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Research

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Functional comparison of *Clostridium butyricum* and sodium butyrate supplementation on growth, intestinal health, and the anti-inflammatory response of broilers

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Abstract

Background: Butyrate has been reported to promote proliferation of colonic epithelial cells and maintain intestinal barrier integrity in broilers. Although supplementation of *Clostridium butyricum* and sodium butyrate have been shown to confer benefits on broilers, their effects and mechanisms have not been compared.

Results: In this study, *C. butyricum* and sodium butyrate were added into the basal diet of broilers and their effects on growth performance, intestinal health, and anti-inflammatory response were analyzed. It was found that both *C. butyricum* and sodium butyrate showed good probiotic effects on broilers. Their effects on growth rate and expression of inflammation related genes were even superior to that of antibiotic. Besides, the two dietary supplements improved intestinal structure integrity and secretion of inflammatory cytokines, although the antibiotic had negative effects. Comparison of the two supplements revealed that sodium butyrate more effectively improved the growth and intestinal structure of broilers than *C. butyricum*. On the contrary, *C. butyricum* was superior to sodium butyrate in promoting tight junction protein expression, SCFAs production, and anti-inflammatory response.

Conclusions: In summary, this study demonstrates the positive effects of *C. butyricum* and sodium butyrate on broilers, and will serve as a reference for selection of appropriate butyrate supplementation for broilers in the breeding industry.

Keywords: *Clostridium butyricum*; sodium butyrate; broiler; growth performance;

41 intestinal health; anti-inflammatory response

Background

Short-chain fatty acids (SCFAs), a class of organic acids that includes acetate, propionate, and butyrate, are important metabolites produced by intestinal microbial anaerobic fermentation (1). Numerous studies currently show that SCFAs exert diverse functions on the host (2). SCFAs, for example, contribute to the integrity of the gut structure by meeting a significant portion of the energy requirements of colonic epithelial cells (3). Additionally, SCFAs promote intestine health in a variety of other ways, including phagocytosis, intestinal dynamic balance, and immune regulation (4). Butyrate, a typical C_4 SCFA, has garnered much attention in recent years for its beneficial effects on intestinal health (5). Butyrate is the most important energy substance of colon cells, promoting epithelial cell growth and differentiation (6, 7). Some studies have also shown that butyrate, as an anti-inflammatory agent, plays an essential role in modulating immune response and intestinal barrier function (4, 8).

Because of its positive effects on the host, including butyric acid into dairy diets to promote animal growth has become a widely adopted strategy in the feeding industry (9, 10). There are different approaches for increasing the amount of butyric acid in the diet, including dietary fiber, butyrate, and butyrate-producing bacteria. *Clostridium butyricum* spores and sodium butyrate are the two most common butyric acid supplements in the market. *C. butyricum*, a butyric acid-producing anaerobic bacterium, is widely distributed in animal guts and the natural environment (11). Due to its beneficial properties, *C. butyricum* has been recognized as a typical probiotic on

a global scale. *C. butyricum* has been shown in previous research to significantly improve broiler growth performance, nutritional metabolism, intestinal morphology, and intestinal immune dynamic balance (12-14). Additionally, it enhances intestinal barrier function and inhibits the inflammasome signaling pathways in weaned piglets challenged with enterotoxigenic *Escherichia coli* K88 (15). Numerous studies have reported that sodium butyrate, the sodium salt of butyric acid, has positive effects on growth performance and intestinal integrity in piglets and broiler chickens when used as a feed supplement (16-18). Additionally, it has been shown to repair the imbalanced gut flora caused by a high-fat diet in mice (19).

Although the effects of *C. butyricum* and sodium butyrate on broiler's growth performance and intestinal health have been extensively investigated, the similarities and differences in their probiotic function remain unknown. In this study, we investigated the impact of *C. butyricum* and sodium butyrate on broilers. Our findings indicated that both had varying degrees of positive effects on broilers and comparing their functional differences may help guide market selection of butyric acid supplements.

Methods

Experimental design

A total of 360 one-day-old Cobb500 broilers were randomly assigned to four groups with each group consisting of 15 birds and 6 replicates for each group (Table 1). These four groups were set as follows: basal diet (Control) and a basal diet

supplemented with 100 g/t (1.0×10^9 CFU/g) *C. butyricum* spores (CB), 500 g/t sodium butyrate (SB), or 200 g/t oxytetracycline (Antibiotic), respectively. Table 2 summarizes the composition and nutritional content of the basal diet. *C.butyricum* and SB were obtained from Wuhan SunHY Biology Co., Ltd.

Broilers were raised in wire cages with sufficient ventilation and water supply, and the room temperature was maintained between 22-25°C. Before the experiment, the equipment was cleaned and disinfected, particularly, it was fumigated with potassium permanganate and formaldehyde after cleaning and drying. Immunization and deworming procedures were conducted concurrently with the farm routine. Continuous feeding three times daily at a set time was conducted. Throughout the trial period, the feeding and health of broilers were monitored and documented. The breeding experiment was conducted at SunHY Biology Co., Ltd's Huanghu breeding facility.

Sample collection

Before the experiment started, twelve broilers (two from each replicate) were selected at random from each group to be weighed. The feeding of an additional twelve broilers (selected as above) was stopped at 9:00 p.m. on the 21st and 42nd days of the experiment, respectively, and their water supply was stopped at 7:00 a.m. on the 22nd and 43rd days. The body weight was recorded, they were killed, and the abdominal cavity was rapidly opened to separate jejunum, ileum, and cecum. Samples of the jejunum, ileum, and cecum (about 1-2 cm from the midpoint) were fixed in 4%

paraformaldehyde for tissue section preparation and examination of intestinal morphology. The jejunum was cut, washed with sterile normal saline, scraped, and frozen at -80°C for DNA extraction and gene expression analysis. Ileum and cecal chyme were collected and stored at -20°C for subsequent analysis of volatile SCFAs. Meanwhile, cecum digested samples were taken out and stored at -80°C for 16s rDNA high-throughput sequencing.

Growth performance measurement

Throughout the study, the daily feed consumption, body weight, and health of the broilers were all documented. The following formula was used to determine feed intake (FI), body weight gain (BWG), and feed to gain ratio (F/G).

FI (g/d·bird) = ∑ [(Feed amount - Residual amount) / Number of broilers] / Days.

BWG (kg/d·bird) = (Final average weight - Initial average weight) / Days.

F/G = Total feed consumption / Total weight gain.

Analysis of intestinal histomorphology

Jejunum, ileum, and cecum segments were fixed in formaldehyde and embedded in paraffin. Consecutive sections (5 mm) were stained with eosin-methylene blue for morphological observations using an optical microscope. From each section, fifteen villi were randomly selected and their villi height (V) and crypt depth (C) were measured. Villus height refers to the distance from the apex of the villus to the

entrance of the crypt, while crypt depth refers to the distance between the base of the villus and the basal mucosa. The following formula was used to determine the villi height to crypt depth ratio (V/C).

V/C = Villi height / Crypt depth.

16S rDNA sequencing

The variable region V3 of the cecal microflora 16s rDNA gene was sequenced using 454 high-throughput sequencing technology. The software (Mothur) was used to remove the low-quality DNA sequences, and then the distance between the sequences was calculated. Operational taxonomic units (OTUs) were determined as filtered sequencing clusters with a 97% similarity level. The microbial diversity of various treatments was investigated and compared using the Sliva and RPD databases. Sangon Biotech (Shanghai) Co., Ltd performed the sequencing.

Determination of SCFAs concentrations

Gas chromatography-mass spectrometry (GC-MS) was used to determine the concentrations of standard solutions and volatile SCFAs in the ileum and cecum chyme. Acetic acid, propionic acid, or butyric acid were dissolved in ether to form standard solutions of varying concentrations. 50 mg of chyme was dissolved in a mixture of 50 μL phosphoric acid (15%), 100 μL isohexanoic acid (125 μg/mL), and 400 μL ether. The sample was then vortexed and centrifuged at 13,000 g for 10 min at 4°C. For analysis, the supernatant was injected into the chromatographic column.

SCFAs were analyzed using a Thermo TRACE 1310-ISQ GC-MS system, equipped with an Agilent HP-INNOWAX column (30 m × 0.25 mm ID × 0.25 μm). The split injection was carried out using a 1 μL injection volume (split ratio 10:1). The inlet and transmission line temperature was 250°C, the ion source temperature was 230°C, and the quadrupole temperature was 150°C. The carrier phase was helium at a flow rate of 1.0 mL/min. MS was carried out using an electron bombardment ionization (EI) source and SIM scanning mode. The electron energy was 70 eV.

Expression of intestinal inflammatory factors and tight junction protein genes

RNA from the jejunum samples was extracted, and cDNA was obtained by reverse transcription from total RNA. Occludin, ZO-1, TAK1, NF-kB, IL-1β, IL-6, and TNF-α gene expression levels were then determined by real-time PCR. β-actin expression gene was used as the reference gene. Table 3 lists all of the primers used.

Statistical analysis

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for variance analysis, and Duncan was used for multiple comparisons. The data were expressed as mean ± SD. $p < 0.05$ was considered to be statistically significant.

Results

Sodium butyrate promotes growth performance more effectively than C. butyricum

The feed to gain ratio (F/G) of four diets (Control, CB, SB, Antibiotic, Table 1) was maintained at 1.42-1.47 over 1-21 d (Table 4). However, when the feed additive (*C. butyricum*, sodium butyrate, or oxytetracycline) was introduced, the value significantly decreased ($p < 0.05$) from 2.05 to 1.90-1.92 during 22-42 d and from 1.86 to 1.74-1.75 during 1-42 d (Table 4). Specifically, the SB group's body weight gain (BWG) significantly increased ($p < 0.05$) from 56.31 g to 62.16 g over the 22-42 d period and from 42.13 g to 45.76 g throughout the overall period (1-42 d), while feed intake (FI) remained constant in the Control group (Table 4). Although *C. butyricum* supplementation did not affect BWG in the CB group, FI was significantly decreased ($p < 0.05$) from 115.68 g to 106.57 g during 22-42 d and from 78.38 g to 73.73 g during 1-42 d (Table 4). Besides, there were no significant differences ($p > 0.05$) in BWG and FI between the Antibiotic group and the other two groups (CB, SB), respectively (Table 4). When compared to the CB group, the addition of sodium butyrate significantly increased BWG and FI ($p < 0.05$) throughout 22-42 d and the overall period (1-42 d), but there were no discernable changes in F/G ($p > 0.05$, Table 4).

C. butyricum and sodium butyrate improves intestinal health

Antibiotic supplementation had a negative effect on virtually all indices of intestinal structural integrity, including villus height (V), crypt depth (C), and the ratio of villi height to crypt depth (V/C) (Fig. 1 and Table 5), as well as damaged intestinal morphology (Fig. 1). Broilers fed with diets supplemented with CB or SB, however,

196 had longer, wider villi (Fig. 1 and Table 5), and more goblet cells (Fig. 1). After 21
197 days, V and V/C of the jejunum and cecum in the CB and SB groups were both
198 significantly increased ($p < 0.05$) than in the non-supplemented Control group (Fig. 1
199 and Table 5). Specifically, V of jejunum samples was increased from 711.73 μm to
200 987.79 μm and 985.14 μm, respectively, while that of cecum increased from 143.95
201 μm to 166.58 μm and 187.70 μm at the same time (Table 5). V/C levels increased
202 from 4.88 to 7.62 and 8.41 in the jejunum, and from 1.13 to 1.45 and 1.74 in cecum
203 samples from two supplemented diets (CB, SB, Table 5). Furthermore, V/C of the
204 ileum was increased from 4.07 to 6.70 and 7.08, with significant statistical differences
205 ($p < 0.05$), and C was reduced from 127.23 μm to 76.58 μm in the CB group and
206 100.37 μm in the SB group ($p < 0.05$, Table 5). At 42 d, we observed that the V/C of
207 the jejunum, ileum, and cecum were almost all significantly increased ($p < 0.05$) in
208 the CB and SB diets (Table 5). In particular, the V/C of the jejunum increased from
209 5.43 to 6.93 in the SB group, that of the ileum increased from 5.39 μm to 6.58 μm and
210 6.73 μm, while that of the cecum samples increased from 0.93 to 1.50 and 1.75 (Table
211 5). Furthermore, it was clear that V/C in the SB group increased more apparent than
212 in the CB group, not only in various segments of the small intestine but also
213 throughout different development stages of broilers (Table 5).

214 Real-time PCR findings revealed that broilers fed the SB diet had higher levels
215 of Occludin gene expression ($p < 0.05$) than those in the Control group at all stages
216 (Fig. 2). Furthermore, *C. butyricum* supplementation significantly increased both
217 Occludin and ZO-1 expression ($p < 0.05$) at 42 d (Fig. 2). Specifically, the expression

level increased by 2.56 and 2.64, respectively (Fig. 2). However, all tight junction protein genes were lowly expressed in the antibiotic-supplemented group, with Occludin levels falling by 1.34-1.96, and ZO-1 levels falling by 1.35-1.55 compared to the Control group (Fig. 2).

Chao1 and Shannon indexes were used to express the alpha diversity of microbiology communities in the cecum (Fig. 3A). *C. butyricum*, sodium butyrate, and oxytetracycline supplementation altered the community's species diversity as compared to the Control group (Fig. 3A). The principal coordinates analysis (PCoA) based on uniFrac distance was used to assess the community structure differences of four groups. The findings revealed that four diets clearly separated the microbiota (Fig. 3B). According to the Venn diagram, there were 1004 universal OTUs shared by all four groups, as well as 2689, 2516, 3911, and 3442 unique OTUs in the Control, CB, SB, and Antibiotic groups, respectively (Fig. 3C). Furthermore, linear discriminant analysis (LDA) showed that *Tannerellaceae* and *Parabacteroides* were considerably more abundant in the CB group (Fig. 3D), while the addition of SB increased the abundances of *Campylobacter*, *sulfurimonas*, and *Paludibacter* (Fig. 3D).

At the phylum level, the relative abundance of microbial communities revealed that *Firmicutes* and *Bacteroidota* were the most abundant orders (Fig. 4A). Among these, the *Firmicutes*/*Bacteroidota ratio* was lower in the CB group, while it was close to the Control group in the other two groups (SB, Antibiotic) (Fig. 4A). At the genus level, *Rikenellaceae*, *Alistipes*, and *Coprobacter* were the most predominant

240 orders in the Control group (Fig. 4B). The findings showed that the addition of *C.*
241 *butyricum* produced the most significant change in microbial structure among the
242 three treatments, with the abundance of *Rikenellaceae* increasing (Fig. 4B).
243 Furthermore, the abundance of *Alistipes*, *phascolarctobacterium* and *Coprobacter* was
244 decreased in the CB, SB, and Antibiotic groups (Fig. 4B).
245
246 ***C. butyricum promotes SCFAs production more effectively than sodium butyrate***
247 Data revealed significant changes ($p < 0.05$) in concentrations of all identified SCFAs
248 (acetic acid, propionic acid, and butyric acid) in broiler ileum chyme across three
249 supplementation groups (CB, SB, Antibiotic) compared to the Control group (Fig. 5).
250 In particular, the concentration of acetic acid was increased from 40.85 μg/g to 283.42
251 μg/g in the SB group at 21 d, which showed the most significant differences (Fig. 5).
252 Besides, data revealed that sodium butyrate supplementation promoted SCFAs
253 production better than *C. butyricum* (Fig. 5).
254 It was clear that concentrations of SCFAs in cecal chyme were considerably
255 higher than those in the ileum (Fig. 5 and Fig. 6). Moreover, compared with the basal
256 diet group, the supplementation of *C. butyricum*, sodium butyrate, or oxytetracycline
257 all increased the concentrations of SCFAs to a similar extent in the cecum of 21 days
258 and 42 days old broilers ($p < 0.05$, Fig. 6).
259
260 ***C. butyricum improves anti-inflammatory response more than sodium butyrate***

Broilers fed with CB diet or antibiotic diet exhibited lower concentrations of TAK1 and NF-kB ($p < 0.05$) in jejunal mucosa than those fed with basal diet or SB diet at 21 days of age (Fig. 7A). Among these, the addition of *C. butyricum* decreased the expression level of TAK1 by 2.23 times (Fig. 7A). At 42 d, the three treatments (CB, SB, Antibiotic) showed various degrees of inhibitory effects on the expression of two genes (Fig. 7A). It's worth mentioning that SB-supplementation decreased the expression of TAK1 and NF-kB at 42 d, while there was no expression inhibition on 21-day old broilers (Fig. 7A). The inhibitory effect of CB on the expression of two genes was more significant than SB (Fig. 7A).

As for inflammatory factors, the supplementation of oxytetracycline did not inhibit the expression of inflammatory factors (Fig. 7B). CB diet decreased the expression levels of IL-1β and IL-6 during 1-21 d ($p < 0.05$, Fig. 7B) and that of IL-1β, IL-6, and TNF-α during 1-42 d ($p < 0.05$, Fig. 7B). Especially, the expression of IL-6 decreased by 2.37 times when compared with the Control group at 42d (Fig. 7B). For the SB-supplemented group, other than the expression of IL-6 at 21d which was increased, the rest were all decreased (Fig. 7B). Although both *C. butyricum* and sodium butyrate were helpful to the broilers, the benefits of *C. butyricum* were more apparent (Fig. 7B).

Discussion

According to the findings (Table 4), there were no significant differences ($p > 0.05$) in broiler growth indicators between the CB or SB groups and the Antibiotic group. The

283 probiotic effect of *C. butyricum* and sodium butyrate on growth performance is
284 consistent with earlier findings (20), suggesting that these two butyrate supplements
285 may be used instead of growth-promoting antibiotics. Notably, the feed to gain ratio
286 (F/G) in the CB group significantly decreased ($p < 0.05$) throughout the experimental
287 period (1-42 d), although body weight gain (BWG) remained constant (Table 4). We
288 hypothesized that supplemented strain's vector reduced feed intake (FI), however,
289 butyric acid generated by *C. butyricum* supplied approximately 10-30% of the energy
290 needs for broilers (21), and improved food digestibility by lowering intestinal pH and
291 inhibiting pathogenic bacteria (22). There have also been reports that a *C. butyricum*
292 or sodium butyrate diet has no impact on animal growth performance (23), which may
293 be related to the effective dosage, diet structure, animal health, and even
294 environmental factors.

295 Sodium butyrate significantly increased FI and BWG as compared to the *C.*
296 *butyricum* group ($p < 0.05$, Table 4). Sodium butyrate, whose probiotic function is
297 mostly dictated by dosage, may stimulate growth stably if taken in adequate
298 quantities. The probiotic effect of *C. butyricum*, on the other hand, will be affected by
299 a variety of factors, including viable count and intestine physiological state.
300 Furthermore, strain in the gut consumes enteral nutrients, reducing growth
301 performance. According to these findings, sodium butyrate is a more stable butyrate
302 supplementation for broiler growth performance than *C. butyricum.*

303 Intestinal health is defined as having a fully functional intestinal structure,
304 intestinal mucosal immune balance, and intestinal microbial balance. In this study, the

intestinal morphological structure was damaged by antibiotics as shown by decreased V/C at different periods (Fig. 1 and Table 5). However, *C. butyricum* and sodium butyrate diets both significantly improved the villus height (V), crypt depth (C), and V/C of jejunum, ileum, and cecum ($p < 0.05$, Table 5), preserving the normal morphology of the intestinal epithelial cell (Fig. 1), which is consistent with the previous findings (24). Furthermore, when compared to *C. butyricum,* the findings showed that sodium butyrate was more beneficial for maintaining intact intestinal structure (Table 5). We hypothesized that since sodium butyrate was in close contact with the intestinal tract, it would help to restore the intestinal barrier more rapidly.

The major tight junction proteins in the animal gut, Occludin and ZO-1, are important markers for assessing intestinal permeability and integrity (25). Li et al found that *C. butyricum* could significantly increase the expression of Occludin, ZO-1, and Claudin-3 in pigs infected with *Escherichia coli* K88 (15). In this study, *C. butyricum* and sodium butyrate both increased the expression of Occludin and ZO-1 in jejunal epithelial cells (Fig. 2), resulting in decreased permeability of intestinal epithelial cells, which prevented the passage of toxins and pathogens and decreased disease occurrence. Simultaneously, *C. butyricum* exhibited a more pronounced expression-promoting effect than sodium butyrate (Fig. 2). We hypothesized that *C. butyricum* improved intestinal barrier function more continuously through both live bacteria and its metabolites.

In this study, a sodium butyrate diet increased the species diversity of the cecal community, as reflected by the Chao1 index and Venn diagram (Fig. 3A and C).

Paludibacter, whose abundance increased in the SB group, has been identified as a propionate-producing bacteria (26) and may help to enhance the species diversity of the cecal community via the probiotic function of propionate. However, compared to the Control group, the species diversity of the sample in the CB group was decreased (Fig. 3A and C), which differs from the study in *C. butyricum*-supplemented laying hens (27). Furthermore, we hypothesized that *C. butyricum* might be viable in broiler guts but does not colonize, therefore *C. butyricum* was not included in the list of dominating species in the CB group (Fig. 3D).

There are many and complex microbiotas in the poultry intestine, the composition, and diversity of which are affected by a variety of factors (28). Meanwhile, the intestinal microbiota has an impact on host health (29). Wu et al. found that 800 mg/kg sodium butyrate significantly decreased the relative abundance of *Enterobacteriaceae* but increased that of *Lachnospiraceae* and *Rikenellaceae* in broilers cecum (30). *Rikenellaceae*, the main component at the genus level in this study, was similarly increased in supplemented groups (CB, SB) compared to the Control group (Fig. 4). Because of its high carbohydrate fermentation capacity, this species could generate butyrate and therefore exert a probiotic function on the host (31). When butyrate supplementation was administered, other dominating bacteria, such as *Clostridial* and *Lactobacillus*, which could both generate SCFAs increased (Fig. 4). Furthermore, the detection of SCFAs concentrations revealed that SCFAs in the CB and SB groups were much higher than that in the Control group (Fig. 5 and Fig. 6), supporting the conclusion that SCFAs-producing bacteria were the dominant

intestinal microbiota and significantly contributed to host health.

TAK1 is an IKK kinase involved in the NF-kB signaling pathway (32). NF-kB is an important transcription factor that promotes the expression of a variety of inflammatory and immune-related genes (33). TAK1 and NF-kB expression levels were significantly lower in the treatment groups (CB, SB, Antibiotic) than that in the Control group ($p < 0.05$) (Fig. 7A), suggesting that *C. butyricum*, sodium butyrate, and oxytetracycline may all inhibit the activation of the NF-kB pathway to reduce inflammation. Besides, CB supplementation outperformed the SB diet in terms of inhibiting the inflammatory response (Fig. 7A).

TNF-α, IL-1β, and IL-6 are the three main inflammatory cytokines that reflect the host's inflammatory state (34). Chen et al. found that 0.4% *C. butyricum* significantly decreased the expression of TNF-α and increased the expression of the anti-inflammatory cytokine IL-10 in weaned piglet ileum mucosa (35). Ni et al. reported that butyric acid up-regulated the production of IL-10 and inhibited the production of TNF-α, IL-1β, and NO (36). In this study, *C. butyricum* and sodium butyrate both decreased TNF-α, IL-1β, and IL-6 expression (Fig. 7B), alleviating inflammation and promoting intestinal homeostasis in broilers. Furthermore, *C. butyricum* decreased the expression of inflammatory cytokines more than sodium butyrate in most cases (Fig. 7B), suggesting that *C. butyricum* has more apparent anti-inflammatory benefits than sodium butyrate, which was consistent with the results on the expression of signaling pathway-related genes (Fig. 7A). We hypothesized that the numerous SCFAs generated by *C. butyricum*, including not only butyric acid but also

371 acetic acid and propionic acid would have additive effects on the inhabitation of
372 expression of inflammation related genes, enhancing the host's inflammatory
373 regulation.
374
375 **Conclusion**
376 *C. butyricum* or sodium butyrate supplementation in the basal diet showed beneficial
377 effects on broiler's growth performance, intestinal health, and anti-inflammatory
378 response. However, these two supplements have varying degrees of probiotic
379 functions in various aspects. Specifically, sodium butyrate promoted broiler growth
380 and maintained intact intestinal structure more effectively, while *C. butyricum* is more
381 beneficial for production of tight junction protein and SCFAs, as well as anti-
382 inflammatory response. Based on the functional comparison of these two butyrate
383 supplements, we believe that our study will offer references for appropriate selection
384 and support the growth of the broiler breeding industry.
385
386 **Abbreviations**
387 CB: *Clostridium butyricum*; SB: Sodium butyrate; SCFAs: Short-chain fatty acids; FI:
388 Feed intake; BWG: Body weight gain; V: Villi height; C: Crypt depth; OTUs:
389 Operational taxonomic units; GC-MS: Gas chromatography-mass spectrometry; EI:
390 Electron bombardment ionization; ZO-1: Zonula occludens-1; TAK1: TGF-β activated
391 kinase-1; NF-kB: Nuclear factor kappa B; IL-1β: Interleukin-1β; IL-6: Interleukin-6;
392 TNF-α: Tumor necrosis factor-α; PCoA: Principal coordinates analysis; LDA: Linear

discriminant analysis; IKK: Inhibitor of nuclear factor kappa B kinase; IL-10: Interleukin-10.

Declarations

Ethics approval and consent to participate

All experiments were performed in accordance with the ethical standards of Huazhong Agriculture University's Laboratory Animal Center (HZAUCH-2019-008).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LL and HL carried out the animal experiments and data analysis, and drafted the manuscript. WZ and YZ participated in the animal trial. YL and NP helped with study design. SZ designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Authors' information

Not applicable.

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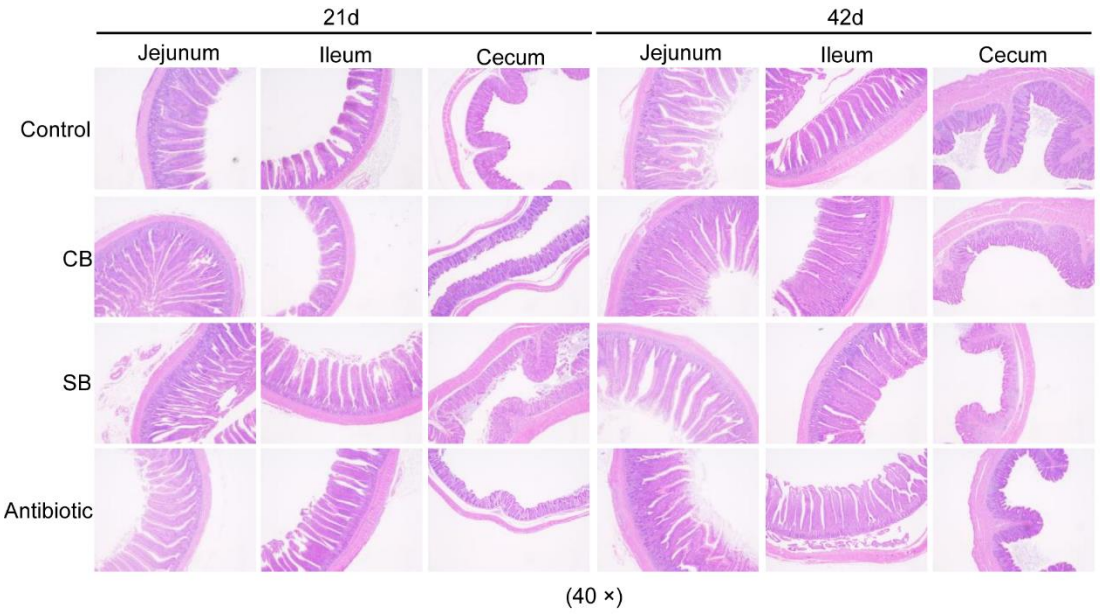


535

536 **Fig 1. Intestinal tissue morphology.** Samples of the jejunum, ileum, and cecum

537 (approximately 1-2 cm obtained from the midpoint) at 21 d and 42 d were fixed. Villi

538 and goblet cells were observed from eosin-methylene blue-stained sections of samples

539 by optical microscopy at 40 ×.

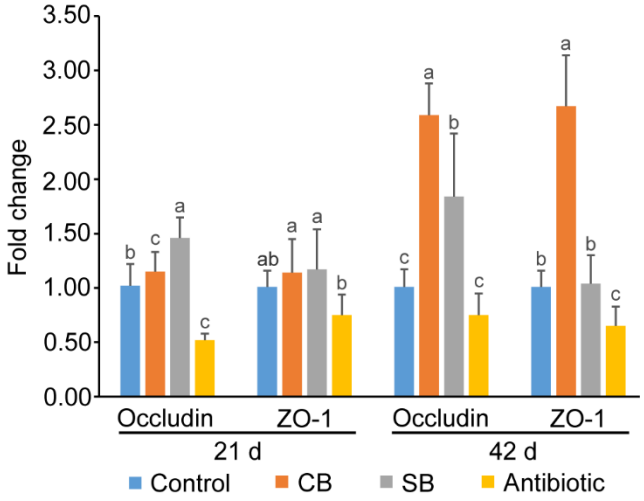


Fig 2. Tight junction protein expression in broiler jejunum. Significant differences are shown by bars labeled with various letters.

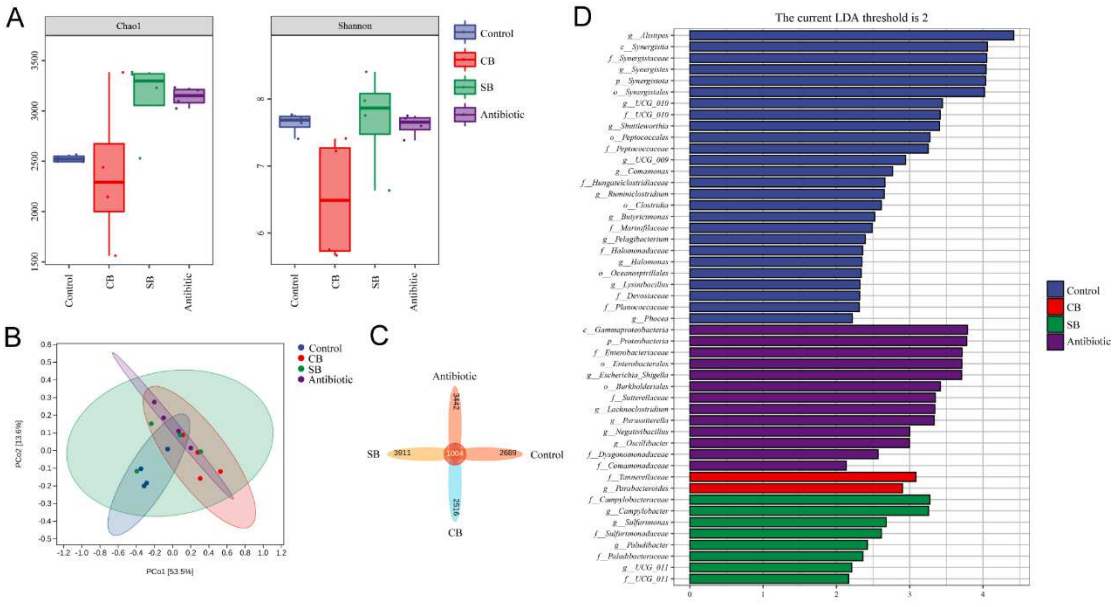


Fig 3. Effects of different feed additives on intestinal microbial community structure in the cecum. (A) Alpha diversity was assessed by Chao1 and Shannon indexes. (B) Principal coordinates analysis (PCoA) based on uniFrac distance was used to represent the community structural differences of four groups. (C) OUT Venn diagram. (D) Linear discriminant analysis (LDA) scores for various taxa abundances. Data were presented as mean ± SD.

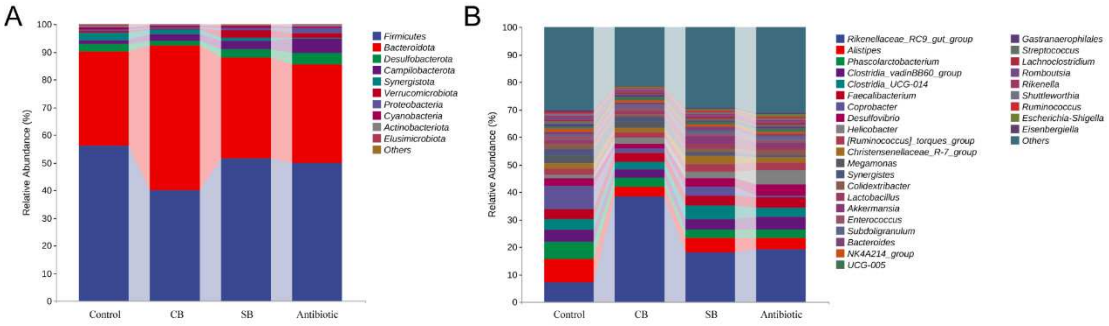


Fig 4. Taxonomic differences in broiler caeca microbial community. (A) Phylum levels. (B) Genus levels. Data were presented as mean ± SD.

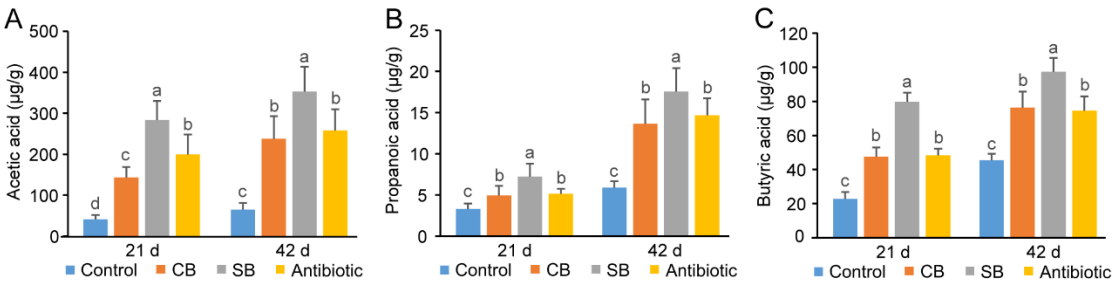


Fig 5. SCFAs concentrations in ileum chyme of broilers. (A) Acetic acid. (B) Propanoic acid. (C) Butyric acid. SCFAs concentrations were expressed in microgram per gram of chyme sample (μg/g). Significant differences are shown by bars labeled with various letters.

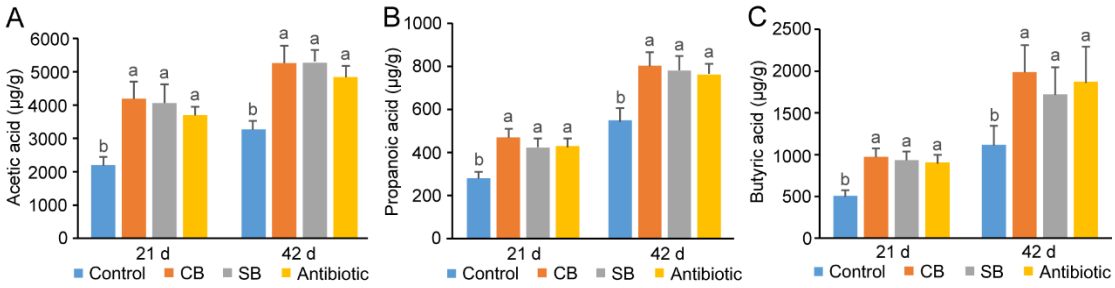
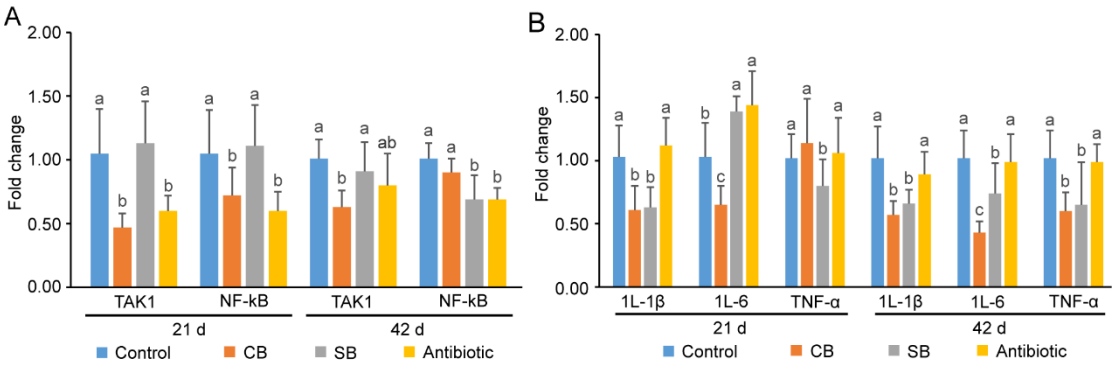


Fig 6. SCFA concentrations in broiler cecum chyme. (A) Acetic acid. (B) Propanoic acid. (C) Butyric acid. SCFAs concentrations were expressed as micrograms per gram of chyme sample (μg/g). Significant differences are shown by bars labeled with various letters.



563

564 **Fig 7. Expression of inflammatory and immune-related genes in broiler jejunal**

565 **mucosa.** (A) Signaling pathway-related protein. (B) Inflammatory cytokines.

566 Significant differences are shown by bars labeled with various letters.

567 **Table 1. Groups and diets**

Groups	Diets
Control	Basal diet
CB	Basal diet + *C. butyricum* (1.0×10^9 CFU/t)
SB	Basal diet + sodium butyrate (500 g/t)
Antibiotic	Basal diet + oxytetracycline (200 g/t)

568

569 **Table 2. Nutrient levels and composition of basic diet**

Item (% unless noted)	Starter (1-21 d)	Grower (22-42 d)
Ingredients		
Corn (7.8%, crude protein)	530	540
Soybean meal (43%, crude protein)	360	342.50
Rapeseed meal	0	20
Fish powder (68%)	30	0
Soybean oil	40	60
Limestone	12	13
CaHPO4	14	13
Lysine (70%)	4	2.80
Methionine	2	1.50
Salt	3	3
Choline Chloride (50%)	1	1
Rice bran	0.80	0
Multivitamin*	2	2
Multimineral†	1.20	1.20
Total	1,000	1,000
Calculated nutrient levels		
Metabolic energy (Mcal/kg)	3,030	3,150
Crude protein	22	20
TP	0.69	0.60
AP	0.45	0.35
Ca	1	0.88

Lys	1.45	1.20
Met	0.55	0.45
Met+Cys	0.88	0.78
Thr	0.92	0.84
Trp	0.28	0.25
Arg	1.20	1.12

570 *Supplied per kilogram of diet: retinyl acetate 5,000-10,000 KIU; vitamin D3 2,000-
571 5,000 KIU; DL-α-tocopheryl acetate≥25,000 mg; menadione≥2,400 mg; thiamine
572 nitrate≥2,000 mg; riboflavin≥6,000 mg; vitamin B6≥3,500 mg; cyanocobalamin≥
573 12 mg; nicotinamide≥30,000 mg; D-biotin≥75 mg; D-calcium pantothenate≥8,000
574 mg; folic acid≥950 mg.
575 †Supplied per kilogram of diet: copper 6,000-18,000 mg; iron 30,000-150,000 mg;
576 manganese 60,000-125,000 mg; zinc 50,000-100,000 mg; iodine 400-900 mg;
577 selenium 150-300 mg.

578 **Table 3. Primers used in this study**

Primers	Sequence (5'-3')
ZO-1-F	TCGGGTTGTGGACACGCTAT
ZO-1-R	TTCATAGGCAGGGAACTTTGTCT
Occludin-F	GTTCCTCATCGTCATCCTGCTC
Occludin-R	CGTTCTTCACCCACTCCTCCAC
TAK1-F	ATGATAATGATTGTCCTACTGCCCC
TAK1-R	GGCAGGCTCAAATGGTAGGC
NF-kB-F	ATGCTCACAGCTTGGTGGGTAA
NF-kB-R	TCATGCGTGTTTCCAGAGTTTC
IL-1β-F	ATGACCAAACTGCTGCGGAG
IL-1β-R	AAGGACTGTGAGCGGGTGTAG
1L-6-F	GGTGATAAATCCCGATGAAGTGG
1L-6-R	AGGCACTGAAACTCCTGGTCTT
TNF-α-F	GGAATGAACCCTCCGCAGTA
TNF-α-R	GCAACAACCAGCTATGCACCC
β-actin-F	CTGACTGACCGCGTTACTCC
β-actin-R	TTGCACATACCGGAGCCATT
341F	CCTACGGGAGGCAGCAG
534R	TAGATTACCGCGGCTGCT

579

580 **Table 4. Effects of diet on the growth of broilers**

	Control	CB	SB	Antibiotic
1-21 d				
FI	41.09 ± 0.97	40.89 ± 0.91	41.54 ± 0.79	40.95 ± 1.06
BWG	27.94 ± 0.69^{b}	28.82 ± 0.84^{ab}	29.37 ± 0.98^{a}	28.78 ± 0.61^{ab}
F/G	1.47 ± 0.06	1.42 ± 0.06	1.42 ± 0.04	1.42 ± 0.04
22-42 d				
FI	115.68 ± 5.95^{a}	106.57 ± 7.91^{b}	118.80 ± 6.32^{a}	114.85 ± 5.77^{a}
BWG	56.31 ± 2.75^{b}	56.16 ± 3.30^{b}	62.16 ± 5.27^{a}	60.45 ± 3.76^{ab}
F/G	2.05 ± 0.04^{a}	1.90 ± 0.08^{b}	1.92 ± 0.07^{b}	1.90 ± 0.04^{b}
1-42 d				
FI	78.38 ± 3.19^{a}	73.73 ± 3.81^{b}	80.17 ± 3.52^{a}	77.90 ± 3.31^{a}
BWG	42.13 ± 1.32^{b}	42.49 ± 1.96^{b}	45.76 ± 2.71^{a}	44.61 ± 2.16^{ab}
F/G	1.86 ± 0.04^{a}	1.74 ± 0.05^{b}	1.75 ± 0.04^{b}	1.75 ± 0.04^{b}

581 FI, feed intake (g); BWG, body weight gain (g); F/G, feed to gain ratio.

582 **Table 5. Effects of diet on structure of intestinal villi in broilers.**

		Control	CB	SB	Antibiotics
21 d					
	V	711.73 ± 124.66^{b}	987.79 ± 124.66^{a}	985.14 ± 221.50^{a}	628.08 ± 62.30^{b}
Jejunum	C	151.82 ± 46.53^{ab}	129.24 ± 19.89^{b}	120.60 ± 33.68^{b}	173.74 ± 27.38^{a}
	V/C	4.88 ± 0.91^{b}	7.62 ± 1.06^{a}	8.41 ± 1.77^{a}	3.68 ± 0.71^{b}
	V	549.93 ± 63.83^{b}	504.15 ± 26.07^{b}	705.65 ± 132.81^{a}	523.35 ± 38.44^{b}
Ileum	C	127.23 ± 12.19^{a}	76.58 ± 9.15^{c}	100.37 ± 11.83^{b}	132.22 ± 12.22^{a}
	V/C	4.07 ± 0.24^{b}	6.70 ± 1.15^{a}	7.08 ± 1.37^{a}	4.00 ± 0.58^{b}
	V	143.95 ± 25.52^{b}	166.58 ± 27.15^{ab}	187.70 ± 17.85^{a}	140.79 ± 11.51^{b}
Cecum	C	128.07 ± 20.29	115.80 ± 15.16	111.82 ± 28.91	142.61 ± 29.28
	V/C	1.13 ± 0.20^{bc}	1.45 ± 0.27^{ab}	1.74 ± 0.34^{a}	1.03 ± 0.27^{c}
42 d					
	V	1,015.09 ± 176.38^{bc}	1,138.61 ± 157.01^{ab}	1,201.12 ± 92.46^{a}	903.92 ± 102.50^{c}
Jejunum	C	185.74 ± 17.82^{b}	279.79 ± 38.46^{a}	184.77 ± 58.36^{b}	246.79 ± 54.33^{a}
	V/C	5.43 ± 0.59^{b}	4.11 ± 0.67^{c}	6.93 ± 1.63^{a}	3.79 ± 0.82^{c}
	V	754.30±119.87^{b}	936.03 ± 129.26^{a}	795.69 ± 113.05^{b}	672.43 ± 78.85^{b}
Ileum	C	142.10±27.63^{ab}	145.51 ± 31.00^{ab}	120.57 ± 29.15^{b}	173.00 ± 31.22^{a}
	V/C	5.39±0.83^{b}	6.58 ± 1.29^{a}	6.73 ± 1.18^{a}	4.07 ± 1.10^{c}
	V	152.05±36.18^{b}	195.83±10.99^{a}	193.05±18.61^{a}	147.59 ± 19.09^{b}
Cecum	C	165.19±40.80^{ab}	135.46±27.13^{bc}	111.43±13.46^{c}	174.54 ± 33.77^{a}
	V/C	0.93±0.13^{b}	1.50±0.35^{a}	1.75±0.24^{a}	0.88 ± 0.23^{b}

583 V, villus height (μm); C, crypt depth (μm); V/C, villus height to crypt depth.