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Weizmannia coagulans NCU-33 improves rheumatoid arthritis through anti-inflammatory and immunomodulatory effects

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ABSTRACT: The limitations of current rheumatoid arthritis (RA) therapies, including substantial adverse effects and economic burden, necessitate the development of safer, cost-effective adjunct treatments. Probiotic-based interventions targeting the gut-joint axis hold therapeutic promise, yet their precise mechanisms of action remain incompletely understood. This study investigated the therapeutic efficacy and underlying mechanisms of *W. coagulans* NCU-33 in a collagen-induced arthritis (CIA) mouse model. Intervention with NCU-33 significantly attenuated clinical arthritic scores, joint swelling, and histopathological damage. Mechanistically, NCU-33 exerted a coordinated, multi-pronged effect: in joint tissue, it suppressed TLR2/MyD88/NF- κ B signaling, reducing pro-inflammatory cytokines TNF- α and IL-6; systemically, it modulated Notch signaling in splenic lymphocytes, thereby rectifying the pathogenic Th17/Treg imbalance; and in the gut, it restructured the microbiota by enriching beneficial *Lactobacillus* spp., which enhanced intestinal barrier integrity and elevated short-chain fatty acid levels. In summary, *W. coagulans* NCU-33 ameliorates experimental arthritis through an integrated, multi-target mechanism that concurrently regulates local inflammation, systemic immune homeostasis, and gut ecosystem equilibrium. These findings provide robust preclinical evidence supporting the clinical development of *W. coagulans* NCU-33 as a novel, mechanism-driven probiotic therapy for RA via modulation of the gut-joint axis.

Keywords: Rheumatoid arthritis, *Weizmannia coagulans*, Probiotics, Gut microbiota

1. Introduction

RA is a chronic autoimmune disorder pathologically characterized by synovial hyperplasia and bone erosion, clinically presenting with symmetric joint swelling, morning stiffness, and progressive pain.^[1]

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Advanced disease stages often culminate in irreversible joint deformities and multisystem involvement.^[2] Despite the widespread clinical utilization of NSAIDs (Non-steroidal anti-inflammatory drugs), glucocorticoids, and biological agents, their therapeutic utility remains constrained by critical limitations including gastrointestinal bleeding, metabolic disturbances, and notably the prohibitively high treatment costs of biologics.^[3-5] Consequently, the development of novel, cost-effective adjuvant therapeutic strategies with low toxicity profiles holds considerable significance for optimizing the clinical management of RA.

The CIA model, induced by immunization with type II collagen, serves as a pivotal preclinical tool that closely mirrors the autoimmune pathogenesis of human RA.^[6] It recapitulates the breach of immune tolerance, leading to a synergistic autoimmune attack involving both anti-collagen antibody production and the activation of pro-inflammatory T helper cells, notably Th1 and Th17 subsets.^[7] This cascade drives robust synovial inflammation, characterized by the secretion of cytokines such as TNF- α , IL-1 β , and IL-6, and results in the hallmark pathologies of pannus formation, cartilage degradation, and bone erosion.^[8] Critically, CIA development is closely linked to concomitant gut microbiota dysbiosis and intestinal barrier impairment. This dysbiosis diminishes the production of immunomodulatory metabolites and, together with a leaky gut, facilitates the systemic translocation of microbial components. These events perpetuate systemic immune activation and joint inflammation, forming a pathogenic feedback loop that exacerbates disease.^[9] The CIA model provides a highly relevant system for investigating the interconnected roles of gut microbiome dysregulation, systemic immune imbalance, and joint-specific inflammation. This makes it an ideal model for evaluating therapeutic interventions, like probiotics, that target the gut-joint axis.

The pathogenesis of RA entails complex interactions between genetic susceptibility and environmental factors. Mounting evidence implicates gut microbiota dysbiosis as a critical environmental determinant contributing to RA development and progression.^[10] Jiang et al. (2022) reported significant enrichment of *Prevotella* species within the gut microbiota of patients with early-stage RA.^[11] Complementing these findings, Liu et al. (2023) documented marked reductions in bacterial populations responsible for producing short-chain fatty acids (SCFAs), indole derivatives, and secondary bile acids in murine models of RA.^[12] This dysbiotic milieu is increasingly recognized as a central driver of RA pathogenesis.^[13] Critically, microbiota dysbiosis induces intestinal barrier impairment, facilitating the systemic translocation of microbial products such as lipopolysaccharide (LPS) and peptidoglycan.^[14, 15] These pathogen-associated molecular patterns (PAMPs) activate innate immune responses predominantly through the NF- κ B and MAPK pro-inflammatory signaling pathways.^[16-18] Sustained inflammatory signaling promotes fibroblast-like synoviocyte (FLS) proliferation and cartilage degradation via the overproduction of matrix metalloproteinases (MMPs).^[19] Concurrently, this dysregulation perturbs the Th17/Treg cell balance through dendritic cell (DC)-mediated, Notch signaling-dependent upregulation of the transcription factor ROR γ t.^[20, 21] Collectively, these mechanisms highlight the therapeutic potential of gut microbiota modulation and intestinal barrier restoration for mitigating immune dysregulation in RA.^[22]

Probiotics, defined as live microorganisms conferring health benefits when administered in adequate amounts, demonstrate therapeutic potential in RA.^[23–25] Preclinical studies indicate that *Lactobacillus* species ameliorate joint pathology by modulating gut microbiota composition and restoring immune homeostasis.^[26] Specifically, supplementation with *Lactocaseibacillus casei* significantly reduced toe erythema, swelling, and serum levels of pro-inflammatory cytokines (IL-1 β , TNF- α) in a rat model of CIA.^[27] Similarly, administration of *Lactiplantibacillus plantarum* exerted comparable therapeutic effects in CIA mice.^[28] *W. coagulans*, a Gram-positive commensal bacterium isolated from healthy human intestines, enhances microbial ecology through competitive pathogen exclusion and intestinal barrier reinforcement while regulating mucosal immunity to maintain homeostasis.^[29, 30] Preliminary clinical trials confirm its efficacy in reducing joint swelling and pro-inflammatory cytokines in RA patients, though the molecular mechanisms underlying its specific immunomodulatory effects on articular inflammation require further elucidation.^[31]

This study investigated the therapeutic mechanisms of *W. coagulans* NCU-33, a strain isolated from healthy human fecal samples, using a CIA murine model.^[32] Histopathological and molecular analyses demonstrated that *W. coagulans* NCU-33 significantly attenuated joint damage and suppressed hyperactivation of NF- κ B and Notch signaling pathways. Furthermore, 16S rRNA sequencing revealed the strain's capacity to reverse gut dysbiosis while selectively enriching SCFA-producing bacterial taxa. By elucidating dual mechanisms targeting articular inflammation-immunomodulation and gut-microbiota homeostasis restoration, these findings provide a mechanistic foundation for developing *W. coagulans* NCU-33 as a probiotic therapeutic against RA.

2. Materials and methods

2.1 Bacterial Culture and Probiotic Property Assessment

The bacterial strain *W. coagulans* NCU-33 (CGMCC No.31779) used in the research was isolated from fecal samples of healthy human donors and cultured by the laboratory of Professor Tingtao Chen's team at Nanchang University. This study was reviewed and approved by the Ethics Committee of Jiangxi University of Chinese Medicine Affiliated Reproductive Hospital (No. 2023097).

W. coagulans NCU-33 was cultured aerobically at 37°C in MRS medium (Qingdao Haibo Bio-Technology) prepared by dissolving premix powder in distilled water (pH 6.2–6.5), followed by autoclaving for liquid medium or solid plates with 1.5% agar. For probiotic characterization, activated cultures (37°C, 24 hour) were subjected to acid tolerance testing through serial dilution in PBS (pH 1.0–7.0) with 4-hr incubation and viable counts determination; bile salt tolerance was assessed by resuspending centrifuged pellets (7,000 rpm, 3 min) in PBS containing 0–0.5% bile salts followed by 4 hour incubation and plate counting. Antibacterial activity against foodborne pathogens (*Escherichia coli* O157, *Listeria monocytogenes* ATCC 19111, *Salmonella enterica* serovar Typhimurium ATCC 13311, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Shigella flexneri* ATCC 12022, *Staphylococcus aureus* Cowan 1, β -hemolytic *Streptococcus* spp., *Candida albicans* SC531) was evaluated via Oxford cup assay using 250 μ L of cell-free supernatant, which was prepared by centrifugation, filtration, and heat

inactivation of the bacterial culture, on lawns of the indicator pathogens seeded in LB agar plates, with sterile MRS broth serving as the negative control.^[33] For the antioxidant assay, *W. coagulans* NCU-33 was cultured overnight and subsequently reconstituted to assess its ability to scavenge superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), and DPPH radicals, chelate ferrous ions (Fe^{2+}), and exhibit total reducing power. Hemolytic activity was tested by spotting PBS-washed cells on BHI-blood agar (5% sheep blood) alongside *S. aureus* controls with 24-hr incubation. Antimicrobial susceptibility employed disc diffusion on MRS agar with ten antibiotics (Penicillin, Ampicillin, Tetracycline, Erythromycin, Ciprofloxacin, Lincomycin, Compound sulfamethoxazole, Chloramphenicol, Gentamicin), measuring inhibition zones after 16–18 hour incubation.^[34]

2.2 Animal Experiments

Male DBA/1 mice (SPF grade, 6-8 weeks old; SpePharm (Beijing) Biotechnology Co., Ltd.) were acclimatized for 7 days and then randomly assigned to four groups (n=8/group): the control Control (Group C); the CIA model group received saline gelatin for 6 weeks (Group M); the model group treated with methotrexate (Group MB), which received methotrexate (7.6 mg/kg body weight) by gavage twice per week from weeks 2 to 7; the model group treated with a high concentration of *W. coagulans* (Group MH), which received a bacterial suspension (resuspended in saline containing 0.01% gelatin)(1×10^9 CFU/mL in 200 μ L) by daily gavage from weeks 2 to 7.^[35] CIA was induced using bovine type II collagen dissolved in 0.05 molarity acetic acid (4 mg/ml) and emulsified with an equal volume of Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA) via homogenization (10,000-30,000 rpm, 2 min twice) on ice; emulsion stability was confirmed by a dispersion test in deionized water. Mice received a primary subcutaneous immunization (100 μ L collagen-CFA emulsion) at the tail base on Day 7, followed by a booster immunization (100 μ L collagen-IFA emulsion) at the same site on Day 21. Arthritis was monitored weekly via body weight measurement, visual assessment of joint swelling and deformities, paw swelling measurement, and a standardized clinical arthritis score (0-4 per limb). At the experimental endpoint, mice were euthanized by cervical dislocation under isoflurane anesthesia (5% induction, 2% maintenance). All procedures complied with the guidelines of the Nanchang University Laboratory Animal Science Center and were approved by the Nanchang University Medical Laboratory Animal Ethics Committee (approval number : NCULAE-20221228050).

2.3 Measurement of Paw Swelling and Arthritis Scoring

Paw swelling was measured weekly after primary immunization using a digital caliper at sites consistently marked on each paw with picric acid. Arthritis severity was scored independently for each limb (0-4) based on clinical criteria: 0 = normal; 1 = mild erythema/swelling in toes/forepaws/ankles; 2 = moderate swelling in multiple digits/joints; 3 = severe swelling involving the entire paw and ankle; 4 = severe swelling with joint deformity . The total arthritis score (maximum 16) was calculated by summing all limb scores, where higher scores indicate greater disease severity.

2.4 Histopathological examination

Hind limb joints harvested from euthanized male DBA/1 mice were fixed in 4% paraformaldehyde (24 h), decalcified in 10% EDTA (2–4 weeks), paraffin-embedded, and sectioned (4–5 μm). Histopathological assessment included: (1) Hematoxylin and eosin staining to evaluate synovitis, cartilage/bone destruction, and joint architecture; (2) Modified Safranin O-Fast Green staining to quantify glycosaminoglycan loss, cartilage structural damage, and osteochondral junction abnormalities; (3) TRAP staining (37°C, 30–60 min with naphthol AS-BI phosphate/Fast Red TR/tartrate buffer, pH 5.0) to detect osteoclast-mediated bone resorption; and (4) Goldner's trichrome staining to distinguish trabecular bone, osteocytes, and cartilage degeneration. All stained sections were comprehensively examined under a light microscope (40–400 $\times$ magnification) for qualitative and descriptive assessment of arthritic pathological features, including synovitis, cartilage/bone damage, proteoglycan loss, osteoclast activity, and osteoid deposition.

2.5 Flow Cytometry

Splenic single-cell suspensions from euthanized male DBA/1 mice underwent red blood cell lysis (ACK buffer), followed by surface staining with FITC-anti-CD4 and APC-anti-CD25 antibodies (Elabscience). After fixation/permeabilization, intracellular staining was performed using PE-anti-IL-17A and PE-anti-Foxp3 antibodies (Elabscience). CD4⁺ T cells were gated to quantify Th17 (CD4⁺IL-17A⁺) and Treg (CD4⁺CD25⁺Foxp3⁺) cell subpopulations by flow cytometry.

2.6 Western Blot

Protein extracts from ankle joint, spleen and colon tissues of CIA model mice were quantified by BCA assay, separated via SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with primary antibodies against: β -actin (1:20,000, 66009-1-Ig, Proteintech), occludin (1:10,000, 66378-1-Ig, Proteintech), Claudin1 (1:2,000, ab180158, Abcam), DLL4 (1:1,000, ab183532, Abcam), Notch1 (1:1,000, ab52627, Abcam), Hes1 (1:1,000, 11988S, CST), TLR2 (1:500, ab213676, Abcam), MYD88 (1:2,000, 67969-1-Ig, Proteintech), NF- κ B (p65, 1:2,000, 10745-1-AP, Proteintech), and p-NF- κ B (p-p65, 1:2,000, 82335-1-RR, Proteintech), followed by species-matched HRP-conjugated secondary antibodies. Protein expression was visualized by chemiluminescence.

Protein expression levels were quantified using densitometric analysis in ImageJ software (National Institutes of Health, v1.53t). The relative expression of target proteins was normalized to β -actin (loading control) within the same membrane. Data from three independent experiments were expressed as the target/ β -actin ratio.

2.7 RT-qPCR

Spleen and ankle joint tissues (80–100 mg) were homogenized in Trizol reagent for RNA extraction via chloroform separation, isopropanol precipitation, ethanol washing, and dissolution in RNase-free water. RNA purity (A260/A280 ratio) was spectrophotometrically assessed. After genomic DNA removal (Takara, RR092A, Japan), cDNA was synthesized and qPCR performed using gene-specific primers (Supplementary Table S1) and premix reagents (Takara, RR820A, Japan) with melt curve validation. Tissue gene expression was normalized to β -actin. All data were analyzed using the $\Delta\Delta\text{Ct}$ method.

2.8 ELISA

Serum isolated from clotted blood samples of DBA/1 mice was analyzed by ELISA (Sizhengbai Biotechnology Co., Ltd., China) to quantify anti-type II collagen antibody, anti-citrullinated peptide antibody, and cytokines (IL-1 β , IL-6, IL-10, IL-17A, TGF- β 1, TNF- α). Concentrations were derived from standard curves generated by plotting absorbance against known standards and applying optimal curve-fitting models.

2.9 High-throughput sequencing

Bacterial genomic DNA was extracted (Tiangen Biotech Co., Ltd, Beijing, China), and the V4 hypervariable region of 16S rRNA was amplified prior to Illumina Novaseq sequencing. Raw data were processed using DADA2 to generate Amplicon Sequence Variants (ASVs). Taxonomic composition, intergroup differences, α -diversity, and β -diversity were analyzed through combined ASV/Operational Taxonomic Unit (OTU) approaches. All FASTQ-format sequencing data were deposited in the NCBI database.

2.10 Data Analysis

Data from this experiment are expressed as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad 9 software, and all data were analysed using one-way ANOVA or two-way ANOVA, the Kruskal–Wallis rank sum test and Dunn's test were used for 16sRNA data, and Tukey's test was used for all other experimental data to determine significant differences between groups. $*P < 0.05$ was defined as statistically significant ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$).

3. Results

3.1 Evaluation of Probiotic Properties of *W. coagulans* NCU-33

To evaluate aerobic growth performance, OD_{600nm} was monitored over 36 hours. *W. coagulans* NCU-33 exhibited logarithmic growth between 8–16 hours, reaching stationary phase at 26 hours with an OD_{600nm} of 2.35, indicating robust growth (Fig. 1A). Under gastric conditions, viable counts reached 10⁹ CFU/mL at pH 7.0, and remained at 10⁶ CFU/mL even at pH 1.0 (Fig. 1B, D). Similarly, bile salt tolerance (0.3%) sustained viability at 10⁶ CFU/mL (Fig. 1C, D). The strain consistently inhibited pathogens including *Escherichia coli* O157, *Listeria monocytogenes* ATCC 19111, *Salmonella enterica* serovar Typhimurium ATCC 13311, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Shigella flexneri* ATCC 12022, *Staphylococcus aureus* Cowan 1, β -hemolytic *Streptococcus* spp., *Candida albicans* SC531 (Fig. 1E). Additionally, the antioxidant properties of *W. coagulans* NCU-33 were assessed, revealing substantial scavenging rates against DPPH (91.48%), -OH (69.58%), and O²⁻ (65.21%), a high Fe²⁺ chelating rate of 61.13%, and a total reducing power of 0.759 (Fig. 1F).

Safety assessments showed no hemolytic activity on 5% sheep blood agar (Fig. 1G), and susceptibility to multiple antibiotics including penicillin, ampicillin, tetracycline, erythromycin, ciprofloxacin, lincomycin, sulfamethoxazole, chloramphenicol, and gentamicin (Fig. 1H).^[36] These results affirm *W. coagulans* NCU-33 as a promising probiotic with favorable functional properties.

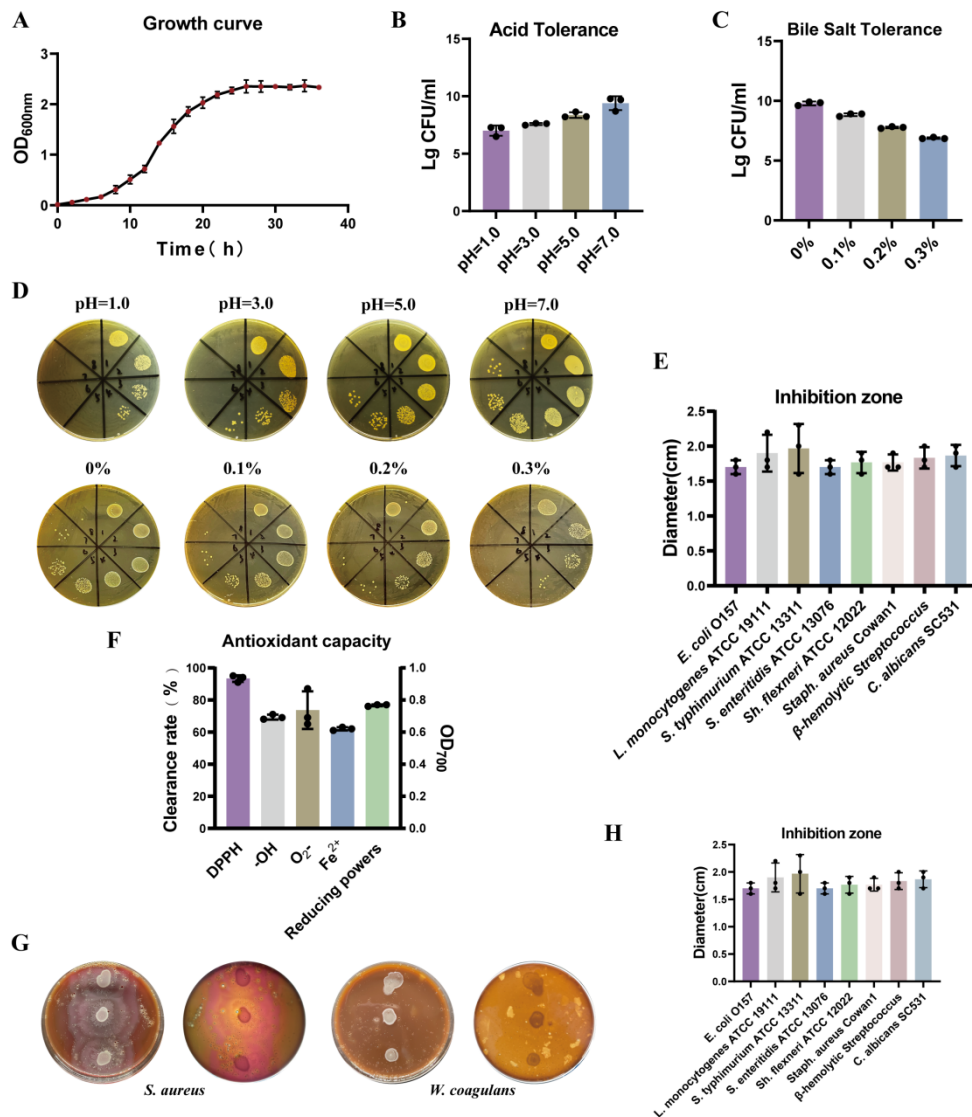


Fig.1 Assessment of the probiotic characteristics of *W. coagulans* NCU-33. (A) Growth curve of *W. coagulans* NCU-33 (n=3); (B-D) The tolerance of *W. coagulans* NCU-33 to hydrochloric acid with different pH values (n=3); (C-D) The tolerance of *W. coagulans* NCU-33 to bile salts at different concentrations (n=3); (E) The inhibitory effect of *W. coagulans* NCU-33 on the growth of intestinal pathogenic bacteria (n=3); (F) Antioxidant capacity test of *W. coagulans* NCU-33 (n=3); (G) The hemolytic test of *W. coagulans* NCU-33 and *Staphylococcus aureus*; (H) The antibiotic resistance test of *W. coagulans* NCU-33 (n=3). Data are presented as mean ± standard deviation.

3.2 *W. coagulans* NCU-33 exhibits a protective effect against arthritis development in CIA mice.

To evaluate the therapeutic effects of *W. coagulans* NCU-33 on CIA, mice were divided into different intervention groups (Fig. 2A). Arthritis hallmarks such as toe swelling and redness were observed in CIA model mice. Both MTX and *W. coagulans* NCU-33 treatments markedly alleviated these symptoms (Fig. 2B). Significant reductions in arthritis scores and toe swelling were observed in the Group MB and Group MH groups compared to the Group M, indicating effective suppression of disease progression (Fig. 2C, D).^[37] Moreover, compared with the other groups, the Group MB exhibited a significant reduction in body weight. (Fig. 2E).

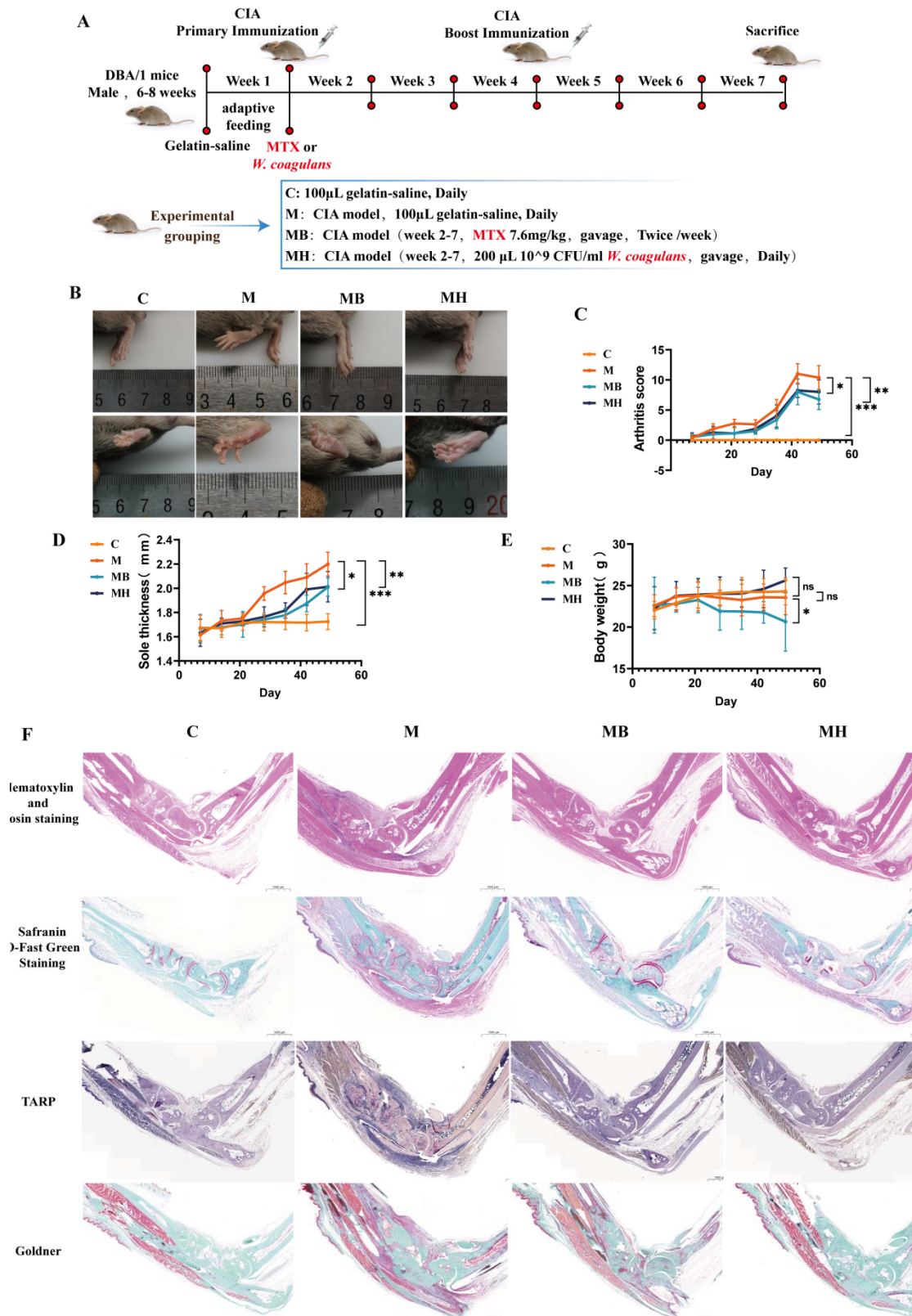


Fig.2 *W. coagulans* NCU-33 significantly improved arthritis symptoms and histopathological changes in CIA mice.

(A) After the first immunization in the second week, CIA male mice were daily gavaged with gelatin-saline, methotrexate (Week 2-7, 7.6mg/kg, Gavage, Twice/week) (MB), or *W. coagulans* NCU-33 (Week 2-7, 200μl 10⁹ CFU/ml, Gavage, Daily) (MH). DBA/1 male mice as control group were daily gavaged with gelatin-saline; (B) Representative images of mouse toes; (C) Arthritis score (0-16) (n=8); (D) Footpad swelling degree (n=8); (E) Body weight changes of mice during the experiment (n=8); (F) Histopathological images (Pathological Analysis of Joints: Hematoxylin and Eosin 1.2×; Modified Safranin O-Fast Green Cartilage 1.2×; Osteoclast Tartrate-Resistant Acid Phosphatase 1.2×; Goldner's Trichrome 1.2×). C: control group, M: model group, MB: methotrexate intervention group, MH: probiotic intervention group. Statistical analysis was showed using one-way repeated measures ANOVA, Data are presented as mean ± standard deviation. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Histological analysis of ankle joints was performed to qualitatively assess arthritic pathology. Representative images (Fig. 2F) revealed the following observations: Histological analysis of ankle joints revealed decreased synovial lymphocyte infiltration in the Group MH compared to the Group M (H&E staining). Safranin O/Fast Green staining indicated more proteoglycan loss in the Group M, while TRAP staining showed reduced osteoclast activity in the Group MH. Goldner's Trichrome staining demonstrated increased osteoid deposition in the Group M relative to the Group MH. Together, these qualitative histopathological observations support that treatment with *W. coagulans* NCU-33 is associated with a marked amelioration of joint pathology in CIA mice.

3.3 *W. coagulans* NCU-33 ameliorates inflammatory arthritis by mediating the NF- κ B pathway

Building upon the protective effects of *W. coagulans* NCU-33 on joint integrity in CIA mice, we investigated its underlying molecular mechanisms. Serum analysis showed systemic reduction of pro-inflammatory cytokines IL-17A, TNF- α , IL-6, and IL-1 β following probiotic treatment (Fig. 3A–D).^[38] In ankle tissues, mRNA levels of MMP1a, MMP2, and MMP13 were significantly downregulated, with MMP13, a key mediator of cartilage degradation, exhibiting the most marked suppression (Fig. 3E–G).^[39] Concurrent decreases in chemokines CXCL9, CXCL10, and CXCL11 indicated impaired inflammatory cell recruitment and osteoclast activation (Fig. 3H–J).^[40]

The TLR2/NF- κ B axis is a central pathogenic regulator in RA, supported by clinical evidence of its significant upregulation in synovial tissues (especially in eroded areas) and correlation with disease activity.^[41, 42] To investigate whether the anti-arthritic effect of *W. coagulans* NCU-33 on CIA mice was mediated through the TLR2/MyD88 pathway, Western blot analysis was conducted. The results demonstrated that *W. coagulans* NCU-33 downregulated the expression of TLR2 and MyD88 and reduced the phosphorylation of NF- κ B p65 (Fig. 3K–N). This suppression of the TLR2/MyD88/NF- κ B axis correlated with diminished transcriptional activity of NF- κ B, indicating a mechanism through which NCU-33 attenuates inflammation and cartilage degradation.

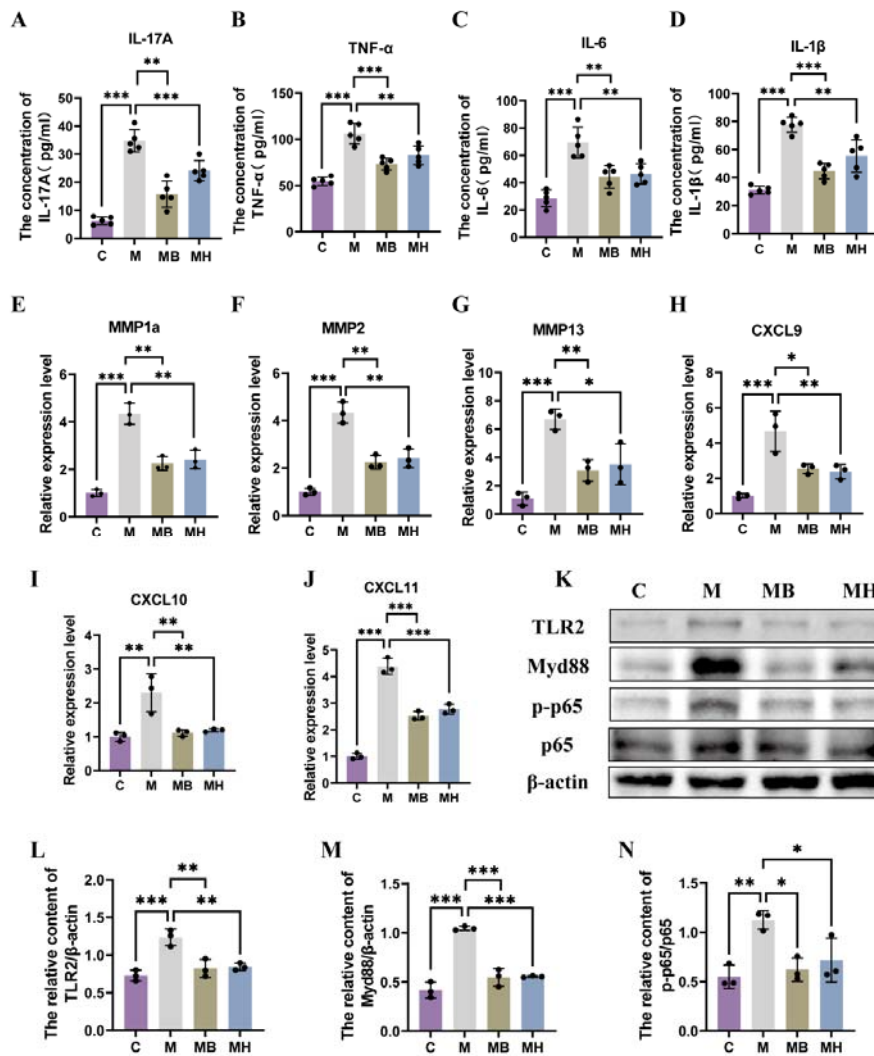


Fig.3 *W. coagulans* NCU-33 improves joint inflammation and reduces joint damage in CIA mice. (A-D) Determination of serum inflammatory cytokine (IL-17A、TNF- α 、IL-6、IL-1 β) concentrations in mice (n=5); (E-J) The relative expression levels of matrix metalloproteinases (MMP1a、MMP2、MMP13) and chemokine (CXCL9、CXCL10、CXCL11)-related mRNA in joints (n=3); (K-N) Detection of NF- κ B signaling pathway-related protein expression in mouse joint (TLR2、Myd88、p-p65、p65) (n=3). C: control group, M: model group, MB: methotrexate intervention group, MH: probiotic intervention group. Statistical analysis was showed using one-way repeated measures ANOVA, Data are presented as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3.4 *W. coagulans* NCU-33 mitigates autoimmune arthritis via restoring Th17/Treg equilibrium.

RA is closely associated with dysregulated immune status, as reflected by key Immune indicators.^[43] Serum levels of anti-CII antibodies and ACPAs (Anti-citrullinated peptide antibody) were significantly elevated in Group M compared to Group C, while *W. coagulans* NCU-33 treatment effectively reduced their levels (Fig. 4A, B). Group M also exhibited splenomegaly, indicated by increased spleen indices, which was mitigated by NCU-33 administration (Fig. 4C, D). Further analysis revealed a pronounced Th17/Treg imbalance in the spleens of Group M, characterized by increased Th17 and decreased Treg cell proportions among CD4⁺ T cells. NCU-33 restored this balance (Fig. 4G,H, I) and elevated serum levels of IL-10 and TGF- β 1, indicating a shift away from a Th17-polarized state (Fig. 4E, F).

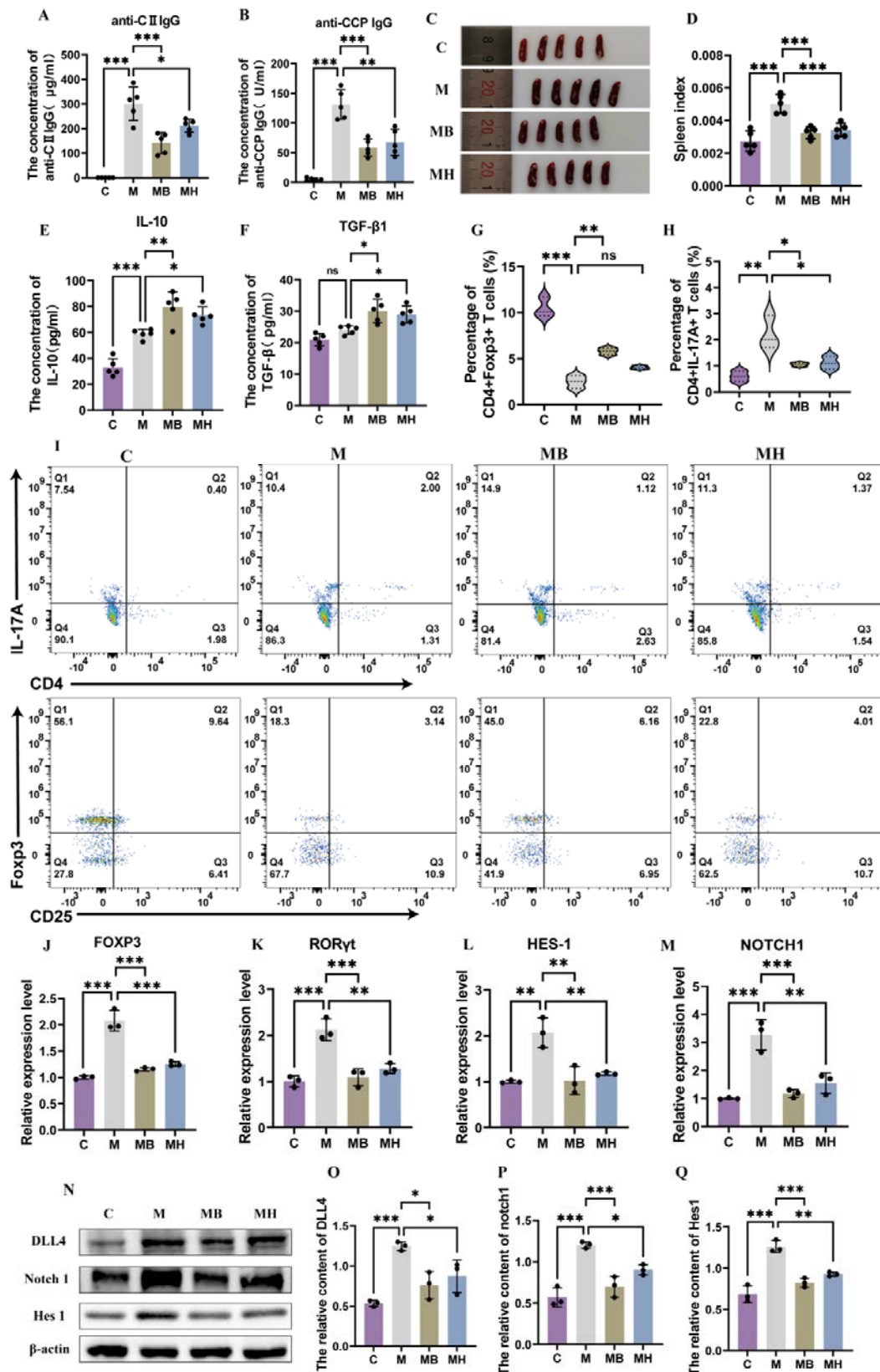


Fig.4 *W. coagulans* NCU-33 restored immune dysregulation. (A-B) Quantitative Analysis of Autoantibody (anti-CII IgG, anti-CCP IgG) Concentrations (n=5); (C-D) Spleen Morphology and Spleen Index in Mice (n=5); (E-F) Determination of serum inflammatory cytokine (IL-10, TGF-β1) concentrations in mice (n=5); (G-H) Proportion of Th17 and Treg cells among CD4⁺ T cells in the spleen (n=3); (I) Flow cytometry analysis results of Th17 and Treg cells among CD4⁺ T cells in mice spleen; (J-M) The relative expression levels of mRNAs related to Th17 and Treg cell differentiation in mouse joint (n=3); (N-Q) Detection of Notch signaling pathway-related protein expression in spleen (DLL4, Notch1, Hes1) (n=3). C: control group, M: model group, MB: methotrexate intervention group, MH: probiotic intervention group. Statistical analysis was showed using one-way repeated measures ANOVA, Data are presented as mean ± standard deviation. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Given the role of Notch signaling in promoting Th17 differentiation, western blot analysis showed upregulated DLL4, Notch1, and Hes1 in Group M spleens, which was suppressed by NCU-33 (Fig. 4N–Q).^[44, 45] Consistent with this, qPCR of ankle joints showed that abnormal expression of Th17/Treg-related transcription factors (ROR γ t and Foxp3) and Notch pathway genes (Notch1 and Hes1) was normalized by NCU-33 treatment (Fig. 4J–M). These results demonstrate that *W. coagulans* NCU-33 rectifies Th17/Treg imbalance and alleviates autoimmune responses via modulation of the Notch signaling pathway.

3.5 *W. coagulans* NCU-33 restores the gut microbiome in CIA mice

Gut microbiota dysbiosis is closely associated with RA.^[46] To assess the influence of *W. coagulans* NCU-33 on the gut microbiome in CIA mice, we conducted 16S rRNA sequencing of fecal samples. Further analysis at the phylum levels showed that Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria constituted over 90% of the microbiota (Fig. 5A). Consistent with earlier reports, CIA mice exhibited elevated Firmicutes and reduced Bacteroidetes, a shift reversed by *W. coagulans* NCU-33 treatment (Fig. 5B, C). Analysis at the genus level revealed that key genera included *Lactobacillus*, *Oscillospira*, *Bacteroides*, *Adlercreutzia*, and *Rikenella*. Group M displayed decreased *Lactobacillus* compared to group C, trends that were counteracted by NCU-33 supplementation (Fig. 5D, E).

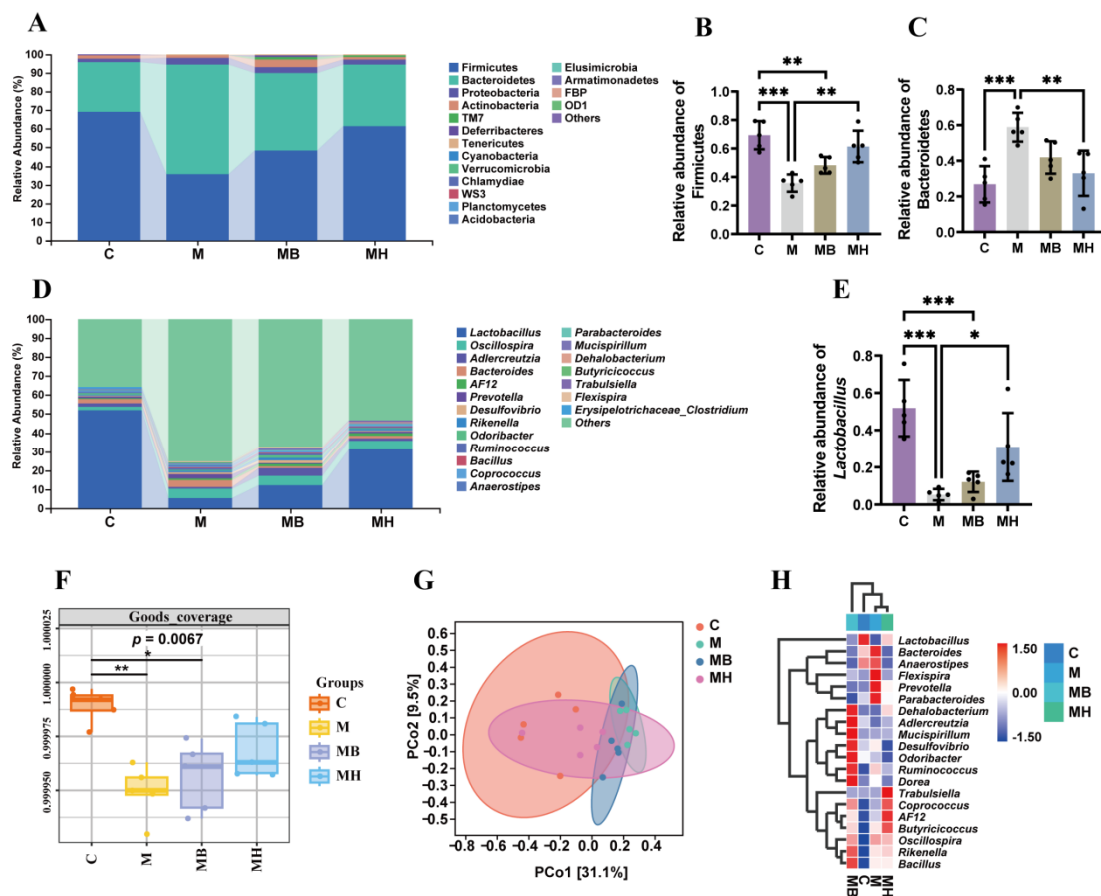


Fig.5 The amelioration of gut microbiota dysbiosis in CIA mice by *W. coagulans* NCU-33. (A)The phylum-level distribution of the gut microbiota (n=5); (B,C)The relative abundance of *Firmicutes* and *Bacteroidetes* at the phylum level (n=5); (D)The genus-level distribution of the gut microbiota (n=5); (E)The relative abundance of *Lactobacillus* at the genus level (n=5); (F) The α -diversity of gut microbiota; (G) Principal Coordinate Analysis of β -diversity indices (n=5); (H)The species composition heatmap analysis (n=5). C: control group, M: model group, MB: methotrexate intervention group, MH: probiotic intervention group. Statistical analysis was showed using one-way repeated measures ANOVA, Data are presented as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Alpha diversity (Goods coverage index) showed that microbial richness in group MH closely resembled that of group C, which suggest that *W. coagulans* NCU-33 restored the alpha diversity of the gut microbiota in CIA mice. (Fig. 5F). In addition, the results from PCoA and species composition heatmap analysis revealed significant distinctions between group M and the other groups.(Fig. 5G, H). These findings imply that *W. coagulans* NCU-33 contributes to restoring microbial balance in CIA mice.

3.6 *W. coagulans* NCU-33 alleviates intestinal barrier impairment and inflammation.

Previous studies have demonstrated that RA progression is associated not only with gut microbiota dysbiosis but also with compromised intestinal barrier function, a physiological defense that prevents the translocation of toxins and harmful substances across the intestinal epithelium.^[47] To evaluate whether *W. coagulans* NCU-33 mitigates intestinal barrier damage in collagen-induced arthritis (CIA) mice, we examined the expression of the tight junction proteins claudin-1 and occludin in colonic tissues by Western blotting. The results indicated that *W. coagulans* NCU-33 significantly restored the expression of these proteins compared with the model group (Fig. 6A–C).

Given that intestinal barrier impairment is often accompanied by inflammation, we further assessed the colonic mRNA levels of key pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) using qRT-PCR.^[48] Our data showed that *W. coagulans* NCU-33 significantly downregulated the elevated expression of these inflammatory mediators in the colons of CIA mice relative to the model group (Fig. 6D–F). Collectively, These findings indicate that *W. coagulans* NCU-33 restores intestinal barrier function and reduces inflammation.

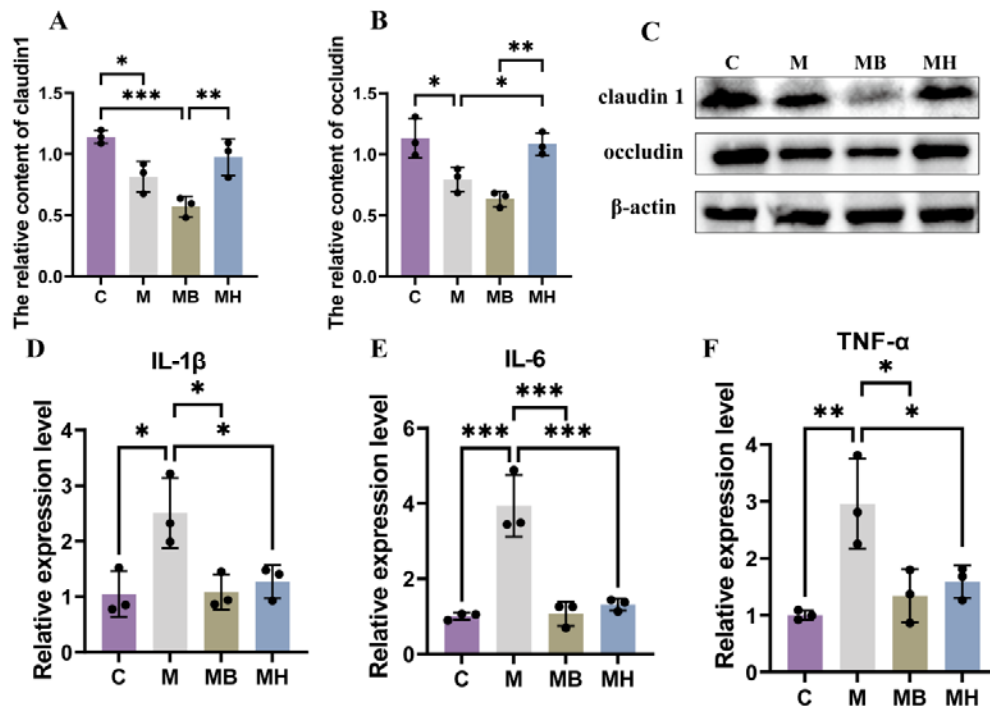


Fig.6 *W. coagulans* NCU-33 promotes the restoration of healthy intestinal barrier function. (A-C)Detection of intestinal barrier function-related (claudin 1、occludin) proteins. (D-F)The relative expression levels of inflammatory cytokines (IL-1 β 、IL-6 and TNF- α) in the colon. C: control group (n=3), M: model group (n=3), MB: methotrexate intervention group (n=3), MH: probiotic intervention group (n=3). Statistical analysis was showed using one-way repeated measures ANOVA, Data are presented as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3.7 *W. Coagulans* NCU-33: Mechanistic Insights into Ameliorating Rheumatoid Arthritis

In the CIA model, *W. coagulans* NCU-33 significantly reduces serum levels of pro-inflammatory cytokines while alleviating synovial inflammation and tissue damage via suppression of the TLR2/NF- κ B signaling pathway in joints. Concurrently, this strain corrects Th17/Treg cell imbalance by inhibiting the Notch signaling pathway and reduces autoantibody levels. Furthermore, NCU-33 reverses arthritis-associated gut microbiota dysbiosis, improving intestinal barrier function and ultimately exerting systemic therapeutic effects through the "gut-joint axis." (Fig. 7).

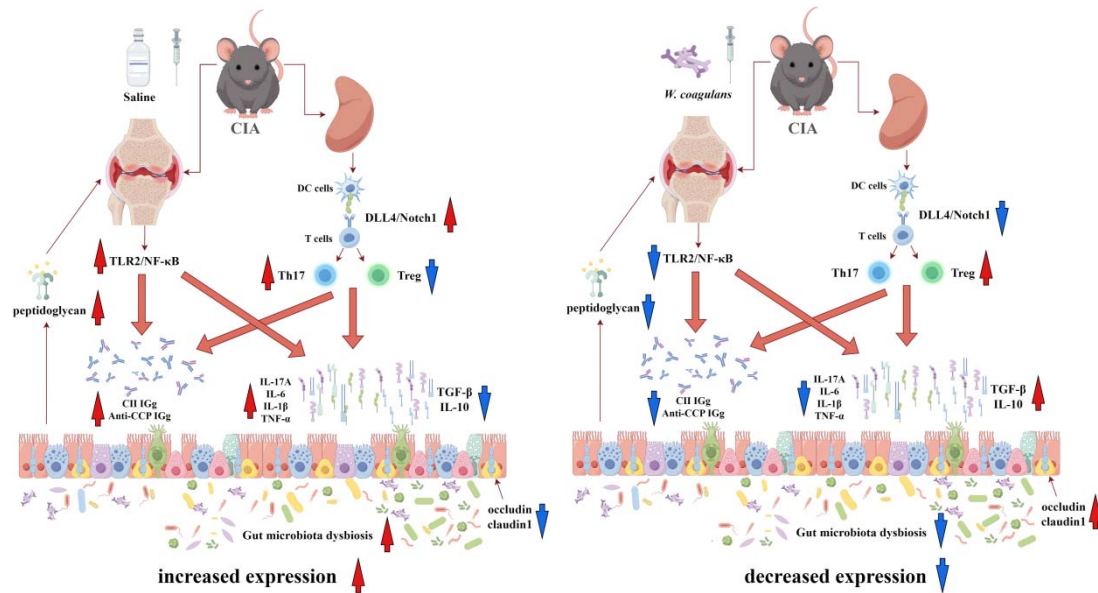


Fig.7 *W. coagulans* NCU-33 mitigates arthritis progression in CIA mice through multi-target mechanisms.

4. Discussion

RA, a complex autoimmune disease, continues to pose significant challenges in clinical management.^[49] Current treatment strategies, primarily involving non-steroidal anti-inflammatory drugs, glucocorticoids, and biologics, have achieved certain progress in controlling disease progression. However, their long-term use is often associated with considerable side effects, and the high cost of some agents considerably limits their broad application.^[50, 51] Therefore, there is a pressing need to explore novel therapeutic approaches that are safe, effective, and cost-effective. In recent years, as research into the pathogenesis of RA deepens, the role of gut microbiota in the development and progression of the disease has gained increasing attention, particularly its function in immune regulation and systemic inflammation, offering new perspectives for RA intervention.^[52, 53] Against this backdrop, probiotics have emerged as a potential adjuvant therapy for RA, capitalizing on their advantages of minimal adverse effects and low cost.^[54]

Among various probiotics, *W. coagulans* has in recent years been included in the list of probiotics approved for use in food, further confirming its safety and functional properties.^[55] This strain has been reported to possess notable anti-inflammatory and immunomodulatory capabilities.^[56] Nevertheless, research on its role in RA remains in its early stages, with the underlying mechanisms still poorly

understood.^[57] A systematic investigation into the beneficial effects of *W. coagulans* in RA would not only facilitate its industrial development as a functional strain but also provide new insights for the clinical management of RA. Based on previous clinical findings which observed that *W. coagulans* could reduce serum levels of inflammatory cytokines in RA patients,^[58] this study further employed a well-established CIA mouse model combined with molecular biology techniques. Our objective is to systematically elucidate the potential mechanisms through which this strain ameliorates RA, thereby providing a scientific foundation for its future clinical application.

In the evaluation of probiotic properties, *W. coagulans* NCU-33 demonstrates outstanding performance across multiple functional domains.^[59] The strain exhibits excellent growth characteristics and substantial tolerance to gastrointestinal stressors, including low pH and bile salts. Its spore forming capability serves as a fundamental protective mechanism,^[60] ensuring high viability during gastrointestinal transit and subsequent intestinal colonization. Beyond its resilience, *W. coagulans* NCU-33 shows broad spectrum antimicrobial activity against common intestinal pathogens, primarily through secretion of bacteriocins and lipopeptides that disrupt microbial membrane integrity.^[61] Notably, the strain possesses significant antioxidant capacity, demonstrating substantial free radical scavenging ability against DPPH, hydroxyl, and superoxide anions. This activity appears to derive from both direct production of antioxidant metabolites and potential modulation of host antioxidant pathways. Safety assessments confirm its favorable profile, with appropriate antibiotic susceptibility and absence of hemolytic activity. Regulatory approvals including GRAS status from the U.S. FDA and approval as a food strain by China's National Health Commission in 2016 further support its suitability for human consumption.^[62] Collectively, the gastrointestinal tolerance, antimicrobial efficacy, antioxidant potential, and established safety profile of *W. coagulans* NCU-33 highlight its strong potential for modulating gut microbiota and promoting intestinal health. These comprehensive properties provide a solid foundation for its development as a novel probiotic candidate and support further investigation into its potential role in managing RA.

Notably, *W. coagulans* NCU-33 shares core functional commonalities with well-studied probiotics in RA management: all target the gut-immune axis by reinforcing intestinal barrier integrity, regulating immune cell responses, and suppressing pro-inflammatory cytokines. However, NCU-33 exhibits distinct characteristics that distinguish it from *Lactobacillus* species and other probiotics. First, its spore-forming nature enables superior tolerance to gastric acid and bile salts, a trait that supports higher viability during gastrointestinal transit compared to non-spore-forming *Lactobacillus* strains.^[63] This stability is a key advantage for gut colonization, as it ensures the strain can reach the intestinal tract in sufficient quantities to exert biological effects. Second, unlike most *Lactobacillus* strains that primarily act via short-chain fatty acids or single signaling pathways,^[64] NCU-33 mediates dual regulation of the TLR2/MyD88/NF- κ B and Notch pathways. This unique mode of action addresses both local joint inflammation and systemic immune dysregulation, which is rarely reported in other probiotic strains used for RA intervention. Third, NCU-33 can exert effects at a relatively moderate dose range, a feature closely linked to its spore-forming advantage

that ensures high viability without the need for extremely high dosing. These similarities confirm the validity of gut-targeted probiotic therapy for RA, while the unique traits of NCU-33, particularly its gastrointestinal tolerance and multi-pathway regulatory capacity, underscore its potential as a strain-specific candidate for clinical translation.

The CIA model stands as one of the most extensively utilized and well-characterized experimental systems for studying RA pathogenesis and evaluating potential therapeutic interventions.^[65] This model is established by immunizing genetically susceptible animals, such as DBA/1 mice, with an emulsion of CII and adjuvant. This immunization provokes a specific autoimmune response against articular cartilage CII, ultimately leading to chronic inflammatory joint pathology that closely mirrors key features of human RA.^[66] The CIA model effectively recapitulates several critical aspects of the human disease, including activation of both humoral and cellular immune pathways, sustained joint inflammation, synovial hyperplasia, and progressive bone and cartilage destruction.^[67] To further elucidate the potential therapeutic role of *W. coagulans* NCU-33 in RA, we administered a high concentration (1×10^9 CFU/mL) of this probiotic as an intervention in a well-established CIA model using DBA/1 mice, with methotrexate serving as a positive control. Our results demonstrated that *W. coagulans* NCU-33 significantly ameliorated the arthritic phenotypes in the model mice. This improvement was evidenced by reduced arthritis scores, decreased paw swelling, and mitigation of body weight loss. Histopathological analysis further confirmed that *W. coagulans* NCU-33 intervention effectively diminished inflammatory cell infiltration and alleviated cartilage and bone erosion within the joints. The protective effects observed in our study may be attributed to the ability of *W. coagulans* NCU-33 to modulate immune responses. The CIA model is known for its immune dysregulation, often involving an imbalance between pro-inflammatory and anti-inflammatory T helper cell responses.^[68] Although the specific immunomodulatory mechanisms of *W. coagulans* NCU-33 require further investigation, its amelioration of joint inflammation and destruction in our CIA model suggests a potential role in restoring immune homeostasis. This is consistent with the recognized capacity of certain probiotic strains to influence host immunity.

Dysbiosis of the gut microbiota is increasingly recognized as a critical factor in the pathogenesis of RA, primarily through mechanisms involving molecular mimicry, impaired intestinal barrier function, and the production of pro-inflammatory metabolites.^[69-71] In particular, gut dysbiosis in RA patients is associated with elevated intestinal permeability, which promotes systemic translocation of microbial components such as LPS, endotoxin, and peptidoglycan.^[72] Peptidoglycan, a major PAMP derived from bacterial cell walls, is recognized by TLR2 expressed in synovial tissues.^[73] This interaction initiates intracellular signaling cascades, largely dependent on the adaptor protein MyD88, leading to activation of the NF- κ B pathway.^[74]

The TLR2/MyD88/NF- κ B axis holds significant biological relevance in RA pathogenesis beyond its general pro-inflammatory role.^[75] Clinically, TLR2 expression is markedly upregulated in RA synovial tissue and correlates positively with disease activity scores (DAS28) and serum levels of C-reactive protein.^[76] MyD88-dependent signaling is indispensable for inflammatory arthritis development, as MyD88-deficient

mice are resistant to both CIA and serum-transfer arthritis models. Furthermore, NF- κ B hyperactivation in RA synoviocytes creates a self-perpetuating inflammatory loop, driving not only cytokine production but also synovial hyperplasia and pannus formation.^[77] Thus, pharmacological disruption of this axis represents a biologically significant therapeutic strategy, not merely a downstream anti-inflammatory effect.

The NF- κ B signaling pathway plays a central role in RA pathogenesis by driving macrophages to secrete substantial quantities of pro-inflammatory cytokines, including TNF- α and IL-6. Furthermore, NF- κ B activation promotes autoantibody production by B cells, enhances inflammatory cell infiltration, stimulates pathological synovial fibroblast proliferation, increases the release of MMPs, and activates osteoclast differentiation.^[78] Collectively, these processes contribute to progressive synovitis and joint destruction. In the present study, intervention with *W. coagulans* NCU-33 significantly reduced serum levels of key inflammatory cytokines, including TNF- α and IL-6, in a CIA mouse model. It also downregulated the expression of destructive and chemotactic mediators such as MMP1, MMP2, and CXCL9 in joint tissues, indicating attenuation of joint tissue degradation. Further mechanistic investigation revealed that *W. coagulans* NCU-33 suppressed the expression of critical proteins within the NF- κ B pathway, specifically TLR2, MyD88, and phosphorylated p65, in local joint tissue. Suppression of the TLR2/MyD88/NF- κ B pathway likely operates through a dual mechanism rooted in gut microbiome restoration. First, by significantly increasing the abundance of *Lactobacillus* and other beneficial genera, NCU-33 intervention is poised to elevate the levels of microbiota-derived SCFAs, such as butyrate. SCFAs are well-established histone deacetylase inhibitors and ligands for specific G-protein-coupled receptors, which can directly inhibit NF- κ B activation in immune cells and exert broad anti-inflammatory effects.^[79] Second, the restoration of intestinal barrier integrity, evidenced by the upregulation of tight junction proteins, would effectively reduce the systemic translocation of immunostimulatory bacterial products like peptidoglycan.^[80] This decrease in ligand availability would consequently dampen the chronic activation of the TLR2/MyD88/NF- κ B axis in distant joints. Therefore, the therapeutic effects of *W. coagulans* NCU-33 may be mediated by a synergistic action: reducing the upstream trigger (microbial translocation) while simultaneously enhancing systemic anti-inflammatory tone (via SCFAs), ultimately leading to the downregulation of the central pro-inflammatory pathway observed in our study. This positions *W. coagulans* NCU-33 as a promising candidate for further development as an adjunctive therapy for RA.

Our study confirmed that *W. coagulans* NCU-33 suppresses the activation of the TLR2/MyD88/NF- κ B pathway in local joint tissue. This finding not only reveals a direct anti-inflammatory mechanism within the joint but also suggests the initiation of broader immunomodulatory effects, as chronic joint inflammation and systemic immune dysregulation are intricately linked in RA.

Notably, the TLR2/MyD88/NF- κ B and Notch signaling pathways exhibit extensive crosstalk in RA-related immunomodulation, forming a pro-inflammatory regulatory network that amplifies immune imbalance and joint damage.^[81] Mechanistically, NF- κ B activation can directly upregulate the transcription of Notch ligand DLL4 in dendritic cells and synovial fibroblasts—key cells involved in RA pathogenesis.

Elevated DLL4 then binds to Notch receptors on T cells, activating the Notch pathway to promote the transcription of ROR γ t, the master regulator of Th17 cell differentiation, while inhibiting Foxp3 expression and Treg cell induction.^[82] Conversely, persistent Notch pathway activation can also reinforce NF- κ B signaling by upregulating TLR2 expression and enhancing MyD88-dependent downstream cascades, creating a positive feedback loop that sustains systemic inflammation and Th17/Treg imbalance.^[83] This reciprocal crosstalk highlights that targeting either pathway alone may not fully abrogate the pro-inflammatory network, whereas simultaneous suppression of both pathways can more effectively disrupt the inflammatory amplification loop, which is critical for alleviating RA pathology. Against the backdrop of this NF- κ B/Notch crosstalk, we further investigated the immunomodulatory effects of NCU-33 at the systemic level. Immune cell imbalance is a central event in the pathogenesis of RA.^[84] In this study, we focused on the disruption of Th17/Treg balance and evaluated the immunomodulatory potential of *W. coagulans* NCU-33. A key aspect of RA pathogenesis involves the synergistic roles of ACPAs and anti-CII antibodies. ACPAs, targeting citrullinated self-proteins, serve as a highly specific serological marker for RA and actively contribute to joint inflammation and bone erosion through immune complex formation, complement activation, and Fc receptor-mediated immune cell activation. In contrast, anti-CII antibodies directly recognize type II collagen, the major structural component of articular cartilage, initiating complement activation and inflammatory cell recruitment, ultimately leading to cartilage degradation.^[85, 86] In the CIA mouse model, simultaneous assessment of both antibodies is methodologically essential. Treatment with *W. coagulans* NCU-33 reduced serum levels of anti-CII antibodies and ACPA, biomarkers indicative of disease severity, and significantly lowered the splenic index, providing preliminary evidence of systemic immunomodulation. Further flow cytometric analysis revealed a pronounced increase in the Th17/Treg ratio in CIA mice, which was effectively reversed by *W. coagulans* NCU-33 intervention. Th17/Treg imbalance is a hallmark of RA pathogenesis: Th17 cells promote a pro-inflammatory microenvironment, activate synovial fibroblasts and osteoclasts, and drive synovitis, cartilage breakdown, and bone erosion. A persistent inflammatory milieu can induce dendritic cells to express DLL4, which upregulates the key Th17 transcription factor ROR γ t via the Notch signaling pathway, thereby exacerbating the Th17/Treg imbalance.^[20, 87] Consistent with this, we observed upregulation of Notch pathway components, including DLL4, in the spleens of CIA mice, accompanied by decreased mRNA expression of the Treg master regulator Foxp3 and increased ROR γ t mRNA. *W. coagulans* NCU-33 intervention significantly reversed these alterations, indicating that its therapeutic effect is mediated, at least in part, through suppression of the Notch pathway and restoration of Th17/Treg balance.

The restoration of Th17/Treg balance and the modulation of the Notch pathway by NCU-33 may be mechanistically linked to its ability to restore gut microbial homeostasis and elevate SCFA levels. SCFAs, particularly butyrate, are potent histone deacetylase (HDAC) inhibitors. Research indicates that butyrate can specifically inhibit class I HDAC isozymes, leading to targeted histone acetylation at the Foxp3 gene promoter and subsequent enhancement of Foxp3 expression and Treg differentiation.^[88] Concurrently,

SCFAs can also influence Th17 cell differentiation through HDAC-dependent and independent pathways, contributing to a rebalanced immune milieu. The impact of SCFAs on these key transcription factors may extend to the Notch signaling pathway, which is a known regulator of lymphocyte fate. It is plausible that the HDAC inhibitory activity of SCFAs, or their signaling through G-protein coupled receptors (GPCRs) like GPR43, could modulate the expression of Notch receptors or the stability of the Notch intracellular domain (NICD), thereby attenuating the aberrant Notch activation observed in RA.^[89] Therefore, the increase in SCFA-producing bacteria induced by NCU-33 may drive these epigenetic and signaling alterations. The coordinated suppression of the Notch pathway and correction of the Th17/Treg imbalance likely represent interconnected downstream effects of a restored gut-derived metabolic landscape. This multifaceted immunomodulatory action, stemming from gut microbiota remodeling, positions *W. coagulans* NCU-33 as a promising therapeutic candidate that targets the “gut-joint axis” in RA by simultaneously engaging local immune regulation and systemic metabolic homeostasis.

Therefore, the NCU-33-induced increase in SCFA-producing bacteria, such as *Lactobacillus*, may constitute an upstream regulatory event. The resulting microbial metabolites, like SCFAs, could provide a plausible metabolic basis—through epigenetic and signaling pathways—for the downstream observations, including Notch pathway suppression, Th17/Treg rebalancing, and the attenuation of joint inflammation. Research demonstrates a close association between RA and gut dysbiosis.^[90] Significant alterations in gut microbiota composition are observed in RA patients and their high-risk relatives, characterized by increased Prevotellaceae and decreased Faecalibacterium, among others.^[91, 92] In CIA mice, dysbiosis is detectable even prior to arthritis onset, manifesting as elevated Bacteroidetes and Proteobacteria, alongside reduced Firmicutes. FMT (Fecal microbiota transplantation) from healthy donors has been shown to ameliorate arthritis in animal models and improve clinical symptoms in RA patients, suggesting microbiota modulation as a potential therapeutic strategy. Notably, FMT derived from high-risk RA individuals increases intestinal permeability in recipient mice, indicating that dysbiosis and intestinal barrier damage are interconnected and jointly contribute to RA pathophysiology.^[93] Utilizing high-throughput sequencing, this study found that *W. coagulans* NCU-33 intervention effectively restored both α - and β -diversity of the gut microbiota in CIA mice. It reversed the dysbiotic trends observed in the model, specifically counteracting the decrease in *Firmicutes* and the increase in *Bacteroidetes*. Critically, *W. coagulans* NCU-33 significantly increased the abundance of *Lactobacillus*, a primary producer of SCFAs. We speculate that this shift in microbial composition may contribute to the observed benefits by elevating SCFA levels, which are known for their anti-inflammatory and immunomodulatory properties.

The integrity of the intestinal barrier is crucial for host health.^[94] Its physical barrier function is maintained by tight junction proteins between intestinal epithelial cells, including Claudin-1, Occludin, and ZO-1. SCFAs, as key microbial metabolites, enhance the expression of tight junction proteins and restore the impaired intestinal barrier through mechanisms such as inhibition of HDAC. This study found that NCU-33 intervention significantly restored the abundance of major SCFA-producing bacterial genera. Notably, this

restoration was associated with the upregulated expression of key intestinal barrier proteins. Based on the established role of SCFAs in enhancing tight junction protein expression, we speculate that the barrier-repairing effects of NCU-33 may be partly mediated by promoting the production of beneficial metabolites such as SCFAs, a possibility that warrants future investigation. This restoration was accompanied by the upregulated expression of intestinal barrier function-related proteins, demonstrating its efficacy in repairing intestinal barrier function.

In summary, the action of *W. coagulans* NCU-33 in the CIA model presents a multi-layered, integrated mechanism (as illustrated in the Graphical Abstract). It initiates the remodeling of gut microbiota, enriching beneficial taxa and repairing the intestinal barrier. This improvement in the gut environment, potentially mediated by an increase in metabolites like SCFAs, systemically modulates host immunity, evidenced by the suppression of the Notch pathway, restoration of Th17/Treg balance, and reduction in autoantibody levels. Concurrently, its spore-forming nature may ensure sufficient viable components reach systemic circulation to directly or indirectly dampen the local TLR2/MyD88/NF- κ B inflammatory axis in the joints. These interconnected events collectively delineate a holistic picture of how NCU-33 alleviates RA via the ‘gut-joint axis’.

Although this study confirmed the arthritis-alleviating effects of *W. coagulans* NCU-33 in CIA mice, certain limitations exist. First, future research is required to elucidate the synergistic mechanisms between MTX and this probiotic in RA, providing a scientific basis for its clinical application as an adjunctive therapy. Second, the CIA model has inherent limitations, such as incomplete recapitulation of human RA pathology due to species differences and dependence on specific animal strains.^[95] Moreover, the current study has methodological constraints. Our analysis of the gut microbiota relied solely on 16S rRNA gene sequencing, which offers genus-level resolution but lacks functional insights. The absence of metabolomic data prevents us from directly quantifying microbial metabolites, such as SCFAs, and from establishing a definitive causal link between the observed microbial shifts and the host’s metabolic and immunomodulatory phenotypes. This also leaves unresolved the fundamental question of whether the microbial alterations are a driving cause or a secondary effect of disease modulation, thereby limiting a mechanistic interpretation of the “gut-joint axis.” To address these gaps and to pinpoint the specific bacterial components or metabolites responsible for the therapeutic effects, future work should integrate targeted metabolomics and functional approaches such as FMT experiments. In summary, while this work supports the therapeutic potential of *W. coagulans* NCU-33 in experimental arthritis, future research must continue to unravel the complex dialogue within the “microbiota-gut-joint axis” to fully elucidate its underlying mechanisms.

5. Conclusions

In this study, we employed a CIA mouse model to investigate the therapeutic potential of *W. Coagulans* NCU-33 in RA. Our results demonstrate that *W. coagulans* NCU-33 exhibits favorable probiotic properties and significantly alleviates arthritic symptoms in CIA mice. At the mechanistic level, this study reveals for

the first time that this strain mitigates local joint inflammation and tissue damage by suppressing the TLR2/NF- κ B signaling pathway. Furthermore, *W. coagulans* NCU-33 restores immune balance by modulating the Notch signaling pathway, thereby rectifying the Th17/Treg imbalance closely associated with RA. Additionally, *W. coagulans* NCU-33 shows remarkable capacity to modulate the gut microbiota, restore microbial composition, enhance intestinal barrier integrity, and reduce gut inflammation. In summary, this study elucidates the multi-faceted mechanisms through which *W. Coagulans* NCU-33 ameliorates RA. We demonstrate that its therapeutic effect is mediated through a coordinated action that concurrently engages the gut ecosystem, systemic immunity, and local joint inflammation. By delineating this integrative mechanism through animal models and molecular analyses, our work provides mechanistic support for the “gut-joint axis” in probiotic intervention against RA. Furthermore, it contributes preclinical data to support the continued development of *W. coagulans* NCU-33 as a safe and effective adjuvant therapeutic strategy.(Fig. 7).

Data availability

The data used to support the findings of this study are available from the corresponding author upon request, and the raw 16S rRNA sequencing data generated in this study have been deposited in the NCBI database under accession number PRJNA1357298 <https://www.ncbi.nlm.nih.gov/>.

Conflicts of interest

There are no conflicts to declare.

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