# Effects of *Clostridium butyricum* on intestinal environment and gut microbiome under *Salmonella* infection

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**ABSTRACT** Salmonellosis causes massive economic losses globally every year. Especially in poultry, numerous drug-resistant bacteria have emerged; thus, it is imperative to find alternatives to antibiotics. As a probiotic, *Clostridium butyricum* (***C. butyricum***) provides the latest strategy for inhibiting the proliferation of *Salmonella*. This study aimed to evaluate the effects of *C. butyricum* on intestinal environment and gut microbiome under *Salmonella* infection. In this study, we modeled the infection of *Salmonella* using specific pathogen-free (**SPF**) chicks and found that the use of *C. butyricum* directly reduced the number of *Salmonella* colonizations in the spleen and liver. It also alleviated the histopathological changes of the liver, spleen, and cecum caused by *Salmonella* Enteritidis (***S.* Enteritidis**). In addition, *S.* Enteritidis increased the expression of pro-inflammatory *IL-6* in the cecum on day 6 postinfection. Interestingly, we found that *C. butyricum* changed *PPAR-γ* transcript levels in the cecum on day 6 postinfection. Analysis of the chick gastrointestinal microbiome showed that *Salmonella* infection increased the relative abundance of *Subdoligranulum variabile.* Further analysis found that *Salmonella* challenge significantly reduced the relative abundance of *Faecalibacterium prausnitzii* and *C. butyricum* increased the relative abundance of anaerobic bacteria in the gut on day 6 postinfection. Moreover, early supplementation of *C. butyricum* restored the epithelial hypoxia in *S.* Enteritidis infection in chicks. The results suggest that *C. butyricum* restores epithelial hypoxia caused by *S.* Enteritidis, improves the stability of intestinal flora, and inhibits the proliferation of *Salmonella*.

**Key words:** *Salmonella* Enteritidis, *Clostridium butyricum*, chick, inflammatory response, intestinal microbiome

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## INTRODUCTION

Salmonellosis is among the most frequent zoonotic diseases found in humans and is inseparable from food safety and hygiene. Poultry is considered to be the main source of *Salmonella* Enteritidis (***S.* Enteritidis**) infection (Cox and Pavic, 2010), and when *Salmonella* is ingested the infection enters by mouth and travels through the digestive tract to the intestine. When *Salmonella* enters the intestine, it comes into contact with the intestinal epithelial cells and triggers a large-scale inflammatory response (Hapfelmeier et al., 2005; Bakowski et al., 2008). In recent years, as a result of large-scale and long-term use and abuse, antibiotics have produced a series of negative effects, including the generation of resistant strains, thereby destroying the balance of intestinal flora and even endangering human health (Eng et al., 2015). This situation has made the prevention and treatment of *Salmonella* disease even more difficult.

At present, probiotics are used as additives for an effective way to prevent and control *S.* Enteritidis (Casey et al., 2007). *Lactobacillus, Bacillus subtilis,* and numerous other probiotics have achieved good research results in the treatment of *Salmonella* infection (Higgins et al., 2007; Thirabunyanon and Thongwittaya, 2012). In addition, *Clostridium butyricum* (***C. butyricum***) has been widely used to treat intestinal inflammation in animals because of its ability to inhibit inflammation in the intestine and promote homeostasis of the intestinal flora (SEKI et al., 2003; Jia et al., 2017). The study showed that *C. butyricum* and its spent culture supernatants exhibited significant inhibitory activity on *S.* Enteritidis and that the ability of *Salmonella* to invade the intestinal epithelium was reduced (Gao et al., 2013). Moreover, *C. butyricum* has been shown to reduce the secretion of IL-6 in acute pancreatitis, thereby maintaining intestinal homeostasis in mice with acute pancreatitis (Mudter

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and Neurath, 2007). In addition, when *C. butyricum* was depleted in mice, butyric acid levels decreased, followed by an increase in *Salmonella* (Rivera-Chavez et al., 2016). *C. butyricum* can also reduce inflammatory bowel disease and related diarrheal diseases caused by the use of antibiotics (Matsuoka and Kanai, 2015). In the inflammatory response, PPAR-$\gamma$ not only inhibits the inflammatory response by competitively inhibiting the production of inflammatory signal pathways and inflammatory mediators but can also downregulate the production of pro-inflammatory cytokines in organs and tissues (Kelly et al., 2003). As it is strictly anaerobic, *C. butyricum* can grow in the intestine and produce a large number of short-chain fatty acids, such as butyrate and acetate (Waligora-Dupriet et al., 2009). Numerous studies have demonstrated that short-chain fatty acids (**SCFAs**) can improve gut health by maintaining an anaerobic gut environment (Zhang et al., 2019) and preventing facultative anaerobic pathogens from multiplying (Jha et al., 2019). As a complex and independent ecosystem, the gut microbiota is essential to ensure the health of poultry (Kogut, 2019). In healthy chicks, the anaerobic environment created by strictly anaerobic bacteria ensures the stability of the vivo environment (Shin et al., 2015). *S.* Enteritidis uses the body's own inflammatory response to compete with other bacterial flora in the intestine to endanger the health of the body and cause the number of obligate anaerobes in the intestine to sharply reduce the proliferation of aerobes and facultative anaerobes and destroy the intestine's anaerobic environment, leading to an increase in the oxygen content in the intestine and the imbalance of intestinal homeostasis (Ohland and Jobin, 2015). Additionally, some obligate anaerobic bacteria prevent the expansion of facultative anaerobic *Enterobacteriaceae* by limiting the production of host-derived oxygen (Spees et al., 2013). The purpose of this experiment was to explore the inhibitory effect of *C. butyricum* on *Salmonella* by changing the intestinal flora and intestinal environment.

## MATERIALS AND METHODS

### Ethics Statement

The protocol for the experimental animal procedures was completed in the Laboratory Animal Center of Shandong Agricultural University Science and Technology Innovation Park. This study was approved by the Shandong Agricultural University Animal Experiment Ethics Committee (the license number: SDAUA-2021-036).

### Bacterial Strains

*C. butyricum* was used in the study and obtained from the Dalian Sanyi Animal Medicine Company (China). The *C. butyricum* was cultured at 37°C on Clostridia Medium broth (RCM, Biokar Diagnostics) under anaerobic conditions. In this study, *C. butyricum* spores were used, and the content was $8 \times 10^{10}$ CFU/g ([colony-forming unit]/g).

*S.* Enteritidis (CVCC3377) was isolated from a diseased chicken from the Avian Disease Centre of Shandong Agricultural University. To cultivate *S.* Enteritidis, it was grown in Luria broth (LB culture medium) cultures for 16 h at 37°C. After overnight incubation, *Salmonella* growth was determined by serial dilution and plating of samples on xylose lysine deoxycholate agar plates (XLD; catalog no. C7322, Criterion, Hardy Diagnostics, Santa Maria, CA), incubating at 37°C for 24 h. All cultures of isolates were stored in 50% glycerol at −80°C.

### Experimental Design and Animal Management

Specific pathogen-free chicks (**SPF**) were obtained from Jinan SPAFAS Poultry Company (Jinan, China). Two independent trials were conducted to evaluate the effect of prophylactic oral administration of *C. butyricum* spores to reduce the pathogenicity of *S.* Enteritidis in SPF chicks. Chicks were given unlimited access to feed and water. Three-day-old chicks were divided into 4 groups (8 chicks/group). As shown in Table 1, Group CB and Group CBS was orally inoculated with the same volume of *C. butyricum* spore oral liquid (0.5 mL $4 \times 10^8$ CFU/chick) for 3 consecutive days. Group CON and group S were orally inoculated with the same volume of bacteria-free saline (0.5 mL/chick) for 3 consecutive days. Three days later, both Group CBS and Group S were orally infected with 1 mL *Salmonella* $2 \times 10^9$ CFU/chick, and the other 2 groups (Groups CON and CB) were orally inoculated with the same volume of bacteria-free saline. Sample collections were performed on d 3 and d 6 postinfection.

**Table 1.** Experimental design of *C. butyricum* treatment in SPF chicks.

| Group | Treatment | Doses | | | Treatment schedule of *C. butyricum* | Age of *Salmonella* infection | Time of euthanasia (day) |
|---|---|---|---|---|---|---|---|
| | | Saline | *Salmonella* | *C. butyricum* | | | |
| CON | Oral saline | 0.5 mL | - | - | - | - | 9, 12 |
| CB | Only *C. butyricum* treated | | - | 0.5 mL $4 \times 10^8$ CFU/chick | 3-5 nd day | - | 9, 12 |
| CBS | *C. butyricum* treated and *Salmonella* challenged | | 1 mL $2 \times 10^9$ CFU/chick | 0.5 mL $4 \times 10^8$ CFU/chick | 3-5 nd day | 6 nd day | 9, 12 |
| S | *Salmonella* challenged | | 1 mL $2 \times 10^9$ CFU/chick | - | - | 6 nd day | 9,12 |

### Sample Collection and Processing

We counted the liver and spleen immune organ index of each chick on d 6 postinfection, $n = 8$ chicks. Samples of livers and whole spleens of the chicks were taken in order to determine the number of *Salmonella* bacteria, $n = 4$ chicks. The liver, spleen, and cecum samples were fixed by 4% paraformaldehyde for 24 h at room temperature. Then samples were embedded in paraffin, and slides were stained with hematoxylin-eosin (**HE**) stain. After picking up the cecum, we dehydrated it with different concentrations of sucrose and made frozen sections for immunohistochemistry. The cecum of per chicks were collected in multiple cryogenic tubes and placed in a liquid nitrogen tank and then preserved at −80°C. The contents of the cecum will be used to perform the 16S rRNA amplicon sequencing and bioinformatic analyses of the gut microbiota. Other remaining cecum parts will be used for quantitative real-time PCR (**QPCR**).

### Real-Time PCR

For chick RNA isolation, cecum and cecum tissue were homogenized in a tissue homogenizer (BioSpec Products, China), and RNA was isolated by Trizol reagent (Invitrogen, China) following the manufacturer's protocol and then stored at −80°C for QPCR; $\beta$-actin was used as an internal reference. QPCR was performed using SYBR Green (Applied Biosystems), PCR mix, and the appropriate primer sets (Table 2). QPCR reactions were run in a 10-$\mu$L reaction mixture using an ABI 7500 Detection System (Applied Biosystems, Carlsbad, CA, United States). The RNA was solubilized in RNase-free water. RNA quantity and quality were evaluated using a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), followed by cDNA synthesis via the Transcriptor First-Strand cDNA Synthesis Kit (Roche, China) using 2 $\mu$g RNA template. The PCR procedure consisted of 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s.

### Hypoxyprobe-1 Kit

Using imaging for the detection of hypoxia, chicks were treated with 60 mg/kg of pimonidazole HCl (Hypoxyprobe-1 kit, Hypoxyprobe) via intraperitoneal injection 1 h prior to euthanasia. Under hypoxic conditions, nitroreductase enzymes reduce pimonidazole to hydroxylamine intermediates, which bind irreversibly to nucleophilic groups in proteins or DNA (Kizaka-Kondoh and Konse-Nagasawa, 2009). After the cecum was removed, it was dehydrated with different concentrations of sucrose, and frozen sections were prepared for immunohistochemistry.

**Table 2.** Primers used for real-time PCR analysis.

| | Gene name | Primer sequerence(5′-3′) |
|---|---|---|
| 1 | *IL-6-F* | TGGTGATAAATCCCGATGAAG |
| 2 | *IL-6-R* | GGCACTGAAACTCCTGGTCT |
| 3 | *β-actin-F* | CTGGCACCTAGCACAATGAA |
| 4 | *β-actin-R* | CTGCTTGCTGATCCACATCT |
| 5 | *PPAR-γ-F* | TGGTTGACACAGAAATGCCGT |
| 6 | *PPAR-γ-R* | CCATTTTGATTGCACTTTGGC |
| 7 | *NF-κB-F* | CTCTCCCAGCCCATCTATGA |
| 8 | *NF-κB-R* | CCTCAGCCCAGAAACGAAC |
| 9 | *TNF-α-F* | GAGCGTTGACTTGGCTGTC |
| 10 | *TNF-α-R* | AAGCAACAACCAGCTATGCAC |

### Bacterial Burden and Histopathology

On d 3 and d 6 postinfection, four chicks were randomly selected from each group. The liver and whole spleen were removed, weighed, and homogenized in PBS, and serial dilutions of the homogenates were plated onto xylose lysine deoxycholate plates to count bacteria. On d 3 and d 6 postinfection, the liver, spleen, and cecum from each group were fixed using 4% paraformaldehyde for 24 h at room temperature. Then the liver samples were embedded in paraffin, and slides were stained with HE stain.

### Analysis of the Effects of C. butyricum on Intestinal Flora

The cecum contents of 1-wk-old chicks were homogenized in PBS, and serial dilutions of the homogenate were inoculated onto LB agar plates and incubated in anaerobic and aerobic cultures. Bacterial species were analyzed using the IVD MALDI Biotyper system. We evaluated the effects of *C. butyricum* on the microbiota in chicks' ceca using Illumina sequencing of the 16S rRNA V3 and V4 regions (Takahashi et al., 2014).

### Statistical Analysis

Data were analyzed using a one-way ANOVA model, and differences between means were compared using Tukey's multiple comparisons test when the main effect of treatment was significant. Differences were considered significant at $P < 0.05$.

## RESULTS

### Growth Performance After Infection

As shown in Figure 1, *Salmonella* infection had not affected the body weight of the chicks on d 6 postinfection (Figure 1A). However, chicks challenged with *Salmonella* showed changes in both liver/body (Figure 1B) and spleen/body (Figure 1C), and weight ratios on d 6 postinfection were comparable to those of the CON group ($P > 0.05$).

### S. Enteritidis Translocation

As shown in Figure 2, by measuring the bacterial load of *Salmonella* in the liver and whole spleen on d 3 and 6 postinfection, we observed that the *C. butyricum*

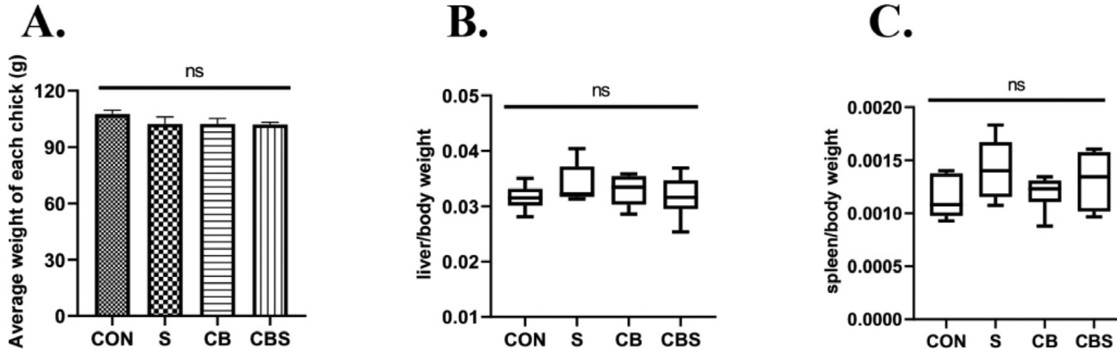


**Figure 1.** *C. butyricum* therapeutic effect on body weight, liver, and spleen of chicks infected with *S. Enteritidis*. (A) The weight average of each group before collecting samples. Liver (B) and spleen (C) organ index on 6 postinfection. *, $P < 0.05$ (One-way ANOVA); ns, no significance.

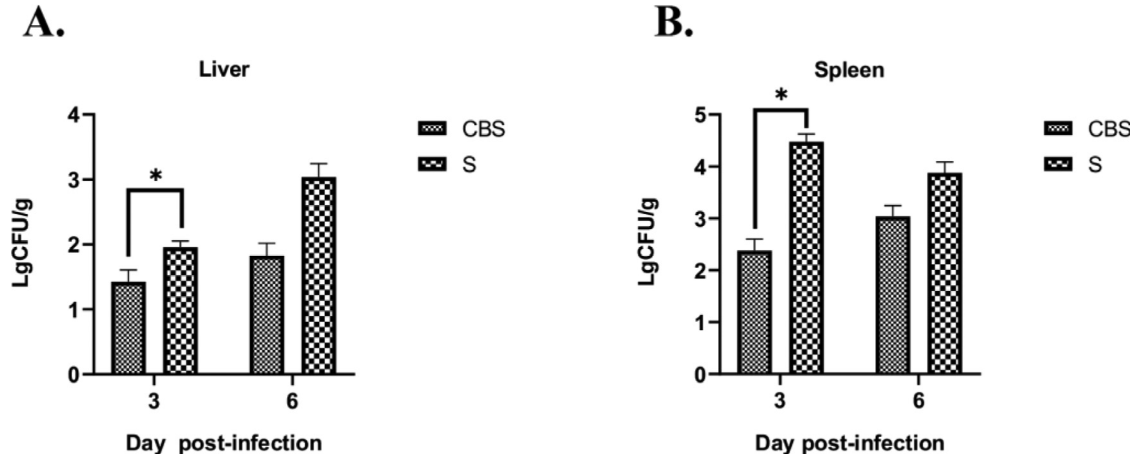


**Figure 2.** Effect of *C. butyricum* on the reduction of *S.* Enteritidis counts. Bacterial loads in liver (A), spleen (B) on d 3 and 6 postinfection, $n = 4$ chicks. *, $P < 0.05$ (One-way ANOVA); ns, no significance.

treatment group had adequately inhibited the proliferation of *Salmonella*. The average loads of *S.* Enteritidis in the liver ($P < 0.05$) and whole spleen ($P < 0.05$) decreased significantly in the CBS group on d 3 postinfection, and no significant difference was found in *Salmonella* numbers on d 6 postinfection (Figures 2A and 2B).

## C. butyricum *Therapeutic Effect on the Liver, Spleen, and Cecum of Chicks Infected With* S. *Enteritidis*

As shown in Figure 3A, no abnormal lesions were found in the CON and CB groups. A small number of lymphocyte infiltration foci were observed in the CBS group, as well as focal infiltration of lymphocytes and infiltration of heterophilic granulocytes in the S group on d 3 and 6 postinfection in the liver. No abnormal lesions were found in either the CON or CB group. The red pulp of the spleen was slightly congested, and a small amount of heterophilic granulocyte infiltration was observed in the CBS group as well as mild red-pulp congestion in the spleen and infiltration of a small amount of heterophilic granulocytes in the S group on d 3 and 6 postinfection in the spleen (Figure 3B). No abnormal lesions were found in the CON and CB groups; the goblet cells in the epithelium of the cecum had increased and secreted exuberant in the CBS group, and congestion and hemorrhage were noted in the lamina propria of the cecum mucosa and lymphocytic infiltration in the S group on d 3 and 6 postinfection in the cecum (Figure 3C).

## *Detection of Relative Expression Levels of IL-6 and PPAR-γ in the Cecum*

On d 6 postinfection, the hypothesis that early preconditioning of chicks with *C. butyricum* may alter cytokine production in gut tissue following *S.* Enteritidis challenge was evaluated by measuring cytokine levels. The gene expression levels of cytokines *IL-6* in the cecum were evaluated and found to be was significantly elevated in the S group compared to the CON group ($P < 0.01$; Figure 4A). The results showed that the expression level of *PPAR-γ* in the cecum was decreased in the CBS group compared to the CB group ($P < 0.05$); however, there was no significant difference between the CON and S groups ($P > 0.05$; Figure 4B). The mRNA expression of *NF-κB* and *TNF-α* were not significantly different between all groups (Supplementary Figure S1A, B).

## *Analysis of the Effects of* C. butyricum *on Intestinal Flora*

The cecum contents of a 1-wk-old chick were anaerobic and aerobic cultured on autoclaved LB solid agar

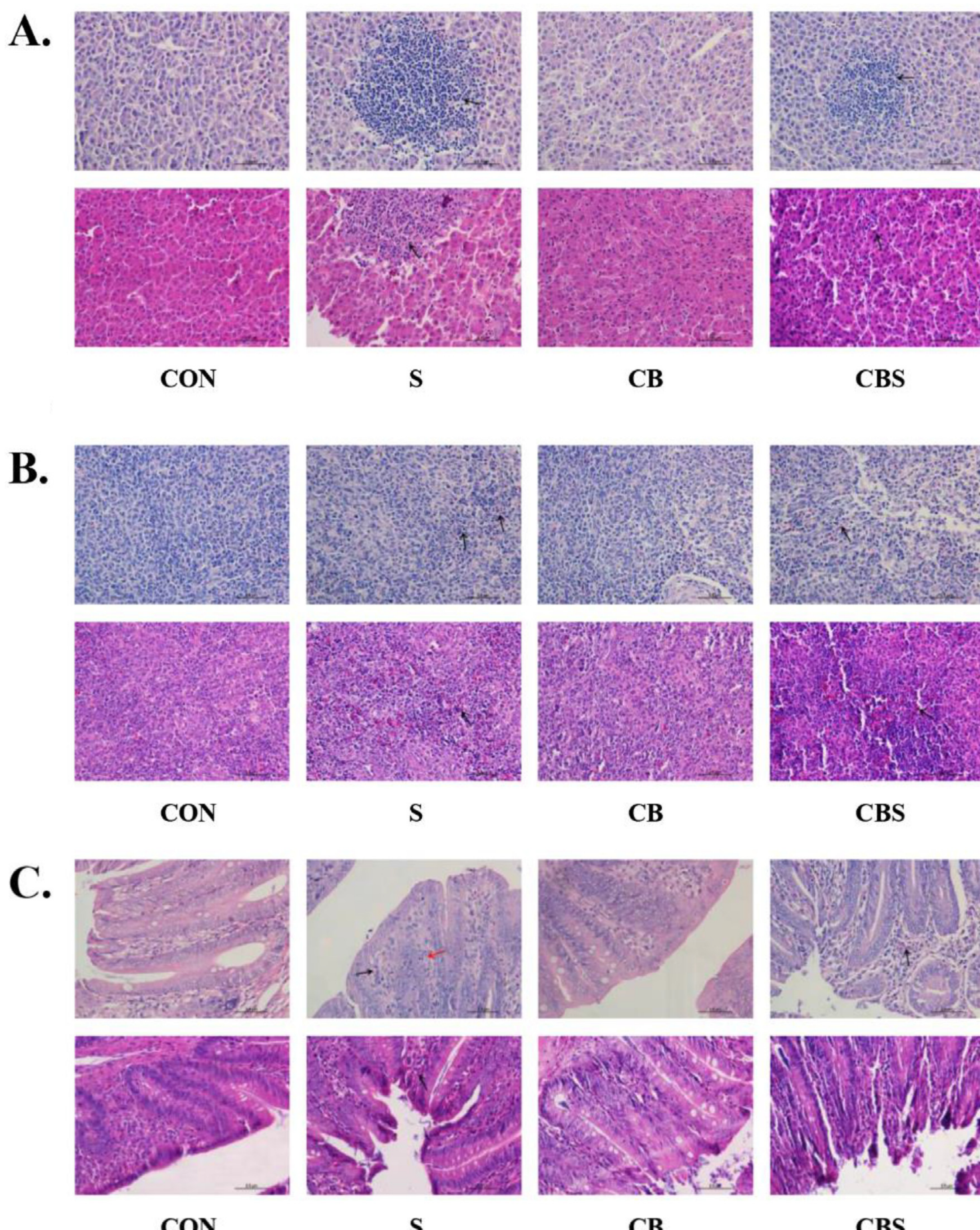


**Figure 3.** *C. butyricum* therapeutic effect on pathological changes of organs. Pathological sections of liver (A), spleen (B) and cecum (C) tissue on d 3 (top row) and 6 (bottom row) post-nfection (400 ×).

under anaerobic and aerobic conditions, following the IVD MALDI Biotyper System analysis. The colonies found were mainly *Enterococcus faecium* and *Escherichia coli*, in addition to a small amount of *Enterococcus faecalis, Enterobacter cloacae,* and other bacteria. The major colonies were identified as members of the *Escherichia* and *Enterococcus* families, and they are facultative anaerobes (Figure 5A). Anaerobic bacteria among the top 20 bacteria with the highest relative abundance on d 6 postinfection, the results showed that the number of anaerobic bacteria in the S group was significantly reduced and that the use of *C. butyricum* had restored the abundance of anaerobic bacteria (Figure 5B). According to relative abundance analysis at the genus level, *Faecalibacterium* and *Lactobacillus* were the 2 most-abundant bacterial genera on d 3 postinfection (Figure 5C). However, *Faecalibacterium* and *Subdoligranulum* were the 2 most-abundant bacterial genera, and the relative abundance of *Ruminococcus* in the CON group was 1.61%; in the CB group, it was 1.87%,

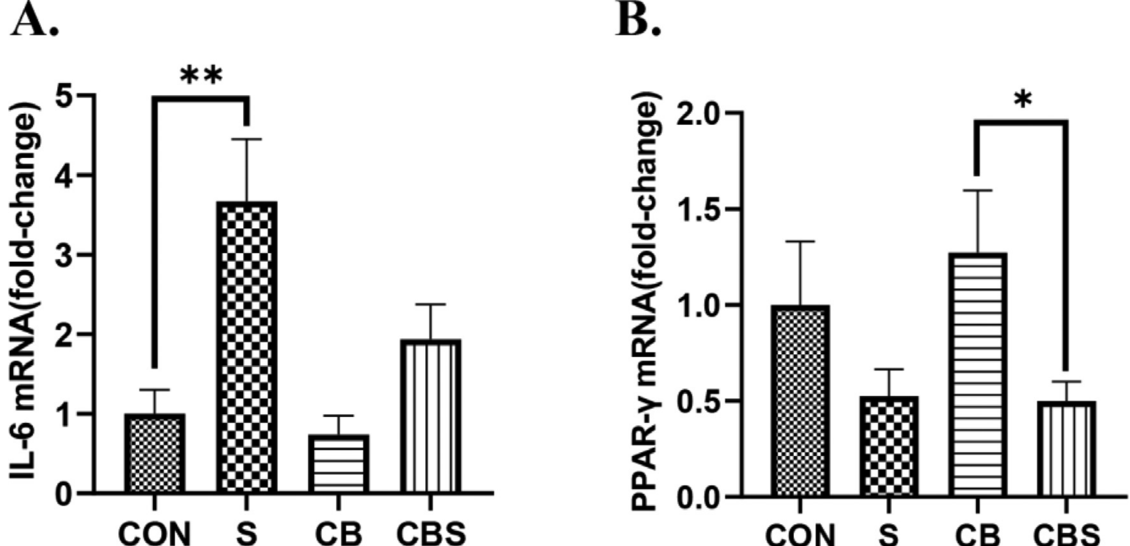


**Figure 4.** Detection of relative expression levels of *IL-6* and *PPAR-γ* in the cecum, $n = 8$ chicks. Three-day-old chicks were infected with $2 \times 10^9$ CFU of the indicated *S.* Enteritidis strains. Relative changes in transcript levels of *IL-6* (A) and *PPAR-γ* (B) were determined by quantitative real-time PCR using RNA isolated from cecum on day 6 post-infection. **, $P < 0.01$ *, $P < 0.05$ (One-way ANOVA); ns, no significance.

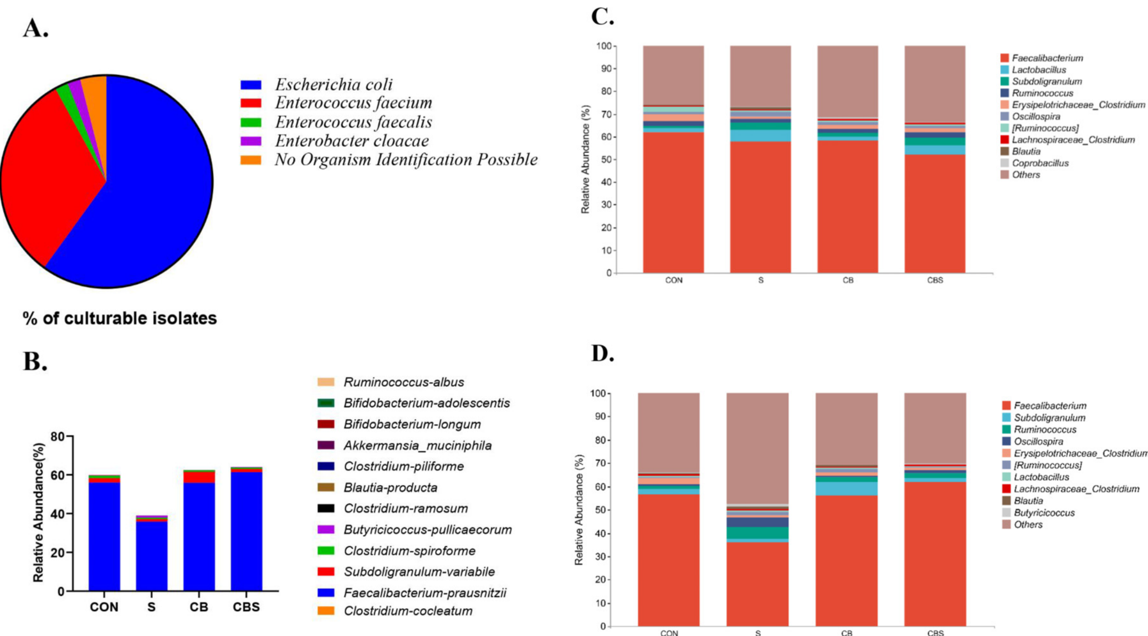


**Figure 5.** (A) Cecum contents of a 1-wk old chick were cultured on LB solid agar under anaerobic and aerobic conditions using IVD MALDI Biotyper System. (B) Relative abundance of anaerobic bacteria species in the gut on d 6 postinfection. (C) Relative abundance of the most abundant bacterial genus on d 3 postinfection. (D) Relative abundance of the most abundant bacterial genus on d 6 postinfection.

in the CBS group 2.26%, and in the S group 5.17%. The relative abundance of *Ruminococcus* increased considerably in Group S on d 6 postinfection (Figure 5D). Diversity analysis of gut bacterial communities revealed differences between groups (Supplementary Figure S2).

## *Effects of* C. butyricum *on Intestinal Oxygen Content*

The results showed that strong pimonidazole staining of the cecum epithelium of the CON group chicks suggested that the cecum was lined with a hypoxic surface (Figure 6A). Importantly, colonocytes of mock-infected chicks exhibited hypoxia, which was eliminated during *S.* Enteritidis infection (Figure 6D) but could be restored by inoculation with *C. butyricum* (Figure 6C).

## DISCUSSION

Currently, *Salmonella* infections cause serious public health safety problems in many countries (Barrow et al., 2012). Therefore, reducing the spread of *Salmonella* in

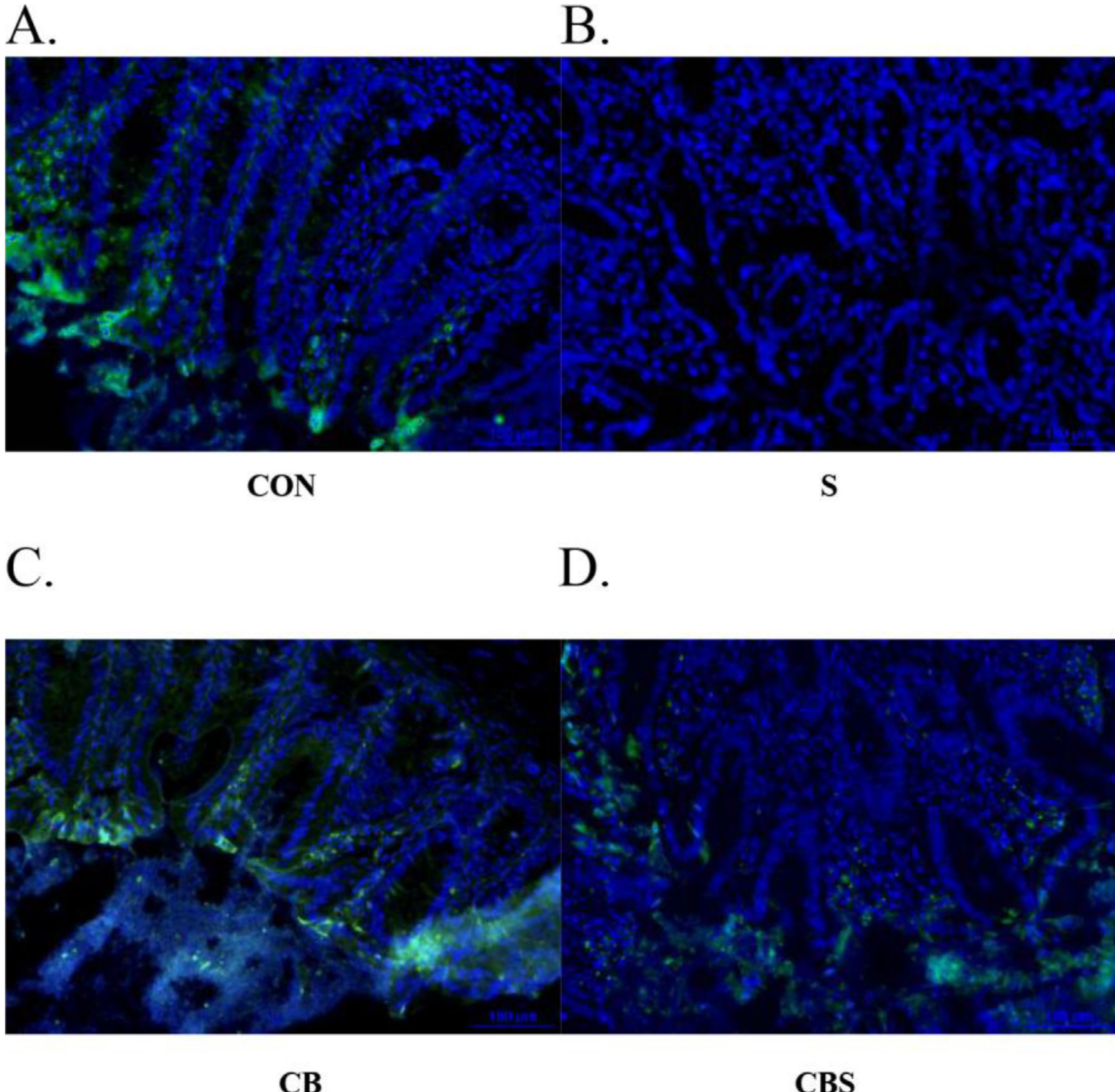


**Figure 6.** The cecum of each group of chicks was elected by immunohistochemistry on d 6 postinfection. Representative images of pimonidazole (green) staining detected in cecum sections counter stained with DAPI nuclear (blue). Representative images are shown.

poultry and preventing the outbreak of food-borne *Salmonella* is an urgent problem that should be solved on a global scale.

*S.* Enteritidis in the gut causes intestinal inflammation, intestinal barrier damage, and digestive disturbances (Yan and Ajuwon, 2017). Studies have shown that the number of heterophil cells in the spleen and liver increased significantly after infection with *Salmonella* in both resistant and susceptible chicken lines (Kogut et al., 2005). The occurrence of inflammation leads to the secretion of the pro-inflammatory cytokine IL-6. Mudter and Neurath found that the invasion of *Salmonella* Typhimurium and *S.* Enteritidis caused an 8-to-10-fold increase in production of the pro-inflammatory cytokine *IL-6* (Mudter and Neurath, 2007). Nowadays, the restoration of altered intestinal microbes using probiotics is considered a potential strategy for the prevention and treatment of *Salmonella* infection (Mukherjee et al., 2018). Since its discovery, *C. butyricum* has been widely used, and numerous studies have demonstrated that it has many positive effects on the growth and immunity of chicks (Yang et al., 2012). Moreover, it has been shown to promote intestinal microecological balance through intestinal microbiota (Chen et al., 2019). Consistent with previous research findings in the laboratory (Zhao et al., 2017), our study showed that the use of *C. butyricum* can reduce the bacterial load of *Salmonella* in the liver and spleen and effectively prevent its further spread. By analyzing pathological sections, our study showed that heterophile antibodies increased in the spleen and liver of chicks after challenge with *Salmonella*. In the present study, *C. butyricum* was found to inhibit the secretion of IL-6 and reduce the inflammatory response. It is also worth noting that our results showed that *C. butyricum* changes the relative expression content of *PPAR-$\gamma$* in the chick cecum as one of the important intracellular pathways.

As most gut bacteria are obligate or facultative anaerobes, this not only ensures protection of the body's digestion, absorption, and metabolism but also prevents

the reproduction of pathogenic bacteria (Parada Venegas et al., 2019). Therefore, ensuring an anaerobic environment in the intestine is critical. *S.* Enteritidis is a facultative anaerobic bacterium, and *Salmonella* entering the intestine destroys its anaerobic environment by producing exogenous electron acceptors, thus promoting its colonization in the gut. Importantly, the oxygenated microenvironment in the gut is particularly conducive to the proliferation of *Enterobacteriaceae* (Zeng et al., 2017). Previous studies have demonstrated that, commensally, *Enterobacteriaceae* protect against *Salmonella* colonization through oxygen competition (Litvak et al., 2019). In the present study, *C. butyricum* as a probiotic can effectively alleviate changes in the intestinal oxygen environment caused by *Salmonella* after entering the body and plays an important role in maintaining an anaerobic intestinal environment. Interestingly, the cecum contents of 1-wk-old chicks were cultured and bacterial colonies were facultative anaerobes. Additional studies are necessary to further explore the potential mechanism of *C. butyricum* in intestinal immunity. As a complex ecosystem, the population density and composition of intestinal microbes are constantly changing as animals grow and develop (Lawley and Walker, 2013). Gut microbiota plays an irreplaceable role in the immune system, metabolic system, and nervous system, among others. Intestinal flora disorder caused by *S.* Enteritidis infection is another key problem to be solved in regard to the cecum. Intestinal colonization by *S.* Enteritidis not only causes intestinal inflammation but also leads to dysbiosis of intestinal flora, disruption of intestinal epithelial structure, and reduced intestinal barrier function (Bjerrum et al., 2005). It is well-known that *C. butyricum* in the intestinal tract can promote digestion and absorption and ensure the stability of intestinal flora. The relative abundance of anaerobic bacteria can serve as a measure of physical health, and previous studies have shown that the relative abundance of anaerobic bacteria is closely related to intestinal homeostasis (Liu et al., 2018). Our present findings suggest that *C. butyricum* can effectively prevent *Salmonella*-induced reduction in the relative abundance of anaerobic bacteria in the cecum and increase the relative abundance of anaerobic bacteria in the gut, thereby leading to intestinal homeostasis.

In conclusion, *C. butyricum* changed the oxygen environment in the intestine, increased the relative abundance of anaerobic bacteria, and effectively inhibited the proliferation of *Salmonella*. This finding reveals in greater depth the interaction between the flora from the perspective of gut microbes and provides a theoretical basis for the prevention and treatment of salmonellosis.

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## DISCLOSURES

The authors declare that they have no competing interests.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102077.

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