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Capsaicin Alleviates Ankylosing Spondylitis in Mice: A Study on the NLRP3/Caspase-1/GSDMD Pyroptosis Pathway and Intestinal Barrier Repair

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Background: Cell pyroptosis and dysbiosis of the gut microbiota are closely related to the pathogenesis of Ankylosing Spondylitis (AS). Capsaicin, an active component in chili peppers, has demonstrated anti-inflammatory and antioxidant potential. This study aims to explore the therapeutic effects of capsaicin on a mouse model of AS and its underlying mechanisms.
Methods: The AS mouse model was established and divided into control, AS model, capsaicin-treated, and sulfasalazine (positive control drug) treated groups. Cytokines in serum were detected by Enzyme-Linked Immunosorbent Assays (ELISA). The activation status of pyroptosis-related proteins and the nuclear factor kappa-B (NF-κB) pathway in spinal joint tissues were analyzed by Western blot. The function of the intestinal mucosal barrier was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Additionally, the composition of the gut microbiota was analyzed.
Results: Capsaicin inhibited tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-17A in the serum of AS mice and increased IL-10 ($p < 0.01$). In the spinal joint tissues, capsaicin effectively inhibited pyroptosis-related proteins such as NOD-like receptor thermal protein domain associated protein 3 (NLRP3), caspase-1, and Gasdermin D (GSDMD) ($p < 0.01$), thus reducing cell pyroptosis. Furthermore, capsaicin inhibited the activation of the NF-κB pathway ($p < 0.01$), improved the function of the intestinal mucosal barrier, increased levels of beneficial probiotics such as *Lactobacillus* and *Bifidobacterium* ($p < 0.001$), and decreased levels of harmful bacteria including *Enterococcus faecalis* ($p < 0.05$) and *Escherichia coli* ($p < 0.001$).
Conclusion: This study confirms that capsaicin alleviates inflammation and pathological damage in AS mice by inhibiting the pyroptosis pathway, repairing the intestinal barrier, and regulating the composition of the gut microbiota.

Keywords: capsaicin; Ankylosing Spondylitis; NLRP3 inflammasome; intestinal barrier; gut microbiota

Introduction

While the precise cause of Ankylosing Spondylitis (AS) is not fully understood, growing evidence indicates that an unhealthy gut microbiome is strongly linked to the onset of AS [1,2]. Additionally, damage to the intestinal barrier is considered one of the key factors in the pathogenesis of AS [3–5]. Thus, finding therapeutic strategies that can simultaneously repair the intestinal barrier, modulate the gut microbiota, and alleviate inflammation is of significant importance for the treatment of AS [6–8].

The NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome is a group of proteins capable of detecting pathogens and stress signals, triggering caspase-1 activation, and consequently leading to the development and secretion of inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [9,10]. In recent years, the role of the NLRP3 inflammasome in autoimmune diseases such as AS has received extensive attention [11,12]. Gasdermin D (GSDMD) is a key protein in executing pyroptosis, which, under the action of caspase-1, is cleaved to form pores resulting in cell death and further exacerbating the inflammatory response [13,14]. In recent years, capsaicin, the primary active ingredient found in chili peppers, has garnered significant interest for its potential effects on reducing inflammation and modulating the immune system [15,16]. Studies have indicated that capsaicin can control the immune system by different means, such as blocking the creation of inflammatory cytokines, adjusting T-cell activity, and impacting the gut microbiota composition [17,18].

Recent findings indicate that capsaicin exerts its biological activity by affecting various cellular signaling pathways, including the activation pathway of the NLRP3 inflammasome [19,20]. Activation of the NLRP3 inflammasome leads to the conversion of pro-caspase-1 to caspase-1, triggering the release of inflammatory cytokines and cell pyroptosis. Furthermore, capsaicin has been found to repair intestinal barrier function and regulate the gut microbiota [17,21]. The precise ways in which capsaicin works in AS, especially its effects on the NLRP3/caspase-1/GSDMD py-

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roptosis pathway in spinal joints, and how this is connected to healing the intestinal barrier and adjusting the gut microbiota, remain unknown at this time [22]. Additionally, capsaicin has been shown to improve the strength of the intestinal barrier, decreasing the movement of inflammatory mediators across cell membranes [16,23]. Although the potential therapeutic role of capsaicin in inflammatory diseases has been extensively studied, its application in AS remains a research gap. Further research is needed to explore how capsaicin relieves symptoms of AS by blocking the NLRP3/caspase-1/GSDMD pyroptosis pathway in the spine, as well as its impact on repairing the intestinal barrier and regulating gut microbiota.

The objective of this research is to investigate how capsaicin reduces AS in mice by blocking the pyroptosis pathway in spinal joints, restoring the intestinal barrier, and adjusting the gut microbiota. We plan to evaluate the effects of capsaicin on spinal joint inflammation and its potential influence on the intestinal barrier and gut microbiome using a mouse model. The results of this research could offer a fresh approach to the treatment of AS, particularly for individuals who do not respond well to traditional therapies or suffer from significant adverse reactions. Furthermore, this study will provide fresh perspectives on how capsaicin functions in controlling the immune system and promoting gastrointestinal well-being.

Materials and Methods

AS Animal Model

Thirty female C57BL/6 mice, aged 7–8 weeks and weighing approximately 20 g, were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). These mice were free of specific pathogens and were housed at the Animal Research Center. All procedures were approved by the Ethics Committee of Luoyang Orthopedic-Traumatological Hospital (No. CS2023–091).

The AS model was induced using cartilage proteoglycans (PGs) and Complete Freund's Adjuvant (CFA). Mice received immunization via intraperitoneal injection of 100 µL CFA (Lot: 2109, Sigma-Aldrich, St. Louis, MO, USA) and 200 µL emulsion with 100 µg cartilage PGs (Lot: 3942, Sigma-Aldrich, St. Louis, MO, USA) at weeks 0, 3, and 6. The mice in this group did not receive any specific treatment during the experimental period. A successful AS mouse model exhibits pathology, including joint inflammation, cartilage and ligament damage, and spinal bone proliferation, validated by HE staining. The success rate of modeling AS in mice is 90%.

In the Capsaicin-Treated Group, starting from the 4th week after PG induction, capsaicin (Lot: 14321, Sigma-Aldrich, St. Louis, MO, USA) was administered continuously for 4 weeks. The dose of capsaicin was 25 mg/kg body weight per day.

In the Sulfasalazine-Treated Group, sulfasalazine (Lot: 43224, Sigma-Aldrich, St. Louis, MO, USA) was administered at a dosage range of 50–200 mg/kg body weight per day. The treatment duration and timing aligned with the capsaicin-treated group. The chosen doses may have been determined through a dose-response curve analysis and previous studies, aiming to identify the concentration range that elicits the desired biological effects without causing significant toxicity. All mice were randomly divided into 6 mice in each group. Specific groups are as follows:

Control group: Healthy mice, normal diet.

Capsaicin-treated group: From the fourth week, the mice were given capsaicin 25 mg/kg body weight per day.

Sulfasalazine-treated group: Sulfasalazine-treated AS group as a positive control, 50–200 mg/kg body weight per day.

AS model group: Mice were intraperitoneally injected with 100 µL and 200 µL emulsions containing 100 µg chondropg at week 0, 3, and 6.

Capsaicin-treated AS group: Mice were intraperitoneally injected with 100 µL and 200 µL emulsions containing 100 µg chondropg at week 0, 3, and 6. From the fourth week, the mice were given capsaicin 25 mg /kg body weight per day.

At the end of the 14th week, mice were sacrificed by CO_2 asphyxiation (Lot: 2791, Ambion, Austin, TX, USA), and blood samples, spinal joint tissues, and intestinal mucosa were collected for further analysis. Blood samples from mice were collected using sterile needles and syringes, then transferred to tubes for plasma separation. Spinal joint tissues were dissected under aseptic conditions. Intestinal mucosal samples were carefully isolated and rinsed with phosphate buffer saline (PBS). Each sample was appropriately stored for further experiments.

ELISA

Enzyme-Linked Immunosorbent Assays (ELISA) kits for Mouse IL-1β, IL-6, tumor necrosis factor-α (TNF-α), IL-17A, interferon (IFN)-γ, IL-10, and C-reactive protein (CRP) were acquired from eBioscience (San Diego, CA, USA) with the following lot numbers: 151906029, 153149017, 161044013, 137148393, 182937193, 146382942, 153714595. Serum was collected, and assays were performed strictly according to the instructions provided with the purchased mouse ELISA kits (Lot: 1935, QIAGEN, Duesseldorf, Germany) and the protein quantification Bicinchoninic Acid Assay (BCA) kit (Lot: 34452, QIAGEN, Duesseldorf, Germany). The enzyme-linked immunodetection instrument (model550, Bio-Rad, Hercules, CA, USA) was used to measure absorbance at 450 nm, recorded as OD values. The content of the above indicators in the mouse samples of each group was calculated using the standard curve, followed by statistical analysis.

Table 1. The primer sequences used.

	Forward	Reverse
Occludin	ACTCCTCCAATGGACAAGTG	CCCCACCTGTCGTGTAGTCT
ZO-1	CCACCTCTGTCCAGCTCTTC	CACCGGAGTGATGGTTTTCT
β-actin	GCTGAGAGGGAAATCGTGCGTG	CCAGGGAGGAAGAGGATGCGG

ZO-1, zonula occluden-1.

Fecal DNA Extraction

200 mg sample was placed in a 2 mL sterile tube. 900 μL of PBS (pH 8.0, 0.1 M) was mixed and vortexed for 5 minutes to ensure thorough mixing. After centrifugation for 5 minutes, the supernatant was discarded, and the precipitate was collected. 800 μL of PBS and 20 μL of lysozyme (50 mg/μL) were combined with the precipitate, ensuring thorough mixing, and incubated in a water bath at 37 °C for 30 minutes. 50 μL of 20% sodium dodecyl sulfate (SDS) solution (containing 0.25 molar NaCl, 0.1 molar Ethylene Diamine Tetraacetic Acid (EDTA), and 4% SDS) was combined with 100 μL of GITC solution (5 molar), and then placed in a water bath at 65 °C for half an hour to incubate, and centrifuged at 10,000 r/min for 10 minutes. The liquid floating above the sediment was gathered, combined with 0.125 times the amount of potassium acetate solution (5 molar concentration) and 0.42 times the amount of polyethylene glycol 8000 solution (40%), stirred thoroughly, and placed in an ice bath for 15 minutes, and then centrifuged at 14,000 r/min for 10 minutes. 700 μL of Tris-EDTA buffer (TE) was combined with the precipitate, followed by an equal amount of chloroform-isoamyl alcohol (24:1 ratio), and gently mixed by inverting and then centrifuged at 12,000 revolutions per minute for 5 minutes. The supernatant was collected, added an equal volume of isopropanol, and placed in an ice bath for 30 minutes and then centrifuged at 12,000 r/min for 10 minutes. The solid was rinsed with 70% ethanol and allowed to dry in the air at room temperature. Finally, the DNA was dissolved in 50 μL TE buffer.

16S rDNA Gene Amplification

Polymerase chain reaction (PCR) amplification was performed on the extracted DNA. The amplification system included 2 μL of template (concentration: 50 ng/μL), 2 μL of primers (each concentration: 10 pmol/μL), 0.4 μL of Takara Taq DNA polymerase (equivalent to 2 U), 4 μL of deoxy-ribonucleoside triphosphate (dNTP) Mix (each concentration: 2.5 mmol/L), 5 μL of 10× buffer, and 34.6 μL of ddH_2O, making a total volume of 50 μL. PCR amplification was carried out with an initial denaturation step at 94 °C for 5 minutes, followed by denaturation. The amplification products were detected by 1% agarose gel electrophoresis and gel imaging.

Real-Time PCR

Total RNA was extracted and Real-time PCR was conducted strictly adhering to the instructions provided with the purchased kits. Total RNA extraction involved the following processes: Trizol homogenization (Lot: 3021, Invitrogen, Carlsbad, CA, USA), sequentially adding chloroform and isopropanol, centrifuging, discarding the supernatant, washing the precipitate with anhydrous ethanol, dissolving RNA in DEPC water, and storing at –80 °C for later use. For reverse transcription, 12 μL of RNA-Primer Mix was combined with 5 μL of 5× RT Reaction Buffer, 1 μL of 25 mM dNTPs, 1 μL of 25 U/μL RNase Inhibitor, 1 μL of 200 U/μL M-MLV Rtase, 1 μL of Oligo (dt) 18, and 4 μL of ddH_2O (DNase-free), making a total volume of 25 μL. The reaction was incubated at 37 °C for 60 minutes, then heated at 85 °C for 5 minutes, cooled at 4 °C for 5 minutes, and finally stored at –20 °C. For the Real-time PCR amplification, a SYBRGreenMix from Thermo (Lot: 72718, Waltham, MA, USA) with 12.5 μL was used, along with 0.5 μL of the upstream primer F, 0.5 μL of the downstream primer R, 9.5 μL of ddH_2O, and 2 μL of the cDNA template, making a total volume of 25 μL. This was followed by a final extension step at 95 °C for 15 seconds, annealing at 60 °C for 1 minute, and a final extension step at 95 °C for 15 seconds, annealing at 60 °C for 15 seconds. The primer sequences used are listed in Table 1. qPCR data were quantified, ΔCt was calculated (difference between target gene Ct and housekeeping gene Ct), then ΔΔCt was performed (difference between sample ΔCt and control ΔCt). The relative expression was determined using $2^{-\Delta\Delta Ct}$ to compare gene expression levels. The analysis of data was conducted utilizing the integrated software ABI Prism 7300 SDS Software (Version 1.4, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Western Blot

Following the separation of proteins with varying molecular weights through SDS-PAGE, the proteins were then transferred onto a PVDF membrane (Φ = 0.25 mm, Lot: 2618391, Bio-Rad, Hercules, CA, USA) using the Bio-Rad electrophoretic transfer system (Lot: 2931, Bio-Rad, Hercules, CA, USA). The transfer process was carried out according to the specified conditions, which included a transfer buffer containing Tris-base 3 g, glycine 14.4 g, SDS 4 g, and 200 mL M-OH per 1 L, as well as transfer settings of a constant voltage of 100 V for 2 hours. Following the

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transfer, the membrane was placed in a solution to block (TBST buffer with 5% skim milk) at room temperature and gently shaken for 1 hour.

Electrophoresis (1658034, Bio-Rad, Hercules, CA, USA, mini protean 3 cells) and electrophoretic transfer (Lot: PS-9, Dalian Jima Technology Co., Ltd., Dalian, China) were employed. For antibody incubation, primary antibodies were diluted in a blocking solution under specific conditions. Antibodies used in the experiment included NLRP3 (1:1000, Lot: 15101, Cell Signaling Technology, Danvers, MA, USA), apoptosis-associated speck-like protein containing a CARD (ASC) (1:3000, Lot: 67824, CST, Temecula, CA, USA), Cleaved caspase-1 (1:1000, Lot: 89332, CST, Temecula, CA, USA), Cleaved GSDMD (1:2000, Lot: 36425, CST, Temecula, CA, USA), p-p65 (1:5000, Lot: 3033, CST, Temecula, CA, USA), p65 (1:1000, Lot: 8242, CST, Temecula, CA, USA), p-inhibitor of nuclear factor kappa-B (NF-κB) (IκB)-α (1:1000, ab133462, Abcam, Cambridge, MA, USA), IκB-α (1:1000, ab32518, Abcam, Cambridge, MA, USA), zonula occluden-1 (ZO-1) (1:2000, ab96587, Abcam, Cambridge, MA, USA), Occludin (1:1000, Lot: 91131, CST, Temecula, CA, USA), and β-actin (1:5000, Lot: 4970, CST, Temecula, CA, USA). The membranes were fully immersed in the diluted primary antibody solution and incubated at room temperature for 2 hours.

Following the primary antibody incubation, the membranes underwent 3 washes lasting 5–10 minutes each. Horse radish peroxidase (HRP)-conjugated secondary antibodies (Lot: 1375, Beyotime, Shanghai, China) were diluted 100,000-fold and incubated on a shaker at room temperature for 1 hour. Following incubation with the secondary antibody, the membranes underwent a washing step. The HRP-ECL chemiluminescence method was used for color development and identification, followed by exposure. To quantify Western blot (WB) data, band intensity for the target protein and control was measured using Image-Pro Plus software (Version 6.0, Media Cybernetics, Silver Springs, MD, USA). The protein band intensities were normalized, relative expression was calculated by comparing to a control, and statistical analysis was conducted for significance. Optical density scanning and analysis were performed using the imaging system's built-in software (Lot: X1820, Bio-Rad, Hercules, CA, USA).

Statistical Analysis

The data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and displayed as the average plus or minus the standard deviation. Quantitative data that adhered to a normal distribution and had homogeneity of variance underwent a one-way analysis of variance (ANOVA), while data that did not adhere to a normal distribution underwent a non-parametric (rank-sum) test. In the case of significant results from ANOVA, post hoc multiple comparisons were conducted to determine which group means were significantly different. Common post hoc multiple comparison methods included Least Significant Difference (LSD) and Tukey Honestly Significant Difference (Tukey HSD), which were used to compare group means while controlling for overall significance level. The *t*-test was utilized to compare the two groups. A *p*-value less than 0.05 was considered as statistical significance.

Results

Capsaicin Inhibits the Production of Inflammatory Cytokines in Mice Suffering from AS

In the AS model group, levels of pro-inflammatory cytokines were higher than in both the control and capsaicin-treated groups, indicating that the AS model successfully induced an inflammatory response. In normal mice, there was no significant difference in these pro-inflammatory cytokines between the capsaicin-treated group and the control group, indicating that capsaicin does not have a significant effect on the body. Compared to the AS model, the capsaicin and sulfasalazine-treated AS model groups had pro-inflammatory cytokine levels close to those of the control group ($p < 0.01$), further validating the anti-inflammatory effect of capsaicin similar to known anti-inflammatory drugs (Fig. 1A–D). In the AS + capsaicin treatment group, IL-10 was higher than that in the AS model group ($p < 0.01$), indicating that capsaicin can not only inhibit pro-inflammatory cytokines but also increase the production of anti-inflammatory cytokines, thus exerting an anti-inflammatory effect (Fig. 1E). The trend of CRP levels was similar to that of pro-inflammatory cytokines, that is, the AS model group was higher than the control group and the capsaicin treatment group ($p < 0.01$), further confirming the anti-inflammatory effect of capsaicin (Fig. 1F).

Capsaicin Inhibits the Pyroptosis Process in the Vertebral Joint Tissues of Mice with AS

Furthermore, we analyzed pyroptosis-related protein markers in the spinal joint tissues through Western blot. NLRP3, ASC, Cleaved caspase-1, and Cleaved GSDMD proteins were higher in the AS model group than in the control (CTRL) group and the capsaicin-treated group, indicating that the AS model successfully induced the activation of the pyroptosis process. Compared to the AS model, AS + capsaicin treatment reduced NLRP3, ASC, Cleaved caspase-1, and Cleaved GSDMD ($p < 0.01$), suggesting that capsaicin mainly inhibited the activation of the NLRP3/caspase-1/GSDMD pathway to reduce the pyroptosis process. The sulfasalazine treatment group showed effects similar to the capsaicin treatment group, further confirming the anti-inflammatory and anti-pyroptotic effects of capsaicin (Fig. 2A–E). The results indicate that capsaicin can inhibit the pyroptosis process in the vertebral joint tissues of mice with AS by suppressing the activation of NLRP3 and reducing pyroptosis-related protein markers.

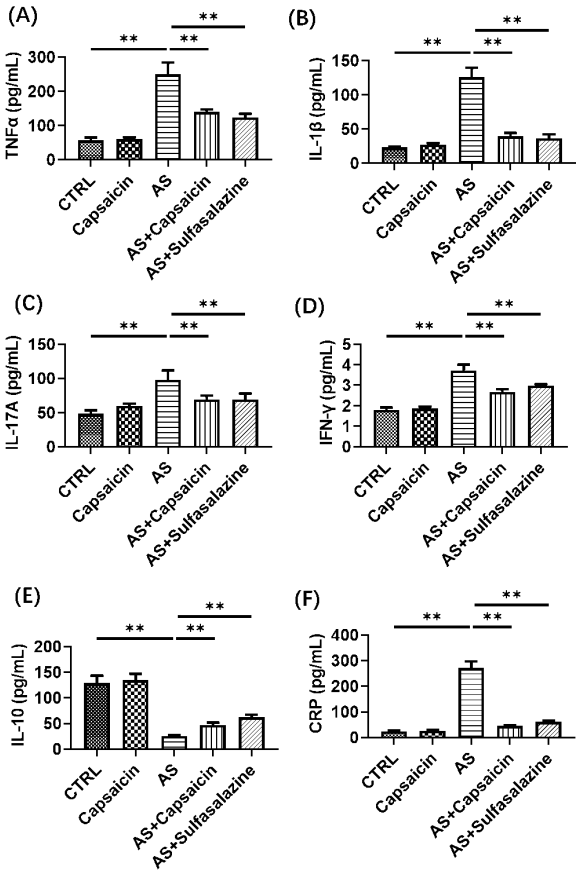

Fig. 1. Capsaicin suppresses inflammatory cytokines in Ankylosing Spondylitis (AS) mice, with sulfasalazine serving as a positive control. (A–F) Levels of tumor necrosis factor-α (TNF-α) (A), interleukin (IL)-1β (B), IL-17A (C), interferon (IFN)-γ (D), IL-10 (E), and C-reactive protein (CRP) (F) in the serum of different animal groups were measured using Enzyme-Linked Immunosorbent Assays (ELISA). Data were expressed as the mean $\pm$ standard deviation (SD) (n = 6). $^{**}p < 0.01$.

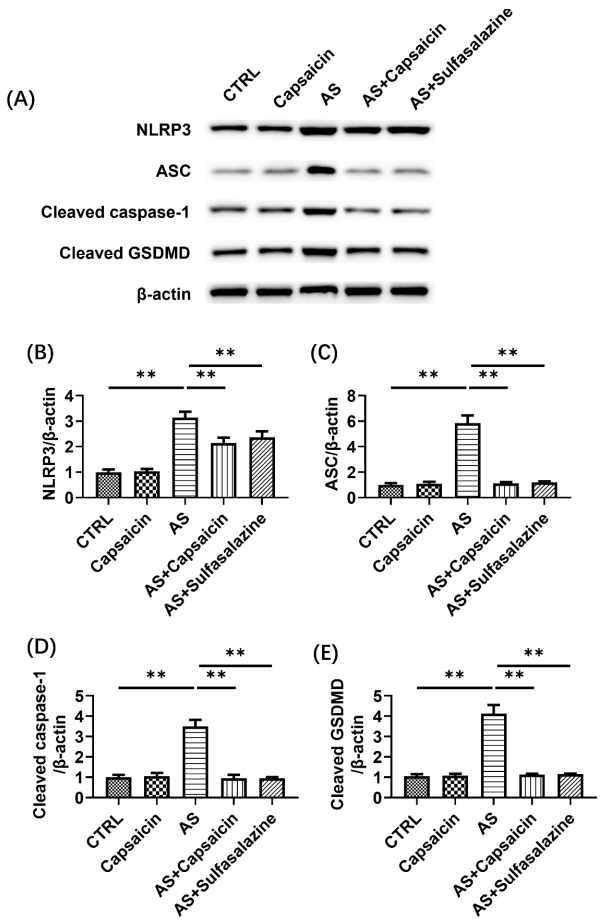

Fig. 2. Capsaicin inhibits the process of pyroptosis in the vertebral joint tissues of AS mice. (A) Pyroptosis-related proteins in mouse spinal joint tissues were detected by Western blot. (B–E) Grayscale analysis was used to determine NOD-like receptor thermal protein domain associated protein 3 (NLRP3) (B), apoptosis-associated speck-like protein containing a CARD (ASC) (C), Cleaved caspase-1 (D), and Cleaved Gasdermin D (GSDMD) (E). Data were expressed as the mean $\pm$ standard deviation (SD) (n = 6). $^{**}p < 0.01$. CTRL, control.

Capsaicin Inhibits the Activation of the NF-κB Pathway in Mice with AS

To study the nuclear factor kappa-B (NF-κB) pathway, we assessed the effect of capsaicin by measuring the phosphorylation levels of key proteins in spinal joint tissues. In the AS model group, p-p65 was higher than in the CTRL group ($p < 0.01$), while p65 remained unchanged across all groups. This indicates that the NF-κB pathway is activated under the conditions of AS. Treatment with capsaicin and sulfasalazine reduced p-p65. Furthermore, p-IκB-α in the AS model group was higher than in the control group ($p < 0.01$), while IκB-α remained unchanged across all groups. This further confirms the activation of the NF-κB pathway under AS conditions. Treatment with capsaicin and sulfasalazine inhibited the activation of NF-κB by reducing p-IκB-α (Fig. 3A–C). These results suggest that capsaicin can alleviate the symptoms of AS in a mouse model by inhibiting inflammation-related protein markers.

Capsaicin Improves Intestinal Mucosal Barrier Function

Using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot techniques, we assessed the impact of capsaicin on intestinal mucosal barrier function. The mRNA levels of *ZO-1* and *occludin* showed that, in the AS model group, the mRNA levels of *ZO-1* and *occludin* were lower than in the control group, indicating impaired intestinal barrier function under the conditions of AS. Treatment with capsaicin and sulfasalazine increased the mRNA expression levels of these tight junction proteins ($p < 0.01$), suggesting that these treatment strategies may help restore or enhance intestinal barrier function (Fig. 4A,B). ZO-1 and occludin were consistent with the trends observed in mRNA levels. In the AS model group, ZO-1 and occludin were lower than in the control group

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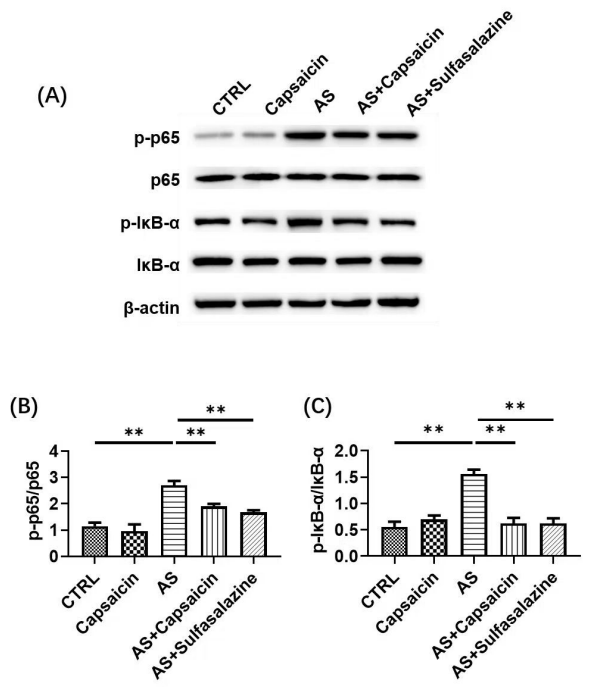

Fig. 3. Capsaicin suppresses the activation of the nuclear factor kappa-B (NF-κB) pathway in AS mice. (A) p-p65, p65, p-inhibitor of NF-κB (IκB)-α, and IκB-α in mouse spinal joint tissues were detected using Western blot; (B,C) Grayscale analysis was used to determine the ratios of p-IκB-α/IκB-α and p-p65/p65. Data were expressed as the mean ± standard deviation (SD) (n = 6). $^{**}p < 0.01$. CTRL, control.

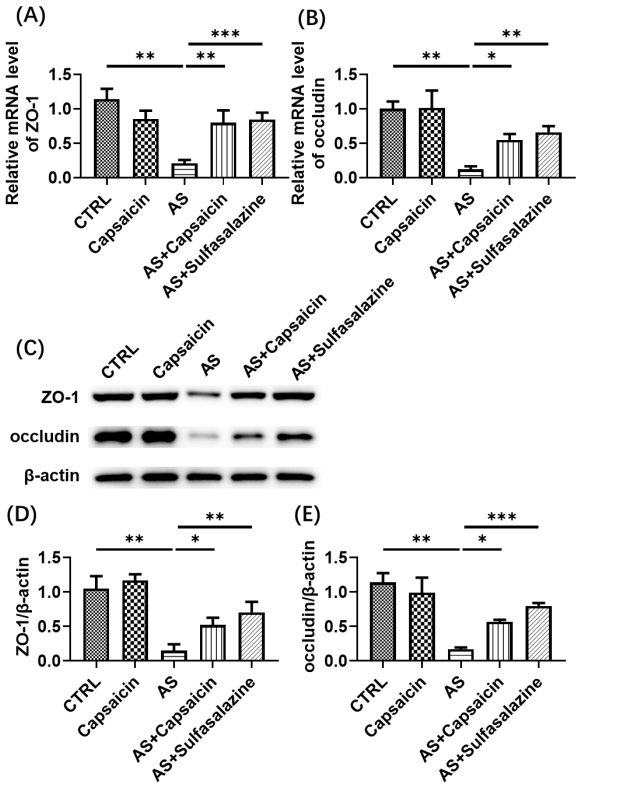

Fig. 4. Capsaicin improves intestinal mucosal barrier function. (A,B) *ZO-1* (A) and *occludin* (B) in mouse ileum tissues were detected using qRT-PCR; (C–E) ZO-1 and occludin were detected using Western blot. Data were expressed as the mean ± standard deviation (SD) (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

($p < 0.01$). Treatment with capsaicin and sulfasalazine increased these tight junction proteins, further confirming their positive impact on intestinal barrier function (Fig. 4C–E).

Capsaicin Enhances Probiotics and Reduces Pathogenic Bacteria in Mice with AS

Finally, we assessed the impact of capsaicin on the intestinal microbiota using RT-qPCR technology. *Lactobacillus* and *Bifidobacterium* are considered major probiotics in the intestine, contributing to the balance of the intestinal microecology, promoting digestion and absorption, and enhancing the immune system. Quantifying them through RT-qPCR technology can reflect the health status of the intestine. In the AS + Capsaicin group, *Lactobacillus* and *Bifidobacterium* were higher than in the AS model group ($p < 0.001$), indicating that capsaicin can effectively increase the abundance of these probiotics, helping restore and maintain the balance of the intestinal microecology. This may be achieved by capsaicin's ability to inhibit inflammatory responses and repair intestinal barrier function, thus creating favorable conditions for the growth of probiotics (Fig. 5A,B). *Enterococcus faecalis* and *Escherichia coli* are generally considered opportunistic pathogens that exist at low levels in the intestine under normal conditions, but their numbers may increase when the intestinal microecology is imbalanced, leading to inflammation or infection. In the AS + Capsaicin group, *Enterococcus faecalis* ($p < 0.05$) and *Escherichia coli* ($p < 0.001$) were lower than in the AS model group, indicating that capsaicin can effectively reduce the abundance of these harmful bacteria. This result further supports the role of capsaicin in regulating the balance of the intestinal microbiota, contributing to the alleviation of inflammation associated with AS (Fig. 5C,D). Capsaicin, by increasing probiotics *Lactobacillus* and *Bifidobacterium*, while reducing pathogenic bacteria *Enterococcus faecalis* and *Escherichia coli*, aids in regulating the intestinal microbiota and repairing the intestinal barrier, thereby alleviating AS in a mouse model.

Discussion

This study unveiled the potential therapeutic effects of capsaicin in a mouse model of AS, particularly its role in suppressing inflammatory responses, repairing damage to the intestinal barrier, and modulating dysbiosis of the gut microbiota. We observed that capsaicin alleviated spinal inflammation in AS model mice, a discovery with significant implications for understanding the pathogenic mechanisms

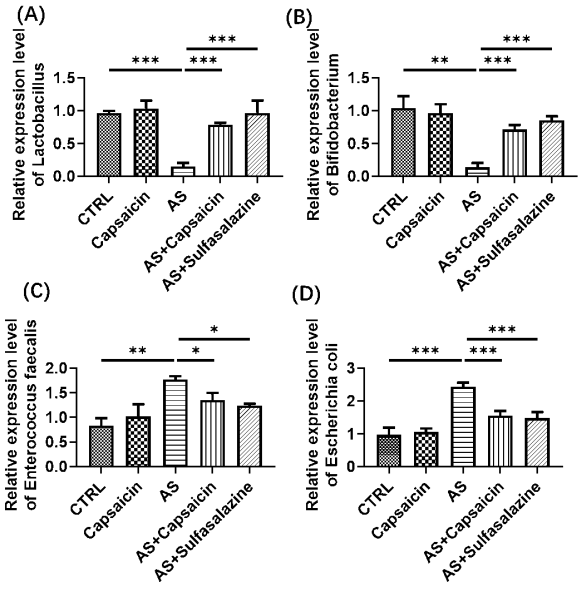

Fig. 5. Capsaicin enhances beneficial gut microbiota *Lactobacillus* and *Bifidobacterium* and reduces harmful bacteria *Enterococcus faecalis* and *Escherichia coli* in AS mice. (A–D) Changes in *Lactobacillus* (A), *Bifidobacterium* (B), *Enterococcus faecalis* (C), and *Escherichia coli* (D) in the mouse cecum were determined by RT-qPCR. Data were expressed as the mean ± standard deviation (SD) (n = 6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

of AS and identifying new therapeutic approaches. Additionally, the effects of capsaicin on repairing the intestinal barrier and modulating the gut microbiota may offer a novel perspective for the atypical treatment of AS, underscoring the importance of gut health in the disease process of AS.

Capsaicin, as a bioactive compound in chili peppers, has garnered attention for its potential effects on the gut microbiota and its relationship with inflammatory conditions such as Ankylosing Spondylitis (AS). Studies have suggested that capsaicin may influence host immune and inflammatory responses by modulating the balance of gut microbiota. The antimicrobial and anti-inflammatory properties of capsaicin make it a promising candidate for improving gut microbiota composition and immune function.

The regulation of gut microbiota by capsaicin is a complex and intricate process that warrants further exploration. While some research has pointed to the beneficial effects of capsaicin on gut health, the specific molecular mechanisms underlying this interaction remain poorly understood. Future studies utilizing advanced molecular biology and metabolomics techniques could provide valuable insights into how capsaicin interacts with gut microbiota and modulates the host immune response.

In the context of AS, capsaicin's anti-inflammatory properties hold promise for managing the systemic inflammation associated with the condition. By reducing overall inflammation, capsaicin may potentially alleviate joint symptoms and improve the quality of life for AS patients. However, the exact mechanisms through which capsaicin exerts its therapeutic effects in AS are still unclear and require further investigation.

Capsaicin has been extensively studied, and its anti-inflammatory effects have been validated in multiple disease models [24,25]. However, studies on the role of capsaicin in AS, especially its action through the NLRP3/caspase-1/GSDMD pyroptosis pathway, are relatively scarce. Previous findings have indicated that the balance between pro-inflammatory and anti-inflammatory cytokines plays a key role in the pathogenesis of AS [26,27]. Our findings are consistent with previous discoveries, further confirming the potential utility of capsaicin in modulating this balance.

The NLRP3/caspase-1/GSDMD pyroptosis pathway is considered a key factor in the progression of inflammatory diseases such as AS [28,29]. By inhibiting this pathway, our study reveals a new mechanism by which capsaicin mitigates the pathological changes in AS. The NF-κB pathway is a critical regulatory factor in many inflammatory diseases, and the inhibitory effect of capsaicin on this pathway aligns with previous research findings, emphasizing its central role in the inflammatory response. Studies [28,29] showed that capsaicin can inhibit the degradation of IκB-α, thus preventing the activation of NF-κB.

Normally, NF-κB forms a complex with IκB proteins, keeping NF-κB in an inactive state. However, upon degradation of IκB-α, NF-κB is released and translocated into the nucleus, activating the transcription of inflammatory genes. The action of capsaicin can hinder the degradation of IκB-α, retaining NF-κB in the cytoplasm, thereby suppressing its transcriptional activity and its ability to initiate inflammatory responses. Moreover, capsaicin can modulate the transcriptional activity of NF-κB by influencing its binding to DNA. NF-κB needs to bind to DNA to transcribe specific genes involved in the inflammatory process. Capsaicin can interfere with the binding of NF-κB to DNA, reducing its role in gene regulation.

Compared to existing study, our research not only confirms the anti-inflammatory effects of capsaicin but also further reveals its specific mechanisms of action in AS, providing a theoretical basis for the application of capsaicin in the treatment of AS [30]. Increasing evidence suggests a close link between gut health and autoimmune diseases such as AS [3,7,31]. The regulatory effects of capsaicin on the intestinal barrier and microbiota demonstrated in this study offer a new perspective for treating AS.

The activation of the NLRP3 inflammasome plays a crucial role in the inflammatory response in AS, with caspase-1 and GSDMD being important effector molecules following the activation of the NLRP3 inflammasome. Our study found that capsaicin could inhibit the activation of the NLRP3 inflammasome as well as caspase-1 and GSDMD, thereby mitigating the inflammatory response. The discovery of this mechanism provides a new theoretical basis for the application of capsaicin in treating AS.

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Despite some meaningful findings, this study has several limitations. First, the study was confined to AS model mice, thereby limiting the generalizability of its results to human AS patients. It is crucial to underscore that the direct applicability of these findings to human AS necessitates further elucidation through extensive clinical experimentation. The models and outcomes of the current study are exclusively based on mouse models, underscoring the imperative for translation and validation in human clinical trials.

To translate the effects of capsaicin on alleviating AS in mouse models into clinical practice, the differences between the immune response and the gut microbiota need to be fully considered. Given the substantial interspecies variations in these aspects between mice and humans, comprehensive investigations are warranted to validate the efficacy and safety of capsaicin in AS treatment. Clinical trials need to be designed to specifically evaluate the effects of capsaicin on NF-κB signaling and inflammatory responses in AS patients, as well as to study the effects of capsaicin on human gut microbiota and immune responses to better understand its therapeutic potential in clinical settings.

Second, the study failed to comprehensively elucidate the precise influence of capsaicin on the gut microbiota, nor did it address how these alterations impact the therapeutic efficacy for AS. Finally, the study overlooked the potential impact of capsaicin dosage on therapeutic outcomes; hence, future research should explore the optimal therapeutic effects of different capsaicin dosages.

Furthermore, careful consideration of the generalizability of the results across diverse populations is imperative to ascertain the broad applicability of capsaicin in AS treatment. Thus, more comprehensive studies investigating responses in varied populations are warranted. Additionally, a discussion on the long-term effects of capsaicin treatment is essential to evaluate its durability and efficacy as a therapeutic intervention for AS. An in-depth exploration of the long-term anti-inflammatory effects of capsaicin, potential side effects, and overall impact on disease progression throughout extended treatment durations is necessary to comprehensively assess its potential for clinical application.

Considering the intricate pathology of AS and the multifaceted nature of its treatment, it is essential to discuss these findings in a more critical and nuanced manner. A thorough examination of the interplay among genetic, environmental, and immune factors in AS pathogenesis is imperative to assess the potential of capsaicin as a therapeutic intervention. By candidly acknowledging the limitations, biases, and long-term effects of research, a more comprehensive and balanced perspective can be attained, thereby enhancing the translational relevance of research findings for clinical application.

Based on the findings of this study, we suggest that future research should focus on a comprehensive examination of the impact of capsaicin on the gut microbiota and its potential implications for AS pathogenesis via the gut-joint axis. Furthermore, investigating the therapeutic efficacy of varying doses of capsaicin to establish an optimal treatment protocol is recommended. Finally, considering the importance of gut health in AS, future studies should also explore the combined effects of capsaicin with other interventions targeting gut health regulation.

Conclusion

The study indicates that capsaicin achieves these effects by targeting the pyroptosis pathway, enhancing the integrity of the intestinal barrier, and modulating the composition of the gut microbiota. By inhibiting key inflammatory cytokines in the serum, suppressing pyroptosis-related proteins in spinal joint tissues, and regulating the NF-κB pathway, capsaicin demonstrates promise in attenuating cell pyroptosis and improving overall gut health. The differential impact on beneficial probiotics and harmful bacteria further underscores the multifaceted benefits of capsaicin in the context of AS. These findings pave the way for future research exploring the therapeutic potential of capsaicin in managing AS and related inflammatory conditions.

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting the first author if needed.

Author Contributions

XQW and HLS designed the research study. LLZ and SS performed the research. WL analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures were approved by the Ethics Committee of Luoyang Orthopedic-Traumatological Hospital (No. CS2023–091).

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Conflict of Interest

The authors declare no conflict of interest.

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