RESEARCH ARTICLE

J Anim Sci Technol 2025;67(5):1033-1049

https://doi.org/10.5187/jast.2024.e71

JAST

Journal of Animal Science and Technology

pISSN 2672-0191 eISSN 2055-0391

Supplemental illite or in combination with probiotics improves performance and intestinal health in broiler chickens challenged with *Salmonella typhimurium*

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Received: Apr 15, 2024
Revised: Jun 19, 2024
Accepted: Jul 7, 2024

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Abstract

This study investigated the impact of dietary illite alone or in combination with a *Clostridium butyricum* and *Bacillus subtilis* (CB) complex on growth performance and intestinal health in broiler chickens challenged with *Salmonella enterica* serotype *Typhimurium* (ST). A total of 72 one-day-old Arbor Acres broilers with initial body weight (BW) of 35.28 ± 0.34 g were used in a 4-week experiment and assigned to four treatment groups (six replications, three birds each per cage): 1) NC, non-challenged control fed a basal diet; 2) CC, ST-challenged control fed a basal diet; 3) IA, CC supplemented with 1% illite (10 g/kg); 4) ICB, IA supplemented with 0.1% CB (1×10^8 CFU/kg). In the ST challenge treatments, broilers were orally inoculated with 1.5 mL and 2.1 mL of ST (1×10^7 CFU/mL) on days 8 and 15, respectively, for 3 consecutive days. The ST challenge reduced ($p < 0.05$) broiler performance, dry matter digestibility, and villus height (VH) and increased the levels of heterophil, interleukin-6, and tumor necrosis factor-α in serum, and crypt depth (CD). However, additives counteracted ST-induced impairments ($p < 0.05$) in broilers, the IA and ICB showed higher body weight gain (BWG) at 15 to 21 d, and lower feed conversion ratio (FCR) at 15 to 21 d and 1 to 28 d compared with the CC. Also, the IA and ICB showed lower ($p < 0.05$) CD and higher ($p < 0.05$) VH to CD ratio, and count of *Lactobacillus* in feces than the CC at 28 d. Additionally, unlikely IA, the ICB increased ($p < 0.05$) the BW at 21 d and the dry matter digestibility at 28 d, while decreasing ($p < 0.05$) the FCR at 8 to 14 d and count of *Salmonella* in feces at 14 and 28 d. Overall, illite alone and in combination with CB can effectively alleviate ST infection in broiler chickens, suggesting their potential as feed additives to improve growth performance, fecal microflora and intestinal morphology in ST-challenged broilers.

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Competing interests
No potential conflict of interest relevant to this article was reported.

Funding sources
Following are results of a study on the "Leaders in Industry-university Cooperation 3.0" Project, supported by the Ministry of Education and National Research Foundation of Korea.

Acknowledgements
Not applicable.

Availability of data and material
All data generated or analyzed during this study are included in this published article.

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Ethics approval and consent to participate
The experimental protocol was approved (CBNUA-2148-23-01) by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea.

Keywords: *Bacillus subtilis*, Broiler performance, *Clostridium butyricum*, Illite, *Salmonella typhimurium*, feed additive

INTRODUCTION

Salmonella enterica serotype *typhimurium* (ST) is a gram-negative pathogen in the *Salmonella* spp., which is rapidly contagious and can also be transmitted vertically from hens to chicks through eggs [1–5]. The ST infection can occur in broilers at any age, and it can result in high mortality among young chicks [6]. Additionally, in older broilers, ST can cause intestinal inflammation, damage intestinal epithelial cells, generate oxidative stress, and ultimately reduce growth performance [7–10]. Controlling ST has become an important issue due to its significance in both the economy and public health [11]. Accordingly, antibiotics have been widely used as additives to promote the healthy development of the poultry industry [12,13]. However, since 2006, the European Union has banned the use of antibiotics as growth promoters in animal feed to address the growing threat of multidrug-resistant bacteria [14,15]. Therefore, the poultry industry has conducted studies on antibiotic alternatives to enhance growth performance and mitigate pathogen infections [16].

Clay minerals are composed of aluminosilicate molecules with an intermediate layer of phyllosilicate. The layer of phyllosilicate contains internal pores and channels that enhance the electronic charge [17,18]. Among silicates, illite is characterized by its relatively high surface area and cation exchange capacity, which contributes to its high utility [19]. These properties enable to facilitate ion exchange, thereby assisting in the reduction of harmful substances by adsorbing enteric toxins on their surfaces and improving the gut environment [20,21]. Also, aluminosilicate could strengthen the immune response and decrease inflammation, thereby improving broiler performance [22,23]. Previous studies have showed that supplementing with 1% of illite to diet significantly increased the body weight gain (BWG) in broilers [24]. Additionally, the inclusion of 0.6% illite to diet enhanced the levels of immunoglobulin G, as well as egg production and feed conversion ratio (FCR) in laying hens [25]. Therefore, illite has been used in poultry diets due to its positive effects on poultry performance, making it a valuable addition [26,27].

Probiotics have been steadily used as an alternative to antibiotics. The potential of probiotics is determined by factors such as the number of viable cells, resistance to acid and bile salts, production of antimicrobial metabolites, and ability to form colonies [28]. *B. subtilis* and *C. butyricum* can form endospores tolerant to low pH and bile [29-32]. These characteristics can greatly aid in colonizing the intestines with probiotics [33]. Also, *B. subtilis* exhibits antibacterial properties in the intestines of broilers, thereby inhibiting the proliferation of harmful bacteria such as *Escherichia coli* [34]. *C. butyricum* is a gram-positive anaerobic bacterium that produces butyric acid, inhibiting pathogen bacteria within the intestines [35].

However, there are few studies that identify the effects of adding illite to broilers that are challenged with ST. Additionally, research on the use of a combination of *C. butyricum* and *B. subtilis* (CB) is also limited. Therefore, we hypothesize that the adsorption properties of illite could alleviate the effects of ST and have a synergistic effect when combined with the antibacterial properties of CB in the intestine. This study aimed to investigate the impact of illite alone (IA) and in combination with CB (ICB) on growth performance, frequency of diarrhea, nutrient digestibility, blood profiles, and intestinal morphology in broilers that have been challenged with ST.

https://doi.org/10.5187/jast.2024.e71

MATERIALS AND METHODS

Animal ethics

The experimental protocol was approved (CBNUA-2148-23-01) by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea.

Source of illite, probiotics and bacterial strains

The chemical composition of illite, which provided by Garam is shown in Table 1. In this study, 1×10^8 CFU/kg of *C. butyricum* and *B. subtilis* were used (Garam). The ST was provided in stock form. The ST was thawed, and ten microliters were mixed with 10 mL of nutrient broth, cultivated at 37°C for 24 h, and then sub-cultured at approximately 1.0×10^7 CFU/mL.

Animals and experiment design

A total of 72 one-day-old Arbor Acres broilers were randomly assigned to three groups based on their initial body weight (BW) of 35.28 ± 0.34 g, with six replicate cages (W: 173 cm, D: 63 cm, H: 55 cm) per group and three birds per replicate. The experimental period lasted for 28 d. Dietary treatments included the following: 1) NC, non-challenge control, birds fed with basal diet; 2) CC, ST challenge control, birds fed with basal diet; 3) IA, the CC with 1% illite alone (10 g/kg); 4) ICB, the IA with 0.1% CB (1×10^8 CFU/kg). The experiment initiation temperature was 31 ± 1°C, and then the temperature was gradually lowered to maintain 22 ± 1°C. All broilers except NC group were orally inoculated with a total of 1.5 mL and 2.1 mL ST (1×10^7 CFU/mL) for 3 consecutive days on 8 and 15 d, respectively. All diets were formulated to meet or exceed the nutrient requirements for poultry by the NRC [36]. Compositions of basal diets are shown in Table 2. Broilers were fed *ad libitum* diet and water.

Growth performance

At 7, 14, 21, and 28 d, all broilers and remaining diet in the cages were weighed at each time point to determine the BW, BWG, feed intake (FI), and FCR. The BWG was calculated as the BW of the previous time point was subtracted from the BW of the current time point. The FI was calculated by subtracting the remaining diet amount from the initial diet amount, and FCR was calculated by dividing FI by BWG.

Table 1. Chemical composition of illite

Items	Content
Ingredient (%)	
SiO_2	67.40
Al_2O_3	20.30
K_2O	5.50
Fe_2O_3	2.35
Na_2O	0.54
Ti_2O	0.27
MgO	0.24
CaO	0.04
P_2O_5	0.04
MnO	0.01

Table 2. Ingredient composition of experimental diets

Items	Pre-starter, d 1–7	Starter, d 8–14	Grower, d 15–21	Finisher, d 22–28
Ingredients (%)	100.0	100.0	100.0	100.0
Corn	37.6	41.6	45.2	48.9
Wheat fine	15.3	15.1	15.6	15.2
Rice pollards	2.4	2.5	2.5	2.6
Soybean meal (45% CP)	26.9	21.0	17.7	15.5
Cookie wheat flour	1.9	2.0	2.0	2.0
DDGS	5.0	7.0	6.0	5.0
Animal protein	6.3	6.1	6.4	6.2
Animal fat	1.7	1.9	1.9	1.9
L-lysine	0.6	0.6	0.6	0.5
DL-methionine	0.4	0.3	0.3	0.4
L-threonine	0.2	0.1	0.1	0.1
L-tryptophan	0.1	0.1	0.1	0.1
Salt	0.2	0.2	0.2	0.2
Limestone	0.5	0.6	0.5	0.5
MDCP	0.2	0.2	0.2	0.2
Liquid-Choline	0.1	0.1	0.1	0.1
Vitamin premix[1)]	0.3	0.3	0.3	0.3
Mineral premix[2)]	0.3	0.3	0.3	0.3
Chemical composition				
AMEn (kcal/kg)	3,000	3,020	3,070	3,100
CP (%)	23.3	21.3	20.2	19.1
Ether extract (%)	5.5	5.9	6.0	5.8
Crude fiber (%)	3.4	3.4	3.2	3.0
Crude ash (%)	5.8	5.3	5.1	4.8
Calcium (%)	0.9	0.8	0.8	0.7
Phosphorus (%)	0.5	0.6	0.5	0.5
Lysine (%)	1.5	1.3	1.2	1.1
SAA (%)	1.1	1.0	1.0	1.0

[1)]Supplied per kg diet: vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 48 mg; vitamin K, 3 mg; thiamin, 1.8 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin B_{12}, 0.012 mg; niacin, 42 mg; folic acid, 1.2 mg; biotin, 0.24 mg; pantothenic acid, 12 mg.
[2)]Supplied per kg of diet: manganese, 120 mg; zinc, 100 mg; iron, 80 mg; copper, 20 mg; iodine, 2 mg; selenium, 0.3 mg; cobalt, 0.5 mg.
CP, crude protein; DDGS, dried distiller's grains with soluble; MDCP, mono-dicalcium phosphate; SAA, sulfur amino acids; AMEn, nitrogen-corrected apparent metabolizable energy.

Nutrient digestibility

Broilers were fed diets mixed with 0.2% chromium oxide (Cr_2O_3) for 3 consecutive days from 11 d and 25 d, and fecal samples were collected during that period. At the same time, diet samples were collected. After collection, fecal and diet samples were stored in a freezer at −20°C, immediately. At the end of the experiment, fecal samples were dried at 70°C for 72 h and then crushed on a 1 mm screen. The procedures utilized for the determination of dry matter (DM) and crude protein (CP) digestibility were conducted with the methods by the AOAC [37]. The gross energy (GE) of the diets and feces was analyzed by using an adiabatic oxygen bomb calorimeter (Parr 6400, Parr Instruments, USA). Chromium levels were determined via UV absorption spectrophotometry

https://doi.org/10.5187/jast.2024.e71

(UV-1201, Shimadzu) using the Williams et al. [38] method. The apparent total tract digestibility (ATTD) percentage was calculated using the following equation:
ATTD, % = 100 – [100 × (Cr_2O_3 in diet / Cr_2O_3 in fecal) × (nutrient in fecal / nutrient in diet)].

Fecal score

The fecal scores were individually recorded at 08:00 and 17:00 by the same person during the entire experimental period. The fecal score was scored using a method used by Cooper et al. [39]. The fecal score was as follows: 0, normal dropping; 1, normal to pasty; 2, liquid; 3, liquid with blood; 4, bloody droppings.

Blood profile

At 14 and 28 d, blood samples (2 mL each) were collected from the brachial wing vein into a sterile syringe. At the time of collection, blood samples were collected in a vacuum tube containing K_3EDTA for complete blood count analysis and nonheparinized tubes for serum analysis, respectively. After collection, blood samples were centrifuged at 12,500×g at 4°C for 20 min. Red blood cell, white blood cell, heterophil, and lymphocyte were analyzed with a hematology analyzer (XE2100D, Sysmex). interleukin (IL)-6 and tumor necrosis factor α (TNF-α) concentrations were determined using commercially available ELISA kits (Quantikine, R&D Systems), and the absorbance was measured at 450 nm.

Bacteria counts

At 14 and 28 d, fecal samples were collected in conical tubes. Fecal samples were stored on ice and analyzed immediately. From the sample, 0.1 g was suspended in 1 × phosphate buffered saline (PBS; GenDEPOT), homogenized, and diluted from 10^{-4} to 10^{-7} to count the number of bacteria. Evenly spread 100 μL of the diluted solution on the agar. Brilliant Green (BG) Sulfa agar (KisanBio) was used for *Salmonella*, and De Man–Rogosa–Sharpe (MRS) agar (KisanBio) was used for *Lactobacillus*. *Salmonella* was cultured for 24 h 37°C, and *Lactobacillus* was cultured for 48 h 37°C. Immediately after removal from the incubator, *Salmonella* and *Lactobacillus* were counted, and statistical analysis was performed by converting them to logs.

Intestinal morphology

Six broilers per treatment were sacrificed at the end of the experiment to collect ileal tissue samples. The tissue sample for morphological measurements was taken from the ileal segment (2 cm anterior to the ileocecal valve), rinsed clean with 10% neutral buffered formalin (NBF; Sigma-Aldrich). The intestinal segment was submerged in approximately 20 mL of 10% NBF for 24 h. Slides of intestinal cross-sections (5 μm thick) were treated with paraffin and stained with hematoxylin and eosin. The slides were examined using an inverted phase-contrast microscope (Olympus IX51, Olympus Corporation). The villus height (VH) was measured from the tip of the villus to the crypt orifice. The crypt depth (CD) was measured from the junction of the villus to the crypt base. And then, the VH to CD ratio (VH:CD) was calculated.

Statistical analysis

All data except for frequency of diarrhea were analyzed by one-way ANOVA using JMP (JMP Pro version 16.0.0, SAS Institute), using each pen as the experimental unit. The results are presented as the mean ± standard error of the mean. Differences between treatment means were determined using Tukey's multiple range test. A probability level of $p<0.05$ was indicated to be statistically significant, and a level of $0.05 \leq p < 0.10$ was considered to have such a tendency. The frequency

of diarrhea was analyzed contingency analysis to test the relationship between categorical variables (scores) and the different combinations tested in this study. A Chi-square test was performed to determine if the different combinations had an effect on the categorical variables repartition with significance accepted at $p \leq 0.05$, and visualized using GraphPad Prism 9.5.1 (GraphPad, San Diego, CA, USA).

RESULTS

Growth performance

The CC group showed lower ($p < 0.05$) BW compared with the NC group at 21 d (Table 3). Additionally, during the 1st challenge period and over the entire period, the CC group showed a higher ($p < 0.05$) FCR compared to the NC group. While the ICB group showed increased ($p <$

Table 3. Effects of illite and probiotics supplementation on growth performance of *Salmonella enterica* serotype *typhimurium*-challenged broilers

Items	NC	CC	IA	ICB	SE	*p*-value
BW (g)						
Initial	35.22	35.35	35.22	35.33	0.152	0.890
7 d	166.67	155.33	160.25	162.00	4.191	0.319
14 d	482.19	446.97	458.51	466.45	13.723	0.349
21 d	1,027.70[a]	891.75[b]	1,011.56[ab]	1,019.50[a]	31.414	0.019
28 d	1,655.00	1,455.58	1,610.28	1,601.11	51.649	0.066
1–7 d						
BWG (g)	131.45	119.99	126.61	126.67	3.954	0.267
FI (g)	145.50	148.33	141.39	143.12	4.935	0.774
FCR (g/g)	1.11	1.24	1.12	1.14	0.037	0.086
1st challenge[1)]						
8 to 14 d						
BWG (g)	315.25	288.94	297.19	304.45	11.553	0.443
FI (g)	403.61	459.77	448.33	435.85	16.478	0.124
FCR (g/g)	1.28[b]	1.60[a]	1.51[ab]	1.44[b]	0.047	0.001
15 to 21 d						
BWG (g)	545.50[a]	438.56[b]	546.92[a]	548.89[a]	29.722	0.040
FI (g)	738.34	861.28	800.37	778.25	45.174	0.304
FCR (g/g)	1.36[b]	1.99[a]	1.46[b]	1.44[b]	0.084	< 0.001
2nd challenge[2)]						
22 to 28 d						
BWG (g)	627.31	563.83	598.72	581.61	25.663	0.369
FI (g)	1,093.73	1,149.94	982.09	969.84	67.261	0.201
FCR (g/g)	1.74	2.05	1.64	1.69	0.116	0.091
1 to 28 d						
BWG (g)	1,619.78	1,420.07	1,574.97	1,565.78	51.653	0.065
FI (g)	2,381.50	2,620.07	2,385.70	2,328.72	101.328	0.211
FCR (g/g)	1.47[b]	1.85[a]	1.52[b]	1.50[b]	0.066	0.001

[1)]1st challenge: *Salmonella enterica* challenge at 1×10^7 CFU/mL with 1.5 mL for 3 consecutive days on 8 d.

[2)]2nd challenge: *Salmonella enterica* challenge at 1×10^7 CFU/mL with 2.1 mL for 3 consecutive days on 15 d.

[a,b]Means within column with different superscripts differ significantly ($p < 0.05$).

NC, non-challenge control, birds fed with basal diet; CC, *Salmonella enterica* serotype *typhimurium* challenge control, birds fed with basal diet; IA, CC with 1% illite alone; ICB, IA with 0.1% of *Bacillus subtilis* (1×10^8 CFU/kg) and *Clostridium butyricum* (1×10^8 CFU/kg), respectively; BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

https://doi.org/10.5187/jast.2024.e71

0.05) BW compared with the CC group at 21 d. Also, compared with CC group, there was a higher ($p < 0.05$) BWG and a lower ($p < 0.05$) FCR in broilers fed IA and ICB at 15 to 21 d. During the entire experimental period, the IA group and ICB group showed a higher tendency ($p = 0.065$) for BWG and lower ($p < 0.05$) FCR than the CC group.

Nutrient digestibility

At 14 and 28 d, the CC group and IA group showed lower ($p < 0.05$) ATTD of DM than the NC group (Table 4). However, group of broilers fed diets with ICB increased ($p < 0.05$) ATTD of DM compared with CC group at 28 d.

Fecal score

These observed fecal score was statistically different among the four dietary treatments ($p < 0.05$; Fig. 1). The CC group showed the highest score 2 (35.71%), which is considered as diarrhea, and ICB group showed a lower level at 12.8% compared to the CC group.

Blood profile

At 14 d, the CC group had higher ($p < 0.05$) heterophil and TNF-α than the NC group (Table 5). Additionally, the CC group was significantly higher ($p < 0.05$) IL-6 and TNF-α than the NC group at 28 d. On the other hand, the IA group and ICB group showed no significant differences ($p > 0.05$) in TNF-α, and IL-6 compared with the NC group.

Bacteria counts

At 14 and 28 d, the CC group showed a higher ($p < 0.05$) *Salmonella* count and lower ($p < 0.05$) *Lactobacillus* count in feces than the NC group (Table 6). However, the ICB group showed a lower ($p < 0.05$) *Salmonella* count in feces than the CC group at 14 and 28 d. Also, at 28 d, the IA group and ICB group had a significantly higher ($p < 0.05$) *Lactobacillus* count in feces than the CC group.

Intestinal morphology

Compared with the NC group, the CC group showed decreased ($p < 0.05$) VH and VH:CD and increased ($p < 0.05$) CD (Table 7). However, the IA group and ICB group had no significant difference ($p > 0.05$) on the CD and VH:CD compared to the NC group. Moreover, the IA group and ICB group showed a lower ($p < 0.05$) CD and a higher ($p < 0.05$) VH:CD than the CC group.

Table 4. Effects of illite and probiotics supplementation on nutrients digestibility of Salmonella enterica serotype *typhimurium*-challenged broilers

Items (%)	NC	CC	IA	ICB	SE	*p*-value
14 d						
DM	75.40^{a}	73.04^{b}	73.56^{b}	74.15^{ab}	0.369	0.002
CP	77.21	76.31	76.49	77.17	0.412	0.318
GE	78.89	78.46	78.68	78.85	0.270	0.664
28 d						
DM	75.22^{a}	72.84^{c}	73.86^{bc}	74.44^{ab}	0.324	< 0.001
CP	77.60	77.13	77.07	77.77	0.413	0.558
GE	79.89	79.24	79.32	79.36	0.284	0.382

$^{a-c}$Means within column with different superscripts differ significantly ($p < 0.05$).

NC, non-challenge control, birds fed with basal diet; CC, *Salmonella enterica* serotype *typhimurium* challenge control, birds fed with basal diet; IA, CC with 1% illite alone; ICB, IA with 0.1% of *Bacillus subtilis* (1×10^8 CFU/kg) and *Clostridium butyricum* (1×10^8 CFU/kg), respectively; DM, dry matter; CP, crude protein; GE, gross energy.

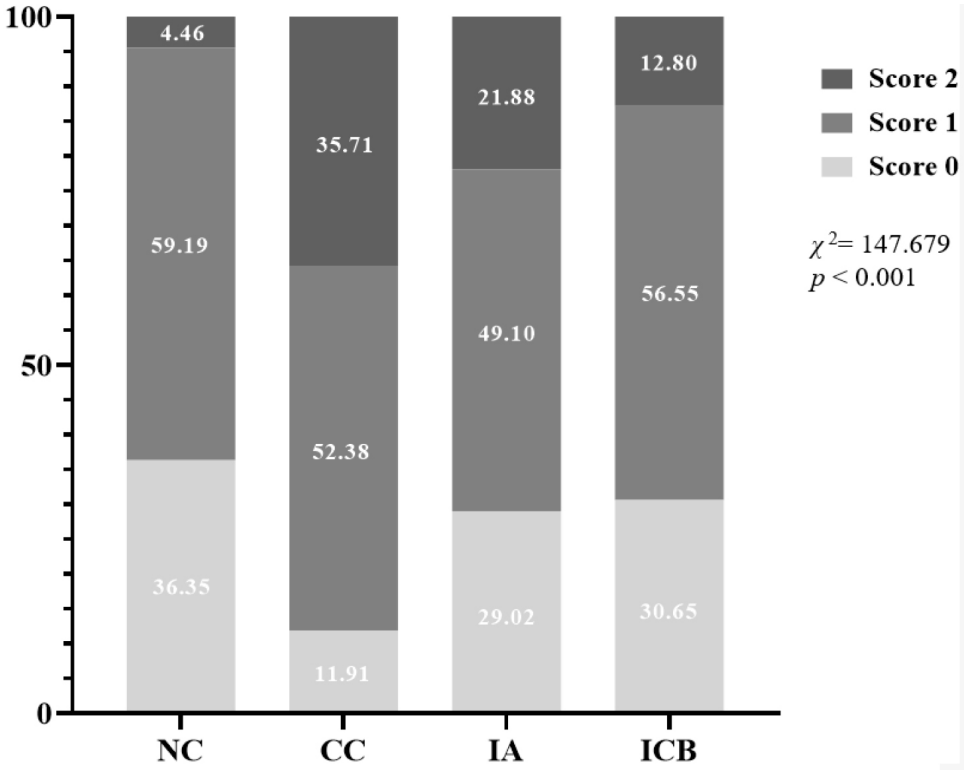

Fig. 1. Effects of illite and probiotics supplementation on fecal score of *Salmonella enterica* serotype *typhimurium*-challenged broilers Score 0, normal dropping; 1, normal to pasty; 2, liquid; 3, liquid with blood; 4, bloody droppings. χ^2= 147.679, $p < 0.001$. NC, non-challenge control, birds fed with basal diet; CC, *Salmonella enterica* serotype *typhimurium* challenge control, birds fed with basal diet; IA, CC with 1% illite alone; ICB, IA with 0.1% of *Bacillus subtilis* (1×10^8 CFU/kg) and *Clostridium butyricum* (1×10^8 CFU/kg), respectively.

Table 5. Effects of illite and probiotics supplementation on blood profile of *Salmonella enterica* serotype *typhimurium*-challenged broilers

Items	NC	CC	IA	ICB	SE	*p*-value
14 d						
RBC (10^6/μL)	2.56	2.45	2.85	2.78	0.263	0.688
WBC (10^3/μL)	23.11	33.73	28.64	24.50	3.704	0.207
Heterophil (10^3/μL)	9.85^b	25.36^a	20.97^{ab}	17.54^{ab}	3.386	0.028
Lymphocyte (10^3/μL)	10.01	4.34	6.62	5.35	1.770	0.155
IL-6 (pg/mL)	150.78	175.08	168.59	165.84	10.050	0.393
TNF-α (pg/mL)	176.76^b	243.89^a	211.73^{ab}	205.05^{ab}	13.644	0.021
28 d						
RBC (10^6/μL)	1.95	1.99	2.31	2.08	0.234	0.718
WBC (10^3/μL)	17.41	18.83	17.95	18.13	2.581	0.984
Heterophil (10^3/μL)	3.41	2.88	3.65	3.25	0.673	0.876
Lymphocyte (10^3/μL)	11.15	16.67	13.21	14.17	2.327	0.426
IL-6 (pg/mL)	151.12^b	200.97^a	185.40^{ab}	175.39^{ab}	11.166	0.034
TNF-α (pg/mL)	131.16^b	174.70^a	152.98^{ab}	141.10^{ab}	10.222	0.039

^{a,b}Means within column with different superscripts differ significantly ($p < 0.05$).

NC, non-challenge control, birds fed with basal diet; CC, *Salmonella enterica* serotype *typhimurium* challenge control, birds fed with basal diet; IA, CC with 1% illite alone; ICB, IA with 0.1% of *Bacillus subtilis* (1×10^8 CFU/kg) and *Clostridium butyricum* (1×10^8 CFU/kg), respectively; RBC, red blood cell; WBC, white blood cell; IL, interleukins; TNF-α, tumor necrosis factor-α.

https://doi.org/10.5187/jast.2024.e71

Table 6. Effects of illite and probiotics supplementation on bacteria counts of *Salmonella enterica* serotype *typhimurium*-challenged broilers

Items (/Log CFU/g)	NC	CC	IA	ICB	SE	*p*-value
14 d						
Salmonella	3.16[b]	5.31[a]	4.92[ab]	4.47[b]	0.131	< 0.001
Lactobacillus	6.68	6.20	6.33	6.49	0.134	0.104
28 d						
Salmonella	2.80[c]	4.68[a]	4.22[ab]	3.89[b]	0.124	< 0.001
Lactobacillus	6.83[a]	6.19[b]	6.73[a]	6.77[a]	0.125	0.005

[a–c]Means within column with different superscripts differ significantly ($p < 0.05$).

NC, non-challenge control, birds fed with basal diet; CC, *Salmonella enterica* serotype *typhimurium* challenge control, birds fed with basal diet; IA, CC with 1% illite alone; ICB, IA with 0.1% of *Bacillus subtilis* (1×10^8 CFU/kg) and *Clostridium butyricum* (1×10^8 CFU/kg), respectively.

Table 7. Effects of illite and probiotics supplementation on small intestinal morphology of *Salmonella enterica* serotype *typhimurium* challenged broilers

Items	NC	CC	IA	ICB	SE	*p*-value
VH (μm)	1,074.53[a]	686.01[b]	712.73[b]	776.63[b]	27.615	< 0.001
CD (μm)	85.70[b]	120.66[a]	91.71[b]	86.84[b]	5.343	< 0.001
VH:CD	12.54[a]	5.87[c]	7.83[b]	9.01[b]	0.418	< 0.001

[a–c]Means within column with different superscripts differ significantly ($p < 0.05$).

NC, non-challenge control, birds fed with basal diet; CC, *Salmonella enterica* serotype *typhimurium* challenge control, birds fed with basal diet; IA, CC with 1% illite alone; ICB, IA with 0.1% of *Bacillus subtilis* (1×10^8 CFU/kg) and *Clostridium butyricum* (1×10^8 CFU/kg), respectively; VH, villus height; CD, crypt depth; VH:CD, VH to CD ratio.

DISCUSSION

Growth performance

Numerous studies have showed that ST challenges cause poor performance by inhibiting nutrient digestion, absorption [40–42]. Correspondingly, in our study, ST challenge decreased ATTD of DM, thereby resulting the impaired performance. Consistent with our study, Alkhulaifi et al. [7] has demonstrated that ST challenge exhibited an 11% decrease of ADG and a 9% increase of FCR compared to the non-challenged group in broilers at 11 to 25 d. Moreover, Shao et al. [43] has reported that ST challenge caused 7% reduction of BWG in broilers. However, in our study, supplementation with IA and ICB to diet in ST-challenged broilers improved FCR during the entire period, with no significant difference in the non-challenged group. This alleviation might be attribute to the antimicrobial properties of illite and CB. The Al_2O_3 and Fe_2O_3 are key elements for their antimicrobial efficacy, which is a main component of illite [44]. Also, previous study has reported that *B. subtilis* exerts beneficial effects on performance in weaning pigs through the production of antimicrobials [45]. Additionally, Zhang et al. [46] have found that *C. butyricum* produced substances that suppress pathogens, decreasing *E. coli* count in the cecal of broilers challenged with *E. coli* at 21 d. Similarly, the antimicrobial effect of ICB reduced the count of *Salmonella* in feces on 14 and 28 d. Also, IA and ICB have decreased the frequency of diarrhea by 13.83% and 22.91%, respectively. Diarrhea occurs due to disruption of the intestinal acid-base equilibrium balance caused by ST, and a decrease in the frequency of diarrhea indicates alleviated ST infection [47,48]. Therefore, our result revealed that dietary IA and ICB improved growth performance in ST-challenged broilers by suppressing *Salmonella* infection. However, contrary to

our findings, previous research has shown that supplementation with *C. butyricum* and *B. subtilis* did not impact the performance of broilers challenged with *Salmonella* [49,50]. This reason could be attributed to differences in the time point of the challenge, animal model, as well as the dosages of *Salmonella* and probiotics used.

Nutrient digestibility

The digestion and absorption of dietary nutrients primarily occur in the small intestine [51]. The improvement of feed efficiency in broilers could partly explained by enhanced VH which leads to increase the capacity for absorbing nutrients [52,53]. Several studies have revealed that adding illite improved VH and nutrient digestibility (such as DM, CP, and GE) in broilers and pigs [54–56]. Nevertheless, our study did not show the effects of illite supplementation on the VH as well as nutrition digestibility in broilers. However, inconsistent with IA, supplementation with ICB to diet significantly increased the ATTD of DM at 28 d, possibly due to the complementary effect of illite and probiotics. According to the previous studies, Zhang et al. [32] and Mohamed et al. [57] have reported that *B. subtilis* and *C. butyricum* enhance digestion by boosting the activity of enzymes in the gastrointestinal tract and might be involved in improving digestion and absorption. Silicates also produce sticky mucus, which slow down the transit time of digesta [58]. This effect might be further amplified when combined with the enzyme activity of probiotics. The exact mechanism of increased nutrition digestion by the synergy effect has not been documented. However, we speculate that an elevated ATTD of DM could be attributed to distinct mechanisms of illite and probiotics.

Intestinal morphology

The ST induces intestinal inflammation through the production of enterotoxins and compromises the integrity of the intestinal epithelium, leading to villus atrophy [59,60]. Thereby, enterocyte proliferation occurs in the crypts, resulting in a deeper crypt [61,62]. In brief, deeper CD suggests the presence of harmful bacteria and toxins in the intestines. In the present study, ST challenge damaged intestinal mucosa, as observed by decreased VH and increased CD, which is consistent with previous studies [63,64]. However, dietary IA and ICB alleviate negative effects (including an increase in CD and a decrease in VH:CD) caused by ST, suggesting a reduction in intestinal *Salmonella* bacterial load and toxin activity. The Al^{3+} and Fe^{2+} cations present in the interlayer space structure of illite can primarily bind to the lipopolysaccharide molecules produced by gram-negative bacteria (such as ST), thereby contributing to the overall health of the gut [65,66]. Also, numerous studies have showed that supplementation with probiotics improves intestinal morphology by decreasing the number of *Salmonella* in the intestines [67–70]. Actually, in this study, the addition of IA and ICB reduced the counts of *Salmonella* in the feces, which can support the intestinal morphology findings of this study. Also, reduction in *Salmonella* count by ICB may emerge from a complementary effect of combining clay mineral and probiotics. Previously, Han et al. [71] has stated that the adsorption ability of clay mineral from lipopolysaccharides could more effectively help probiotic colonization in the intestines. This could be the reason why the combination of illite and CB was more effective than illite alone in reducing the *Salmonella* count in this study. Additionally, this mechanism may explain why supplementing with IA and ICB to diet in ST-challenged broilers has a higher count of *Lactobacillus* than the CC group in the feces at 28 d.

Blood profile

Pro-inflammatory cytokines are essential in initiating immune responses of the host [72,73].

https://doi.org/10.5187/jast.2024.e71

However, their exaggerated or prolonged secretion may harm the host [74]. The increased levels of heterophils, IL-6, and TNF-α after the ST challenge suggest that the immune and inflammatory responses were overly activated [75–77]. Consistent with our results, Olfati et al. [78] and Milby-Blackledge et al. [79] have stated that ST challenge could lead to the release of pro-inflammatory cytokines in broilers. However, our study showed that both IA and ICB group led to a numerical reduction in the secretion of pro-inflammatory factors IL-6 and TNF-α compared with CC group in the serum. This result suggests that systemic inflammation was effectively reduced [80]. Consistently, previous studies have shown that silicate can reduce the activation of TNF-α and IL-6 by increasing antibody production and enhancing humoral immune function [81,82]. Also, our result is partially correlated with previous study's evidence, suggesting that the dietary *B. subtilis* and *C. butyricum* reduce the TNF-α and IL-6 level in serum and liver, respectively, in *Salmonella*-challenged broilers [75,83]. According to [84,85], dietary *B. subtilis* and *C. butyricum* increased goblet cell production, inhibiting the binding of ST from epithelial cells, ultimately alleviating ST infection. However, other studies have showed that the supplementation with *C. butyricum* changed the immune sensitivity of broilers by increasing TNF-α and IL-6 mRNA expression, respectively [32]. Additionally, there are few studies about dietary illite supplementation on the cytokine level in animals infected with ST, thereby an exact mechanism is not presented for the anti-inflammatory effects of illite. This lack of mechanistic understanding hampers the ability to optimize dosages and combinations of these supplements for maximal efficacy. Also, hyperimmune of illite and CB may result in autoimmunity or conflicting interactions with the host immunity [86]. Therefore, our study suggested that additional research is needed on the mechanisms by which illite and CB supplementation affect the broiler immune system.

CONCLUSION

In broilers infected with ST, the addition of illite alone or in combination with CB alleviated the negative effects of ST on growth performance, fecal score, fecal bacteria count, and intestinal morphology. Also, the ICB more effectively improved digestibility and reduced the number of *Salmonella* in the feces compared with IA. Therefore, the ICB was suggested to be a more effective alternative than illite alone in ST-infected broilers.

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