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Pretreatment with probiotics *Enterococcus faecium* NCIMB11181 attenuated *Salmonella Typhimurium*-induced gut injury through modulating intestinal microbiome and immune responses with barrier function in broiler chickens

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Abstract

Background: Preventing *Salmonella* infection and colonization in young birds is key to improving poultry gut health and reducing *Salmonella* contamination of poultry products and decreasing salmonellosis for human consumption (eggs and meat). Probiotics can improve poultry health. The present study was conducted to investigate the impact of a probiotics, *Enterococcus faecium* NCIMB11181 (*E. faecium* NCIMB 11181) on the intestinal mucosal immune responses, microbiome and barrier function in the presence or absence of *Salmonella* Typhimurium (*S.* Typhimurium, ST) infection.

Methods: Two hundred and forty 1-day old *Salmonella*-free male broiler chickens (Arbor Acres AA^+) were randomly allocated to four groups with 6 replicate cages of 10 birds each. The four experimental groups were follows: (1) negative control (NC), (2) *S.* Typhimurium challenged positive control (PC), (3) the *E. faecium* NCIMB 11181-treated group (EF), (4) the *E. faecium* NCIMB 11181-treated and *S.* Typhimurium challenged group (PEF).

Results: Results indicated that, although continuous feeding *E. faecium* NCIMB 11181 did not obviously alleviate growth depression caused by *S.* Typhimurium challenge (P >0.05), *E. faecium* NCIMB 11181 addition significantly lessened intestinal inflammation by competitively blocking *Salmonella* intestinal colonization and inhibiting *TLR4*-medicated immune inflammatory responses through upregulating intestinal mucosa *Tollip* mRNA levels (P < 0.05). Moreover, supplemental *E. faecium* NCIMB 11181 to the infected chickens remarkably attenuated gut morphological structure damage and decreased intestinal cell apoptosis as evidenced by increasing gut villous height, and reducing *Salmonella* translocation and intestinal TUNEL-positive cell numbers (P < 0.05). Also, *E. faecium* NCIMB 11181 administration notably enhanced humoral immunity through promoting the production of anti-*Salmonella* specific antibodies in intestinal mucosa and serum of the infected birds (P < 0.05). Additionally, 16S rRNA sequencing analysis revealed that *E. faecium* NCIMB 11181 supplementation ameliorated *S. Typhimurium* infection-induced gut microbial dysbiosis by enriching *Lachnospiracease* and A*listipes* levels, and suppressing *Barnesiella* abundance. Predicted function analysis (PICRUSt) indicated that the functional genes of cecal microbiome involved in C5-branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; glycerolipid metabolism and lysine biosynthesis were enriched; while alanine, asparate and glutamate metabolism; MAPK signal pathway-yeast; ubiquine and other terpenoid-quinore biosynthesis,

protein processing in endoplasmic reticulum; as well as glutathione metabolism were suppressed by *E. faecium* NCIMB 11181 supplementation.

Conclusion: Collectively, our data suggested that dietary *E. faecium* NCIBM 11181 supplementation could ameliorate *S.* Typhimurium infection-induced gut injury in broiler chickens. Our findings also suggest that *E. faecium* NCIMB 11181 may serve as an effective non-antibiotic feed additive for improving gut health and immune defense to *Salmonella* infection.

Keywords: Broiler chickens*, Enterococcus faecium*, Gut health, *Salmonella*

Background

Salmonella enterica var. Typhimurium (*S*. Typhimurium), a rod-shaped, flagellated, aerobic, gram-negative intracellular pathogen, which is one of the most prevalent serotypes of *Salmonella* in broiler chickens, and cause gastroenteritis in the human by entering the human food chain through animal products, particularly raw poultry products[1,2] *S.* Typhimurium infection in chicks younger than 2 weeks old results in poor growth rate, severe enteric and systemic disease with a high mortality rate along with persistent *Salmonella* infection in tolerant chickens, while infection in older chickens results in asymptomatic cecal colonization and persistent shedding of the organisms in feces, resulting in *Salmonella* contamination of poultry products, vertical transmission to offspring along with a cumulative economic loss [3-6]. Prolonged persistent infection with *S.* Typhimurium in the GI tract of chickens throughout their lifespan could alter the development of gut microbiota and have detrimental effect on the overall gut health of the chicken host, especially exposed to stress stimulation or other pathogens challenge. Therefore, reducing *S.* Typhimurium in the intestinal tract of chickens could not only reduce morbidity and mortality caused by *Salmonella* infection in young chicks, but also reduce *Salmonella* prevalence in poultry along with decrease contamination of poultry products and salmonellosis in humans.

Various alternative or preventive strategies, which include cleaning up the breeding herbs, general strict hygiene and biosecurity measures in the farm, vaccination, genetic selection of chicken lines with improved immunity, supplementation with feed additives, such as organic acids, short- and -medium-chain fatty acids, essential oils, yeast cell wall extract, bacteriophages, prebiotics, probiotics and synbiotics, have been made by farmers and food processors, both pre- and post-

harvest, to prevent *Salmonella* contamination of food products, and reduce drug-residue and frequency multi-drug resistant bacteria occurrence[5,7]. Among these above strategies, probiotics and their metabolites could confer the health benefit on the host through several mechanisms including inhibition of pathogenic bacteria through competitive exclusion, production of bacteriostatic and bactericidal substances against pathogens, modulation of innate and acquired immunity, enhancement of intestinal barrier function, as well as maintain gut homeostasis when administered in adequate amounts in chickens [8,9], being considered as one of the promising and useful measures employed in controlling *Salmonella* infections and colonization in poultry thanks to the prophylactic and therapuetic use of antibiotics will be further restricted or banned starting in 2022 [10-14].

Enterococcus faecium was Gram-positive and facultative anaerobic bacteria and belong to lactic acid bacteria for their ability to produce lactic acid and enterococcin during the growth. Although some strains of the genus *Enterococcus* are pathogenic to human or animals, many strains such as *Enterococcus faecium* NCIMB 11181, NCIMB10415, *E. faecium* SF68 and *E. faecium* M-74 are nonpathogenic and even some are used as commercial probiotics in medicine, food and animal feed because of their resistance to low pH, bile salts and encountered in digestion and produce enterocins [15-17].

Previous studies have demonstrated that feed or drinking water supplementation with *E. faecium* strain facilitates systemic and intestinal local mucosal immune responses [18-20]. *E. faecium* also increases the absorptive and secretory capacity of jejunal mucosa, improves intestinal barrier function [21], enhances disease resistance to pathogenic infection, partially prevents or treats diarrhea in pigs [18,22-25]. Furthermore, the addition of *E. faecium* to pig directly or indirectly modifies intestinal bacterial communities by increasing the prevalence of beneficial bacteria and reducing pathogenic bacteria load and/or increases growth performances [22,26-29]. Results from poultry experiments have revealed that supplementation of the diet with *E. faecium* strain improves growth performance, eggshell quality and modulates intestinal microflora composition [30-33]. This supplementation also results in the regulation of intestinal mucosal immune responses and enhanced chicken resistance to intestinal pathogen infection including infections caused by *Salmonella* [34-36], *E. coli* [37,38], *Clostridium perfringens* [33] and *Eimeria* . Probiotic strains differ regarding the properties and clinical effects that they elicit; these differences are even observed when the

strains belong to the same bacterial species. *E. faecium* strain NCIMB 11181 (isolated from pigs) is currently authorized by the EFSA Panel on Additives and Products or Substances used in Animal Feed as a supplement for fattening and improving the performance of animals [39]. This strain has been shown to effectively increase daily weight gain and improve feed conversion, and enhance gut health in pigs. Additionally, our previous studies also demonstrated that dietary *E. faecium* strain 11181 addition could improve growth performance, enhance cellular and humoral immunity of broiler chickens reared under non-challenged conditions [33], ameliorate necrotic enteritis-induced intestinal barrier injury in broilers [32], and improved growth and reduced the death rate together with maintaining the intestinal integrity in *Escherichia coli* O78-challenged-challenged broiler chickens[38]. Probiotics, including lactic acid bacteria have been repeatedly proven as an alternative approach for the treatment of various infections. Nevertheless, it is unknown whether dietary *E. faecium* NCIMB 11181 addition could be helpful for protecting intestinal health in broiler chickens infected with *S. Typhimurium.* Therefore, this study was conducted to investigate the effects of *E. faecium* NCIMB 11181 addition on *Salmonella* colonization and invasion, development of intestinal pathological lesions, intestinal immune response, together with intestinal barrier function in broiler chickens challenged with *Salmonella Typhimurium*. In addition, we further assess the shifts in intestinal microbial community structure induced by dietary treatment and /or ST challenge to explain the possible protective effects of *E. faecium* NCIMB 11181 addition on broilers infected with ST.

Materials and methods

Animal Ethics Statement

All animal experiments were approved by the China Agricultural University Animal Care and Use Committee, Beijing, P. R. China.

Experimental design, birds, diets and animal management

One hundred and twenty (n = 120) 1-day old *Salmonella*-free male broiler chickens (Arbor Acres AA^+) were purchased from a local supplier (Beijing Arbor Acres Poultry Breeding Company, Beijing, China). Birds were used to evaluate the protective efficacy of *E. faecium* NCIMB 11181 feed supplementation against ST infection. Meconium from each individual chicken was collected and checked it for *Salmonella* negativity using the plating method. Samples were pre-enriched with

tetrathionate broth (CM 203-01, Land Bridge Technology Ltd., Beijing, China) at 37^0C for 24h, and then streaked on Bismuth sulfite agar (CM 207, Land Bridge Technology Ltd.) to confirm that the chicks were free of *Salmonella.* Subsequently the chicks were randomly divided into four experimental groups. These 120 two-day old *Salmonella*–negative chickens was randomly assigned into 4 groups including: no additive and no challenge with *S*. Typhimurium (negative control, **NC**); no additive but challenged with *S*. Typhimurium (positive control, **PC**); *E. faecium*-supplemented but uninfected (**EF**); *E. faecium*-supplemented and infected with *S. Typhimurium* (**PEF**). Each group contained three replicate pens with 10 birds per pen and fed a balanced, un-medicated corn and soybean meal-based pelleted diet that contained either 0 or 200 mg/kg *E. faecium* NCIMB11181**,** (viable count $\geq 2 \times 10^9$ CFU/g; manufactured by Probiotics International Ltd. Co., UK). To avoid cross-contamination, all uninfected birds were reared in one clean separate room, whereas all infected birds were housed in another room under the same environmental conditions. Antibiotic-free and coccidiostat-free corn-soybean meal-based pelleted diets were formulated to meet or exceed National Research Council (1994) requirements (23% protein, 3,030 kcal/kg metabolizable energy, 1.12% lysine, 0.42% methionine, 0.97% calcium, 0.43% available phosphorus) and tested for the presence of *Salmonella.*

The composition of the basal diet and nutrient levels are presented in **Table 1**. The experimental diet was formulated by mixing the basal diet with 200 mg of *E. faecium* (2×10^9 CFU/g of the product) to reach 2×10^8 CFU/kg of diet. To ensure the homogeneity of the additives, approximately 5 kg of the basal diet mixed with the additive were thoroughly mixed using a plastic bucket. The feed samples were taken and the *E. faecium* number was counted by *Enterococcus faecium* Agar (Bile Aesculin Azide Agar, HB0133-3) to ensure the probiotic dosages were performed correctly. Prior to the experiments, feed samples were collected and checked for the presence of *Salmonella* by bacterial culture as described above.

The chicks were reared on net floor cages in a closed and ventilated house. Each pen had a floor space of 7, 200 (120×60) cm^2 and was equipped with a separate feeding trough. Water was supplied through nipple drinkers. Water and feed were provided *ad libitum*. In accordance with the AA+ Broiler Management Guide, all chicks received continuous light for the first 24 h, and were then maintained under a 23-h light/1-h dark cycle for the remainder of the study. The room temperature was maintained at 33-34°C on the first 3 days, and then gradually decreased by 2°C/week until a

final room temperature of 22-24°C of reached. The relative humidity was kept at 60-70% during the first week and then 50-60% thereafter.

Salmonella typhimurium challenge

The *Salmonella enteritis serotype Typhimurium* CVCC2232 was obtained from the China Veterinary Culture Collection Center (Beijing, China). The frozen culture was recovered by using sterile buffered peptone water (CM201, BPW, Land Bridge Technology Ltd.). ST pre-culture was transferred to 100 mL of tryptone soy broth (CM201, TSB, Land Bridge Technology Ltd.) and incubated at 37°C with orbital shaking for 16 to 18 h. The concentration of viable ST in the culture was counted on Bismuth sulfite agar at 37°C for 24 h and the stock culture was adjusted to a final concentration of 1×10^9 CFU/mL ST. At 9 to 10 d of age, birds in the ST-challenged groups were inoculated with 1 mL of bacterial suspension containing approximately 1×10^9 colony forming units (**CFU**) of ST suspension by gavage. Unchallenged groups received 1.0 mL of PBS without ST on the same date. Feed was withdrawn from all birds 10 h before challenge.

Measurement of growth performance

Body weight of broilers was measured individually at 1, 11 and 21 days of bird age. The weight of feed consumed in each pen was recorded. Body weight gain (DWG) during different periods (during d 1 to 11, and d 12 to 21).

Samples collection

On days 7 after the *S.* Typhimurium challenge, all birds from each group were euthanized via cervical dislocation, blood was collected for serum anti-*Salmonella* specific IgG analysis, and livers, spleen and left cecal contents were aseptically harvested and assessed for ST concentration as soon as possible. The right cecal contents were aseptically collected, snap-frozen in liquid nitrogen and then stored at -80 °C for intestinal microbial 16Sr DNA-based analysis. Proximal ileum segments were flushed with 0.05 M PBS, pH7.2 and fixed in 4% (w/vol) polyoxymethylene solution for histological and immunohistochemistry examination. Distal ileum parts were collected, washed for 2 times with ice-cold PBS, and then snap-frozen in liquid nitrogen for mRNA determination. Ileal mucosa from each bird was collected, homogenized in ice-cold PBS (pH 7.2), centrifuged, and then the supernatant was collected and stored at –20°C for anti-*Salmonella* specific IgA analysis.

Detection of *Salmonella* in cecum content and internal organs

Salmonella numbers in cecal contents and internal organ was determined as described previously

[5]. Briefly, samples of liver, spleen and cecal contents were weighed, homogenized in BPW (10% w/v suspensions) for 1 min using a Stomacher respectively and serially diluted 10-fold (1: 10) with sterile PBS to appropriate levels for *Salmonella* numeration on xylose lysine tergitol 4 (XLT4) agar plates containing 100 μg/ml nalidixic acid. The number of black bacterial colonies was determined counted on XLT4 agar plates after incubation for 24 h at 37°C and expressed as mean ± standard error of the mean log10 CFU/g feces or tissues. Samples that were positive only after enrichment with tetrathionate broth (TTB) and then streaked into XLT4 agar plated containing 100 μg/ml nalidixic acid solution were counted as 1 CFU/g, and samples that yielded no *Salmonella* growth after enrichment were counted as 0 CFU/g. A *Salmonella*-positive bird was defined based on recovery of *Salmonella* from any of the internal organs (liver, spleen) studied in an assay. The percent efficacy of protection for a particular group was calculated based on the number of *Salmonella*-positive birds out of the total number of birds in a group .

Intestinal histology and immunohistochemical staining analysis

Jejunal samples were collected and fixed in 4% neutral buffered formalin after postmortem examination, and then processed, trimmed, and embedded in paraffin by routine methods. The serial paraffin sections (5 μm) were prepared and stained with hematoxylin-eosin (HE) for histological (Villous height (VH) and crypt depth (CD), magnification × 40) [5]. In addition, HE-stained 5-μm-thick sections was determined intestinal inflammation or pathological scores as previously described [4,40] using a light microscope (Leica model DMi8, Leica, Wetzlar, Germany) with an image analysis software (version 4.2, Leica application suite, Leica, Wetzlar, Germany) at magnification of × 200. All scores were obtained in a blinded fashion by two independent investigators. The TUNEL assay of jejunum-tissue sections was performed by using immunostaining following the same procedure as described in our previous study [33]. The IOD of TUNEL-positive cells in the jejunum was assessed by a digital microscope and camera system (Nikon DS-Ri1, Japan). Positive cells (the red arrow points out a typical positive cell in the jejunal crypt and the jejunal villus) of from five fields from each area of the image in each section were measured in high-power fields selected at random (200 × magnification, 50 × 50 μm) with a computerized image-analysis system (MultiScanBase v. 14.02, Computer Scanning System, Warsaw, Poland). Accumulated integral optical density (IOD) for positive staining in each image was analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., MD, USA).

240 **Quantitative real-time PCR**

241 Total RNA isolation, reverse transcription, and real-time PCR were carried out as previously
242 described [33]. The primers for real-time PCR are listed in **Table 2**.The efficiency of all tested genes
243 was between 90% and 110%. All the tissue samples for the cDNA synthesis and in the following
244 PCR amplifications were run in triplicate. Gene expression for immune-related genes (*TLR2, TLR4,*
245 *MyD88, NF-κB, IFN-γ, IL-1β, IL-6, IL-8, TNF-α, TGF-β4, A20*, and *Tollip*), tight junction proteins-
246 related genes (*claudin-1, occludin, ZO-1, ZO-2* and *MLCK*) was analyzed using glyceraldehyde-3-
247 phosphate dehydrogenase (*GAPDH*) as an endogenous control. The method of $2^{-\Delta\Delta Ct}$ was used to
248 analyze the real-time PCR data [41] and results were expressed as the fold change relative to the
249 average value of the negative control group (the non-treated and non-challenged control).

250 **Measurement of anti-*Salmonella* specific antibody in the intestine and serum**

251 Briefly, ST CVCC 2232 (10^8 cfu/mL) cells were washed 3 times and lysed by an ultrasonic
252 processor 250, USA at 85 Watts and 30-second intervals on ice for 5 min. The lysed cells were
253 centrifuged at 10,000×g for 10 min, and the resultant supernatant was collected and stored at -70ºC
254 until use. Flat-bottomed 96-well ELISA microplates (Corning, NY, USA) were coated with 100 µL
255 of 20 µg/mL of the antigen diluted in 0.1 M carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM
256 $NaHCO_3$, 0.3 mM NaN_3) and analyzed for anti–ST specific IgG in the serum and specific IgA in
257 intestinal mucosa homogenate, respectively, using an indirect enzyme-linked immunosorbent assay
258 (ELISA) as described earlier [4,42]. Serum samples were diluted 1:100 and intestinal wash samples
259 were diluted 1:5 in PBST with 1% BSA. Absorbance values (optical density, OD) were read at 490
260 nm using an automatic ELISA reader (Bio-Tek EL311sx autoreader, Bio-Tek, USA). Each serum
261 sample or intestinal sample was tested in duplicate.

262 **Microbial DNA extraction, 16S rRNA amplification and sequencing and bioinformatic**
263 **analysis**

264 Microbial DNA was extracted from cecum contents of broilers using QIAamp DNA Stool Mini
265 Kits protocol (Qiagen Inc) according to the manufacturers' protocol. The quality and quantity of
266 DNA samples were determined using a Nanodrop ND-1000, and agarose gel electrophoresis was
267 used to confirm the absence of degradation. An aliquot of each of the extracted high-quality DNA
268 samples was sent for PCR amplification of the 16S rRNA gene, library preparation, and DNA
269 sequencing to a commercial provider (Beijing Mega-genomics Technology Co. Ltd, Beijing, China).

The primer set F341/R806 (F341: ACTCCTACGGGRSGCAGCAG, R806: GGACTACVVGGGTATCTAATC) which targets the V3-V4 region of the bacterial 16S rDNA was chosen by adding index and linker sequence adequate to HiSeq 2500 PE250 sequencing (Illumina, San Diego, United States) at Biomarker Technology Co., Ltd. (Beijing, China). FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies) was applied for the assembly of resulting 300-bp paired-end reads [43]. Additional sequence read processing, which included quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds, was performed within QIIME (version 1.9.1) [44]. USEARCH (version 7, 64-bit) was utilized for denoising, chimera detection, and clustering into operational taxonomic units (OTUs) (97% identity) [45,46]. All of the effective reads from each sample were clustered into operational taxonomic units (OTUs) based on a 97% sequence similarity identified by UCLUST in QIIME v1.8.0 [46]. Taxonomic classification at different taxonomic levels of OUT sequences were performed by comparing sequences to the GreenGene v13.8 database [47]. Shannon and Simpson indices, Chao1 and ACE estimators were included in a-diversity analysis by using the MOTHUR v1.31.2 [48]. The principal coordinates analysis (PCoA) and partial least squares discriminant analysis (PLS-DA) plots based on weighted and unweighted Unifrac distance matrices were used to estimate pairwise distances among samples and to establish β-diversity. Analysis of similarities (ANOSIM) with 999 permutations was used to detect statistical significances between microbial communities in different groups. This test measures a value of R, normally scaled from 0 to 1, which is based on the average rank similarity among groups and replicates within each group [49]. R = 0 indicates that two groups are similar, whereas R = 1 shows a perfect separation between groups. Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) and non-parametric *t*-test (with Metastats software) were further employed to identify the biological differences in the microbial composition among groups [50]. LDA was performed from the phylum to genus level, and LDA scores ≥ 4.0, and p values < 0.05 were selected for plotting and further analysis.

Functional analysis of the gut microbiota

Metagenome functional content from high-quality 16S rDNA was predicted using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software (http://picrust.github.io/picrust/) [51], based on the Kyoto Encyclopedia of Genes and

Genomes (KEGG) Orthology database (https://www.genome.jp/kegg/ko.html). Significance analysis were performed by two-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data were then analyzed with Statistical Analysis of Taxonomic and Functional Profiles (STAMP) version 2.1.3 [52]. Differentially represented functional pathways (level 2 in hierarchy, representing KEGG pathways) between the two conditions (presented in extended error bar plots) were analyzed with two-sided Welch's t-test on every pair of means where P <0.05 was considered significant. Confidence intervals of 95% were obtained by inverting the Welch's tests.

Statistical analysis

The growth performance data, intestinal structure data, intestinal apoptosis index, intestinal and internal organs salmonella numbers, antibody levels and gene expression data were subjected to 2-way ANOVA by using the GLM procedure of the SPSS, version 18.0 (SPSS Inc., Chicago, IL). The model included the main effect of probiotics treatments, *Salmonella challenge* and their interaction. The relative abundance of microorganisms obtained from 16SrRNA sequencing was analyzed using the Kruskal-Wallis rank sum test to compare the difference between the comparison groups. Significance was set at $P < 0.05$, and a trend towards significance at $P < 0.10$ was seen. Data in the tables were expressed as means and pooled SEM.

Results

Growth performance

Data on growth performance including body weight (BW), body weight gain (BWG) of different phase are shown in **Table 3**. ST challenge resulted in a significant reduce in BW (at d 21) and BWG (during d 12 to 21) compared with the non-challenged birds ($P < 0.05$), while the addition of *E. faecium* NCIMB 11181 in feed had no remarkable influence on growth performance (BW and BWG) in broiler chickens irrespective of ST challenge ($P > 0.05$). No significant difference for mortality rate was observed in all groups during the experimental period ($P > 0.05$).

Intestinal histopathological scores and intestinal cell apoptosis index

A small number of heterophils were found only in the ileum of the ST group, indicating a mild inflammatory reaction at 7 hpi, while no severe inflammation and infiltration of heterophils (polymorphonuclear cells) were found in the other three groups (**Fig. 1**). Moreover, ST strongly increased the inflammation score, and TUNEL-positive cell numbers, significantly decreased

villous height, and the ratio of villous height to crypt depth crypt (V/C) (P<0.05) as compared to the non-infected groups. *E. faecium* NCIBM 11181 pretreatment significantly decreased the inflammation score and TUNEL-positive cell content, moreover, promoted the growth of villous height irrespective of ST infection (P <0.05), but the crypt depth and V/C was not affected by *E. faecium* NCIBM 11181 (P >0.05) (**Table 4; Fig. 2**). However, no significant interaction effect was observed in intestinal histopathological scores, cell apoptosis rates at 7 dpi in the jejunum among the four treatment groups (P >0.05).

Salmonella numbers in cecal contents and internal organs

It is known that bacterial translocation occurs when the barrier function of the intestine is impaired. Therefore, we checked the number of colonies in the intestines and internal organs. All samples that were taken from uninfected control chicks (NC group) were negative for *S. typhimurium*. The efficacy of *E. faecium* NCIMB 11181 supplementation in reducing *Salmonella* colonization and invasion was evaluated by bacterial counting of the ST challenge strain in liver, spleen and cecal content of broiler chickens (**Table 5**). **Table 5** shows that at 7 days post-challenge, *Salmonella* was detected in the liver, spleen and cecal content only after enrichment in all challenged groups (PC and EFP) with significant differences in ST numbers (burden) in the liver and cecum compared with un-challenged groups (P < 0.05). Challenged-birds fed diets with *E. faecium* NCIMB 11181 resulted in a significant reduction in *Salmonella* counts in the cecum in comparison with the challenged but un-supplemented birds. ST load in the liver of the *E. faecium* NCIMB 11181-treated birds tended to be lower than that of the single ST-challenged group, though not statistically significantly. After direct enrichment cultures, the number of ST-positive birds (liver) was significantly lower in the *E. faecium* NCIMB 11181-treated birds (6/29) than in the single challenged control (14/29) at days 7 post-challenge, whereas no significant difference was found in the spleen. The results showed that compared with the ST group, the number of the liver and cecum in the ST-infected birds fed *E. faecium* NCIMB 11181 group was significantly lower in both liver tissue and cecum content, indicating that *E. faecium* NCIMB 11181 can reduce bacterial colonization and prevent bacterial translocation.

Ileum immune-related genes expression

To explain the anti-inflammatory action of *E. faecium* NCIMB 11181, the mRNA levels of TLR-mediated signal pathway molecules, i.e. *TLR4, MyD88, NF-κB, IL-1β, IL-6, IL-8, TNF-α, TGF-β4,*

IFN-γ, pIgR and negative regulators *A20, Tollip* and *PI3K* in the chicken ileum mucosa were measured at 7 days post ST infection. Results showed that the expression levels of *MyD88, NF-κB, IFN-γ, pIgR* and negative regulators *Tollip* and *PI3K* in the chickens of the infected group were significantly upregulated ($P < 0.05$) than those of the non-challenged control groups (**Table 6**). ST infection also showed an increased trend for *TLR4* (P = 0.099) and *TNF-α* (P = 0.096) mRNA levels. While these genes mRNA levels except for *pIgR* and *Tollip* genes were not affected by the addition of *E. faecium* NCIMB 11181 to the diets. Significant upregulation of *pIgR* and *Tollip* mRNA expression level in the ileum was observed in the *E. faecium* NCIMB 11181-treated groups compared with the non-supplemented birds. However, there was no significant cooperative effects on immune-related molecules expression between ST challenge and *E. faecium* NCIMB 11181 addition.

Gene expressions of intestinal tight junction

In order to investigate why the intestinal permeability was changed by *S*. Typhimurium and *E. faecium* NCIMB 11181, expression of selected *MLCK* and TJ genes was measured by RT-PCR. As showed in **Table 7**, in comparison with the non-challenged group, *Salmonella* infection significantly decreased mRNA levels of tight junction *Claudin-1, Occludin*, *ZO-1 and ZO-2* while increased the mRNA level of *MLCK* ($P < 0.05$). No significant difference in *MLCK, CLDN-1, Occludin, ZO-1* and *ZO-2* at the mRNA level was observed in the *E. faecium* NCIMB 11181 group compared with the un-supplemented group regardless of ST infection ($P > 0.05$). Moreover, there was no significant interactive effects on *MLCK* and these TJ molecules expression at 7 DPI between ST challenge and *E. faecium* NCIMB 11181 supplementation.

Humoral immune response

The induction of humoral immune responses against the *S. typhimurium*-specific antigen was monitored during the weeks after ST infection to evaluate the immune-regulatory capacity of the *E. faecium* NCIMB 11181. As illustrated in **Table 8**, intestinal mucosa anti-*Salmonella* sIgA and serum anti-ST specific IgG levels were significantly elevated ($P < 0.05$) at 7 days following ST infection in broiler chickens. *E. faecium* NCIMB 11181 addition remarkably promoted intestinal mucosa anti-*Salmonella* specific antibody production compared with those in the non-supplemented groups ($P < 0.05$). Moreover, there was significant cooperative effects on intestinal mucosa anti-*Salmonella* IgA titers between ST challenge and *E. faecium* NCIMB 11181 supplementation. Infected birds

given *E. faecium* NCIMB 11181 displayed the highest anti-*Salmonella* IgA content compared with the other three groups ($P < 0.05$), infected birds alone showed higher IgA content as compared to that of the non-infected groups.

Cecal microbiome bacterial diversity and community composition

As shown in **Table 9,** a total of 542,151 high-quality sequences were obtained from 4 groups, with an average of 22,590 sequences per sample. All of OTUs were defined at 97% species similarity level, 25,765 OTUs were obtained from cecal digesta samples, with an average of 1074 OTUs per sample. We obtained the species richness (observed OTUs, Chao, good-coverage) and the community diversity (Shannon, Simpson) was not influenced by ST challenge, *E. faecium* NCIMB 11181 supplementation or the interaction between *E. faecium* NCIMB 11181 and ST challenge (**Fig. 3A-E**).Venn diagram (**Fig. 3F**) indicated 4,786 common core OTUs were shared among all groups, while 17014, 21904, 16981, and 17776 OTUs were unique to groups NC, PC, EF and PEF, respectively. PCA, PCoA and NMDS plots (weighted and unweighted UniFrac distances for the PCA) was to be visualized beta diversity (**Fig.4A-B**). Results showed that there was no obvious clustering tree (no obvious segregation of the microbiota) associated with dietary treatments or *S.* Typhimurium infection. Conversely, the infected birds fed *E. faecium* NCIMB 11181 form a unique cluster separated from all other three groups, especially separated from the single ST-infected birds. The single ST-infected birds displayed little similarity with the other 3 groups ($0 < R = 0.3704 < 1$; $P = 0.011$). However, no distinct separation for cecal microbiota was found between the EF and NC groups. Differences in microbial community abundance (at the phylum and genus level) between all groups were shown in **Fig. 5.** At phylum level, the Top 5 dominant phylum includes *Firmicute, Bacteriodetes,Cyanobacteria,, Proteobacteria and Tenericutes.* Higher abundance of *Firmicutes* ($P = 0.085$) and lower abundance of *Bacteriodetes (P=0.061)* were detected in PEF compared to the PC control. The top 10 microbes at the genus level (**Fig. 6**) had *Alistipes*, *Barnesiella*, *Bacterioides*, *Lachonclostridum*, *Faecalibacterium*, *Ruminococcaceae-UCG-014, Ruminiclostridium-5, Ruminococcaceae-UCG-005, Phascolarctobacterium* and *Subdoligranulum.* The abundance of genus *Alistipes* at 7 days after ST infection was significantly suppressed in the ST-challenged birds but remarkably increased by *E. faecium* NCIMB 11181 addition compared with the PC control ($P < 0.05$). The infected birds treated with *E. faecium* NCIMB 11181 showed an increased trend for *Lachnoclostridum*, while/ reduced the percentage of the genus *Barnesiella* (*P* <

0.05) and display a reduced trend in the abundance of *Bacterioides(0.05<P<0.1)*. LEFsE analysis (**Fig. 7**) highlighted that the infected birds fed *E. faecium* NCIMB 11181 showed higher abundance in the genus *Lachnospiracease*, genus A*listipes*, Rikenellaceae family and *Lachonclostridum*, which was similar to the changes of intestinal microbial communities of the non-infected control compared with the single ST-infected control. Under non-infected conditions, *E. faecium* NCIMB 11181 addition enriched cecal *Anaerotruncus* and *Flavonifractor* abundance as compared to the NC group.

Predicting the function of intestinal bacteria based on 16S rDNA data (PICRUSt analysis)

The functions of the intestinal microbiota genomes in different treated groups were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) from the Kyoto Encyclopedia of Genes and Genomes pathways, to identify significant differences ($P < 0.05$) in biological processes and pathways (**Fig.** 8). At KEGG level 2, STAMP analysis revealed that pretreatment with *E. faecium* NCIMB 11181 enriched the abundance of functional genes related to flavanoid biosynthesis pathway ($P = 0.035$) but suppressed novobiocin pathway ($P = 0.041$) of cecal microbiota of the non-infected birds. As compared to the single ST-infected group, the abundance of the metabolic pathway functions, including C5-branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis, methane metabolism, glycerolipid metabolism and lysine biosynthesis were notably enriched ($P<0.05$), while alanine, asparate and glutamate metabolism, RNA degradation, transcription machinery, MAPK signal pathway-yeast, ubiquine and other terpenoid-quinore biosynthesis, protein processing in endoplasmic reticulum; as well as glutathione metabolism were remarkably suppressed ($P<0.05$) in the cecal microbiota in the ST-infected birds fed diets supplemented with *E. faecium* NCIMB 11181. Thus, dietary supplementation with *E. faecium* NCIMB 11181 affected important predicted functions of the intestinal microbiota of the ST-infected birds.

Discussion

Foodborne pathogens, such as *Salmonella* have been, for decades, a major concern for public health and environment. Decreasing the *Salmonella* burden in chicken flocks, is a promising strategy to promote food safety. Our results have found that dietary *Enterococcus faecium* can enhance cellular and humoral immune functions [32] and mitigated gut barrier impairment caused by *Clostridium perfrigens* [33]. However, whether dietary *E. faecium* NCIMB 11181 addition could be

helpful for controlling *Salmonella Typhimurium* infection and protecting intestinal health in broiler chickens remains unknown. In this research, the protective effects of *E. faecium* 11181 addition on intestinal health of broiler chickens challenged with *Salmonella Typhimurium* and its underlying action mechanisms were investigated. Our results showed that *Salmonella typhimurium* challenge caused a significant negative effect on broiler growth performance, which was similar to previous findings [5,53-55]. However, feeding *E. faecium* NCIMB 11181 had no remarkable growth-improving influences on chicken growth performance regardless of ST challenge. In contrast to our findings, some previous studies have showed that appropriate dose of probiotic *Enterococcus faecium* supplementation has positively affected broiler performance by increased weight gain or decreased FCR under non-challenged rearing environments [30,56-62]. Similarly, our previous results demonstrated that dietary supplementation of low dose of *E. faecium* NCIMB 11181 (50 mg/kg) remarkably improved growth performance, and high dose of *E. faecium* NCIMB 11181 (200 mg/kg) notably enhanced immunity of broiler chickens under non-challenged conditions [32]. Additionally, Cao et al. [37] and Mountzouris et al. [63] reported that *E. coli* K88-infected broiler chickens fed *Enterococcus faecium* showed increased growth performance, improved intestinal morphology and cecal microflora. Furthermore, our research group had reported that pretreatment with *E. faecium* NCIMB 11181 could alleviate the growth suppression caused by *Eimeria spp/ Clostridium perfringens* co-challenge in broilers [33] and *E.coli* O78-challenged birds [38]. The inconsistent results in growth performance was possible attribute to the difference in biological features and properties of probiotics strain *Enterococcus faecium*, additive amount of *E. faecium*, feeding schedules, age of broiler chickens, challenged or not, as well as type of pathogens used for infection. In view of results of growth performance in this study, we suggested that dietary *E. faecium* NCIMB 11181 addition (200 mg/kg) did not mitigate the adverse effects of *Salmonella typhimurium* on broiler growth performance.

Intestinal morphology (villus height, crypt depth and the VH/CD ratio), lesion scores, histopathological grades, bacterial colonization and translocation together with intestinal cell proliferative and apoptosis indices are important indicators of intestinal health, mucosal barrier function, integrity, permeability and recovery [64]. Additionally, intestinal epithelial cells apical junctional proteins including Claudins, Occludins, ZOs, junctional adhesion molecules (JAMs) and E-cadherins, also play a vital role in regulating intestinal permeability and maintaining gut barrier

integrity, defense pathogens infection and inflammation response [65]. In this study, histopathological and immunohistochemistry examination revealed that ST infection led to intestinal inflammation and gut wall impairment, as evidenced by extensive oedema and a thickened appearance associated with an influx of leukocytes in the ileum, atrophied villus height, longer crypt depths, reduced VH/CD ratio, increased TUNEL-positive cell numbers and higher *Salmonella* load in cecal content and liver, together with downregulated tight junction *CLDN-1, Occludin*, and *ZO-1* mRNA levels and upregulated *MLCK* mRNA level in the ileum in the single *Salmonella*-infected chickens. These observation was partially or totally in consistent with the results of previous studies [5,53,61,66,67] in chickens, suggesting that *Salmonella* infections induced the damage of intestinal morphology, promoted villus cells apoptosis, compromised the intestinal barrier integrity, and increased gut permeability of broiler chickens, thereby bacterial translocation. However, these adverse changes caused by ST were partially alleviated or abolished by including *E. faecium* NCIMB 11181 in the diet, thus indicating that *E. faecium* NCIMB 11181 addition could prevent *Salmonella typhimurium* intestinal colonization and proliferation, decrease intestinal inflammation scores, and limit liver invasion of intestinal *Salmonella*, which may be attributed to the alleviation of the mucosal atrophy and the inhibition of epithelial cell necrosis induced by ST challenge, and restoration of the integrity of the gut barrier. Consistent with our findings, other *Enterococcus faecium* strains addition were reported to have the ability to improve gut morphological structure or increase villus lengths and VH/CD ratios of broiler chickens reared under non-challenged conditions [57,58,60], and /or decrease gut gross lesions and histopathological scores, reduce intestinal pathogenic bacteria such as *C. perfringens* and *E. coli* numbers [59,68], preventing *Salmonella* colonization and invasion together with attenuated gut mechanical barrier injury in chicks challenged with *Salmonella* [34-36,55,69,70], *Clostridium perfringens* [33] and *Eimeria* [71]. Hence, it also can be concluded that the addition of the probiotic product *E. faecium* NCIMB 11181 can be effective in controlling *Salmonella* infection of chickens and restoring intestinal barrier integrity. This protective and anti-*Salmonella* action induced by probiotics *Enterococcus faecium* may be dependent on its strong antibacterial activity, and associated with its producing organic acids such as lactic acid and antimicrobial substances such as hydrogen peroxide, bacteriocin /enterocins and adhesion inhibitors [9,70,72].

Intestinal inflammation will lead to destruction of intestinal epithelial integrity, changes in the

expression of tight junction proteins, and decreased expression of tight junction proteins will aggravate intestinal inflammation. To elucidate clearly the mechanism by which dietary probiotic *E. faecium* NCIMB 11181 supplementation affects intestinal barrier function and health, we further evaluated the changes in the intestinal mucosal toll-like receptors (TLR) and their downstream targets along with humoral immune responses in ST-infected broiler chickens. TLR-mediated signaling pathways are involved in regulating intestinal mucosal immune defense and epithelial barrier integrity as well as maintaining maintain mucosal and commensal homeostasis [73]. Overexpression of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-1β have been reported to increase intestinal permeability and tissue damage via the dysregulation of TJPs [74,75], while various regulatory peptides including anti-inflammatory cytokines (TGF-β, IL-4 and IL-10), growth factors (EGF, GLP-2 and IGF-2), negative regulators (Tollip, PI3K, and A20) of the TLR signaling pathway have been demonstrated to protect intestinal barrier function by regulating tight junction expression and facilitating the repair of damaged gut tissue [76]. In this study, our results revealed that infection with ST remarkably /significantly upregulated or changed intestinal cytokines expression profiles, as indicated by not only upregulating *MyD88, NF-κB, IFN-γ, pIgR*, and the negative regulators (*Tollip* and *PI3K*) mRNA levels; but also showing an increased trend for *TLR4* and *TNF-α* in the gut of the infected chickens at the early stage of infection; which was in similar with observations of previous studies in chickens [4,54,77-83]. Meanwhile, intestinal mucosa anti-*Salmonella* IgA and serum anti-ST specific IgG levels were significantly elevated following ST infection in broiler chickens, which was in consistent with previous results [3,54]. Our above observation showed that *Salmonella* infection not only triggered intestinal local inflammation by over-activating TLR4-mediated inflammatory signal pathway, thereby causing the disruption of intestinal barrier structure and the increase of gut permeability, resulting in intestinal *Salmonella* translocation into liver and spleen as well as systemic inflammatory response. Interestingly, the present study found that *E. faecium* NCIMB 11181 addition only upregulated *pIgR* and *Tollip* mRNA expression level, but did not alter other genes expression profiles in the ileum of TLR-related signal pathway regardless of *Salmonella* infection when compared with the non-supplemented group birds. On the contrary, results from some studies have demonstrated that inclusion of *E. faecium* in the diet remarkably altered the genes expression profiles of intestinal TLR-mediated signal pathway and its downstream molecules in gut tissues when subjected to pathogen challenge in chickens [33-

38,69,71]. The discrepancy of these findings was attributed to the variation in chicken age, strains and administration dose of probiotics *E. faecium*, strains type and virulence of challenged *Salmonella*, and the post-infection sampling time-point and sampled tissues. The accurate reason for these difference should need to further be investigated. *Tollip* is a negative modulator which can suppress activation of *TLR*-related signal pathway. Increased *Tollip* expression in the ileum of the *Salmonella* pathogen challenged chickens treated with *E. faecium* NCIMB 11181 indicated that *E. faecium* 11181 had the capability to prevent exacerbated gut inflammatory reaction caused by *Salmonella* and suppress intestinal inflammatory responses through the inhibition of *TLR* signal pathway over-activation. Additionally, administration of *E. faecium* NCIMB 11181 promoted *Salmonella*-specific IgA production in intestinal mucosa of *Salmonella*-infected broiler chickens. Similarly, Beirao et al. [34] reported that in-feed *E. faecium* NCIMB 10415 probiotic increased the production of *Salmonella*-specific mucosal IgA following immunization with an attenuated *S. Enteritidis* vaccine in laying hens. Here, our results suggested that *E. faecium* NCIMB 11181 administration could promote specific secretary IgA secretion in the gut when subjected to *Salmonella* challenge. Secretory IgA (sIgA) and its transcytosis receptor, polymeric immunoglobulin receptor (*pIgR*), along with mucus form the first lines of intestinal mucosal defenses, mainly defensing or neutralizing pathogenic bacteria and enteric toxins [4,79,80,84,85]. In this study, increased intestinal *pIgR* expression here may mean more mucosal secretory IgA antibody production in the gut, which help in reducing cecal *Salmonella* load and facilitating *Salmonella* elimination from the gut lumen during the recovery phase of infection. Higher sIgA levels in the ileum together with lower *Salmonella* burden in the intestine and liver in the infected chickens fed the diets with *E. faecium* NCIMB 11181, support our notion that feeding *E. faecium* 11181 had ability to provide protection against *Salmonella* infection and decrease the disease severity of *Salmonella* infection through upregulating *pIgR* gene expressions and enhancing specific sIgA production. Collectively, elevated expressions of the Tollip and pIgR gene along with abundant sIgA content here may also confirmed that the inclusion of *E. faecium* NCIMB 11181 in chicken's diet could reveal the protective and anti-inflammatory potential when broiler chickens were confronted with *Salmonella* infection. Above these changes further showed that pretreatment with probiotic *E. faecium* NCIMB 11181 could alleviate *Salmonella*-induced intestinal injury, possibly attributable to inhibiting intestinal inflammation or differentially regulating intestinal mucosal

immune responses as well as promoting humoral immunity of chickens.

The chicken gastrointestinal tract is colonized by trillions of microorganisms, constituting a dynamic ecosystem with significant impacts on host metabolism, productivity, immune responses and health status including gut health. Consequently, modulation of the gut microbiota and modification of the intestinal microenvironment could assist in preventing animal colonization by the pathogen [1]. More importantly, changes in gut microbe populations may be closely related to the degree of intestinal inflammation and disease resistance, which is one of the characteristics of *S. typhimurium* infection [1,85-87]. In the current study, our results revealed that neither *S. Typhimurium* infection nor dietary probiotics *E. faecium* NCIMB 11181 treatments significantly altered α-diversity of chicken caecal microbiota, indicated that the caecal microbiota diversity remained relatively stable. Nevertheless, *S.* Typhimurium infection significantly modified the indigenous microbiota composition and relative abundance of some bacterial species in the cecum of chickens, as showed by increasing relative abundance of *Barnesiella,* whereas decreasing *Alistipes* abundances. In similar to our findings, some previous studies also reported that *S. Typhimurium* infection disturbed microbial composition of gut microbiota of chickens, as evidenced by expanding relative abundance of potential harmful bacteria such as *Enterobacteriaceae*, whereas decreasing potential beneficial bacteria including (i.e., Butyrate-producing bacterira *Lachnospiracease*, *Bifidobacterium* and *Lactobacillus*) abundances [88,89]. Thus, above findings showed that *S. Typhimurium* infection disrupted microbial composition of gut microbiota of chickens, besides impairment in intestinal barrier structure. Additionally, taxonomic analysis showed that *E. faecium* NCIMB 11181 addition enriched the relative abundance of the phylum *Firmicutes* and the genera *Alistipes* while suppressed the relative population of the genera *Barnesiella* of the cecal microbiota of the infected birds when comparing with the single *S. Typhimurium* infected control. LEFsE analysis also indicated /highlighted that the infected birds received *E. faecium* NCIMB 11181 showed higher abundance in the genus *Lachnospiracease*, A*listipes*, Rikenellaceae family and *Lachonclostridum*, which was similar to the changes of intestinal microbial communities of the non-infected control compared with the single ST-infected control. Almost similar to our observations, but not completely consistent, Wang et al. [62] reported that dietary supplementation with *E. faecium* changed the gut microbiota populations of broilers, as showed by an increase in the relative abundance of the following genera: *Alistipes*, *Eubacterium*,

Rikenella and *Ruminococcaceae,* and a decrease in the relative abundance of *Faecalibacterium* and *Escherichia-Shigella,* which was in consistent with Bednorz et al' findings in piglets fed with the probiotic *E. faecium* NCIMB 11181 [21]. Similarly, treatment with an attenuated *Salmonella* enterica serovar Typhimurium strain resulted in a significant increase of the genus *Alistipes* and *Lactobacillus* in chickens, along with a faster clearance after *Salmonella* infection [1]. Out study demonstrated that *E. faecium* NCIBM 11181 treatment modified the structure of the gut microbiome of the *Salmonella*-infected chickens. *Alistipes* is a relatively recent sub-branch genus of the *Bacteroidetes* phylum, which are highly relevant in dysbiosis and disease, and can be either beneficial or harmful roles in human clinical studies. Intriguingly, some studies have shown that *Alistipes* may have protective effects against some diseases, including liver fibrosis, inflammatory colitis, cancer immunotherapy, and cardiovascular disease [90]. Bacterial species *Alistipes finegoldii* had been associated with a low food conversion rate (FCR) in broilers [91]. Butyrate-producting bacteria *Lachnospiraceae* was reported to be positively correlated with good FCR performance and gut health [92], and an increase in its abundance has shown to limit expansion of aerobic enteric pathogens, reduce inflammatory diseases and prevent gut barrier dysfunction in HFD-induced metabolic endotoxemia and systemic chronic lower-grade inflammation mice [93,94]. The genus *Barnesiella*, an obligate anaerobic bacteria, one genus of the *Porphyromonadaceae* family, were negatively linked with anti-inflammatory responses but strongly correlated with proinflammatory responses in chickens. Thus, higher proportion of *Lachnospiracease*, A*listipes* and lower abundance of *Barnesiella*, accompanied by reduced *Salmonella* colonization and invasion in the cecum of *Salmoenlla*-challenged broiler chickens following *E. faecium* NCIMB 11181 administration, suggesting that pretreatment with probiotics *E. faecium* NCIMB 11181 competitively exclude *Salmonella* infection and promotes intestinal health, possibly via modulating gut microbiome or increasing the growth of potential gut healthy bacteria or restoring intestinal microbiota hemostasis. These data also indicated that the anti-inflammatory and barrier-protecting effects of *E. faecium* NCIMB 11181 is possibly associated with the improvement of intestinal microbiome and microenvironment.

PICRUSt analysis reveled the enrichment of functional genes related with C5-branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; methane metabolism; glycerolipid metabolism and lysine biosynthesis; while depletion of genes involved in alanine, asparate and

glutamate metabolism; RNA degradation, transcription machinery; MAPK signal pathway-yeast; ubiquine and other terpenoid-quinore biosynthesis, protein processing in endoplasmic reticulum; as well as glutathione metabolism in response to *E. faecium* NCIMB 11181addition in gut microbiome of the *Salmonella*-infected chickens, suggested that functional changes of intestinal microbiota induced by *Salmonella* infection could altered by probiotics *E.* faecium NCIMB 11181. Amino acids metabolism was closely related to metabolic disorder, especially during pathogen infection. Amino acids supply was associated with energy supply, immune regulation and damage repair of gut cells, especially under challenge conditions [95,96]. *Salmonella* infection induced up-regulation of glycolytic process and the catabolism of amino acids at the middle and later of infection, resulting in exhaustion of energy and amino acids in chickens [97]. Such increase in amino acids biosynthesis and C5-branched dibasic acid metabolism might suggest that feeding probiotics *E. faecium* NCIMB 11181 to *Salmonella*-infected chickens potentially promoted amino acids biosynthesis processes of intestinal microbe, thereby contributing to energy supply of gut cells and repairmen of gut barrier impairment as well as dampening of *Salmonella*-induced intestinal inflammatory responses in broiler chickens. Asparate and glutamate metabolism, glutathione metabolism and ubiquinone biosynthesis, protein processing in endoplasmic reticulum, and other terpenoid-quinore biosynthesis reported to be involved in host nucleotide synthesis, energy metabolism of mitochondria, and redox status. Overactivation of these pathways meant that host was being exposed to stress stimulus and in a state of the imbalance of redox, resulting in oxidative stress. We further identified that inhibition of MAPK signal pathway-yeast by *E. faecium* NCIMB 11181 might also contribute to prevention of *Salmonella*-induced intestinal inflammation and barrier dysfunction. Hence, the increased amino acids biosynthesis, and the decreased MAPK signal pathway and redox pathway suggested that *E. faecium* NCIBM 11181 may play a role in alleviating *Salmonella*-induced intestinal inflammation by regulating gut microbiome, which in turn affects amino acids biosynthesis, redox pathway metabolism and MAPK signal pathway. Further experiments would be essential to confirm this possibility.

Conclusion

In summary, *Salmonella* Typhimurium infection induced intestinal inflammation, resulting in gut barrier injury and bacterial translocation in broiler chickens by disrupting the balance of gut

microbiota, activating TLR4-mediated immune inflammatory responses, increasing gut cells apoptosis index, damaging gut morphological structure, together with downregulating tight junction proteins *Claudin-1, Occludin* and ZO-1 mRNA levels. Moreover, continuous feeding *Enterococcus faecium* NCIMB 11181 could protect broiler chickens from *Salmonella*-induced intestinal inflammation and mucosal barrier impairment through modulating gut microbiota composition and functions, decreasing the *Salmonella* colonization and invasion, inhibiting TLR4-mediated inflammatory responses through upregulating the expression of negative regulatory molecules, promoting intestinal specific anti-*Salmonella* IgA production, along with inhibiting intestinal cells apoptosis. The results provide new information on the critical role played by dietary *Enterococcus faecium* NCIBM 11181 in controlling *Salmonella* infection in broiler chickens.

DATA AVAILABILITY STATEMENT

This study's microbial raw sequencing data can be found in the NCBI Sequence Read Archive database.

ETHICS STATEMENT

The animal study was reviewed and approved by all experimental protocols of the Animal Care and Use Committee of China Agricultural University (CAU-2018-006) and carried out in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

Z.W. conceptualized the project and designed the study. R. Z., F. G. and Y. S. contributed to performing animal experiments, analyzing samples, doing statistical analysis, and preparing the original manuscript draft. Y. G. and Z. W. made insightful edits for the manuscript. All authors reviewed the manuscript and approved the final submission.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

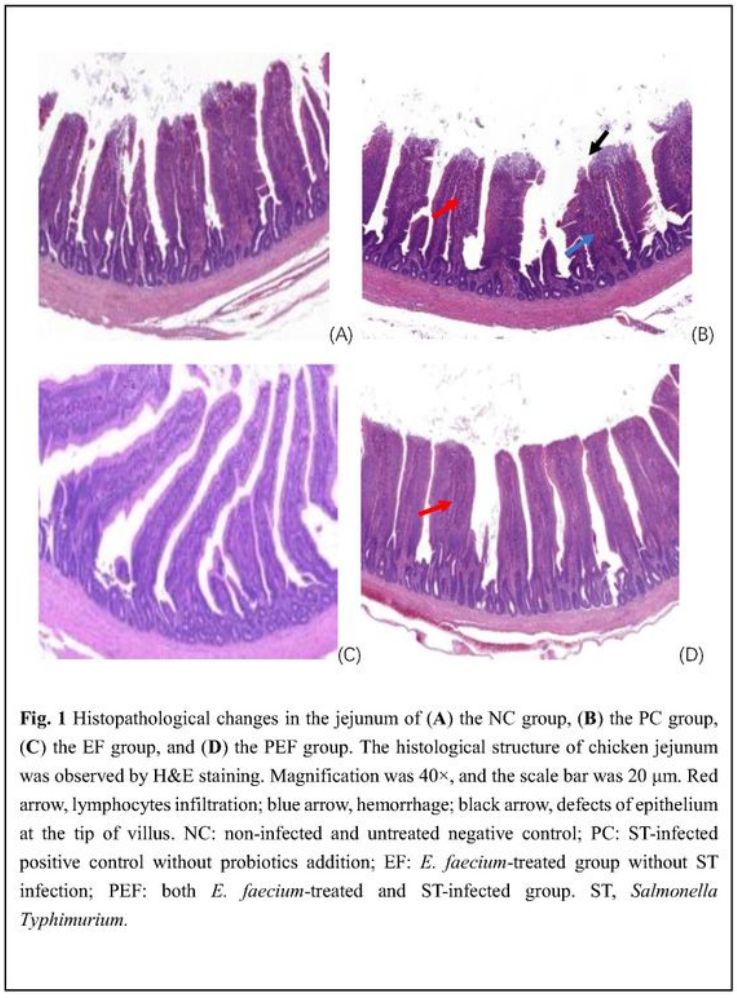

Fig. 1 Histopathological changes in the jejunum of (**A**) the NC group, (**B**) the PC group, (**C**) the EF group, and (**D**) the PEF group. The histological structure of chicken jejunum was observed by H&E staining. Magnification was 40×, and the scale bar was 20 μm. Red arrow, lymphocytes infiltration; blue arrow, hemorrhage; black arrow, defects of epithelium at the tip of villus. NC: non-infected and untreated negative control; PC: ST-infected positive control without probiotics addition; EF: *E. faecium*-treated group without ST infection; PEF: both *E. faecium*-treated and ST-infected group. ST, *Salmonella Typhimurium*.

Figure 1

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Figure 2

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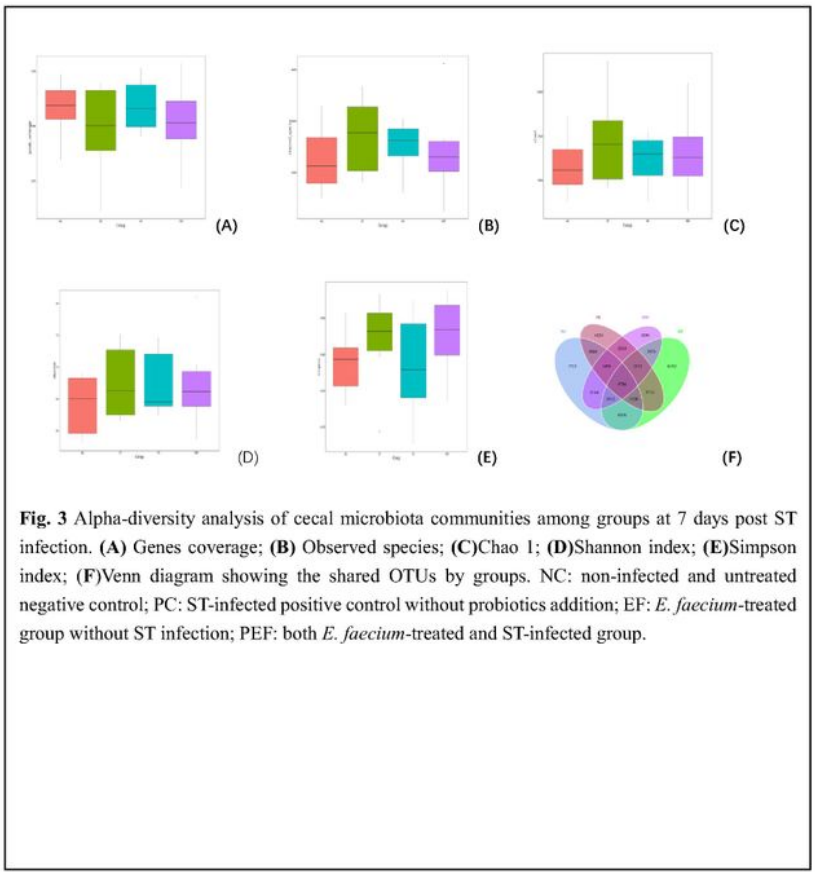

Fig. 3 Alpha-diversity analysis of cecal microbiota communities among groups at 7 days post ST infection. **(A)** Genes coverage; **(B)** Observed species; **(C)**Chao 1; **(D)**Shannon index; **(E)**Simpson index; **(F)**Venn diagram showing the shared OTUs by groups. NC: non-infected and untreated negative control; PC: ST-infected positive control without probiotics addition; EF: *E. faecium*-treated group without ST infection; PEF: both *E. faecium*-treated and ST-infected group.

Figure 3

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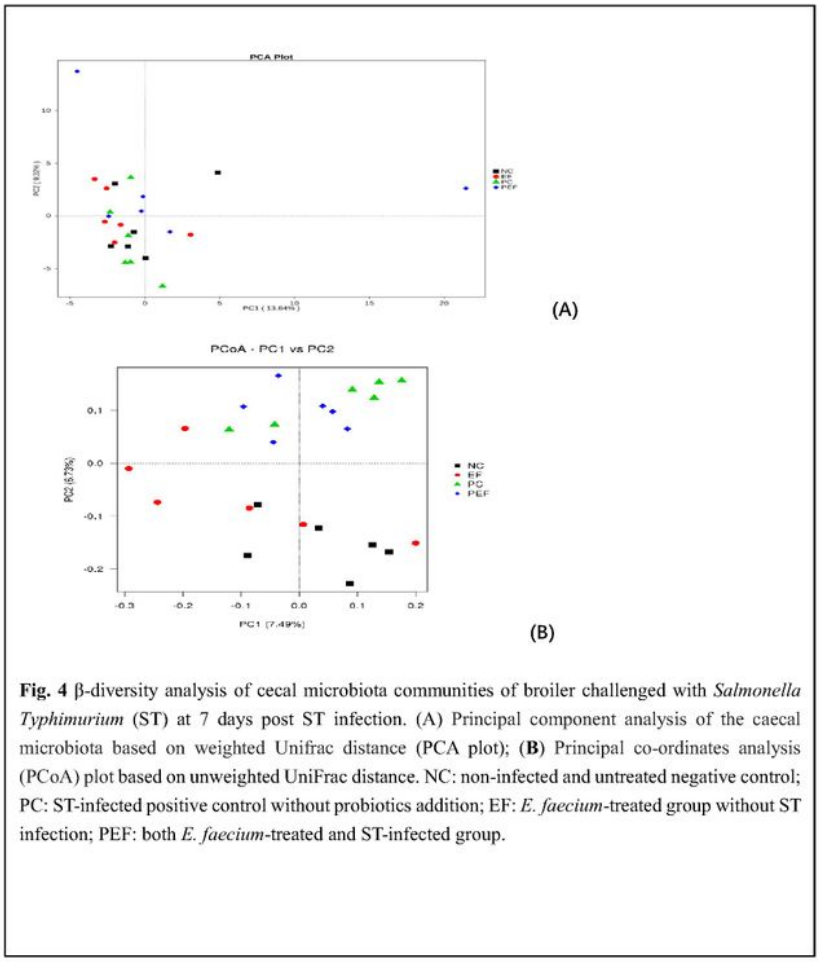

Fig. 4 β-diversity analysis of cecal microbiota communities of broiler challenged with *Salmonella Typhimurium* (ST) at 7 days post ST infection. (A) Principal component analysis of the caecal microbiota based on weighted Unifrac distance (PCA plot); (**B**) Principal co-ordinates analysis (PCoA) plot based on unweighted UniFrac distance. NC: non-infected and untreated negative control; PC: ST-infected positive control without probiotics addition; EF: *E. faecium*-treated group without ST infection; PEF: both *E. faecium*-treated and ST-infected group.

Figure 4

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Figure 5

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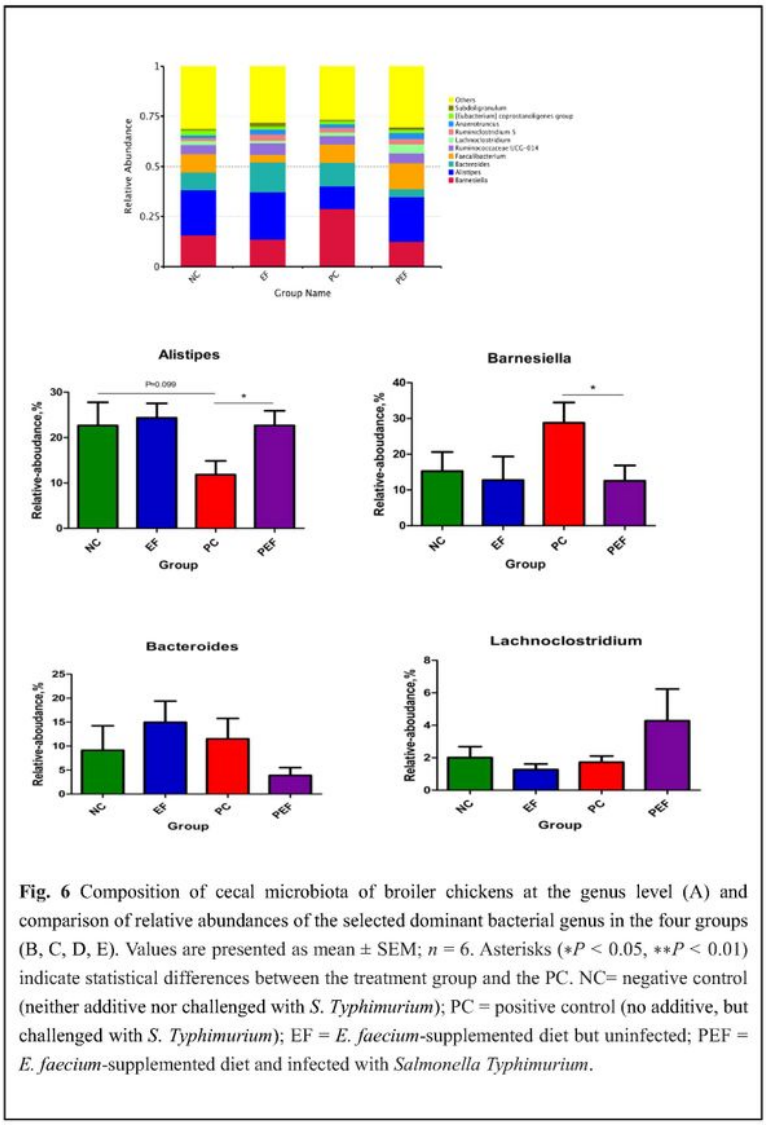

Fig. 6 Composition of cecal microbiota of broiler chickens at the genus level (A) and comparison of relative abundances of the selected dominant bacterial genus in the four groups (B, C, D, E). Values are presented as mean ± SEM; $n = 6$. Asterisks ($*P < 0.05$, $**P < 0.01$) indicate statistical differences between the treatment group and the PC. NC= negative control (neither additive nor challenged with *S. Typhimurium*); PC = positive control (no additive, but challenged with *S. Typhimurium*); EF = *E. faecium*-supplemented diet but uninfected; PEF = *E. faecium*-supplemented diet and infected with *Salmonella Typhimurium*.

Figure 6

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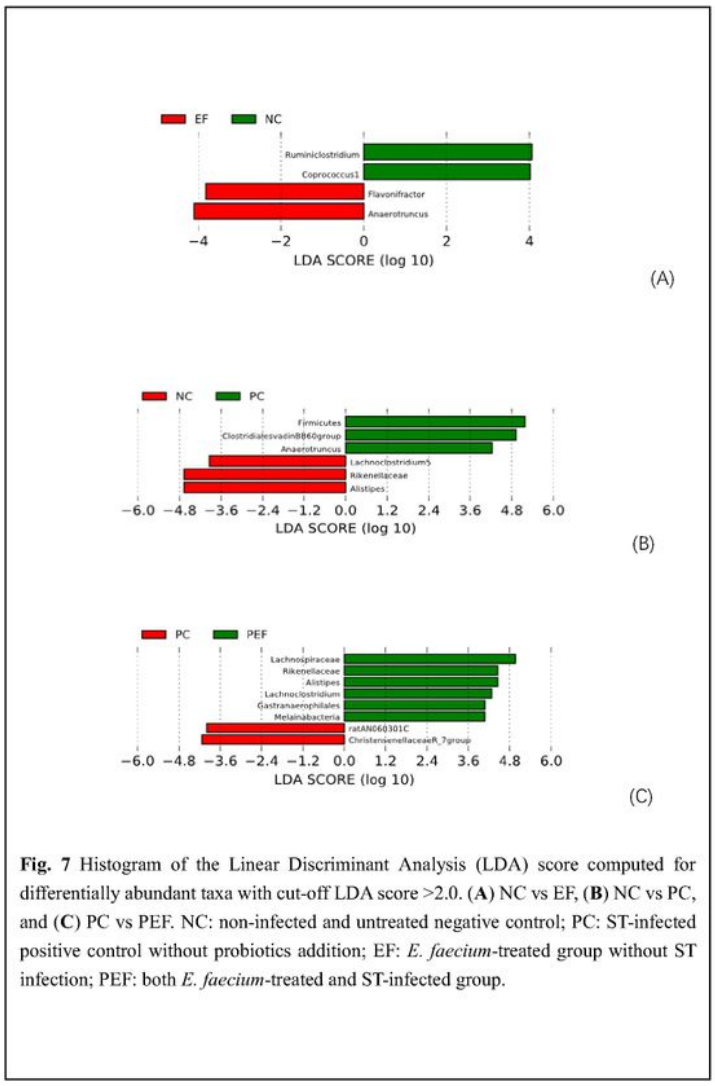

Fig. 7 Histogram of the Linear Discriminant Analysis (LDA) score computed for differentially abundant taxa with cut-off LDA score >2.0. (**A**) NC vs EF, (**B**) NC vs PC, and (**C**) PC vs PEF. NC: non-infected and untreated negative control; PC: ST-infected positive control without probiotics addition; EF: *E. faecium*-treated group without ST infection; PEF: both *E. faecium*-treated and ST-infected group.

Figure 7

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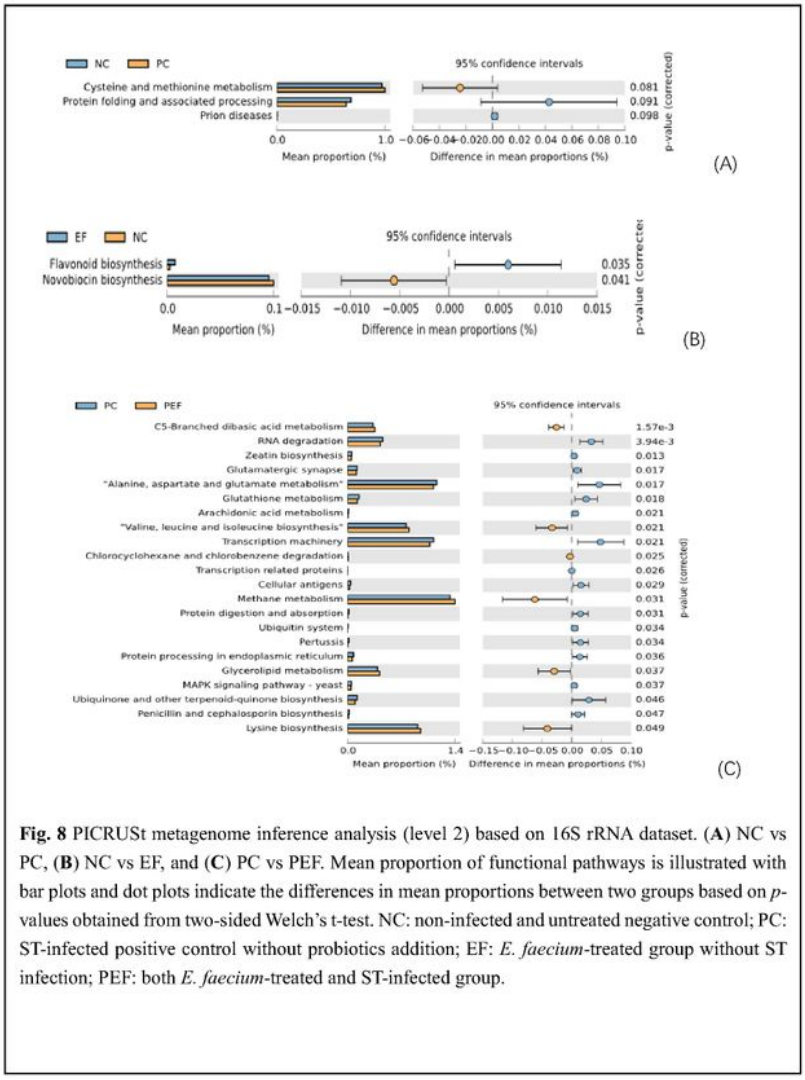

Fig. 8 PICRUSt metagenome inference analysis (level 2) based on 16S rRNA dataset. (**A**) NC vs PC, (**B**) NC vs EF, and (**C**) PC vs PEF. Mean proportion of functional pathways is illustrated with bar plots and dot plots indicate the differences in mean proportions between two groups based on *p*-values obtained from two-sided Welch's t-test. NC: non-infected and untreated negative control; PC: ST-infected positive control without probiotics addition; EF: *E. faecium*-treated group without ST infection; PEF: both *E. faecium*-treated and ST-infected group.

Figure 8

See image above for figure legend.

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