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Effect of intestinal microbiota on duck short-beak and dwarf syndrome caused by novel goose parvovirus

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ABSTRACT Short-beak and dwarf syndrome (**SBDS**) is caused by infection with novel goose parvovirus (**NGPV**), which leads to intestinal dysbiosis, developmental delay, short beak, lameness, and paralysis in ducks and is the cause of skeletal health problems. NGPV infection can cause intestinal microbial disturbances, but it is still unclear whether the intestinal microbiota affects the pathogenicity of NGPV. Here, the effects of intestinal microbiota on NGPV-induced SBDS in Cherry Valley ducks were assessed by establishing a duck model for gut microflora depletion/reestablishment through antibiotics (**ABX**) treatment/fecal microbiota transplanted (**FMT**). By measuring body weight, beak length, beak width and tarsal length, we found that SBDS clinical symptoms were alleviated in ducks treated with ABX, but not in FMT ducks. Next, we conducted a comprehensive analysis of bone metabolism, gut barrier integrity, and inflammation levels using quantitative real-time PCR (**qPCR**), enzyme linked immunosorbent assay (**ELISA**), biochemical analysis and histological analysis. The results showed that ABX treatment improved bone quality reduced bone resorption, mitigated tissue lesions, protected intestinal barrier integrity, and inhibited systemic inflammation in NGPV-infected ducks. Moreover, cecal microflora composition and short-chain fatty acids (**SCFAs**) production were examined by bacterial 16S rRNA sequencing and gas chromatography. The results revealed that ABX treatment mitigated the decreased abundance of *Firmicutes* and *Bacteroidota* in NGPV-infected ducks, as well as increased SCFAs production. Furthermore, ABX treatment reduced the mucosa-associated lymphoid tissue lymphoma translocation protein 1 (***Malt1***) and nuclear factor κB (***NF-κB***) expression, which are correlated with systemic inflammation in SBDS ducks. These findings suggested that intestinal microflora depletion alleviated NGPV-induced SBDS by maintaining intestinal homeostasis, inhibiting inflammatory response and alleviating bone resorption. These results provide evidence for the pivotal role of intestinal microbiota in the process of SBDS and contribute a theoretical basis for the feasibility of microecological preparation as a method to control SBDS.

Key words: short-beak and dwarf syndrome, novel goose parvovirus, intestinal microbiota, inflammation, bone resorption

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INTRODUCTION

Short-beak and dwarf syndrome (**SBDS**) is a highly contagious disease caused by novel goose parvovirus (**NGPV**), with a high incidence rate and low fatality in ducks. Ducks affected by SBDS exhibit growth retardation, smaller beak and tarsus, leg swelling, and weakness (Shang, et al., 2023). Furthermore, mixed infection involving NGPV and other pathogens has a detrimental effect on the emergence rate of meat ducks and leads to significant financial losses for the waterfowl breeding industry (Yang, et al., 2020). There is presently no efficacious remedy available for NGPV. Therefore, by studying the pathogenic mechanism of NGPV, we can identify ways to alleviate SBDS and reduce the losses of farmers.

An existing study has shown that the infection of NGPV disrupts the gut microbiota by diminishing the prevalence of dominant genera and compromising microbial diversity (Luo et al., 2019). Therefore, in addition to bone development abnormalities, it is likely that ducks infected with NGPV experience health and performance issues as a result of a disrupted microbiota profile. The microorganisms in the gut are crucial in maintaining

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intestinal homeostasis and preventing inflammation. For example, diabetic mice treated with prebiotics showed a reduced inflammatory response, linked to improved tight junction proteins (**TJPs**) expression and decreased permeability of the intestines (Cani et al., 2009). Several studies have proposed that disruption to intestinal integrity and an imbalance in gut microbiota can initiate systemic inflammation, which promotes enhanced bone resorption and reduced bone mass and strength (Jhong et al., 2022; Qiao et al., 2022; Zhang et al., 2022b). Moreover, bone samples obtained from Germ-free mice exhibit a reduction in osteoclast population, along with reduced levels of interleukin-6 (**IL-6**) and tumor necrosis factor-alpha (**TNF-α**) (Sjögren, et al., 2012; Ohlsson, et al., 2017). All of this suggests that gut microbes are involved in bone development, and this phenomenon is commonly referred to as the "gut-bone" axis (Zhang et al., 2021). In addition, the microbiota metabolite short-chain fatty acids (**SCFAs**) influence bone density by modulating immune responses (Yan et al., 2018). Apart from acidifying the gut environment to promote mineral release, such as calcium (**Ca**) and phosphorus (**P**), it has been demonstrated that SCFAs exert protective effects on bone density by inhibiting osteoclast differentiation and reducing bone resorption (Lucas et al., 2018). These studies indicate that the disruption of microbiota may contribute to bone health deterioration by activating the immune system. However, whether the intestinal dysbiosis caused by NGPV is related to abnormal skeletal development in SBDS ducks is unclear. NGPV has been found to modify the microbiota, disrupt the integrity of the intestinal barrier, and lead to immune dysfunction in ducks (Luo et al., 2021). This evidence suggests an important link between gut microbes and NGPV pathogenicity.

Therefore, our study aimed to investigate the influence of intestinal microbiota on the development of SBDS in ducks infected with NGPV through antibiotic (**ABX**) -induced microflora depletion. Furthermore, the important molecules and penitential mechanisms that determine how the intestinal microbiota influences the progression of bone abnormalities were explored.

MATERIALS AND METHODS

Virus and Animals

The NGPV virus propagated in our laboratory, and genes were sequenced and submitted to the National Center for Biotechnology Information (**NCBI**) GenBank (No. KU516831), the virus propagation by SPF duck embryo.

Eighty newly hatched unvaccinated Cherry Valley ducks were obtained from Hebei Le Shou Duck Industry Co. Ltd (Hebei, China).

Experimental Modeling

Zero-day-old male Cherry Valley ducks were used in this study, and all the ducks were kept in a constant temperature and humidity environment with unrestricted access to food and water. The grouping and experimental design of ducks are shown in Figure 1A.

Intestinal microflora depletion model: Ampicillin, neomycin sulfate, and metronidazole were added to the drinking water with 1 g/L final concentration for 3 d, and ampicillin (200 mg/kg), neomycin sulfate (200 mg/kg), metronidazole (200 mg/kg) and vancomycin hydrochloride (40 mg/kg) were administered intragastrically (mg drug/kg body weight), once a day for 3 d.

Intestinal microflora reestablishment: Fecal samples (2 g) were collected from untreated ducks, diluted with 5 mL normal saline, homogenized at 45 Hz for 1 min with a sterile steel ball, and filtered through a 70-μm filter. The fecal microbiota transplanted (**FMT**) group ducks were fed filtered fecal homogenate, 0.5 mL/duck, once a day for 2 d.

NGPV infection model: 6-day-old ducks (treated with antibiotics for 3 d and discontinued for 2 d to eliminate the influence of antibiotics on the experimental results) were intramuscularly injected with $10^{4.35}$ EID_{50}, along with oral gavage of $10^{4.35}$ EID_{50} allantoic fluid containing NGPV per duck, kept in isolation, and observed continuously for 28 d.

Identification of Intestinal Microflora Depletion/Re-establishment

Two hundred milligrams of duck feces were collected diluted with 0.5 mL normal saline and homogenized with sterile steel balls at 45 Hz for 1 min and filtered through a 70-μm filter. Subsequently, a 50 μL aliquot of fecal homogenate was evenly distributed onto a brain-heart infusion (**BHI**) agar plate supplement with 10 % sheep blood. The plate was then incubated at 37 °C under anaerobic conditions for 48 h, followed by an additional day of aerobic incubation at the same temperature, to observe colony growth on the plate. In addition, nucleic acid was extracted from anal swabs in 10 ABX-treated ducks using the DNA extraction Kit (TransGen Biotech, Beijing, China). The bacterial universal V3-V4 region of the 16S rRNA gene was amplified using the primers shown in Table 1.

Sample and Data Collection

At 1, 3, 5, 7, and 14 d postinfection (**dpi**), 8 ducks were selected at random from each group, and anal swabs were collected for viral load testing. At 1, 7, 14, 21, and 28 dpi, 500 μL of blood was drawn from the jugular vein of the 8 ducks after they fasted for 6 h, after which they were centrifuged at 4,000 *g* for 15 min at 4 °C to separate serum. Eight selected ducks at 14 and 28 dpi were sacrificed, and the duodenum, terminal ileum, cecal contents, bone marrow, and the proximal end of tarsal bone were stored at -80 °C. Another tarsal bone, 1 cm of duodenum, and ileum were dissected and promptly submerged in 4 % paraformaldehyde for histological examination.

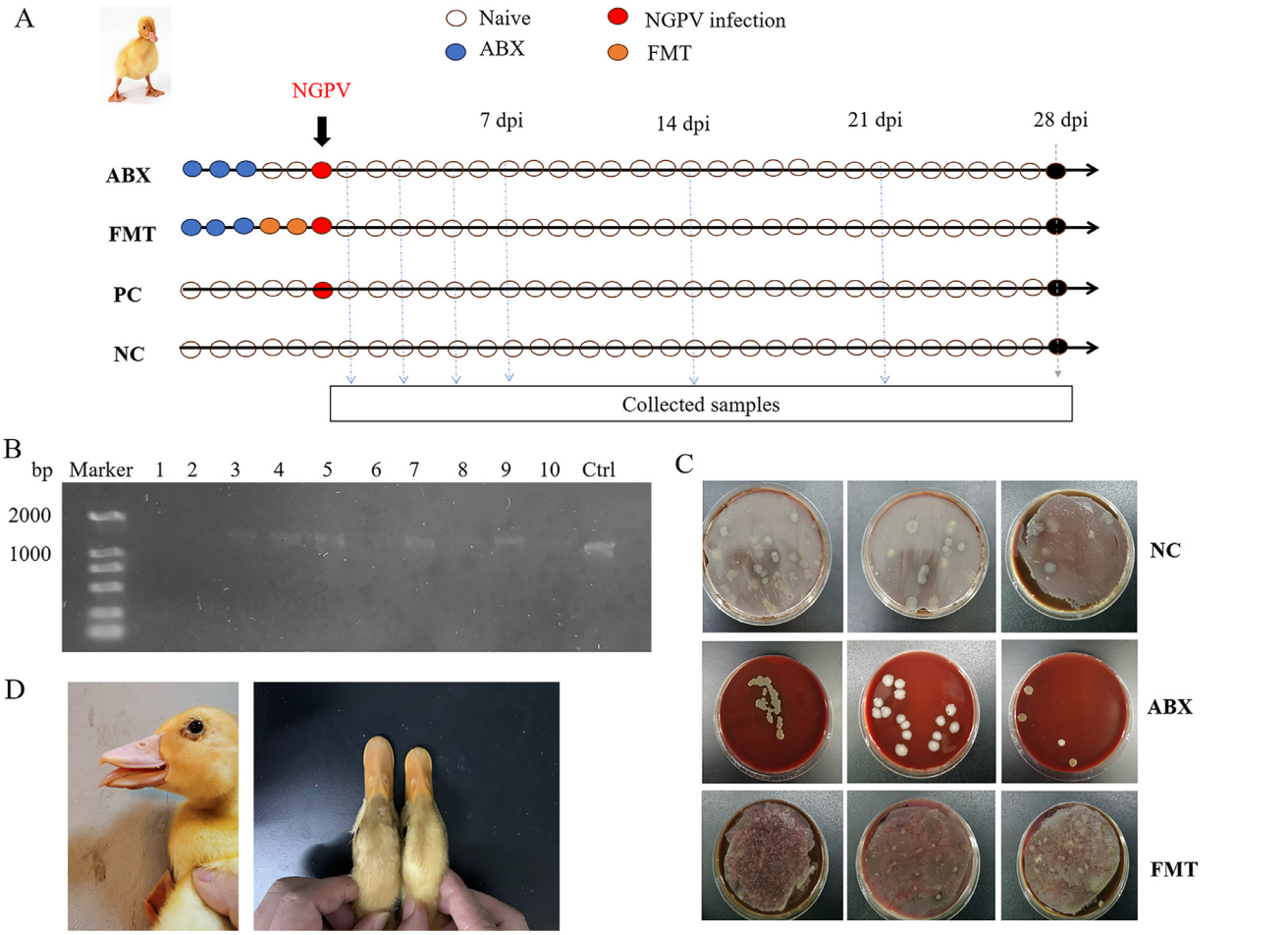

Figure 1. Intestinal microflora depletion/reestablishment in SBDS duck models were established. (A) Experimental design. Two groups of WT ducks (n = 20/group) were given antibiotics once a day for 3 d, after which one group was fed with a pre-antibiotics fecal sample for 2 d (FMT), and the other received no intervention (spontaneous recovery) (ABX). The third (n = 20) and fourth groups (n = 20) did not receive antibiotics, and on the sixth day, the first 3 groups were infected with NGPV, while the fourth group was not. Viral load, fecal samples and intestinal tissues were collected at the various experimental stages. (B) Lanes 1 to 10 were collected from the anal swabs of 10 different ABX-treated ducks, and lane-ctrl samples were collected from NC ducks on the fifth day. Bacteria in the anal swabs were detected by PCR. (C) Colony growth of a BHI agar plate containing 10% sheep blood with feces homogenized in each group; 3 repetitions per group. (D) NGPV-infected ducks exhibit the classic symptoms of SBDS: the beak becomes shorter, and the tongue is exposed.

Viral Load Detection

The duck anal swabs were ground and homogenized, and DNA was extracted with a DNA extraction kit (TransGen Biotech, Beijing, China). Quantitative real-time PCR (**qPCR**) was performed on a detection system (Bio-Rad, CA), qPCR products were stained with SYBR green (Vazyme, Nanjing, China). The primer sequences are shown in Table 1.

Sequencing of Cecal Microbiota

The DNA in the cecal content at 28 dpi was isolated and subjected to sequencing using an Illumina PE 150 platform (Novogene, Beijing, China). The sequences were subjected to processing using the DADA2 method, which is mainly used for denoising. It no longer clusters based on similarity, only removing duplicates or essentially clustering at 100 % similarity. Each unique sequence generated after denoising with DADA2 is called an amplicon sequence variant (**ASV**), also known as a feature sequence. At the same time, it produces fewer false sequences, and using ASVs instead of operational taxonomic units (**OTUs**) improves the accuracy, comprehensiveness, and reproducibility of marker gene data analysis.

Cecal SCFAs Analysis

The cecal content (approximately 0.5 *g*) was thoroughly mixed with 2 mL of ultrapure water, subsequently, vortex oscillation for 5 min and deposition for 15 min at 4 °C. Next, the samples were subjected to centrifugation at 12,000 *g* for 10 min. Following this step, one milliliter of supernatant was combined with 0.2 mL of a metaphosphoric acid solution of 25 % (w/v). After another round of centrifugation using the same force and duration as previously mentioned, gas chromatography was employed to separate and quantify the SCFAs contents in samples.

Table 1. Primer design.

Name	Sequence(5′-3′)	Tm(°C)	Product size (bp)
Bacteria	Forward: AGAGTTTGATCCTGGCTCAG Reverse: GGTTACCTTGTTACGACTT	58	1,500
NGPV	Forward: TGCCGATGGAGTGGGTAAT Reverse: GAAGTGGCAGTGGAAGCGATT	56	99
TNF-α	Forward: CCGCCCAGTTCAGATGAGTTGC Reverse: GCCACCACACGACAGCCAAG	56	97
IL-1β	Forward: TCGACATCAACCAGAAGTGC Reverse: GAGCTTGTAGCCCTTGATGC	56	185
IL-6	Forward: TGGCTTCGACGAGGAGAAATG Reverse: TATCGTCGTTGCCAGATGCT	56	151
IL-10	Forward: AGCTGCCTCCACTTGTCTGA Reverse: GTTCATCGTCTTGGGATTGAAAGT	56	100
Claudin1	Forward: GCCGTGACTGGCATGAAATG Reverse: CCAATGCTGACAAACCTGCAAT	56	112
Occludin	Forward: TGAAGGACTTGAAGCGGAGCC Reverse: TCGTAGTCGCTCACCATGC	56	103
ZO-1	Forward: GCCATCTCTACGCCTGTGAA Reverse: ACTGGCTTTGGTTCTGGGAG	56	110
MUC-2	Forward: AGTTCTTGCCTAATTCCTCAGTCT Reverse: TTGCCGTTCATATCCAGGTTCA	56	128
OPG	Forward: GAAGGTCTGCTCTTGCGAAC Reverse: GCCTAACTGGCTGAACTTGC	56	106
RANKL	Forward: TAAGTTTGCCTGGCCTTTGT Reverse: GCCTTTTGCCCATCTCATTA	56	100
Malt1	Forward: TTGTGCAGGGGATTGGTAAT Reverse: GAGCGTTTTCAAGAGGTTGC	56	118
NF-κB	Forward: AGGGATCTTCTCCTGCCATT Reverse: GAGCGTTTTCAAGAGGTTGC	56	123
GPR41	Forward: TGGTGAGGTAGATGCTGGTG Reverse: ACTGACGTCCTCCTCCTCAA	56	160
GPR43	Forward: GTGGAATATTAGGCCGAGCA Reverse: AGCAGCTGAGCTTTGTCCTC	56	129
GAPDH	Forward: GCTTTCCCGTGTGCCAACCC Reverse: GCCCATCAGCAGCAGCCTTC	56	116

Histological Analysis

The tarsus was immersed in a 4 % paraformaldehyde solution for 24 h, followed by decalcification using ethylene diamine tetraacetic acid. Subsequently, the tarsus was embedded in paraffin and sectioned into 5 μm sections for staining with tartrate-resistant acid phosphatase (**TRAP**) stain kit (Solarbio, Beijing, China). Three fields were randomly selected for each sample and were used to count the number of osteoclasts (**N.Oc/BS**).

Digital radiography (**DR**) photos of duck legs were taken at the local animal hospital.

Duodenal and ileal tissues from ducks were fixed with formalin, dehydrated with graded ethanol, and embedded in paraffin after clearing with xylene. Five-micron of tissue sections were stained with hematoxylin-eosin (**H&E**) stain kit (Solarbio, Beijing, China). Sections were observed under a microscope. Intestinal villus length and crypt depth were analyzed using Image J 1.41o software.

Serum Biochemistry and Cytokine Detection

The concentrations of the cytokines IL-1β, IL-6, TNF-α, C-terminal cross-linked telopeptide of type I collagen (**CTx1**), and procollagen type I N-propeptide (**P1NP**) were measured using an enzyme linked immunosorbent assay (**ELISA**) kit (Meimian Biotechnology, Jiangsu, China). The levels of calcium (**Ca**), phosphorus (**P**) and alkaline phosphatase (**ALP**) in serum were assessed using a biochemical analysis instrument (Rayto Life and Analytical Sciences, Shenzhen, China).

Gene Expression Assays

The RNA in the duodenum, ileum, tarsus, and bone marrow were isolated using TRIzol (Takara Bio, Beijing, China) according to the protocol. Subsequently, cDNA synthesis was using a Vazyme reverse transcription kit. The primer sequences are shown in Table 1. The products are stained with SYBR green (Vazyme, Nanjing, China). Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) is the housekeeping gene. The average Ct values of the 3 technical replicates were used for data analysis, and relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism Version 9.0.0 software, which facilitated the generation of graphs. The plotted values correspond to the average of all replicates performed, while the error bars represent the standard deviation. Correlation analysis was performed using Pearson analysis. The P value was calculated by the independent sample t-test, and a P value less than 0.05 was regarded as significant.

RESULTS

NGPV-Induced Short-Beak and Dwarf Syndrome Can be Alleviated by Intestinal Microflora Depletion

To investigate the impact of the intestinal microbiota on NGPV-induced SBDS. The Cherry Valley ducks

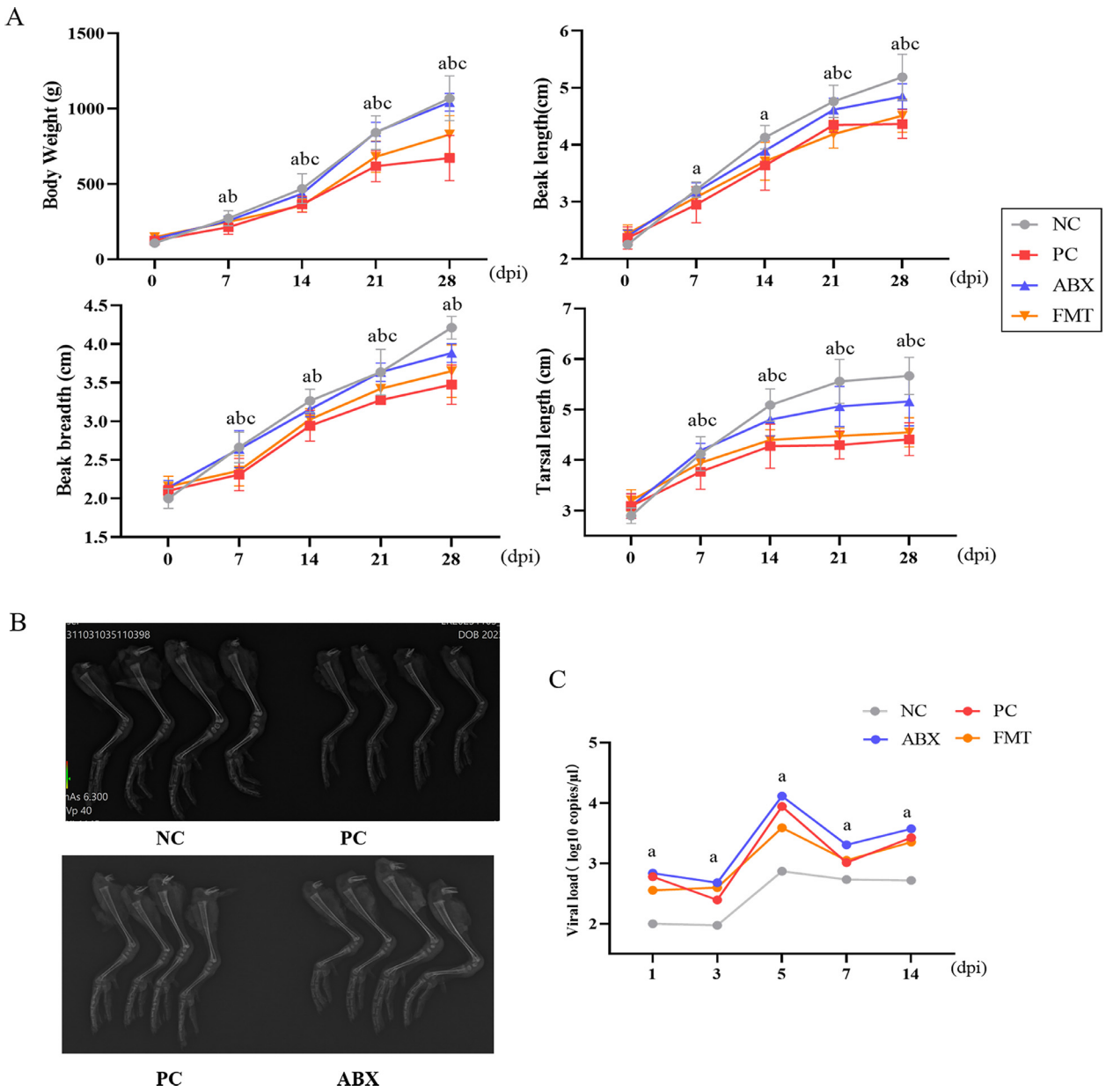

Figure 2. Effects of intestinal microflora depletion on the growth and development of NGPV-infected ducks. (A) Changes in duck body weight, beak length, beak width and tarsal length after NGPV infection. (B) The bone structure of NGPV-infected duck legs was detected by DR. (C) Viral load in anal swabs after NGPV infection was detected by qPCR. (a: PC compared with the NC, $P < 0.05$; b: ABX compared with the PC, $P < 0.05$; c: FMT compared with ABX, $P < 0.05$).

were randomly divided into 4 groups, namely the negative control (**NC**) group, positive control (**PC**) group, ABX group, and FMT group, and the experimental design is shown in Figure 1A. Ducks were administrated with an ABX cocktail (ampicillin, neomycin sulfate, metronidazole and vancomycin hydrochloride) to consume intestinal microflora, nucleic acid was extracted from ABX-treated duck anal swab for PCR, and the results showed that intestinal microflora was depleted (Figure 1B). Fecal homogenate plate coating results showed that intestinal microflora consumed by ABX was successfully reestablished in the FMT group (Figure 1C). In addition, the animal model of SBDS caused by NGPV infection was successfully constructed (Figure 1D). We found that NGPV infection decreased body weight, beak length, beak breadth and tarsal length, while gut microbial depletion alleviated this phenomenon (Figure 2A). Compared with the PC group, the ABX group had strong legs and greater bone quality under conditions of NGPV infection (Figure 2B). No significant differences in viral load were detected among the NGPV-infected groups (Figure 2C). What's more, there was no significant difference between FMT and PC ducks. Overall, these results suggested that the SBDS caused by NGPV infection could be relieved by microflora depletion due to ABX treatment.

Intestinal Flora Depletion Alleviates NGPV-Induced Bone Loss by Inhibiting Bone Resorption

The histological and biochemical assessment was conducted to evaluate the impact of the intestinal

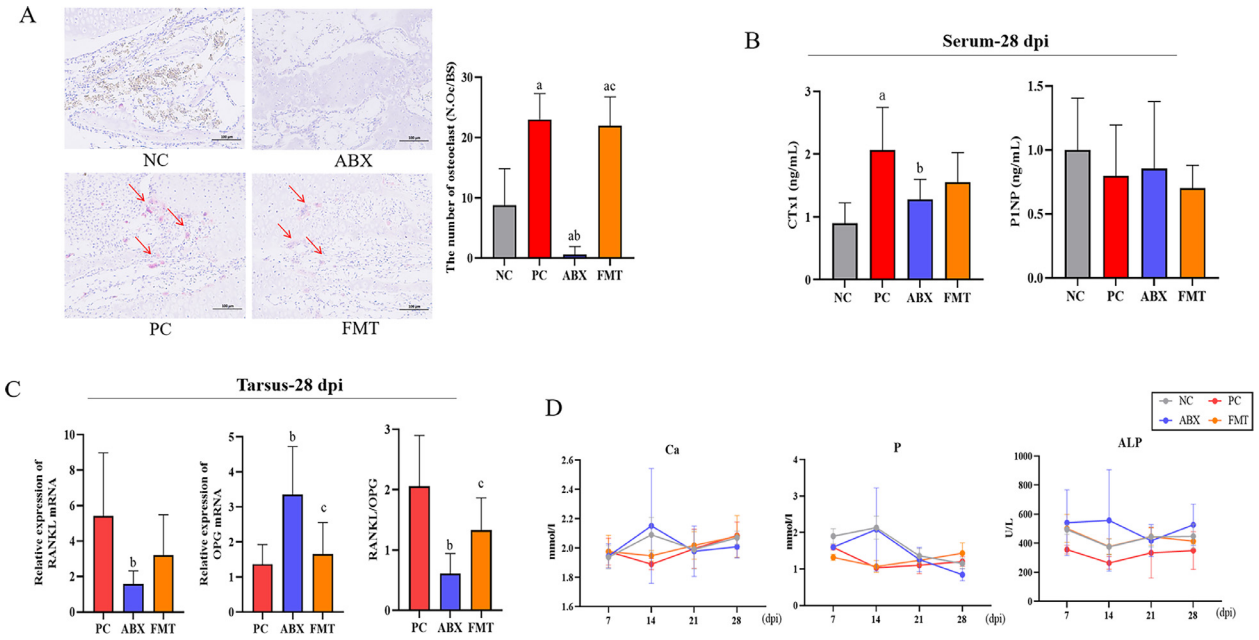

Figure 3. Effects of intestinal microflora depletion on the bone metabolism of NGPV-infected ducks. (A) TRAP staining of a tarsal section and the N.Oc/BS in proximal tarsi stood determined by histomorphometry. (B) Serum P1NP and CTx1 concentrations were evaluated. (C) qPCR analysis of the relative mRNA expression of RANKL and OPG in the proximal end of tarsi was performed, and the ratio of RANKL/OPG was calculated. (D) Serum ALP, Ca and P were evaluated. (a: compared with the NC group, $P < 0.05$; b: compared with the PC group, $P < 0.05$; c: compared with the ABX group, $P < 0.05$).

microbiota on bone resorption. TRAP-positive cells were observed in the proximal tarsus region of NGPV-infected ducks (Figure 3A). The high level of N. Oc/BS ratio in SBDS ducks was subsequently decreased by ABX administration, while that in the FMT group did not decrease (Figure 3A). Compared with that in the NC group, the circulating bone resorption marker CTx1 in the PC group was markedly increased, while in the ABX group was decreased (Figure 3B). Furthermore, we found that ABX treatment upregulated osteoprogerin (**OPG**) mRNA level, while downregulated the receptor activator for nuclear factor-κB ligand (**RANKL**) mRNA level. Significantly, thereby decreasing the high ratio of RANKL/OPG induced by NGPV infection, while FMT did not (Figure 3C). In addition, the effects of intestinal microflora depletion on bone formation were evaluated through analysis of serum biochemical parameters. Notably, there were no significant differences observed in the serum markers of osteogenesis, such as P1NP (Figure 3B) and ALP (Figure 3D). There were no significant differences in the serum Ca or P concentration among the groups (Figure 3D). These data indicate that the alleviation of NGPV-induced bone loss by intestinal microflora depletion could be mainly attributed to the inhibition of bone resorption.

Intestinal Microflora Depletion Changes Microbial Composition and SCFAs Production in NGPV-Infected Ducks

We next determined the impact of ABX treatment on the microbial community of the duck cecal contents following NGPV infection. Disruption of the cecal bacterial community caused by NGPV infection was confirmed by unweighted UniFrac-based PCoA (Figure 4A). The gut microbiota composition at the phylum and family levels is shown in Figure 4B. Compared with those in the NC group, the relative abundances of *Bacteroidota* and *Firmicutes* decreased in NGPV-infected ducks, but ABX treatment mitigated this decrease (Figure 4C). We used the linear discriminant analysis effect size (**LEfSe**) to assess and compare the gut microbiota among the 4 groups to identify the specific microbiota associated with ABX treatment. The differential strains were mainly concentrated in the ABX group (Figure 4D). The G protein-coupled receptor 41(**GPR41**) and GPR43, are SCFA receptors, measured in the ileum, duodenum and bone marrow. The GPR41 in the ileum and bone marrow and the GPR43 in the duodenum and bone marrow were greater in the ABX group than the PC group (Figure 4E). However, there were no obvious differences between the PC group and the FMT group (Figure 4E). Furthermore, the decrease in SCFAs production due to NGPV infection, such as propionic acid and butyric acid, was increased by ABX treatment (Figure 4F). These results suggest that depletion in the gut microbiota via ABX contributes to an increase in SCFAs levels in NGPV-infected ducks.

Intestinal Microflora Depletion Enhances Intestinal Integrity in NGPV-Infected Ducks

Intestinal barrier integrity is associated with systemic inflammation. Therefore, we evaluated the effect of microflora depletion on the intestinal pathological features of NGPV-infected ducks. H&E staining revealed

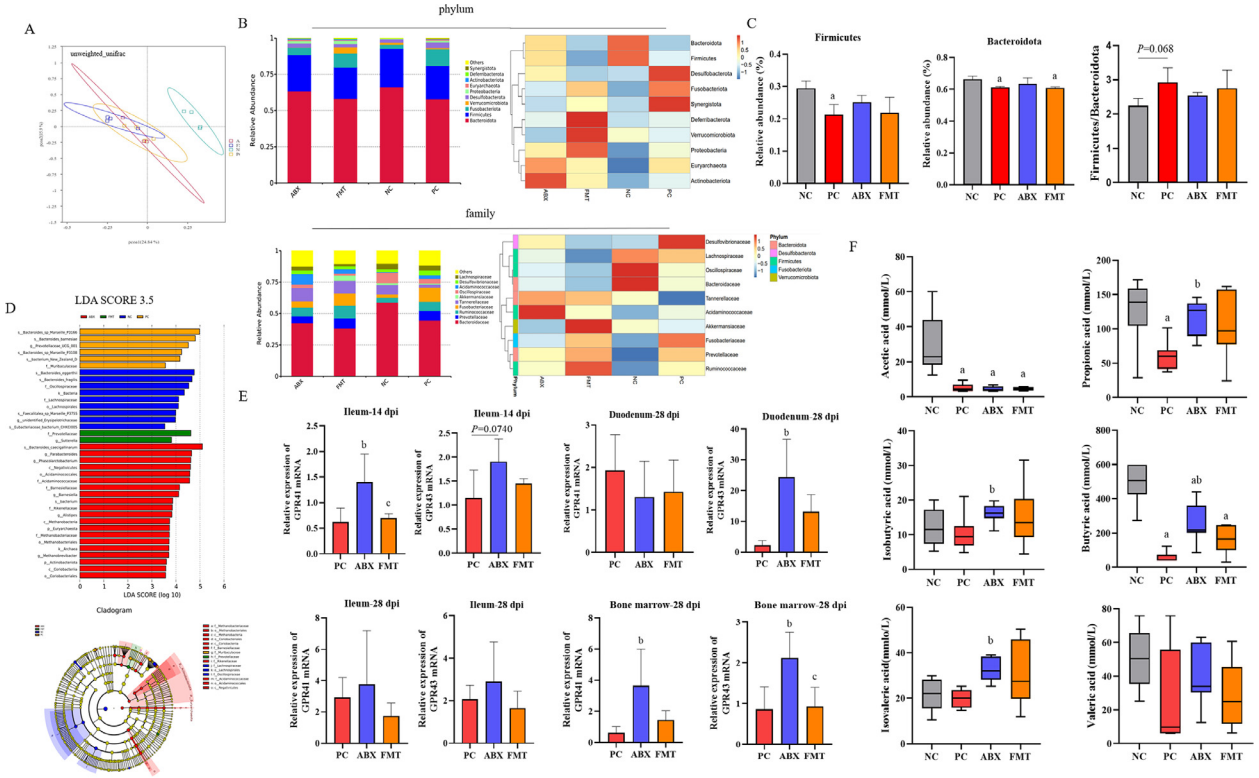

Figure 4. Intestinal microflora depletion caused changes in microbial composition and short-chain fatty acid. (A) PCoAs of beta diversity based on Bray−Curtis dissimilarities of bacterial operational taxonomic units. (B) Relative abundance of gut bacteria at the phylum and family levels and clustering heatmaps at the phylum and family levels. (C) *Firmicutes* and *Bacteroidota* relative abundance, and ratio of *Firmicutes* and *Bacteroidota*. (D) LEfSe analysis (LDA score>3.5). (E) SCFA receptor gene expression, including that of GPR41 and GPR43, in the ileum, duodenum and bone marrow. (F) SCFAs production in cecal contents. (a: compared with the NC group, $P < 0.05$; b: compared with the PC group, $P < 0.05$; c: compared with the ABX group, $P < 0.05$).

that NGPV infection resulted in an irregular arrangement of intestinal villi and epithelial cell shedding in the duodenum and ileum, while intestinal lesions were relieved in the ABX group (Figure 5A). Regarding intestinal integrity, there were differences between the NC, PC, ABX and FMT groups regarding villus length and crypt depth in the duodenum and ileum. For example, the length of villi in the duodenum and ileum decreased in NGPV-infected ducks, while that in the ABX group was significantly greater than that in the PC group. The ratio of villus length to crypt depth in the ABX group increased, while that in the FMT group was always similar to that in the PC group (Figure 5B). Moreover, gut microflora depletion significantly increased the mRNA levels of TJPs, such as Claudin-1, Occludin, zona occludens-1 (**ZO-1**), and mucin-2 (**MUC-2**), in the ileum to prevent increased permeability caused by NGPV infection (Figure 5C). Notably, FMT and PC groups have no difference. Overall, intestinal microflora depletion can enhance intestinal integrity in NGPV-infected ducks.

Intestinal Microflora Depletion Allevaties System Inflammatory Reactions in NGPV-Infected Ducks

Considering the crucial involvement of cytokines in the development of NGPV-induced bone loss, we further investigated the impact of intestinal microflora depletion on pro-inflammatory cytokine production. IL-1β, IL-6, and TNF-α mRNA levels in the ileum were increased in NGPV-infected ducks, however, microflora depletion decreased IL-6 and TNF-α mRNA levels in the ileum at 14 dpi, and IL-1β at 28 dpi (Figure 6A). In the duodenum, compared with the NC group, the TNF-α mRNA levels in the PC and FMT ducks were markedly increased, but in the ABX group, there was no increase compared with the NC group (Figure 6B). In addition, ABX treatment also reduced the significant increase in the serum IL-1β and TNF-α levels caused by NGPV infection at 28 dpi (Figure 6C). The IL-1β, IL-6, TNF-α, mucosa-associated lymphoid tissue lymphoma translocation protein 1 (***Malt1***), and nuclear factor κB (***NF-κB***) mRNA levels were increased in the bone marrow of NGPV-infected ducks were decreased by ABX treatment (Figures 6D and 6E). Notably, none of these proinflammatory cytokines were significantly different between the FMT group and the PC group. To acquire a more comprehensive understanding of the association between modified microorganism levels and inflammatory cytokine expression in bone marrow, we conducted a Pearson correlation analysis. Our results demonstrated a significant positive correlation between 4 bacterial phyla and pro-inflammatory factors (Figure 6F). These results indicated that gut microbial depletion can inhibit NGPV-induced systemic inflammation.

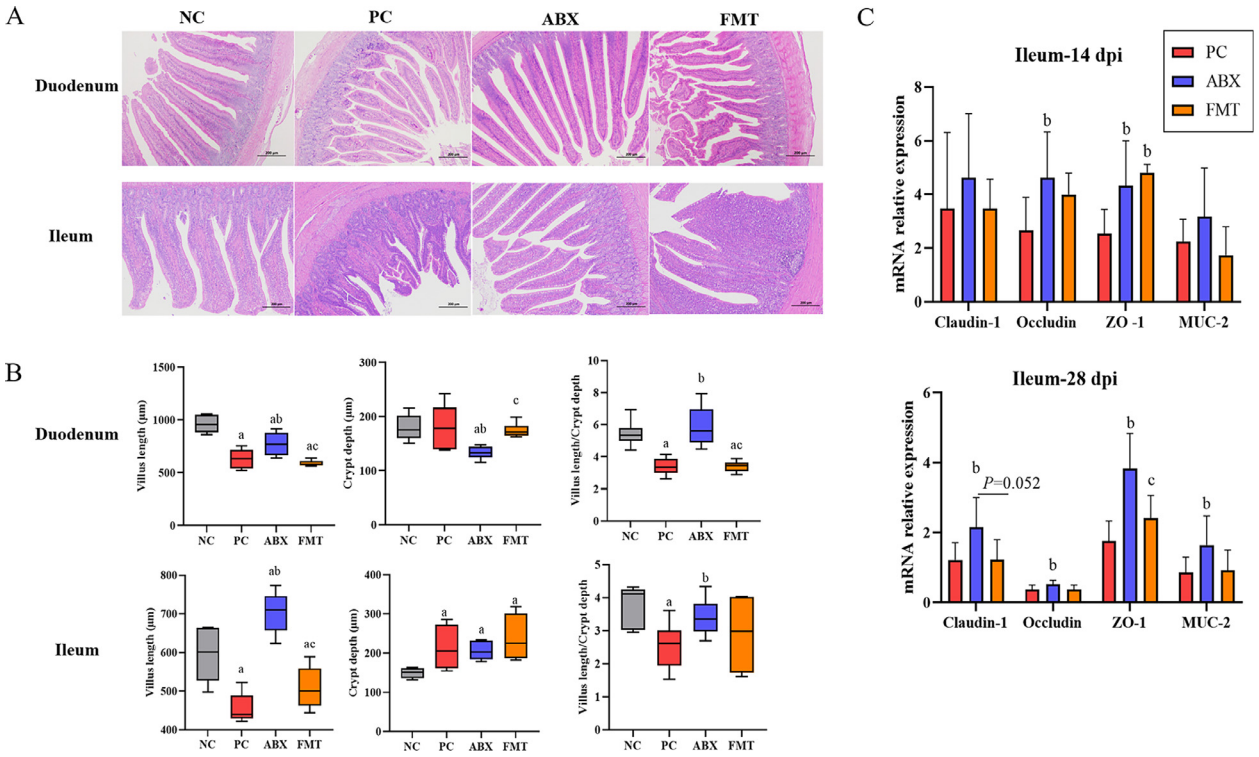

Figure 5. Effects of intestinal microflora depletion on the intestinal mucosal integrity of NGPV-infected ducks. (A) Morphology of the duodenum and ileum in ducks at the endpoint. (B) Villus length, crypt depth and the ratio of villus length to crypt depth in the duodenum and ileum. (C) The mRNA levels of TJPs, including Claudin-1, Occludin, ZO-1 and MUC-2, in the duodenum and ileum at 14 and 28 dpi. (a: compared with the NC group, $P < 0.05$; b: compared with the PC group, $P < 0.05$; c: compared with the ABX group, $P < 0.05$).

DISCUSSION

Intestinal microorganisms, referred to as "forgotten organs" or "virtual metabolic organs", modulate the immune response in mucosal tissues and regulate inflammation, which can be employed as a tactic to combat various pathogens (Derovs, et al., 2019). Intestinal microbiota manipulation by the consumption of antibiotics, changes in dietary habits, and the use of prebiotics and probiotics may affect bone health (D'Amelio and Sassi, 2018). Here, we report the ability of microflora depletion to alleviate SBDS development in NGPV-infected ducks, which is attributed to maintaining intestinal homeostasis, inhibiting inflammation, and suppressing bone resorption.

The NGPV exhibits a high level of pathogenicity in Cherry Vally ducks, resulting in the development of atrophic beaks in experimentally infected individuals (Luo et al., 2019). The growth retardation and skeletal dysplasia observed in these infected ducks are comparable to those observed in naturally affected ones (Chen et al., 2016; Ning et al., 2018). However, the mechanism of bone dysplasia caused by NGPV infection has not been elucidated. ALP and P1NP are used as markers for bone formation, CTx1 is a bone resorption marker that can sensitively measure bone damage, and together they are used as biochemical bone turnover markers for assessing bone turnover to reflect the status of bone health (Shankar and Hosking, 2006; Dharmapatni, et al., 2015). In our study, bone loss was observed in SBDS ducks, while there was no significant difference in P1NP, ALP, serum P, and Ca levels, but the number of osteoclasts and serum CTx1 was increased, indicating that bone loss in SBDS ducks was related to osteoclast differentiation and enhanced bone resorption, while, these bone loss can be mitigated by ABX. Furthermore, the differentiation of osteoclast is induced by RANKL binds to RANK, which is expressed on the surface of osteoclasts (Yamamoto et al., 2006), whereas, OPG, which is secreted by osteoblasts/stromal cells, is an important receptor antagonist of RANKL, effectively inhibiting its interaction with RANK and thereby decreasing the formation of osteoclasts (Boyce and Xing, 2007). In our study, increased RANKL expression and increased RANKL/OPG ratios in NGPV-infected ducks were reduced by ABX treatment, which suggests that gut microflora depletion can reduce NGPV-induced osteoclast differentiation. Following previous studies, manipulating the gut microbiota via oral antibiotics promotes bone regeneration (Yan and Charles, 2017). Similar to other studies, we found that gut microflora depletion mitigates NGPV-induced bone damage by reducing osteoclastic bone resorption, which could be the key to mitigating the SBDS process.

In addition to bone abnormalities, NGPV infection can target the intestinal tract, cause gut microbiota dysbiosis and decrease SCFA production (Luo et al., 2019). These results were also observed in our study. The intestinal microbiota plays a crucial role in maintaining bone health, influencing the growth and decline of skeletal structure during postnatal development (Contino et al., 2024). For example, the depletion of gut microbiota was

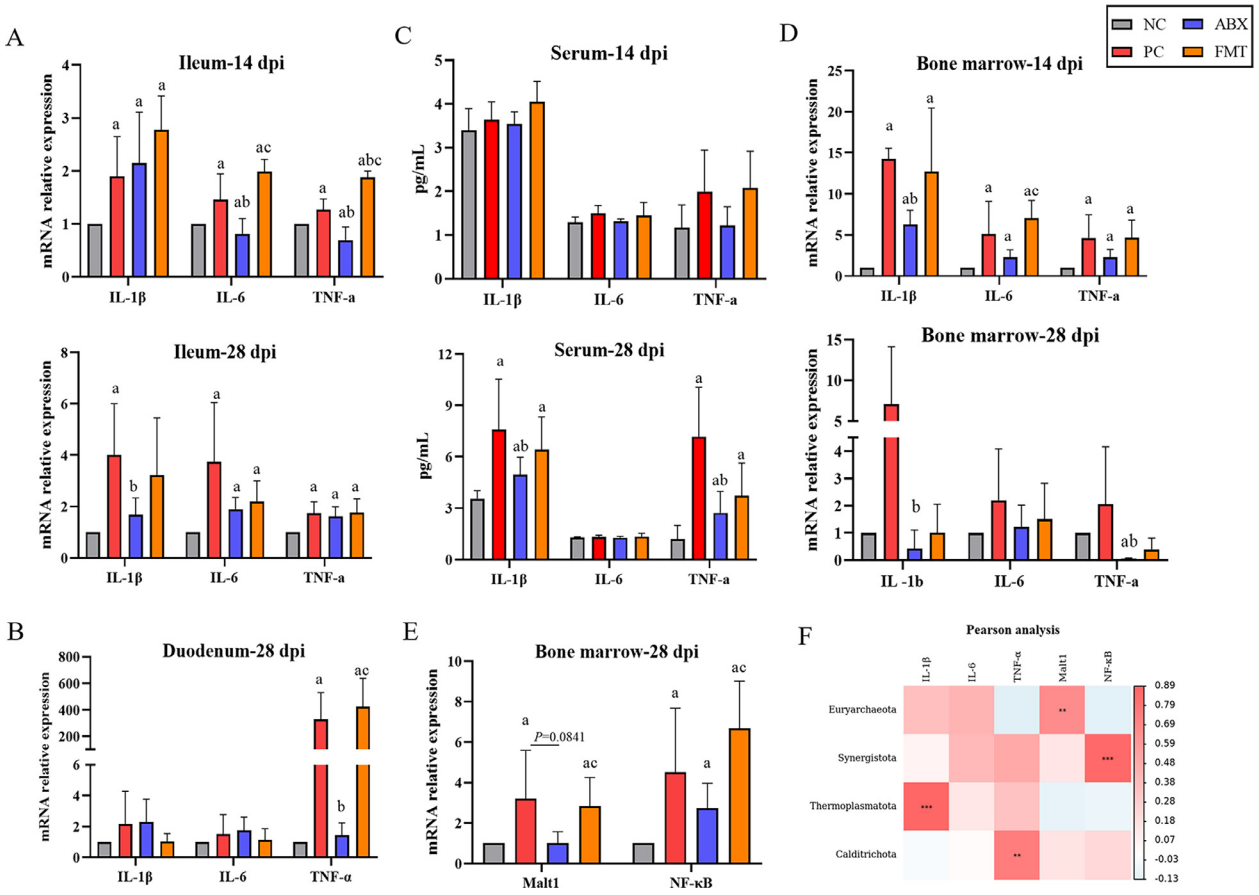

Figure 6. Effect of intestinal microflora depletion on systemic inflammation in NGPV-infected ducks. (A) Relative mRNA expression of IL-1β, IL-6 and TNF-α in the ileum at 14 and 28 dpi. (B) The relative mRNA expression of IL-1β, IL-6 and TNF-α in the duodenum at 28 dpi. (C) The levels of IL-1β, IL-6 and TNF-α in serum from different groups at 14 and 28 dpi. (D) The relative mRNA expression of IL-1β, IL-6 and TNF-α in the bone marrow at 14 and 28 dpi. (E) The relative mRNA expression of *Malt1* and *NF-κB* in the bone marrow at 14 dpi. (F) Correlations between the relative abundance of intestinal bacterial genera and the mRNA levels of IL-1β, IL-6, TNF-α, *Malt1* and *NF-κB* in the bone marrow. (a: compared with the NC group, $P < 0.05$; b: compared with the PC group, $P < 0.05$; c: compared with the ABX group, $P < 0.05$. Pearson analysis: $^{**}P < 0.01$; $^{***}P < 0.001$).

found to have a protective effect on bone loss and cartilage degradation in individuals with osteoporosis and osteoarthritis (Yin et al., 2024). And a decrease in the *Firmicutes*/*Bacteroidetes* ratio was found to be associated with the decreased bone loss observed in mice (Schepper et al., 2019). The *Firmicutes*/*Bacteroidetes* ratio in our study increased in the NGPV-infected group and decreased in the ABX group, which supports the concept that NGPV infection-induced intestinal microbial disturbance promoted bone loss. In general, we observed a greater standard deviation than that observed in other experiments. However, this was expected, as farm-provided meat ducks show high donor variability.

Recently, the gut microbiota has been discovered to generate significant regulatory metabolites known as SCFAs through the process of fermenting complex carbohydrates (Wang et al., 2023). SCFAs, specifically butyrate, are important to promote effective bone regeneration by regulating crucial cells engaged in the process of fracture healing (Wallimann et al., 2021). In our study, early microbial consumption was found to enhance the production of SCFAs in the cecum and increase SCFA receptor expression in bone marrow during the later stage of NGPV infection, suggesting that intestinal microbiota modulate NGPV-induced bone loss potentially through SCFAs. Furthermore, intestinal microbiota and SCFAs are involved in numerous chronic inflammatory diseases and act as mediators in the gut-bone signaling axis (Tousen et al., 2019; Langan et al., 2021). Their ability to ameliorate inflammation and mitigate damage to the intestinal barrier has been widely reported (Gasaly et al., 2021; Zhu et al., 2022; Guo et al., 2023). GPR41 and GPR43 are SCFA receptors, which can be activated by SCFA to regulate immune responses. For example, actication of GPR43 exerts anti-inflammatory effects in the colon (Singh et al., 2014). Therefore, we assessed the influence of intestinal depletion on both immune status and integrity of the intestinal barrier. As anticipated, the impairment of the intestinal barrier caused by NGPV is consistent with previous findings in ducks (Luo et al., 2021), and was reversed by microflora depletion, as indicated by intestinal pathological examination and upregulated mRNA levels of TJPs. When gut integrity is impaired, bacteria and their components possess the capability to translocate across the intestinal barrier, eliciting systemic inflammation (Bianchi, 2010). Furthermore, the

expression of RANK and RANKL is modulated by inflammatory mediators and their associated peptides, which also exert an impact on osteoblasts, osteoclasts, and various other immune cells (Epsley et al., 2021). In our study, microflora depletion can inhibit the systemic inflammation caused by NGPV infection in ducks. Therefore, dysregulation of intestinal microflora is the important factor leading to systemic inflammation in SBDS ducks, moreover, the inflammation may potentially aggravate bone resorption.

Malt1, as a signaling protein within cells, plays an important role in both innate and adaptive immune responses (Afonina et al., 2015). *Malt1* can act as a scaffold to activate *NF-κB* signaling, a key regulator of inflammatory cytokine secretion. The proteolytic activity of *Malt1* also governs the regulation of gene expression in myeloid cells (Liu et al., 2016; Zhang et al., 2022a). The paracaspase activity of *Malt1* facilitated *NF-κB* activation by cleaving substrates, which have previously been implicated in osteoclastogenesis (Matmati et al., 2011). Therefore, *Malt1/NF-κB* can induce inflammation and the consequent promotion of osteoclastogenesis. In our study, the depletion of microflora reduced *Malt1/NF-κB* expression in bone marrow, potentially contributing to the alleviation of inflammation and bone loss. However, whether *Malt1/NF-κB* induced inflammation is involved in bone resorption in SBDS ducks needs to be further investigated.

CONCLUSIONS

Our study showed that NGPV-induced intestinal microflora disturbance promotes the SBDS process. Intestinal microflora depletion alleviates the development of SBDS by, maintaining intestinal homeostasis, inhibiting systemic inflammation, and reducing bone resorption in meat ducks. Our findings support the notion that intervention strategies targeting the intestinal microbiota hold significant value, suggesting that microbial products serve as a potential adjunctive therapy for SBDS in ducks.

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Author Contributions: The work was mainly conceived and designed by Mandi Liu, Mandi Liu performed the experiments; Mandi Liu, Yongzhi Xue, Maoyuan Sun and Fengjun Xiang collected and analyzed experimental data; Kuan Zhao, Wuchao Zhang, Baishi Lei, Chuanchuan Shang, Yibin Hu contributed material and analysis tools; Limin Li supervised and reviewed the experiments; the manuscript was mainly written by Mandi Liu and revised by Limin Li and Wanzhe Yuan. All authors read and approved the manuscript.

Ethical Statement: Animals were used following the Laboratory Animal Guidelines for Ethical Review of Animal Welfare in China (GB/T 42011-2022) and approved by the Animal Welfare and Ethics Committee at the Laboratory animal center of Hebei Agricultural University (2023006). All husbandry practices and euthanasia were performed with full consideration of animal welfare.

DISCLOSURES

The authors declare no conflicts of interest.

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