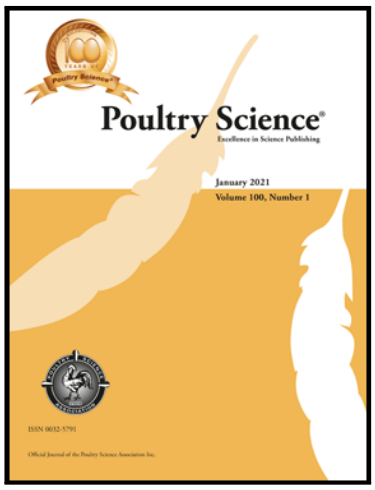

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Evaluation of stimbiotic on growth performance and intestinal development of broilers fed corn- or wheat-based diets

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ABSTRACT: In the antibiotics-free era, stimbiotic (**STB**) has been suggested as a new alternative of antibiotic growth promoters to modulate intestinal health via stimulating dietary fiber utilization in poultry production. The aim of this study was to evaluate the effects of STB supplementation in corn- or wheat- basal diet on growth performance, intestinal development and function of broilers. A total of 512, One-day-old Arbor Acres（**AA**）broilers were randomly allocated four treatments, including corn group (**CG**), corn + 100 g/t STB (**CG+STB**), wheat group (**WG**), wheat + 100 g/t STB (**WG+STB**). The broilers were weighed at the days of 14, 28 and 42, of which 8 repetitions per treatment were randomly selected to determine the intestinal morphology, intestinal barrier, and cecal microbiota and metabolites. Our data showed that STB increased ($P < 0.05$) feed intake, body weight and reduced FCR for the overall period (0-42 days). At 28 days of age, significant increases in villus height and the villus height-to-crypt depth ratio (**V/C**) were found in the STB supplementation groups ($P < 0.05$). Addition of STB significantly increased intestinal mucosal DAO and AMPK enzyme activity and the gene expression of *OCLN*, *CLDN1*, *ZO1*, *MUC2*, *SGLT1*, *PEPT1*, *FABP2*, *Ghrelin* and *GCG* in Jejunum ($P < 0.05$), and significantly decreased the expression of the *PYY* gene. In addition, STB increased the relative abundance of beneficial bacteria, such as *Akkermansia*, *Bifidobacterium*, and *Oscillospirales* ($P < 0.05$). A significant increase in cecal short-chain fatty acid (**SCFAs**) concentration was also observed in the STB supplementation groups. At the cellular level, STB cannot directly increase the expression of small intestinal epithelial cells, and may indirectly improve intestinal barrier function by increasing the level of sodium butyrate. Overall, these results indicated that STB supplementation could improve the growth performance, intestinal development and barrier functions, and fiber fermentation in cecum of broiler chickens.

Key words: broilers, stimbiotic, growth performance, intestinal barrier, microbiota

INTRODUCTION

The topic of gut health has always been a subject of great public interest. In recent years, as research on intestinal microecology has deepened, people have gained a more profound understanding of the significance of probiotics and prebiotics (Quigley, 2019; Sanders, et al., 2019). Probiotics, which encompass microorganisms that are advantageous to the host, include *lactic acid bacteria* and *bifidobacterial* (Hidalgo-Cantabrana, et al., 2017; Kleerebezem and Vaughan, 2009; Reuben, et al., 2019). Studies have revealed that probiotics can enhance gut health through various mechanisms. Firstly, they competitively inhibit the proliferation of harmful bacteria, thereby maintaining the delicate balance within the intestinal microecosystem(Suez, et al., 2019). Secondly, probiotics bolster the intestinal barrier function, shielding the mucosa from harmful substances (Shen, et al., 2018). Additionally, probiotics are also capable of modulating the immune system and heightening the body's resistance against pathogenic microorganisms (Lin, et al., 2021; Marchesi, et al., 2016).

Prebiotics, on the other hand, encompass substances that foster the growth and activity of probiotics (Holscher, 2017). Common examples include cellulose, fructooligosaccharides, and inulin, etc (Slavin, 2013). Scientific investigations have found that prebiotics can facilitate a healthy gut in numerous ways (Gill, et al., 2021; Venter, et al., 2022). Firstly, prebiotics serve as a fuel source for probiotics, propelling their growth and proliferation (Yadav, et al., 2022). Secondly, prebiotics can regulate the pH level of the intestinal tract, maintaining it within an optimal range that is conducive to probiotic growth (Sorboni, et al., 2022). Moreover, prebiotics can

enhance the diversity of beneficial bacteria within the intestine, thereby improving intestinal functionality (Holscher, 2017).

The intestinal mucosa functions as a protective barrier within the intestinal tract, playing a pivotal role in isolation and defense (Jankowski, et al., 1994). Extensive research has demonstrated that both the morphology and functionality of the intestinal mucosa are of paramount importance in promoting gut health (Blikslager, et al., 2007; Shi, et al., 2017). The microbiome refers to the collective term for microorganisms that reside within the gut (Kuziel and Rakoff-Nahoum, 2022). Together with their host organisms, they form a complex and stable micro-ecosystem. Studies have revealed that the microbial community actively participates in processes such as food digestion, nutrient absorption, production of short-chain fatty acids (SCFAs), maintenance of intestinal pH stability, and regulation of the immune system (Belizário, et al., 2018; de Vos, et al., 2022).

There exist interrelationships and intrinsic connections between probiotics, prebiotics, mucosal morphology and functionality, as well as the intestinal microbial community. Probiotics and prebiotics can regulate the delicate balance within the intestinal microecosystem, thereby improving mucosal morphology and functionality, and promoting the restoration and maintenance of barrier function. Simultaneously, a healthy mucosal morphology and functionality aid in the colonization and growth of probiotics and prebiotics. A well-balanced microbial community can generate beneficial metabolites like SCFAs, which promote mucosal well-being and sustain intestinal barrier function. Therefore, probiotics, prebiotics, mucosal morphology and

functionality, and the microbiota are inextricably linked and mutually influence gut health (Rose, et al., 2021).

STB (stimbiotic) is a new functional feed additive (González-Ortiz, et al., 2019), which is able to accelerate fiber-degrading microbiota establishment in GIT to maximum the utilization of dietary fiber, especially arabinoxylans, which is the mayor component of dietary fiber in practical diets. It has been recently reported that STB supplementation could modulate intestinal inflammatory response and therefore improves broilers performance in an experimentally-induced necrotic enteritis infection model (Lee, et al., 2022). STB improves nutrient digestibility, and increases the abundance of beneficial bacteria, that reflects on improved technical performance of broilers and pigs (González-Ortiz, et al., 2021; Morgan, et al., 2021). The current study was designed to evaluate the effect of STB on the intestinal morphology and intestinal functions in broiler chickens. Furthermore, it tests the hypothesis that STB help increase the abundance of beneficial bacteria through stimulating dietary fiber utilization and fermentation in cecum, eventually improving the growth performance and gut health of broilers.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by the Animal Care Committee of the College of Animal Science and Technology, Henan Agricultural University, and were performed following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of China. All efforts were made to minimize animal suffering.

Experimental design and dietary treatments

A total of 512 one-day-old Arbor Acres (AA) male broiler chicks (initial body weight 40.78 ± 0.37g) were randomly divided into 4 groups with 8 replications with 16 birds per replicate. Experimental design is a full a 2x2 factorial arrangement with two cereals i.e., corn and wheat (**CG** and **WG**) supplemented or not with stimbiotic (Vistapros, AB Vista, UK) at 0.01% (**CG + STB** and **WG + STB**) Experimental diets were fed in a three-phase feeding program i.e., day 1 to 14 (brooder phase), day 14 to 28 (grower phase) and from day 28 to 42 (finisher phase). All experimental animals were raised in an environmentally controlled house in an AA broilers chicken farm in Henan, where the temperature of the first week was constant at 35°C, and then lowered 3°C weekly until the temperature reached 26°C. The basic feed is formulated based on NRC nutritional recommendations (NRC ,1994), as shown in Table 1.

Growth performance determination

Feed and water were provided *ad libitum*. Each cage of chickens was weighed on the mornings of 1, 14, 28, and 42 as feed cunsumption. Feed intake and body weight (**BW**) were recorded per cage and average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**) and body weight corrected FCR (**bwcFCR**) were calculated. Mortality was recorded as it occurred. European Production Efficiency Factor (**EPEF**) was calculated with the following formula: (ADG per day x livability %) / FCR x 10.

Sample collection

The samples were respectively collected on days 14, 28 and 42. For each sampling time point, 8 broilers per group with body weights close to the average were randomly selected and then euthanized by cervical dislocation followed by decapitation. The small intestine was extracted, and two segments (1 cm in length) of the midduodenum, jejunum, and ileum were excised, rinsed gently with sterile phosphate-buffered saline (PBS) to remove intestinal chyme, and one of them was fixed in the Morphological examination in 4% paraformaldehyde (Beijing Suo Leibao Technology Co., Ltd., Beijing, China); another copy immediately frozen in liquid nitrogen, kept in a freezer (−80°C) for subsequent experiments. The intestinal mucosae were scraped off with a glass slide, rapidly frozen in liquid nitrogen tank, and then transferred to − 80 °C freezer for storage. The bilateral ceca were split with sterile scissors and forceps. Then, the caecal digesta were scraped to frozen tubes and stored at − 80 °C for 16S rRNA amplicon sequencing and metabolomics analysis.

Intestine morphological analyses and observation

Use paraformaldehyde-fixed jejunum sections to observe intestinal morphology. Intestinal sample tissue was removed from the fixative and embedded in paraffin after trimming, dehydration, clearing, and waxing. After the wax block was made, the sample was sectioned and stained with hematoxylin and eosin (H&E). The small intestine slides were photographed by a light microscope (Wuhan Hundred Thousand

Degree Biotechnology Co., LTD Wuhan China). Morphometric analysis was performed by CaseViewer software (3DHISTECH LTD). Villus height was measured from the villus tip to the villus-crypt junction, and the crypt depth was defined as the length from the villus-crypt junction to the base of the crypt. Furthermore, the villus height-to-crypt depth ratio (V/C) was obtained according to the means of villus height and crypt depth.

Three slices were made for each chicken, each slice counted 6 villi, and the average value of the three slices was used as the villi statistical value of the chicken.

mRNA extraction and gene expression analysis

According to the manufacturer's instructions, total RNA was extracted from intestinal tissue using Trizol reagent (Vazyme, Nanjing, China). The integrity of all RNA samples was determined by 1.5% agarose gel electrophoresis, and the concentration was determined by measuring the 260/280 nm absorbance ratio with a Nano Photometer spectrophotometer (Implen, CA, USA). The primers were designed by Primer 5.0 and synthesized by Sangon Biotech (Shanghai, China), in which beta-actin (*ACTB*) was selected as the internal standard. The qRT–PCR volume was 10 μl, including 5 μl of ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China), 0.5 μl each of the forward and reverse primers, 1 μl of cDNA and 3 μl of RNase free water. The PCR instrument was QuantStudio^{TM} 5 (Thermo Fisher, USA). The qPCR conditions were as follows: pre denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at

72°C for 30 seconds; extension at 72°C for 10 min. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Serum Diamine Oxidase (DAO), D-lactate acid (D-LA) and AMP-activated protein kinase (AMPK) assay

Preparation of intestinal tissue homogenate: a part of intestinal tissue sample was weighed and placed into a 1.5-mL centrifuge tube filled with steel balls, add 9 times the volume of ice saline, with automatic quick grinding apparatus of grinding 90 S (55 Hz), after removal, the supernatant was extracted into a new 1.5 mL centrifuge tube, completes the tag, store in the refrigerator at 4°C for later use. The intestinal samples were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Jiangsu Meimian Industrial Co., Ltd)., and the content of DAO (Serum Diamine Oxidase), D-LA (D-lactate acid) and AMPK (AMP-activated protein kinase) activities were calculated.

16S rRNA amplicon sequencing analysis

To analyze the profiles of bacterial communities in fecal samples, the genomic DNA was extracted with CTAB (hexadecyltrimethylammonium bromide) methods, which was carried out according to previous study. The V3-V4 fragment of 16S rRNA gene was amplified using the barcoded primer set 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). Afterwards, a small fragment library was

constructed using NEBNext® Ultra™ II DNA Library Prep Kit (Illumina, USA), and each sample was sequenced on Illumina NovaSeq 6000 platform in Novogene Co., Ltd. (Beijing, China).

Paired-end reads were assigned to samples based on their unique barcodes and were truncated by cutting off the barcodes and primer sequences. Paired-end reads were merged using FLASH (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/)(Magoč and Salzberg, 2011), a very fast and accurate analysis tool designed to merge paired-end reads when at least some of the reads overlap with the reads generated from the opposite end of the same DNA fragment, and the splicing sequences were called Raw Tags. Quality filtering on the raw tags were performed using the fastp (Version 0.20.0) software to obtain high-quality Clean Tags. The Clean Tags were compared with the reference database (Silva database https://www.arb- silva.de/ for 16S/18S, Unite database https://unite.ut.ee/ for ITS) using Vsearch (Version 2.15.0) to detect the chimera sequences, and then the chimera sequences were removed to obtain the Effective Tags [2]

For the Effective Tags obtained previously, denoise was performed with DADA2 or deblur module in the QIIME2 software (Version QIIME2-202006) to obtain initial ASVs (Amplicon Sequence Variants), and then ASVs with abundance less than 5 were filtered out. Species annotation was performed using QIIME2 software. For 16S/18S, the annotation database is Silva Database, while for ITS, it is Unite Database. In order to study phylogenetic relationship of each ASV and the differences of the dominant species among different samples(groups), multiple sequence alignment was performed

using QIIME2 software. The absolute abundance of ASVs was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on the output normalized data. Alpha diversity indices (e.g., observed ASVs, Chao1, Shannon and Pielou's evenness) were also calculated by QIIME 2. Beta diversity analysis of bacterial community was performed by principal coordinate analysis (PCoA) based on based on unweighted unifrac distances. The linear discriminant analysis (LDA) effect size (LEfSe) was performed to identify the differentially abundant taxa (phylum to genera) of bacteria among the different groups (LDA score > 3.5, $P < 0.05$).

Gas chromatographic determination of short-chain fatty acids

The cecal contents had been dissolved in an internal standard solution containing 16.7% isohexanoic acid, and then centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant had been taken and tested on the machine. Pretreated caecal content samples had been analyzed using an Agilent 7890 gas chromatograph system (Agilent, USA). The SCFAs measured had primarily included acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid.

Isolation and culture of small intestinal epithelial cells

Inside a cell culture hood, the surface of fertilized chicken eggs was wiped with 75% ethanol. After breaking the eggshell, the chicken embryo was transferred into a laminar flow hood. The small intestine of the chicken embryo was removed and

placed in a sterile Petri dish. The small intestine tissue was washed repeatedly with a solution containing 100 U/mL penicillin and 0.1 mg/mL streptomycin until the washing liquid turned clear. The mesentery of the small intestine was detached and the small intestine tissue was cut into small pieces using sterile scissors until it turned into a homogenate. The homogenate was transferred into a centrifuge tube using a pipette, and 5 mL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) cell culture medium containing fetal bovine serum [45 mL DMEM/F12 cell culture medium + 5 mL fetal bovine serum + 500 μL penicillin-streptomycin mixed solution (100×)] was added at room temperature for 3 minutes. The supernatant was discarded, 5 mL of 1 mg/mL type I collagenase was added to the centrifuge tube, and digestion took place for 50 minutes in a 37°C water bath, with the tube gently tapped every 5-10 minutes. The centrifuge was set at 1000 rpm for 10 minutes and the supernatant was discarded. 5 mL of DMEM cell culture medium was added to the centrifuge tube, allowed to stand for 5 minutes, and the upper layer of suspended cells was carefully pipetted. The sample was then centrifuged at 1200 rpm for 5 minutes and the supernatant was again discarded. The cell suspension was filtered through a 100-mesh cell filter and the cell solution was transferred into a 75 mL cell culture flask. After 2 hours, the cell suspension was transferred to a 12-well cell culture plate to achieve preliminary separation of epithelial cells and fibroblasts. After 48 hours of culture, STB (which was filtered through a 0.22μm bacterial filter to remove bacteria) was added to the cells at concentrations of 25μg/mL, 50μg/mL, and 100μg/mL, and the expression levels of genes related to intestinal nutrient absorption and barrier were

measured. The cells were cultured in a 37°C, 5% CO2 incubator and the medium was changed every 48 hours.

The identification of IECs

The small intestine epithelial cells were identified using alkaline phosphatase and immunofluorescence staining methods. In the case of alkaline phosphatase staining, the cells were first fixed in 4% paraformaldehyde. They were then stained using the BCIP/NBT alkaline phosphatase color development kit (Beyotime Biotech, C3026, China) as per the manufacturer's protocol (Chen, et al., 2019). The images were acquired using a microscope. For immunofluorescence staining, after 48 hours of isolation and culture of IECs, the cells were washed once with PBS and left to stand for 5 minutes. After discarding the PBS, 500 μl of pre-cooled 4% cell tissue fixation solution was added to each well, and left to stand at room temperature for 20 minutes. The fixation solution was then discarded, and 1 mL of PBS residual liquid was added to each well and repeated three times. After discarding the PBS, 500 μl of 1% Triton membrane-breaking solution was added to each well, and incubated at room temperature for 10 minutes. The membrane-breaking solution was then discarded, and 1 mL of PBS residual liquid was added to each well and repeated three times. 500 μl of 10% blocking goat serum (Bio-Rad) was added to each well, and incubated at room temperature for 1 hour. After discarding the blocking solution, 300 μl of Cytokeratin 18 Antibody (CK-18; clone C-04; Santa Cruz Biotechnology, Dallas, TX, USA) was added to each well and incubated at 37°C for 1 hour (Jin, et al., 2022; Nguyen, et al.,

2021). 1 mL of PBS residual liquid was added to each well and repeated three times. Then, 300 μl of Cy3-conjugated goat anti-mouse (Beyotime Biotechnology; catalog A0521) was added to each well and incubated at 37°C in the dark for 1-2 hours(Bao, et al., 2021). 1 mL of PBS residual liquid was added to each well and repeated three times. Finally, 200 μl of 1 μg/mL DAPI (Sigma-Aldrich) was added to each well and incubated for 5 minutes, followed by the addition of 1 mL of PBS residual liquid and repeated three times. Images were obtained using a confocal microscope (SP8, Leica Microsystems).

Cellular level mRNA extraction and gene expression analysis.

The medium was changed every 24 hours; on the second day after the isolation of small intestinal epithelial cells was completed, STB at different concentrations (such as 0, 5, 10, 20 mg/ml) and sodium butyrate at different concentrations (such as 0, 0.5, 1, 2 mM/ml) were added to the cultured cells. 48 hours later, the cells were harvested. Total RNA was extracted from the cells. The expression levels of intestinal barrier-related genes and digestion and absorption-related genes in the cells were detected. The effects of different concentrations of STB on the expression of these genes were analyzed.

Statistical Analysis

Performance data were submitted to a 2-way ANOVA (JMP 16.2.0) with the effect of STB and diets and their interaction. When interactions were found, means were separated using Student's T-test. Significance was accepted when $P \leq 0.05$, and

trends were accepted when $0.05 \leq P \leq 0.1$. All the other data were analysed using independent samples t-test in SPSS 22.0 and expressed as Mean ± SEM. GraphPad Prism 8.0 and Adobe Illustrator 2022 were used for data and graphics processing.

RESULTS

Growth performance

The effect of STB on the broiler growth performance is summarized in Table 3. The initial weight of the broilers of different experimental groups was identical ($P > 0.05$). In the corn group, the final weight at 42 days was significantly higher in the CG+STB group than in the CG group ($P < 0.05$). The ADG and ADFI from 28-42 days were significantly higher than in the CG group ($P < 0.05$), but there was no statistically significant difference in the FCR between the groups ($P > 0.05$). In the wheat group, the WG+STB group had weights significantly higher than those of the WG group at 14, 28, and 42 days ($P < 0.01$). The ADG in the WG+STB group was significantly higher than in the WG group during the periods of 0-14 days, 14-28 days, 28-42 days ($P < 0.01$), and throughout the entire experimental period. The FCR in the WG+STB group was significantly lower than that in the WG group during 0-14 days, 14-28 days ($P < 0.05$). However, throughout the entire experimental period, the FCR in the WG+STB group was significantly lower than in the WG group ($P < 0.01$). An interaction was observed between the corn group and the wheat group in terms of FCR during 28-42 days and throughout the entire experimental period ($P < 0.01$). The interaction effect between

diet and STB is nearly not significant. Therefore, the subsequent data will be analyzed using independent samples t-test.

Intestine barrier function

DAO enzyme activity and D-LA content in the jejunal mucosa. Figure 1A shows the activity of DAO and the content of D-LA in the jejunal mucosa, in which the DAO activity in the intestinal mucosa was increased in the STB group ($P < 0.05$) from 14d onwards except for birds fed corn-based diet at 28d. However, there was no significant difference in D-LA in jejunal mucosa ($P > 0.05$).

Statistic of jejunum villi structure. As shown in Table 4, in corn groups, the results showed that villus height in STB group was significantly increased at 28 and 42 days ($P < 0.05$), crypt depth (μm) was decreased at 14 and 28 days ($P < 0.05$). The ratio between villus height/crypt depth was higher than the control group at 14 and 28d ($P < 0.05$). In wheat groups, the results showed that the villus height and the villus height/crypt depth ratio in STB group was significantly increased during the whole experiment period ($P < 0.05$). Crypt depth (μm) was only decreased at 28 days ($P < 0.05$).

The expression of the intestinal barrier-related genes detected in jejunum. As shown in Figure 1 C and D, in corn groups, the STB treatment significantly up-regulated the gene expression of jejunum *OCLN*, *CLDN1*, *ZO1* from day 1 to 14,

CLDN1, *ZO1* from 14 to 28 and *MUC2* from 28 to 42($P < 0.05$); In wheat group, STB treatment significantly up-regulated the gene expression of jejunum *CLDN1* at 28d ($P < 0.05$).

The expression of the intestinal nutrient digestion and absorption genes detected in jejunum. As shown in Figure 2 A, B, C, D, E and F, in corn groups, the STB treatment significantly up-regulated the gene expression of *FABP2* at 14d, tended to up-regulate the expression of *GCG* at 14d. At 42d, *SGLT1* expression was up regulated ($P < 0.05$) while *PYY* expression down-regulated ($P < 0.05$). There was a tendency to promote gene expression of *PEPT1* at 42 days ($P < 0.1$). In wheat groups, the STB treatment showed a significant increase in the gene expression of *SGLT1*, *PEPT1*, Ghrelin and *GCG* at 14 days. *FABP2* was up-regulated at 28d while *PYY* expression was reduced at 28 and 42d ($P < 0.05$).

In addition, the STB supplementation significantly increased intestinal AMPK activity of broilers at 14 and 28d ($P < 0.01$) in both corn and wheat groups, while there was no difference ($P > 0.05$) at 42 days regardless cereal based diets (shown in Figure 2 G, H and I).

Intestinal microbial diversity and community

The relative abundance of the broilers' caecal microbiota in level phylum and Genus was observed in Figure 3. The most abundant phylum was Firmicutes, followed by Bacteroidetes. Those two phyla dominated with around 90% of the bacterial

community throughout the whole experiment period. In the remaining 10% of the bacterial population, Proteobacteria, Actinobacteria and Desulfobacterota were common. Notably, Verrucomicrobiota had a higher relative abundance in the STB treatment groups ($P < 0.05$). At the genus level, the most abundant genus were *Clostridia_UCG-014* and *Aistipes*.

Figure 4 and 5 described alpha diversity analysis of broiler cecum contents in corn and wheat respectively. Figure 4 and 5 A and B described alpha diversity analysis of broiler cecum contents in wheat groups. Chao1 and Shannon indexes were used to express the alpha diversity of microbial community in cecum. There were no significant differences in Chao1 index, Simpson index and Shannon index between the STB treatment groups and the control groups. Figure 4 D, F and G and Figure 5 C, E and G described beta diversity analysis of broiler cecum contents in corn and wheat groups. The principal coordinates analysis (PCoA) based on unweighted unifrac distances was used to assess the community structure differences. According to the PCoA principal coordinate analysis, at 28 d, there were significant differencesin the intestinal microbiota between the CG group and the CG + STB group ($P < 0.05$), as well as between the WG group and the WG + STB group ($P < 0.05$).

Figure 4 C, E and H and Figure 5 D, F and H are LEfSe differential analysis in corn and wheat groups. LEfSe analysis further revealed increased relative abundance of the genus *Akkermansia, Christensenellaceae_R_7_group, Bifidobacterium, Intestinimonas* and *Parabacteroides* in STB supplemented groups ($P < 0.05$), which were known as probiotics or potential probiotics. In addition, we also found an

increase in fiber-degrading bacteria such as the genus *Ruminococcus* and *Bacteroides* in STB supplemented groups.

Short-chain fatty acids (SCFAs) analysis

The total SCFAs were considered as the sum of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. Overall, there is an increase of SCFAs production with the age of the animals. In both corn and wheat groups, main of the effects were observed at 42d. Nevertheless, in corn group hexanoate was increased ($P < 0.05$) at 14d and butyrate and valerate were increased ($P < 0.05$) at 28d by the STB supplementation. At 42d, acetate, propionate, butyrate, valerate and hexanoate were increased ($P < 0.05$) by the STB. In wheat group, at 14d acetate, butyrate and valerate were increased ($P < 0.05$) by STB supplementation, no effect was shown at 28d. At 42d, all fatty acids analyzed in this study were increased by the STB vs control ($P < 0.05$).

Correlation analysis between gut microbiota with SCFAs and growth performance

The correlation of intestinal flora with metabolites was assessed by calculating the Spearman coefficient of microorganisms with metabolites in caeca (shown in Figure 7). In the results, the phylum Bacteroidota, Verrucomicrobiota, Desulfobacterota, Campilobacterota and Cyanobacteria are positively correlated with SCFAs and growth performance (Figure 7A). However, the phylum Firmicutes and Proteobacteria, Gemmatimonadota were negatively correlated with SCFAs and growth performance. The phylum Actinobacteriota and Acidobacteriota were not

associated with SCFA production and growth performance. In Figure 7 B, the family Akkermansiaceae, Rikenellaceae, Bacteroidaceae, Barnesiellaceae, Oscillospiraceae, Tannerellaceae were positively correlated with SCFAs and growth performance, while the family of Lachnospiraceae, Ruminococcaceae and Enterobacteriaceae were negatively correlated SCFAs and growth performance. In Figure 7 C, the genus *Akkermansia*, *Alistipes*, *Bacteroides*, *Barnesiella*, and *Parabacteroides* were positively correlated with SCFAs and growth performance. Besides, the genus *Clostridia_UCG_014*, *Shuttleworthia*, *Escherichia.Shigella* and *Ruminococcus_torques_group* were negatively correlated with SCFAs and growth performance. The genus *Clostridia_vadinBB60_group* showed no correlation with SCFAs and growth performance.

Identification of small intestinal epithelial cells

In the process of isolation and culture of small intestine epithelial cells, it will be interfered by fibroblasts and other miscellaneous cells. Therefore, we stained small intestinal epithelial cells by immunofluorescence method and evaluated the cell purity. After purification by differential adhesion method, the cell purity could reach 60%-70%. As shown in Figure 8 C, the coincidence part of CK18 and DAPI was small intestinal epithelial cells, and the excess part of nuclear staining in Figure 8 B. DAPI was fibroblasts and other heterocells compared with Figure 8 A. Figure 8 D, E, and F showed the results of alkaline phosphatase staining of small intestinal epithelial cells at different multiples. It can be seen from Figure 8 G and H that the Upper-right

area (dark purple part) is small intestinal epithelial cell, while the lower left regions (transparent unstained part) are fibroblasts and other miscellaneous cells.

Gene expression analysis (cellular level)

As shown in the results in Figure 9 A, adding different concentrations of STB to small intestinal epithelial cells could not directly affect ($P > 0.05$) the expression of *MUC2*, *CLDN1*, *OCLN*, *ZO1* and other related genes. However, the addition of 1 mM/mL sodium butyrate significantly ($P < 0.05$) increased the expression of *OCLN* and the addition of 2 mM/mL sodium butyrate significantly ($P < 0.05$) increased the expression of *MUC2* and *OCLN* in small intestinal epithelial cells. There was a tendency to promote the expression of the *ZO1* gene, but no significant effect was observed.

DISCUSSION

The primary function of the small intestine is to ingest, digest and absorb nutrients to support animal growth and physiological function, while protecting the host from harmful compounds i.e., toxins, microorganisms, dietary antigens, etc. The digestion and absorption of dietary nutrients primarily occur in the small intestine (Kawalilak, et al., 2010).The increased feed efficiency may be partly explained by the enhanced intestinal morphology, which improves the animals' nutrient absorption.

It’s well known that tight junctions are important physical barriers, such as *CLDN1*, *Occludin* and *ZO1*, that are the main transmembrane proteins to constitute tight junctions and play an important role in maintaining the intestinal barrier (Ali, et

al., 2022; Gong, et al., 2020). Many studies have shown that the addition of xylanase to diet has no effect on the expression of genes related to the intestinal barrier in broilers (Wang, et al., 2021a). However, the addition of xylanase and β-glucanases to the diet significantly increased the expression of *CLDN1* mRNA in broiler jejunum (Wang, et al., 2021b). Tiwari et al reported in 2018 that xylanase and mannanase supplementation increased *CLDN1*, *OCLN*, and *ZO1* mRNA expression in the jejunum of nursey pigs fed corn distiller's dried grain with soluble source of fibre (Tiwari, et al., 2018). The addition of STB significantly reduces the depth of intestinal crypts at 14 and 28 days, but by 42 days, the addition of STB does not significantly affect crypt depth. At 28 and 42 days, the addition of STB significantly increases the height of the villi in both the corn and wheat groups, but at 14 days, this significant difference was only observed in the corn group. This situation may be related to age. Based on this study, we speculate that the improvement of intestinal crypts mainly occurs in the early development stage of broilers, while the intestinal villi continue to change in the replacement process of small intestinal epithelial cells, a process that seems unrelated to age. Healthy gut morphology is essential to maintain an effective gut barrier and reasonable gut permeability. The intestinal barrier is a physical barrier, composed of intestinal epithelial cells and their tight junctions, that prevents harmful microorganisms and substances from entering the body while facilitating the absorption of nutrients (Bao, et al., 2021; Zhang, et al., 2021). When intestinal morphology is impaired, such as damaged epithelial cells or weakened tight junctions, intestinal barrier function may be affected, leading to increased intestinal permeability,

which is called "leaky gut" (Camilleri, et al., 2012). Increased gut permeability may allow harmful substances and bacteria to enter the bloodstream, triggering an immune response and inflammation that may be linked to the development of several diseases (Odenwald et al., 2013; Suzuki, 2020). In our study, the addition of excitation prebiotics to feed could significantly reduce the depth of Jejunum crypt in broilers, significantly increase the villi height and the ratio of villi height to crypt depth, reduce intestinal permeability and promote intestinal barrier function. STB up-regulated main of those gene that could have activate the immune response to prevent pathogen invasion, improve the intestinal barrier function of animals, and maintain intestinal homeostasis. AMPK, as an energy sensor and regulator in intestinal cells, has been demonstrated to regulate glucose absorption (Koffert, et al., 2017), and when AMPK is activated, it can promote the expression of intestinal *GLUT2* gene (Sakar, et al., 2010; Walker, et al., 2005). Recent advance indicates that AMPK activation stimulates intestinal amino acid absorption with the upregulation of amino acid transporters, and enhances intestinal lipid absorption (Chopra, et al., 2011). Futhermore, AMPK plays a critical role in regulating epithelial barrier function. AMPK activation enhances intestinal barrier function by promoting the assembly of tight junctions (Nozu, et al., 2019; Peng, et al., 2009). AMPK activation also regulates epithelial barrier function through upregulating tight junction protein expression, such as *ZO1*, *OCLN*, and *CLDN1* (Rowart, et al., 2018; Wu, et al., 2020). In this study, the supplementation of STB promoted the improvement of intestinal AMPK enzyme activity and the expression of genes related to intestinal nutrient digestion and absorption in different

periods. These results suggested that STB could enhance the ability of broiler chickens to digest and absorb nutrients and gut barrier function.

In the microbiota analysis, no difference was observed on the alpha diversity and richness of broiler fed the diets with or without STB at day 14, 28 and 42 of age, while the structure of broiler cecal microorganism was shifted when fed STB at day 28 of age. To further explore the effects of STB on the gut microbes of broiler chickens, alterations in cecal microbiota were also analyzed by the LEfSe method on d 14, 28, and 42. Interestingly, the phylum *Verrucomcrobiota*, including the genus *Akkermansia* of the phylum in the STB-treated group were significantly higher than in the control group, which was defined as the next generation of promising probiotics (Cani, et al., 2022), and played a key role in host health, especially for the reduction of intestinal diseases and inflammation(Liu, et al., 2022; Zhao, et al., 2023). In addition, a shift of bifidobacterium and high amount of fiber-degrading bacteria, like *Christensenellaceae_R_7_group* and *Intestinimonas* was observed in the hindgut of broilers treated the diet with STB, and it was also found that the genus of *Intestinimonas, Bacteriods, Parabacteroides* and Oscillospirales etc were enriched in the broilers fed the diet with STB supplement, which were closely associated with the production of short-chain fatty acids (Lee, et al., 2017). Those results support the fact that STB is playing a role in modulating the hindgut microbiome in a more fibrolytic environment that potentially translates in higher dietary fiber utilisation in the ceaca. STB was effective in low vs high soluble fibre diet illustrated by corn vs wheat. It's not only a better use of fibre but also a stimulation of a good microbiome that ensure

good gut function avoiding dysbiosis. It has been proved that dietary fiber was the main carbon and energy source for proliferation of good microbiota, and can produce SCFAs by the fermentation and utilization of fiber-degrading bacteria flora, which showed multiple benefits for gut health, such as decreasing the incidence of intestinal inflammation, enhancing the body's intestinal barrier function, and promoting overall body homeostasis (McNabney and Henagan, 2017; Vinolo, et al., 2011) (Makki, et al., 2018). All of them were consistent with the findings in the present study that high amount SCFAs during the whole period in STB group and further revealed the consequence mechanism of stimbiotic that accelerated the growth of fiber-degrading bacteria to utilize dietary fiber and increased the production of SCFAs to improve broiler performance and gut health. In this study, supplementing corn-, and wheat-diets with stimbiotic improved growth performance of broilers that was already observed in previous studies. It has been proposed that an improved growth performance with stimbiotic was a result of improved fermentation of dietary arabinoxylan.

To gain a deeper understanding of the relationship between gut microbes, SCFAs, and growth performance, we conducted a Pearson correlation analysis of the three variables. At the phylum level, the phylum Bacteroides, Verrucomicrobiota, Desulfobacterota, Campilobacterota and Cyanobacteria are positively correlated with the production of SCFAs and growth performance. At the family level, the family Akkermansiaceae, Rikenellaceae, Bacteroidaceae, Barnesiellaceae, Oscillospiraceae, Tannerellaceae are positively correlated with SCFAs and growth performance. At the

Genus level, the Genus Alistipes, Bacteroids, Barnesiella, Pa*rabacteroides* are positively correlated with the production of SCFAs and growth performance. However, only the family *Oscillospiraceae* is positively related to butyric acid production. *Oscillospira* was named as a candidate for the next generation of probiotics (Yang, et al., 2021), which were positively associated with leanness and health (Gophna, et al., 2017; Konikoff and Gophna, 2016). Some studies speculated that *Oscillospira* probably produces the short-chain fatty acid butyrate (Yang, et al., 2021), which is consistent with our correlation analysis.

Small intestinal epithelial cells include a variety of cell types, which jointly perform physiological functions such as digestion and absorption, endocrine regulation and barrier protection, and maintain the normal structure and function of the small intestine. These cells coordinate with each other to jointly regulate the homeostasis of the small intestine. IECs provide a physical and biochemical barrier that segregates host tissue and commensal bacteria to maintain intestinal homeostasis(Peterson and Artis, 2014). Xylo-oligosaccharides are able to regulate intestinal epithelial cell proliferation or apoptosis and intestinal goblet cell differentiation through the Notch and Wnt/β-catenin signaling pathways (Tang, et al., 2022). In this study, STB did not directly affect the expression of intestinal barrier-related genes, but the addition of sodium butyrate could significantly improve the expression of intestinal barrier-related genes. In the in vivo experiment of this experiment, the addition of STB can significantly increase the production of sodium butyrate in the intestine (show in Figure 6). This suggests that STB may improve the

advocacy barrier function by enhancing the production of intestinal sodium butyrate. We deduced the mechanism of action of STB in broiler intestines: STB increased the proliferation and colonization of short-chain fatty acid producers (such as Oscillospirales, etc.), increased the content of short-chain fatty acids such as butyric acid produced by intestinal flora, indirectly regulate the expression of intestinal barrier and related genes.

CONCLUSION

The results showed that STB improves the development of intestinal structure of broilers, enhances the intestinal barrier function of broilers, increases the abundance of beneficial intestinal flora, promotes the colonization of beneficial intestinal flora, increases the concentration of short-chain fatty acids, and improves the intestinal health of broilers. Altogether may have conducted to broiler's growth performance improvement. Our results contribute important insights into the mechanism of action of STB in broiler intestines. These findings have the potential to inform the development of new strategies for improving gut health and performance in broiler chickens.

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DISCLOSURES

The authors declare no conflict of interest.

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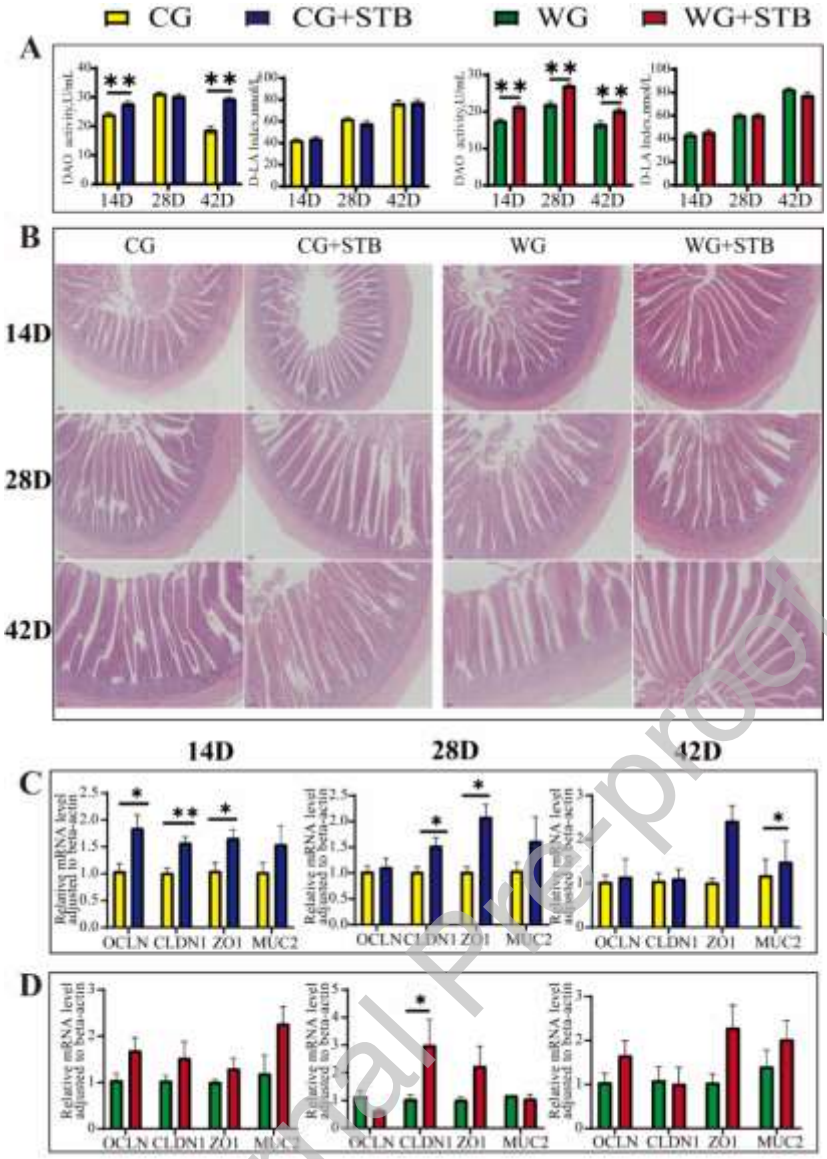

Figure 1 Stimbiotic can improve broiler intestinal barrier function. The data are presented as means ± SEM (*n* = *6*). Asterisks (* and **) represent significant differences with $P < 0.05$ and $P < 0.01$. **A** DAO enzyme activity and D-LA content in the jejunal mucosa. **B** Morphological observation of the epithelial tissue in jejunum with HE staining. All sections were observed under a light microscope at 60× amplification. **C and D** Relative gene expression of *OCLN*, *CLDN1*, *ZO1* and *MUC2* in jejunum of broilers. CG: corn control group; CG+STB: corn add STB group; WG: wheat control group; WG+STB: wheat add STB group. *OCLN*: occluding, *CLDN1*: claudin1, *ZO1*: tight junction protein, *MUC2*: mucin2. HE staining: hematoxylin and eosin staining.

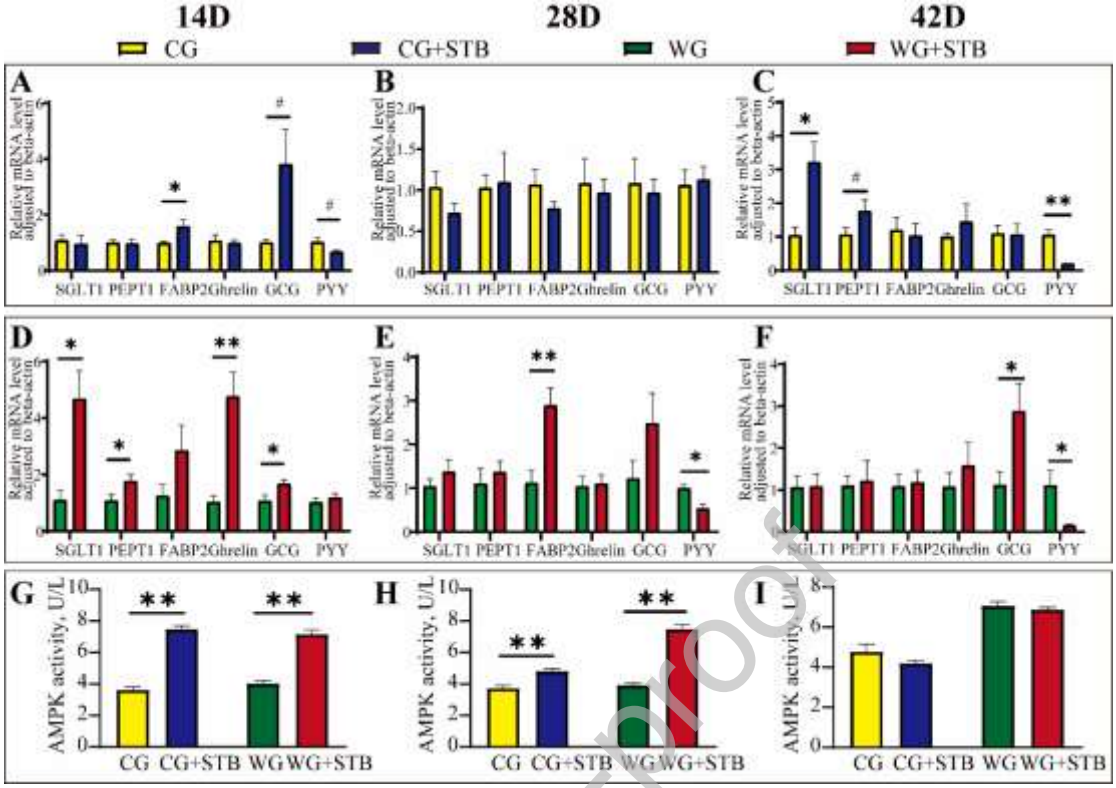

Figure 2 Stimbiotic can improve animal nutrient digestion and absorption. **A, B, C, D, E and F** Relative gene expression of *SGLT1*, *PEPT1*, *FABP2*, *Ghrelin*, *GCG* and *PYY* in jejunum of broilers. G, H and I The data are presented as means ± SEM ($n = 6$). Asterisks (* and **) represent significant differences with $P < 0.05$ and $P < 0.01$, pound sign (#) stands for $0.05 < P < 0.1$. *SGLT1*: sodium/glucose cotransporter 1, *PEPT1*: peptide transporter 1, *FABP2*: fatty acid-binding protein 2, *Ghrelin*: ghrelin, *GCG*: glucagon, *PYY*: peptide YY, AMPK: AMP-activated protein kinase. CG: corn control group; CG+STB: corn add STB group; WG: wheat control group; WG+STB: wheat add STB group.

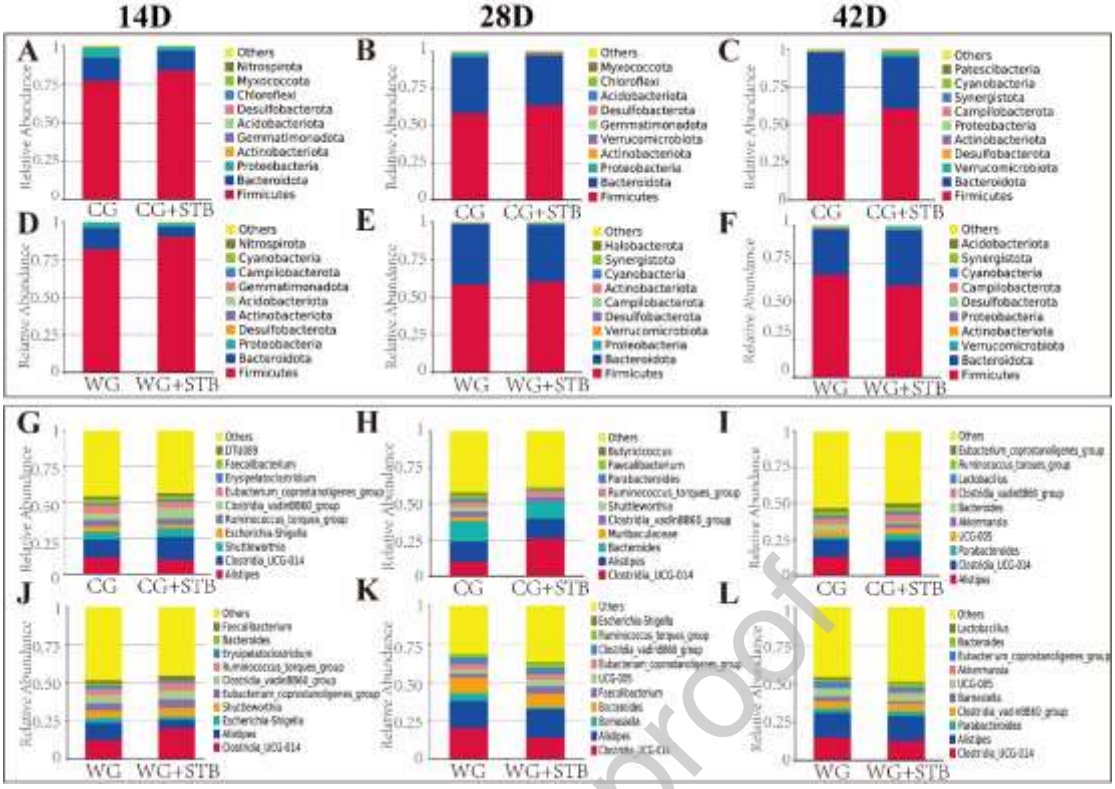

Figure 3 Relative abundance of the broilers' caecal microbiota in level phylum and Genus (top 10). Each mean represents six samples. CG: corn control group; CG+STB: corn add STB group; WG: wheat control group; WG+STB: wheat add STB group.

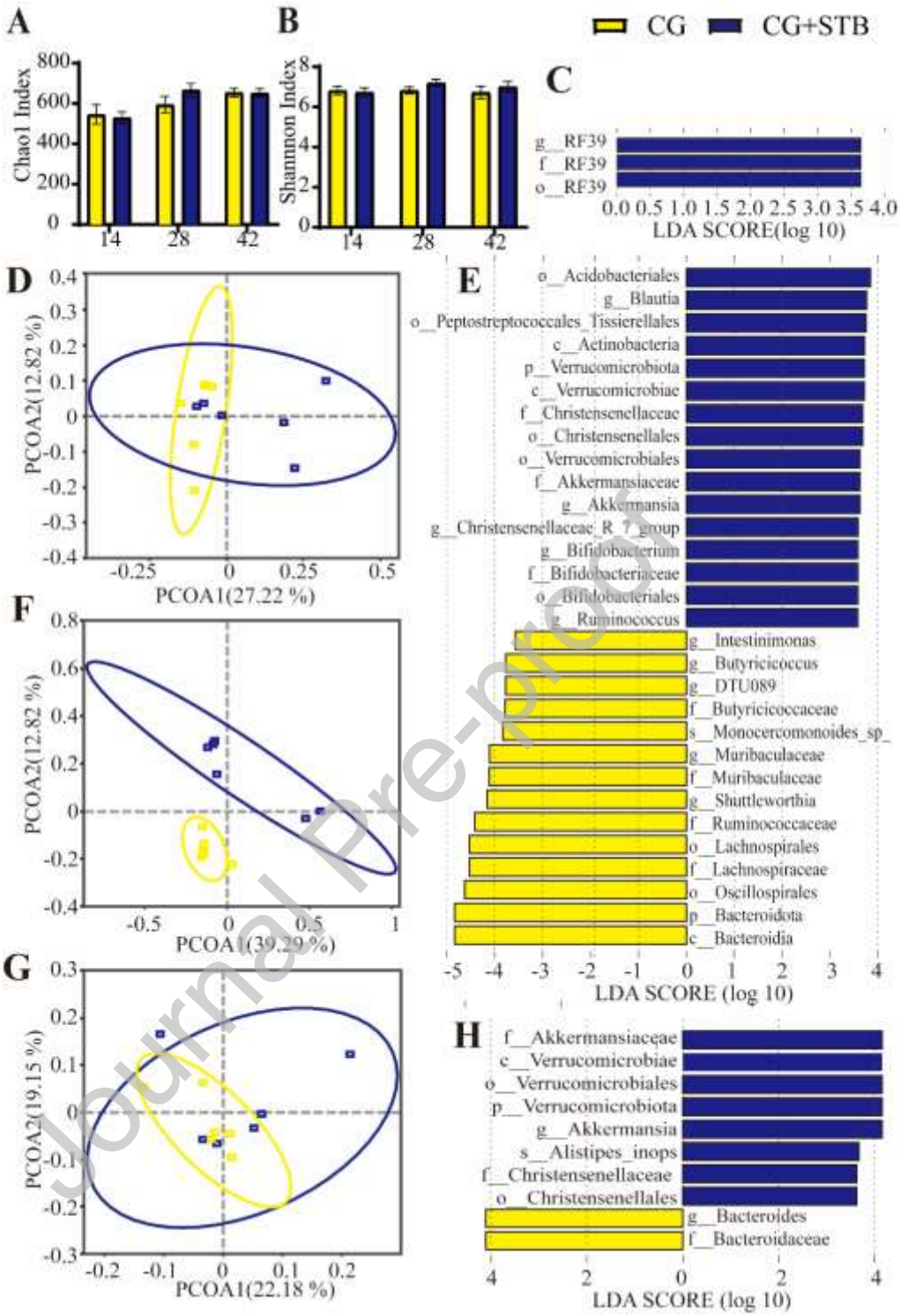

Figure 4 Microbial diversity analysis of broilers intestine in corn basal diet groups. **A** Chao1 index on the ASVs level; **B** Shannon index on the ASVs; **D, F and G** 14, 28 and 42 days principal coordinate analysis (PCoA) based on the UniFrac; **C, E and H** 14, 28 and 42 days linear discriminant analysis effect size of intestinal microbiota (LDA > 3.5, $P < 0.05$); CG: corn control group; CG+STB: corn add STB group.

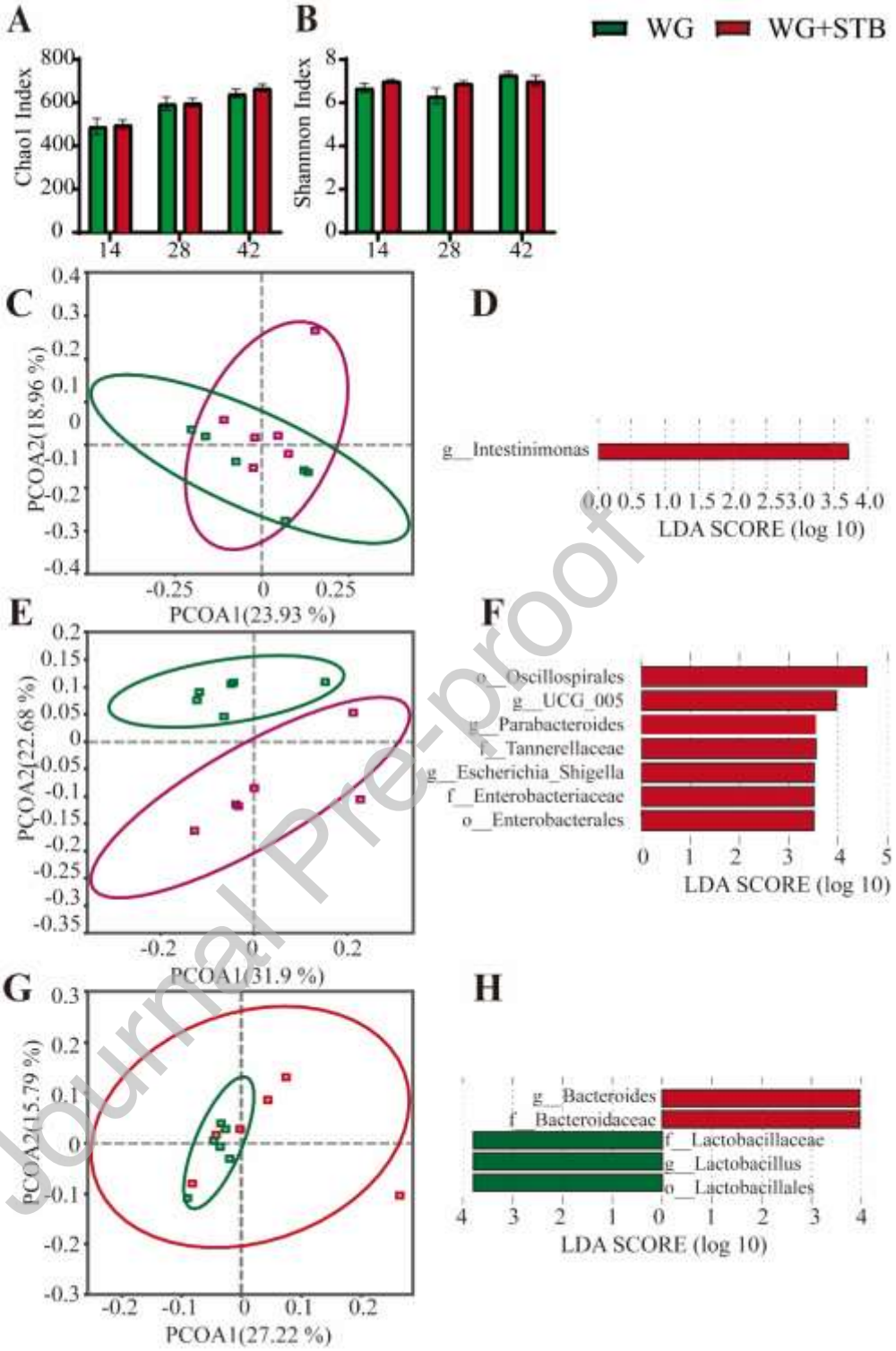

Figure 5 Microbial diversity analysis of broilers intestine in wheat basal diet groups. **A** Chao1 index on the ASVs level. **B** Shannon index on the ASVs. **C, E and G** 14, 28 and 42 days principal coordinate analysis (PCoA) based on the UniFrac; **D, F and H** 14, 28 and 42 days linear discriminant analysis effect size of intestinal microbiota (LDA > 3.5, $P < 0.05$). CG: corn control group; CG+STB: corn add STB group.

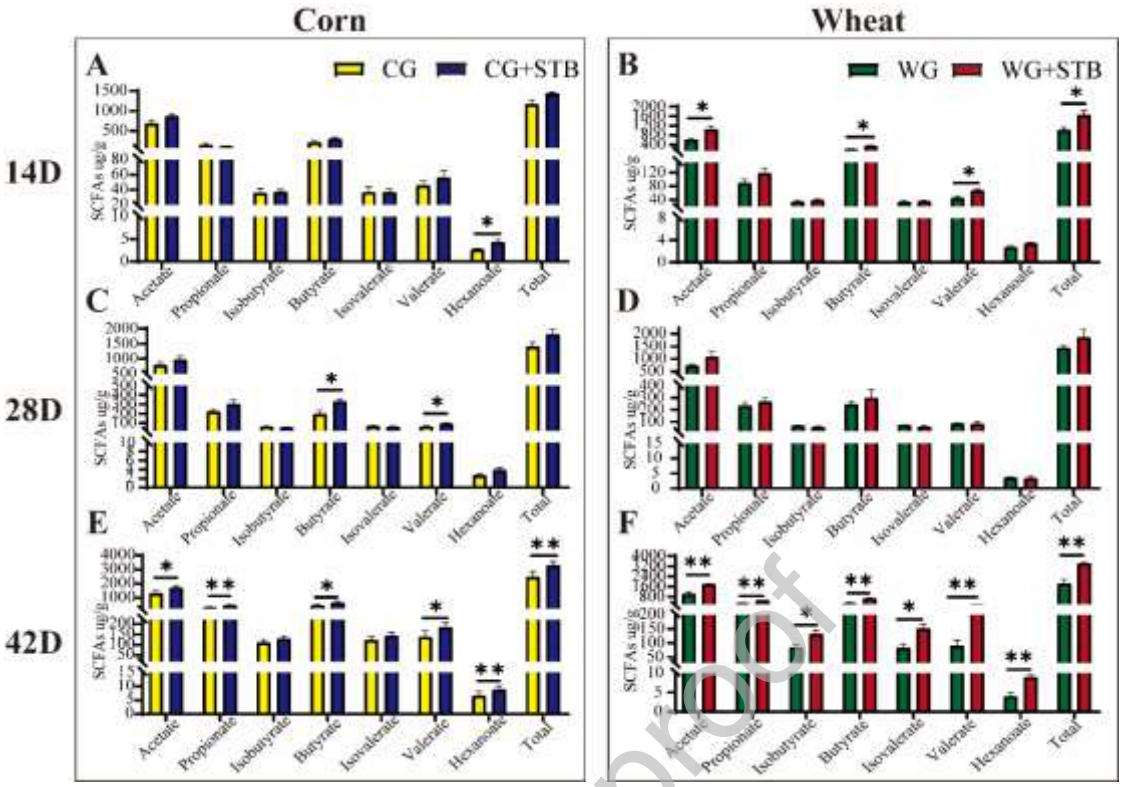

Figure 6 Analysis of SCFAs in broilers intestine. The data are presented as means ± SEM ($n = 6$). Asterisks (* and **) represent significant differences with $P < 0.05$ and $P < 0.01$. **A, C and E** 14, 28 and 42 days analysis of SCFAs in corn groups. **B, D and F** 14, 28 and 42 days analysis of SCFAs in corn groups. CG: corn control group; CG+STB: corn add STB group; WG: wheat control group; WG+STB: wheat add STB group.

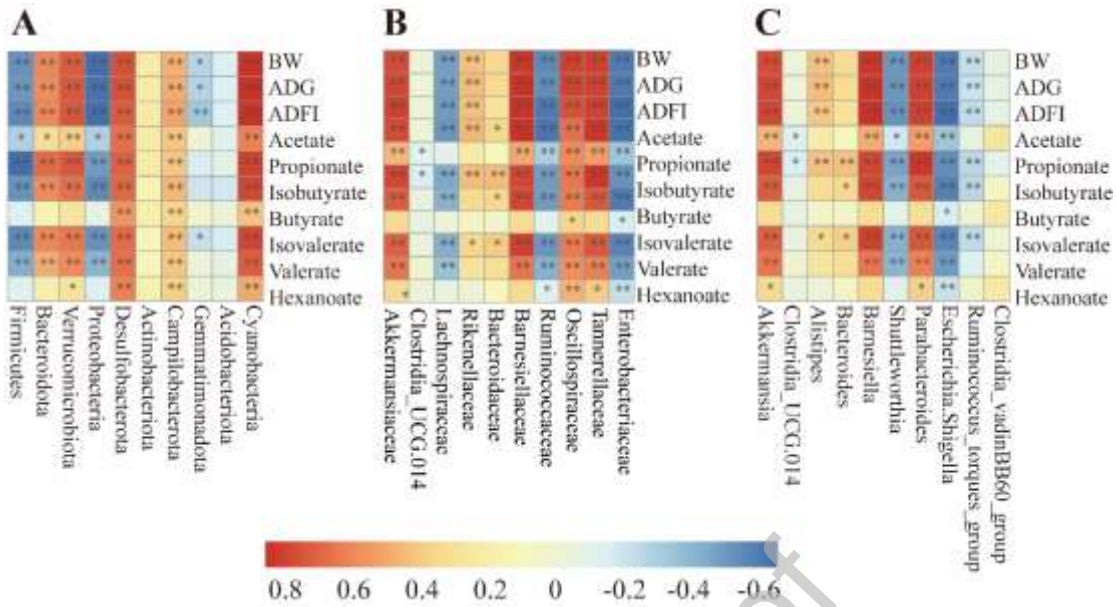

Figure 7 Correlation analysis of gut microbiota with SCFAs and growth performance. **A** Correlation analysis between phylum level and SCFAs and growth performance. **B** Correlation analysis between class level and SCFAs and growth performance. **C** Correlation analysis between genus level and SCFAs and growth performance. Red and blue colors represent positive and negative correlation, respectively, and color gradation indicates the size of the correlation coefficient.

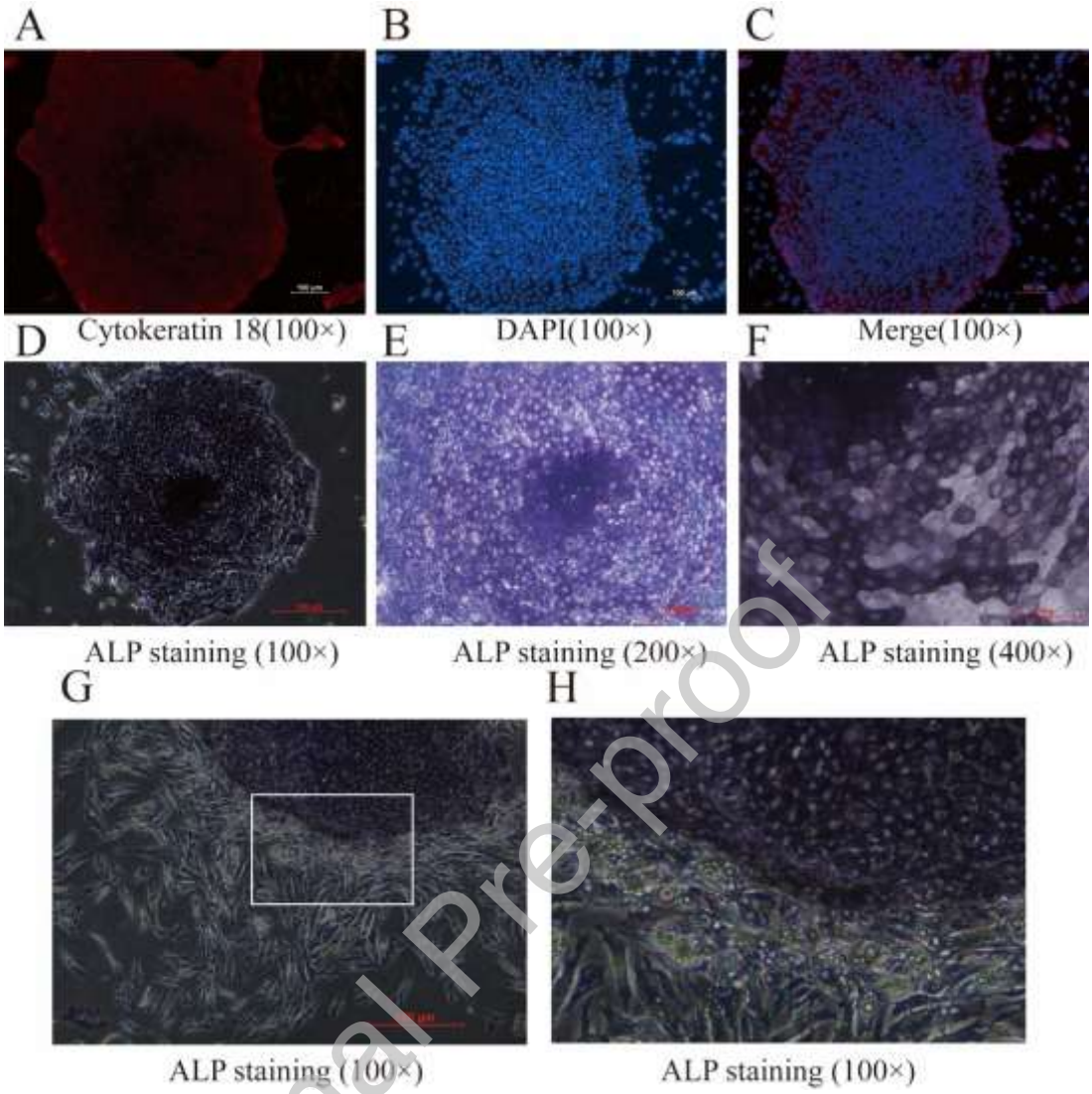

Figure 8 Identification of Chicken Embryo Small Intestinal Epithelial Cells. **A** Cytokeratin 18 immunofluorescence staining. **B** DAPI immunofluorescence staining. **C** A combined with B. **D** Alkaline phosphatase staining at 100x magnification. **E** Alkaline phosphatase staining at 200x magnification. **F** Alkaline phosphatase staining at 400x magnification. **G** Alkaline phosphatase staining at 100x magnification. **H** Enlarged area of the white box in G.

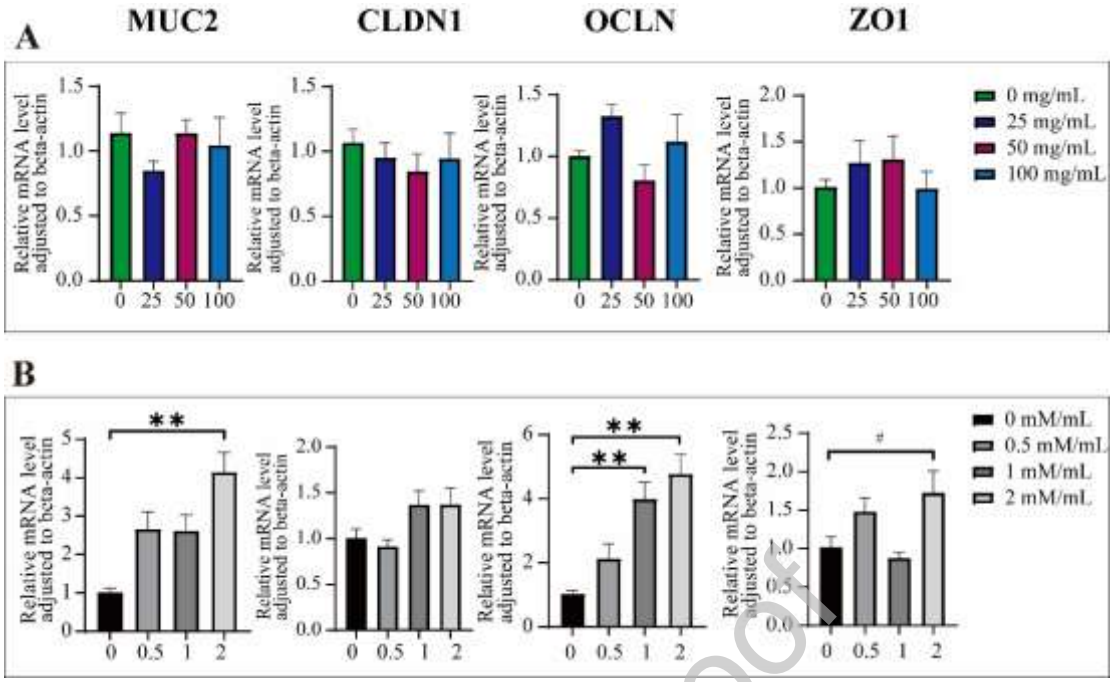

Figure 9 Cellular level mRNA extraction and gene expression analysis. The data are presented as means ± SEM ($n = 6$). Asterisk (**) represent significant differences with $P < 0.01$, pound sign (#) stands for $0.05 < P < 0.1$. **A** Relative gene expression of *MUC2*, *CLDN1*, *OCLN*, *ZO1* of different concentrations STB supplementation in IECs. **B** Relative gene expression of *MUC2*, *CLDN1*, *OCLN*, *ZO1* of different concentrations sodium butyrate supplementation in IECs.

Table 1. Feed formulation used in the three feeding phases

Ingredient, %	Brooder phase		Grower phase		Finisher phase	
	Corn	Wheat	Corn	Wheat	Corn	Wheat
Corn (7.34% CP)	46.00	-	48.5	-	52.60	-
Wheat (13.58% CP)	-	57.00	-	59.96	-	65.00
Soybean meal (45.43% CP)	42.00	31.6	38.5	27.60	33.6	21.8
DCP[1]	1.63	1.60	1.60	1.60	1.60	1.60
Limestone	1.30	1.15	1.07	1.00	0.84	0.72
DL-methionine	0.45	0.43	0.42	0.40	0.38	0.35
L-lysine HCL	0.24	0.52	0.16	0.46	0.15	0.46
L-threonine	0.08	0.20	0.05	0.18	0.03	0.17
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Soy oil	7.00	6.20	8.40	7.50	9.5	8.60
0.5% premix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Bile acid/Emulsifier	0.50	0.50	0.50	0.50	0.50	0.50
Sum	100	100	100	100	100	100
Nutrition levels						
ME, kcal/kg[3]	3.02	3.02	3.12	3.12	3.20	3.20
Crude protein, %	23.06	23.06	21.55	21.55	19.50	19.50
Met, %	0.74	0.74	0.69	0.69	1.15	1.15
Met + Cys, %	1.05	1.05	0.99	0.99	0.63	0.62
Lys, %	1.44	1.44	1.29	1.29	0.90	0.90
Thr, %	0.97	0.96	0.88	0.88	0.78	0.78
Try, %	0.26	0.26	0.24	0.24	0.22	0.22

[1] DCP: CaHPO4·2H2O

[2] The premix provided the following per kg of diets：vitamin A 8 000 IU，vitamin D_3 3 000 IU，vitamin E 20 IU, vitamin K_3 2 mg, vitamin B_1 4 mg, vitamin B_2 3.6 mg, vitamin B_6 4 mg, vitamin B_{12} 0.02 mg, biotin 0.15 mg, nicotinic acid 40 mg, pantothenic acid 12 mg, folic acid 1 mg, choline 300 mg，Cu 10 mg，Fe 80 mg，Zn 75 mg，Mn 80 mg，I 0.4 mg，Se 0.3 mg，phytase 2 000U/g.

[3] ME was a calculated value, while the others were measured values.

Table 2. Primer sets for quantitative real-time PCR

Gene	GenBank number	Primer	Sequence of nucleotide (5´-3´)	Size (bp)
CLDN1[1]	NM_001013611.2	F	CTTCGTCATGCTCATCGCCT	149
		R	TGATCAGCAGTGCAATTCCT	
OCLN[2]	NM_205128.1	F	CTGCAACAACACAGACAGCC	171
		R	GGGCTTGTAGAAGGGGTGAC	
ZO1[3]	XM_040706827.2	F	GACATGTTGATGCCAGCCTT	115
		R	AGCAGGCAGGCTCCTAAAAC	
SGLT1[4]	NM_001293240.2	F	AAGGTTTGCACGAGCCAGAT	176
		R	TGTGGACACCAAATGCTTCA	
PEPT1[5]	NM_204365.2	F	TCTCTGTCCGTCCCTCGGT	282
		R	GGATGGGCGTCAAGTAGCA	
FABP2[6]	NM_001007923.2	F	GAAGCAATGGGCGTGAATGT	203
		R	CCTTCCAGGTTCCAAGAGCC	
Ghrelin	NM_001001131.2	F	AACCTGGTGAAGTTTCAGAGCA	119
		R	AGGCAGTGCTTCAAATGTGT	
GCG[7]	NM_001190165.5	F	CCGAAGACATGCAGATGGCA	258
		R	CGGCATGCAAGGTGACATAG	
PYY[8]	NM_204786.1	F	TGCCACCGAGGGATCAGG	218
		R	AGGTCGTTGTAGAAGCGGATG	
ACTB[9]	NM_205518.2	F	TTGTTGACAATGGCTCCGGT	153
		R	TCTGGGCTTCATCACCAACG	

1. *CLDN1*: claudin 1; 2. *OCLN*: occluding; 3. *ZO1*: zona occludens 1; 4. *SGLT1*: sodium-dependent glucose transporter 1; 5. *PEPT1*: peptide transporter 1; 6. *FABP2*: fatty acid binding protein 2; 7.*GCG*: glucagon; 8. *PYY*: peptide YY; 9. *ATCB*: bate-actin

Table 3. Effects of corn and wheat diets supplemented with STB on Growth performance of Broiler chicken

Items	Diets		SEM	STB		SEM	P-value		
	Corn	Wheat		-	+		Diets	STB	Diet*STB
BW[1](g/bird)									
0	40.73	40.81	0.054	40.75	40.79	0.054	0.260	0.626	0.871
14	491.18	493.21	5.880	486.68	497.71	5.880	0.808	0.196	0.048
28	1733.85	1765.63	16.600	1716.05	1783.43	16.600	0.187	0.008	0.099
42	3217.06	3311.35	24.521	3175.47	3352.94	24.521	0.010	<.0001	0.874
ADG[2](g/d/bird)									
0-14	32.18	32.31	0.420	31.85	32.64	0.420	0.817	0.197	0.048
15-28	88.76	90.89	1.070	87.81	91.84	1.070	0.173	0.013	0.292
28-42	104.22	110.41	1.670	102.52	112.11	1.670	0.014	0.000	0.087
0-42	75.63	77.87	0.580	74.64	78.86	0.580	0.010	<.0001	0.873
ADFI[3](g/d/bird)									
0-14	33.68	33.92	0.268	33.79	33.80	0.268	0.534	0.971	0.050
14-28	114.50	117.24	1.895	115.83	115.91	1.895	0.315	0.978	0.385
28-42	185.48	182.78	1.929	178.48	189.79	1.929	0.324	0.000	0.004
0-42	109.99	109.57	0.960	108.26	111.30	0.960	0.761	0.033	0.064
FCR[4]									
0-14	1.048	1.051	0.009	1.063	1.037	0.009	0.786	0.051	0.249
14-28	1.291	1.292	0.020	1.320	1.263	0.020	0.974	0.049	0.083
28-42	1.833	1.657	0.229	1.793	1.697	0.229	<.0001	0.005	0.185
0-42	1.468	1.408	0.012	1.464	1.412	0.012	0.002	0.006	0.214
Bwc FCR[5]	1.490	1.395	0.018	1.499	1.386	0.018	0.001	0.000	0.537
EPEF[6]	507.94	538.18	8.148	500.95	545.17	8.148	0.013	0.001	0.769

1: body weight; 2: average daily gain; 3: average dialy feed intake; 4: feed conversion ratio; 5: body weight corrected FCR; 6: European Production Efficiency Facto. SEM = standard error of mean, n = 8.

Table 4. Effects of diets on Jejunum villi structure in broilers

Day		Corn					Wheat			
		CG	CG+STB	SEM	P value		WG	WG+STB	SEM	P value
14	V[1]	730.77	695.09	21.46	0.127	V	661.04	927.6	34.85	0.000
	C[2]	139.73	116.39	9.15	0.029	C	187.04	173.14	12.93	0.308
	V/C[3]	5.30	6.01	0.38	0.090	V/C	3.56	5.42	0.32	0.000
28	V	964.18	1122.72	41.20	0.003	V	888.33	1195	38.56	0.000
	C	194.72	153.12	14.16	0.015	C	225.77	170.99	14.14	0.003
	V/C	5.01	7.47	0.57	0.002	V/C	3.96	7.23	0.72	0.001
42	V	1252.78	1348.17	28.94	0.002	V	1054.69	1218.5481	27.5676	0.000
	C	219.39	225.80	8.38	0.462	C	247.07	230.7539	13.9664	0.270
	V/C	5.74	6.01	0.26	0.321	V/C	4.31	5.3556	0.2519	0.002

1: villus height; 2: crypt depth; 3: the villus height-to-crypt depth ratio