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Bacillus subtilis HW2 enhances growth performance and alleviates gut injury via attenuation of endoplasmic reticulum stress and regulation of gut microbiota in broilers under necrotic enteritis challenge

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ABSTRACT This study investigated the effects of *Bacillus subtilis* HW2 on the growth performance, immune response, endoplasmic reticulum (ER) stress, and intestinal health in broilers with necrotic enteritis. Three hundred 1-day-old male Cobb 500 broilers (33.88 ± 2.34 g) were randomly allocated to 5 groups including non-infected control (NC group), basal diet + necrotic enteritis challenge (NE group), basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge (L-Pro group), basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge (M-Pro group), and basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge (H-Pro group), with 6 replicates per group. All broilers except NC group were orally given with sporulated coccidian oocysts at day 14 and *Clostridium perfringens* from days 19 to 21. Results showed that L-Pro and M-Pro groups improved growth performance and intestinal morphology in necrotic enteritis-challenged broilers, and L-Pro, M-Pro, and H-Pro groups improved intestinal barrier function and immune response and decreased ER stress in necrotic enteritis-challenged broilers. Analysis of the gut microbiota revealed that L-Pro group increased the abundances of *Alistipes, Coprobacter, Barnesiella*, and *Limosilactobacillus*, decreased *Erysipelatoclostridium* abundance on day 42 in necrotic enteritis-challenged broilers. M-Pro group increased *Turicibacter* abundance on day 28 and the abundances of *Alistipes, Barnesiella*, and *Limosilactobacillus* on day 42 in necrotic enteritis-challenged broilers. H-Pro group decreased *Romboutsia* abundance on day 28 and *unidentified_Clostridia* abundance on day 42 in necrotic enteritis-challenged broilers. Analysis of short-chain fatty acids (**SCFAs**) revealed higher isobutyric acid and isovaleric acid levels in L-Pro and M-Pro groups than NE group. Correlation analysis revealed the correlations between the biochemical parameters and gut microbiota as well as SCFAs, especially *Romboutsia, Barnesiella, Coprobacter,* isobutyric acid, and isovaleric acid. Overall, our results indicated that ***B. subtilis* HW2 supplementation could ameliorate necrotic enteritis infection-induced gut injury. The optimal dietary supplementation dosage of *Bacillus subtilis* HW2 was 5×10^6 CFU/g.**

Key words: *Bacillus subtilis,* broiler, necrotic enteritis, endoplasmic reticulum stress, gut microbiota

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INTRODUCTION

Necrotic enteritis is a common intestinal bacterial disease in broilers, which is mainly caused by the conditional pathogenic bacteria *Clostridium perfringens* (Mehdizadeh et al., 2021) and often associated with one or more factors such as *Eimeria* and high-protein fish meal (Rajput et al., 2020; Khan et al., 2021). Necrotic enteritis causes intestinal injury, leading to decreased growth performance and elevated mortality in broilers (Coles et al., 2021). The onset of necrotic enteritis is closely linked to a shift in the gut microbiota (Stanley et al., 2012; Antonissen et al., 2016). After *C. perfringens* and *Eimeria maxima* challenge, the abundance of *Clostridiales* significantly increased, but the abundances of *Bacillales* and *Lactobacillaceae* decreased in broilers (Kim et al., 2015; Huang et al., 2019). In addition, the abundances of *Lactobacillus reuteri, Subdoligranulum variable, Mediterraneibacter, Straphylococcus, Coryne-bacterim*, and *Kocuria* were progressively declined as necrotic enteritis was aggravated in broilers (Yang et al., 2021). Short chain fatty acids (**SCFAs**) are the primary end-products of gut microbiota fermentation of nondigestible carbohydrates. Studies showed that necrotic enteritis decreased the abundance of butyrate-producing

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Ruminococcaceae family and the levels of acetate and butyrate in cecal content of broilers (Antonissen et al., 2016; Kheravii et al., 2018; Kumar et al., 2022).

Endoplasmic reticulum (**ER**) stress plays crucial roles in the pathogenesis of necrotic enteritis, as it can activate nuclear factor kappa B (**NF-κB**) through inositol-requiring enzyme 1α/tumor necrosis factor receptor associated factor 2 (**IRE1α/TRAF2**) signaling pathway to regulate the secretion of inflammatory factors (Keestra-Gounder et al., 2016; Mora et al., 2020). Recent studies have indicated that there are relationships between ER stress and gut microbiota or their metabolites (Eugene et al., 2020; Ke et al., 2021). Wang et al. (2022a) revealed that transplantation of fecal microbiota from amyloid precursor protein/presenilin 1 mice and Alzheimer's disease patients enhanced ER stress in the cerebral cortex of wild-type mice. Host-microbiota crosstalk via epithelial-specific ER stress sensor ERN2/IRE1β effects development of colon goblet cells (Grey et al., 2022). Therefore, decrease ER stress by regulation of the gut microbiota and SCFAs seems to be a way to attenuate necrotic enteritis.

Probiotics are widely used to benefit the intestinal health by regulating the gut microbiota (Li et al., 2021). *Bacillus subtilis* have garnered attention due to their higher resistance to harsh environments and ability to be stored for long periods at ambient temperature (Gao et al., 2017). Previous findings demonstrated that *B. subtilis* improved growth performance and intestinal inflammation by increasing the abundances of beneficial commensal microorganisms and related metabolites in broilers with necrotic enteritis (Liu et al., 2021b; Wang et al., 2021b). Our previous works found a probiotic, *B. subtilis* HW2, isolated from intestinal mucosa of healthy broilers (Qingdao, China), which had anti-*C. perfringens* activities in vitro and could protect primary chicken intestinal epithelial cells against *C. perfringens* infection (unpublished data in Table S1 and Figure S1). However, the effects of *B. subtilis* HW2 on the growth performance and intestinal health of broilers with necrotic enteritis need to be further determined. Therefore, we evaluated the effects of *B. subtilis* HW2 on the growth performance, immune response, and intestinal health of broilers with necrotic enteritis. Furthermore, ER stress, gut microbiota, and SCFAs were analyzed to clarify the underlying mechanisms.

MATERIALS AND METHODS

Materials

The genomic sequence of *Bacillus subtilis* HW2 was deposited into China General Microbiological Culture Collection Center database under accession CGMCC 26159. Cryopreserved *B. subtilis* HW2 was activated and cultured in liquid beef extract peptone medium (Haibo Biotechnology Co., Ltd., Qingdao, China) at 37° C for 16 h, and was diluted until the concentration of bacteria reached to required concentration. We purchased a NetB toxin-positive type A *C perfringens* (CVCC 2030) strain that derived from the intestine of broiler with clinically diagnosed with necrotic enteritis from China Institute of Veterinary Drug Control (Qing et al., 2017). The preparation of *C. perfringens* was conducted following the methods by McReynolds et al. (2004). Four *Eimeria* species (*Eimeria tenella, Eimeria necatrix, Eimeria acervuline,* and *Eimeria maxima*) were supplied by the Laboratory of veterinary parasitology at Qingdao Agricultural University, China.

Animals and Experimental Design

The study protocol was approved by Institutional Animal Care and Use Committee of Qingdao Agricultural University (No. 20220506062). A total of 300 1-day-old male Cobb 500 broilers (33.88 ± 2.34 g) were randomly divided into 5 groups consisting of 6 replicate cages of ten broilers each. The duration of the feeding period was 42 d. The 5 groups included noninfected control (NC group), basal diet + necrotic enteritis challenge (NE group), basal diet + 1×10^6 CFU/g *B subtilis* HW2 + necrotic enteritis challenge (L-Pro group), basal diet + 5×10^6 CFU/g *B subtilis* HW2 + necrotic enteritis challenge (M-Pro group), and basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge (H-Pro group). The basal diet was formulated in accordance with the nutritional requirements based on the management guideline of Cobb 500 broiler (Cobb-Vantress, 2022) (Table 1), and contained no coccidiostats or antibiotics. The broilers in the L-Pro, M-Pro, and H-Pro groups were given diets containing *B. subtilis* HW2 daily during days 0 to 42. According to the method of Pham et al. (2020), on day 14, all broilers except NC group were orally given 1 mL saline solution containing 4×10^4 sporulated oocysts (*Eimeria acervuline*, 1×10^4; *Eimeria tenella*, 1×10^4; *Eimeria maxima*, 1×10^4; *Eimeria necatrix*, 1×10^4), and broilers in NC group were orally given with the same amount of saline solution. From days 19 to 21, all broilers in necrotic enteritis challenge groups were daily given orally with 1 mL of freshly prepared *C. perfringens* CVCC 2030 at 4×10^8 CFU/mL, and the broilers in NC group were orally given the same amount of normal sterile medium. The temperature and lighting conditions were managed according to the Cobb Broiler Management Guide (Cobb-Vantress, 2022).

Growth Performance

Feed intake and body weight was recorded on days 0, 14, 28, and 42. The mortality of broilers was recorded daily. Average daily gain (**ADG**), average daily feed intake (**ADFI**), and the feed/gain ratio (**F/G**) were calculated based on a replicate basis.

Chemical Analysis

Metabolizable energy was measured according to Larbier and Leclercq (1994). The crude protein, calcium,

Table 1. Ingredients and nutrient composition of the basal diet (air-dry basis) %.

Items	D 0−14	D 14−28	D 28−42
Ingredients			
Corn	59.27	63.17	65.27
Soybean meal	33.83	29.43	27.10
Soybean oil	2.69	3.50	3.99
Limestone	2.00	1.80	1.60
$CaHPO_4$	0.50	0.35	0.30
NaCl	0.30	0.30	0.30
DL-methionine	0.18	0.18	0.20
L-lysine HCl	0.16	0.20	0.20
L-threonine	0.07	0.07	0.04
Premix[1]	0.50	0.50	0.50
Multivitamin[2]	0.50	0.50	0.50
Total	100	100	100
Nutrient composition			
Metabolizable energy, kcal/kg [3]	2966	3047	3095
Crude protein, % [4]	21.58	19.83	18.88
Calcium, % [4]	0.99	0.86	0.76
Available phosphorus, % [3]	0.61	0.46	0.41
Lysine, % [4]	1.28	1.18	1.12
Methionine, % [4]	0.52	0.50	0.51
Threonine, % [4]	0.87	0.80	0.74
Tryptophan, % [4]	0.27	0.24	0.23

[1]Per kilogram of dietary supply: iron, 40 mg; manganese, 90 mg; zinc, 75 mg; copper, 8 mg; iodine, 0.20 mg; and selenium, 0.40 mg.

[2]Per kilogram of dietary supply: vitamin A (trans-retinyl acetate), 7000 IU; vitamin B_1 (thiamin), 0.50 mg; vitamin B_2 (riboflavin), 8 mg; vitamin B_6 (pyridoxine HCl), 4 mg; vitamin B_{12} (cobalamin), 0.02 mg; vitamin D_3 (cholecalciferol), 4,000 IU; vitamin E (all-rac-α-tocopherol acetate), 70 IU; vitamin K_3 (menadione), 3 mg; nicotinic acid, 35 mg; calcium pantothenate, 8.50 mg; folic acid, 2 mg; and biotin, 0.05 mg.

[3]Calculated values.

[4]Measured values.

and amino acid contents in the basal diet was measured according to the methods described by Association of Official Analytical Chemists 2001.11, 968.08, and 999.13, respectively. The crude protein was determined using the auto Kjeldahl nitrogen analyzer (Kjeltec 8400, Foss Co., Ltd., Hilleroed, Denmark). Calcium was measured by an atomic absorption spectrometer (AA6300, Hitachi High-Technologies Corporation, Tokyo, Japan). Tryptophan was determined using a high-performance liquid chromatograph (Aligent 1200 Series, Agilent Technologies, Inc., Santa Clara, CA). Lysine, methionine, and threonine were determined using an amino acid analyzer (LA8080, Hitachi High-Technologies Corporation).

Samples Collection

On days 28 and 42, one broiler was randomly selected from each replicate, fasted for 12 h, and blood samples were taken from the jugular vein into vacuum tubes and centrifuged (10 min, 3,000 × *g*, 4°C). Serum samples were stored at −20°C. Then the selected broiler was killed via cervical dislocation. The lesions of duodenum, jejunum, and ileum were analyzed. The samples of jejunum and ileum were preserved in a 4% formaldehyde solution. The ileal specimens were preserved in a 2.5% glutaraldehyde solution. Ileal mucosa and cecal contents were collected and stored at −80°C.

Eimeria Oocyst Counts and Intestinal Lesion Scores

Each replicated broilers excreta samples were collected on day 19 (day 5 postchallenge) and the oocysts per gram of excreta (**OPG**) calculated according to the method of Youn and Noh (2001). On days 28 and 42, small intestinal lesions were visually examined and scored using according to the method of Johnson and Reid (1970).

Intestinal Morphology

Fixed intestinal segments in 4% formaldehyde were followed by embedding in paraffin wax, sliced into sections, stained with hematoxylin and eosin, and imaged under the microscope (Eclipse 80i, Nikon Inc., Tokyo, Japan). We measured villus height from the tip to the base, crypt depth from the base to the mucosa, and calculated the villus height/crypt depth ratio (**V/C**). Ileal specimens (1 mm^3) were preserved in 2.5% glutaraldehyde solution and sliced into ultrathin sections, which were stained with uranyl acetate and imaged using transmission electron microscope (HT7700, Hitachi High-Technologies Corporation).

Analysis of D-Lactic Acid, Diamine Oxidase (DAO), and Immune-Related Indexes

Serum D-lactic acid and DAO levels, as well as interleukin (**IL**)-1β, IL-8, IL-10, IL-17, tumor necrosis factor-α (**TNF-α**), interferon-γ (**IFN-γ**), and secretory immunoglobulin A (**sIgA**) levels in ileal mucosal were measured using enzyme-linked immunosorbent assay kits (Meimian Industrial Co., Ltd., Yancheng, China). Protein concentration in ileum mucosa was measured using the enhanced BCA protein assay kit (Biyuntian Biotechnology Co., Ltd., Shanghai, China), and the results were expressed as per milligram of protein.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

The ileal mucosa RNA samples were extracted via the Trizol reagent (Tiangen Biochemical Technology Co., Ltd., Beijing, China), which was subsequently assessed for purity and concentration at 260/280 nm. Moloney murine leukemia virus reverse transcriptase (Accurate Bio-Medical Technology Co., Ltd., Hunan, China) was used to reverse-transcribe 1 g of RNA into cDNA. Each RT-qPCR reaction included nuclease-free water, gene-specific forward and reverse primers of each gene, cDNA, and TB Green Premix Ex Taq mix (Takara Biotechnology Co., Ltd., Dalian, China). The primers for RT-qPCR analysis were designed based on sequences retrieved from the GenBank database (Table S2). A denaturation stepped at 95°C for 30 s was followed by 40 cycles at 95°C for 5 s and 60°C for 30 s during the RT-qPCR process. The relative mRNA levels of the

target genes normalized to β-actin were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western Blot Analysis

Total proteins were extracted from ileal mucosa using RIPA lysis buffer (Biyuntian Biotechnology Co., Ltd.). Proteins were separated from each sample in equal amounts by electrophoresis using premade agarose gels (Solarbio Biotechnology Co., Ltd., Beijing, China) and then electroblotted onto nitrocellulose membranes, and blocked with 5% skimmed milk (Biyuntian Biotechnology Co., Ltd.) and probed with primary antibodies against β-actin, zonula occludens-1 (**ZO-1**), occludin, glucose-regulated protein 78 (**GRP78**), phosphor-protein kinase R-like endoplasmic reticulum kinase (**PERK**), p-PERK, activating transcription factor 6 (**ATF6**), IRE1, p-IRE1, TRAF2, p65, and p-p65 overnight at 4°C. The β-actin, ZO-1, and occludin were obtained from Servicebio Technology Co., Ltd. (Wuhan, China), and the GRP78, PERK, p-PERK, ATF6, IRE1, p-IRE1, TRAF2, p65, and p-p65 were obtained from Boosen Biotechnology. Co., Ltd. (Beijing, China). A secondary antibody conjugated to horseradish peroxidase-conjugated was added to the membranes (Biyuntian Biotechnology Co., Ltd.). The blots were developed using the enhanced chemiluminescence kit (Biyuntian Biotechnology Co., Ltd.) and imaged using CanoScan scanner (LiDE 100, Canon Inc., Tokyo, Japan). Using ImageJ software (Version 1.8.0.112), densitometric quantification of band intensities was performed.

Quantification of SCFAs

Quantification of SCFAs was conducted in the same way as previously reported (Zhao et al., 2006). Briefly, the frozen cecal contents were thawed and 20 mg aliquots were transferred to centrifuge tubes in ice bath, dissolved in 1 mL 0.5% phosphoric acid solution, and ultrasonicated for 5 min. Then, the mixture was vortexed and centrifuged (12,000 × g, 10 min, 4°C). Afterward, 0.1 mL supernatant solution was mixed with 0.5 mL methyl tert-butyl ether with an internal standard in ice bath in a 1.5 mL centrifuge tube and centrifuged (12,000 × g, 15 min, 4°C). Then, 200 μL supernatant was placed in a gas chromatograph vial cooled to -20°C and analyzed with the Agilent GC-MS/MS platform (7890B-7000D, Agilent Technologies, Inc., Santa Clara, CA).

Gut Microbiota Analysis

Genomic DNA of the gut microbiota was extracted from cecal contents under sterile conditions at days 28 and 42 using a TIANamp Stool DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). The primer pair 341F/805R was employed to amplify the V3 to V4 regions of the 16S rRNA gene. The PCR products were detected by electrophoresis using a 2% concentration agarose gel. Subsequently, sequencing was carried out using the Illumina NovaSeq6000 platform (Illumina Inc., San Diego, CA). High-quality clean tags were generated by quality filtering of the raw tags according to QIIME (Version 1.2.1). Operational taxonomic units clustering was carried out according to the 97% sequence similarity principle using Uparse software (Version 7.0.1001). The α-diversity (Observed by otus, Shannon, Simpson, Chao1, abundance-based coverage estimator (**ACE**), Goods coverage) was assessed using QIIME software (Version 1.9.1). The β-diversity was visualized by principal coordinates analysis (**PCoA**) using the "stats" package and "ggplot2" package of R software (Version 4.1.2). Linear discriminant analysis effect size (**LEfSe**) was analyzed (LDA score = 4). Functional genes of the intestinal microbiota were predicted with PICRUSt2 software (Version 2.3.0_b) based on the Kyoto Encyclopedia of Genes and Genomes database using the Kruskal-Wallis test. Data on all sequences have been submitted to NCBI under BioProject ID PRJNA982385.

Statistical Analysis

Data analyses were performed for single factor analysis by SPSS Statistics 26.0 (SPSS Statistics, Chicago, IL). The data of growth performance, immune response, intestinal morphology, intestinal permeability, ER stress, and SCFAs were compared with one-way ANOVA. The data of intestinal lesion scores and OPG were analyzed using Kruskal-Wallis test. Duncan's multiple comparison test was used to compare differences among 5 groups. The spearman's correlation coefficient was calculated to assess relationships between the biochemical parameters and gut microbiota as well as SCFAs. The replicate was considered as an experimental unit for statistical analysis of growth performance and OPG data. For the other data, broiler served as an experimental unit for statistical analysis. The $P < 0.05$ was considered statistically significant.

RESULTS

Growth Performance

From days 0 to 14, there were no significant differences in the ADG ($P = 0.685$), ADFI ($P = 0.283$), or F/G ratio ($P = 0.364$) among the 5 groups. From days 14 to 28, the ADG ($P < 0.001$) and ADFI ($P = 0.017$) decreased, while the F/G ratio ($P = 0.003$) increased in NE group compared to NC group. Compared to NE group, the ADG ($P < 0.001$) increased in L-Pro and M-Pro groups, while the F/G ratio ($P = 0.003$) decreased in L-Pro, M-Pro, and H-Pro groups. From days 0 to 42, the ADG ($P = 0.001$) decreased, while the F/G ratio ($P = 0.032$) and mortality increased in NE group compared to NC group. Compared to NE group, the ADG ($P = 0.001$) increased in M-Pro group, and mortality ($P = 0.003$) decreased in L-Pro, M-Pro, and H-Pro groups (Table 2).

Table 2. Effect of *Bacillus subtilis* HW2 on growth performance in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items[1]	NC[2]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 0−14							
ADG, g	34.55	33.61	33.59	32.93	34.73	0.427	0.685
ADFI, g	40.11	38.02	39.51	40.86	41.06	0.484	0.283
F/G	1.17	1.13	1.18	1.24	1.18	0.017	0.364
D 14−28							
ADG, g	72.08^{a}	53.20^{c}	62.44^{b}	62.57^{b}	58.49^{bc}	1.407	<0.001
ADFI, g	119.44^{a}	105.79^{b}	111.48^{ab}	111.40^{ab}	105.37^{b}	1.556	0.017
F/G	1.66^{b}	2.00^{a}	1.79^{b}	1.78^{b}	1.81^{b}	0.030	0.003
D 28−42							
ADG, g	82.68	75.26	75.48	79.69	71.95	1.508	0.183
ADFI, g	176.15	166.35	170.55	171.48	166.20	2.411	0.702
F/G	2.14	2.22	2.26	2.16	2.33	0.037	0.521
D 0−42							
ADG, g	64.66^{a}	55.32^{c}	59.26^{bc}	60.15^{b}	57.29^{bc}	0.819	0.001
ADFI, g	101.69	93.79	100.18	100.11	97.43	0.985	0.079
F/G	1.58^{b}	1.70^{a}	1.69^{a}	1.67^{a}	1.70^{a}	0.015	0.032
Mortality, %	0^{c}	15.00^{a}	6.67^{bc}	3.33^{bc}	8.33^{ab}	1.384	0.003

[1]ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain ratio.
[2]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1 × 10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5 × 10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1 × 10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.
[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

Intestinal Lesion Scores, Eimeria Oocyst Counts, and Intestinal Morphology

On day 28, the duodenal lesion ($P < 0.001$), jejunal lesion ($P = 0.001$), and ileal lesion ($P < 0.001$) scores increased in NE group compared to NC group. Compared to NE group, the duodenal lesion ($P < 0.001$), jejunal lesion ($P = 0.001$), and ileal lesion ($P < 0.001$) scores decreased in M-Pro group. On day 42, compared to NC group, the duodenal, jejunal, and ileal lesion scores ($P < 0.001$) increased in NE group. Compared to NE group, the duodenal and jejunal lesion scores ($P < 0.001$) decreased in L-Pro group, and the duodenal, jejunal, and ileal lesion scores ($P < 0.001$) decreased in M-Pro group. Moreover, on day 19, the OPG ($P < 0.001$) increased in NE group compared to NC group. Compared to NE group, the OPG ($P < 0.001$) decreased in M-Pro group (Table 3).

The jejunal villus height ($P = 0.027$), ileal villus height ($P = 0.002$), and V/C ratio ($P = 0.049$) decreased in NE group compared to NC group on day 28. Compared to NE group, the ileal V/C ratio ($P = 0.049$) increased in L-Pro group, while the ileal villus height ($P = 0.002$) and V/C ratio ($P = 0.049$) increased in M-Pro group. On day 42, the jejunal villus height ($P < 0.001$) and ileal villus height ($P = 0.005$) decreased in NE group compared to NC group. Compared to NE group, the jejunal villus height ($P < 0.001$) increased in L-Pro group, while the jejunal villus height ($P < 0.001$) and ileal villus height ($P = 0.005$) increased in M-Pro group (Figure 1 and Table 4).

Intestinal Permeability

On day 28, serum D-lactic acid ($P = 0.001$) and DAO ($P = 0.008$) levels increased in NE group compared to NC group and decreased in L-Pro and M-Pro groups compared to NE group. On day 42, serum DAO ($P = 0.009$) level increased in NE group compared to NC group, but decreased in L-Pro, M-Pro, and H-Pro groups compared to NE group (Table 5).

Table 3. Effect of *Bacillus subtilis* HW2 on intestinal lesion scores and oocysts per gram of excreta in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items	NC[1]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 19							
Oocysts per gram of excreta, × 10^5/g of excreta	0^{c}	43.79^{a}	7.01^{abc}	6.82^{bc}	7.96^{ab}	3.638	0.021
D 28							
Duodenum lesion score	0^{c}	2.50^{a}	1.33^{ab}	1.33^{b}	1.50^{ab}	4.819	<0.001
Jejunum lesion score	0^{c}	2.83^{a}	1.83^{ab}	1.67^{b}	2.00^{ab}	4.828	0.001
Ileum lesion score	0^{c}	3.00^{a}	1.83^{ab}	1.50^{b}	1.83^{ab}	4.897	<0.001
D 42							
Duodenum lesion score	0^{c}	2.67^{a}	1.33^{b}	1.17^{b}	1.83^{ab}	4.875	<0.001
Jejunum lesion score	0^{c}	3.33^{a}	2.00^{b}	1.83^{b}	2.50^{ab}	4.878	<0.001
Ileum lesion score	0^{c}	3.17^{a}	2.00^{ab}	1.83^{b}	2.67^{ab}	4.899	<0.001

[1]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1 × 10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5 × 10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1 × 10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.
[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

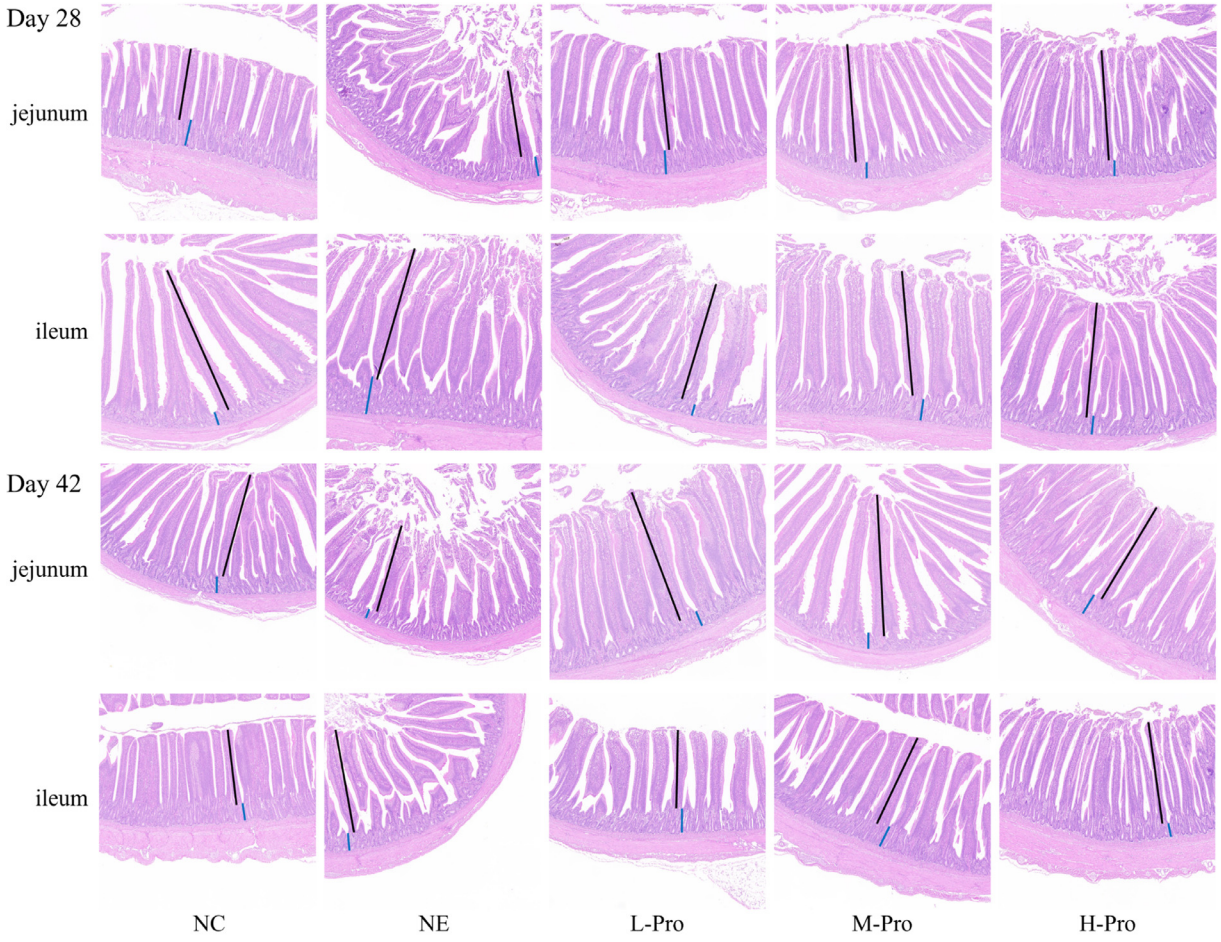

Figure 1. Effect of *Bacillus subtilis* HW2 on intestinal morphology in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). Villus height, black line; crypt depth, blue line; NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge.

Table 4. Effect of *Bacillus subtilis* HW2 on intestinal morphology in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items[1]	NC[2]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 28							
Jejunum							
Villus height, μm	1251.16[a]	993.08[b]	1121.53[ab]	1154.50[ab]	1082.18[b]	27.108	0.027
Crypt depth, μm	178.05	156.77	174.50	171.28	170.09	2.731	0.124
V/C	7.12	6.32	6.43	6.81	6.36	0.173	0.563
Ileum							
Villus height, μm	1091.34[a]	771.17[c]	911.32[bc]	978.77[ab]	881.86[bc]	28.878	0.002
Crypt depth, μm	182.72	176.05	159.45	171.66	166.78	3.643	0.331
V/C	6.02[a]	4.38[b]	5.86[a]	5.70[a]	5.34[ab]	0.198	0.049
D 42							
Jejunum							
Villus height, μm	1561.85[a]	1340.64[c]	1429.40[b]	1430.39[b]	1383.54[bc]	18.703	<0.001
Crypt depth, μm	172.28	163.64	167.01	162.19	160.23	1.840	0.266
V/C	9.06	8.19	8.58	8.84	8.66	0.105	0.091
Ileum							
Villus height, μm	978.67[a]	836.45[c]	889.64[bc]	924.92[ab]	842.56[c]	15.207	0.005
Crypt depth, μm	136.27	117.72	125.42	128.80	127.41	2.261	0.127
V/C	7.22	7.13	7.17	7.20	6.66	0.148	0.761

[1]V/C, villus height/crypt depth.

[2]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.

[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

Table 5. Effects of *Bacillus subtilis* HW2 on D-lactic acid and diamine oxidase levels in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items	NC[1]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 28							
D-lactic acid, nmol/mL	122.50[b]	134.02[a]	122.83[b]	115.16[b]	131.91[a]	1.749	0.001
Diamine oxidase, ng/mL	37.49[c]	43.07[a]	38.59[bc]	37.69[c]	41.61[ab]	0.650	0.008
D 42							
D-lactic acid, nmol/mL	125.27	130.21	127.68	127.76	125.19	3.922	0.995
Diamine oxidase, ng/mL	43.52[b]	48.63[a]	42.80[b]	44.35[b]	44.99[b]	0.591	0.009

[1]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.

[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

On day 28, the protein expressions of ileal occludin and ZO-1 ($P < 0.001$) were higher in NC, L-Pro, M-Pro, and H-Pro groups than NE group, but there were no significant differences between NC and M-Pro groups. On day 42, the protein expression of occludin ($P < 0.001$) was higher in NC, L-Pro, and M-Pro groups than NE group, but there were no significant differences between NC and L-Pro groups. The protein expression of ZO-1 ($P < 0.001$) was higher in NC, L-Pro, M-Pro, and H-Pro groups than NE group (Figure 2).

Expression Levels of Immune-Related Indexes in the Ileal Mucosa

On day 28, the IL-1β ($P < 0.001$), IL-8 ($P = 0.001$), IFN-γ ($P < 0.001$), and TNF-α ($P < 0.001$) levels increased, while the IL-10 ($P < 0.001$) level decreased in NE group compared to NC group. Compared to NE group, the IL-1β ($P < 0.001$), IFN-γ ($P < 0.001$), and TNF-α ($P < 0.001$) levels decreased and the IL-10 ($P < 0.001$) level increased in L-Pro group. In addition, the IL-1β ($P < 0.001$), IL-8 ($P = 0.001$), IFN-γ ($P < 0.001$), and TNF-α ($P < 0.001$) levels decreased and the IL-10 ($P < 0.001$) level increased in M-Pro group, while the IL-1β ($P < 0.001$), IFN-γ ($P < 0.001$), and TNF-α ($P < 0.001$) levels decreased in H-Pro group. On day 42, the IL-1β ($P < 0.001$), IL-8 ($P = 0.003$), IFN-γ ($P < 0.001$), and TNF-α ($P = 0.001$) levels increased and the IL-10 ($P < 0.001$) level decreased in NE group compared to NC group. Also, the IL-1β ($P < 0.001$), IFN-γ ($P < 0.001$), and TNF-α ($P = 0.001$) levels in L-Pro group compared to NE group, while the IL-1β ($P < 0.001$),

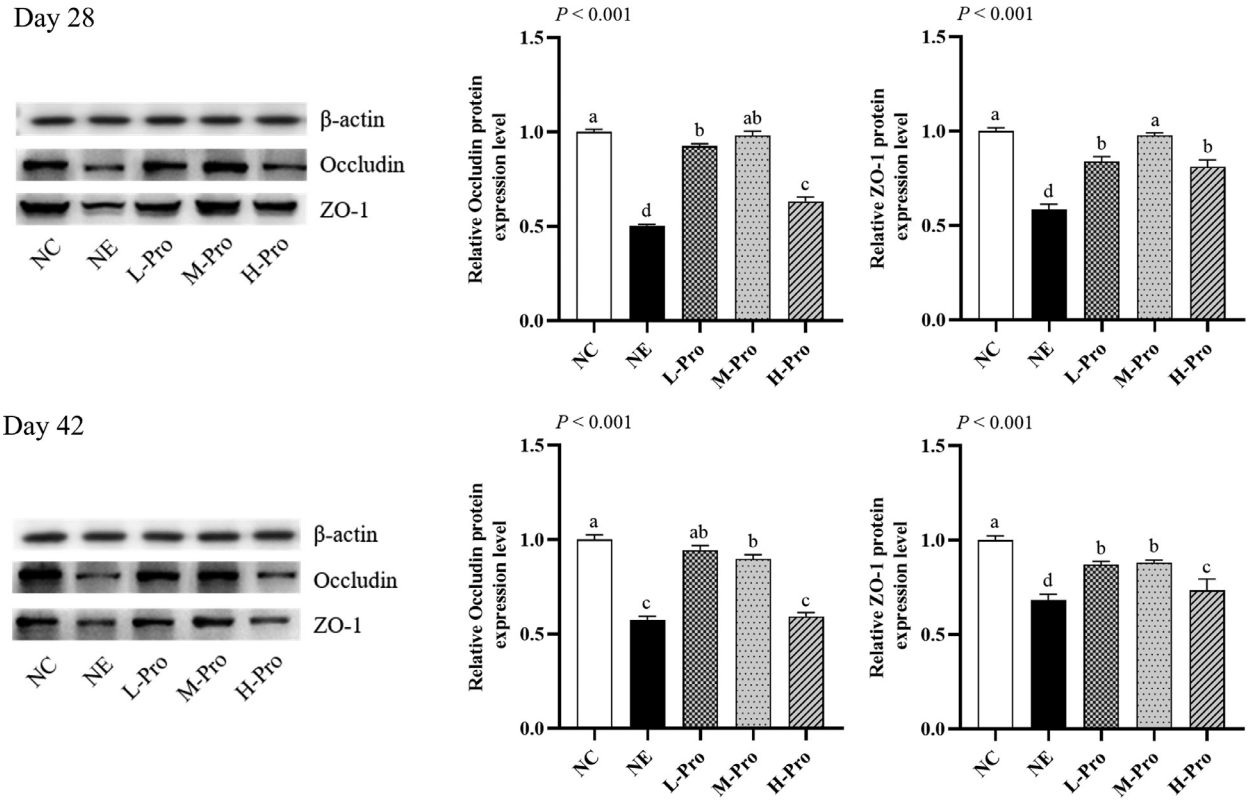

Figure 2. Effect of *Bacillus subtilis* HW2 on tight junction protein expression in ileal mucosa in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge. [a,b,c,d] Different letters superscripts mean significant differences ($P < 0.05$).

Table 6. Effects of *Bacillus subtilis* HW2 on immune-related indexes levels in ileal mucosa in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items[1]	NC[2]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 28							
IL-1β, pg/mg	2.98^{c}	3.82^{a}	3.20^{bc}	2.98^{c}	3.31^{b}	0.066	<0.001
IL-8, pg/mg	2.56^{b}	2.97^{a}	2.90^{a}	2.60^{b}	2.91^{a}	0.045	0.001
IL-10, pg/mg	3.65^{a}	2.59^{c}	2.82^{b}	2.87^{b}	2.76^{bc}	0.074	<0.001
IL-17, pg/mg	0.49	0.57	0.53	0.49	0.54	0.011	0.057
IFN-γ, pg/mg	27.44^{c}	34.74^{a}	29.87^{b}	28.06^{c}	30.43^{b}	0.511	<0.001
TNF-α, pg/mg	2.71^{c}	3.52^{a}	2.90^{bc}	2.73^{c}	3.01^{b}	0.061	<0.001
slgA, μg/mg	1.43	1.43	1.48	1.45	1.41	0.014	0.549
D 42							
IL-1β, pg/mg	2.71^{c}	3.77^{a}	2.98^{b}	2.98^{b}	3.07^{b}	0.068	<0.001
IL-8, pg/mg	2.78^{c}	3.17^{a}	3.04^{ab}	2.80^{c}	2.89^{bc}	0.040	0.003
IL-10, pg/mg	3.14^{a}	2.38^{c}	2.38^{bc}	2.57^{b}	2.31^{c}	0.068	<0.001
IL-17, pg/mg	0.51	0.57	0.52	0.53	0.57	0.007	0.092
IFN-γ, pg/mg	24.14^{c}	31.07^{a}	27.42^{b}	25.93^{bc}	27.51^{b}	0.497	<0.001
TNF-α, pg/mg	2.36^{c}	2.86^{a}	2.50^{c}	2.53^{bc}	2.74^{ab}	0.046	0.001
slgA, μg/mg	1.44	1.43	1.37	1.48	1.43	0.016	0.303

[1]IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α; sIgA, secretory immunoglobulin A.
[2]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.
[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

IL-8 ($P = 0.003$), IFN-γ ($P < 0.001$), and TNF-α ($P = 0.001$) levels decreased and the IL-10 ($P < 0.001$) level increased in M-Pro group, and the IL-1β ($P < 0.001$), IL-8 ($P = 0.003$), and IFN-γ ($P < 0.001$) levels decreased in H-Pro group (Table 6).

On day 28, the mRNA expressions of IL-1β ($P < 0.001$) and TNF-α ($P = 0.001$) were lower in NC, L-Pro, and M-Pro groups than NE group, while there were no significant differences among NC, L-Pro, and M-Pro groups. Also, the mRNA expressions of IL-8 ($P = 0.007$) and IFN-γ ($P = 0.015$) in NC and M-Pro groups were lower than NE group, while there were no significant differences among NC, L-Pro, and M-Pro groups. Meanwhile, the mRNA expression of IL-10 ($P = 0.020$) in NC and M-Pro groups was higher than NE group with no significant differences among NC, L-Pro, M-Pro, and H-Pro groups. Also, the mRNA expression of IL-17 ($P = 0.001$) in NC and M-Pro groups was lower than NE group. On day 42, the mRNA expressions of IL-1β ($P = 0.002$) and IFN-γ ($P = 0.001$) in NC, L-Pro, and M-Pro groups was lower than NE group with no significant differences between NC and M-Pro groups. In addition, the mRNA expressions of IL-8 ($P = 0.001$) and TNF-α ($P = 0.002$) in NC, L-Pro, and M-Pro groups were lower than NE group with no significant differences among NC, L-Pro, and M-Pro groups. Meanwhile, the mRNA expression of IL-10 ($P = 0.049$) in NC group was higher than NE group, while the mRNA expression of IL-17 ($P = 0.013$) in NC and M-Pro groups was lower than NE group with no significant differences among NC, L-Pro, and M-Pro groups (Figure 3).

The ER Stress and Morphology

The ER lumen was dilated and the ER structure was damaged in NE group. However, dietary supplementation with different concentrations of *B subtilis* HW2 improved the ER morphology (Figure 4).

On day 28, compared to NC group, the mRNA expressions of the markers of ER stress upregulated in NE group, which included GRP78 ($P = 0.001$), PERK ($P = 0.015$), ATF6 ($P = 0.014$), IRE1 ($P = 0.003$), TRAF2 ($P = 0.022$), and p65 ($P = 0.009$). Compared to NE group, the mRNA expressions of GRP78 ($P = 0.001$) and IRE1 ($P = 0.003$) in L-Pro group were downregulated, while the mRNA expressions of many markers of ER stress downregulated in M-Pro group, which included GRP78 ($P = 0.001$), PERK ($P = 0.015$), ATF6 ($P = 0.014$), IRE1 ($P = 0.003$), TRAF2 ($P = 0.022$), and p65 ($P = 0.009$), while the mRNA expressions of GRP78 ($P = 0.001$) and IRE1 ($P = 0.003$) downregulated in H-Pro group. On day 42, compared to NC group, the mRNA expressions of many markers of ER stress upregulated in NE group, which included GRP78 ($P = 0.029$), PERK ($P < 0.001$), ATF6 ($P = 0.001$), IRE1 ($P < 0.001$), TRAF2 ($P = 0.002$), and p65 ($P = 0.001$). Compared to NE group, the mRNA expressions of several markers in L-Pro and M-Pro groups were downregulated, including PERK ($P < 0.001$), ATF6 ($P = 0.001$), IRE1 ($P < 0.001$), TRAF2 ($P = 0.002$), and p65 ($P = 0.001$), while the expressions of several downregulated in H-Pro group, which included ATF6 ($P = 0.001$), IRE1 ($P < 0.001$), TRAF2 ($P = 0.002$), and p65 ($P = 0.001$) (Figure 5).

Western blot analysis was performed to further analyze the expression levels of markers of ER stress. On day 28, the protein expressions of GRP78 ($P < 0.001$), ATF6 ($P < 0.001$), p-IRE1 ($P < 0.001$), TRAF2 ($P < 0.001$), and p65 ($P < 0.001$) were increased in NE group compared to NC group. Compared to NE group, the protein expressions of GRP78 ($P < 0.001$), ATF6 ($P < 0.001$), TRAF2 ($P < 0.001$), and p65 ($P < 0.001$) were

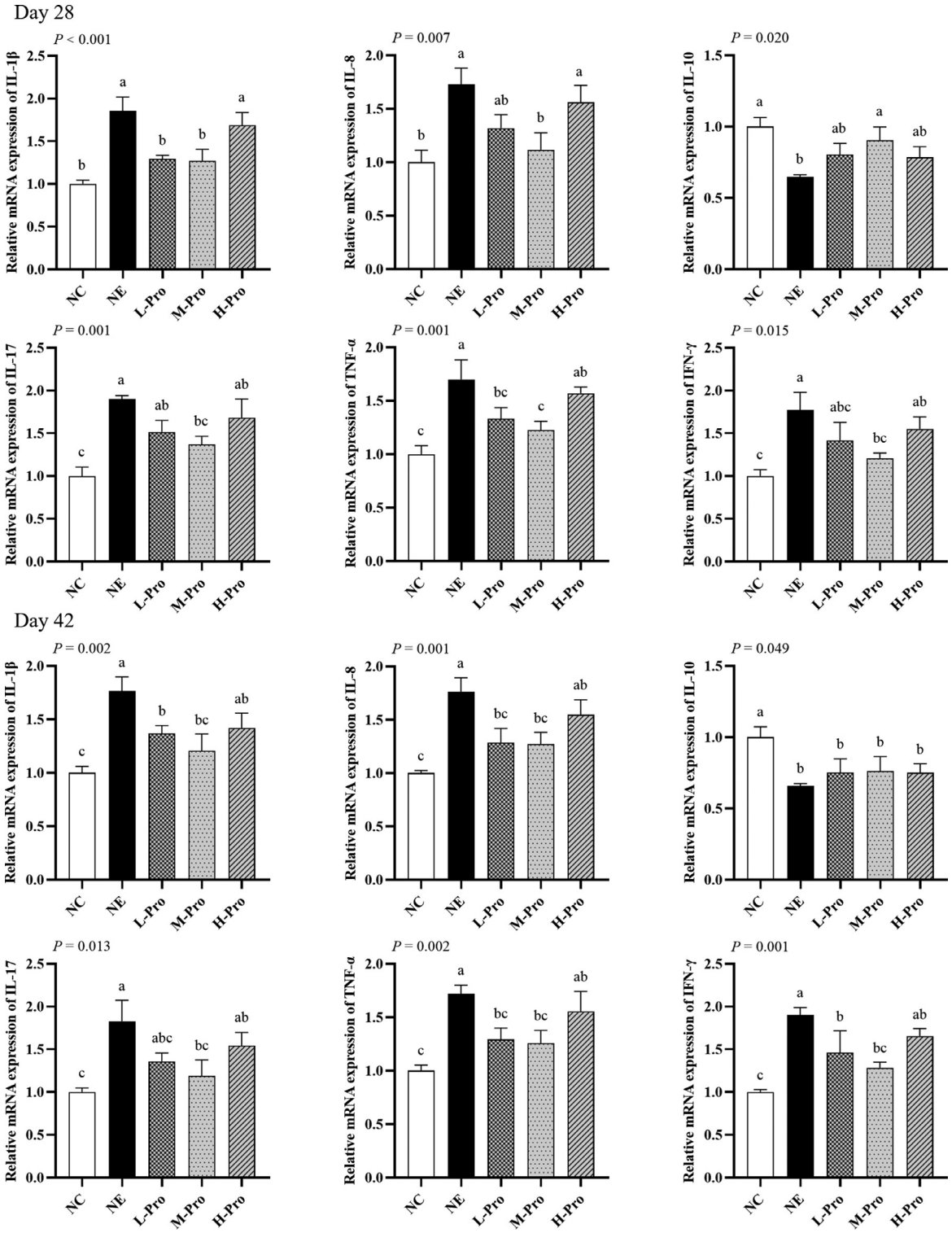

Figure 3. Effects of *Bacillus subtilis* HW2 on relative mRNA expression of immune-related indexes in ileal mucosa in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α. [a,b,c] Different letters superscripts mean significant differences ($P < 0.05$).

decreased in L-Pro group, while the protein expressions of GRP78 ($P < 0.001$), ATF6 ($P < 0.001$), p-IRE1 ($P < 0.001$), TRAF2 ($P < 0.001$), and p65 ($P < 0.001$) were decreased in M-Pro group and the protein expression of GRP78 ($P < 0.001$) was decreased in H-Pro group. On day 42, the protein expressions of GRP78 ($P < 0.001$), p-PERK ($P < 0.001$), ATF6 ($P < 0.001$), p-IRE1 ($P < 0.001$), TRAF2 ($P < 0.001$), and p-p65 ($P = 0.004$) were increased in NE group compared to NC group. Compared to NE group, the protein expressions of GRP78

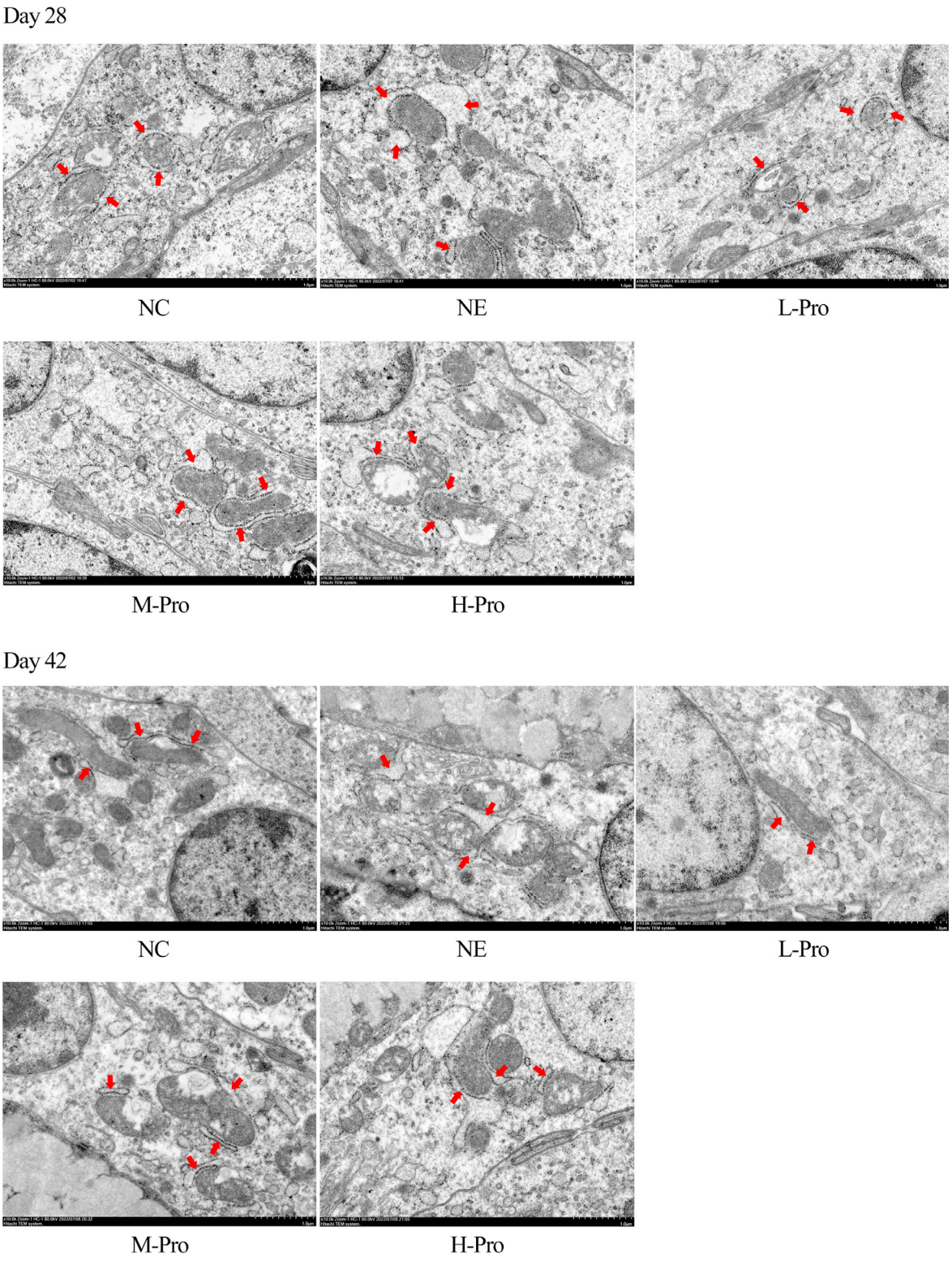

Figure 4. Effects of *Bacillus subtilis* HW2 on intestinal ultrastructure in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; Arrow, ER morphology.

($P < 0.001$), ATF6 ($P < 0.001$), p-IRE1 ($P < 0.001$), and TRAF2 ($P < 0.001$) were decreased in L-Pro group, while the protein expressions of GRP78 ($P < 0.001$), p-PERK ($P < 0.001$), ATF6 ($P < 0.001$), p-IRE1 ($P < 0.001$), TRAF2 ($P < 0.001$), and p-p65 ($P = 0.004$) were decreased in M-Pro group, and the protein expressions of ATF6 ($P < 0.001$) and TARF2 ($P < 0.001$) were decreased in H-Pro group (Figure 6).

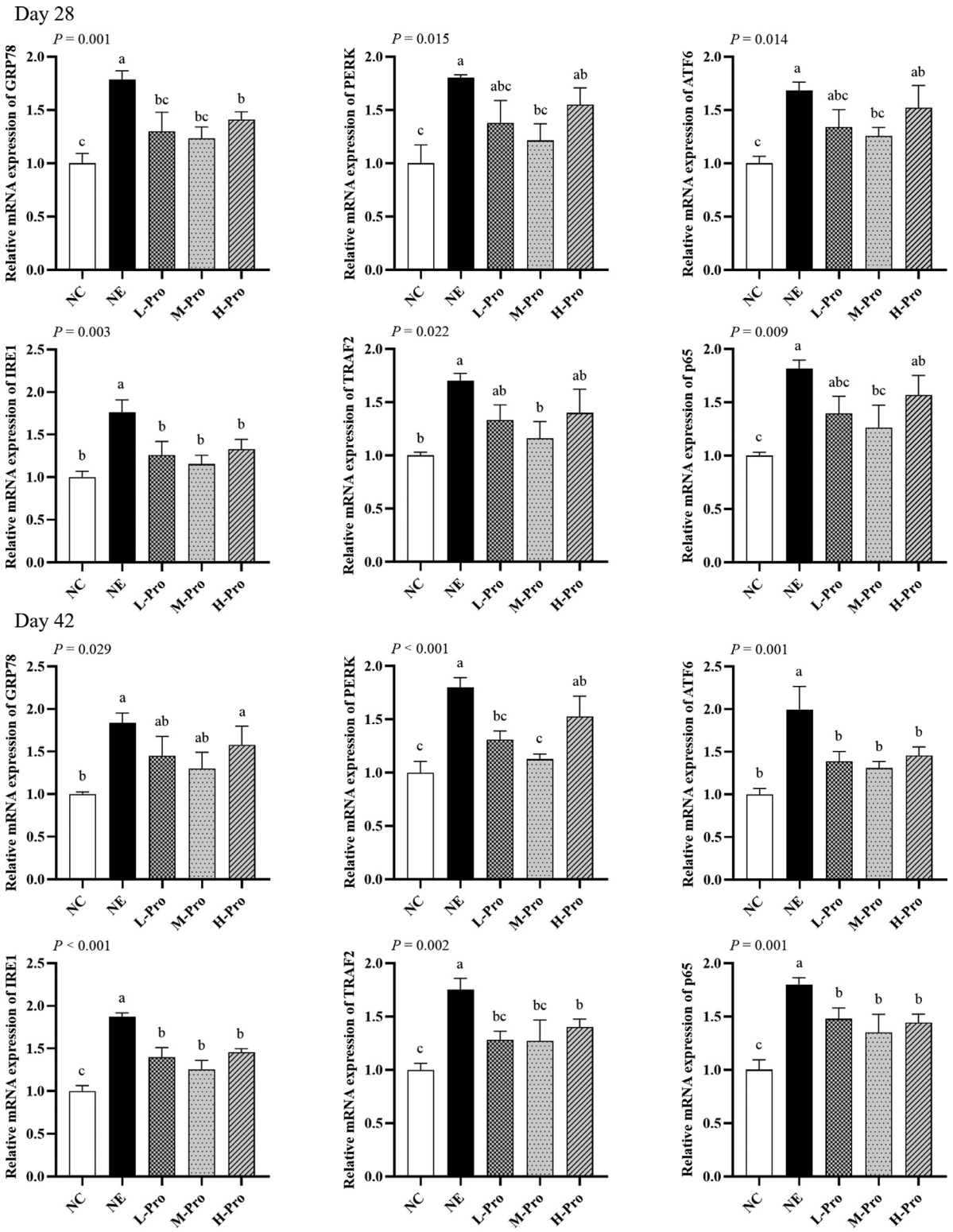

Figure 5. Effects of *Bacillus subtilis* HW2 on endoplasmic reticulum-associated relative mRNA expression in ileal mucosa in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; GRP78, glucose-regulated protein 78; ATF6, activated transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, phosphor-protein kinase R-like endoplasmic reticulum kinase; TRAF2, tumor necrosis factor receptor associated factor 2. [a,b,c] Different letters superscripts mean significant differences ($P < 0.05$).

The α-Diversity and β-Diversity Indices of the Gut Microbiota

On day 28, the Observed otus ($P = 0.015$), Chao1 ($P = 0.012$), and ACE ($P = 0.005$) indices in M-Pro group were higher than NE group (Table 7). The Goods coverage ($P = 0.027$) index was higher in NC group than L-Pro, M-Pro, and H-Pro groups. On day 42, there was no significant differences in α-diversity index among the groups ($P > 0.05$). Moreover, the different groups

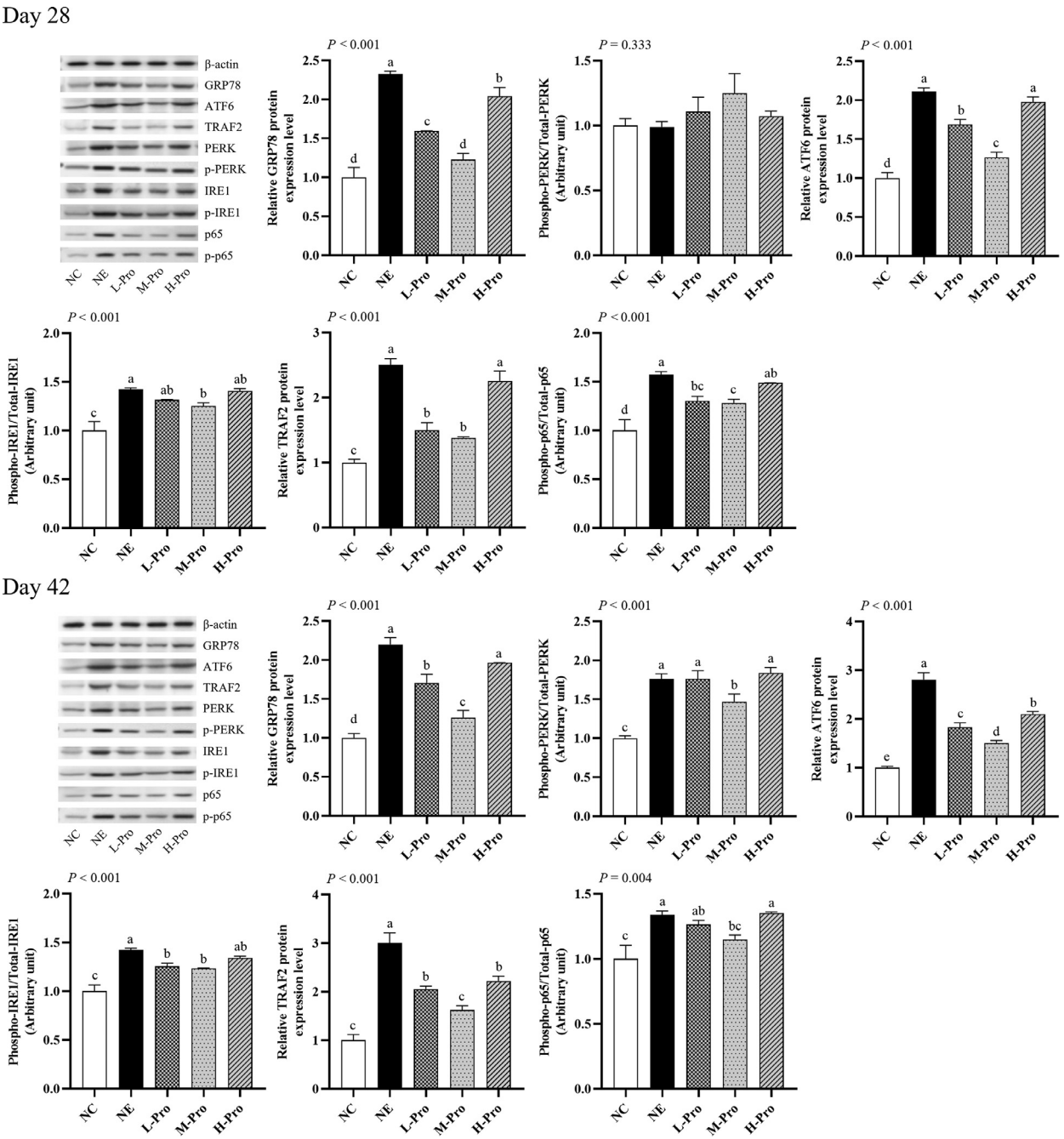

Figure 6. Effects of *Bacillus subtilis* HW2 on endoplasmic reticulum-associated protein expression levels in ileal mucosa in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; GRP78, glucose-regulated protein 78; ATF6, activated transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, phosphor-protein kinase R-like endoplasmic reticulum kinase; TRAF2, tumor necrosis factor receptor associated factor 2. [a,b,c,d,e] Different letters superscripts mean significant differences ($P < 0.05$).

did not significantly alter β-diversity index on days 28 ($P = 0.648$) and 42 ($P = 0.891$) (Figure 7).

Abundance of Gut Microbiota

On day 28, the abundances of f_*Corynebacteriaceae* ($P = 0.021$), f_*Vibrionaceae* ($P = 0.003$), and g_*Turicibacter* ($P = 0.036$) decreased, while that of f_*Erwiniaceae* increased ($P = 0.005$) in NE group compared to NC group. Moreover, compared to NE group, the abundance of g_*Christensenellaceae* ($P = 0.035$) increased in L-Pro group, while the abundances of p_*Actinobacteria* ($P = 0.010$), p_*Cyanobacteria* ($P = 0.034$), f_*Burkholderiaceae* ($P = 0.011$), f_*Aeromonadaceae* ($P = 0.019$), f_*Staphylococcaceae* ($P = 0.024$), f_*Corynebacteriaceae* ($P = 0.029$), f_*Propionibacteriaceae* ($P = 0.033$), f_*Moraxellaceae* ($P = 0.036$), f_*unidentified_Cyanobacteria* ($P = 0.037$), f_*Micrococcaceae* ($P = 0.039$), f_*Erysipelotrichaceae* ($P = 0.042$), f_*Acetobacteraceae* ($P = 0.044$), f_*Listeriaceae* ($P = 0.048$), and g_*Turicibacter* ($P = 0.048$) increased, and the abundance of

Table 7. Effects of *Bacillus subtilis* HW2 on the α-diversity index of cecal microbiota in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items[1]	NC[2]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 28							
Observed otus	567.33^{c}	625.00^{bc}	686.00^{ab}	764.50^{a}	675.83^{abc}	19.802	0.015
Shannon	4.84	4.44	4.80	4.92	5.07	0.110	0.493
Simpson	0.87	0.80	0.86	0.85	0.91	0.012	0.069
Chao1	709.89^{c}	798.59^{bc}	913.77^{ab}	963.53^{a}	848.42^{abc}	26.220	0.012
ACE	704.79^{c}	790.73^{bc}	906.25^{ab}	983.99^{a}	850.28^{abc}	26.998	0.005
Goods coverage	0.99^{a}	0.99^{ab}	0.99^{b}	0.99^{b}	0.99^{b}	0.001	0.027
D 42							
Observed otus	813.83	734.83	819.33	913.50	941.83	31.498	0.233
Shannon	5.85	5.75	5.22	6.20	6.19	0.150	0.220
Simpson	0.92	0.92	0.88	0.93	0.95	0.012	0.414
Chao1	1023.55	909.29	1062.56	1156.28	1165.14	39.900	0.237
ACE	1026.37	921.73	1086.72	1158.42	1192.28	41.035	0.239
Goods coverage	0.99	0.99	0.99	0.99	0.99	0.001	0.183

[1]ACE, abundance-based coverage estimator.
[2]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.
[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

g_*Erwiniaceae* ($P = 0.004$) decreased in M-Pro group. Furthermore, the abundances of f_*Peptostreptococcaceae* ($P = 0.011$), f_*Lactobacillaceae* ($P = 0.018$), and g_*Romboutsia* ($P = 0.016$) decreased and the abundances of f_*Erwiniaceae* ($P = 0.019$) and f_*unidentified_Rhodospirillales* ($P = 0.041$) increased in H-Pro group (Figure 8 and Tables S3–S6).

On day 42, compared to NC group, NE group decreased the abundances of p_*Bacteroidota* ($P = 0.039$), f_*Rikenellaceae* ($P = 0.011$), f_*Barnesiellaceae* ($P = 0.019$), g_*Alistipes* ($P = 0.011$) and g_*Barnesiella* ($P = 0.020$). Compared to NE group, L-Pro group increased the abundances of p_*Bacteroidota* ($P = 0.010$), f_*Rikenellaceae* ($P = 0.007$), f_*Christensenellaceae* ($P = 0.016$), f_*Barnesiellaceae* ($P = 0.016$), f_*Lactobacillaceae* ($P = 0.017$), f_*Hungateiclostridiaceae* ($P = 0.019$), g_*Alistipes* ($P = 0.007$), g_*Coprobacte* ($P = 0.008$), g_*Barnesiella* ($P = 0.017$), and g_*Limosilactobacillus* ($P = 0.047$), and decreased the abundances of p_*Firmicutes* ($P = 0.005$), f_*unidentified_Erysipelotrichales* ($P = 0.027$), f_*Enterococcaceae* ($P = 0.032$), and g_*Erysipelatoclostridium* ($P = 0.034$). Meanwhile, M-Pro group increased the abundances of p_*Bacteroidota* ($P = 0.021$), f_*Rikenellaceae* ($P = 0.011$), f_*Barnesiellaceae* ($P = 0.007$), f_*unidentified_Gastranaerophilales* ($P = 0.025$), g_*Barnesiella* ($P = 0.008$), g_*Alistipes* ($P = 0.011$), and g_*Limosilactobacillus* ($P = 0.015$), while H-Pro group increased the abundance of f_*Hungateiclostridiaceae* ($P = 0.041$) and decreased the abundance of g_*unidentified_Clostridia* ($P = 0.049$) (Figure 8 and Tables S7–S10).

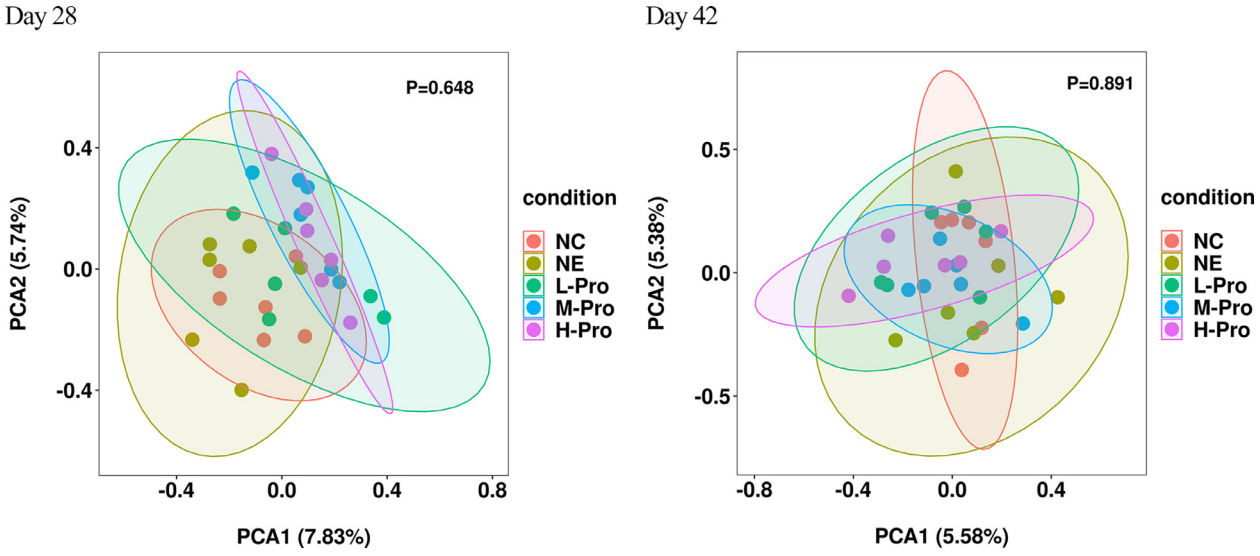

Figure 7. Effects of *Bacillus subtilis* HW2 on the PCoA of gut microbiota in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge.

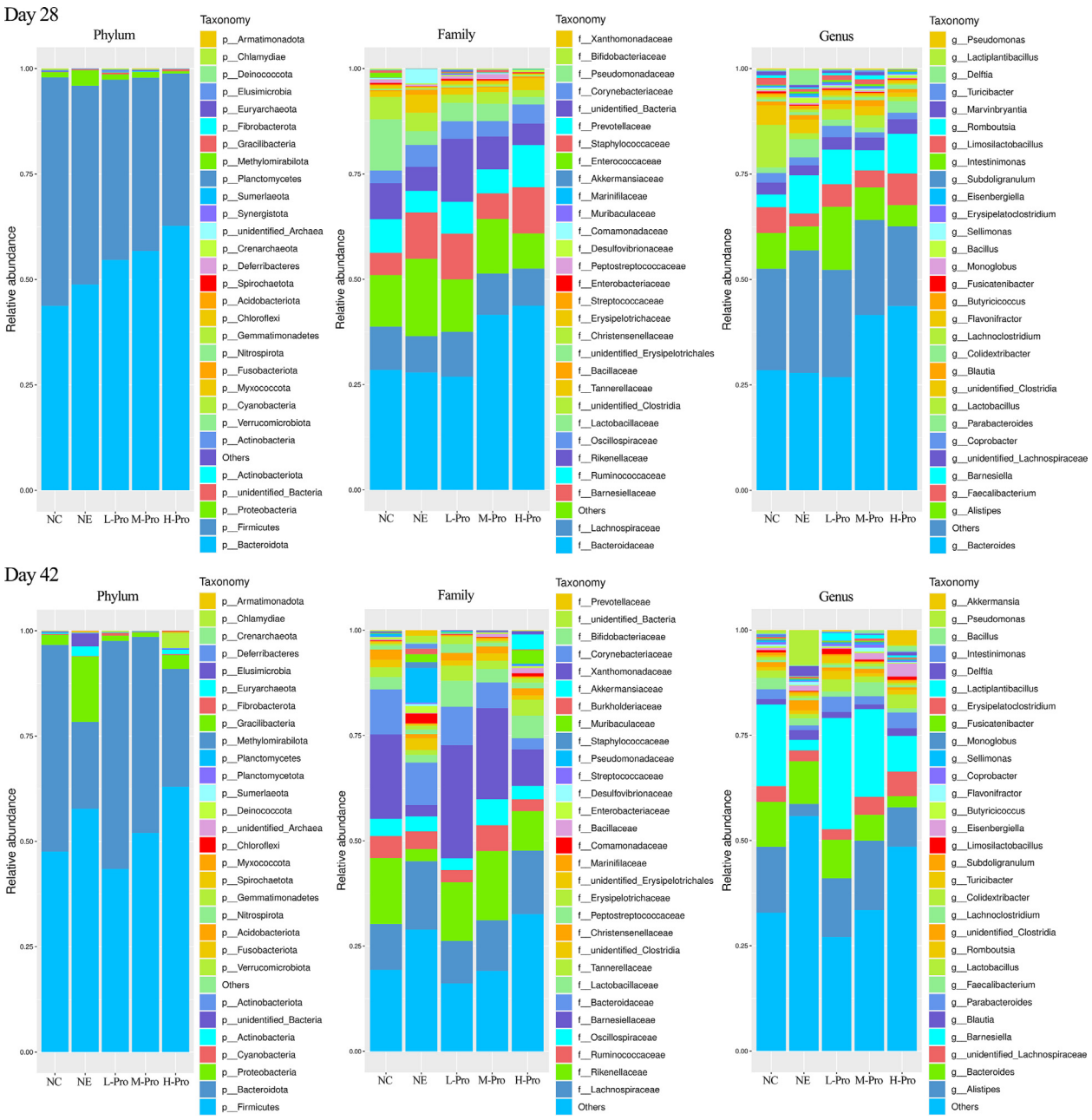

Figure 8. Effects of *Bacillus subtilis* HW2 on the abundance of gut microbiota at phylum, family, and genus levels in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge.

LEfSe Analysis

Taxa that were significantly differentially represented among the groups were examined by LEfSe (LDA score = 4). On day 28, g_*Lactobacillus* was abundant in NC group, while g_*Delftia* and f_*Comamonadaceae* were abundant in NE group. On day 42, o_*Enterobacterales*, g_*unidentified_Bacteria,* p_*unidentified_Bacteria*, f_*Enterobacteriaceae*, g_*Staphylococcus,* g_*unidentified_Clostridia,* c_*unidentified_Actinobacteria*, and p_*Actinobacteria* were abundant in NE group. Meanwhile, o_*Bacteroidales*, p_*Bacteroidota*, c_*Bacteroidia*, c_*Barnesiella*, f_*Barnesiellaceae*, and g_*Lactiplantibacillus* were abundant in L-Pro group, and g_*Alistipes,* f_*Rikenellaceae*, and f_*Ruminococcaceae* were abundant in M-Pro group (Figure 9).

Functional Prediction of Gut Microbiota

The pathways of RNA polymerase ($P = 0.046$) and mismatch repair ($P = 0.047$) were decreased on day 28 in NE group compared to NC group, while the pathways of ethylbenzene degradation ($P = 0.025$), butanoate metabolism ($P = 0.027$), spinocerebellar ataxia ($P = 0.031$), lysine degradation ($P = 0.041$), and phenylalanine metabolism ($P = 0.043$) were increased. Compared to NE group, the pathways of biosynthesis of

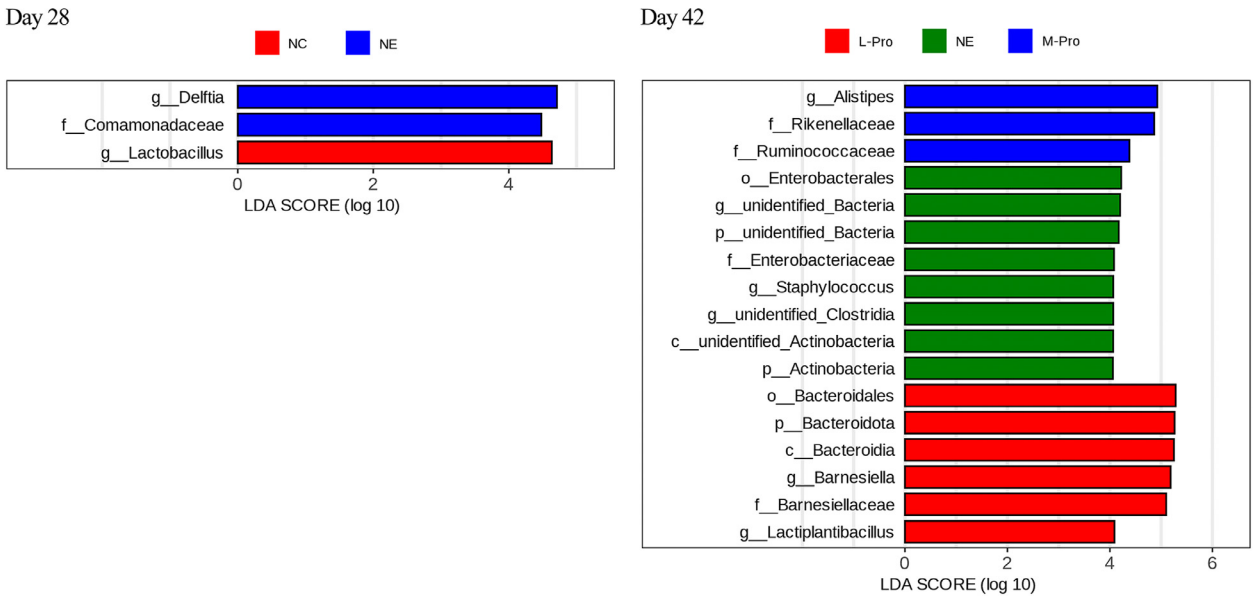

Figure 9. Effects of *Bacillus subtilis* HW2 on linear discriminant analysis effect size of gut microbiota in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge. Default parameters: LDA score > 4 and $P < 0.05$.

various secondary metabolites-part 2 ($P < 0.001$), photosynthesis proteins ($P < 0.001$), photosynthesis ($P < 0.001$), secretion and action ($P = 0.045$), and parathyroid hormone synthesis ($P = 0.045$) were decreased in L-Pro group, while the type I diabetes mellitus pathway ($P = 0.038$) was increased. The M-Pro group decreased the pathways of biosynthesis of various secondary metabolites-part 2 ($P < 0.001$), secretion and action ($P = 0.025$), parathyroid hormone synthesis ($P = 0.025$), *Vibrio cholerae* infection ($P = 0.026$), photosynthesis-antenna proteins ($P = 0.031$), melanogenesis ($P = 0.039$), indole alkaloid biosynthesis ($P = 0.043$), vancomycin resistance ($P = 0.045$), replication and repair ($P = 0.046$), and bacterial invasion of epithelial cells ($P = 0.047$), and increased the MAPK signaling pathway − plant ($P = 0.047$). Meanwhile, H-Pro group decreased the pathways of toluene degradation ($P < 0.001$), mitochondrial biogenesis ($P = 0.012$), betalain biosynthesis ($P = 0.022$), secretion and action ($P = 0.024$), parathyroid hormone synthesis ($P = 0.024$), pathways in cancer ($P = 0.041$), indole alkaloid biosynthesis ($P = 0.043$), and pathogenic *Escherichia coli* infection ($P = 0.047$), etc., and increased the pathways of protein processing in ER ($P = 0.012$), glycosaminoglycan degradation ($P = 0.037$), glycosaminoglycan binding protein ($P = 0.045$), glutamatergic synapse ($P < 0.001$), GABAergic synapse ($P = 0.019$), and prokaryotic defense system ($P = 0.050$) (Figure 10).

On day 42, compared to NC group, NE group had no significant differences on the functions of gut microbiota ($P > 0.05$). Compared to NE group, L-Pro group decreased the pathways of histidine metabolism ($P = 0.027$), pantothenate and CoA biosynthesis ($P = 0.030$), leucine and isoleucine biosynthesis ($P = 0.041$), and C5-branched dibasic acid metabolism ($P = 0.050$), and increased the pathways of viral proteins ($P = 0.011$), glutathione metabolism ($P = 0.035$), metabolism of xenobiotics by cytochrome P450 ($P = 0.041$), drug metabolism - cytochrome P450 ($P = 0.042$), dioxin degradation ($P = 0.043$), transport ($P = 0.048$), and purine metabolism ($P < 0.001$). Meanwhile, M-Pro group decreased the pathways of lysine biosynthesis ($P < 0.001$), unclassified viral proteins ($P < 0.001$), pyrimidine metabolism ($P < 0.001$), proteoglycans in cancer ($P < 0.001$), protein processing in ER ($P < 0.001$), and IL-17 signaling ($P < 0.001$), etc., and increased the pathways of protein kinases ($P < 0.001$), sulfur metabolism ($P < 0.001$), cofactor metabolism ($P < 0.001$), nitrogen metabolism ($P < 0.001$), and 2-component system ($P < 0.001$). Besides, H-Pro group increased the pathways of cofactor metabolism ($P = 0.024$) and viral proteins ($P = 0.033$), and decreased the pathways of carbon fixation in photosynthetic organisms ($P = 0.027$), mannose type O-glycan biosynthesis ($P = 0.037$), other types of O-glycan biosynthesis ($P = 0.037$), pyrimidine metabolism ($P = 0.042$), and histidine metabolism ($P = 0.042$) (Figure 10).

Levels of SCFAs

On day 28, isobutyric acid ($P = 0.013$) and isovaleric acid ($P = 0.020$) levels were decreased in NE group compared to NC group. In addition, isobutyric acid ($P = 0.013$) and isovaleric acid ($P = 0.020$) levels were increased in L-Pro and M-Pro groups compared to NE group. On day 42, isobutyric acid ($P = 0.020$) and isovaleric acid ($P = 0.006$) levels were decreased in NE group compared to NC group. In addition, isobutyric acid ($P = 0.020$) and isovaleric acid ($P = 0.006$) levels were increased in M-Pro group compared to NE group (Table 8).

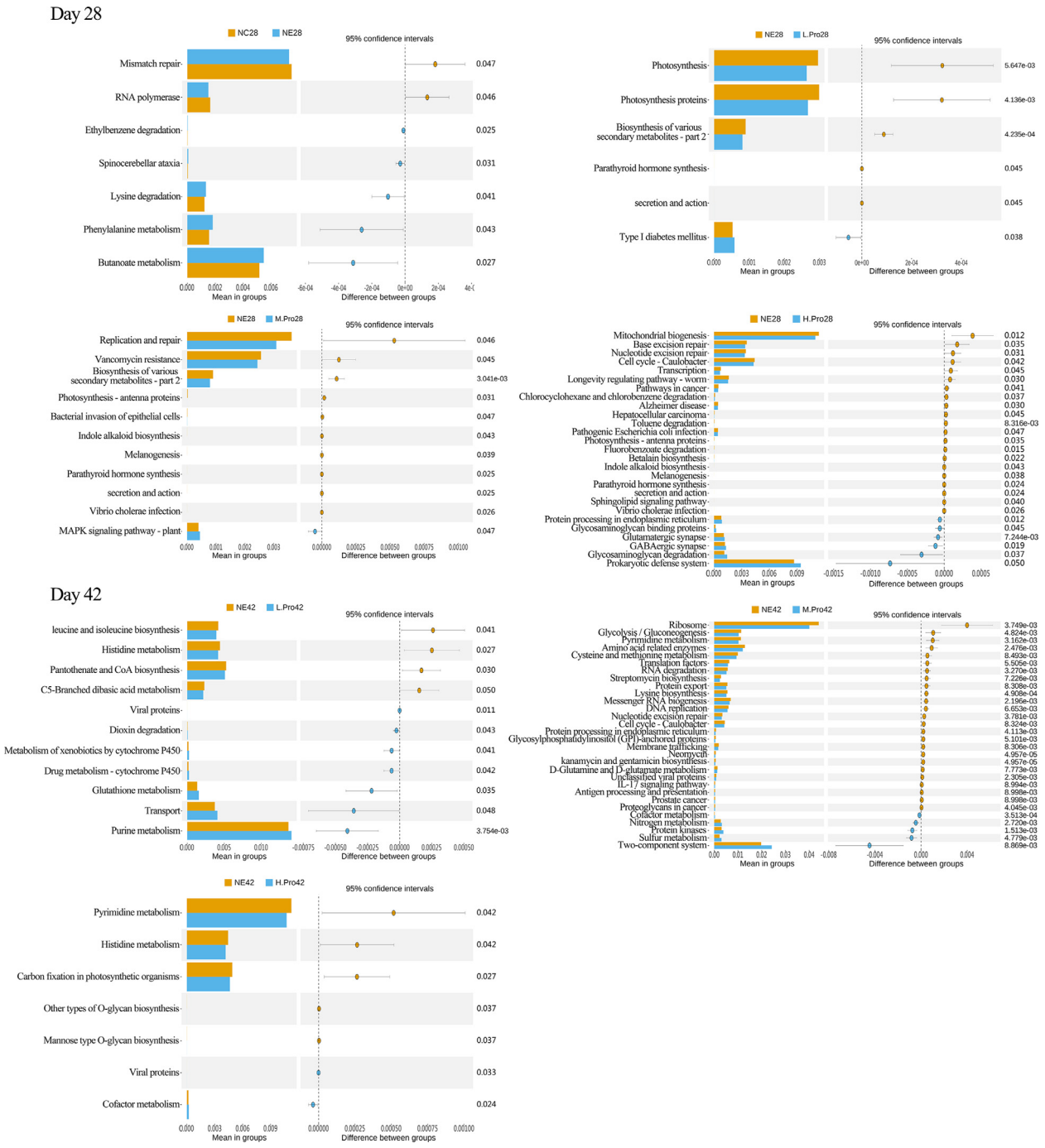

Figure 10. Functional prediction of gut microbiota in necrotic enteritis-challenged broilers ($n = 6$ per experimental group). NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *B. subtilis* HW2 + necrotic enteritis challenge.

Spearman Correlation Analyses

Potential correlations among the biochemical indices, abundance of gut microbiota, and levels of SCFAs were investigated. A correlation analysis was conducted on factors with correlation coefficients (r) that were greater than 0.6 or less than -0.6. On day 28, the abundance of *Romboutsia* was correlated negatively with ileal protein expression of IRE1 ($r = -0.62$, $P = 0.016$). The level of isobutyric acid was negatively correlated with ileal levels of IL-1β ($r = -0.82$, $P < 0.001$), IL-17 ($r = -0.68$, $P = 0.007$), and TNF-α ($r = -0.77$, $P = 0.001$), and protein expressions of GRP78 ($r = -0.69$, $P = 0.005$), ATF6 ($r = -0.70$, $P = 0.005$), IRE1 ($r = -0.67$, $P = 0.008$), TRAF2 ($r = -0.67$, $P = 0.008$), and p65 ($r = -0.71$, $P = 0.004$), but positively correlated with ADG ($r = 0.68$, $P = 0.007$), ADFI ($r = 0.70$, $P = 0.005$), and ileal expression levels of occludin ($r = 0.73$, $P = 0.003$) and ZO-1 ($r = 0.67$, $P = 0.008$). The level of isovaleric acid was correlated negatively with ileal levels of IL-1β ($r = -0.78$, $P < 0.001$) and TNF-α ($r = -0.68$, $P = 0.007$) and protein expressions of ATF6 ($r = -0.63$, $P = 0.014$) and TRAF2 ($r = -0.65$, $P = 0.011$), but correlated positively with ADG ($r = 0.60$, $P = 0.020$), ileal

Table 8. Effects of *Bacillus subtilis* HW2 on short chain fatty acids levels in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

Items, μg/g	NC[1]	NE	L-Pro	M-Pro	H-Pro	SEM	*P*-value
D 28							
Acetic acid	884.79	869.61	857.57	872.02	843.81	21.221	0.987
Butyric acid	181.37	174.62	173.70	170.66	168.00	8.680	0.995
Caproic acid	2.73	2.24	2.41	2.39	2.24	0.073	0.188
Isobutyric acid	42.67^{ab}	25.44^{c}	42.32^{ab}	47.57^{a}	31.95^{bc}	2.602	0.013
Isovaleric acid	27.35^{ab}	18.03^{c}	32.45^{a}	27.88^{ab}	22.75^{bc}	1.618	0.020
Propionic acid	223.55	206.83	214.29	215.18	210.27	7.849	0.982
Valeric acid	47.34	39.19	40.73	40.25	39.36	2.030	0.753
D 42							
Acetic acid	658.69	591.75	627.96	637.49	606.58	20.211	0.894
Butyric acid	207.93	195.93	204.07	206.73	204.77	3.199	0.836
Caproic acid	2.47	2.29	2.30	2.56	2.24	0.079	0.718
Isobutyric acid	55.66^{a}	23.20^{c}	41.79^{abc}	46.32^{ab}	27.17^{bc}	3.974	0.020
Isovaleric acid	47.84^{a}	21.94^{b}	34.86^{ab}	41.91^{a}	19.29^{b}	3.460	0.006
Propionic acid	179.33	146.09	157.93	172.53	150.38	7.163	0.591
Valeric acid	49.06	44.31	46.57	47.32	45.06	1.243	0.821

[1]NC, basal diet; NE, basal diet + necrotic enteritis challenge; L-Pro, basal diet + 1×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; M-Pro, basal diet + 5×10^6 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge; H-Pro, basal diet + 1×10^7 CFU/g *Bacillus subtilis* HW2 + necrotic enteritis challenge.

[a,b,c]Different letters superscripts mean significant differences ($P < 0.05$).

sIgA level (r = 0.82, $P < 0.001$), and ZO-1 protein expression (r = 0.62, $P = 0.016$) (Figure 11 and Table S11).

On day 42, the abundance of *Barnesiella* was correlated negatively with ileal level of IFN-γ (r = −0.60, $P < 0.001$), while the abundance of *Coprobacter* was correlated negatively with ileal protein expression of p65 (r = −0.63, $P = 0.014$), but positively correlated with ileal protein expression of ZO-1 (r = 0.60, $P = 0.020$). The level of isobutyric acid was negatively correlated with F/G ratio (r = −0.61, $P = 0.018$), ileal levels of IL-1β (r = −0.80, $P < 0.001$) and IFN-γ (r = −0.88, $P < 0.001$), and protein expressions GRP78 (r = −0.75, $P = 0.002$), ATF6 (r = −0.76, $P = 0.001$), IRE1 (r = −0.75, $P = 0.002$), and TRAF2 (r = −0.71, $P = 0.004$), but was positively correlated with ileal IL-10 level (r = 0.65, $P = 0.011$) and protein expressions of occludin (r = 0.73, $P = 0.003$) and ZO-1 (r = 0.74, $P = 0.002$). The level of isovaleric acid was negatively correlated with F/G ratio (r = −0.61, $P = 0.019$), ileal levels of IL-1β (r = −0.78, $P = 0.001$) and IFN-γ (r = −0.80, $P < 0.001$), and protein expressions of GRP78 (r = −0.79, $P < 0.001$), ATF6 (r = −0.77, $P = 0.001$), IRE1 (r = −0.77, $P = 0.001$), TRAF2 (r = −0.72, $P = 0.003$), PERK (r = −0.61, $P = 0.018$), and p65 (r = −0.64, $P = 0.013$), but positively correlated with ileal IL-10 level (r = 0.65, $P = 0.010$) and protein expressions of occludin (r = 0.72, $P = 0.003$) and ZO-1 (r = 0.76, $P = 0.002$) (Figure 11 and Table S12).

DISCUSSION

Necrotic enteritis is characterized by the sudden onset of diarrhea and mucosal necrosis caused by the overgrowth of *C. perfringens* in the small intestine, leading to the decreased growth performance of broilers (Musa et al., 2019). In our study, the impaired growth performance of broilers in NE group was improved by dietary supplementation with 1×10^6 and 5×10^6 CFU/g *B. subtilis* HW2. Similarly, Jayaraman et al. (2013) found that dietary supplementation with *B. subtilis* increased the ADG and decreased the F/G ratio in broilers with necrotic enteritis. Besides, in this study, necrotic enteritis also caused serious intestinal lesions and increased oocysts number in excreta, while dietary supplementation with 5×10^6 CFU/g *B. subtilis* HW2 decreased intestinal lesion scores. In line with our results, Bortoluzzi et al. (2019) found that dietary supplementation of *B. subtilis* DSM 32315 decreased the intestinal lesions of broilers with necrotic enteritis.

Intestinal morphology is a key factor in maintaining intestinal health and integrity (Costa et al., 2019). Necrotic enteritis decreased villus height, but dietary supplementation with *B. subtilis* HW2 at 5×10^6 CFU/g increased villus height, which was consistent with the report by Zhao et al. (2020) showing that *B. licheniformis* H2 increased ileal villus height and V/C ratio of broilers with necrotic enteritis. These results indicated that *B. subtilis* HW2 could mitigate gut impairment caused by necrotic enteritis infection. Tight junctions, as the most important components of intestinal epithelial cell barrier, prevent the entry of pathogenic materials (Sun et al., 2020). Serum D-lactic acid and DAO levels are also important symbols of intestinal permeability and damage (Liu et al., 2020). In this study, necrotic enteritis decreased ileal expressions of occludin and ZO-1, and increased serum D-lactic acid and DAO levels. However, *B. subtilis* HW2 increased the tight junction proteins and decreased serum D-lactic acid and DAO levels. Previous research also revealed that *B.* increased the expressions of ileal tight junction proteins in broilers (Bilal et al., 2021), and also decreased D-lactic acid and DAO levels to some extent (Zhang et al., 2022). Moreover, the results of this study showed that lower dosage of *B. subtilis* HW2 had more beneficial effects on intestinal permeability.

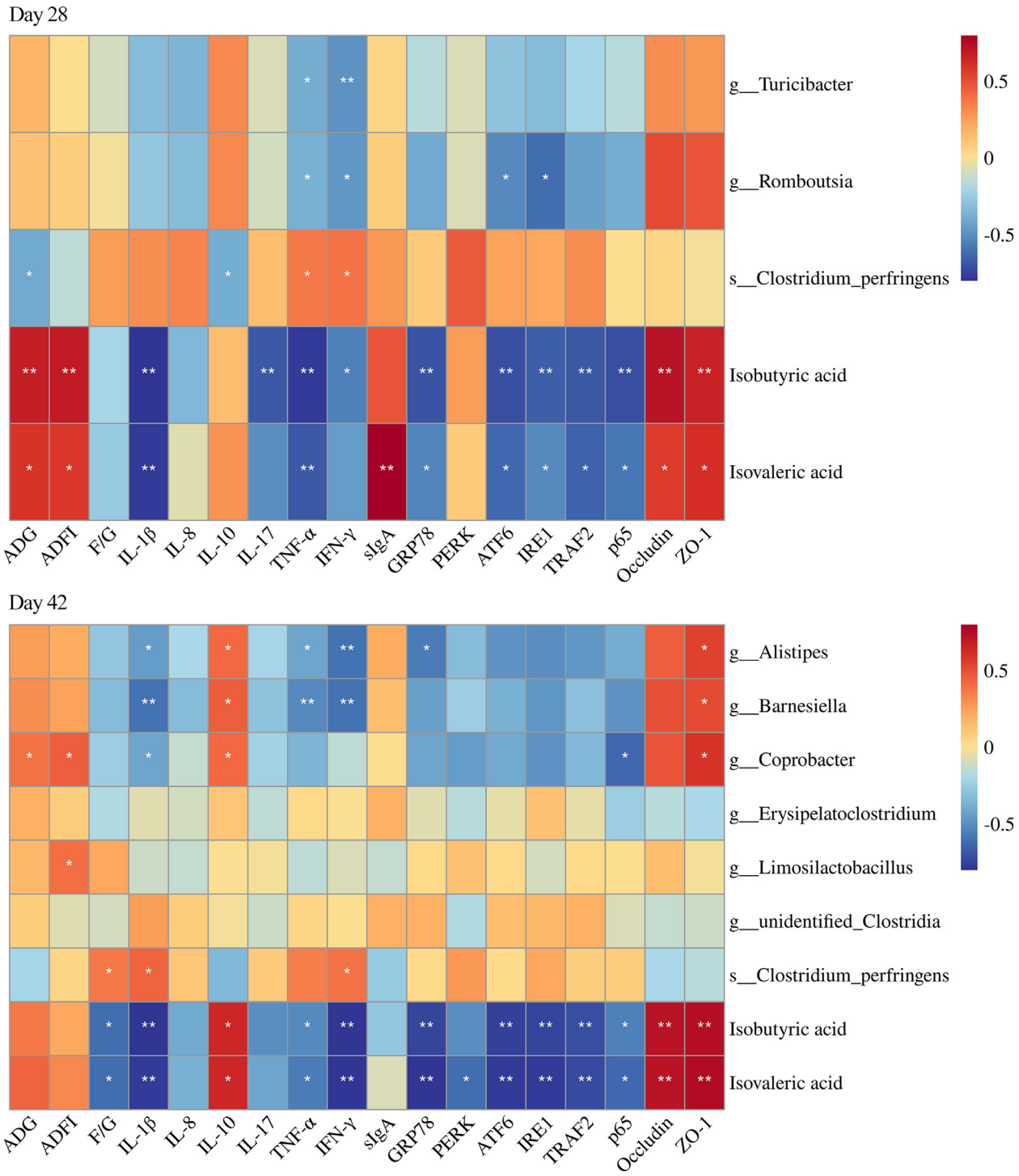

Figure 11. The spearman correlation analyses of the between the biochemical parameters and gut microbiota as well as short-chain fatty acids in necrotic enteritis-challenged broilers ($n = 6$ per experimental group).

ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain ratio; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α; sIgA, secretory immunoglobulin A; GRP78, glucose-regulated protein 78; ATF6, activated transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, phosphor-protein kinase R-like endoplasmic reticulum kinase; TRAF2, tumor necrosis factor receptor-associated factor 2.

Inflammatory cytokines play important roles as communication signals in the regulation of inflammation response (Sun et al., 2017; Fasina and Lillehoj, 2019). Here, we found that necrotic enteritis increased ileal levels of IL-1β, IL-8, IFN-γ, and TNF-α and gene expressions of IL-1β, IL-8, IL-17, IFN-γ, and TNF-α, but decreased the IL-10 level and gene expression. Studies conducted by Lee et al. (2013) and Yu et al. (2022) also reported elevated intestinal inflammation in necrotic enteritis-challenged broilers. On the contrary, *B. subtilis* HW2 decreased the levels of IL-1β, IL-8, IFN-γ, and TNF-α, and gene expressions of IL-1β, IL-8, IL-17, IFN-γ, and TNF-α, and increased the IL-10 level and gene expression. Previous studies also found that *B. amyloliquefaciens* decreased the intestinal gene expression of IFN-γ, IL-6, and IL-17 in broilers (Kan et al., 2021;

Zhang et al., 2022). Our results implied that *B. subtilis* HW2 reduced inflammatory responses and therefore relieved the inflammatory damage in necrotic enteritis-challenged broilers.

Harmful stimuli, like infection, hypoxia, oxidative stress, and harmful metabolites, disrupt homeostasis and cause dysfunction and structural damage in ER which further induces ER stress (Zhu et al., 2020a). Our results showed that necrotic enteritis caused the damage of ER ultrastructure, enlarged lumen and vacuolated. However, dietary *B. subtilis* HW2 supplementation restored the ER ultrastructure. In addition, ER stress and unfolded protein response (**UPR**) activation are important mechanisms for intestinal inflammation (Hosomi et al., 2015; Kim et al., 2019). The GRP78 is a major ER chaperone as well as a master regulator of UPR (Pfaffenbach and Lee, 2011). The IRE1, PERK, and ATF6 function as 3 distinct ER stress sensors to cope with complex UPR scenarios (Chen and Brandizzi, 2013). Moreover, IRE1 has been associated with activation of NF-κB (Kaser et al., 2011; Chen and Brandizzi, 2013), a vital transcription factor related to inflammation. In line with the inflammatory results, necrotic enteritis also increased expressions of ER stress-related genes and proteins, such as GRP78, ATF6, PERK, IRE1, TRAF2, and p65. In contrast, dietary supplementation with *B. subtilis* HW2 decreased the expressions of above genes and proteins. Kim et al. (2019) demonstrated that *Lactobacillus acidophilus* exerted an anticolitic effect by ameliorating ER stress and suppressing NF-κB activation in HT-29 cells and mice. Zhang et al. (2021) found that *L. johnsonii* attenuated *Citrobacter rodentium*-induced colitis via regulation of inflammatory responses and ER stress in mice. In broilers, Yang et al. (2020) also found that *Lactobacillus johnsonii* L531 suppressed ER stress, which contributes to the ameliorate intestinal inflammation caused by *Salmonella*. Additionally, Zhou et al. (2015) revealed that necrotic enteritis challenge increased protein expression of p65 in rats, but the supplementation with *Bifidobacteria* significantly decreased the p65 expression. Therefore, *B. subtilis* HW2 can alleviate intestinal inflammation and injury in broilers with necrotic enteritis by decreasing ER structural damage and attenuating ER stress.

The gut microbiota assumes a crucial part in maintaining intestinal health (den Besten et al., 2013; Cheng et al., 2021) and affects ER stress pathways (Wang et al., 2022a). In our study, although necrotic enteritis had no significant differences on the α-diversity index of gut microbiota, *B. subtilis* HW2 increased Observed otus, Chao1 and ACE indices on day 28, which was consistent with a study by Hadieva et al. (2021), in which *B. subtilis* GM5 increased Observed otus and Chao1 indices of the gut microbiota in broilers. As for the gut microbiota composition, necrotic enteritis decreased the abundance of *Turicibacter* and increased the abundance of *Erwiniaceae* on day 28, decreased the abundances of *Barnesiellaceae, Barnesiella*, and *Alistipes* on day 42. Although the roles of *Turicibacter* remain unclear, Jiao et al. (2018) found that the decreased *Turicibacter* is consistent with elevated inflammation. *Erwiniaceae* was found to be abundant in patients with presence of coronary calcium but without previous cardiovascular disease (Ortega-Madueño et al., 2022). Besides, Weiss et al. (2014) reported that *Barnesiella* could turn fucosyllactose into an energy source to colonize the intestine and improve its anti-inflammatory ability. Zhu et al. (2020b) also demonstrated that *Alistipes* was negatively correlated with lipopolysaccharide and TNF-α levels, demonstrating that *Alistipes* might suppress diabetic inflammation. Therefore, the above alterations to the abundance of the gut microbiota were in accordance with elevated inflammation in broilers challenged with necrotic enteritis. Additionally, we also observed increased *Christensenellaceae* abundance on day 28, increased *Barnesiellaceae, Barnesiella, Alistipes, Lactobacillaceae* and decreased *Enterococcaceae* on day 42 in L-Pro group. Similarly, M-Pro group decreased *Erwiniaceae* abundance and increased *Turicibacter* abundance on day 28, increased the abundances of *Barnesiellaceae, Barnesiella*, and *Alistipes* on day 42. H-Pro group increased the abundances of *Lactobacillaceae* and *Erwiniaceae* on day 28, increased *Hungateiclostridiaceae* abundance and decreased *unidentified_ Clostridia* abundance on day 42. *Christensenellaceae* are considered a signature taxon of healthy gut and are depleted in conditions associated with inflammation (Mancabelli et al., 2017). Study showed that *Christensenellaceae* was positively associated with the antioxidant genes (SOD2, TXN, and PRDX1) in broilers (Wasti et al., 2021). *Enterococcaceae* was identified as a potential biomarker of intestinal bowel disease (Lo Presti et al., 2019). Moreover, *Hungateiclostridiaceae* abundance increased in mice after oral administration of heat-killed *Latilactobacillus sakei* (Chung et al., 2021). Strains of *Clostridia*, like *C. difficile*, was mediators for inflammation (Shen, 2012). Thus, these findings indicated that *B. subtilis* HW2 supplementation had beneficial effects on regulating gut microbiota in associated with inflammation or diseases.

Functional prediction of the gut microbiota indicated that NE group decreased the mismatch repair pathway on day 28, but had no significant differences on gut microbiota function on day 42, suggesting that the adverse effects of necrotic enteritis on gut microbiota function decreased with time extended. Compared to NE group, L-Pro group increased pathways including glutathione metabolism, metabolism of xenobiotics by cytochrome P450, and drug metabolism-cytochrome P450 on day 42, which were closely related to the antioxidant and anti-inflammatory function (Wu et al., 2004; Guo et al., 2020; Stipp and Acco, 2021). M-Pro group decreased the pathways of bacterial invasion of epithelial cells on day 28 and the IL-17 signaling on day 42, implying that *B. subtilis* HW2 played vital roles in regulating bacterial infection via regulating cytokines, including IL-17. Furthermore, H-Pro group decreased the pathways related to diseases and infections, such as Alzheimer disease, pathways in cancer, hepatocellular carcinoma, pathogenic *E. coli* infection on day 28,

confirming the beneficial role of *B. subtilis* HW2. Moreover, the pathways of mannose type O-glycan biosynthesis and other types of O-glycan biosynthesis were decreased in H-Pro group on day 42. Report indicated that changes in O-glycans biosynthesis were found in many diseases, like intestinal bowel disease and malignant melanoma (Akiyoshi et al., 2020). Therefore, decreased the pathway related to O-glycan biosynthesis also implied the beneficial roles of *B. subtilis* HW2.

The SCFAs, produced during bacterial fermentation in the cecum of broilers, play crucial roles in energy metabolism, intestinal functionality, and intestinal pathogen reduction (Oladokun et al., 2021; Dai et al., 2022). Moreover, the study also indicated that ER stress can be modulated by SCFAs (Ke et al., 2021). Kushwaha et al. (2022) demonstrated that sodium butyrate treatment could reduce the ER stress markers p-PERK in high-fat diet-fed rats. Thus, the altered intestinal health, ER stress level, and gut microbiota structure of broilers prompted us to further detect the SCFAs levels in the cecal content. Here, we found that the decreased isobutyric acid and isovaleric acid levels in NE group were increased by *Bacillus subtilis* HW2 supplementation. Wang et al. (2021a) also found that *L. plantarum* increased the total SCFAs, acetate, lactate, and butyrate in broilers infected with *C. perfringens*. However, according to the study by Nylen et al. (2014), 2 mM isobutyric acid and 0.5 mM isovaleric acid increased the expression of antimicrobial peptide LL-37, which could increase upon induction of the ER stress pathway (Park et al., 2011). The different results of this study may be due to different levels of isobutyric acid and isovaleric acid.

To figure out the possible roles of gut microbiota and SCFAs in regulating growth performance and intestinal injury, correlation analysis was performed. On day 28, *Romboutsia* was negatively correlated with IRE1 expression. On day 42, *Barnesiella* and *Coprobacter* were also negatively correlated with inflammation-related parameters. Similarly, reports found that *Romboutsia* was strongly correlated with anti-oxidant and anti-inflammatory effects in high-fat diet fed rats (Wang et al., 2022b), *Barnesiella* was positively correlated with the ADG, villus height, and V/C ratio of broilers (Zhao et al., 2023), and *Coprobacter* was also negatively correlated with inflammatory factors in broilers (Kong et al., 2021). Besides, in present study, isobutyric acid and isovaleric acid were negatively correlated with pro-inflammatory cytokines and ER stress-related proteins, and positively correlated with ADG and tight junction proteins on days 28 and 42. Previous study also showed that SCFAs increased intestinal barrier function and attenuated intestinal inflammation in broilers (Liu et al., 2021a). Furthermore, ER stress was reported to activate NF-κB via the IRE1α/TRAF2 signaling pathway. The SCFAs were also significantly negatively correlated with p-p65 (Yuan et al., 2021). Therefore, the inactivation of p65 in *B. subtilis* HW2-treated groups and increased SCFAs levels may be related to the decrease of ER stress.

CONCLUSIONS

The results showed that *B. subtilis* HW2 improved growth performance, intestinal morphology, barrier function, and immune response in necrotic enteritis-challenged broilers. We found significant attenuation of ER stress and reshaping of the gut microbiota, especially *Romboutsia, Barnesiella*, and *Coprobacter*, along with changes in the SCFAs levels of cecal contents (including isobutyric acid and isovaleric acid). Besides, significant correlations were observed between the biochemical parameters and gut microbiota as well as SCFAs, indicating that the ER stress, gut microbiota, and SCFAs contents play crucial roles in enhancing growth performance and alleviating gut injury in *B. subtilis* HW2-treated broilers with necrotic enteritis. Under the conditions of this experiment, the optimal dietary supplementation dosage of *B. subtilis* HW2 is 5×10^6 CFU/g.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Peng Chen: Conceptualization, Data curation, Writing − original draft. **Huimin Lv:** Investigation, Methodology, Formal analysis. **Mengmeng Du:** Investigation, Formal analysis. **Weiyong Liu:** Visualization. **Chuanyan Che:** Investigation. **Jinshan Zhao:** Investigation. **Huawei Liu:** Conceptualization, Writing − review & editing, Funding acquisition.

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DISCLOSURES

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.103661.

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