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BACILLUS STRAINS ALLEVIATE STRESS IN BROILERS Bacillus pumilus TS1 and Bacillus amyloliquefaciens B64 alleviate corticosterone-induced oxidative stress and intestinal injury in broilers

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Abstract

This study investigated the protective effects of *Bacillus pumilus* TS1 and *Bacillus amyloliquefaciens* B64 on broilers subjected to corticosterone-induced oxidative stress. A total of 102 one-day-old Arbor Acres broilers were randomly assigned to six treatment groups. Birds received drinking water containing either TS1 or B64 for three weeks, followed by subcutaneous corticosterone administration (4 mg/kg) to induce oxidative stress. Growth performance, antioxidant capacity, intestinal morphology, microbial composition, and expression of intestinal barrier and antioxidant-related genes and proteins were evaluated. Supplementation with TS1 and B64 significantly improved body weight gain compared with corticosterone-treated controls. Both strains enhanced serum total antioxidant capacity and reduced malondialdehyde, lactate dehydrogenase, and creatine kinase levels, indicating mitigation of oxidative damage. Histological analysis revealed that TS1 and B64 protected duodenal and jejunal villus structure and preserved mucosal integrity. 16S rRNA sequencing showed that corticosterone disrupted intestinal microbial balance, while both Bacillus strains restored microbial diversity and increased beneficial genera such as *Lactobacillus and Akkermansia*. At the molecular level, TS1 and B64 upregulated the transcription and expression of intestinal tight junction proteins (Claudin-1, Claudin-3, ZO-1, and Mucin-2), thereby enhancing barrier function. Both strains activated the KEAP1/NRF2 signaling pathway, evidenced by increased expression of NRF2, HO-1, and NQO1, and suppressed KEAP1 expression, suggesting improved antioxidant defense. Among the two strains, B64 exhibited slightly stronger regulatory effects on antioxidant and tight junction markers. In conclusion, *Bacillus pumilus* TS1 and *Bacillus amyloliquefaciens* B64 effectively alleviate corticosterone-induced oxidative stress and intestinal injury in broilers by modulating the intestinal microbiota, enhancing antioxidant capacity, and activating the KEAP1/NRF2 pathway. Supplementation with these Bacillus strains can improve oxidative resilience and intestinal health in broilers under stress, providing a potential alternative to antibiotics for promoting performance and gut integrity in commercial poultry production.

Introduction

In poultry production, various stressors such as noise, handling, and transportation can trigger complex pathophysiological responses in birds, including increased aggression, feather-pecking behavior, reduced growth rate, impaired feed conversion, immune dysfunction, and disturbances in the intestinal microbiota. These effects not only compromise animal welfare but also lead to substantial economic losses in the broiler industry and, in severe cases, even mortality (NAWAB A, 2018). Therefore, developing effective strategies to mitigate stress is essential, and the use of feed additives represents one promising approach. For decades, antibiotics have served as the cornerstone of poultry production (Seven P, 2008). However, their excessive and indiscriminate use has led to ecological and environmental pollution, antimicrobial resistance, and significant economic concerns. Consequently, many countries, including China, have moved toward restricting or phasing out antibiotic use in animal feeds. Probiotics have emerged as a safe and effective alternative, these beneficial microorganisms help maintain intestinal microecological balance, enhance nutrient digestion and absorption, inhibit pathogenic colonization, and strengthen intestinal barrier function, thereby improving overall performance and health in poultry (De Filippis F, 2020; Vemuri R, 2017; Suez J, 2019).

According to previous studies, corticosterone (**CORT**) is commonly used to establish a chronic stress model in animals (Dallman M F, 1973; Costantini David, 2008). Oxidative stress results in the excessive generation of reactive oxygen species (**ROS**), which damage cellular structures and impair physiological functions. The enzymatic antioxidant defense system plays a central role in maintaining redox balance and protecting the body from oxidative injury. Total antioxidant capacity (**T-AOC**) serves as a key indicator reflecting the overall efficiency of

the organism's defense mechanisms against free radicals. Classic markers of oxidative damage include malondialdehyde (**MDA**), which reflects the degree of lipid peroxidation, and catalase (**CAT**), a major peroxisomal enzyme responsible for hydrogen peroxide decomposition. In addition, lactate dehydrogenase (**LDH**), a key glycolytic enzyme that indicates cellular membrane integrity, and creatine kinase (**CK**), whose serum concentration correlates with intestinal mucosal injury and microbial imbalance, were also measured. Therefore, these biochemical indicators were analyzed in the serum of broilers to evaluate the protective effects of Bacillus supplementation (MOOLI R G R, 2022; CHENG D Y, 2024; Altan O, 2003).

Our previous research identified two probiotic strains, *Bacillus pumilus* TS1 and *Bacillus amyloliquefaciens* B64, with promising biological functions. Feeding medium to high doses of *Bacillus pumilus* TS1 has been shown to effectively regulate key proteins such as Nuclear factor E2 related factor 2 (**Nrf2**), p38 mitogen-activated pro-tein kinase(**p38**), mitogen-activated protein kinase,**MAPK**, Nuclear factor-kappa B,NF-κB**NF-κB**, and heme oxygenase-1 (**HO-1**) within inflammatory signaling pathways, thereby enhancing antioxidant and anti-inflammatory responses. Meanwhile, *Bacillus amyloliquefaciens* has been reported to improve growth performance, enhance digestive enzyme activity, and strengthen immune function in broiler chickens (Liu Y, 2024; Zhu Yongming, 2023; RAMLUCKEN U, 2020; Bampidis V, 2021; Bampidis V, 2022). Building on these findings, the present study aimed to further investigate whether supplementation with *Bacillus pumilus* TS1 and *Bacillus amyloliquefaciens* B64 could alleviate corticosterone (CORT)-induced oxidative stress through modulation of the KEAP1/NRF2 antioxidant signaling pathway.

Previous studies have demonstrated that CORT induces oxidative stress and apoptosis both *in vivo* and *in vitro*, leading to excessive free radical generation and cellular injury. In this experiment, Bacillus strains were administered via drinking water. Specifically, *Bacillus pumilus* TS1 (1.4×10^7 CFU/mL), isolated from yak, and *Bacillus amyloliquefaciens* B64 (5.0×10^7 CFU/mL), isolated from cattle, were used to colonize the intestinal tract of broilers. The intestinal microbiota plays a central role in maintaining host health by regulating nutrient metabolism, immune function, and intestinal homeostasis. Disruption of microbial balance can lead to bacterial translocation, intestinal barrier dysfunction, epithelial injury, and increased permeability, which amplify stress responses and mucosal damage (HEZT, 2022; CUI M X, 2020).

To evaluate whether TS1 and B64 could mitigate such intestinal injury, this study analyzed intestinal villus height and crypt depth, assessed mucin production, and quantified key tight junction proteins, including occludin, claudin, and zonula occludens (**ZO-1**), which are essential for maintaining epithelial barrier integrity (PARADIS T, 2021). Additionally, the KEAP1/NRF2 pathway, a critical endogenous antioxidant defense system, was examined. Activation of NRF2 and suppression of its negative regulator KEAP1 promote the transcription of cytoprotective genes such as HO-1 and NAD(P)H quinone oxidoreductase 1 (**NQO1**), enhancing cellular antioxidant capacity and reducing oxidative injury (Xin X, 2024; Niu B, 2024; Shen Y, 2019).

In summary, this study investigates the protective effects of *Bacillus pumilus* TS1 and *Bacillus amyloliquefaciens* B64 on the intestinal health of broilers, focusing on their ability to mitigate CORT-induced oxidative stress and elucidating the underlying KEAP1/NRF2-mediated mechanisms.And evaluate which of Bacillus subtilis and Bacillus amyloliquefaciens has stronger stress relieving ability.

Materials and Methods

Experimental design and animal preparation

A total of 102 one-day-old Arbor Acres (**AA**) broiler chicks were obtained from Nanjing Te Awesome Planting Cooperative (Nanjing, China). Upon arrival, all birds were individually weighed, wing-banded for identification, and each group is randomly assigned to 3–4 cages to ensure equal average initial body weight among groups. The chicks were allowed a 7-day acclimation period before the start of the experiment, during which body weights were recorded at predetermined intervals. Birds were housed in environmentally controlled rooms equipped with wire-floor cages. The ambient temperature was maintained at 30 ± 3°C during the first week and gradually reduced to 25 ± 3°C thereafter. Relative humidity was controlled at 60–70%, and natural ventilation was provided daily to ensure adequate air exchange. Feed and water were available ad libitum throughout the trial. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Nanjing Agricultural University (Nanjing, China).

Bacillus colonization and CORT treatment

Table 1 Experimental Groups

As shown in Fig. 1, bacterial suspensions were prepared by dissolving the Bacillus cultures in distilled water to achieve the desired concentrations. Following a one-week adaptation period, birds were provided drinking water containing the designated bacterial preparations according to their respective treatment groups. A total of six groups were established for administration, The following groups are respectively(CON group、CON+CORT group、TS1 group、TS1 + CORT group、B64 group and B64 + CORT group). After three weeks of Bacillus supplementation, birds received subcutaneous injections of CORT powder (Sigma-Aldrich, St. Louis, MO, USA) dissolved in physiological saline with anhydrous ethanol as a solvent at a dose of 4 mg/kg body weight. The injections were administered once daily for seven consecutive days to induce oxidative stress. At the end of the treatment period, birds were fasted for 8 hours before sample collection.

Sample collection

Broiler chickens were euthanized by neck breakage.Blood samples were immediately collected, and serum was separated by centrifugation and stored at − 80°C until analysis. Subsequently, portions of major organs, including intestinal tissues, were divided into two parts: one was snap-frozen in liquid nitrogen and stored at − 80°C for biochemical and molecular analyses, while the other was fixed in 10% neutral-buffered formalin (Sinopharm Chemical Reagent Co., Shanghai, China) for histological examination.

Enzyme-linked immunosorbent assay (**ELISA** *)*

Serum levels of creatine kinase-MB (CK-MB), malondialdehyde (MDA), superoxide dismutase (SOD), lactate dehydrogenase (LDH), and total antioxidant capacity (T-AOC) were determined using commercial ELISA kits (Beyotime Biotechnology, Nanjing, China). All assays were performed in accordance with the manufacturer's protocols. Measurements were conducted at the School of Veterinary Medicine, Nanjing Agricultural University (Nanjing, China), and absorbance was read using a microplate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

Hematoxylin and eosin (H&E) staining

Following euthanasia by carotid artery exsanguination, intestinal tissue samples were immediately collected and processed according to standard histological procedures. Fresh tissues were fixed in 10% neutral-buffered formalin (Sinopharm Chemical Reagent Co.) for 6–12 h, then dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections of 4–5 μm thickness were mounted on glass slides. After deparaffinization and rehydration, the sections were stained with hematoxylin for 5 min, differentiated in acid alcohol, and blued in alkaline solution. Cytoplasmic counterstaining was performed with eosin for 1 min, followed by dehydration in graded ethanol, clearing in xylene, and sealing with neutral resin. Under microscopic examination, nuclei appeared blue-purple, whereas cytoplasm and collagen fibers appeared pink, allowing clear visualization of histological features. This classical and widely applied staining method enabled evaluation of pathological alterations and provided morphological evidence supporting experimental results.

High-throughput analysis of microorganisms

Intestinal contents from the six experimental groups were collected under sterile conditions and subjected to 16S rRNA gene next-generation sequencing. Total microbial DNA was extracted from each sample using a commercial DNA extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified by PCR (Eppendorf™, Model # AG 22331, Hamburg, Germany), and the amplicons were purified, quantified, and normalized prior to library construction. Sequencing libraries were prepared using the Illumina TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina NovaSeq 6000 platform. Raw sequence data were processed through quality control and paired-end read assembly to remove low-quality and chimeric sequences. Operational taxonomic units (**OTUs**) were clustered based on 97% sequence similarity and annotated using the SILVA reference database. Taxonomic classification and inter-group community comparisons were performed at both the phylum and genus levels. α-diversity indices, including Shannon, ACE, and Chao1, were calculated to evaluate microbial diversity among samples, and visualization of relative abundance distributions was generated in bar and petal plots for comparative analysis.

Immunohistochemical assay

Paraffin-embedded intestinal tissue sections were first incubated at 60°C for 1 h to melt residual paraffin, followed by deparaffinization in xylene and rehydration through a graded ethanol series. Antigen retrieval was carried out in preheated sodium citrate buffer (pH 6.0) using a water bath, after which the sections were rinsed three times with phosphate-buffered saline (**PBS**) for 5 min each. A hydrophobic barrier was drawn around the tissue using an immunohistochemical pen. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min at room temperature, followed by three additional PBS washes. Non-specific binding was blocked with normal goat serum (Beyotime Biotechnology) for 10 min at room temperature. Sections were then incubated overnight at 4°C with the appropriately diluted primary antibodies prepared in antibody dilution buffer (P0023A, Beyotime Biotechnology). After washing with PBS, the slides were incubated with a biotinylated secondary antibody for 30 min at room temperature, followed by treatment with avidin–biotin complex solution and visualization using 3,3'-diaminobenzidine (**DAB**) chromogen. Counterstaining was performed with hematoxylin, followed by differentiation, bluing, dehydration, and mounting with neutral resin. Immunostaining was examined under a fluorescence microscope (Imager A2, Zeiss, Oberkochen, Germany). Brownish-yellow staining was interpreted as a positive reaction, whereas blue staining indicated cell nuclei.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from approximately 50 mg of intestinal tissue using FreeZol Reagent (Novozymes, Nanjing, China) according to the manufacturer's protocol. Briefly, 500 μL of reagent was added to each sample for lysis, followed by a 5 min incubation at room temperature. After centrifugation for 5 min, the supernatant was mixed with Dilution Buffer (5:1, v/v) and centrifuged for 15 min. The resulting supernatant was transferred to a new microcentrifuge tube, and an equal volume of isopropanol was added to precipitate RNA. Following a 10 min incubation at room temperature, the mixture was centrifuged again, and the pellet was washed with 1 mL of 75% ethanol, centrifuged for 3 min, air-dried, and dissolved in 20–100 μL of DEPC-treated water. RNA purity and concentration were determined spectrophotometrically using a NanoDrop 2000 (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from total RNA using a reverse transcription kit (CW3360, Kangwei Century, Taizhou, China). Quantitative PCR was performed using SYBR Green Master Mix (Qingke Biotechnology, Beijing, China) with gene-specific primers (Table 1) on a real-time PCR system (Eppendorf AG). The amplification program consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s. Gene expression levels were determined based on Ct (threshold cycle) values, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the internal reference. Statistical analysis of gene expression data was conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of Bacillus supplementation on body weight and serum antioxidant indices

Figure 2 (A) Body weight change trend (B-F) Serum stress factors detected by ELISA: T-AOC, MDA, CAT, LDH, CK

As shown in Fig. 2A, the body weight of broilers increased linearly during the experimental period. On day 14, birds in the TS1 group exhibited a significant increase in body weight compared with the control ($P < 0.05$), and this difference became highly significant on day 21 ($P < 0.01$). By day 35, both the CORT and control groups showed pronounced weight loss ($P < 0.001$), reflecting the inhibitory effect of stress on growth performance. In contrast, birds supplemented with TS1 maintained higher body weights, although a slight reduction was still observed ($P < 0.05$). Similarly, the TS1 + CORT and CON+CORT groups exhibited mild but significant decreases in body weight ($P < 0.05$).

As illustrated in Figs. 2B–F, CORT administration markedly elevated serum MDA levels ($P < 0.001$) and significantly increased CAT, LDH, and CK activities ($P < 0.05$), indicating enhanced oxidative stress and tissue damage. Conversely, supplementation with TS1 or B64 effectively restored antioxidant balance. The TS1 + CORT and CON+CORT groups displayed significant increases in T-AOC ($P < 0.01$), accompanied by marked reductions in MDA and CAT levels ($P < 0.01$) and a strong suppression of CK activity ($P < 0.001$). Similarly, birds in the B64 + CORT and CON+CORT groups exhibited a highly significant enhancement of T-AOC ($P < 0.001$) and reduced MDA and CAT expression ($P < 0.05$). These findings indicate that both Bacillus strains enhanced the systemic antioxidant defense, mitigated oxidative damage, and improved overall physiological resilience under CORT-induced stress.

Effects of Bacillus supplementation on intestinal morphology in CORT-treated broilers

Figure 3 (A-B) HE-stained sections of duodenum and jejunum (C) Quantitative analysis of intestinal villi (D) Quantitative analysis of intestinal crypts (E) Statistical analysis of villus-to-crypt ratio.

As shown in Fig. 3, supplementation with TS1 or B64 effectively mitigated intestinal structural damage caused by CORT exposure. In the duodenum, villus height was significantly greater in the TS1 + CORT and CON+CORT groups compared with the control ($P < 0.05$), while the B64 + CORT group exhibited a highly significant increase in villus length ($P < 0.001$). No notable differences were observed in crypt depth or the villus-to-crypt ratio among the duodenal samples. In the jejunum, however, CORT treatment resulted in a pronounced shortening of villi ($P < 0.001$) and a reduction in crypt size ($P < 0.05$) compared with the CON group, indicating structural injury due to oxidative stress. Supplementation with Bacillus strains significantly reversed these changes: both the CON+CORT and B64 + CORT groups displayed a marked increase in crypt depth ($P < 0.01$), restoring intestinal morphology toward normal levels. These results suggest that TS1 and B64 supplementation contributes to maintaining villus architecture and mucosal integrity in broilers under CORT-induced stress.

Effects of Bacillus supplementation on cecum microbial diversity and community composition

Figure 4 Cecal microbiota α-diversity analysis (A-C) Shannon, ACE, and Chao indices (D) Total number of microbial communities in each group (E) Microbial community analysis at the phylum level (F) Microbial community analysis at the genus level.

As shown in Figs. 4A–D, α-diversity analysis of the cecum microbiota revealed distinct differences among the treatment groups. According to the Shannon index, microbial diversity in the CON and B64 groups was significantly lower than that in the CORT-treated groups ($P < 0.001$), while both the TS1 and B64 groups showed a marked reduction in microbial abundance compared with the latter ($P < 0.01$). Consistent patterns were observed in the ACE and Chao1 indices, where the number of microbial species in the CON and CON+CORT groups decreased significantly compared with the other treatments ($P < 0.01$), and the B64 group exhibited the lowest richness ($P < 0.001$).

At the phylum level (Figs. 4E–F), *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were dominant taxa in the intestinal communities. Their relative abundances were notably reduced in the TS1 + CORT and B64 + CORT groups compared with the CORT group, suggesting that Bacillus supplementation helped restore microbial balance disrupted by oxidative stress. At the genus level, CORT exposure led to increased proportions of *Akkermansia* and *Lactobacillus*, accompanied by a reduction in *Erysipelotrichaceae* species. Treatment with TS1 and B64 partially reversed these changes, indicating that both strains promoted a more favorable microbial structure conducive to intestinal health and resilience against CORT-induced dysbiosis.

Effects of Bacillus supplementation on tight junction and antioxidant-related gene expression

Figure 5 (A-C) Detection of mRNA expression levels of Claudin-3, ZO-1, Mucin2, Occludin, NRF2, HO-1, NQO1, SOD, CAT, and GSH-PX in the duodenal intestine by qPCR (D-F) mRNA transcription levels of Claudin-1, Claudin-5, ZO-1, Mucin2, NRF2, HO-1, NQO1, SOD, CAT, and GSH-PX in the jejunal intestine.

As shown in Fig. 5A, supplementation with TS1 and B64 significantly enhanced the transcription of tight junction and mucin-related genes in the duodenum. Compared with the CORT and CON groups, ZO-1 expression was

significantly upregulated ($P < 0.05$). In the TS1 + CORT group, Claudin-3 transcription increased ($P < 0.05$), while ZO-1 and Mucin-2 levels rose markedly ($P < 0.01$). Similarly, in the B64 + CORT group, Claudin-3 and Mucin-2 were significantly upregulated ($P < 0.01$), ZO-1 expression showed a strong increase ($P < 0.001$), and Occludin expression also rose ($P < 0.05$). Moreover, when comparing TS1 + CORT and B64 + CORT, the latter exhibited higher Claudin-3 expression ($P < 0.01$), indicating that B64 more effectively preserved tight junction integrity.

Figures 5B and 5C demonstrate that CORT exposure markedly suppressed NRF2 transcription ($P < 0.01$) and downregulated antioxidant genes SOD and CAT ($P < 0.05$) in the duodenum. However, TS1 and B64 supplementation effectively counteracted these effects. In the TS1 + CORT group, HO-1 ($P < 0.05$), CAT ($P < 0.001$), and GSH-PX ($P < 0.05$) transcription levels significantly increased compared with CORT alone. In the B64 + CORT group, NRF2 and HO-1 expression were strongly upregulated ($P < 0.001$), while NQO1 and CAT also increased ($P < 0.05$), and SOD and GSH-PX levels rose significantly ($P < 0.01$). Notably, B64 supplementation produced greater increases in NRF2 ($P < 0.05$), HO-1, SOD, and CAT ($P < 0.01$) compared with TS1, suggesting superior antioxidant activation.

As shown in Fig. 5D, similar patterns were observed in the jejunum. The TS1 + CORT group exhibited significantly higher Claudin-1, Claudin-5, and Mucin-2 transcription ($P < 0.01$) and increased ZO-1 expression ($P < 0.05$) compared with CORT. The B64 + CORT group also displayed elevated Claudin-5 and Mucin-2 ($P < 0.05$) and enhanced ZO-1 ($P < 0.01$). In Figs. 5E and 5F, CORT markedly suppressed NRF2 expression in the jejunum ($P < 0.01$), but both Bacillus strains reversed this effect. TS1 + CORT increased NRF2 ($P < 0.01$), HO-1 ($P < 0.001$), and NQO1 ($P < 0.05$), whereas B64 + CORT induced robust upregulation of NRF2, HO-1, NQO1, and CAT ($P < 0.001$), with additional increases in SOD and GSH-PX ($P < 0.05$). Compared with TS1 + CORT, the B64 + CORT group showed higher SOD and GSH-PX expression ($P < 0.05$) and a markedly greater rise in CAT transcription ($P < 0.001$). These findings indicate that both Bacillus strains enhanced intestinal barrier function and activated KEAP1/NRF2-mediated antioxidant defense, with B64 exhibiting a stronger regulatory effect.

Immunohistochemical analysis of intestinal oxidative stress and junction protein expression

Figure 6 Immunohistochemistry detection of NRF2 (A-B), KEAP1 (C-D), NQO1 (E-F), Claudin-1 (G-H), and Mucin2 (I-J) expression levels in the duodenum and jejunum, and Image J statistical analysis of the duodenum (K-M) and jejunum (L-N).

As shown in Figs. 6A–N, immunohistochemical staining revealed clear differences in the expression patterns of oxidative stress–related and intestinal barrier–associated proteins among the treatment groups. In both the duodenum and jejunum, the CORT and CON groups exhibited lighter positive staining for NRF2、KEAP1、NQO1、Claudin-1 and Mucin-2, whereas TS1 and B64 supplementation markedly enhanced the intensity of these signals, indicating increased protein expression. Conversely, the KEAP1-positive staining was more intense in the CORT group, reflecting elevated KEAP1 expression, while it was noticeably lighter in the TS1 and B64 groups, suggesting that both strains suppressed KEAP1 expression and mitigated oxidative stress.

Quantitative analysis using ImageJ further supported these observations. In the duodenum, CORT treatment led to a downward trend in NRF2 expression, a significant increase in KEAP1 ($P < 0.01$), and NQO1 declined compared with CON. In the jejunum, NRF2 expression decreased markedly ($P < 0.001$), while KEAP1 increased ($P < 0.05$)and a significant reduction in NQO1 ($P < 0.01$) compared with CON. Supplementation with TS1 + CORT reversed these effects, showing elevated NRF2 and KEAP1 levels in the duodenum ($P < 0.05$) and a highly significant increase in

NRF2 in the jejunum ($P < 0.001$). Compared with TS1 + CORT, the B64 + CORT group displayed lower NRF2 expression in the duodenum ($P < 0.05$) but higher NRF2 levels in the jejunum ($P < 0.05$).

Regarding intestinal barrier proteins, Claudin-1 expression in the duodenum tended to decrease in the CORT group but significantly increased in B64 + CORT ($P < 0.05$), accompanied by a marked rise in Mucin-2 expression ($P < 0.01$). Similarly, in the jejunum, CORT treatment reduced both Claudin-1 and Mucin-2 expression ($P < 0.05$), whereas Bacillus supplementation restored their levels. These results confirm that TS1 and B64 alleviated CORT-induced oxidative stress by downregulating KEAP1 and enhancing NRF2-mediated antioxidant activity while simultaneously reinforcing intestinal barrier integrity through upregulation of Claudin-1 and Mucin-2.

DISCUSSION

Body weight gain or loss serves as a direct indicator of the physiological and health status of broilers during experimental evaluation. Under oxidative stress, broilers typically exhibit reduced feed intake, decreased water consumption, and a lowered metabolic rate, all of which contribute to inhibited growth performance (Zhao G L, 2022;YANG X, 2010). Previous studies have demonstrated that *Bacillus* supplementation can enhance growth performance by improving nutrient utilization and maintaining intestinal integrity. In this study, consistent with these findings, the B64 group showed a significant increase in body weight as early as day 7, while the TS1 group exhibited notable gains on days 14 and 21, indicating that both strains exerted growth-promoting effects at different stages. Conversely, broilers in the CORT group experienced a pronounced reduction in body weight by day 35, confirming that CORT exposure induced anorexia and growth suppression. These results suggest that TS1 and B64 supplementation effectively counteracted the adverse effects of oxidative stress on growth performance, thereby supporting their potential use as functional probiotics to maintain productivity in stressed broilers.

Intestinal oxidative stress increases the production of reactive oxygen species, impairing cellular function, disrupting the body's antioxidant defense balance, and leading to ROS-induced damage. This process also results in the release of toxic metabolites into circulation, which alters the activity of key antioxidant markers such as CAT, SOD, and GSH-Px (Altan O, 2003;BAI K, 2018PETERSON L W, 2014). In the present study, subcutaneous administration of CORT significantly elevated serum MDA, CAT, and LDH levels, accompanied by an upward trend in T-AOC, confirming the activation of oxidative stress pathways. Following probiotic colonization with TS1 and B64, the levels of LDH and MDA declined markedly, whereas T-AOC significantly increased, indicating mitigation of oxidative damage. Gene transcription analysis further supported these findings: SOD, CAT, and GSH-Px expression decreased notably in the duodenum and jejunum of the CORT group but showed a clear recovery or upward trend in the TS1 + CORT and B64 + CORT groups. These results demonstrate that both TS1 and B64 effectively suppressed oxidative stress and enhanced the antioxidant defense system in CORT-induced broilers. Meanwhile, the antioxidant capacity of TS1 is stronger than that of B64.The pronounced antioxidant effects observed in this study are consistent with previous reports confirming the protective role of Bacillus species in reducing oxidative injury and improving redox homeostasis (SZETO H H, 2006; WANG Y, 2018).

The intestine functions as a critical barrier that protects the host from bacterial toxins and other harmful agents. Structural parameters such as villus height, crypt depth, and the villus-to-crypt ratio are key morphological indicators of intestinal health. Longer villi provide a larger absorptive surface area, thereby improving feed utilization efficiency and nutrient absorption, ultimately supporting growth performance in broilers (CASPARY W F, 1992; Seppi M, 2023). In the present study, histopathological observations revealed that the intestinal tissue of the CON group displayed a well-organized architecture with intact mucosa, orderly epithelial alignment, and no signs

of cellular degeneration or necrosis. In contrast, the CORT group exhibited marked histological alterations, including villus disruption in both the duodenum and jejunum, crypt atrophy, mucosal defects, and distorted epithelial morphology, hallmarks of stress-induced intestinal injury.

Supplementation with TS1 and B64 notably alleviated these pathological changes. Both TS1 + CORT and B64 + CORT groups showed significant increases in duodenal villus height compared with CORT, while jejunal villi were also lengthened, although crypt depth in the duodenum remained unchanged. In the jejunum, CORT exposure caused a significant reduction in crypt size, whereas TS1 + CORT and B64 + CORT treatments restored crypt depth to near-normal levels. No significant difference was observed in the villus-to-crypt ratio. These findings suggest that colonization with TS1 and B64 effectively mitigated CORT-induced mucosal injury and maintained intestinal structural integrity, consistent with previous reports that *Bacillus* supplementation enhances gut morphology and barrier resilience under stress conditions (Liu Y, 2023).

The intestinal microbiota plays a vital role in maintaining host nutrition, immunity, metabolism, and disease resistance. A stable microbial community is essential for intestinal homeostasis and optimal growth performance in animals, while a reduction in microbial diversity disrupts this balance and compromises gut health. As a primary target of oxidative stress, alterations in the intestinal microbiota are key indicators of redox imbalance. The intestinal microbial community is predominantly composed of *Actinobacteria*, *Bacteroidetes*, *Bacteroidota*, and *Verrucomicrobiota* (Zhao Y, 2020; Yaklai K, 2021; XU ZR, 2003; Liu JH,2020). Under stress conditions, the equilibrium of this ecosystem is disturbed, leading to compositional changes that adversely affect host physiology. The ratio of *Firmicutes* to *Bacteroidetes*, a well-established marker of intestinal health, has dual pathological significance: an elevated ratio is associated with metabolic dysfunction, while a decreased ratio indicates inflammatory injury. *Bacillus* species can help reestablish microbial balance by modulating the intestinal environment, forming a biological barrier, suppressing pathogenic colonization, and restoring microbial homeostasis (ZHANG B, 2011; GREINER T B, 2011; AMOAH K, 2020;GU Y F, 2022).

In this study, α-diversity analysis revealed that microbial richness decreased in both the TS1 and B64 groups, suggesting that single-strain colonization modestly influenced the native microbial structure. However, a marked reduction in microbial abundance was observed in the CORT group, indicating that CORT disrupted gut microbial stability. At the phylum level, the CORT group exhibited increased proportions of *Actinobacteria*, *Bacteroidota*, and *Proteobacteria*, which are associated with dysbiosis, metabolic disturbances, and compromised mucosal integrity, potentially heightening the risk of inflammatory bowel conditions. Conversely, in the TS1 + CORT and B64 + CORT groups, the relative abundance of *Actinobacteria* and *Bacteroidota* declined, suggesting partial restoration of microbial equilibrium. The elevated abundance of *Proteobacteria* in these groups may represent a compensatory mechanism supporting epithelial repair and crypt regeneration, while the enrichment of *Bacteroides*, *Verrucomicrobiota*, and *Actinomycetes* contributed to microbiota stabilization (Yang H, 2019).

Notably, the increase in *Akkermansia* in the TS1 + CORT and B64 + CORT groups is indicative of improved digestive capacity, reduced intestinal permeability, and enhanced anti-inflammatory activity, thereby strengthening the mucosal barrier and nutrient absorption (Huck O, 2020; Keshavarz Azizi Raftar S, 2021). The concurrent rise in *Lactobacillus*, a well-known probiotic genus, further supports intestinal homeostasis by inhibiting pathogenic bacteria and promoting intestinal motility (WANG Y, 2017). Meanwhile, the decline of *Erysipelotrichaceae*, an opportunistic pathogen within *Firmicutes*, implies reduced harmful bacterial proliferation and alleviation of intestinal stress, consistent with earlier findings. The distinct microbial patterns observed between the TS1 and

B64 treatments may be attributed to inherent differences in their spore-forming properties and colonization dynamics.

The intestinal epithelial barrier plays a crucial role in maintaining cellular integrity and selective permeability, thereby preventing the invasion of harmful substances, preserving microbial balance, and sustaining a stable environment for bacterial symbiosis. Tight junctions form the first line of defense within the intestinal epithelium and consist of transmembrane proteins (Occludin, Claudin), peripheral membrane scaffolding proteins (ZO-1), and mucin glycoproteins (Mucin-2). Occludin is a multifunctional transmembrane protein involved in intercellular adhesion and permeability regulation, while ZO-1, a cytoplasmic member of the zonula occludens family, links Claudin proteins to the actin cytoskeleton and plays an essential role in maintaining epithelial integrity, permeability, and cell differentiation (Saitou M, 1997; MEI M, 2016). Members of the Claudin family are integral components of tight junctions: Claudin-1 regulates epithelial permeability, Claudin-3 forms a continuous sealing layer that limits paracellular transport, and Claudin-5 tightens intercellular junctions in epithelial and endothelial tissues (Gong Y, 2016; SUZUKI K, 2023; AMASHEH S, 2005). Mucin-2, secreted by goblet cells, serves as a protective mucosal layer that strengthens barrier function, suppresses inflammation, and prevents pathogen invasion.

Disruption of these proteins can increase mucosal permeability, compromise barrier integrity, and exacerbate oxidative stress, leading to inflammatory injury and impaired intestinal homeostasis (HE L Q, 2017; PARADIS T, 2021). Oxidative stress has also been shown to inhibit intestinal stem cell proliferation, disrupt tight junction continuity, and heighten susceptibility to intestinal inflammation. Probiotics and their metabolites, however, can enhance immune cell activity, support epithelial regeneration, and mitigate stress-induced damage (XU ZR, 2003; GRONDIN J A,2020).

In the present study, transcriptional analysis revealed that the expression of Claudin-3, ZO-1, Mucin-2, and Occludin decreased in the duodenal CORT group, with Claudin-1 and Mucin-2 showing a downward trend. Similarly, in the jejunum, Claudin-1, Claudin-3, ZO-1, and Mucin-2 were downregulated, with Claudin-1 and Mucin-2 significantly reduced, indicating compromised barrier integrity and enhanced inflammatory susceptibility. Conversely, TS1 + CORT treatment increased the expression of Claudin-1 and Mucin-2 in both the duodenum and jejunum, while B64 + CORT supplementation significantly upregulated Mucin-2 expression in the duodenum. The consistent transcriptional and protein-level results confirm that TS1 and B64 effectively repaired intestinal barrier damage and restored mucosal function, At the same time, The repair ability of B64 in the duodenum is stronger than TS1, while the repair ability of the two in the jejunum is similar, findings that align with previous reports on the barrier-protective roles of *Bacillus* probiotics under oxidative stress conditions.

The Keap1/Nrf2 signaling pathway is a pivotal endogenous defense mechanism against oxidative stress. Nrf2, a key transcription factor within this pathway, regulates the expression of antioxidant genes and is closely associated with SIRT3-mediated redox balance in the nicotinamide adenine dinucleotide-dependent deacetylase family, while Keap1 serves as its upstream negative regulator (Wang L, 2022; Xin X, 2024; Niu B, 2024). Under physiological conditions, Nrf2 and Keap1 remain bound in the cytoplasm, maintaining redox homeostasis. When oxidative stress occurs, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it activates the transcription of downstream antioxidant genes such as HO-1 and NQO1, thereby restoring the cellular oxidative–antioxidant equilibrium (Shen Y,2019;Xin X,2024;Niu B,2024).

In this study, CORT administration suppressed the transcription and protein expression of Nrf2 and NQO1 in both the duodenum and jejunum, reduced HO-1 transcription, and increased Keap1 expression, indicating excessive oxidative stress and impaired antioxidant signaling. However, supplementation with TS1 + CORT and B64 + CORT reversed these effects. Both treatments upregulated the transcription and expression of Nrf2 and NQO1, elevated HO-1 levels, and inhibited Keap1 expression. These results demonstrate that colonization with TS1 and B64 effectively reactivated the Keap1/Nrf2 signaling pathway, thereby mitigating CORT-induced intestinal oxidative injury.,two types of Bacillus have comparable stress relieving abilities.The findings are consistent with previous studies highlighting the capacity of *Bacillus* species to modulate Nrf2-mediated antioxidant defenses and protect intestinal integrity under stress conditions.

CONCLUSION

In this study, both Bacillus pumilus TS1 and Bacillus amyloliquefaciens B64 promoted growth performance in broiler chickens, with TS1 exerting a more pronounced effect. Under CORT-induced stress, TS1 notably improved body weight gain, and both strains demonstrated strong antioxidant properties. Supplementation with TS1 and B64 positively influenced intestinal morphology by increasing villus height and crypt depth while maintaining a balanced villus-to-crypt ratio. Both strains also modulated the intestinal microbiota by suppressing harmful bacteria, enhancing the abundance of beneficial genera such as Lactobacillus, and optimizing the relative proportion of Bacteroidetes.

Furthermore, TS1 and B64 enhanced the transcription and expression of key intestinal barrier proteins, including Claudin, ZO-1, Occludin, and Mucin-2, thereby reinforcing epithelial integrity. Under CORT-induced oxidative stress, the KEAP1/NRF2 signaling pathway was activated, and Bacillus supplementation significantly enhanced antioxidant defenses by upregulating NRF2 and its downstream targets (HO-1 and NQO1) while suppressing KEAP1 expression.

Overall, *Bacillus pumilus* TS1 and *Bacillus amyloliquefaciens* B64 exerted marked protective and antioxidant effects against CORT-induced intestinal injury, overall, TS1 has stronger antioxidant capacity than B64suggesting their potential as effective probiotic feed additives for improving intestinal health and oxidative resilience in broiler production, and further research on related oxidative stress pathways can be conducted to improve its function

Declarations

Ethics Statement

This study was approved by the Ethics Committee of the College of Arts, Univeristy of College of Veterinary Medicine, Nanjing Agricultural University.All procedures performed in this study involving human participants were conducted in accordance with the ethical standards of the institutional research committee .

Consent to Publish

All participants provided written consent for the publication of anonymised data collected during the study. No identifiable information about participants will be disclosed in this publication.

Consent for publication

of case study details, including anonymised narratives, was obtained from all individuals involved.

Informed consent

was obtained from all individual participants included in the study. Participants were provided with detailed information about the study objectives and procedures prior to their voluntary consent.

Data availability statements

The datasets generated and analysed during the current study are not publiclyavailable due to the experiment is not yet fully completed and is still in the confidential stage but are available fromthe corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest regarding this research.

Author Contribution

Chen zhuoying wrote the main manuscript text and Hang jingjing and Liang wanqing prepared figures 1-3. All authors reviewed the manuscript

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Tables

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Figures

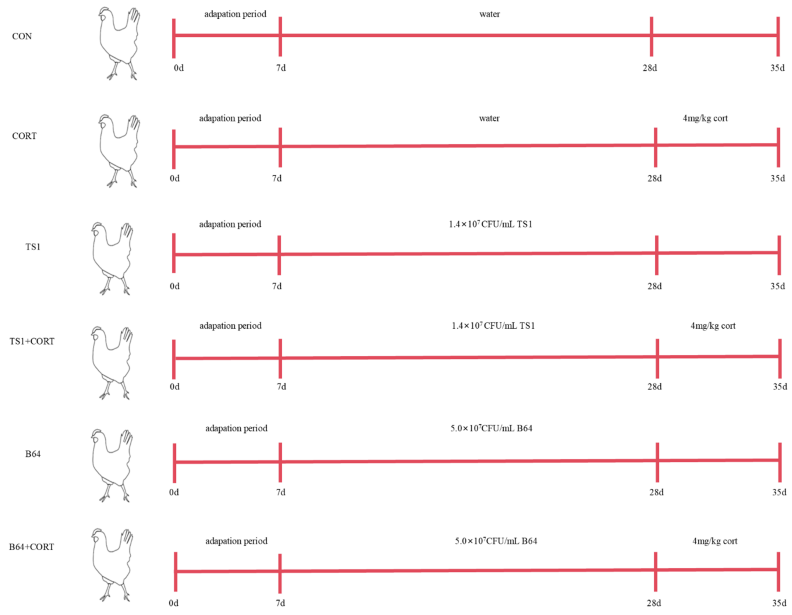


Figure 1

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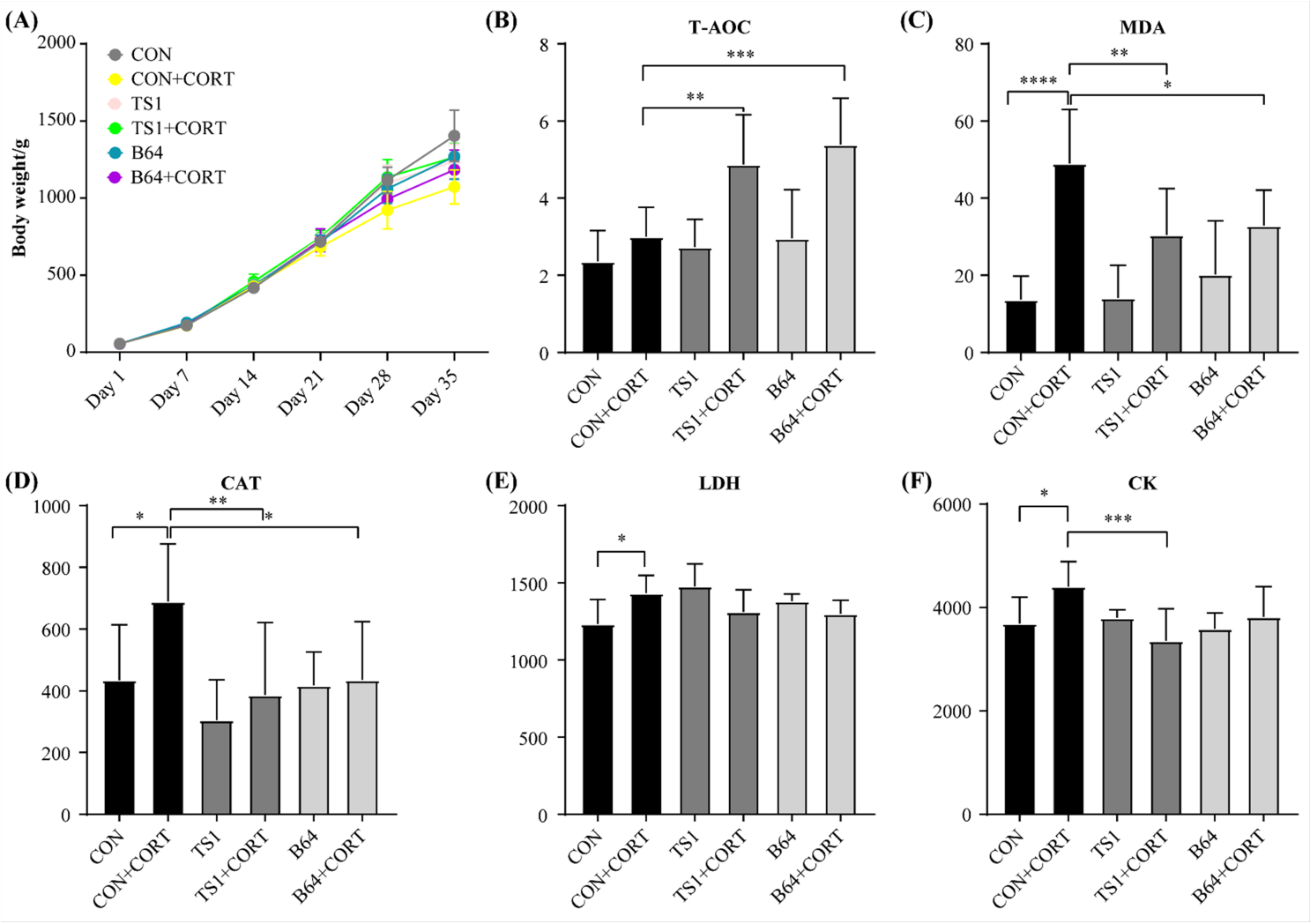


Figure 2

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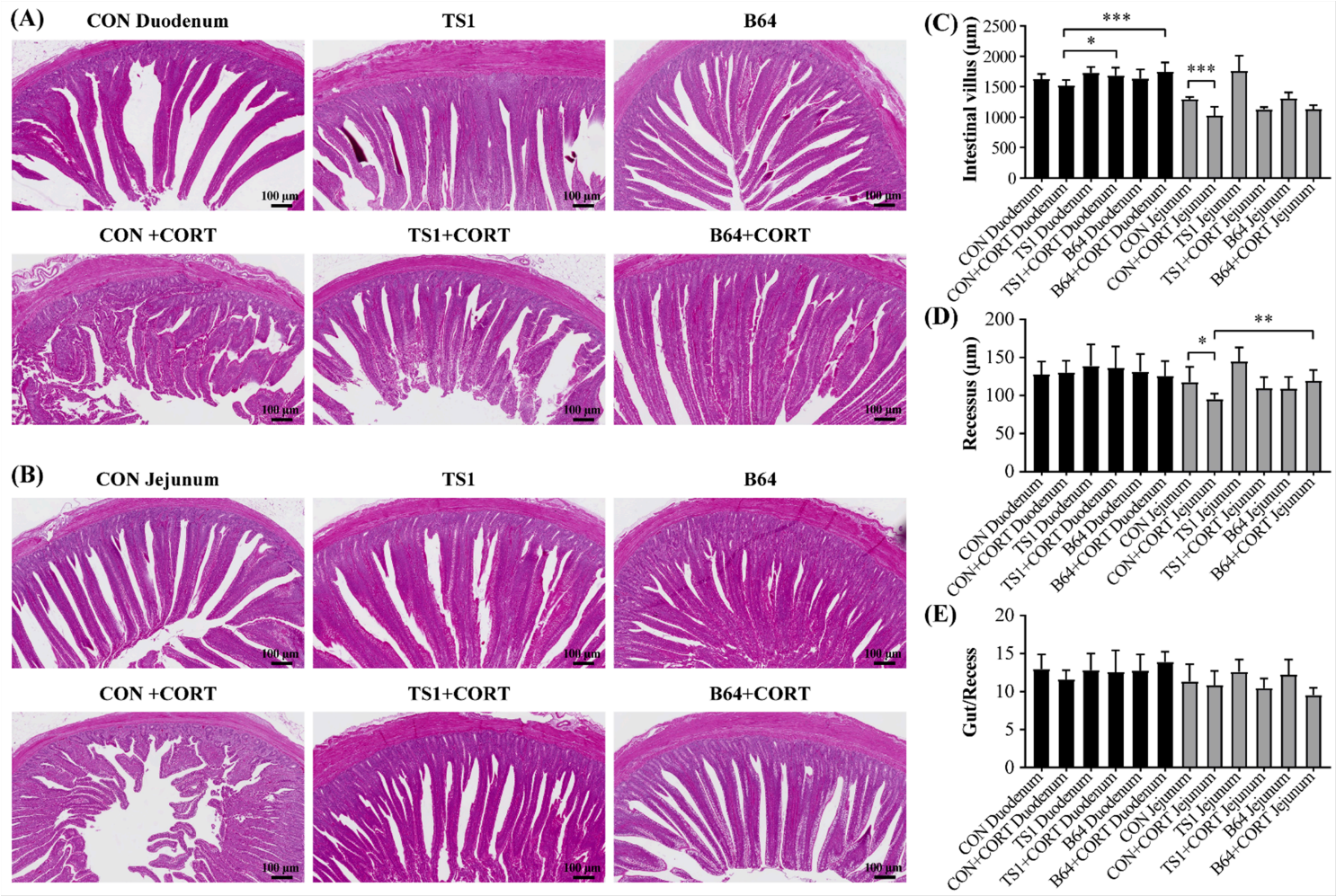


Figure 3

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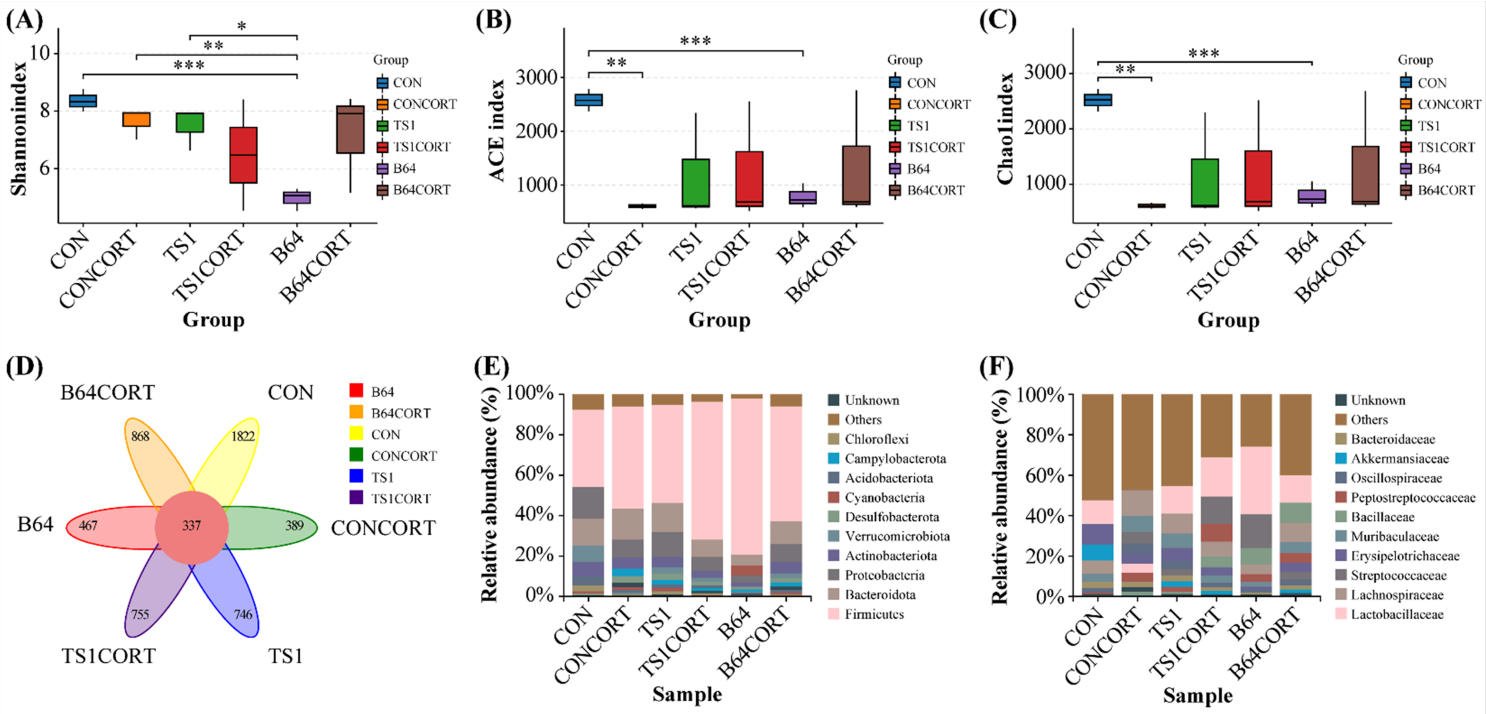


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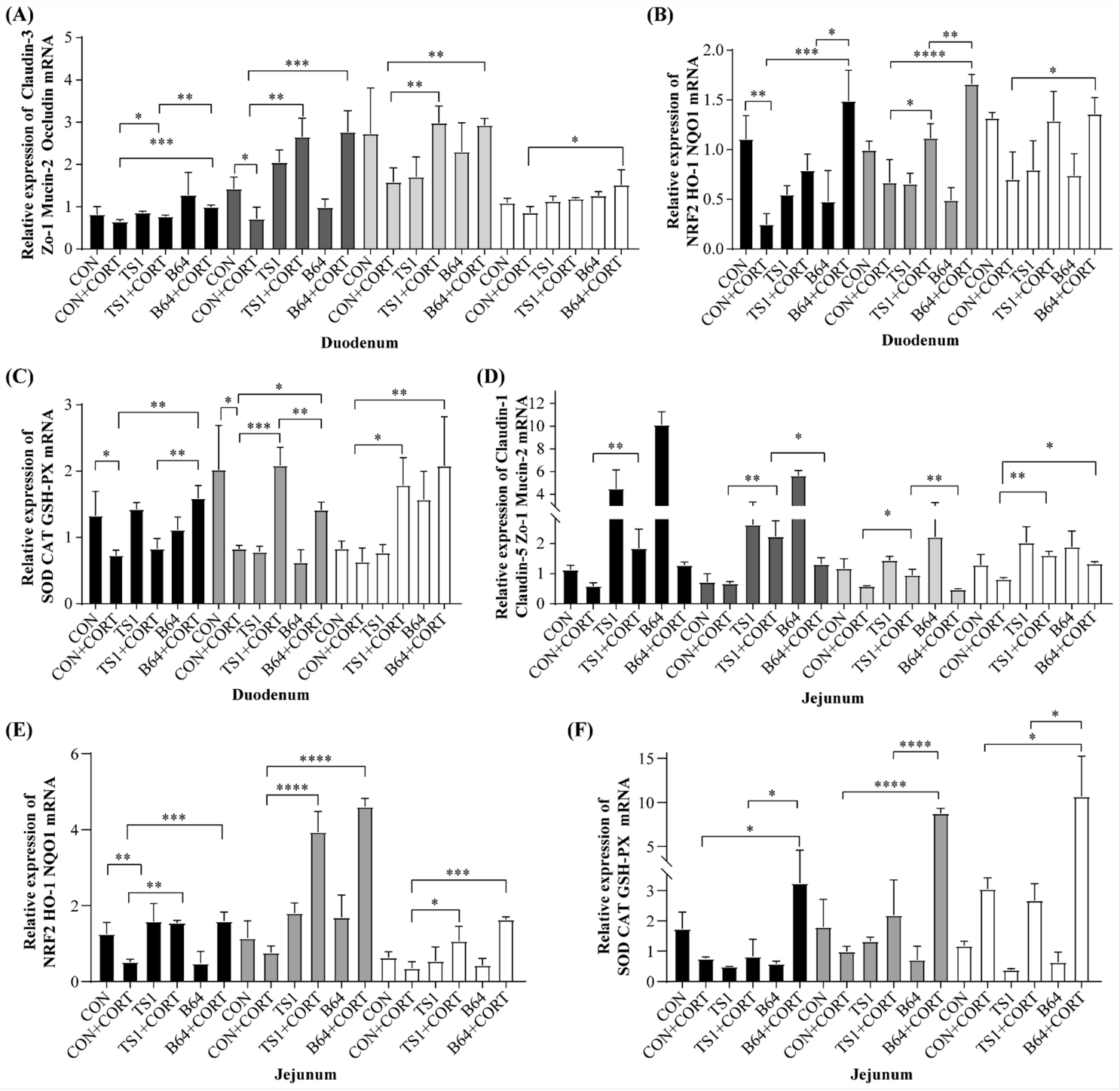


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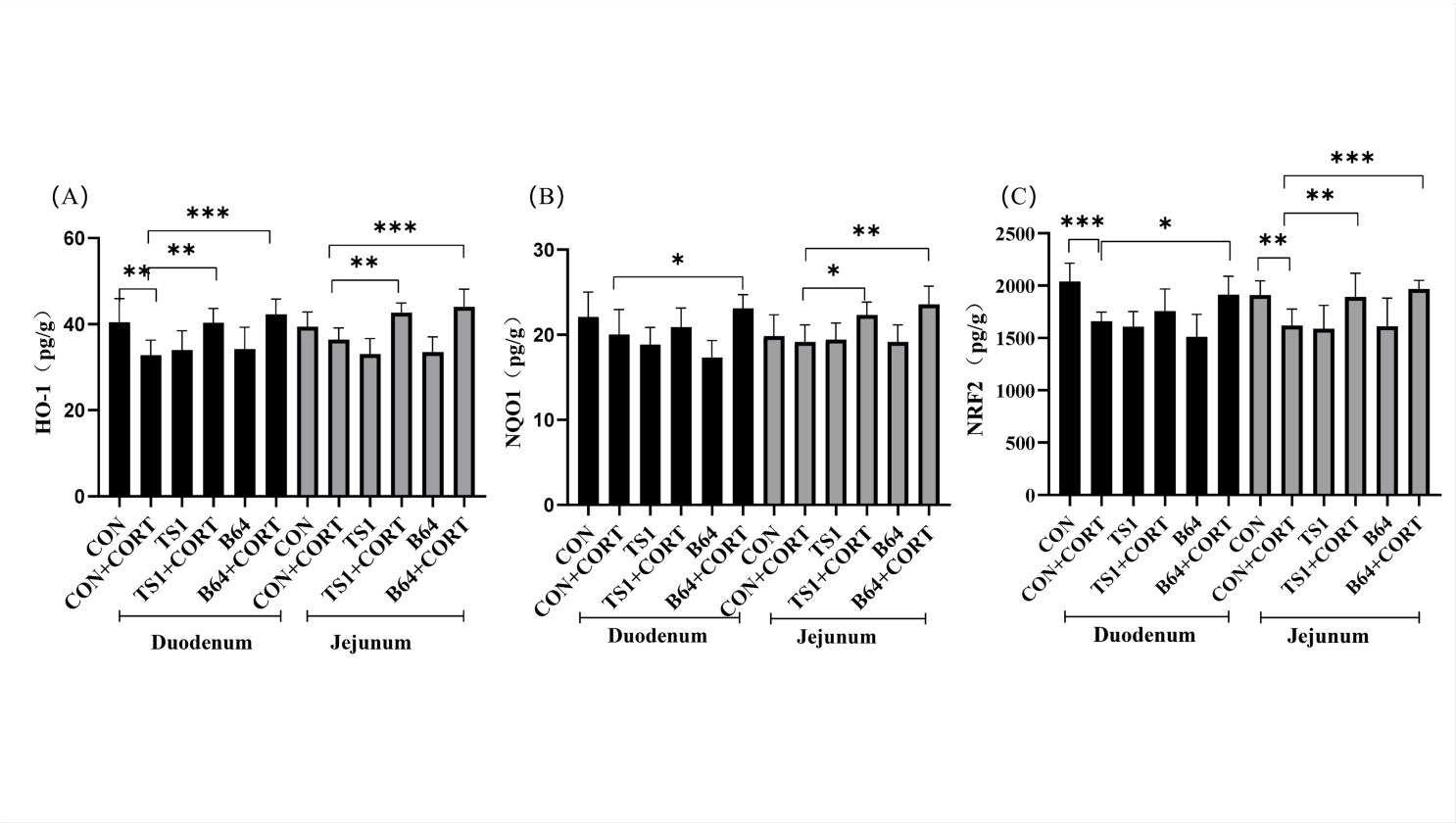


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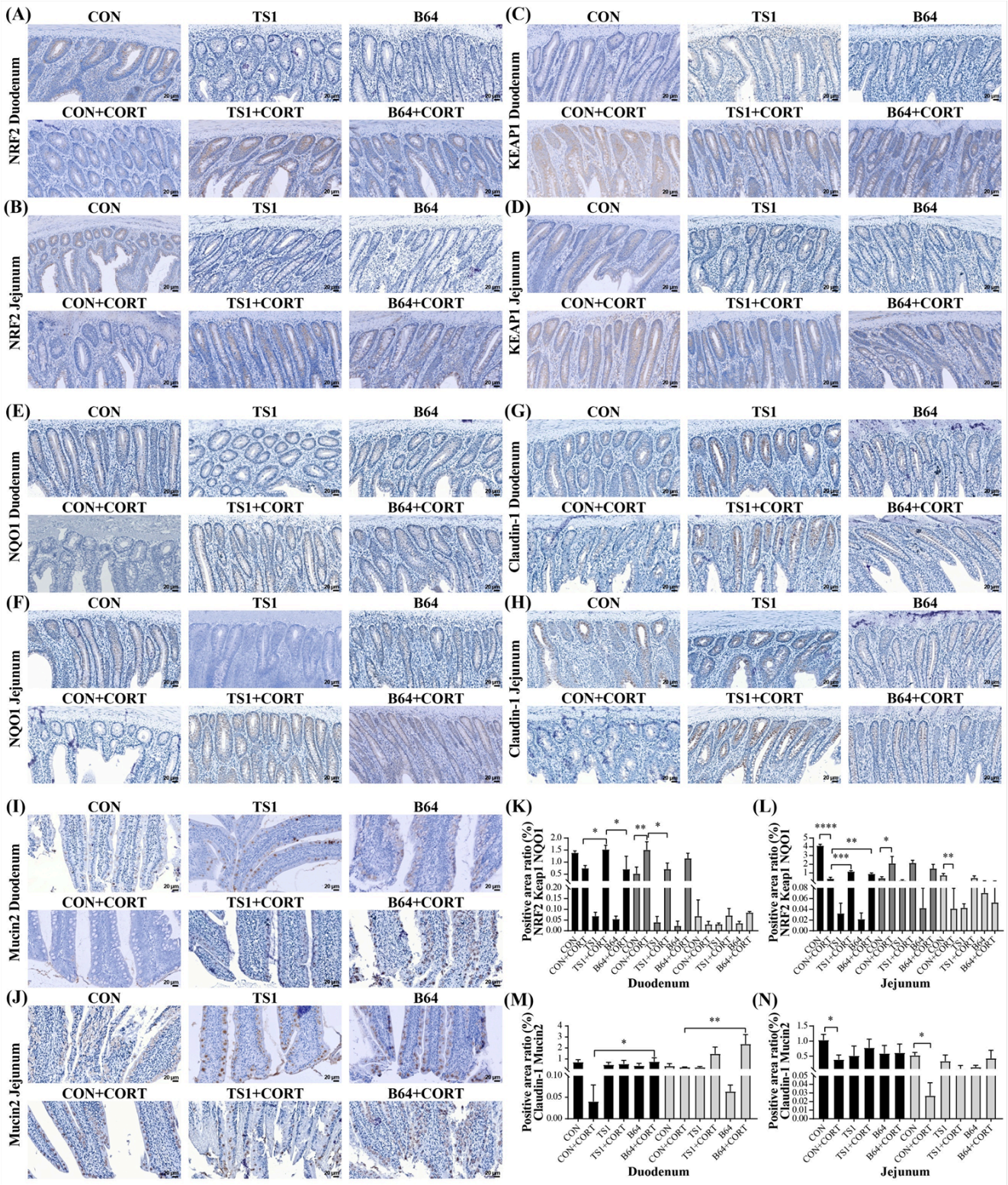


Figure 7

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