104 (2025) 105189

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj

Music intervention mitigates LPS-induced gut barrier disruption and immune stress in broilers via TLR4/NF-κB regulation

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ARTICLE INFO

Keywords:
Lipopolysaccharide
Music-enriched environment
Intestinal barrier function
Inflammation
TLR4/NF-4 κB signaling pathway

ABSTRACT

Immune stress induced by harsh environment in intensive farming can impair broiler intestinal health. Although music as an environmental intervention can alleviate short-term stress injury, its long-term regulatory mechanism on intestinal inflammation has not been clarified. In this study, we investigated the effects of a music-enriched environment on growth performance, intestinal barrier function, and inflammatory responses in lipopolysaccharide (LPS)-induced immunostressed broilers. AA broilers were randomly divided into four groups: control group (**CON**), music-enriched environment group (**MUC**), LPS-induced immune stress group (**LPS**) and music-enriched environment + LPS group (**MUC+LPS**). On the 14th, 16th and 18th days, the LPS and MUC+LPS groups were injected intraperitoneally with 500 μg of LPS to construct an immune stress model, and the CON and MUC groups were injected with an equal amount of saline. On day 28, the birds were sacrificed to detect the indicators associated with intestinal barrier and inflammation. The LPS group showed a significant decrease in performance from 14 to 28 days, with elevated serum levels of CORT, ACTH, DAO, and D-LA, and a decrease in the activity of intestinal mucosal SOD/GSH-Px, and impaired gut morphology. impaired; music remission significantly alleviated the decline in production performance, reduced the levels of stress hormones and markers of intestinal barrier damage, while elevating jejuno-ileal GSH-Px activity and improving intestinal morphology. Significant inflammatory gene expression characteristics were observed in jejunum and ileum tissues after LPS injection: upregulation of TLR4, NF-κB, TNF-α, IL-1β, and IL-6, and significant suppression of jejunal IL-10 expression. Notably, IL-10 and IFN-γ expression in the ileum did not show statistical differences. Inflammation-related gene expression showed an overall down-regulation trend after the music intervention, but was still significantly different from the control group. Music intervention on the regulation of jejunal MYD88 and ileal TNF-α - the LPS group did not show statistically significant differences in the expression of these two key inflammatory nodes with the LPS+MUS group. Mechanistic studies have shown that LPS triggers an oxidative stress cascade through activation of the TLR4/NF-κB signaling axis, leading to disruption of intestinal barrier integrity. In contrast, music exposure exerts a protective effect through a dual mechanism: on the one hand, it helps to enhance the expression of the tight junction protein ZO-1/Occludin to repair the physical barrier; on the other hand, it inhibits the activation of the TLR4/NF-κB pathway, which can effectively alleviate LPS-induced immunopathological damage.

Introduction

Under the intensive farming model, broilers are placed in a high-density rearing environment and at the same time are faced with the market demand for continuous increase in production, and this dual pressure significantly exacerbates the physiological and psychological stress responses of broilers. Specifically, the limited activity space not only restricts broilers' natural behavioral expressions, such as foraging and walking, but also leads to a decline in air quality and drastic fluctuations in temperature and humidity in the house, creating multiple

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https://doi.org/10.1016/j.psj.2025.105189
Received 11 November 2024; Accepted 18 April 2025
Available online 22 April 2025

environmental stressors. In addition, nutrient-enhanced feeds designed for rapid growth accelerate the development process of broilers, but may exceed their physiological regulatory capacity, triggering intestinal metabolic burdens and digestive system dysfunction (Nasr et al., 2021; Sugiharto, 2022). In poultry production, the health of the chicken's intestinal tract is directly related to the overall production performance of the flock (Yegani and Korver, 2008; Yu et al., 2023).The chicken intestine is the largest immune organ in the body, capable of producing a significant amount of antibodies (Cardoso et al., 2023). The intestinal tract contains numerous immune cells and immune factors that can recognize and eliminate harmful substances, thereby protecting the organism from disease (Kurashima et al., 2013; Peterson and Artis, 2014; Zundler et al., 2023). A balanced intestinal micro-ecosystem is crucial for maintaining the health of chickens, as it helps them resist pathogen invasion and promotes the absorption and utilization of nutrients (Bindari and Gerber, 2022). Broilers in intensive farming environments are challenged by multiple stressors: high stocking densities lead to significant increases in house temperatures, exacerbating the heat stress response; dense accumulation of excreta increases the concentration of ammonia and other noxious gases, directly stimulating the respiratory system and weakening the immune barrier; and at the same time, imbalances in the environmental microbial community abnormally increase the abundance of pro-inflammatory pathogens, which further induces the risk of inflammatory diseases of the intestinal tract (Liu et al., 2020; Zhou et al., 2021). When intestinal barrier function is compromised, the resulting inflammatory response not only exacerbates intestinal damage but may also affect the function of other organs through the circulatory system (Khoshbin and Camilleri, 2020), potentially leading to systemic inflammatory response syndrome or multiple organ dysfunction syndrome (Baue et al., 1998). Adding antibiotics to animal feed can help reduce stress responses caused by adverse environmental factors. However, the extensive use of antibiotics may lead to antibiotic residue issues, further endangering human and animal health (Vidovic and Vidovic, 2020; Chen et al., 2021). In addition, as an innovative environmental enrichment strategy, music therapy has shown unique value in intensive farming systems. Studies have shown that music interventions with specific frequencies and rhythms can effectively regulate the physiological rhythms of animals and reduce the levels of stress hormones triggered by high-density feeding and human handling. For example, in broiler farming, playing soothing classical music or natural rhythms not only slows down the heart rate and stabilizes the respiratory rhythm, but also promotes feed intake and digestive efficiency, creating a virtuous growth cycle (Ciborowska et al., 2021). This non-invasive intervention compensates for the monotony of sensory stimulation in intensive environments by simulating natural soundscapes, which helps animals to restore their psychological balance and reduce the occurrence of abnormal behaviors. Long-term implementation of music therapy can significantly improve animal welfare, reduce disease susceptibility and antibiotic use, and provide a scientific and humanistic management solution for the sustainable development of intensive farming (Gustavson et al., 2021; Fu et al., 2023).

Music is a universal language, and music therapy offers broad applicability with advantages such as low cost, no side effects, and ease of use. Although still a subject of debate, many studies suggest that music may help alleviate stress responses (Ciborowska et al., 2021; Marchetto et al., 2021; Snowdon, 2021). In humans, music is commonly employed to improve well-being, alleviate stress, and divert attention from uncomfortable symptoms. (Linnemann et al., 2015; Ginsberg et al., 2022). In recent years, researchers have explored the effects of music on the physiology and behavior of various animals, finding that music can reduce anxiety, stress, and aggressive behaviors by masking potential background noise and providing auditory enrichment, thereby improving animal health and welfare (Amaya et al., 2020; Lindig et al., 2020). Similarly, music enrichment is often incorporated in recirculating aquaculture systems for fish and in the milk production process in automated dairy systems (Guh et al., 2021; Zhao et al., 2023). Additionally, music interventions have been shown to enhance immune function and influence neuroendocrine responses (Pant et al., 2022). Previous research in our lab has shown that extended exposure to classical music can increase positive emotional expressions in piglets and raise levels of immunoglobulin G (**IgG**), interleukin-4 (**IL-4**), and interferon-gamma (**IFN-γ**), thus improving their immune status (Nian et al., 2023). Moreover, our recent findings indicate that long-term music exposure effectively alleviates inflammation in immune organs induced by acute noise stress (Wang et al., 2024). However, whether music can mitigate LPS-induced immune stress has not yet been reported.

Mucosal barrier disruption due to intestinal diseases is a major constraint in the poultry industry, and the increased permeability it triggers not only results in decreased efficiency of nutrient absorption, but also threatens systemic health through enterogenic routes of infection (Wickramasuriya et al., 2022). As a major virulence factor of Gram-negative bacteria, LPS plays a key role in the development of intestinal diseases, and it triggers an inflammatory cascade by activating the TLR4/NF-κB signaling axis, which is a core mechanism of immune injury in sensitive intestinal segments such as jejunum and ileum (Yang et al., 2021;Cardoso et al., 2023). Although the centrality of the chicken intestinal mucosal immune system in the animal immune defense network has been widely recognized, there is still a paucity of research on the mitigating effects and mechanisms of music intervention on intestinal stress injury. In view of the close association between barrier function damage due to intestinal stress and reduced poultry performance, it is of great scientific value to explore the regulatory mechanisms of music intervention on intestinal inflammation. In the present study, we selected Mozart K448 music with specific frequency patterns, and systematically investigated the regulatory effects of this music intervention on the integrity of the chicken intestinal barrier, the expression profiles of inflammatory factors, and the TLR4/NF-κB signaling pathway by constructing an LPS-induced immune stress model. This study not only provides an experimental basis for the development of environmentally friendly intestinal health regulation programs but also reveals the potential of interdisciplinary application of music intervention in enhancing animal welfare under intensive farming conditions, and opens up innovative pathways for stress relief.

Materials and methods

The experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University in Heilongjiang Province, China (Approval No. NEAUEC20210234) (Chang et al., 2024).

Grouping and handling of test animals

A total of 192 male AA broilers with similar body weights (36 ± 0.5 g) were randomly assigned to four groups, each with 6 replicates of 8 chickens, for a 28-day feeding trial. The experiment was conducted in four adjacent, environmentally controlled rooms with identical rearing conditions. Throughout the experiment, the broilers' drinking water was supplied by a running water supply system to ensure the freshness and cleanliness of the drinking water, and at the same time to promote the frequency of drinking and intake of the broilers. The feed ratios followed a strict scientific formula, and the specific ingredients and ratios are shown in the attached **STable 1**. The room temperature was set at 35°C for days 1-3, 32-33°C for days 4-7, and then gradually decreased by 2-3°C every three days until it stabilized at 20°C, where it was maintained for the remainder of the experiment (**STable 2**). The relative humidity was kept at 50 %. Each room was equipped with two light fixtures to ensure uniform light distribution across the entire chicken cage, and each room was connected to a dimmer and timer. There was no sound interference between the rooms, and the noise level in the environmentally controlled rooms was kept below 45 dB(TASI, Jiangsu, China).

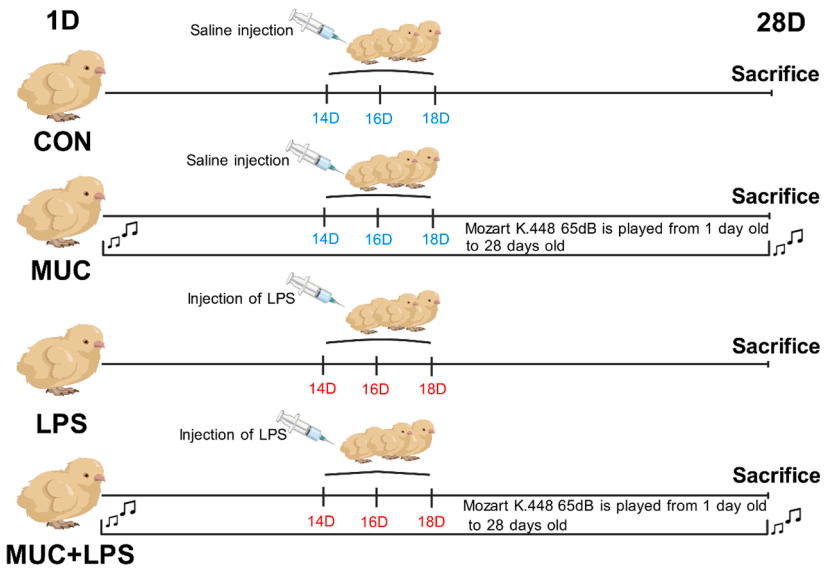

Fig. 1. Processing of experimental broilers and flowchart of sample collection times.

The MUS group and the MUS+LPS group were exposed to 6 h of music stimulation (Mozart K.448) daily (from 10:00 to 16:00) starting from day 1, with the sound level controlled between 65 and 75 dB, until the end of the trial on day 28. The positions of the music players and the chickens were kept constant throughout the experiment. On days 14, 16, and 18, the LPS immune stress group and the MUS+LPS group received an intraperitoneal injection of 500 μg/kg LPS dissolved in saline to induce an immune stress model (Rajput et al., 2013), while the control group and the music stimulation group received an equal volume of saline. The specific experimental treatments are illustrated in Fig. 1. During the experiment, the positions of the audio players and the chickens remained unchanged. The LPS solution was prepared by diluting LPS crystals (Solarbio, Beijing) (Escherichia coli serotype O55: B5) in saline, with the dosage and administration route based on the previous study by Rajput et al.

Growth performance measurements

At days 0, 14, and 28 of the experiment, broilers in each replicate were weighed after a 12-hour fasting period, and the feed intake of each replicate was recorded on days 14 and 28 to calculate the average daily gain (**ADG**), average daily feed intake (**ADFI),** and feed conversion ratio (**F/G**) for each period. Mortality was recorded daily, and performance parameters were subsequently adjusted for mortality.

Ultrastructural analysis of the intestinal tract

Scanning Electron Microscopy (**SEM**): Segments of the jejunum and ileum, washed with physiological saline, were prepared for observation. The tissue samples were cut to a thickness of 2-3 mm, a length of 3-4 mm, and a width of 2 mm, with orientation specified as the villous surface being the top (positive side) and the serosal surface being the bottom (negative side). Each group consisted of at least three replicates, with 5-6 tissue samples per replicate. The samples were fixed in 2.5 % glutaraldehyde for 2-4 h, then rinsed three times with 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 15 min each time. The samples were then fixed in 1 % osmium tetroxide at room temperature in the dark for 1-2 h, followed by another three rinses with 0.1 M PBS (pH 7.4) for 15 min each. The tissues were dehydrated through an ethanol gradient of 30 %, 50 %, 70 %, 85 %, 95 %, and 100 % (twice), with each step lasting 15 min. The samples were then immersed in a 1:1 mixture of isoamyl acetate and ethanol for 10 min, followed by immersion in pure isoamyl acetate for another 10 min. The samples were transferred to a sample basket and placed in a pre-cooled critical point dryer, where liquid carbon dioxide was introduced. The temperature was raised to 15°C and maintained for 10 min, then further increased to 35°C to allow for CO_2 gasification. After venting, the samples were removed and mounted on sample stubs using conductive adhesive. The samples were coated using an ion sputter coater and stored in a desiccator at room temperature. Images were acquired using a scanning electron microscope. The instruments and reagents used in the SEM procedure are detailed in **STable 3 and 4**.

Transmission Electron Microscopy (**TEM**): Fresh jejunum and ileum tissues were collected and cut into small pieces of approximately 1 mm^3. The tissue samples were fixed in 2.5 % glutaraldehyde at 4°C for 2-4 h. The samples were then placed in 1 % osmium tetroxide at 4°C for 1 h. Following fixation, the samples were dehydrated through a graded ethanol series of 30 %, 50 %, 70 %, 85 %, 95 %, and 100 % (twice), with each step lasting 15 min. The samples were then infiltrated overnight with a mixture of acetone and embedding medium in equal proportions. The samples were embedded in embedding molds and placed in a 60°C oven for 48 h to allow complete resin polymerization. Ultrathin sections of 70 nm thickness were obtained from the desired regions and collected onto copper grids. The sections were stained with 3 % uranyl acetate in saturated ethanol for 8 min, followed by three washes in 70 % ethanol and three washes in ultrapure water. The sections were then stained with 2.7 % lead citrate solution for 8 min, followed by three washes in ultrapure water and slight blotting with filter paper. Observations and image acquisition were conducted using a transmission electron microscope. The equipment and reagents used for the TEM procedure were referenced from a previous study (Wang et al., 2024).

Antioxidant activity assay

To further assess the antioxidant activity of the jejunum and ileum, the samples were placed in grinding tubes, and the intestinal tissues were homogenized and centrifuged. The assay kits were sourced from Nanjing Jiancheng, and the experimental procedures were strictly conducted according to the manufacturer's instructions. Approximately 0.3 g of mucosal tissue from the jejunum and ileum were collected on ice and homogenized with 9 mL of 0.9 % sodium chloride solution, followed by centrifugation at 4°C (3,000 rpm, 15 min) to obtain the supernatant. Protein concentration was determined using the BCA method. The activities of superoxide dismutase (**SOD**) and glutathione peroxidase (**GSH-PX**), as well as the malondialdehyde (**MDA**) content, in the jejunal and ileal mucosal homogenate supernatants were measured using

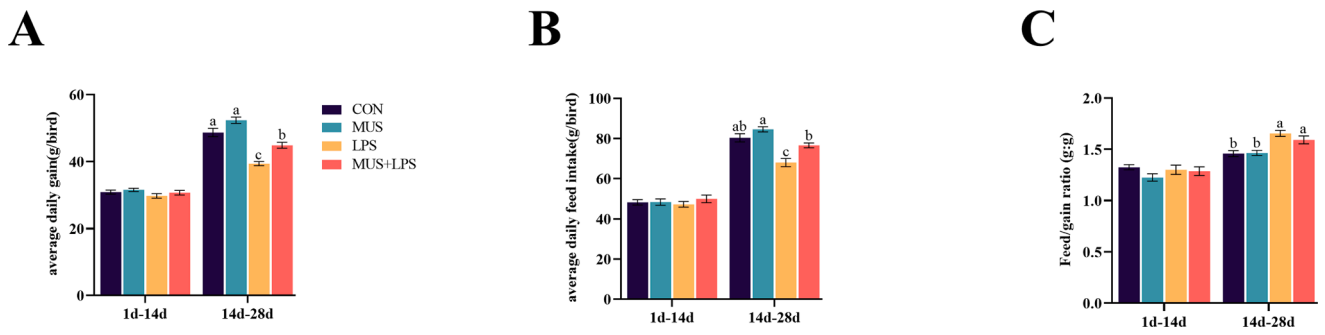

Fig. 2. Long-term musical mitigation of lipopolysaccharide attack on broiler growth performance. (A) 1d-14d, 15d-28d average daily gain (ADG) of broilers. (B) 1d-14d, 15d-28d average daily feed intake (ADFI) of broilers. Bars without the same letter differed significantly ($P < 0.05$).

commercial kits (Nanjing Jiancheng, Nanjing, China). All procedures were performed according to the manufacturer's instructions. The biochemical data were normalized to the protein content of the homogenates.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of Corticosteroids (**CORT**), Adrenocorticotropic Hormone (**ACTH**), Diamine Oxidase (**DAO**), and D-lactic acid (**D-LA**), as well as Interleukin-1β (**IL-1β**), Interleukin-6 (**IL-6**), and Interleukin-10 (**IL-10**) concentrations in the jejunal and ileal mucosal tissues, were measured using commercial ELISA kits (Huijia Biotechnology, Xiamen, Fujian, China). All procedures were carried out according to the manufacturer's instructions.

Gene expression using quantitative real-time PCR

A 0.1 g sample of jejunal and ileal mucosal tissue was weighed using an analytical balance and placed into a 1.5 mL RNase-free grinding tube. Subsequently, 1 mL of RNAiso PLUS reagent (TaKaRa, Dalian, China) and two grinding beads were added. The tube was gently shaken to ensure the sample was fully submerged, and the tissue was then thoroughly ground using a pre-cooled cryogenic grinder. Next, 200 μL of chloroform was added to the tube, which was then manually shaken to mix the contents and incubated at 4°C for 5 min. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C. Afterwards, 400 μL of the supernatant was transferred to a new RNase-free 1.5 mL EP tube, combined with 400 μL of isopropanol, and incubated at 4°C for 10 min. The mixture was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was discarded, and the pellet was washed three times with 75 % ethanol (Wang et al., 2024). The sample was centrifuged at 12,000 rpm for 5 min, resulting in a white precipitate at the bottom of the tube. The supernatant was discarded, and the pellet was dissolved in 50 μL of DEPC-treated water. RNA concentration and purity were determined using a UV spectrophotometer (Eppendorf, Germany) by measuring the OD 260/280 ratio ($1.8 < \text{ratio} < 2.2$). The RNA was then adjusted to the same concentration for subsequent reverse transcription. cDNA was synthesized using the M5 Sprint qPCR RT kit (Toyobo, Japan) according to the manufacturer's instructions. The synthesized cDNA was diluted fivefold with DEPC-treated water for use. Specific primers for both the reference gene and target genes were designed using Primer Premier 6.0 (Premier Biosoft International, USA) and synthesized by Sangon Biotech (Shanghai, China). The primer sequences used for gene detection are provided in Supplementary **STable 5**. Real-time quantitative PCR was conducted using the LightCycler® 96 system (Roche, Switzerland). The PCR conditions were as follows: initial denaturation at 95°C for 60 s, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All PCR reactions were performed in triplicate, with β-actin serving as the internal reference control. The expression levels of the target genes were normalized using the $2^{-\Delta\Delta CT}$ method.

Western blot

A 100 mg sample of intestinal mucosa was weighed from a −80°C freezer and homogenized in 1 mL of Western and IP lysis buffer (containing PMSF). The homogenate was then centrifuged at 4°C for 15 min, and 200 μL of the supernatant was collected. The protein concentration of the sample was determined using a BCA Protein Assay Kit (Beyotime, China) according to the manufacturer's instructions. A 10 % separating gel and a 5 % stacking gel were prepared and poured sequentially. The marker (3 μL) and total protein samples were loaded, followed by electrophoresis at 100 V for 30 min in the stacking gel and 120 V for 90 min in the separating gel. The proteins were transferred to a PVDF membrane at 200 mA for 90 min. The membrane was then blocked in 5 % skim milk at 37°C for 2 h. After blocking, the membrane was incubated with diluted primary antibodies against TLR4, IKB-α, NF-κB, and TNF-α (1:1000, Abmart, China) at 4°C for 12 h. The specific antibodies used are listed in **STable 6**. The membrane was washed three times with TBST for 5 min each, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, Bioss Antibodies) at room temperature for 1 h. The signal was detected using an enhanced chemiluminescence kit (Beyotime, China), and the protein bands were visualized with a grayscale scanner (G: Box Chemi-XX, Cambridge, UK). The intensity of the protein bands was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, MD), and the protein expression levels were quantified as the ratio of the optical density of each target protein to that of β-actin.

Immunofluorescence staining

The collected jejunum and ileum tissues were successively subjected to fixation, dehydration, and embedding. Sections were cut to a thickness of 5-8 mm, followed by antigen retrieval. To prevent nonspecific binding, the sections were blocked with a buffer containing serum or bovine serum albumin (BSA). Immunofluorescence staining was performed with single staining, where each slide was incubated with only one primary antibody, with at least three replicates per group. The primary antibodies used for incubation included claudin-2 (1:800), occludin (1:800), Mucin (1:500), and ZO-1 (1:500) (Proteintech, China). The sections were incubated overnight at 4°C, and unbound primary antibodies were washed off thoroughly with PBS. This was followed by a one-hour incubation with a secondary antibody. DAPI was used for nuclear staining, and the sections were examined microscopically. Grayscale images from the red fluorescence monochrome photographs were analyzed using Image-Pro Plus 6.0 software. The integrated optical density (IOD) and pixel area (AREA) of each positive image were measured, and the average density (IOD/AREA) was calculated.

Statistical analysis

The data obtained from this experiment were initially organized

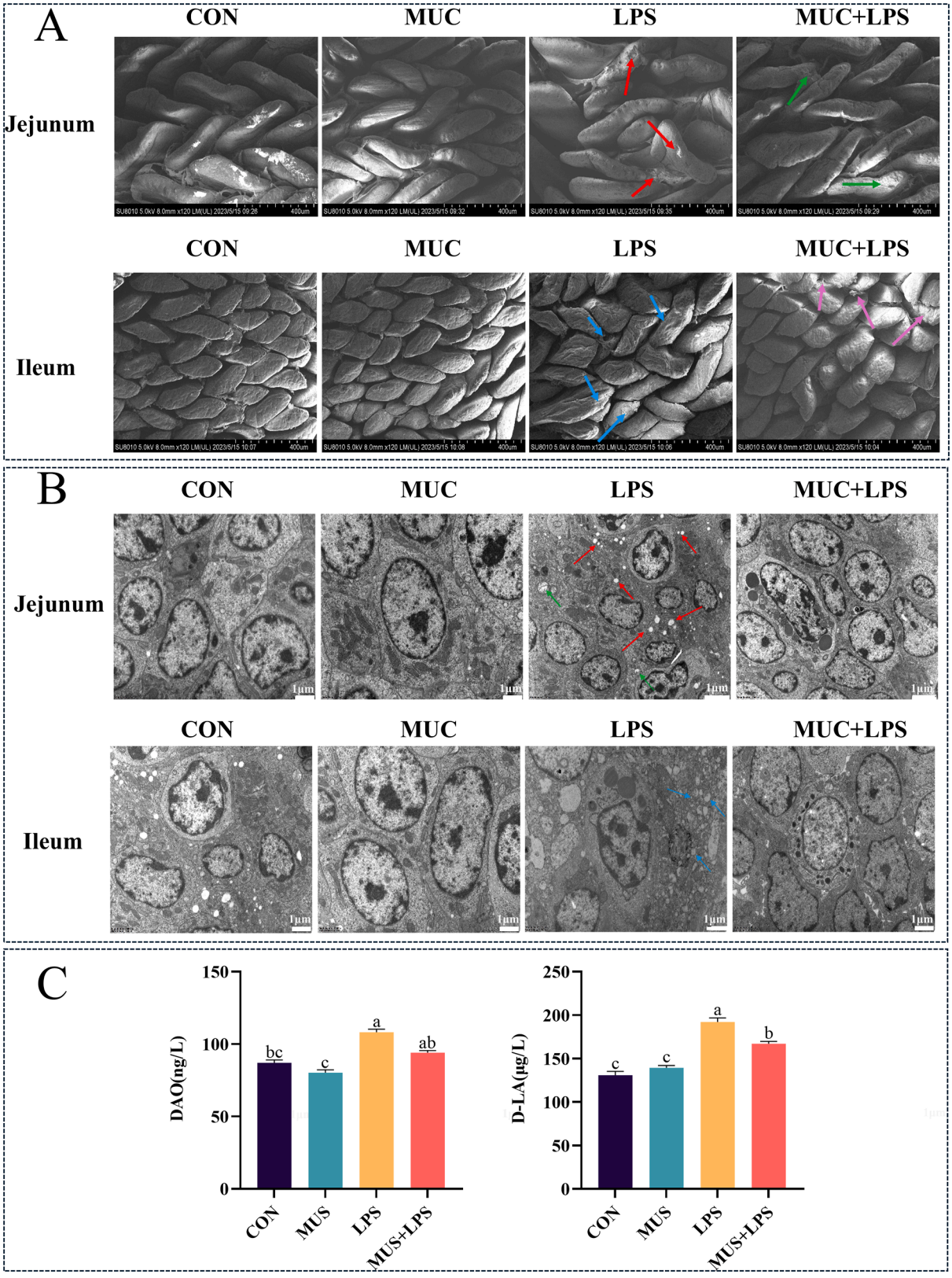

Fig. 3. (A) Scanning electron microscopy (SEM) images of the jejunum and ileum (SU8010, 5.0 kV, 8.0 mm, ×120 LMlUL, 400 μm). In the LPS group for the jejunum, the injury sites are marked with red arrows. In the MUS+LPS group, the injury sites are indicated with green arrows. For the ileum, injury sites in the LPS group are marked with blue arrows, while in the MUC+LPS group, they are marked with purple arrows. (B) Transmission electron microscopy (TEM) results for the jejunum and ileum (magnification: ×15,000). In the LPS group for the jejunum, vacuoles are indicated by red arrows, and mitochondrial cristae disruption is marked with green arrows. In the LPS group for the ileum, mitochondrial damage is marked with blue arrows. (C) The serum levels of DAO and D-LA in broilers. Differences marked with lowercase letters denote a significant difference ($P < 0.05$), while the same letter indicates no significant difference ($P > 0.05$).

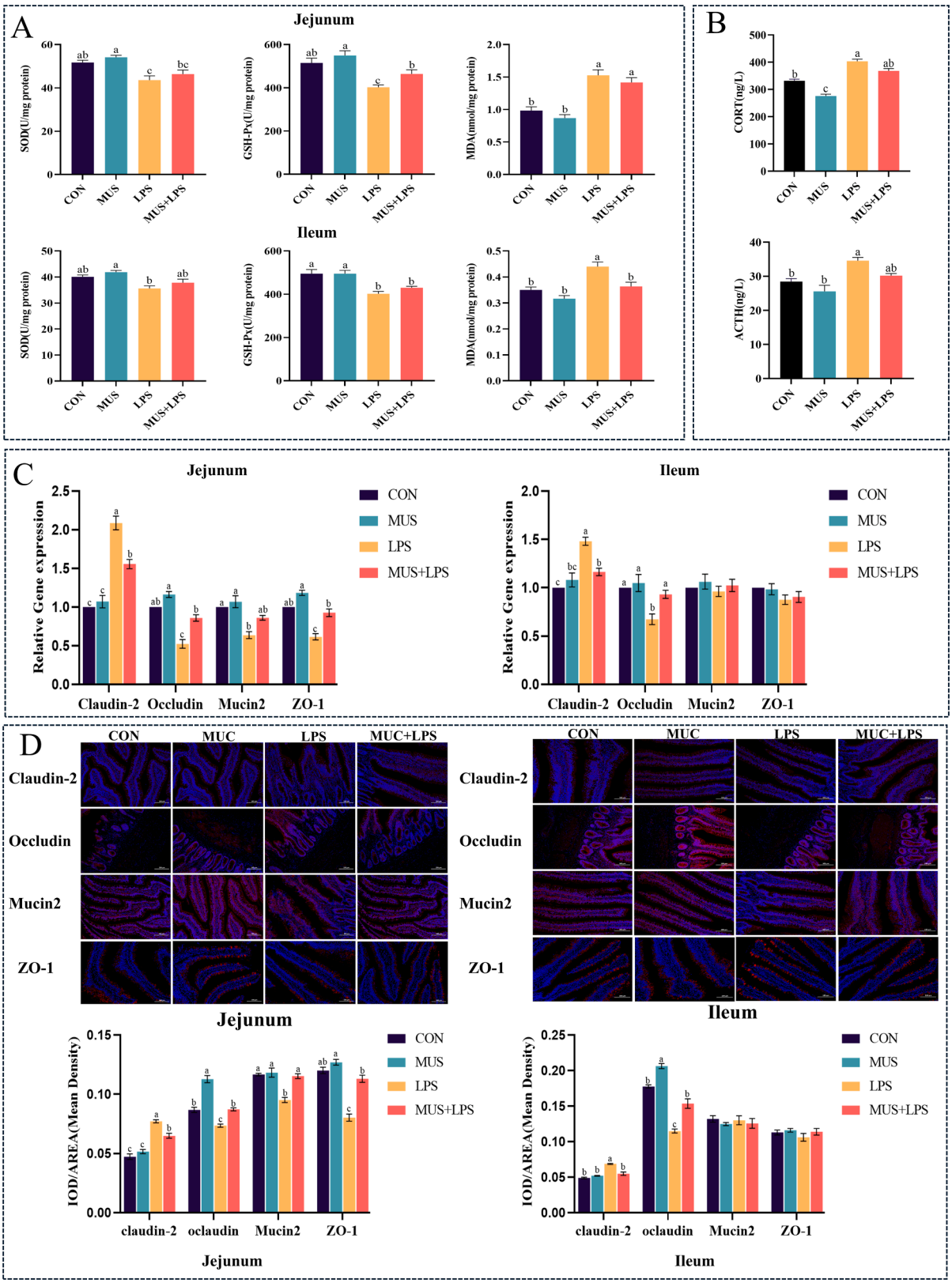

Fig. 4. The Effects of Long-term Music Exposure on Oxidative Stress, Intestinal Barrier, and Intestinal Permeability in Broiler Chickens Injected with Lipopolysaccharide (LPS). (A) SOD activity, GSH-Px activity, and MDA content. (B) Concentrations of CORT and ACTH. (C) Relative mRNA expression of tight junction proteins (Claudin 2, Occludin, ZO-1) and mucin protein Mucin 2 in the jejunal and ileal mucosa of broiler chickens. (D) Immunofluorescence images of tight junction proteins (Claudin 2, Occludin, ZO-1) and mucin protein Mucin 2 in the jejunal and ileal mucosa of broiler chickens. The statistical results of the mean optical density of tight junction proteins (Claudin 2, Occludin, ZO-1) and mucin protein Mucin 2 in the jejunal and ileal mucosa are shown below the immunofluorescence images. Differences marked with lowercase letters denote a significant difference ($P < 0.05$), while the same letter indicates no significant difference ($P > 0.05$).

using Excel 2021 and then analyzed using SPSS 21.0 (IBM, USA). Normality of the data was first assessed using the Kolmogorov-Smirnov test, and non-parametric tests were applied to data that did not follow a normal distribution. Production performance indicators, ELISA results, small intestine morphology statistics, qRT-PCR data, Western blot data, and oxidative stress indicators were all analyzed using one-way ANOVA, followed by multiple comparisons using Duncan's method.

Results

Effect of LPS-induced immune stress in chickens on their growth performance

The growth performance results of broilers from day 1 to 14 and day 15 to 28 are shown in Fig. 2. Before LPS stimulation (days 1-14), there were no significant differences in average daily gain (**ADG**), average daily feed intake (**ADFI**), or feed conversion ratio (**FCR**) among the treatment groups ($P > 0.05$). After LPS stimulation, compared to the control group, the LPS group exhibited significantly reduced ADFI and ADG, and a significantly increased FCR ($P < 0.05$). After prolonged music intervention, the MUS+LPS group showed a significant increase in ADG and ADFI compared to the LPS group ($P < 0.05$), although there was no significant difference in FCR ($P > 0.05$). Compared to the control group, the MUS+LPS group still showed significant differences in ADG and ADFI ($P < 0.05$), but not in FCR ($P > 0.05$). From days 15 to 28, the MUS group exhibited a trend toward higher ADG, ADFI, and FCR compared to the control group, although these differences were not statistically significant ($P > 0.05$).

Effect of long-term musical relief of LPS on histological and structural changes in the jejuno-ileum

The SEM results for the jejunum and ileum tissues of the broilers are presented in Fig. 3A. In the jejunum and ileum, the intestinal villi in the control and MUS groups appeared finger-like, arranged fairly neatly and densely. In the jejunum of the control group, the villi exhibited minor damage, while in the jejunum of the MUS group, as well as in the ileum of both the control and MUS groups, the villi surfaces were relatively smooth, with most showing no detachment or damage. In the jejunum of the LPS group, the villi were irregularly distributed, with noticeable detachment, uneven surfaces, and a reduced number of villi. In the MUS+LPS group, the villi were more orderly arranged, but overall appeared loosely arranged, with a small portion showing detachment. In the ileum, the LPS group exhibited sparse villi with localized disorganization and significant surface damage. In the MUS+LPS group, the villi were sparse and, compared to the LPS group, the surfaces were smoother, with no damage but some detachment. The TEM results showing the cellular morphology of the jejunum and ileum tissues in broilers are presented in Fig. 3B. In the jejunum, the control and MUS groups had well-defined cell boundaries, with tight cell connections and abundant mitochondria around the cells, characterized by dense and intact mitochondrial cristae. In the LPS group, nuclear membrane condensation, large intercellular spaces, visible vacuolation, and fractured mitochondrial cristae were observed, with significant chromatin condensation. In the MUS+LPS group, some cell membranes remained relatively intact, but the number of mitochondria was reduced. In the ileum, the control and MUS groups showed uniform electron density with no significant pathological changes. The nuclear membranes were smooth, chromatin was evenly distributed within the nuclei, and mitochondrial structures were intact with clear cristae. In the LPS group, fractured cristae, some mitochondrial vacuolization, and a significant reduction in mitochondrial volume were observed. In the MUS+LPS group, although distinct cristae fractures were still visible, the severity of the damage was alleviated compared to the LPS group.

Serum diamine oxidase (DAO) and D-lactic acid (D-LA) levels in broilers

When the intestinal mucosal barrier function was impaired, DAO was released into the blood. Similarly, when the intestinal barrier function was lost, permeability increased, and large amounts of D-lactic acid were produced by intestinal bacteria. Therefore, it was crucial to measure the levels of DAO and D-LA in the serum. As shown in Fig. 3C, compared to the control group, the serum levels of DAO and D-LA were significantly increased in the LPS-induced stress group ($P < 0.05$). After music intervention, the levels of DAO and D-LA in the MUC+LPS group showed a significant downward trend compared to the LPS group ($P < 0.05$). Compared to the control group, the D-LA level in the MUC group showed no significant difference ($P > 0.05$), while the DAO level presented a significant downward trend ($P < 0.05$). These research results suggested that LPS injection could induce changes in intestinal tissue permeability, and music intervention could effectively alleviate the LPS-induced changes in intestinal permeability.

Effect of LPS on antioxidant activity of broiler jejuno-ileal tissue

Oxidative stress can induce inflammation and promote the investigation of potential mechanisms in animal models. To further explore the role of LPS in inducing oxidative stress related to inflammation, oxidative stress-related parameters were evaluated to determine whether LPS-induced oxidative stress in the jejunum and ileum could be alleviated by music (Fig. 4A). In the jejunum, compared to the control group, the LPS group showed a significant downregulation in SOD and GSH-Px activities ($P < 0.05$), while MDA levels were significantly upregulated ($P < 0.05$). After prolonged music intervention, compared to the LPS group, the MUS+LPS group showed no significant difference in SOD activity ($P > 0.05$), a significant upregulation in GSH-Px activity ($P < 0.05$), and no significant difference in MDA levels ($P > 0.05$). In the ileum, compared to the control group, the LPS group showed no significant difference in SOD activity ($P > 0.05$), a significant downregulation in GSH-Px activity ($P < 0.05$), and a significant upregulation in MDA levels ($P < 0.05$). After music intervention, compared to the LPS group, the MUS+LPS group showed no significant difference in SOD activity ($P > 0.05$), but MDA levels were significantly downregulated ($P < 0.05$).

Based on the detailed analysis of these oxidative stress markers, it was clearly demonstrated that LPS significantly induces oxidative stress in the jejunum and ileum. However, when evaluating the effects of long-term music therapy in alleviating LPS-induced oxidative stress, the results showed that its mitigation effects were relatively limited, with only general improvement observed. The activation level of ACTH directly influences CORT secretion, which in turn indirectly affects intestinal oxidative stress levels. Accordingly, levels of CORT and ACTH were assessed. The findings revealed that the MUS group presented significantly reduced CORT levels compared to the control group ($P < 0.05$). In contrast, the LPS group demonstrated a notable increase in CORT levels relative to the control ($P < 0.05$). After music intervention, no significant difference was observed between the MUS+LPS group and the LPS group ($P > 0.05$). ACTH levels were significantly elevated in the LPS group compared to the control group ($P < 0.05$). Following music intervention, the MUS+LPS group did not show any significant differences relative to the other three groups ($P > 0.05$). These findings indicate that LPS triggers stress responses in the body. Under such stress conditions, ACTH and CORT collaboratively influence physiological processes, potentially regulating intestinal oxidative stress by affecting the integrity of the intestinal barrier.

Effects of LPS and musical stimulation on the expression of genes and proteins related to tight junction formation

Tight junction proteins played a crucial role in maintaining the integrity and function of various physiological barriers. Therefore,

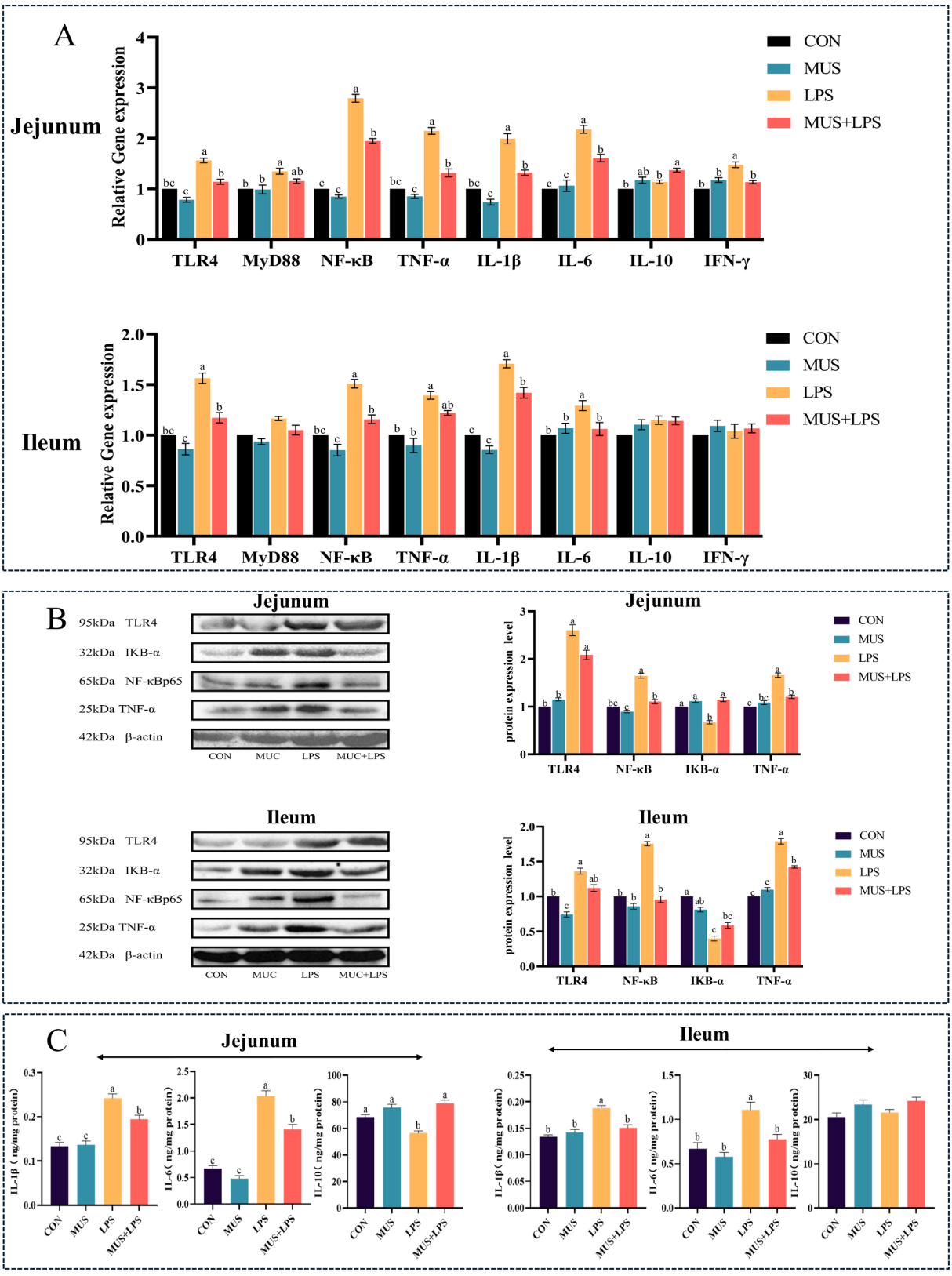

Fig. 5. Music has potential anti-inflammatory effects and can effectively alleviate inflammation in the jejunum and ileum by inhibiting the LPS-triggered TLR4/NF-κB signaling pathway. (A) The figure shows the relative mRNA expression levels of the TLR4/NF-κB inflammatory signaling pathway and related inflammatory factors in the jejunum and ileum of broilers. (B) The figure presents the immunoblot images and corresponding statistical results of TLR4, IKB-α, NF-κB p65, and TNF-α proteins in the jejunum and ileum of broilers. (C) The figure shows the measurement results of IL-1β, IL-6, and IL-10 levels in the jejunum and ileum of broilers across different groups. Differences marked with lowercase letters denote a significant difference ($P < 0.05$), while the same letter indicates no significant difference ($P > 0.05$).

quantitative real-time PCR and immunofluorescence were used to examine the effects on the expression of genes and proteins related to tight junction formation (Fig. 4C and D).

As shown in Fig. 4C and D, LPS induction significantly disrupted the intestinal barrier integrity in the jejunum and ileum. In the jejunum, LPS injection resulted in a significant down-regulation of mRNA expression of the tight junction proteins occludin, mucin2, and ZO-1, whereas the mRNA expression of claudin-2 was significantly up-regulated ($P < 0.05$), suggesting an increase in barrier permeability. Music intervention effectively mitigated this trend: in the MUC+LPS group in the jejunum, the mRNA and protein expression levels of occludin, mucin2, and ZO-1 were significantly up-regulated compared with those in the LPS group, whereas claudin-2 expression was significantly suppressed ($P < 0.05$), suggesting a restoration of barrier function. In the ileum, LPS similarly significantly upregulated the mRNA expression of claudin-2 and downregulated the mRNA expression of occludin ($P < 0.05$). Music intervention significantly reversed this effect, with mRNA expression of claudin-2 significantly reduced and mRNA expression of ocludin significantly increased in the MUS+LPS group ($P < 0.05$). In conclusion, music effectively alleviated LPS-induced intestinal barrier dysfunction by bi-directionally regulating the expression of key tight junction proteins, reflecting its protective effect on intestinal barrier function.

TLR4/NF-κB inflammatory signalling pathway assay results

As shown in Fig. 5A, compared to the control group, the mRNA expression levels of TLR4, MYD88, NF-κB, TNF-α, IL-1β, IL-6, and IFN-γ in the jejunum, and TLR4, NF-κB, TNF-α, IL-1β, and IL-6 in the ileum were significantly upregulated in the LPS-induced stress group ($P < 0.05$). Compared to the LPS-induced stress group, the mRNA expression levels of TLR4, NF-κB, TNF-α, IL-1β, IL-6, and IFN-γ were significantly downregulated ($P < 0.05$), while the mRNA expression level of IL-10 was significantly upregulated in the MUC+LPS group in the jejunum ($P < 0.05$). In the ileum, the expression levels of TLR4, NF-κB, IL-1β, and IL-6 were significantly downregulated in the MUC+LPS group compared to the LPS-induced stress group ($P < 0.05$).

As shown in Fig. 5B, compared to the control group, the protein expression levels of TLR4, NF-κB, and TNF-α were significantly increased ($P < 0.05$), while the protein expression level of IKB was significantly decreased in the LPS-induced stress group in both the jejunum and ileum ($P < 0.05$). Compared to the LPS-induced stress group, the protein expression levels of NF-κB and TNF-α in the jejunum and ileum were significantly reduced in the MUC+LPS group, meanwhile, the protein expression level of IKB-α in the jejunum was significantly increased. ($P < 0.05$).

As shown in Fig. 5C, compared to the control group, the levels of IL-1β and IL-6 in the jejunal and ileal mucosa were significantly increased, while the expression level of IL-10 in the jejunum was significantly decreased in the LPS-induced stress group ($P < 0.05$). Compared to the LPS-induced stress group, the levels of IL-1β and IL-6 in the jejunum and ileum were significantly decreased, and the level of IL-10 in the jejunum was significantly increase under the music environment in the LPS-challenged chickens ($P < 0.05$).The protein expression results were consistent with the gene expression findings. These experimental results indicated that LPS induced the activation of the NF-κB signaling pathway, and music stimulation could suppress the NF-κB signaling pathway.

Discussion

LPS, an endogenous pyrogen, can bind to the lipopolysaccharide receptor complex on the cell membrane, promoting the secretion of proinflammatory cytokines by inflammatory cells and eliciting a robust immune response (Gauthier et al., 2022). Exposure to LPS has been shown to cause damage to tight junctions and thinning of the mucosal layer in the small intestine of poultry, leading to oxidative stress and decreased production performance (Wang et al., 2022; Feng et al., 2023). For instance, intraperitoneal injection of LPS (500 mg/kg body weight) in broiler chickens on days 16, 18, and 20 resulted in significantly reduced ADG, ADFI, and increased FCR from day 16 to day 21. Similarly, studies have found that LPS at a dose of 500 mg/kg significantly impaired the body weight gain of broilers from 14 to 15 days and 17-19 days of age, accompanied by reduced feed intake during these critical growth periods (14-15 days, 15-17 days, and 17-19 days) (Tan et al., 2023). The results of the present study are highly consistent with previous studies, both of which indicated that LPS injection had a significant inhibitory effect on broiler growth performance. Specifically, a significant decrease in ADG and ADFI and a significant increase in FCR were observed after intraperitoneal injection of 500 mg/kg LPS to broilers from 14 to 28 days of age. These finding further confirmed that the negative impact of LPS-induced inflammatory response on broiler performance, and provided an important experimental basis for further exploring the mechanisms linking inflammatory stress and growth performance. Similarly, these results indicate successful modeling of immune stress that can be used in subsequent studies on the health effects of music on broiler chickens. The impairment of LPS on broiler growth performance can be attributed to immune stress-induced energy and nutrient depletion, which interferes with their normal growth and development. Studies have shown that upbeat music can have a positive impact on animal appetite and feeding behavior (Spence, 2012). In this study, we found that music intervention was effective in mitigating LPS-induced decreases in ADG and ADFI in broilers, but did not fully restore them to normal levels. Notably, music did not significantly ameliorate the LPS-induced elevation of FCR. This finding is more consistent with previous findings that musical stimulation partially counteracts the negative effects of immune stress on feeding behavior by inducing a positive emotional state that enhances the animal's pleasure and overall preference for food (Mathiesen et al., 2020). Although music shows some potential to improve broiler welfare, its restorative effect on growth performance remains limited, suggesting that a combination of multidimensional intervention strategies will be needed to fully optimize stress management in the future.

The comprehensive development of the gastrointestinal tract in broilers plays a crucial and decisive role in their subsequent digestive and absorptive capacity (Ravindran and Abdollahi, 2021). The small intestine, as the core component of the digestive and absorption process, is an indispensable prerequisite for the efficient digestion and absorption of nutrients to promote optimal growth in broilers, as a healthy and structurally intact intestinal mucosa is essential (Montoro-Huguet et al., 2021). In this study, we confirmed the negative impact of LPS stimulation on the morphological structure of the intestine. Scanning electron microscopy observations showed that compared to the LPS-induced immune stress group, the MUS+LPS group had longer and more organized villi in the jejunum and ileum, indicating better development. Ultra structural observations revealed that the control, MUS, and MUS+LPS groups had well-preserved cell nuclei, prominent nucleoli, and clear cell boundaries, while the LPS group showed marginalization of nucleoli and the presence of obvious vacuoles in the cytoplasm of the jejunum and ileum tissues. Therefore, the music-enriched environment alleviated the damage to the intestinal morphological structure caused by LPS-induced immune stress in broilers. DAO is a highly active intracellular enzyme secreted by intestinal epithelial cells (Kettner et al., 2022). In some cases, the necrosis and shedding of intestinal mucosal cells into the intestinal lumen can lead to increased DAO activity in the lumen (Hou et al., 2012). DAO can also enter the intercellular spaces, lymph, and bloodstream of the mucosal barrier, resulting in a significant increase in plasma DAO levels (Ji et al., 2013). D-LA is the end product of intestinal bacterial metabolism, and it can enter the bloodstream through the intestinal epithelium when the intestinal barrier is compromised, leading to an increase in its blood concentration (Ma et al., 2022). Therefore, changes in plasma DAO and D-LA levels are ideal indicators of intestinal permeability and mucosal function damage

(Ouyang et al., 2023). This study found that LPS stimulation significantly increased the serum levels of DAO and D-LA in broilers, indirectly indicating that LPS can severely damage the intestinal mucosal structure and barrier function. Compared to the LPS group, the MUC+LPS group showed significantly lower serum levels of DAO and D-LA, suggesting that the music environment can to some extent improve the damage to intestinal mucosal structure and barrier function caused by LPS stimulation. These observations indicate that long-term exposure to music can improve the digestive and absorptive function of the intestine, thereby enhancing nutrient absorption and improving growth performance.

Aerobic metabolism, which is essential for the survival of animal cells, generates reactive oxygen species (**ROS**), primarily free radicals such as superoxide and hydroxyl radicals (Checa and Aran, 2020). An imbalance between free radical production and endogenous antioxidant defenses in cells or tissues can lead to oxidative stress, which can cause biological damage and various physiological disorders, and is closely related to the persistence of inflammatory responses and intestinal health (Vona et al., 2021; Sahoo et al., 2023). We evaluated indicators related to oxidative stress. Our results showed that LPS could significantly induce an oxidative stress response in broilers, as evidenced by decreased activities of SOD and GSH-Px, and increased MDA content in the jejunal and ileal mucosa. In contrast, broilers in the music environment had increased activities of SOD and GSH-Px, and decreased MDA content in the jejunal and ileal mucosa. The excessive production of free radicals is a major triggering factor for the activation of inflammatory pathways (Di Meo and Venditti, 2020; Unsal et al., 2021); therefore, we hypothesize that classical music may interfere with free radical generation by enhancing the activities of antioxidant enzymes and attenuating lipid peroxidation in the intestine of broilers, thereby exerting its antioxidant potential. These research findings are consistent with previous studies, indicating that long-term exposure to music has a mitigating effect on oxidative stress (Mishra and Jha, 2019; Zhang et al., 2023).

Maintaining and optimizing intestinal barrier function is of great importance for the health of broilers (Zhu et al., 2021). The intestinal epithelium forms physical and chemical barriers to prevent the dissemination of antigens, such as pathogens, from the intestinal lumen to the mucosal tissues (Gierynska et al., 2022). The lipopolysaccharide expressed on the outer membrane of Gram-negative bacteria can activate Mucin2 expression by activating Toll-like receptors (Burgueno and Abreu, 2020), and Mucin2-deficient mice have been observed to develop spontaneous colitis and increased susceptibility to inflammation-induced colorectal cancer (Tadesse et al., 2017; Gundamaraju and Chong, 2021). Tight junctions are multi-protein complexes located at the apical end of epithelial cell lateral membranes, and the claudin and occludin families, as well as intracellular plaque proteins such as zonula ZO, are important components of the tight junction structure (Zihni et al., 2016). Our results showed that LPS stimulation significantly decreased the mRNA and protein expression of Mucin2 in the jejunum. Studies have suggested that LPS-induced oxidative stress and apoptosis of goblet cells may impair Mucin2 expression and ultimately disrupt the intestinal mucus barrier. In contrast, this study found that the music environment increased the protein expression of Mucin2 in the jejunum of broilers under LPS-induced immune stress, indicating that the music environment can improve the LPS-induced damage to intestinal barrier function, which may be related to the ability of the music environment to increase the activity of antioxidant enzymes, alleviate oxidative stress and apoptosis of goblet cells, and restore the expression of mucins. Furthermore, LPS stimulation disrupted the mRNA and protein expression of tight junction structural proteins. The expression of tight junction proteins plays a critical role in maintaining the integrity of the intestinal barrier and ensuring normal barrier function. The upregulation of Claudin-2 protein expression has been recognized as an important factor in inflammatory bowel disease. Wang et al. found that the mRNA expression of the tight junction proteins Occludin and ZO-2 in the jejunum of LPS-treated broilers was decreased (Wang et al., 2014). In a study of the effect of pathogenic Escherichia coli on the interaction and localization of tight junction proteins, Michelle et al. found structural alterations in the Z0-1, Occludin and Claudin-1 proteins with concomitant loss of barrier function in human intestinal epithelial cells (T84) infected with pathogenic Escherichia coli (Luo et al., 2012). Compared to the LPS-induced immune stress group, the music stimulation was beneficial in restoring the protein and mRNA expression levels of Occludin and ZO-1 in the intestinal mucosa of broilers. This suggests that the music-enriched environment has a protective effect against the LPS-induced disruption of intestinal barrier function, and an intact intestinal barrier can prevent harmful substances in the intestinal lumen from entering the systemic circulation, which is supported by the decreased serum levels of DAO and D-LA in broilers under the music environment. These results further support the notion that the music environment can alleviate the LPS-induced impairment of intestinal barrier function in broilers.

Impairment of the intestinal barrier is often triggered by pathogens or toxins, and this process is commonly accompanied by significant inflammation (Awad et al., 2017). LPS, as a key ligand of TLR4, plays a central role in initiating the NF-κB-related inflammatory cascade reaction (Tong et al., 2020). Specifically, when LPS binds to the extracellular domain of TLR4, this interaction triggers a series of complex intracellular signal transduction cascade reactions (Ciesielska et al., 2021). Our results showed that LPS stimulation upregulated the mRNA expression of TLR4, MyD88, TRAF6, NF-κB p65, TNF-α, IL-1β, and IL-6, and downregulated the mRNA expression of IKB in the jejunal and ileal mucosa, leading to the further dissociation of IKB from NF-κB. In contrast, music stimulation downregulated the mRNA expression of TLR4, MyD88, and TRAF6, and upregulated the mRNA expression of IKBα, thereby suppressing the excessive mRNA expression of NF-κB p65. This indicates that the music environment can play a positive role in reducing the excessive production of proinflammatory cytokines. To further verify the observed effects of music stimulation on the regulation of the TLR4/NF-κB signaling pathway and the downstream inflammatory response, we detected the protein expression of TLR4, NF-κB p65 subunit, IKB, and TNF-α in the jejunal and ileal mucosa. The results showed that the music environment could inhibit the LPS-induced upregulation of TLR4 expression, nuclear translocation of NF-κB p65, degradation of IKB protein, and the release of inflammatory factors such as TNF-α, IL-1β, and IL-6. Although music can effectively reduce the intestinal damage caused by LPS, no reversal of the injury was observed. These findings indicate that music can regulate the expression of inflammatory factors by modulating the NF-κB signaling pathway, although its effect on alleviating LPS-induced intestinal inflammation is limited. Unfortunately, the spatial constraints of the laboratory prevented us from simulating large-scale farming environments. Furthermore, this study only focused on how music acts on the NF-κB signaling pathway after LPS exposure to regulate intestinal inflammation levels, and the regulatory effects of music on other pathways remain unclear. In the future, our goal is to further investigate whether LPS exposure induces oxidative stress to modulate other pathways, such as the PLC-PKC and MAPK signaling pathways. We hope to elucidate the complex relationship between LPS-induced stress and the immune response in poultry, as well as the positive regulatory role of music in this process, providing a solid scientific foundation for optimizing farming environments, reducing stress responses, and improving overall flock health and production performance.

Conclusion

LPS stimulation increased the intestinal permeability, induced intestinal inflammation, and disrupted the integrity of the intestinal barrier in broilers. In contrast, the music-enriched environment alleviated the negative effects of LPS-induced immune stress in broilers. The higher anti-inflammatory and antioxidant activities observed in broilers raised in the music-enriched environment were associated with the

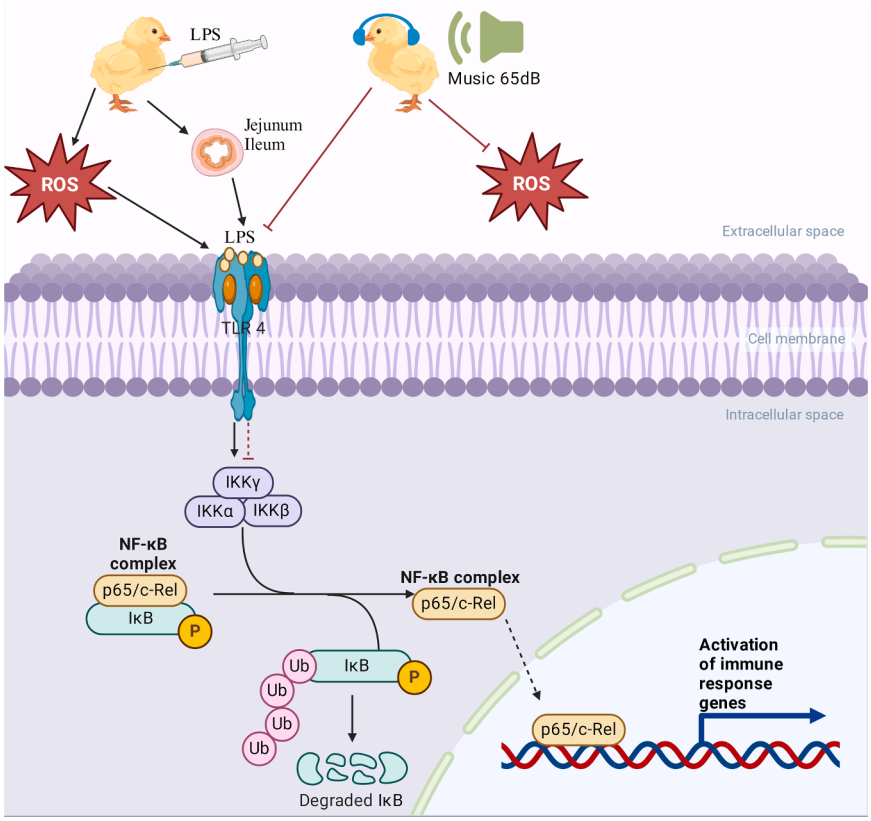

Fig. 6. Prolonged exposure to a music-enriched environment attenuates LPS-induced intestinal inflammation in chickens by modulating the oxidative-antioxidative response and the TLR4/NF-κB signaling pathway in the gut.

suppression of the TLR4/NF-κB signaling pathway and the downstream inflammatory cytokines (Fig. 6). Our study suggests that incorporating music as an environmental enrichment strategy can support the healthy development and enhance the welfare of broilers.

Funding

The study was supported by the National Natural Science Foundation of China (Grant No. 32172788, 31772645).

Declaration of competing interest

The authors confirm that there are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105189.

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