



Full-Length Article

Dietary supplementation with raw potato starch reversed enteritis-induced bone loss via Malt1 paracaspase inhibition in meat ducks

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ABSTRACT

Intestinal inflammation is a key driver of progressive bone deterioration, elevating the risk of fractures and chronic pain in meat ducks. As a fermentable type II resistant starch, raw potato starch (RPS) probably supports gut health and potentially mitigate this inflammatory bone loss. In this study, an enteritis model was established in meat ducks using dextran sodium sulphate (DSS) and to assess its effect on bone metabolism, as well as to determine the role of RPS in enteritis-mediated bone loss. In enteritis-induced meat duck models, dietary supplementation with 12% RPS reversed intestinal inflammation-induced bone loss, an effect accompanied by improved intestinal homeostasis and reduced bone resorption ($P < 0.05$). These changes were associated with alterations in inflammatory cytokine secretion, osteoclastic differentiation, and the mucosa-associated lymphoid tissue 1 (Malt1) mediated nuclear factor kappa-B (NF- κ B) pathway, as supported by transcriptional analysis of the bone marrow and AlphaFold predictions. To elucidate the mechanism by which RPS restores bone mass in the DSS-treated ducks, a specific Malt1 inhibitor (mepazine) was employed. Ducks were fed either a basal diet or a 12% RPS diet, with or without supplemental mepazine at 20 mg/kg body weight, and all were challenged with DSS from d 7 to 21. Herein, with unchanged expressions of regulatory T cell cytokines ($P > 0.05$), Malt1 protease inactivation or dietary RPS supplementation enhanced tibia ash and bone volume in metaphysis (Both $P < 0.05$). Along with increased Firmicutes levels and the production of short chain fatty acids ($P < 0.05$), mepazine or dietary RPS inclusion downregulated NF- κ B inflammatory pathway, resulting in decreased the osteoclastic activity. Co-treatment with mepazine and RPS further amplified this effect, implicating Malt1 protease inhibition as the mechanism by which RPS protects against enteritis-induced osteopenia. These findings revealed that diet contained 12% RPS reverses enteritis-induced bone loss in meat ducks, likely by inhibiting Malt1 paracaspase and subsequently suppressing NF- κ B pathway-mediated bone resorption.

Introduction

Bone abnormalities is a critical health issue with significant implications for both domesticated birds, particularly in the context of chronic inflammatory conditions such as intestinal inflammation. In particular, meat ducks are highly sensitive to environmental stress, pathogens, and feed toxins, these factors known to trigger low-grade intestinal inflammation and damage barrier function (Bi, et al., 2018). This inflammatory state may predispose birds to bone loss, as inflamed

intestinal epithelium in broilers typically exhibits villous blunting and reduced absorptive surface area (Zhang, et al., 2024). Such morphological changes the absorption of critical bone-forming nutrients, including calcium (Ca) and vitamin D, thereby compromising bone quality and resulting in osteoporosis development. Even worse, the chronic release of pro-inflammatory cytokines secreted during enteritis, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α), has been reported to activate the nuclear factor-kappa B (NF- κ B) pathway, which suppresses bone formation and promotes bone

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resorption by altering the receptor activator of nuclear factor- κ B ligand (RANKL) to driving osteoclast differentiation (Irwin, et al., 2013; Targownik, et al., 2013). It was reported that TNF- α could upregulate RANKL expression and enhances its binding affinity, exacerbating osteoclastogenesis (Yamamoto, et al., 2006). Studies in murine colitis models demonstrate that suppressing mediated nuclear factor kappa-B (NF- κ B)-mediated gut inflammation alleviates bone loss (Metzger, et al., 2019). Similarly, in broiler chickens, intestinal barrier dysfunction and elevated ileal inflammatory cytokines correlate with poor tibia quality and reduced bone mass (Zhang, et al., 2021). These findings collectively underscore the critical role of intestinal barrier integrity and inflammation control in maintaining bone homeostasis.

In practice, antibiotics and other biologic agents are commonly used to treat intestinal inflammation, however, their use is associated with adverse effects, most notably intestinal microbial dysbiosis and bone loss (Huang, et al., 2025). These findings emphasize the value of early preventive strategies, such as dietary modification during preclinical intestinal dysfunction, which may halt enteritis progression and its associated bone effects. Dietary resistant starch might represent an effective alternative nutritional strategy, serving as a key fermentable substrate for the hindgut microbiota and supporting intestinal health through beneficial host-microbiome crosstalk (Niu, et al., 2025). A study in weaned pigs found that dietary supplementation with raw potato starch (RPS) increased the abundance of butyrate- and acetate-producing bacteria, which enhanced immune status and conferred protection against *Salmonella* infection (Yi, et al., 2023). Moreover, resistant starch also positively influences bone metabolism. In rodent models, it attenuates bone loss in ovariectomized mice and reduces arthritis-induced bone erosion through microbiota-driven modulation of bone marrow inflammation (Bai, et al., 2021). Outcomes from meat ducks confirmed that diet with 12% RPS could mitigate *Escherichia coli* (*E. coli*) - induced bone loss by suppressing NF- κ B inflammation activation (Zhang, et al., 2022b). However, the potential mechanisms underlying the direct effects on enteritis-induced osteopenia remain unestablished in meat ducks.

In this regard, a reliable chronic enteritis model in meat ducks is needed to elucidate the link between intestinal inflammation and bone metabolism, as well as to evaluate potential dietary interventions. Dextran sodium sulphate (DSS), a polysaccharide toxic to intestinal epithelial cells, is a well-established inducer of experimental colitis in rodents, causing inflammation and dysbiosis (Solomon, et al., 2010). In domestic birds, DSS administration via drinking water or oral gavage has successfully induced small intestinal barrier damage, evidenced by structural disruption, inflammation, and increased permeability, according to published literatures (Chen and Yu, 2022; Chen, et al., 2023; Dal Pont, et al., 2021; Liu, et al., 2022). When considering the inherent variability in waterfowl water consumption, oral gavage is the preferred method over ad libitum drinking water to ensure precise and consistent DSS dosing for establishing a reliable enteritis model in meat ducks. Therefore, the present study initially assessed the effects of RPS and DSS on tibia metabolism in 21-d-old meat ducks, after which a potential mechanism was investigated using a DSS-induced enteritis duck model. The findings would establish a critical foundation for evaluating RPS as a nutritional strategy to prevent or reduce bone loss driven by chronic gut inflammation in poultry.

Materials and methods

Animals, diets, and management

All animal procedures complied with the regulations of the Animal Care and Use Committee of Henan Agricultural University (Approval No. HNND-2025-021). The male Cherry Valley medium-sized meat ducks obtained from a local hatchery was used in the current study. Birds were housed under a 23:1 light-dark cycle with *ad libitum* access to feed and water. With 50%-60% relative humidity, the house

temperature was setup as $32 \pm 2^\circ\text{C}$ from d 1 to 3, followed by linearly decrease to 22°C by d 21. The basal diet was formulated to meet the nutritional requirements of meat ducks (Table 1), and the supplementation level of RPS referred to recently published research (Zhang et al., 2022c).

Study design

Experiment 1 (Expt. 1) was performed to evaluate the effects of RPS and intestinal inflammation in bone mass of meat ducks. According to body weight, a total of 240 one-day-old ducks were assigned to a control group (Ctrl), a DSS challenge group (DSS), and a DSS challenge group fed a 12% RPS diet (DSS+RPS). Each treatment consisted of eight replicates with 10 birds per replicate. DSS (50 kDa, catalog no. S14048) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The ducks in the Ctrl and DSS groups administered a daily oral gavage of phosphate-buffered saline (PBS) and DSS, respectively, at a dose of 1.2 g/kg body weight from d 14 to 21.

Based on transcriptome data of bone marrow in Expt. 1, we hypothesized that mucosa-associated lymphoid tissue 1 (Malt1) activity probably is a potential mediator through which RPS attenuates enteritis-induced osteopenia. To test this, mepazine, a specific Malt1 inhibitor was used to functionally validate its role in Expt. 2. A total of 240 ducklings with similar body weights were randomly allocated to 24 pens (10 birds per pen), representing 4 treatment groups each with 6 replicate pens, including DSS-induced enteritis, DSS and mepazine co-treatment group, DSS treatment combined with dietary 12% RPS supplementation group, and the group receiving DSS, mepazine, and the 12% RPS diet. Of note, mepazine was provided by Eternity Bioscience Inc. (NJ, USA) and dissolved at a concentration of 20 mM in 100% dimethyl sulfoxide (DMSO) as a stock solution, stored at -20°C , and diluted with

Table 1
Dietary formulation and composition (as fed basis).

Item	Basal diet	Raw potato starch diet
Ingredients, %		
Corn	59.82	44.85
Soybean meal	33.22	35.91
Raw potato starch	0.00	12.00
Soybean oil	0.50	1.50
Calcium Carbonate	1.10	1.04
Dicalcium phosphate	1.75	1.82
L-Lysine-HCL	0.12	0.07
DL-Methionine	0.16	0.17
L-Threonine	0.02	0.02
Bentonite	2.33	1.64
Sodium chloride	0.30	0.30
Choline chloride	0.15	0.15
Vitamin premix ¹	0.03	0.03
Mineral premix ²	0.50	0.50
Total	100.0	100.0
Calculated analysis, %		
Apparent metabolism energy, MJ/kg	11.69	11.70
Crude protein	19.48	19.40
Calcium	0.91	0.89
Non-phytate phosphorus	0.44	0.45
Digestibility Lysine	1.04	0.99
Digestibility Methionine	0.43	0.42
Nutrient analysis (%)		
Resistant starch	3.69	7.70
Crude protein	19.50	19.53
Calcium	0.89	0.90
Total phosphorus	0.72	0.69

¹ Provided per kilogram of diet: Cu ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 8 mg; Fe ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 80 mg; Zn ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 90 mg; Mn ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), 70 mg; Se (NaSeO_3), 0.3 mg; I (KI), 0.4 mg.

² Provided per kilogram of diet: retinol, 2.06 mg; cholecalciferol, 0.04 mg; vitamin E, 30.01 mg; thiamine, 1 mg; riboflavin, 3.9 mg; pyridoxine, 3.375 mg; vitamin B₁₂, 0.01 mg; calcium pantothenate, 8.85 mg; folate, 0.5 mg; biotin, 0.1 mg; niacin, 49.25 mg.

medium before each experiment. All meat ducks were consecutively given 1.2 g/kg body weight DSS by oral gavage starting on d 14 to 21 of age. At 15, 17, 19, and 21 d of age, ducks received intraperitoneal injections of mepazine (20 mg/kg body weight) or an equivalent volume of PBS, as referenced in prior studies (Zhang, et al., 2024).

Data and sample collection

Body weights were recorded at 1, 14, and 21 d of age, along with feed intake and the number of dead or culled birds during the trial period. On d 21, one bird with similar body weight in each pen was selected for sampling in Expt.1. Blood was taken from the brachial vein to obtain serum by centrifuging at $4,000 \times g/15\text{min}$ at 4°C , and subsequently the left tibia and mid-ileum mucosa were harvested and stored at -80°C until analysis. Tibial marrows were collected and stored in liquid nitrogen for transcriptomic analysis and inflammatory assessment. In Expt.2, two birds per pen (closest to the average body weight of each pen) were sampled. Following blood collection, the whole duodenum, jejunum, ileum, and cecum were excised from one of birds. The duodenum is isolated as the distinct descending and ascending loops forming a tight U-shape around the pancreas, the jejunum is recognized as the lengthy, highly convoluted mid-intestine extending from the duodenal loop to Meckel's diverticulum, the ileum is taken from Meckel's diverticulum down to the ileocecal junction. The paired ceca, which are long, tapered, sacculated, and microbe-rich for fermenting aquatic vegetation, were also removed. The length and weight of all segments were recorded and normalized to their body weight. The pH of ileal chyme and caecal content was measured immediately using a pH probe (SFK-Technology, Denmark). Mid-ileal mucosa, caecal content, and right tibial bone marrow then snap frozen in liquid nitrogen and stored (-80°C) until analysis. The 2nd bird was also euthanized, and the left tibia was removed for micro computed tomography (Micro-CT) analysis. The right tibia was harvested, length, and diameter (at 50% of length) of tibia were measured after removal of soft tissues. All bone samples were stored at -20°C for determination of tibia characteristics.

Nutrient analysis of study diets

Using a random sampling technique, eight representative feed samples (approximately 1 kg each) were collected during the feed preparation process. The RPS content in the experimental diets was analyzed using a commercial kit (Megazyme Ltd., Wicklow, Ireland) in accordance with AACC Method 32-40.01 (McCleary et al., 2019). The dry matter (method 967.03) and nitrogen (N) (method 990.03) were determined according to the corresponding method of Association of Official Analytical Chemists (AOAC, 2012), and the crude protein (CP) was quantitated using the following equation: $\text{CP} (\%) = \text{N} (\%) \times 6.25$.

Measurement of intestinal permeability

On 21-d-old age, one duck was randomly selected from each pen for oral gavage with fluorescein isothiocyanate-dextran (FITC-d; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 4.16 mg/kg body weight. Blood samples were collected from the jugular vein at 2 h post-gavage. Serum was obtained and the concentration of serum FITC-d was quantified by measuring fluorescence intensity (excitation: 485 nm; emission: 528 nm) against a standard curve generated from serial dilutions of FITC-d, according to a previously described protocol (Zhang et al., 2022c).

Tibial characteristics and micro-structure

The mechanical properties of the tibia were assessed via a three-point bending test to failure using a texture analyser (TA.XT. Plus, Stable Microsystems Ltd., UK) with a constant load cell capacity of 50 kg. From the resulting force-displacement data, the yield load, ultimate

load, and energy to failure (defined as the area under the curve, AUC) were quantified. The stiffness of each bone was subsequently calculated as the slope of the force-displacement curve within its linear elastic region. All fragments from each tibia were pooled, and bone volume was measured using the water displacement method. The samples were then defatted via ethyl ether extraction for 48 h and subsequently oven-dried at 105°C for 24 h to obtain the fat-free dry weight. Therefrom, bone density was calculated as the fat-free weight (g) divided by the tibial volume (cm^3). Hereafter, these defatted, dry bones were then ashed in a muffle furnace at 550°C for 24 h. Ash content was calculated as a percentage of the fat-free weight.

In addition, left tibia subjected to micro-structure analysis according to previous method (Zhang, et al., 2021), to minimize the influence of variable water content, soft tissue was removed from each tibia prior to drying at 65°C . The dried tibiae were then imaged using a GE Explore Locus Micro-CT scanner (GE Healthcare, Piscataway, NJ, USA) at 90 kV. For each scan, at least 2,000 projection images were acquired over a full 360° rotation with an exposure time of 1,000 ms. Projection data were reconstructed into a three-dimensional volume with an isotropic voxel size of $223 \mu\text{m}^3$. Images were analysed using custom scripts in ImageJ (National Institutes of Health, USA) with the thickness plugin in Bone J. A cylindrical volume of interest (VOI) of cortical bone, 37 mm in total length, was selected at the tibial mid-diaphysis. Within this VOI, the diaphysis was automatically segmented to separate cortical from trabecular bone, and the average cortical thickness and bone volume fraction (BV/TV) were measured. To analyse trabecular bone, a VOI was selected from the proximal tibial metaphysis. This region extended 4 mm in length, beginning 9 mm distal to the articular surface of the condyles. BV/TV of metaphysis was calculated for this metaphyseal VOI.

Serum biochemical determination

To quantify the inflammatory status, ileal mucosal supernatants were prepared by homogenizing tissues in ice-cold saline (1:9, w/v) followed by centrifugation. The resulting supernatants were analysed for total protein content using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The concentrations of IL-1 β , IL-6, IL-10, and TNF- α in both serum and these supernatants were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China). Biomarkers of bone turnover in serum were measured with their respective commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), including C-terminal cross-linked telopeptide of type I collagen (CTX), tartrate-resistant acid phosphatase (TRAP), procollagen type I N-terminal propeptide (P1NP), and alkaline phosphatase (ALP). All samples were analysed in triplicate within each assay.

Transcriptome analysis

Total RNA was extracted from bone marrow samples and assessed for quality and integrity. A SMART-Seq Ultra Low Input RNA Kit (Takara Bio Inc., Kusatsu, Japan) was used to generate amplified cDNA from 500 ng of high-quality total RNA. Sequencing libraries were then prepared using the TruSeq Stranded mRNA Library Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina NovaSeq 6000 platform (Novogene Biotechnology Co., Ltd, Beijing, China). Raw reads were quality-filtered by removing low-quality bases (Phred score < 20) and adapters. Clean reads were aligned to the duck reference genome (Assembly accession: GCF_015476345.1) using STAR (v2.7.8a). Gene expression levels were quantified, and differential expression analysis between the DSS and DSS+RPS groups was performed using HISAT2 followed by a statistical analysis package (e.g., DESeq2/edgeR). Genes with an absolute $\log_2$ fold change > 1 and an adjusted P -value < 0.05 were considered differentially expressed (DEGs). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the identified DEGs was conducted using the Database for Annotation, Visualization and Integrated

Discovery (DAVID, v6.8; <https://david.ncifcrf.gov>).

Complex structure prediction by AlphaFold

The structure of the Malt1/NF- κ B complex was predicted using AlphaFold3 (accessed via the AlphaFold Server at <https://alphafold3.ever.com>). The input included the amino acid sequences of Malt1 and NF- κ B p65. Five models were generated, and the top-ranked model based on the predicted interface Template Modelling score (TM-score) and interface predicted alignment error was selected. The model had an overall 0.82 of TM-score of and an average 86.5 of predicted local distance difference test for the protein chains. The predicted interaction interface was analysed for hydrogen bonds and hydrophobic contacts using UCSF Chimera X.

Measurement of Malt1 activity

Malt1 protease activity was measured in ileal mucosal and bone marrow lysates using the fluorogenic Malt1 assay kit (BPS Bioscience, Inc. San Diego, CA). The ileum and bone marrow were homogenized and mixed with lysis buffer containing protease/phosphatase inhibitors. After centrifugation, the total protein of lysates was quantified by BCA protein assay (Pierce, Rockford, IL, USA). Approximately 50 μ g of total protein from each sample was incubated in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% CHAPS, 10 mM DTT) with 200 μ M of the fluorogenic Malt1 substrate Ac-LRSR-AMC in a 96-well plate. To confirm specificity, parallel reactions included the Malt1-specific inhibitor Z-VRPR-FMK (50 μ M). Fluorescence (Excitation: 380 nm, Emission: 460 nm) was kinetically monitored for 90 min at 37°C. Specific Malt1 activity expressed as relative fluorescence units (RFU) was calculated as the inhibitor-sensitive increase in fluorescence per minute, normalized to total protein input.

Cecal microbiota composition

Total genomic DNA was extracted from cecal contents using the PSP Spin Stool DNA Plus Kit (Invitex, Westburg, Netherlands). The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified with the universal primers 338F (5'-GTGCCAGCMGCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR products were purified, quantified, and pooled in equimolar amounts prior to paired-end sequencing (2 $\times$ 250 bp) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). Raw sequence data were processed using QIIME 2 (v2023.5). Sequences were demultiplexed, quality-filtered (with a Phred score threshold of ≥ 20), trimmed, and merged. Chimeric sequences were identified and removed using the DADA2 plugin. Taxonomic assignment was performed against the SILVA (v138) reference database at a 97% similarity threshold. Amplicon sequence variants (ASVs) were generated, and beta diversity metrics were calculated using R software.

Short chain fatty acids (SCFAs) content

The SCFAs content in cecal contents was quantified by gas chromatography (GC), including acetic, propionic, and butyric acid. Approximately 0.5 g of sample was homogenized in 2 mL of ultrapure water and centrifuged at 3,000 $\times$ g for 15 min at 4°C. The supernatant was acidified by mixing with 25% (w/v) metaphosphoric acid in a 5:1 (v/v) ratio, incubated on ice for 30 min, and centrifuged again under the same conditions. The final supernatant was filtered through a 0.22- μ m nylon membrane before injection. Analysis was performed on a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID) and a capillary column. The carrier gas was nitrogen at a constant flow rate of 1.0 mL/min. Quantification of acetic, propionic, and butyric acid was performed by external calibration. A standard curve was constructed by analyzing a series of calibration solutions containing known

concentrations (0.1–50 mM) of each pure SCFA (Sigma-Aldrich) prepared in acidified ultrapure water. Peak areas were integrated using the Galaxie Chromatography Data System (Varian), and analyte concentrations in samples were calculated by linear regression and expressed as μ mol per gram of wet cecal content (μ mol/g). All samples were analyzed in duplicate.

Gene expression analysis by real-time PCR

Total RNA was extracted from ileal mucosa, proximal tibia metaphysis, and flushed bone marrow using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Using a cDNA Reverse Transcription Kit (Takara, Dalian, China), the RNA was reverse transcribed into cDNA and subjected to gene expression analysis. Real-time PCR were performed using the ABI 7900 Sequence Detection System (Applied Biosystems, CA, USA). The primers were designed using online Primer 3 and listed in Table S1. The relative mRNA abundance of the targeted gene was adjusted for the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β -actin.

Statistical analysis

Each pen served as the experimental unit. Statistical power of at least 0.8 (80%) was achieved at a significance level of 0.05, with the effect size determined from the mean and standard deviation using G*Power 3 software (Franz Faul, Christian-Albrechts-Universität Kiel, Kiel, Germany). All of data were assessed the normality of residuals and homoscedasticity using the Shapiro-Wilk and Levene's test, respectively. Differences among treatment groups were determined by the analysis of variance (ANOVA) and Tukey's *post hoc* test. Survival outcomes were analysed with the Kaplan-Meier method, and differences between groups were compared using the log-rank test. For microbial 16S rRNA amplicon sequencing data, community-level statistical analyses were conducted in R using the Microbiota Process package (version 3.17). Alpha diversity was evaluated using Shannon and Simpson index. Beta-diversity was estimated by principal component analysis (PCA). Normality and homogeneity of variance were verified prior to group comparisons, and the differences among treatment groups were analysed via one-way ANOVA followed by Tukey's *post-hoc* test or Kruskal-Wallis H test with multiple pairwise comparisons using R software. Data are presented as mean $\pm$ standard deviation (SD). Differences were considered significant at $P \leq 0.05$ and marginally significant at $0.05 < P < 0.10$.

Results

Diet contained 12% RPS reversed gut leak and outburst of inflammation

As illustrated in Fig. 1A–C, with no apparent changes in the growth performance, including body weight, feed intake, and survival proportions, the RPS treatment tend to enhance ($P = 0.050$) the intestinal integrity compromised by DSS treatment, evidenced by reduced FITC-d concentration in serum (Fig. 1D). In comparison to Ctrl birds, birds challenged with DSS exhibited an increased ($P < 0.05$) the ratio of TNF- α to IL-10 in the ileum of ducks, a marker of immune dysregulation, which was subsequently normalized by RPS supplementation (Fig. 1E). Reflecting to serum, the dietary supplementation of 12% RPS reversed ($P < 0.05$) the increase in pro-inflammatory cytokines induced by DSS injection, including IL-1 β and TNF- α (Fig. 1F).

Dietary RPS supplementation normalized enteritis-induced bone loss via reducing bone resorption

Outcomes of Micro-CT scanning showed that DSS treatment decreased the cortical thickness in diaphysis ($P = 0.055$) and BV/TV in metaphysis ($P < 0.05$) as compared to Ctrl group, whereas dietary RPS

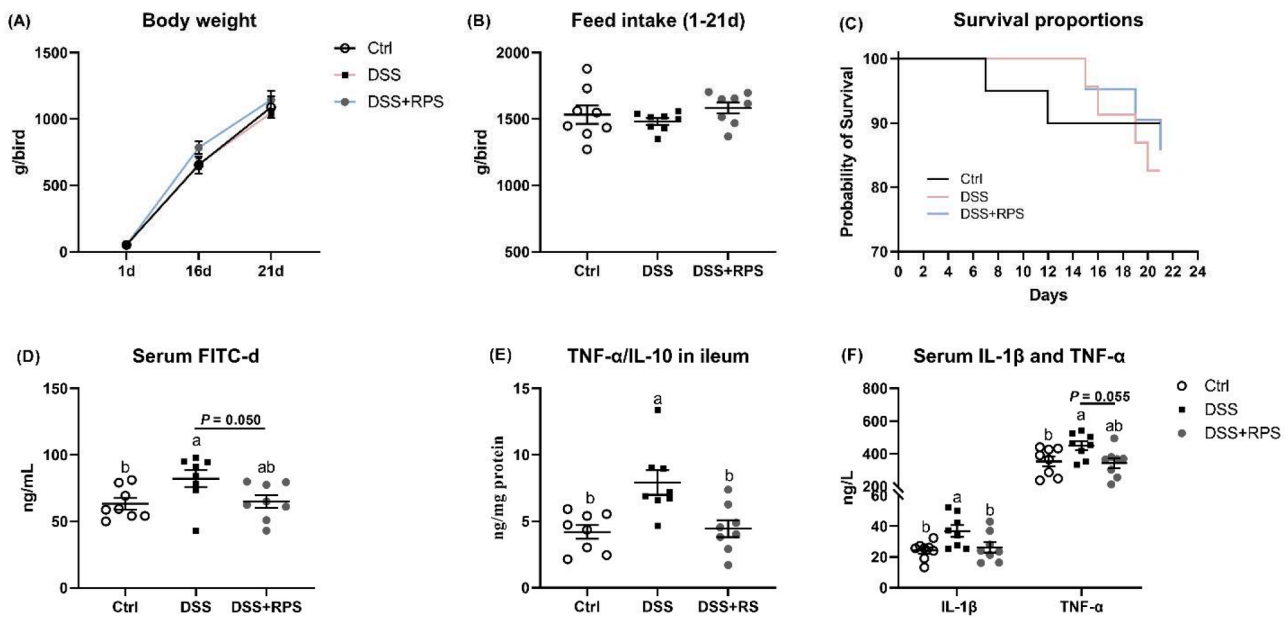


Fig. 1. Effects of dietary raw potato starch (RPS) and dextran sodium sulphate (DSS) on (A) body weight, (B) feed intake, (C) survival proportions, and intestinal barrier function in meat ducks. (D) Gut permeability by serum fluorescein isothiocyanate-dextran (FITC-d) content. (E) ileal inflammation was evaluated by the ratio of pro-inflammatory (Tumor necrosis factor- α , TNF- α) to anti-inflammatory (interleukin-10, IL-10) cytokines. (F) serum concentration of IL-1 β and TNF- α . Data are expressed as mean and standard deviation. Statistical significance at $P < 0.05$.

intervention subsequently increased both parameters. However, the experimental treatment failed to change the BV/TV in diaphysis and tibia ash content (Fig. 2A-E). Considering bone turnover, the levels of serum CTx and TRAP, both are biomarkers of bone resorption, were notably decreased ($P < 0.05$) by dietary RPS manipulation in DSS-treated ducks (Fig. 2F, G). There were no significant alterations in terms of bone formation markers between DSS and DSS+RPS groups, including P1NP and ALP (Fig. 2H-I). On the score, DSS-treated birds exhibited increased serum levels of CTx and TRAP, along with decreased ALP activity relative to the Ctrl birds (Fig. 2F, G, I).

Malt1 protease inactivity probably served as a potential mediator for RPS attenuating enteritis-induced bone loss

To further investigate the protective mechanism of dietary RPS, bone marrow samples from DSS-treated ducks fed either a basal or RPS-supplemented diet were subjected to transcriptome sequencing. The transcriptome data of beta-1,3-galactosyltransferase 5 (**B3GALT5**), solute carrier family (**SLC**)-2, SLC-6, and ghrelin and obestatin prepropeptide (**GHRL**) were consistent with the expressions measured by RT-qPCR (Fig. S1), confirming the reliability of sequencing results in this study. PCA plot revealed clear separation between the two groups (Fig. 3A). A total of 460 genes were significantly downregulated and 263 were upregulated in the RPS-fed birds compared to DSS group (Fig. 3B, C). Functional enrichment analysis indicated that these differentially expressed genes were associated with rheumatoid arthritis, cytoskeletal organization, MAPK/PI3K-Akt signalling, ion channels, NF- κ B signalling, cytokine-cytokine receptor interactions, and osteoclast differentiation (Fig. 3D). Among these, the NF- κ B signalling pathway was one of the most prominently enriched pathways following RPS intervention in DSS-treated ducks (Fig. 3D). Using AlphaFold-based structural bioinformatics, the interaction among key NF- κ B pathway components, i.e., Malt1, tumour necrosis factor receptor-associated factor 6 (**TRAF6**), and NF- κ B, were predicted in the DSS-induced duck enteritis model (Fig. 3E). Subsequent analysis of Malt1 and NF- κ B mRNA expression in bone marrow showed that DSS challenge significantly increased NF- κ B expression ($P < 0.05$), which was reversed by dietary RPS. In contrast, Malt1 transcription remained largely unchanged across groups (Fig. 3F).

Given that Malt1 functions not only as a scaffold but also as a cysteine protease that cleaves specific targets to activate NF- κ B signalling, the enzyme activity was determined and showed the dietary administration of RPS inhibited ($P < 0.05$) the increased Malt1 activity induced by DSS (Fig. 3G).

Inhibition of Malt1 activity or/and dietary RPS supplementation reduced the inflammatory response in the ileum

In the DSS-induced enteritis model, treatment with the Malt1 protease inhibitor mepazine, either alone or combined with dietary RPS inclusion, remarkably reduced Malt1 activity and NF- κ B transcription levels in the ileum ($P < 0.05$), despite comparable Malt1 expression across all four groups (Fig. 4A-C). As expected, Mepazine administration significantly decreased ($P < 0.05$) ileal mRNA expression of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α compared to the DSS-treated control group. Dietary RPS alone or in combination with mepazine reduced ($P < 0.05$) TNF- α mRNA abundance but did not alter IL-1 β or IL-6 levels (Fig. 4D). Although the anti-inflammatory cytokine IL-10 remained at similar levels across groups ($P > 0.05$, Fig. 4E), both Mepazine and the 12% RPS diet significantly lowered ($P < 0.05$) the TNF- α to IL-10 ratio (Fig. 4F). In addition, none of the experimental treatments affected growth performance from 1 to 21 d, small intestine development, or chyme pH in the ileum and cecal content (Fig. S2 and Table 2). The only morphological change observed in DSS-treated ducks was a significant increase ($P < 0.05$) in both absolute and relative ileum weight (Table 2).

Intestinal integrity and caecal microbiota response to Mepazine and dietary RPS treatment

Direct measurement of the concentration of FITC-d, a biomarker of intestinal integrity, suggested that mepazine alone or/and in combination with RPS supplementation significantly decreased ($P < 0.05$) intestinal permeability in the DSS-induced enteritis model (Fig. 5A). Analysis of tight junction protein genes showed that dietary RPS or Mepazine alone numerically increased the expression of zonula occludens-1 (**ZO-1**) and *occludin* compared to the DSS control group.

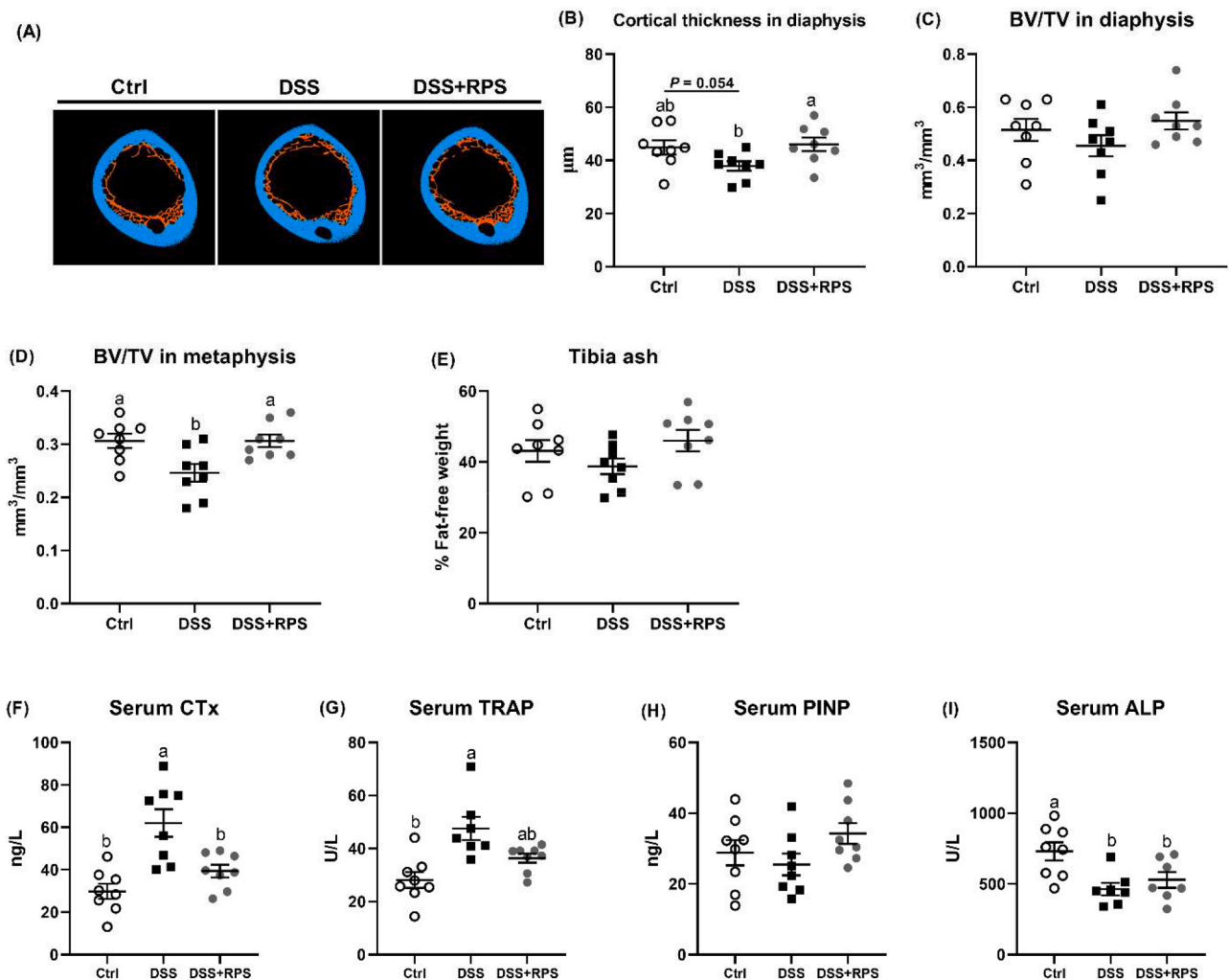


Fig. 2. Response of tibia properties and bone turnover to dietary raw potato starch (RPS) and dextran sodium sulphate (DSS) treatment. (A) Representative micro-CT images and the quantification of (B) cortical thickness in diaphysis, (B, C) bone volume/total volume (BV/TV) in diaphysis and metaphysis. (D) tibia ash content. (F-I) Serum biomarkers of bone turnover, including C-terminal cross-linked telopeptide of type I collagen (CTx) and tartrate-resistant acid phosphatase (TRAP), procollagen type I N-terminal propeptide (PINP), and alkaline phosphatase (ALP). Data are expressed as mean and standard deviation. Statistical significance showed as different letters at $P < 0.05$.

This upregulation was notably enhanced when mepazine and RPS co-treatment were administered together (Fig. 5B). Furthermore, mepazine and RPS manipulation alone significantly increased ($P < 0.05$) *claudin-1* mRNA abundance in ileum of DSS-treated ducks (Fig. 5B).

There were no obvious changes in terms of alpha diversity, showed by similar Shannon and Simpson index (Fig. 5C, D). Beta-diversity assessed via PCA revealed a clear separation between ducks receiving dietary RPS supplementation and the other three treatment groups in the DSS-induced model (Fig. 5E). Distinct differences in microbial composition were also evident at the phylum level (Fig. 5F). Compared to the DSS group (both $P < 0.05$), dietary RPS significantly decreased the abundance of Bacteroidetes and increased that of Firmicutes, thereby elevating the ratio of Firmicutes to Bacteroidetes ratio ($P < 0.05$). In contrast, mepazine treatment had no significant effect ($P > 0.05$) on these phylum-level abundances (Fig. 5F-I). Reflecting on the production of SCFAs, mepazine administration did not alter the proportions of acetic acid, propionate, or butyric acid, nor did it change the total SCFA content. However, dietary RPS supplementation, either alone or in combination with mepazine, significantly increased the concentrations of acetate and butyric acid in the cecal content, leading to a concurrent rise ($P < 0.05$) in total SCFAs of cecal content (Fig. 5J-M).

Inactivation of Malt1 protease and dietary RPS inclusion synergistically improved bone quality through Inhibition of NF- κ B-dependent bone resorption

As presented in Table 3, there was no difference regarding bone growth, instructed by comparable tibia length, perimeter, and fat-free weight ($P > 0.05$). Mepazine treatment increased ($P < 0.05$) tibia ash content, whereas supplementation with RPS alone or in combination with Mepazine further improved both ash level and density relative to the DSS group. In terms of tibial microstructure, either mepazine or dietary RPS supplementation significantly increased the bone volume fraction (BV/TV) in the metaphysis of DSS-treated ducks. Notably, the combined treatment of Mepazine and RPS, but neither intervention alone, increased ($P < 0.05$) cortical thickness in the diaphysis. Furthermore, the bone mechanical property assays showed that Mepazine injection or dietary RPS inclusion promoted the ultimate load and yield load to different degrees. Both parameters were significantly enhanced by the combined administration of mepazine and RPS in the DSS-induced enteritis model (Table 3).

To further unravel the roles of inhibition Malt1 and RPS diet in bone turnover, the inflammatory status was determined and showed that administration with mepazine either alone or combined with dietary

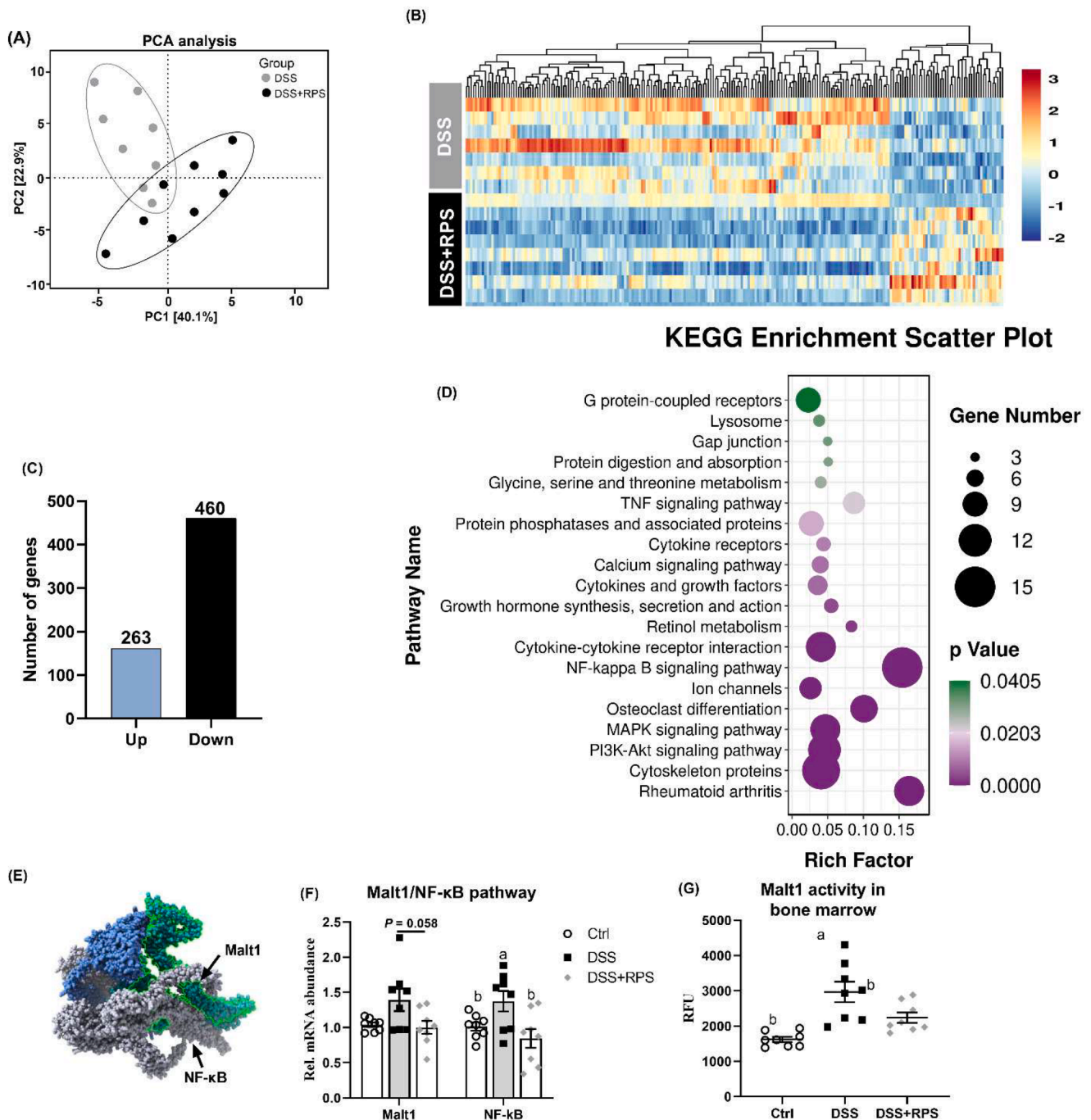


Fig. 3. Transcriptome analysis for differentially expressed genes (DEG) in bone marrow between dextran sodium sulphate (DSS)-treated ducks fed the basal diet and raw potato starch (RPS) diet. (A) Principal component analysis (PCA) of transcript profile. (B) Heatmap with hierarchical clustering analysis of DEGs of the transcriptome. (C) The quantity statistics of DEG. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG)-enrichment analysis of differentially expressed genes. (E) Prediction of nuclear factor-kappa B (NF-κB) pathway by AlphaFold. (F) mRNA abundance of NF-κB and mucosa-associated lymphoid tissue 1 (Malt1) in bone marrow. (G) Malt1 activity in bone marrow. Genes with $\log_2|FC| > 1$ and $P < 0.05$ were considered as significant DEG. Data are expressed as mean and standard deviation. Statistical significance showed as different letters at $P < 0.05$.

RPS inclusion, notably reduced ($P < 0.05$) Malt1 activity in the bone marrow (Fig. 6A). This was accompanied by a marked decrease in mRNA levels of NF-κB and a similar transcription of Malt 1 in the bone marrow of DSS-treated ducks receiving mepazine and/or RPS (both $P < 0.05$, Fig. 6B). Consequently, mepazine and RPS treatment individually or together, downregulated the expression of *IL-1β* and *TNF-α* (Fig. 6C). Notably, these experimental interventions did not alter ($P > 0.05$) the expression of T-regulatory cell cytokines, including *IL-2*, *TGF-β*, and *IL-10* (Fig. 6C). Further evaluation of the osteoclast differentiation

pathway revealed that the expression of *RANKL* was downregulated by Malt1 inhibition alone or in combination with RPS supplementation ($P < 0.05$), whereas osteoprotegerin (*OPG*) mRNA levels showed no significant change. This resulted in a decreased *RANKL/OPG* ratio ($P < 0.05$, Fig. 6D-F). Consistent with this, Mepazine with or without an RPS diet significantly reduced ($P < 0.05$) the transcription level of *cathepsin K* compared to DSS-treated birds (Fig. 6G). These changes indicate that inhibition of Malt1 activity and/or dietary RPS inclusion suppressed bone resorption, evidenced by lower serum CTx concentrations and

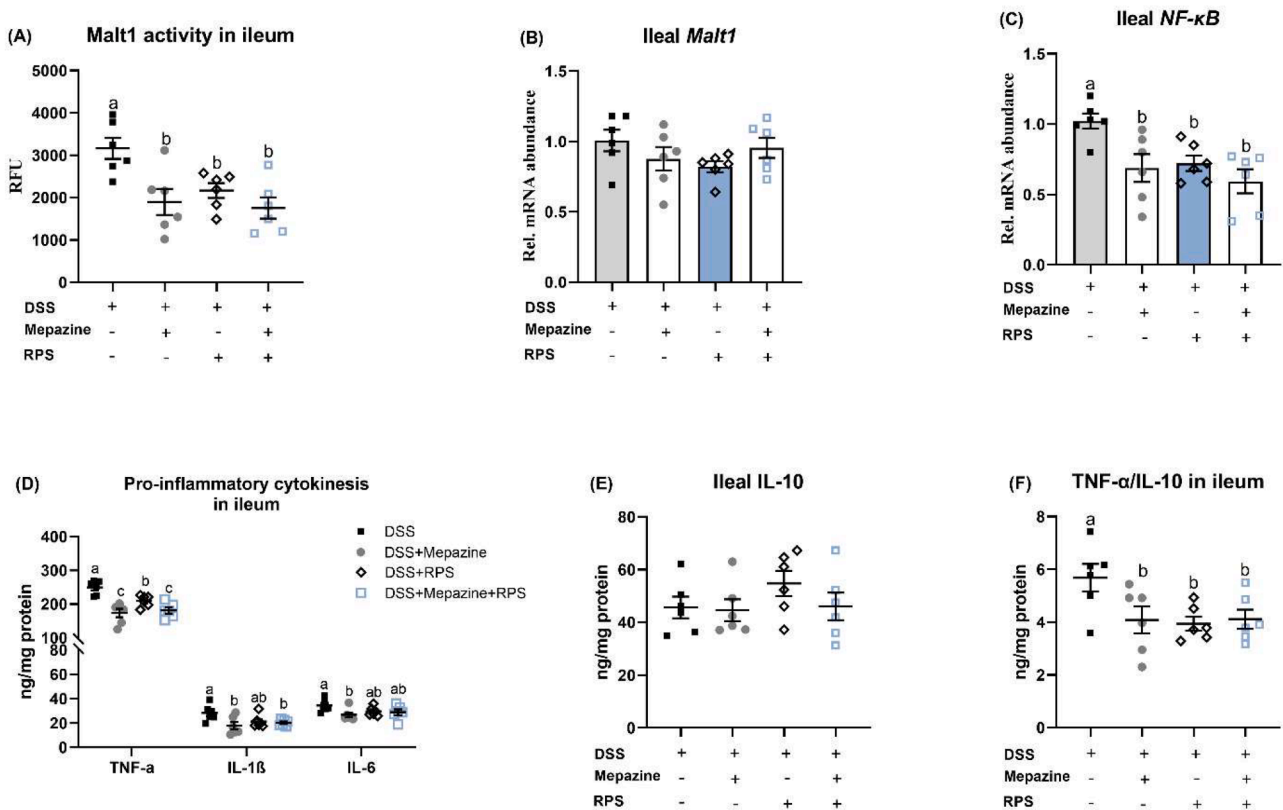


Fig. 4. Effect of dietary raw potato starch (RPS) supplementation and inhibition mucosa-associated lymphoid tissue 1 (Malt1) on ileal inflammation of meat ducks challenged with dextran sodium sulphate (DSS). (A) Malt1 activity in ileum. mRNA abundance of (B) *Malt1*, (C) nuclear factor-kappa B (*NF- κ B*), (D) the concentration of pro-inflammatory cytokines including interleukin (IL-1 β), IL-6, and tumor necrosis factor alpha (TNF- α), (E) anti-inflammatory cytokine IL-10, and (F) its ratio to TNF- α in ileum. Data are expressed as mean and standard deviation. Statistical significance showed as different letters at $P < 0.05$.

reduced TRAP activity in ducks treated with Mepazine and/or RPS when compared to the DSS-only group (Fig. 6H, I). In contrast, markers of bone formation were largely unaffected. There were no significant differences ($P > 0.05$) in serum P1NP levels or in tibial mRNA expression of runt-related transcription factor 2 (*Runx2*) and osteocalcin across experimental groups. The ALP content, however, was significantly increased ($P < 0.05$) in the RPS treatment group relative to both the DSS and Mepazine groups (Fig. 6J-L).

Discussion

The relationship between intestinal inflammation and bone health is a well-established focus in both medical and poultry research, especially in fast-growing meat-type birds. Chronic enteritis can profoundly disrupt tibial bone metabolism, resulting in bone loss and an elevated risk of fractures (Metzger, et al., 2019). Research has shed light on the functional roles of dietary resistant starch supplementation in gastrointestinal disorders, bone loss, and rheumatoid arthritis (Bai, et al., 2021; Zhang et al., 2022c). Thus, RPS intervention, a type II resistant starch, may offer a nutritional strategy to combat intestinal dysfunction and related complications in ducks. Using a DSS-induced enteritis model in meat ducks, the present study demonstrated that a 12% RPS diet could mitigate DSS-induced intestinal permeability and inflammatory onset, thereby suppressing osteoclastic bone resorption driven by pro-inflammatory factors and reversing associated bone loss. The observed regulatory effects on the intestine and bone attributed to gut microbiota modulation and suppression of the Malt1 protease activity-mediated inflammatory response during enteritis.

Evidence from broiler research indicates that intestinal barrier impairment and microbial dysbiosis upregulate pro-inflammatory cytokines in both the ileum and bone marrow, leading to inferior bone

mass and material properties of tibia (Zhang, et al., 2021). The gut-bone axis is further corroborated by clinical evidence linking inflammatory bowel disease to compromised bone health (Targownik, et al., 2013) and murine colitis models, wherein DSS administration reduces bone mass and disrupts microarchitecture (Dobie, et al., 2018; Metzger, et al., 2019). In this study, DSS-treated birds exhibited decreased bone mass, showed by increased cortical thickness in diaphysis and bone mass in metaphysis. The lack of apparent change in tibia ash content might be explained by the relatively short challenge duration. These alterations were accompanied by a significant increase in the TNF- α /IL-10 ratio, a key biomarker of the balance between a pro- versus anti-inflammatory state (Goswami, et al., 2009), and serum pro-inflammatory cytokines (IL-1 β and TNF- α). It has been reported that pro-inflammatory cytokines released during enteritis, such as IL-6 and TNF- α , can suppress bone formation and stimulate bone resorption (Irwin, et al., 2013; Targownik, et al., 2013). In the current study, DSS-induced bone loss in meat ducks likely resulted from inflammatory cytokine-mediated bone resorption, as supported by the observed elevations in serum CTx and TRAP levels. Furthermore, dietary 12% RPS supplementation in this study ameliorated DSS-induced intestinal dysfunction and suppressed the subsequent inflammatory response, thereby inhibiting bone resorption and enhancing tibial mass. These gut-protective effects of RPS are consistent with previous findings. For example, dietary supplementation with 12% RPS reversed *E. coli*-induced bone loss and improved tibial strength in meat ducks (Zhang, et al., 2022b). In ovariectomized mice, the diet supplementation with resistant starch was noticed to attenuate bone loss by regulating the intestinal microbiota and bone marrow inflammation (Tousen, et al., 2019). Given that RPS resists upper gastrointestinal digestion and is fermented by gut microbiota, and considering the efficient fermentative capacity of waterfowl (Józefiak, et al., 2004), it is plausible that RPS-mediated microbial shifts drove the gut-bone

Table 2

Effect of dietary raw potato starch (RPS) supplementation and inhibition Malt1 on intestinal physical characteristics (21 d) of meat ducks challenged with DSS.

Item	DSS	DSS + Mepazine	DSS + RPS	DSS + Mepazine + RPS	P- value
Absolute length, cm					
Duodenum	29.73 ± 2.37	30.77 ± 1.48	31.31 ± 1.93	29.85 ± 0.96	0.684
Jejunum	74.03 ± 4.31	73.25 ± 5.51	74.66 ± 3.97	74.18 ± 4.77	0.517
Ileum	71.20 ± 2.57	73.93 ± 5.40	74.12 ± 4.52	75.70 ± 4.52	0.773
Cecum	33.28 ± 3.19	33.43 ± 3.29	34.43 ± 2.13	34.40 ± 1.43	0.108
Absolute weight, g					
Duodenum	4.79 ± 0.39	5.46 ± 0.69	5.50 ± 0.77	5.22 ± 0.78	0.667
Jejunum	10.98 ± 2.05	11.55 ± 1.87	11.16 ± 1.32	12.28 ± 1.37	0.872
Ileum	9.99 ± 0.63 ^b	12.54 ± 2.25 ^a	12.34 ± 1.35 ^a	12.95 ± 1.01 ^a	0.037
Cecum	2.40 ± 0.29	2.88 ± 0.43	2.93 ± 0.64	2.84 ± 0.31	0.051
Relative length, cm/kg					
Duodenum	30.46 ± 2.43	31.52 ± 1.52	31.86 ± 1.64	30.58 ± 0.98	0.278
Jejunum	75.85 ± 4.42	75.05 ± 5.65	75.46 ± 4.15	76.01 ± 4.88	0.321
Ileum	72.95 ± 2.63	75.75 ± 5.53	75.98 ± 4.76	77.56 ± 4.63	0.583
Cecum	34.10 ± 3.27	34.26 ± 3.38	35.26 ± 3.96	35.25 ± 1.47	0.598
Relative weight, g/kg					
Duodenum	4.48 ± 0.32	5.02 ± 0.49	5.02 ± 0.91	4.54 ± 0.79	0.803
Jejunum	10.26 ± 1.75	10.60 ± 1.21	10.98 ± 1.26	10.64 ± 1.04	0.695
Ileum	9.36 ± 0.44 ^b	11.49 ± 1.56 ^a	11.41 ± 0.65 ^a	11.24 ± 0.88 ^a	0.032
Cecum	2.25 ± 0.30	2.67 ± 0.50	2.71 ± 0.33	2.46 ± 0.20	0.153
pH value					
Ileum	6.35 ± 0.15	6.38 ± 0.22	6.21 ± 0.71	6.46 ± 0.24	0.816
Cecum	5.68 ± 0.36	5.90 ± 0.37	5.71 ± 0.24	5.58 ± 0.25	0.517

Data are expressed as mean ± standard deviation. Statistical significance showed as different letters were assessed by two-way analysis of variance followed by Tukey's *post hoc* test ($P < 0.05$, $n = 6$).

protective effects observed in this study

To elucidate the mechanism by which RPS reversed DSS-induced bone loss in the present study, transcriptomic analysis revealed that dietary RPS intervention in DSS-treated ducks significantly altered bone marrow gene expression, contributing to 263 upregulated and 460 downregulated transcripts. The gene set was enriched for pathways involving cytokines, rheumatoid arthritis, and MAPK/PI3K-Akt signaling, with the NF- κ B pathway, cytokine-cytokine receptor interaction, and osteoclast differentiation being the most prominent. This highlights a key mechanism, as NF- κ B, a master regulator of osteoclastogenesis, drives inflammatory cytokine production and aberrant bone turnover (Boyce, et al., 2015). The observed downregulation of this NF- κ B pathway by dietary RPS manipulation in this study probably provides a direct transcriptional explanation for its protective effect against bone loss (Abu-Amer, 2013; Iotsova, et al., 1997). Foundational studies demonstrate that genetic deficiency of NF- κ B in mice causes severe osteopetrosis due to impaired osteoclast formation (Iotsova, et al., 1997), while its targeted inhibition in the gut rescues bone in colitis (Ke et al., 2019). This mechanism is pathologically relevant, as NF- κ B-dependent cytokines such as TNF- α , IL-1 β , and IL-6 promoted bone resorption in both murine models of colitis-associated osteopenia

(Hamdani, et al., 2008) and IBD patients (Targownik, et al., 2013). Furthermore, the systemic link is underscored by studies where conventional gut microbiota expands bone marrow populations of CD4⁺ T cells and osteoclast precursors, correlating with bone loss (Sjogren, et al., 2012). Collectively, this evidence positions the NF- κ B mediated cytokine as a primary therapeutic target for preserving bone mass in intestinal inflammatory disorders. The application of AlphaFold in this study predicted a structural interaction among Malt1, TRAF6, and NF- κ B, suggesting a potential role of Malt1/NF- κ B pathway in regulating osteoclast frequency and activity. Malt1 is an intracellular signalling protein crucial for immunity, which functions both as a scaffold for NF- κ B activation and as a protease that cleaves inhibitory proteins like A20 and RelB (Afonina, et al., 2015). Pharmacological inhibition of Malt1 blocks NF- κ B activation *in vitro* (Wang, et al., 2020). In the present study, DSS-treated birds exhibited upregulated NF- κ B transcription and increased bone resorption. What is noteworthy is that the specific reduction in Malt1 protease activity, independent of transcriptional change, establishes its proteolytic function as a critical regulatory point. Based on these findings, the current study hypothesizes that dietary RPS supplementation attenuates enteritis-induced bone resorption and osteopenia by inhibiting Malt1 protease activity and suppressing the downstream NF- κ B-mediated inflammatory cascade.

As a matter of course, mepazine, a Malt1 protease inhibitor, was used in this study to evaluate whether inhibition the Malt1 protease and dietary RPS treatment could reverse DSS-induced enteritis and bone loss. Either mepazine or RPS alone suppressed ileal Malt1 protease activity and NF- κ B expression, correlating with a reduction in TNF- α and the TNF- α /IL-10 ratio. The attenuation of DSS-induced enteritis and inflammation observed here via Malt1 inhibition is consistent with prior findings that mepazine alleviates colonic inflammation in mice through NF- κ B suppression (Liu, et al., 2016). This pro-inflammatory cascade also directly compromises intestinal integrity by downregulating tight junction proteins, especially *occludin* and *ZO-1* (Robinson, et al., 2015). Building on evidence that cytokine inhibition can restore the ZO-1 protein level in the large intestinal tissues of DSS-treated mice (Lee, et al., 2018), the direct inhibition of Malt1 protease by Mepazine in previous study could reduce enterocyte apoptosis, improved tight junction integrity, and attenuated inflammatory injury (Liu, et al., 2016). The protective effect on the intestinal barrier was further evidenced by a more pronounced reduction in serum FITC-d content and a greater upregulation of tight junction proteins in the mepazine and RPS co-treatment group compared to either treatment alone. This synergistic effect strongly suggests that the beneficial role of RPS in preserving intestinal integrity is mediated by inhibiting Malt1 protease activity. Parallel to its anti-inflammatory action, RPS treatment supported intestinal barrier function through a prebiotic mechanism involving the gut microbiota (Feng, et al., 2018). Given that Firmicutes are major producers of butyrate and propionate, whereas Bacteroidetes primarily generate acetate and propionate (Kircher, et al., 2022). Consistent with this metabolic profile, the RPS-induced increase in Firmicutes and decrease in Bacteroidetes fostered a metabolic environment conducive to the production of SCFAs, specifically acetic and butyric acid. Butyrate, in particular, plays a vital role by fuelling colonocytes and promoting epithelial cell turnover, thereby directly enhancing intestinal integrity (Abdelqader and Al-Fataftah, 2016). As a result, the addition of RPS at a 12% dietary level was effective in enhancing intestinal barrier integrity in DSS-treated ducks, mediated by the upregulation of tight junction protein transcription.

The closed connection between intestinal homeostasis and bone turnover also prompted us to evaluate the alternation in bone metabolism response to inhibition of Malt1 activity and dietary RPS treatment. In this study, the bone mass, ultimate load, and yield load of tibia in DSS-treated ducks were promoted by mepazine or dietary RPS supplementation, whereas these characteristics were further enhanced by the co-treatment with mepazine and RPS. Homoplasically, *E. coli*-induced bone loss was reversed by Malt1 protease inhibition, which

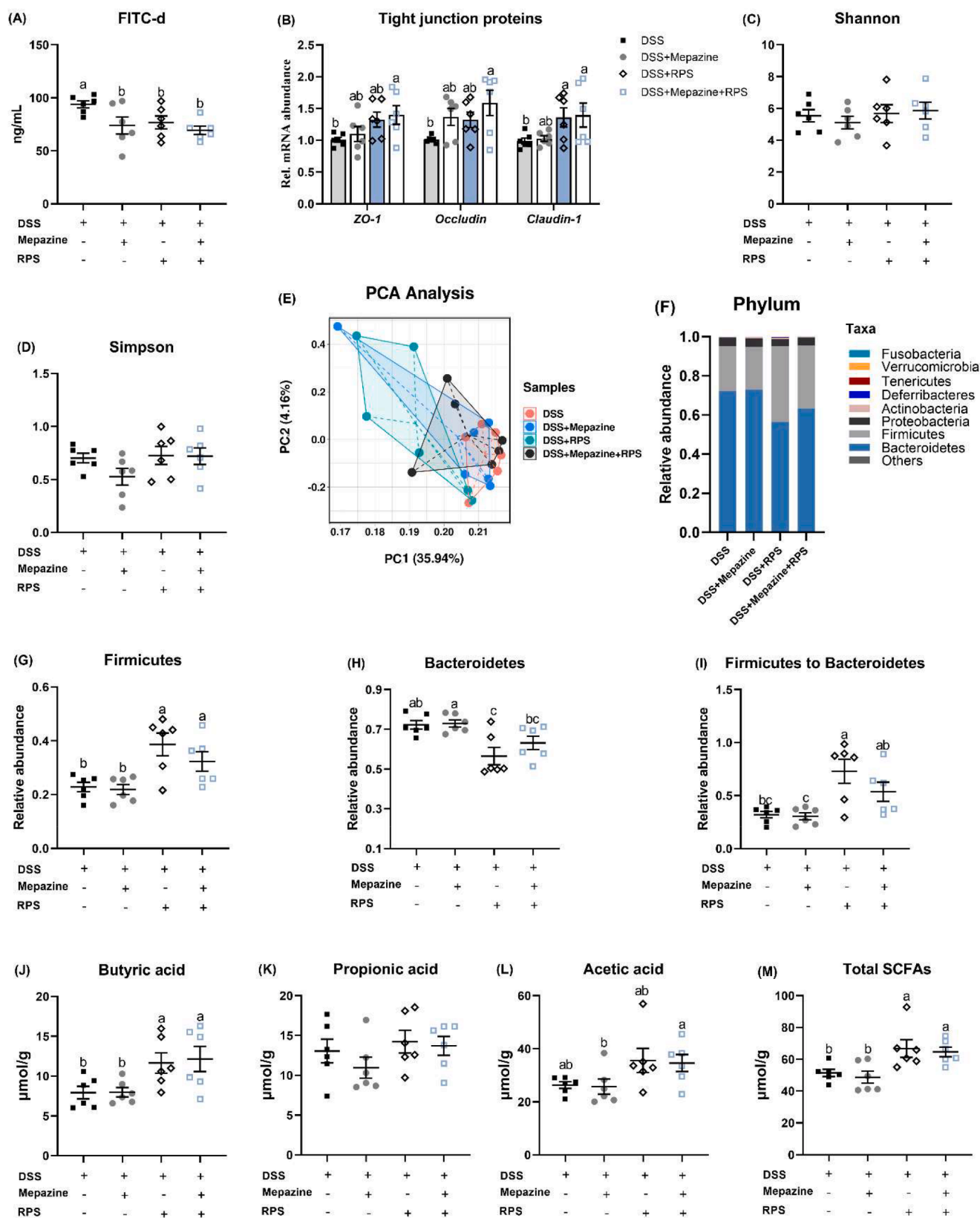


Fig. 5. Intestinal integrity and gut microbiota composition of dextran sodium sulphate (DSS)-treated ducks in response to dietary raw potato starch (RPS) supplementation and inhibition mucosa-associated lymphoid tissue 1 (Malt1) activity. (A) Gut permeability by serum fluorescein isothiocyanate-dextran (FITC-d) content. (B) mRNA abundances of zonula occludens-1 (ZO-1), *occludin*, and *Claudin-1*. (C, D) alpha diversity analysis showed by Shannon and Simpson index. (E) Principal coordinate analyses (PCoA) of beta diversity of bacterial operational taxonomic units. (F) Relative phylum level abundance of gut bacteria. The relative proportion of (G) Firmicutes, (H) Bacteroidetes, and (I) the ratio of Firmicutes to Bacteroidetes. (J-M) The concentration of short-chain fatty acid (SCFA) in caecal content. Data are expressed as mean and standard deviation. Statistical significance showed as different letters at $P < 0.05$.

Table 3

Effect of dietary raw potato starch (RPS) supplementation and inhibition mucosa-associated lymphoid tissue 1 (Malt1) on tibia bone characteristics (d 21) of meat ducks challenged with dextran sodium sulphate (DSS).

Items	DSS	DSS + Mepazine	DSS+RPS	DSS + Mepazine + RPS	p-value
Bone growth					
Length, mm	92.10 ± 2.64	93.60 ± 4.98	93.74 ± 3.64	94.02 ± 2.16	0.986
Diameter, mm	5.99 ± 0.76	6.10 ± 0.34	5.64 ± 0.36	5.91 ± 0.25	0.454
Fat-free weight, g	2.98 ± 0.30	2.84 ± 0.57	3.09 ± 0.47	3.18 ± 0.35	0.576
Bone mass					
Ash, % Fat-free weight	39.57 ± 3.82 ^b	43.47 ± 0.76 ^a	44.72 ± 1.79 ^a	43.95 ± 2.49 ^a	0.031
Density, g/cm ³	0.39 ± 0.04 ^b	0.41 ± 0.06 ^{ab}	0.44 ± 0.09 ^a	0.43 ± 0.05 ^a	0.046
Cortical thickness in diaphysis, µm	37.8 ± 6.29 ^b	39.89 ± 3.25 ^b	40.48 ± 0.93 ^{ab}	45.99 ± 3.54 ^a	0.028
BV/TV in metaphysis, %	0.17 ± 0.06 ^b	0.25 ± 0.05 ^a	0.25 ± 0.02 ^a	0.27 ± 0.08 ^a	0.044
Bone mechanical properties					
Ultimate load, N	101.42 ± 11.35 ^b	122.89 ± 10.42 ^a	119.71 ± 9.62 ^{ab}	134.03 ± 21.79 ^a	0.028
Yield, N	72.03 ± 10.67 ^b	88.84 ± 16.04 ^{ab}	91.86 ± 10.34 ^a	92.57 ± 13.36 ^a	0.500
Slope, N/mm	322.88 ± 46.10	359.42 ± 30.61	363.19 ± 31.73	359.36 ± 35.24	0.373
AUC, N*mm	261.26 ± 30.68	263.87 ± 27.95	259.33 ± 23.12	275.95 ± 32.81	0.858

Data are expressed as mean and standard deviation. Statistical significance showed as different letters at $P < 0.05$ ($n = 6$).

BV/TV Bone volume/Total volume; AUC, Area under the load-displacement curve.

concurrently blocked the NF- κ B-mediated inflammatory pathway and suppressed cytokine-driven osteoclastic resorption (Zhang, et al., 2022b). It is important to distinguish between modifying Malt1 protease activity and deleting the Malt1 gene, as their biological consequences differ significantly. Clinical data also showed that patients with Malt1 deficiency exhibit growth inhibition and severe osteoporosis (Rozmus, et al., 2016). While Malt1 deletion in mouse T cells protected against autoimmune arthritis, it also resulted in spontaneous osteoporosis (Gilis, et al., 2019), and Malt-deficient (Malt1^{KO}) mice develop osteopenia with increased osteoclastogenesis (Monajemi, et al., 2019). One of primary reasons for this phenotype is the alteration of Treg cells, which are well-known inhibitors of osteoclast formation both *in vitro* and *in vivo* (Zaiss, et al., 2007). The absence of regulatory T lymphocytes (Treg cells) in Malt-deficient mice likely drives the osteoporotic phenotype as an indirect effect of gene deletion (Brüstle, et al., 2017; Gilis, et al., 2019). Adoptive transfer experiments of Treg cells in Malt1^{KO} mice could normalize decreased Treg cells account and lower bone density by the abolishment of Malt1 (Gilis, et al., 2019). Here, this study data showed that mepazine and RPS treatment did not change the expression abundances of the related cytokines of Treg cells, including *IL-2*, *IL-10*, and *TGF- β* , indicated that Mepazine and RPS intervene not apparently influenced the Treg cells. On the contrary, administration of mepazine or RPS diet decreased the mRNA level of NF- κ B, as well as downstream *IL-1 β* and *TNF- α* expressions to different extent. The reduced release of pro-inflammatory cytokines in the bone marrow downregulated the expression of *cathepsin K*, a cysteine protease that degrades bone matrix (Novinec and Lenarčič, 2013), leading to decreased osteoclast number and activity. This resulted in lower bone resorption, as evidenced by reduced serum CTx and TRAP levels. Attenuating NF- κ B-mediated inflammation in the gut-bone axis is known to mitigate bone loss by inhibiting osteoclastogenesis and

osteoclast activity (Metzger, et al., 2019). This might be attributed to the depressed the expression of RANKL in DSS-treated ducks, which binds to RANK expressed on the surface of the osteoclast to induce osteoclast differentiation (Yamamoto, et al., 2006). Furthermore, in the process of bone resorption, OPG is secreted by osteoblast and bind with RANK of surface osteoclast to inhibit osteoclastogenesis (Yamamoto, et al., 2006). The lower ratio of RANKL to OPG due to Mepazine or RPS manipulation suggested the inhibitory action on the proliferation of osteoclast in the present study. Notably, the combined treatment of Mepazine and dietary RPS resulted in more pronounced reductions in serum CTx content, and the RANKL/OPG ratio than Mepazine treatment alone. This synergy indicates that dietary RPS inclusion augments the suppression of the Malt1/NF- κ B pathway, thereby more effectively blocking inflammation-induced bone resorption.

Moreover, the potential synergistic effects of mepazine and RPS on bone formation also warrant consideration in the current study. Decreased bone formation is a documented consequence of colitis in both experimental models and patients with inflammatory bowel disease (Croucher, et al., 1993; Hamdani, et al., 2008). The DSS-induced colitis model in mice directly hinders bone formation, reducing key histomorphometry parameters including trabecular double-labelled surface, mineralizing surface, and bone formation rate (Hamdani, et al., 2008). In this study, mepazine, which targets the same Malt1 protease activity as dietary RPS inclusion, failed to elevate serum bone formation markers (P1NP and ALP), suggesting this pathway may not directly stimulate osteogenesis. In contrast, RPS supplementation alone increased serum ALP activity and the osteogenic master regulator *Runx2* expression. As a key mediator of osteoblast differentiation and chondrocyte maturation, *Runx2* transcription was upregulated in early bone formation and stimulated the expression of a wide array of bone-specific genes, including osteocalcin (Komori, 2019). Although higher activity of serum ALP and upregulated *Runx2* mRNA abundance due to alone RPS supplementation was found, the roles of diet containing 12% RPS in bone formation were still suspended in DSS-treated ducks.

The findings of this research should be considered alongside several identified research gaps. The data of RNA-Seq in the current study identified that the tibia morphological changes due to dietary RPS supplementation in DSS-treated birds might be associated with MAPK and PI3K-Akt signalling pathways, whereas these pathways require direct experimental validation. In addition, the specific role of Malt1 in bone metabolism should be further elucidated using genetic models of Malt1 silencing and overexpression to distinguish its effects from broader genetic manipulation.

The present study has several limitations that should be acknowledged. The first is the study design. Since DMSO is required to prepare the mepazine stock solution, DMSO should be added to the PBS group used as the intraperitoneal injection control to ensure experimental consistency. Furthermore, a negative control group should be included in Expt. 2 to elucidate the roles of DSS, RPS, and Malt1 inhibition under identical conditions, given that the effects of DSS on intestinal and bone metabolism were already established in Expt. 1 by comparing alterations between the negative control (Ctrl) and DSS-treated groups. In addition, statistical assessment was limited due to insufficient sample size, leading to some inconsistent results. For instance, while the 12% RPS diet elicited a significant increase in tibial ash content in the DSS model in Expt. 2, the corresponding increase in Expt. 1 failed to reach statistical significance. In addition, the data of RNA-Seq in the current study identified that the tibia morphological changes due to dietary RPS supplementation in DSS-treated birds might be associated with MAPK and PI3K-Akt signalling pathways, whereas these pathways require direct experimental validation. The specific role of Malt1 in bone metabolism should be further elucidated using genetic models of Malt1 silencing and overexpression to distinguish its effects from broader genetic manipulation. Finally, facilitating mineral elements absorption, especially Ca and phosphorus, might present another potential mechanism by which dietary RPS supplementation promotes bone

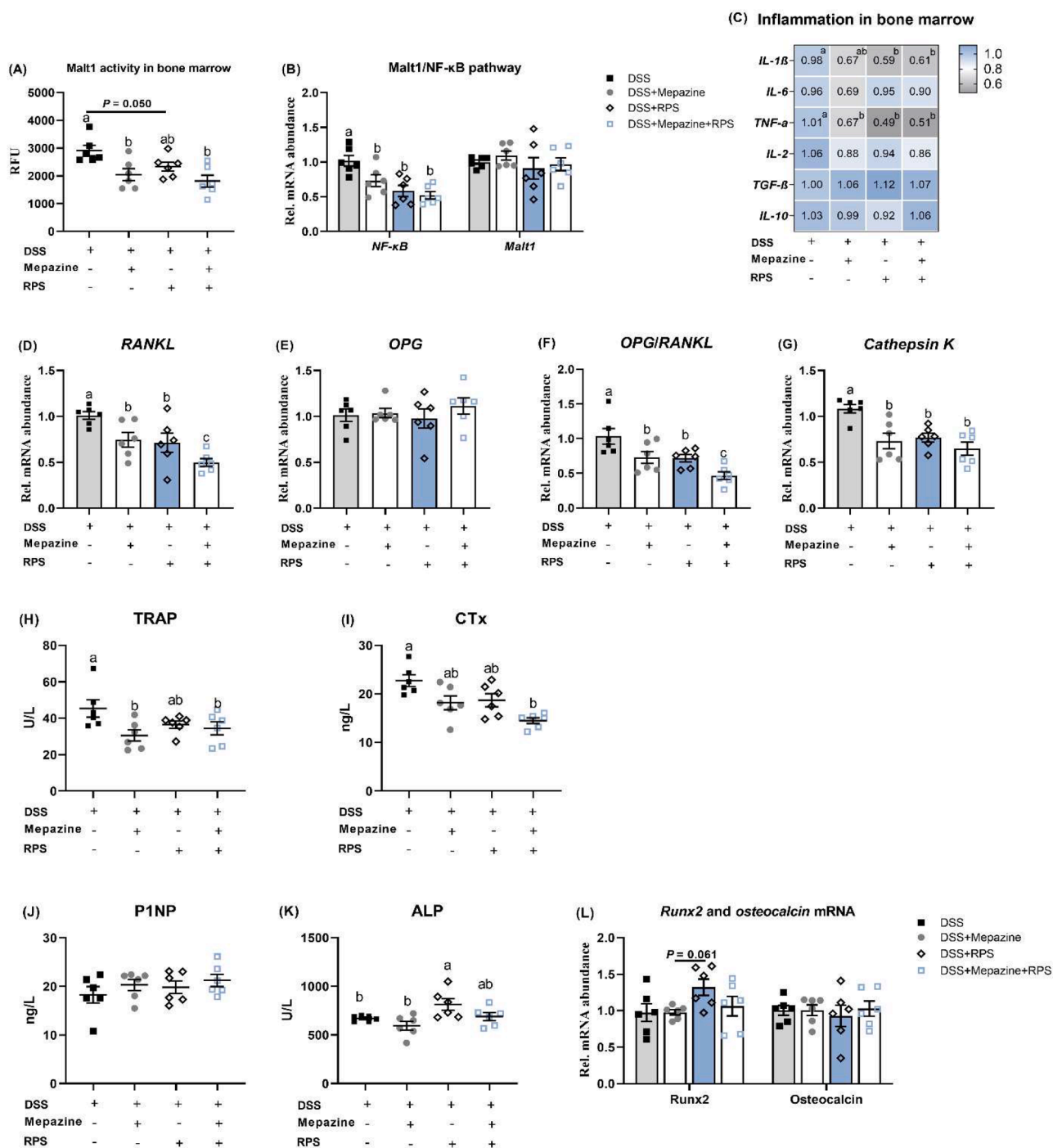


Fig. 6. Effect of dietary raw potato starch (RPS) supplementation and inhibition mucosa-associated lymphoid tissue 1 (Malt1) on tibia inflammation and bone turnover of meat ducks challenged with dextran sodium sulphate (DSS). (A) Malt1 activity and (B) mRNA levels of nuclear factor-kappa B (*NF-κB*) and *Malt1* in bone marrow. (C) mRNA abundance of inflammatory cytokines in bone marrow, including, interleukin (*IL-1β*), *IL-6*, tumour necrosis factor alpha (*TNF-α*), *IL-2*, transforming growth factor beta (*TGF-β*), and *IL-10*. (D-F) Transcription of receptor activator for nuclear factor-κB ligand (RANKL) and osteoprogenitor (OPG) in the proximal end, and the ratio of RANKL/OPG. (G) Expression of osteoclastic Cathepsin K. (H-K) Serum biomarkers of bone turnover, including C-terminal cross-linked telopeptide of type I collagen (CTx) and tartrate-resistant acid phosphatase (TRAP), procollagen type I N-terminal propeptide (P1NP), and alkaline phosphatase (ALP). (L) osteoblastic gene expression, including runt-related transcription factor 2 (*Runx2*) and *osteocalcin*. Data are expressed as mean and standard deviation. Statistical significance showed as different letters at $P < 0.05$.

mineralization in this study. This may be mediated by the SCFAs produced during bacterial fermentation of RPS, which can lower intestinal pH and thereby enhance the solubility and retention of key minerals (Nourmohammadi, et al., 2012; Zhang, et al., 2022a). Overall, we recognize that our conclusions may overestimate or underestimate RPS and DSS effects on tibial mass in meat ducks. Additional research is

required to refine enteritis models and clarify the impacts of RPS and DSS on bone and gut homeostasis in poultry.

In conclusion, using a DSS-induced enteritis model in meat ducks, this study demonstrated that dietary supplementation with 12% RPS improved intestinal barrier function, as evidenced by reduced pro-inflammatory cytokine levels, an effect likely associated with alterations

in the gut microbiota and increased SCFA production. Moreover, RPS inclusion exerted a marked anti-inflammatory effect within the bone marrow microenvironment, thereby suppressing osteoclast formation and activity and subsequently reducing osteoclastic bone resorption. This effectively reversed the osteopenia and poor mechanical bone properties observed in DSS-treated ducks. The proposed mechanism underlying this protective role probably involves inactivation of the Malt1 protease and downregulation of the NF- κ B inflammatory pathway. These findings suggest that dietary modulation of the gut microbiome represents a promising strategy for managing inflammation in meat ducks. Notably, dietary RPS may help prevent inflammatory bone loss and could have potential applications in poultry bone disorders such as including inflammation-associated bone loss.

Author Contribution

Huaiyong Zhang and Wen Chen conceived and designed the experiments. Xin Yue, Yuying Huang, Yuhuang Hou, Xuemeng Si, and Yanqun Huang conducted the broiler study, collected and analyzed the samples. Yuhuang Hou, Minh Tu Nguyen, Xuemeng Si, Rui Zheng, and Huaiyong Zhang analyzed the data and wrote the first draft of the manuscript. All authors read and approved of the final manuscript.

Disclosures

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our 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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.psj.2026.107443](https://doi.org/10.1016/j.psj.2026.107443).

[Table S1](#). Primers for quantitative real-time PCR.

[Fig. S1](#) Verification of transcriptome analysis (RNA-seq) and RT-PCR.

[Fig. S2](#) Dietary RPS and mepazine treatment on growth performance of DSS-treated ducks.

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