**TNF**)-$\alpha$, interleukin (**IL**)-6, IL-10, lipopolysaccharide (**LPS**), were purchased from Beijing Jin Hai Ke Yu Biological Technology (Beijing, China). Superoxide dismutase (**SOD**), total antioxidant capacity (**TAC**) and malondialdehyde (**MDA**) were purchased from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). SCFA standards, including acetic, propionic, and butyric acids were purchased from Sigma Chemical Co. (St. Louis, MO).

### Animals and Design

A total of 192 one-day-old Arbor Acres (**AA**) male broilers were used in the trial. All broilers were randomly divided into three groups with 8 replicates in each treatment and 8 chicks in per cage. Broilers in the treatments were fed with a basal diet (CON), a diet added antibiotics (8 mg/kg of enramycin and 100 mg/kg of aureomycin; **ANT**), or a diet supplemented with 1,000 mg/kg of CSB (CSB), respectively, for d 42. The dosage of antibiotics was referenced on previous study (Zhao et al., 2022) and the dosage of CSB was based on previous study (Wu et al., 2018). The composition of the basal diet, which meets the China (2004) Broiler Feeding Standard, was shown in Table S1. The timeline was shown in Figure 1A.

### Growth Performance Indexes

The amount of feed intake was recorded every day and body weight of each broiler was recorded on d 0, 7, 14, 21, 28, 35, and 42 of the whole experiment and the trial to calculate feed/gain ratio (**F/G**).

### Sample Collections

On the d 14, 21, and 42 of the entire experiment, 8 chicks were slaughtered via exsanguination by cutting the jugular vein after a 12 h fast. The cecal chyme of 3 groups' broilers were aseptically collected in 2-mL sterile tubes, immediately frozen in liquid nitrogen, and then stored at −80°C for 16S rRNA gene sequences analyses and analysis for SCFA concentration. On d 21, blood samples were collected, and then serum was stored at −80°C after centrifugation at 3,000 r/min for 10 min.

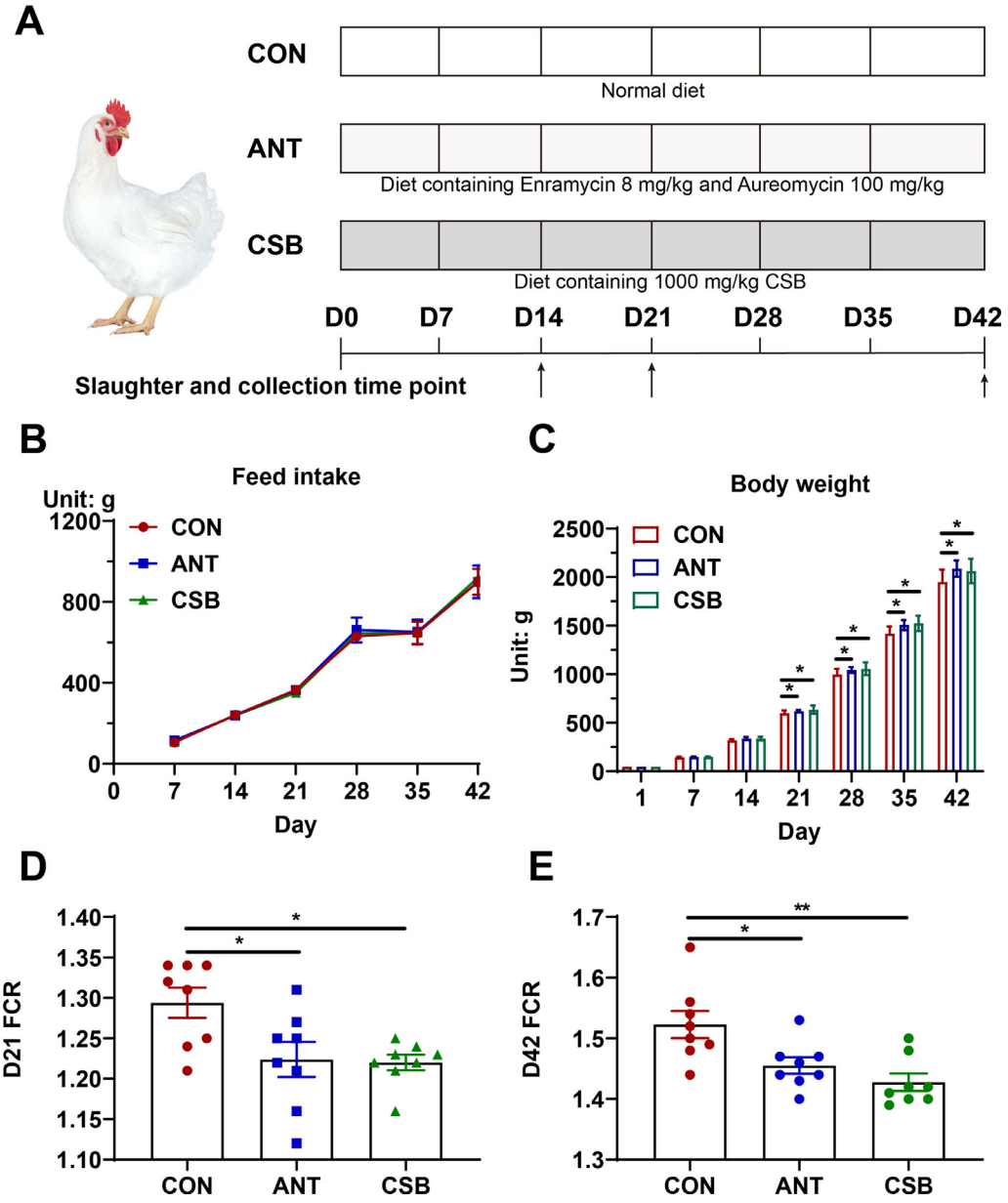


**Figure 1.** Effect of growth performance of long-term CSB or ANT supplementation in broilers. (A) Experimental schedule of CSB and ANT on space variation of intestinal microbiota in broilers. (B) Changes of feed intake. (C) Change of body weight. (D) Feed conversion rate of day 7−21. (E) Feed conversion rate of day 28−42. Data are expressed as mean ± SEM in body weight and F/G. * indicates $P < 0.05$.

## *Gut Microbiota Analysis*

Total genome DNA from cecal samples was extracted using the Fast DNA SPIN for soil kit (MP Biomedicals, Solon, PH). The quality of the DNA was detected by 1% agarose gel, and DNA was quantified by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE). The V3−V4 hypervariable region of the bacterial 16S rRNA gene was amplified with PCR using a primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGT WTCTAAT-3′). The PCR system and amplification conditions are referred to previous reports (Wang et al., 2020a). PCR amplified products were extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (AXYGEN, New York, NY) according to the manufacturer's instructions. After quantified and purified, amplicons were sequenced. The sequences were analyzed and assigned to operational taxonomic units (**OTUs**; 97% identity). The products were directly sequenced by an Illumina MiSeq platform (Illumina, SD) (2 × 300, pair end). After quantified and purified, amplicons were sequenced using Illumina MiSeq platform (Illumina, San Diego, CA, USA ) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) according to standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (**SRA**) database (Accession Number: PRJNA816283).

## *Short Chain Fatty Acids Analysis*

Concentrations of SCFA in cecal contents were measured using gas chromatography (**GC**) based on our previous study (Xia et al., 2022). Briefly, cecal digesta was

weighed into 1.5-mL centrifuge tubes and mixed with 1 mL dd$H_2O$, homogenized, and centrifuged (10,000 rpm, 10 min, 4°C). A mixture of the supernatant fluid and 25% metaphosphoric acid solution (0.9 mL and 0.1 mL, respectively) were vortexed for 1 min and centrifuged (10,000 rpm, 10 min, 4°C) after standing in a 1.5 mL centrifuge tube at 4°C for over 2 h. The supernatant portion was then filtered through a 0.45-$\mu$m polysulfone filter and analyzed using Agilent 6890 gas chromatography (Agilent Tecnologies, Inc, Palo Alto, CA).

### Measurement of Inflammatory Cytokines, Antioxidant, and Immune Indices in Serum

The levels of proinflammatory cytokines (TNF-$\alpha$ and IL-6), anti-inflammatory cytokine (IL-10), and LPS in serum were measured using specific enzyme-immunoassay technique (**ELISA**) following the manufacturer's protocol (Beijing Jin Hai Ke Yu Biological Technology, Beijing, China). Serum levels of antioxidant related parameters (SOD, TAC, and MDA) were tested by using respective assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The ratio of TAC to MDA (TAC/MDA) was calculated to evaluate the antioxidant/oxidative balance of serum.

### Statistical Analysis

Data were presented as the mean ± standard error of the mean (**SEM**). All data were compared by one-way analysis of variance (**ANOVA**) with Tukey's test (SPSS 21.0 software, Chicago, IL). A value of $P < 0.05$ was considered significant. $^*P < 0.05$ indicates a significant difference, and $^{**}P < 0.01$ indicates an extremely significant difference. Plots were performed using GraphPad Prism 8.0.2.

## RESULTS

### The Effect of CSB or ANT on Growth Performance of Broilers

Throughout the entire days 42 experiment. The result showed that feed intake had no significant difference in the 3 groups (Figure 1B). Nonetheless, CSB and ANT groups significantly enhanced body weight gain compared with CON group on d 21, 28, 35, and 42 ($P < 0.05$, Figure 1C). Meanwhile, CSB and ANT groups were significantly improved F/G ratio compared with CON group on d 21, 28, 35, and 42 ($P < 0.05$, Figures 1D and 1E).

### CSB or ANT Supplementation Modulated Composition of Cecal Microbiota in Broilers

Using 16S rRNA amplicon sequencing, the microbiota in the cecal content was analyzed. Each sequence length was 401−440 base pairs. The rarefaction curves observed that most of the microbial diversity and bacterial communities in cecal contents had been sufficiently captured (Figures S1A−C). The Venn diagram showed that broilers in the CON, ANT, and CSB groups contained 405 common OTUs, and 42, 23, and 32 unique OTUs on d 14, respectively (Figure S1D). Similarly, broilers in the CON, ANT, and CSB groups contained 630 common OTUs, and 44, 32 and 31 unique OTUs on d 42 (Figure S1F). Importantly, on d 21, the CSB group contained 65 unique OTUs, which was higher than CON (39 unique OTUs) and ANT (19 unique OTUs) groups (Figure S1E).

Alpha-diversity was presented in Figure 2. The results observed that there was significant difference in the index of ACE among the 3 groups ($P < 0.05$, Figure 2A). Meanwhile, Chao index was significantly decreased in ANT group in comparison with CSB group on the d 14 of chick ($P < 0.05$, Figure 2B). After 3-wk antibiotic exposure also enormously decreased the microbial alpha-diversity (including ACE and Chao indexes) in broilers cecal digesta compared with CSB group ($P < 0.05$, Figures 2A and 2B). In addition, the alpha-diversity results described that after time extension of CON group, antibiotic or CSB administration, the ACE and Chao indexes of cecal digesta were significantly increased in this study ($P < 0.05$, Figures 2A and 2B). $\beta$-diversity was conducted by principal coordinate analysis (**PCoA**) based on weighted unifrac metrics. The results showed that the gut microbiota was significantly different from the broilers after time extension of CON, ANT or CSB treatment ($P < 0.01$, Figures 3A and 3B−3D). Even on the d 14 ($R = 0.0122$, $P = 0.001$, Figures 3A and 3E), d 21 ($R = 0.1350$, $P = 0.017$, Figures 3A and 3F) and d 42 ($R = 0.2248$, $P = 0.017$, Figures 3A and 3G) of time extension of CON or ANT treatments, the cecal microbiota of the 2 groups still demonstrated two notably distinct clusters. Crucially, the gut microbiota was not significantly different from the broilers in the CON and CSB groups ($P > 0.05$).

At the phylum levels, the predominant bacterial communities in CON broilers were *Firmicutes* and *Bacteroidetes* on d 14 to 42 (Figure 4A). However, treatment with ANT led to a significant increase in the relative abundance of *Actinobacteria* compared with CON and CSB groups on d 42 (Figures 4B−4E). Meanwhile, the relative abundance of *Bacteroidetes* was significantly lower in ANT group compared with the CON group on d 42 ($P < 0.01$). Nevertheless, CSB supplementation enhanced the level of *Bacteroidetes* compared with the CON group on d 42 ($P < 0.01$, Figure 4E). The alterations of top 50 genera in relative abundance attracted more attention on d 14, 21, and 42 (Figures 5A and 5B).

At the genus levels, *Akkermansia, Alistipes, Bacteroides, Romboutsia*, and *Shuttleworthia* were differential bacteria in this study (Figure 5C). Some relative abundance of genera was notably decreased after long-term antibiotics exposure on d 42, such as *Akkermansia* (0.003%), *Alistipes* (2.21%), and *Bacteroides* (1.38%). Whereas, CSB supplementation showed a higher relative abundance of *Akkermansia* (1.15%), *Alistipes* (9.94%), and *Bacteroides* (10.68%) on d 42 of whole trial. Meanwhile, the relative abundance of *Romboutsia* was significantly decreased in the ANT group compared with CON ($P < 0.05$) and CSB ($P < 0.05$) groups on d 21, conversely, which was dramatically enhanced in the ANT group in

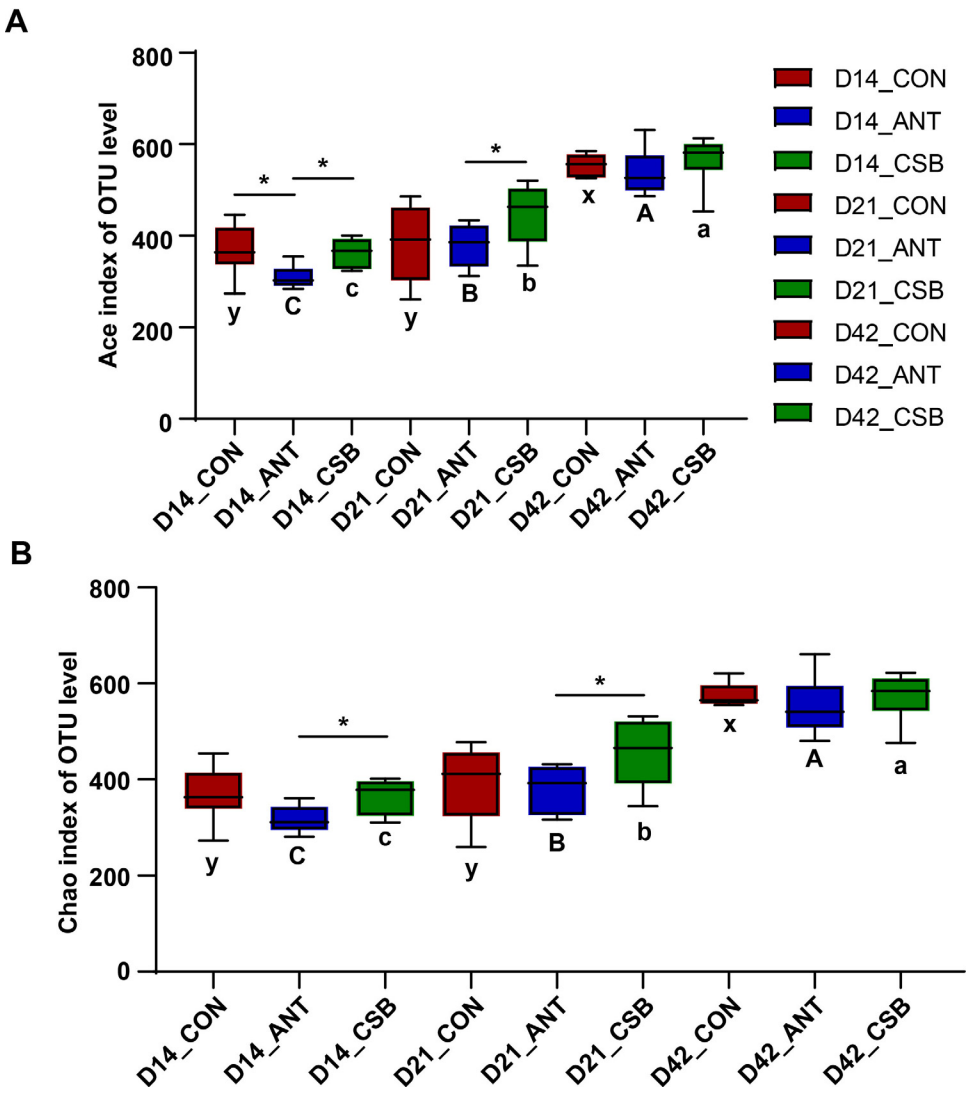


**Figure 2.** Alpha-diversity. The box plot of Ace (A) and Chao (B) index (OUT level) in cecal microbiota. * indicates $P < 0.05$ among CON, ANT and CSB groups (n = 7−8 broilers/group). Boxes (x, y or A, B, C or a, b, c, d) of CON, ANT and CSB groups with different letters are significantly different at $P < 0.05$ among different time points, respectively.

comparison with CSB ($P < 0.05$) group on d 42. Moreover, in contrast to CON group, the relative abundance of *Shuttleworthia* was dramatically higher in the ANT group ($P < 0.05$) and CSB ($P < 0.05$) groups on d 42.

The overall microbial composition in the CON, ANT, and CSB groups differed at the phylum and genus levels. Linear discriminant analysis effect size (**LEfSe**) analysis was performed to evaluate the differentially expressed bacteria. The yellow dots inserted in the circle suggested no significant difference in bacteria among different treatments. LEfSe results observed that 14 (d 14), 7 (d 21), and 36 (d 42) bacterial clades at all taxonomic levels were differentially abundant (LDA > 3.0) in the cecal microbiota on d 14, 21, or 42, respectively (Figure S2A−C).

## CSB or ANT Altered SCFA of the Cecal Chyme

SCFAs in the cecal content were detected. There was an increase in propionic acid ($P < 0.01$) in the CSB group compared with CON and ANT groups. Besides, the level of butyric acid was enhanced in the CSB group in comparison with CON ($P = 0.055$) and ANT ($P = 0.081$) groups, respectively. Crucially, the CSB group dramatically elevated the levels of total SCFA compared with the ANT group ($P < 0.05$), and higher than that of CON group ($P = 0.056$; Figures 5D−5G).

## The Effect of CSB or ANT on Inflammatory Response and Antioxidant Capacity in Broilers

To understand the effect of CSB on the anti-inflammatory capacity on broilers, inflammatory indexes, including TNF-$\alpha$, IL-6, and IL-10 were detected in serum. As shown in Figure 6. The level of TNF-$\alpha$ (Figure 6A) was significantly decreased in CSB group compared to ANT ($P < 0.01$) and CON ($P < 0.05$) groups. Meanwhile, the level of IL-6 (Figure 6B) had a decreased tread in CSB group compared with ANT group ($P = 0.084$). However, the level of IL-10 (Figure 6C) was significantly increased in CSB group

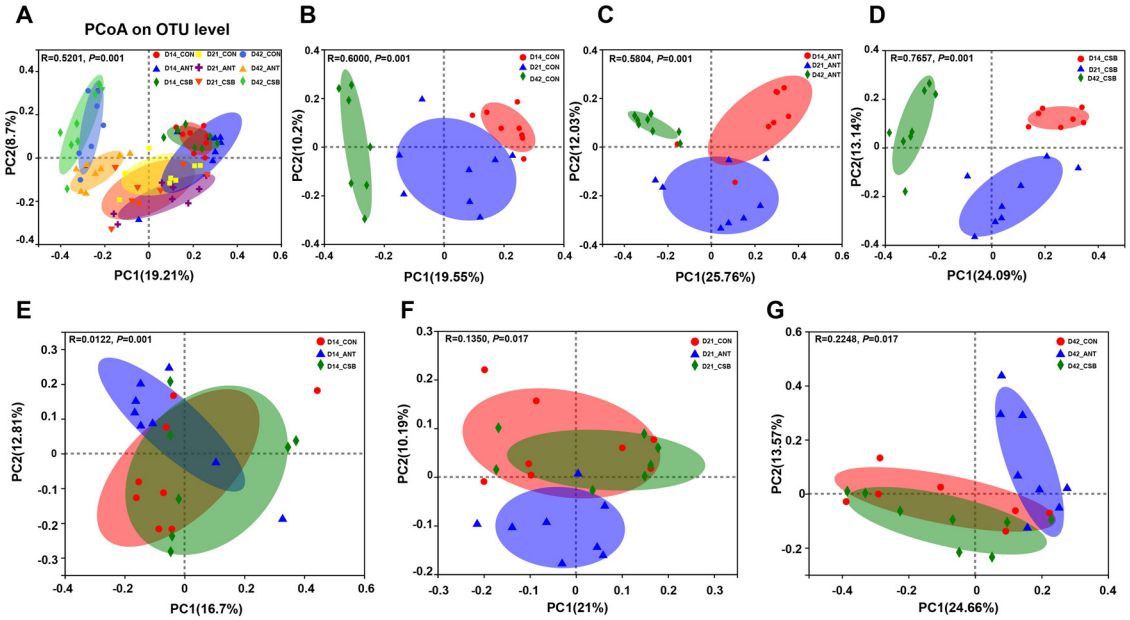


**Figure 3.** Beat-diversity. PCoA (OTU level) of community membership based on the weighted unifrac metrics and ANOSIM test in all groups (A), CON (B), ANT (C), or CSB group (D) at different time points. CON, ANT, and CSB group on day 14 (E), day 21 (F) and day 42 (G) at the same time points. (n = 7–8 broilers/group).

compared to ANT group ($P < 0.01$). Moreover, the level of LPS (Figure 6D) was significantly inhibited in CSB group compared with ANT group ($P < 0.01$).

To understand the effect of CSB on the antioxidant capacity of broilers, antioxidant indicators including TAC, SOD, and MDA were measured. As shown in Figure 6. The level of SOD (Figure 6E), was dramatically decreased in ANT group compared to CSB group ($P < 0.05$). Meanwhile, the level of TAC (Figure 6F) in serum was enhanced in CSB group compared with ANT treatment ($P = 0.094$). Moreover, the CSB treatment had lower level of MDA (Figure 6G) than the CON group ($P = 0.088$) and ANT group ($P = 0.083$). Nonetheless, ratio of TAC/MDA was significantly increased in CSB group in comparison with ANT group ($P < 0.01$, Figure 6H).

## DISCUSSION

Antibiotic as growth promoters into diet has a long history in the animal production. In the present study, the results also demonstrated that improving body weight gain and F/G of broilers of antibiotics (enramycin and aureomycin) supplementation was notably observed. However, the antibiotics had been forbidden for the purposes of enhancing growth rate because of misuse of antibiotic caused antibiotics residues and food security hidden danger (Abdelnour et al., 2019; Li et al., 2022). It is necessary to explore alternative safe and effective feed additives. Sodium butyrate is a new feed additive, and has very extensive application value (Zhao et al., 2022). Many studies have confirmed that different types of sodium butyrate could promote animal growth, which is the main reason that sodium butyrate could be used as growth promoter (Garcia et al., 2019; Melaku et al., 2021). In this study, the results observed that CSB supplementation could improve growth performance of broilers on d 21, 28, 35, and 42, which is consistent with a previous study that dietary supplement of 600 mg/kg CSB enhanced the body weight and F/G of broilers (Lan et al., 2020). The intestine is the main place for nutrient absorption, which is related to gut bacteria (Liu et al., 2014). Our previous study also found that a relationship between growth performance and intestinal microbiota (Zhao et al., 2022). In the present study, CON, CSB, or ANT treatment could dynamically regulate gut microbiota community following the change of time, which formed a new microflora on d 14, 21, and 42. Nevertheless, the new host-microbe homeostasis reshaped by long-term antibiotics administration does seem unfriendly to gut health. This result observed that antibiotics-promoted animal growth was not directly related to intestinal bacteria. Therefore, CSB or antibiotics long-time supplementation could dynamically change the composition of gut microbiota, but the mechanism of action affecting intestinal bacteria was different.

Many studies indicated a link between feed efficiency and intestinal microbiota, the composition of which is influenced by the age of animal and diet (Ramayo-Caldas et al., 2016; McCormack et al., 2018). Further, the relationship between growth performance and composition of gut microbiota should explore at different time points. In this study, compared with the control group on d 14, the microbial diversity was dynamically enhanced at CON group on d 21 and 42. Conversely, antibiotic administration decreased microbial diversity and the cecal microbiota distinctly separated in comparison with CON group on d 21, which demonstrated antibiotics plays an antibacterial function. This result also showed that intestinal microbiota environment was

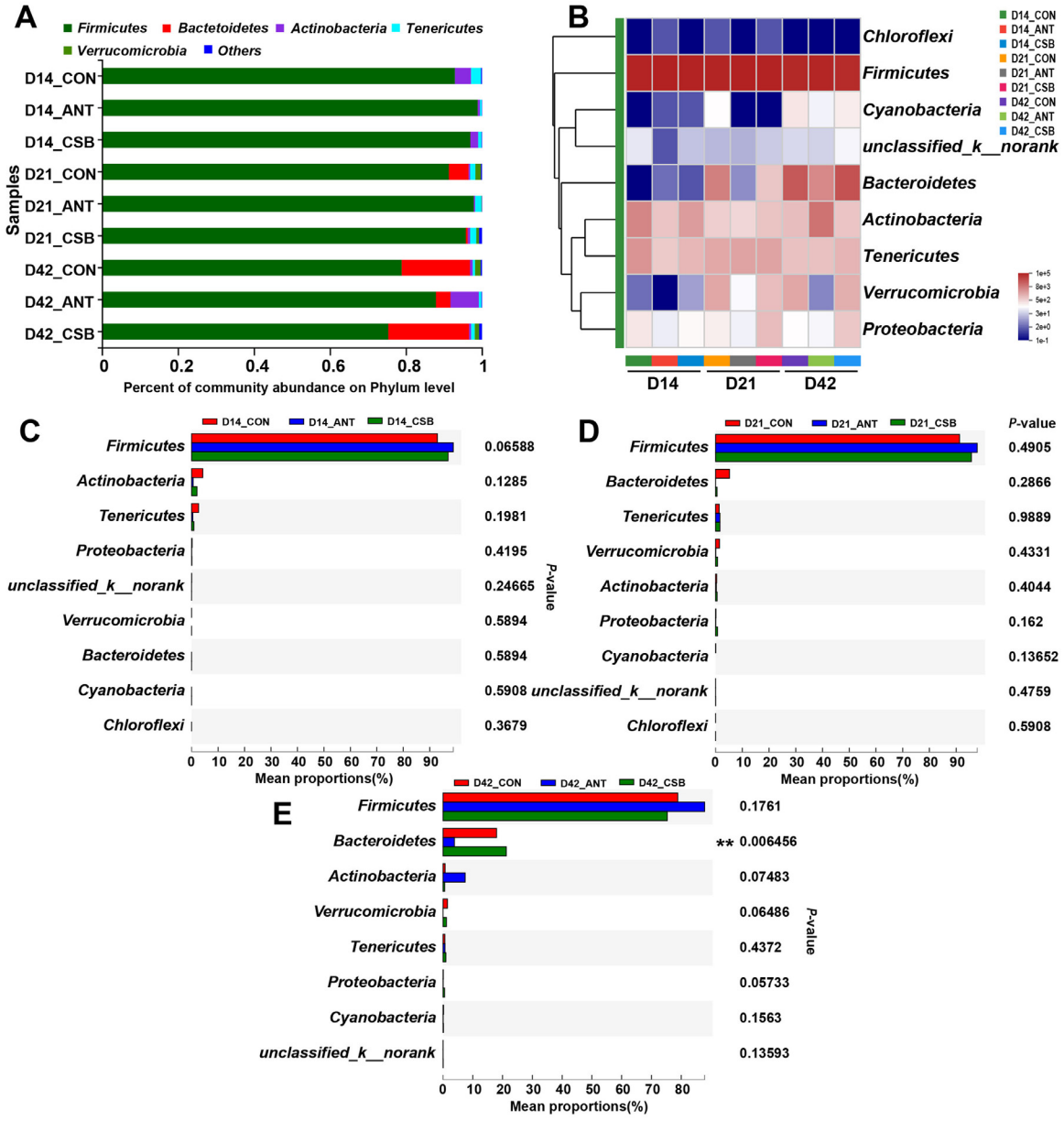


**Figure 4.** Community composition and difference analysis on phylum level. Effects of the CSB or ANT on the relative abundance (A) or trends change (B) of microbiota (phylum level) in cecal microbiota. Differentially abundant phylum from cecal microbiota in CON, ANT and CSB group on day 14 (C), day 21 (D) and day 42 (E). Statistical differences of altered phylum between three groups at day 14, 21 or 42 were calculated by the Kruskal-Wallis H test. ** indicates $P < 0.01$, * indicates $P < 0.05$ (n = 7−8 broilers/group).

destroyed by enramycin and aureomycin long-term exposure. Previous studies investigated that loss of microbial relative abundance and the changes of microbial composition were induced by antibiotic exposure in human (Elvers et al., 2020) and mice (Ramirez et al., 2020). Our team also confirmed that lincomycin exposure for 7 d induced imbalance of intestinal microbiota in young pigs (Tang et al., 2021), which is in concordance with this result. Whereas, the CSB supplementation increased diversity and richness of gut microbiota compared with the ANT group on d 21. This new information in our study contributed to the understanding of the potential effect of the CSB-promoted intestinal health in broilers by increasing the diversity of bacteria. In addition, the persistent increase of cecal microbiota abundance and composition were discovered in all groups on d 42 compared with that of on d 14 and 21. This result reminded that gut microbes were constantly enriched as the days of age increasing in all groups. Nevertheless, the changes of cecal microbiota on different time points after CON, CSB, or ANT administration were different. The above results may be the influence of differently physiological stages and feed of animal (Liu et al., 2019). Particularly, the richness of gut microbiota was gradually decreased and then gradually rose at different time stages after antibiotics long-time treatment. This is an interesting result, which suggested gut bacteria itself could adapt to antibiotics exposure. It's worth noting that the composition of bacteria after CSB supplementation was similar to CON group rather

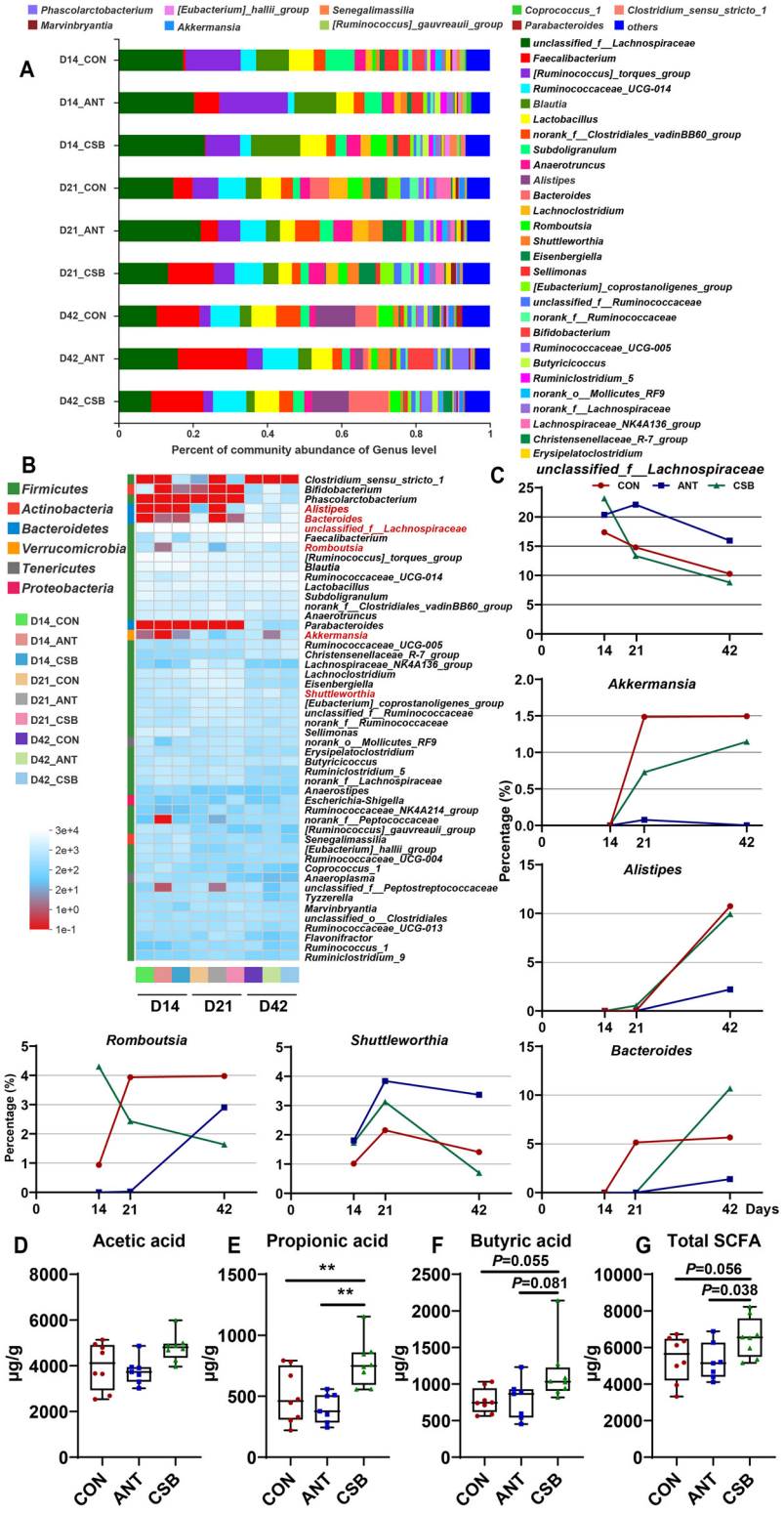


**Figure 5.** Community composition and short-chain fatty acid (SCFA) analysis. Effects of the CSB or ANT on the relative abundance (A) or trends change (B) of microbiota (genus level, top 50) in cecal microbiota. (C) The value was showed as the mean with 7–8 broilers per group. (D) Acetic acid. (E) Propionic acid. (F) Butyric acid. (G) Total SCFA. (n = 7–8 broilers/group).

than ANT group. Altogether, above results indicated that CSB might be more beneficial for intestinal microbiota than ANT treatment.

Given the capacity of CSB to ameliorate the gut microbiota in broilers, we further explored the differential bacteria in the phylum level. A recent study has shown that the increase of pathogenic microorganisms could destroy the intestinal microenvironment after antibiotic treatment (Gjonbalaj et al., 2020; Yang et al., 2021). *Bacteroidetes* is one of the dominant bacteria in the intestine of broilers. Our previous study demonstrated that the abundance of *Bacteroidetes* was decreased by lincomycin exposure for one week in mice (Zhang et al., 2020). In this study, we further found that

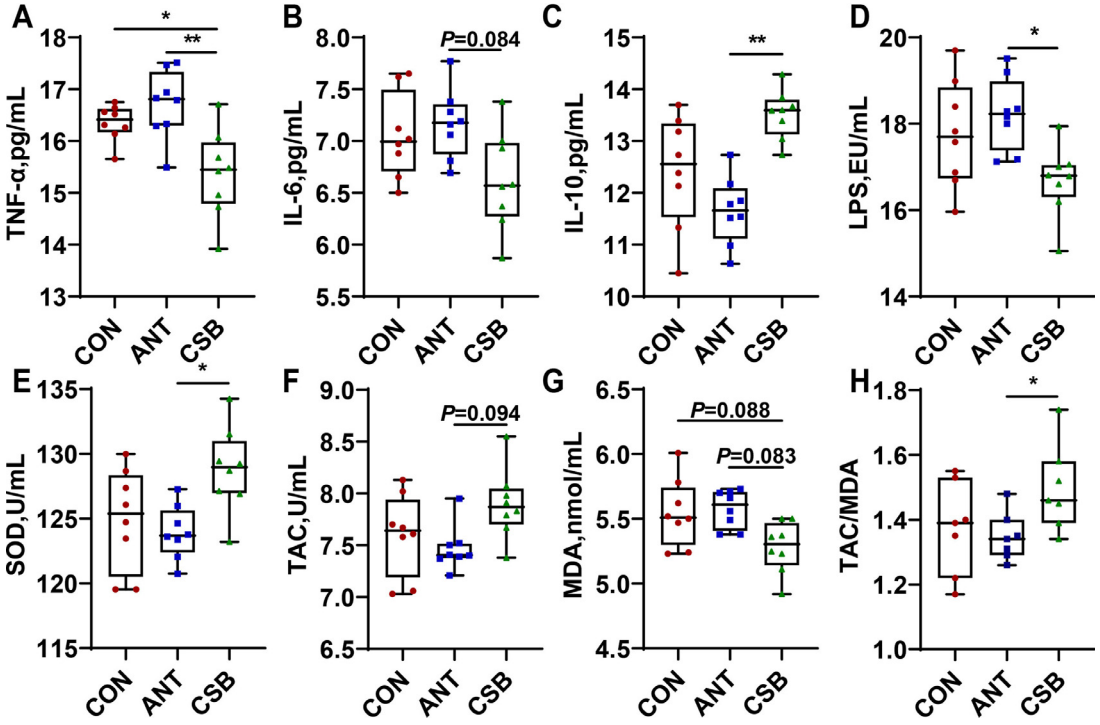


**Figure 6.** Effects of CSB or ANT administration on serum inflammatory and oxidative stress factor parameters. (A) TNF-$\alpha$, (B) IL-6, (C) IL-10, (D) LPS, (E) SOD, (F) TAC, (G) MDA, (H) TAC/MDA as described in the materials and methods section. The values are expressed as mean ± SEM (n = 8 broilers/group). ** $P < 0.01$, * $P < 0.05$.

as time progresses, long-term antibiotics administration is accompanied by a slow enhancement in the abundance of *Bacteroidetes*. This result further indicated that the beneficial microbiota *Bacteroidetes* was restricted after long-term antibiotics treatment, which was similar with previous study. Nevertheless, CSB supplementation could greatly enhance the abundance of *Bacteroidetes*. This beneficial effect was investigated by CSB treatment, indicating the potential of CSB for improving the gut beneficial microbial community. Long-term antibiotics exposure induced disordered gut bacteria, which created a new intestinal homeostasis by increasing the antibiotic-resistant bacteria sharply (Jernberg et al., 2010; Li et al., 2020). Previous research obtained that multiple antibiotics resistance genes such as *Actinoplanes* sp. (*marR* gene), *Collinsella aerofaciens* (*marA* gene), and *Corynebacterium ulcerans* (*marR2* gene) were reported in *Actinobacteria* (Fatahi-Bafghi, 2019). The result verified *Actinobacteria* was a drug-resistant bacteria to resist antibiotic treatment. In this study, the abundance of *Actinobacteria* was increased with antibiotics long-time exposure, which was similar with previous study. Altogether, the time-course variation of cecal bacteria plays an evident characteristic shift pattern of microbes after long-term antibiotic exposure in broilers, while CSB supplementation could perfect intestinal microbiota.

At the genus levels, the diversity of total microbiota was notably reduced after long-term antibiotics exposure in comparison with CON and CSB groups on d 21. The reason may be that antibiotics exposure killed intestinal bacteria. Specifically, beneficial bacteria (e.g., *Akkermansia, Alistipes, Bacteroides*, etc.) was remarkably decreased. *Akkermansia* and the butyrate concentration were reported increasing by the sophorolipid supplementation in broiler chickens, which showed that there was positive correlation between *Akkermansia* and butyric acid (Kwak, et al., 2021). In the present study, the abundance of *Akkermansia* was also enhanced in CSB treatment compared with ANT treatment. *Alistipes*, a new genus of bacteria, belongs to the *Bacteroidetes* phylum, which is highly relevant in gut dysbiosis and diseases (Parker et al., 2020). Our previous study described that the abundance of *Alistipes* was enhanced by the caffeic acid supplementation in DSS-induced colitis mice (Wan et al., 2021), which suggested that *Alistipes* might be inhibit inflammatory response. In the present research, the abundance of *Alistipes* was inhibited by antibiotics treatment, but not changed by CSB treatment. Therefore, the above results can prove that antibiotic exposure reshaped the microbiota composition of broilers, however, the CSB treatment causes some changes to the composition of normal intestinal microbiota of broilers. *Bacteroides* is one of the multifarious genera in the intestinal bacteria having a beneficial impact on the gut microenvironment (Yekani et al., 2020; Yekani et al., 2021). A previous study verified the abundance of *Bacteroides* was reduced after oral antibiotics administration in broilers (Zhang et al., 2021), which is similar to our results. Nevertheless, the abundance of *Bacteroides* was enhanced by the CSB treatment. In addition, long-time exposure of antibiotics could enhance some potential pathogens, such as *Romboutsia* and *Shuttleworthia*. *Romboutsia* was one of main bacterial hosts of antibiotic resistance genes (Wang, et al., 2021). In this study, we found that the

*Romboutsia* was killed by short-term antibiotic administration on d 14 and 21, whereas, the abundance of *Romboutsia* was significantly increased after long-term antibiotic exposure on d 42. This result indicated that *Romboutsia* contains antibiotic resistance genes. Moreover, the abundance of *Shuttleworthia* was increased by *Eimeria* tenella (Chen et al., 2020) or *Salmonella* typhimurium (Khan and Chousalkar, 2020) induced intestinal infection in broilers, which suggested that *Shuttleworthia* may be a detrimental bacteria. In the current study, we further found that the relative abundance of *Shuttleworthia* was dramatically enhanced in the ANT group, while the proportion of *Shuttleworthia* was significantly decreased by the CSB supplementation on d 42. But the mechanism is not distinct. Therefore, more studies are needed to explore the mechanism of *Shuttleworthia*. Altogether, these results verified that the protective effects of CSB supplementation on gut development of broilers could be partly explained potentially by improving the salutary bacteria and inhibiting detrimental bacteria to enhance gut health.

SCFA, one of gut microbiota-derived metabolites, can promote the activation of T cells in the intestinal mucosal tissue to form immune regulatory cells (Bartolomaeus et al., 2019; Schwarz et al., 2021), especially propionic acid could increase the number of regulatory T-cell and the level of IL-10 to adjust intestinal microenvironment (Haghikia et al., 2022). However, the propionic acid was decreased by antibiotic treatment, conversely, supplement of propionic acid could regulate Reg3-associated epithelial regeneration by Reg3-propionate axis to form gut epithelial homeostasis in DSS-induced colitis mice (Bajic et al., 2020). Besides, the specific mechanism of butyric acid was to inhibit G protein-coupled receptor (**GPR**) 43 to inhibit histone deacetylase in regulatory T cells, so that the anti-inflammatory effectiveness can be achieved (Li et al., 2018). Given that the feed formula will be replaced on d 21 and the microbial community composition was obviously enhanced after CSB supplementation, we continued to detect the microbiota-derived SCFA at the end of first phase of feeding. This result demonstrated that propionic acid was decreased in the ANT treatment group, while CSB supplementation can markedly enhance propionic acid level. Moreover, the concentration of total SCFA and butyric acid were increased after CSB supplementation. These data indicated that the beneficial effect of CSB supplementation on gut health in broilers was mechanistically possible to be attributable to the effect of SCFA, particularly butyric acid and propionic acid. Nonetheless, this needs further investigation.

Inflammatory response plays a key role in the activation of manufacture of mature IL-6 and TNF-$\alpha$ in intestinal disorders of animal (Kamada et al., 2013). Usually, the inflammatory response coincides with raised levels of pro-inflammatory cytokines, which could be suppressed by the antibiotic treatment (Hu et al., 2020). Previous studies described that the production of pro-inflammatory cytokines such as IL-6 and TNF-$\alpha$ was restrained after antibiotics treatment in the broilers such as *Enterococcus cecorum* (Schreier et al., 2022) or *Escherichia coli* (Burow et al., 2020) infection. Whereas, long-term antibiotics exposure induced hyperresponsive of intestinal macrophages and T cell dysfunction, in turn producing excess inflammatory cytokines (Scott et al., 2018). In the present study, long-term antibiotics exposure also induced the increasing trend of pro-inflammatory cytokines, but not significantly. A known inflammatory response and oxidative stress were also simultaneously occurring (McGarry et al., 2018; Lin et al., 2019). Recent research also observed that

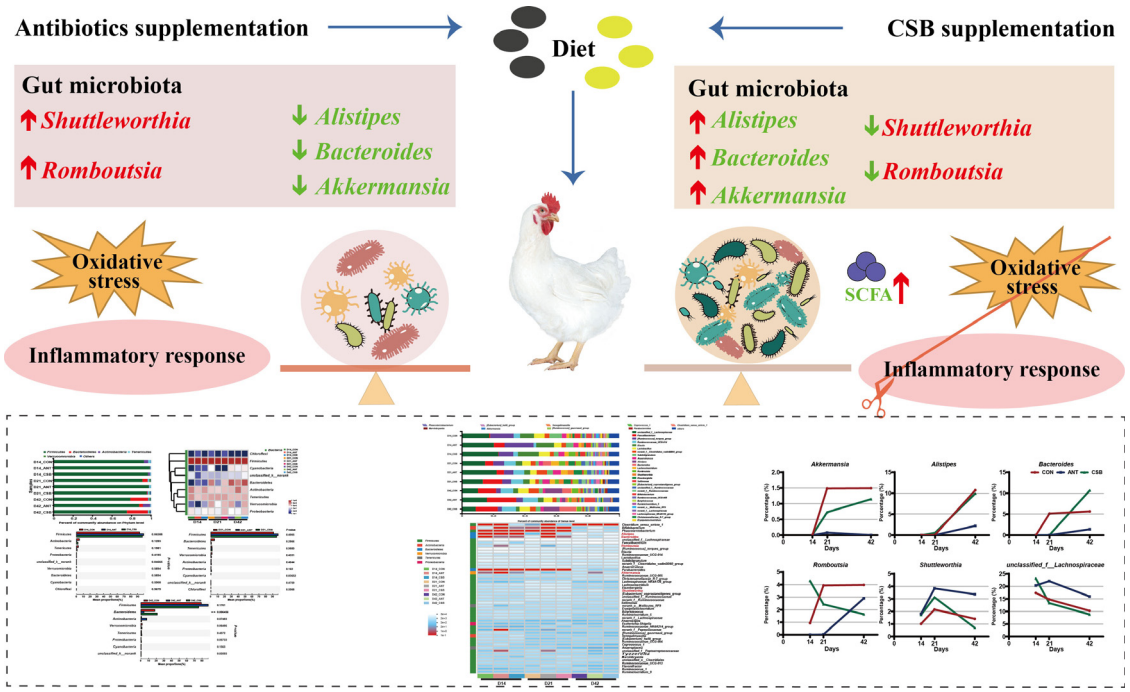


**Figure 7.** Dietary long-term CSB or ANT supplementation dynamically characteristic structural changes of gut microbiota, and CSB has the potential to replace the role of antibiotics by inhibiting oxidative stress and inflammatory response potentially via the modulation of gut microbiota in broilers.

antibiotics such as doxorubicin (Zhao et al., 2018; Song et al., 2019) and ofloxacin (Singh et al., 2019) exposure induced oxidative stress by decreasing TAC and SOD, and increasing MDA level in serum. In this study, we further found that long-term antibiotics exposure induced the decreasing trend of antioxidative enzyme activities. Previous report proved that aureomycin supplementation could improve body weight and F/G because it enhanced the digestibility of nutrients in broilers (Long et al., 2020). In addition, another study indicated that enramycin, as a linear-ring peptide, could improve growth performance by enhancing protein utilization (Wang, et al., 2016). In this study, we also found that aureomycin and enramycin cotreatment also could improve growth performance of broilers. Therefore, we suspected that the positive effects of aureomycin and enramycin are related to promotion with nutrients digestion and absorption, rather than associated with anti-oxidative and anti-inflammatory effects in broilers production. Importantly, the decrease of TNF-$\alpha$ production in the serum was confirmed by CSB supplementation. A previous study showed 300 mg/kg sodium butyrate supplementation could attenuate DSS-induced intestinal inflammation in broilers by enhancing the level of IL-10 (Zou et al., 2019). In the present study, the level of IL-10 in serum has been increased by CSB supplementation, which is similar to the previous study. Besides, antioxidant enzyme activities (CAT and SOD) were activated to against neuron loss and apoptosis by the butyrate supplementation (Li et al., 2021). Our previous study also demonstrated that CSB supplementation could inhibit oxidative stress by increasing the levels of SOD, TAC, and TAC/MDA in jejunum and ileum (Zhao et al., 2022). In this study, similar results were found. Above results observed long-time antibiotics exposure has no significant effect on inflammatory response and oxidative stress, whereas CSB supplementation could inhibit inflammatory response and oxidative stress in broilers production.

In summary (Figure 7), we show for the first time, characteristic changes rule in composition and abundance of gut microbiota throughout different times of broilers under the condition of long-term antibiotics exposure or CSB supplementation. Meanwhile, CSB supplementation showed powerful anti-inflammatory and antioxidative capacity in broilers production. Therefore, we provided a basic data for evaluating the mechanism of action affecting intestinal health by long-time CSB or ANT administration, and CSB has the potential to replace the role of antibiotics.

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## DISCLOSURES

The authors declare that they have no conflict of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102221.

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