

Baicalin nanoengineered probiotics synergistically suppress IL-6/STAT3 signaling and remodel gut microbiota for Crohn's disease therapy

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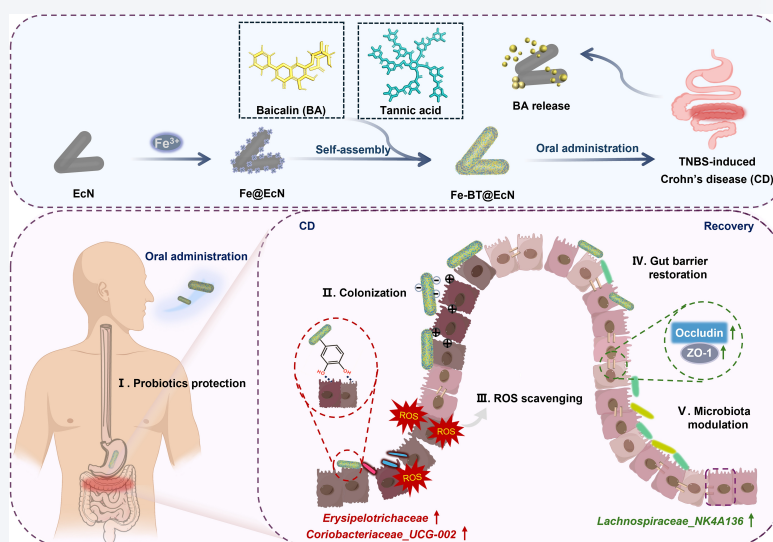


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ABSTRACT: Crohn's disease (CD) is chronic inflammatory bowel disorder characterized by transmural inflammation, intestinal barrier disruption, and gut microbiota dysbiosis. Although probiotics show therapeutic potential for CD through modulating the intestinal flora, conventional oral formulations are constrained by low survival rates in the gastrointestinal tract and limited efficacy. Inspired by the metal-coordinating property and anti-inflammatory effect of baicalin (BA), the nanoengineered probiotics Fe-BT@EcN are developed through synergy with tannic acid and ferric ions, demonstrating enhanced resistance to adverse gastric environment as well as improved intestinal adhesion and colonization. Notably, as confirmed by network pharmacology and molecular docking studies,

BA targets dual therapeutic pathways in CD intervention to elevate antioxidant and anti-inflammatory activities through regulation of reactive oxygen species (ROS) levels and inhibition of the IL-6/STAT3 signaling pathway. In a murine 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced CD model, Fe-BT@EcN exhibited synergistic therapeutic efficacy by alleviating colonic inflammation, restoring epithelial barrier function, and reestablishing microbial homeostasis. This study provides a feasible strategy to develop multifunctional oral microecologies for CD treatment through leveraging different probiotics and natural bioactive small molecules.

KEYWORDS: nanoengineered probiotics, baicalin, IL-6/STAT3 signaling, gut microbiota, Crohn's disease (CD)



1 Introduction

Crohn's disease (CD), a chronic inflammatory bowel disorder, has affected over 3.1 million individuals globally and demonstrated a

200% incidence surge in emerging economies during the first two decades of the 21st century [1–4]. Characterized by transmural inflammation, CD induces severe intestinal mucosal damage that impairs barrier function, thereby facilitating antigen penetration and enterocyte injury coupled with gut microbiota dysbiosis [5–7]. These pathological changes subsequently exacerbate inflammatory response and intestinal barrier destruction, establishing a self-perpetuating vicious cycle that compromises clinical management of CD [8–10]. The gut microbiota, as a complex ecosystem regulating metabolic homeostasis and immune responses, is

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emerging as a critical therapeutic target for CD [11–14]. Notably, the probiotic intervention could promote the restoration of healthy gut microbiota, demonstrating potential therapeutic efficacy against CD [15–18]. Meanwhile, the orally administered probiotics have gained significant momentum in recent years, driven by their superior patient compliance and established safety profiles [19–22]. However, these probiotics are far from perfect in terms of poor bioavailability, limited stability, and single-agent potency in the complicated context of CD [23–26]. Consequently, there is a pressing demand for engineered and multifunctional probiotics to enhance the therapeutic effects on CD.

Current therapeutic approaches for CD encompass corticosteroids, immunosuppressants such as azathioprine and methotrexate, and biologic therapies including anti-tumor necrosis factor- α antibodies [27–29]. While these regimens have proved clinical efficacy in symptom remission and mortality reduction, their long-term application is frequently hindered by acquired drug resistance and dose-limiting toxicities [30–33]. Furthermore, phytochemical-derived bioactive molecules are promising alternatives, benefiting from their superior biocompatibility and potent regulation of redox homeostasis [34–37]. Our previous study demonstrated the excellent therapeutic effect of baicalin (BA) on disuse-induced osteoporosis and bone defect regeneration through suppression of the Wnt/ β -catenin signaling pathway [38].

In addition, oral administration of BA exerted significant remission on dextran sulfate sodium-induced ulcerative colitis through suppression of the NF- κ B signaling pathway [39]. However, its clinical translation faces substantial pharmacokinetic challenges, as BA is classified as a Biopharmaceutics Classification System (BCS) Class IV compound exhibiting low aqueous solubility, membrane permeability, and oral bioavailability [40–42]. The conventional administration strategy for BA predominantly depends on repeated gavage dosing that shows limited lesion accumulation, and the therapeutic potential in CD remains poorly investigated [43–45]. Therefore, the effective integration of BA on probiotics holds great significance for combined therapy of CD.

In this study, we designed nanoengineered probiotics Fe-BT@EcN based on *Escherichia coli* Nissle 1917 (EcN), a non-pathogenic intestinal bacterium, and the coordination-driven assembly of BA and tannic acid with ferric ions (Fig. 1). The formed metal-phenolic network provided effective protection of probiotics against gastrointestinal stresses during oral administration. Through network pharmacology and molecular docking analysis, the BA's action mechanism for CD was elucidated for its dual regulatory effects on reactive oxygen species (ROS) generation and IL-6/STAT3 signaling pathway inhibition, thereby enhancing cellular antioxidant defenses and suppressing inflammatory cascades. Meanwhile, Fe-BT@EcN efficiently

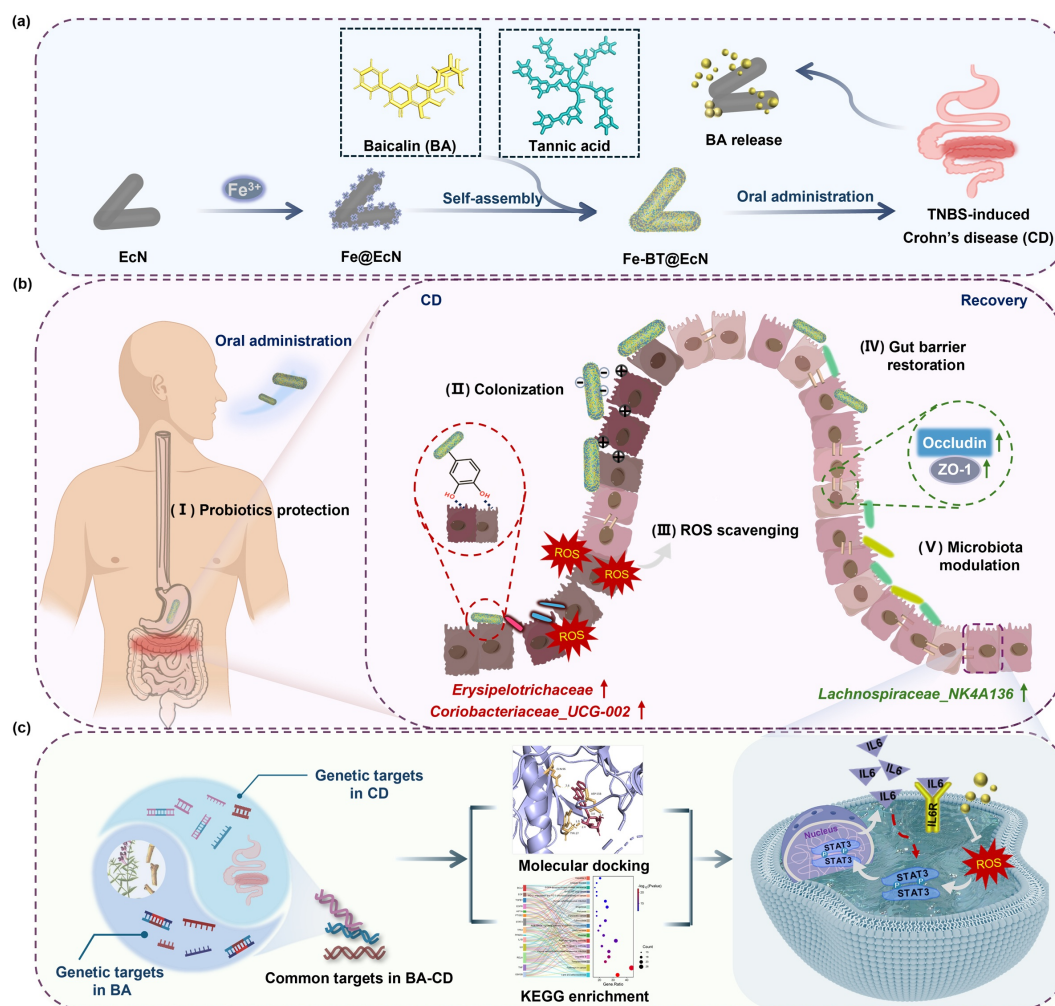


Figure 1 (a) Schematic illustration of the preparation of nanoengineered probiotics Fe-BT@EcN. (b) Treatment mechanism of Fe-BT@EcN for CD after oral administration. (c) Network pharmacology analysis for biological mechanism of BA in treating CD.

eliminated ROS within epithelial cells *in vitro* and guarded the cells from oxidative stress damage. *Ex vivo* fluorescence imaging revealed that Fe-BT@EcN exhibited robust gastric resistance and enhanced intestinal adhesion and colonization. In a murine 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced CD model, Fe-BT@EcN gavage demonstrated significant therapeutic efficacy with a 21.3% enhancement in body weight, a 67.2% reduction in disease activity index, and a 22.3% prolongation in colonic length, as a result of alleviating inflammatory responses, restoring intestinal barrier integrity, and rebalancing gut microbiota composition.

2 Experimental section

2.1 Materials

The used chemicals were collected as below: BA (molecular weight 446.37, Shanghai U-sea Biotech Co., Ltd.), tannic acid (molecular weight 1701.20, Shanghai aladdin Biochemical Technology Co., Ltd.), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Shanghai Titan Technology Co., Ltd.), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, molecular weight 293.17, Sigma-Aldrich Corporation), and kanamycin (Beijing Solarbio Science & Technology Co., Ltd.). The antibodies, anti-MPO, anti-IL6, anti-ZO-1, and anti-Occcludin, were obtained from Wuhan Servicebio Biotechnology Co., Ltd. ROS assay kit and Calcein/PI cell viability/cytotoxicity assay kit were sourced from Shanghai Beyotime Biotechnology Co., Ltd. Simulated intestinal fluid (SIF), simulated gastric fluid (SGF), and simulated colonic fluid (SCF) were formulated following the guidelines of the United States Pharmacopeia, and were sterilized by filtration through a 0.22-micron membrane filter prior to use.

2.2 Bacterial strains and plasmids

EcN was purchased from China General Microbiological Culture Collection Center (GMCC, China) and cultivated in LB liquid medium overnight (220 rpm, 37 °C) before each experiment. The plasmid pBBR1MCS2-Tac-mCherry kanamycin was purchased from domestic supplier and used according to manufacturers' instructions.

2.3 Preparation of Fe-BT@EcN

EcN was cultured in LB broth at 37 °C under shaking condition, followed by three rinses with phosphate-buffered saline (PBS, pH 7.4). EcN (5×10^8 CFU) was resuspended in precooled PBS (900 μL), and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5 mg/mL, 50 μL) was added to the suspension and mixed thoroughly. Afterwards, BA (1.25 mg/mL, 50 μL), tannic acid (1.25 mg/mL, 50 μL), or BA/tannic acid (1.25 mg/mL, 1.25 mg/mL, 50 μL) were respectively added to the suspension with vortex mixing to obtain Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN.

2.4 Characterization of Fe-BT@EcN

The morphology of Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN was examined using scanning electron microscope (SEM, Zeiss, Germany) and transmission electron microscopy (TEM, Hitachi, Japan). The bacterial solution was fixed with 4% paraformaldehyde and then drip-added to the silicon wafer and copper mesh, respectively. After drying completely, the bacteria were respectively placed in 20%, 40%, 60%, 80%, 90%, and absolute ethanol for 30 min for gradient dehydration. Then, the samples on the silicon wafer were air-dried and gold sprayed before observation by SEM.

The copper mesh samples were allowed to air dry and examined by TEM. In addition, the sizes and zeta potentials of Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN were measured with a nanoparticle size analyzer (Malvern Zetasizer Nano ZS, UK).

2.5 Growth curves

EcN, Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN (2×10^8 CFU) were separately resuspended in liquid medium, added to a 96-well plate, and incubated at 37 °C. The absorbance at 600 nm was measured continuously every 30 min for 8 h using a microplate reader (TECAN, Switzerland). The growth curves were plotted based on the absorbance and time. Following a similar procedure, the growth curve of EcN was measured with or without baicalin (0–200 $\mu\text{g/mL}$) for 8 h.

2.6 *In vitro* protective effect

EcN, Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN (10^9 CFU) were separately resuspended into SGF (pH 2.0) and SIF (pH 6.8), followed by incubation on a shaker at 37 °C for preplanned time points. The gradiently diluted bacteria were spread onto LB agar plates, and colony counts were determined after incubation for 12 h.

2.7 *Ex vivo* mucoadhesion

The freshly obtained colonic tissue was sectioned and inverted to expose the mucosal surface. Each tissue was immersed in the bacterial suspension containing the same amount of EcN, Fe-BA@EcN, Fe-TA@EcN, or Fe-BT@EcN (5×10^8 CFU/mL) with mCherry expression, followed by shaking at 20 rpm/min for 1 h. After transferring into PBS (10 mL) and washing twice to remove the unattached bacteria, the colon tissues were imaged using IVIS Lumina Series III (PerkinElmer, USA) to evaluate the mucoadhesive ability of bacteria.

2.8 *In vivo* mucoadhesion

All animal experimental procedures adhered to the animal experimentation guidelines of Northwestern Polytechnical University and were ratified by the University's Animal Ethics Committee. C57BL/6 mice (female, 6–7 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed in a SPF animal laboratory with room temperature of 25 ± 2 °C, humidity of $55\% \pm 10\%$ and 12 h of alternating light and dark, and subjected to adaptive growth for a week. Mice were randomized to receive gavage of EcN, Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN (1×10^9 CFU, expressing mCherry, kanamycin resistance, $n = 3$) under anesthesia after 12 h of starvation treatment. At 2, 4, 24, 72, and 96 h of intragastric administration, the mice were euthanized, and the gastrointestinal tissues were obtained for *in vivo* imaging. In addition, the tissues and contents of stomach, small intestine, cecum, and colon were homogenized, diluted in PBS, and plated on solid LB agar plates for bacterial quantification.

2.9 Prediction of disease and drug targets

Crohn's disease-related targets were searched in six online databases including DrugBank database, TTD database, DisGeNET database, GeneCards database, OMIM database, and PharmGKB database. To identify potential targets for BA, the following databases were searched, Swiss Target Prediction database, TCMSP database, Pharmmapper database, Stitch database, and CTD database.

2.10 Construction of the protein-protein interaction (PPI) network, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

The identified genes that serve as both therapeutic targets and disease-associated targets were uploaded to the STRING database. The database parameters were configured for the species "Homo sapiens" (human), with the interaction confidence score threshold set above 0.7 on a scale from 0 to 1. The Cytoscape 3.8.2 software was then utilized to construct a network highlighting potential key targets. Through the Metascape database, we conducted both Gene Ontology (GO) enrichment analysis, encompassing biological processes (BP), molecular functions (MF), and cellular components (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the selected gene set. The primary signaling pathways were identified through KEGG enrichment to elucidate the potential biological mechanisms underlying BA's therapeutic effects in Crohn's disease.

2.11 Molecular docking verification

The crystal structure of core targets in the topological analysis was downloaded from the PDB database, and the core ligand structure file for BA was obtained from the PubChem database and translated into a 3D structure using Chemdraw software. Subsequently, a docking grid box was generated using AutoDockTools to forecast the binding affinities between BA and the core genes. Thereafter, molecular docking simulations were conducted employing AutoDock Vina. For the visualization of the docking results and their interactions, PyMOL software was utilized. The URLs for all databases are provided in Table S1 in the Electronic Supplementary Material (ESM).

2.12 *In vitro* antioxidant activities

The Rat Small Intestine Crypt Epithelial Cells (IEC-6) were purchased from ATCC and cultured in DMEM-H medium. To detect intracellular oxidative stress, IEC-6 cells were incubated in H₂O₂ (50 μ M) containing medium for 12 h, followed by culturing with BA (5 μ M), tannic acid (5 μ M), or BA (5 μ M)/tannic acid (5 μ M) for 12 h. The cells were then stained with ROS responsive fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), for 30 min, and observed by CLSM under excitation wavelength of 488 nm. To further test the antioxidant activity, the cells were treated with H₂O₂ (0.6 mM), H₂O₂ (0.6 mM) + FeSO₄ (2 μ M), xanthine (0.2 mM) + XO (0.1 U), with or without Fe-BT@EcN for 6 h. After washing with PBS, the cells were stained with Calcein-AM/PI for 15 min and visualized on CLSM.

2.13 Western blotting analysis

The total protein from IEC-6 cells was isolated using RIPA lysis buffer fortified with protease (1%) and phosphatase inhibitors, and the protein concentration was determined using the BCA protein assay kit. Subsequently, protein samples were resolved by SDS-PAGE and electro-transferred onto PVDF membranes, which was placed in a blocking box containing blocking solution and incubated for 2 h to occlude non-specific sites. The diluted primary antibody was added to the antibody incubation box to ensure full contact between the PVDF membrane and the primary antibody, followed by overnight incubation at 4 °C. After incubating with HRP-labeled secondary antibody, the membranes were subjected to chemiluminescence detection by ChemiDoc MP (Bio-Rad) Imaging System.

2.14 Treatment of TNBS-induced CD

The C57 female mice (6 weeks old) were subjected to adaptive growth for one week, followed by the pre-sensitization experiments. The back of mice was depilated and TNBS (1%, 150 μ L) was evenly applied to the depilated position and air-dried. The mice were weighed and anesthetized after 7 days, and TNBS (2.5%, 100 μ L) was slowly injected into the rectum of mice followed by holding upside down for about 1 min. Disease activity index (DAI) score was used to determine the successful establishment of animal models. The mice were randomly allocated into seven groups ($n = 5$ for each group), including control group, TNBS group, EcN group, Fe-BA@EcN group, Fe-TA@EcN group, Fe-BT@EcN group, and i.g.BA group. In the control group, healthy mice were grown freely and gavaged with PBS, while the other groups were treated against the mice with probiotics CD. The amount of EcN in each group was kept the same (10⁹ CFU) and resuspended in PBS. The gavaged BA in the i.g.BA group was 0.8 mg per mouse, which was consistent with the amount of BA in the Fe-BT@EcN group. The mice in each group were given gavage every other day from the day of modeling, and the body weight, fecal consistency, and the presence of fecal blood were monitored from day 1 to day 7. DAI was calculated by averaging the scores of the three parameters: stool consistency (scored 0–4, with 0 indicating normal and 4 indicating watery diarrhea), fecal bleeding (scored 0, 2, or 4, with 0 for no blood, 2 for occult blood, and 4 for visible bleeding), and weight loss (scored 0–4, with 0 for $\leq 1\%$ loss and 4 for $> 15\%$ loss). Additionally, the length and weight of the colons were measured after the mice were euthanized.

2.15 Evaluation of therapeutic efficiency

The collected colonic tissues were fixed with 4% paraformaldehyde and subjected to H&E and immunohistochemistry staining. For antigen retrieval, the colon tissue sections were treated with pepsin solution at 37 °C for 10 min, followed by blocking in a blocking buffer at ambient temperature for 1 h. The sections were then incubated with primary antibodies at 4 °C overnight, including anti-MPO, anti-IL-6, anti-p-STAT3, anti-ZO-1, and anti-Occcludin. Following three washes with PBS, immunohistochemical staining was conducted utilizing a horseradish peroxidase-streptavidin detection system. In the case of immunofluorescence analysis, the sections were subjected to incubation with secondary antibodies conjugated to FITC or Cy3. After three rinses with PBS, the sections were treated with 4',6-diamidino-2-phenylindole (DAPI, 5 μ g/mL) at room temperature for 1 h under dark. The sections were imaged by a fluorescence microscope, and the quantitative fluorescence analysis was performed with Image-J software.

2.16 Sequencing analysis

Upon euthanasia, fecal samples were gathered, flash-frozen in liquid nitrogen, and stored in a -80 °C freezer. To profile the gut microbiota, genomic DNA was extracted from fecal samples. The V3-V4 hypervariable regions of the 16S rRNA gene were then amplified and subjected to paired-end sequencing on an Illumina platform. The generated raw sequencing data were processed using the QIIME 2 pipeline. The feature sequences were taxonomically annotated using the SILVA reference database.

2.17 Statistical analysis

All quantitative results were presented as mean $\pm$ standard deviation (SD). Statistical significance was determined by Student's t-test and one-way analysis of variance (ANOVA). Differences

between groups of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant.

3 Results and discussion

3.1 Preparation and characterization of Fe-BT@EcN

To prepare Fe-BT@EcN, EcN was engineered with BA, that had negligible effect on the probiotic proliferation (Fig. S1 in the ESM), and tannic acid in assistance of Fe^{3+} through the metal-phenolic coordination interaction. Meanwhile, EcN modified only with BA or tannic acid with the same procedure was defined as Fe-BA@EcN or Fe-TA@EcN, respectively (Fig. S2 in the ESM). Scanning electron microscope (SEM) and transmission electron microscope (TEM) were employed to characterize the surface morphology of Fe-BT@EcN, revealing the obvious nanocoating formation on the EcN (Fig. 2(a)). The dynamic light scattering measurement indicated the size of Fe-BT@EcN increased by 281.41 nm, and the zeta potential decreased from -9.05 to -15.21 mV as compared to those of EcN (Figs. 2(b) and 2(c)). Fe-BT@EcN and EcN showed similar photos and amounts of spread plates as well as comparable proliferation profiles in LB medium, which confirmed the nanocoating had negligible influence on EcN bioactivity (Fig. 2(d) and Fig. S3 in the ESM). Moreover, the BA loading amount on Fe-BT@EcN was $73.4 \mu\text{g}/10^8$ CFU as analyzed by UV-vis absorption spectra (Fig. S4

in the ESM). To evaluate the gastrointestinal viability, Fe-BT@EcN was exposed to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (Figs. S5 and S6 in the ESM). After incubation in SGF for 60 min, EcN was completely inactivated, while Fe-TA@EcN demonstrated remarkable acid resistance. Notably, Fe-BT@EcN exhibited an 18.30-fold higher survival rate than Fe-TA@EcN, which might be attributed to the formation of dual metal-phenolic coordination network (Figs. 2(e) and 2(g)). In addition, Fe-BT@EcN maintained similar viability to EcN upon exposure in SIF for 4 h (Figs. 2(f) and 2(g)), and demonstrated enhanced survival rate even after subjection to the H_2O_2 -containing simulated inflammatory colonic fluid for 2 h (Fig. 2(h) and Fig. S7 in the ESM). These results indicated the excellent protective effect of nanocoating for Fe-BT@EcN in the unfavorable environment of gastrointestinal tract.

3.2 Mucoadhesion and biodistribution of Fe-BT@EcN in the gastrointestinal tract

To evaluate the mucoadhesive capability, Fe-BT@EcN was prepared by genetically engineering EcN with the mCherry fluorescent reporter system through pBBR1MCS2-Tac-mCherry plasmid transformation, and incubated with the freshly excised colonic tissues. The stereo microscope image revealed distinct black adhesive of Fe-BT@EcN on the colon surface, and its quantitative fluorescence intensity was 1.10-fold higher compared to that of EcN

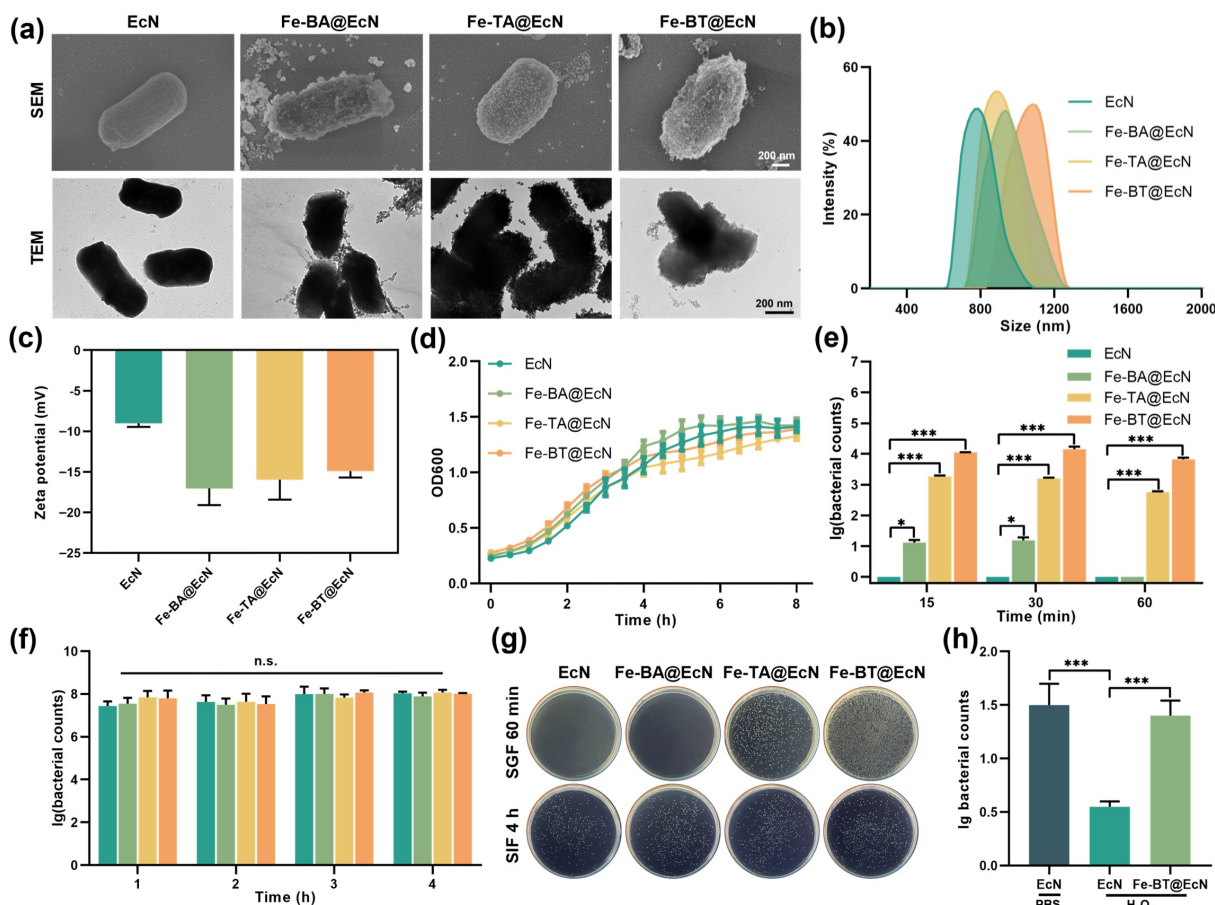


Figure 2 (a) SEM and TEM images, (b) particle size, (c) zeta potential, and (d) growth curves of EcN, Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN. (e) The probiotic amounts after incubation in SGF for 15, 30, and 60 min. (f) The probiotic amounts after incubation in SIF for 1, 2, 3, and 4 h. (g) The spread plates of probiotics after incubation in SGF for 60 min and SIF for 4 h. (h) The amounts of EcN and Fe-BT@EcN after incubation in H_2O_2 -supplemented SCF for 2 h. $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and n.s.: no significance.

(Fig. S8 in the ESM). To further assess the biodistribution in the gastrointestinal tract, Fe-BT@EcN was orally administered to mice. After pre-determined timepoints, the gastrointestinal tract was collected for *in vivo* fluorescent imaging or probiotic quantification (Fig. 3(a)). At 2 h, the Fe-BT@EcN group revealed the maximum probiotic amount in the gastric content and tissue (Fig. S9 in the ESM). Similarly, the highest fluorescent signals were observed in the intestine at 2, 4, 24, 72, and 96 h for the Fe-BT@EcN group, with a 1.56-fold and 2.85-fold increase than the EcN group at 72 and 96 h (Fig. 3(b)). In particular, the significantly enhanced colonization of Fe-BT@EcN was detected in the colon at 24 and 72 h in contrast to the EcN, Fe-BA@EcN, or Fe-TA@EcN group, which was attributed to the strong interaction between the polyphenol on the nanocoating and the colonic mucoproteins (Figs. 3(c) and 3(d)). These results revealed that the dual metal-phenolic network endowed Fe-BT@EcN with superior resistance against the harsh gastric environment and the outstanding mucoadhesion and colonization in the intestine of Fe-BT@EcN upon oral administration.

3.3 Network pharmacology analysis of BA in CD

To systematically investigate the therapeutic mechanism of BA in CD, we employed a multi-database integration strategy. A total of 2035 CD-related targets were curated from six authoritative databases (TTD, DrugBank, PharmGkb, OMIM, GeneCards, and DisGeNET) through standardized data mining protocols (Fig. 4(a)). Concurrently, 257 putative targets of BA were identified through pharmacophore-based virtual screening across five specialized platforms (SwissTargetPrediction, STITCH, PharmMapper, CTD, and TCMSP) (Fig. 4(b)). Intersection analysis revealed 78 common

targets, suggesting their potential as key mediators of BA in the CD treatment (Fig. 4(c) and Table S2 in the ESM). To characterize the protein interaction landscape, a protein-protein interaction (PPI) network was constructed using STRING database and visualized through Cytoscape (Fig. 4(d) and Fig. S10 in the ESM). The gradient color scheme reflected node degree centrality, with darker hues indicating higher network connectivity. MCODE cluster analysis identified two functionally distinct modules, highlighting STAT3, IL6, ALB, and TNF- α as hub nodes with potential therapeutic significance (Fig. 4(e)). Triangulation of three centrality metrics (degree, closeness, and betweenness) showed 15 core targets, including HIF1A, BCL2, TP53, TGFBI, EGFR, PTGS2, ALB, STAT3, IL18, PPARG, TNF, EGF, RELA, IL6, and GSK3B, which were prioritized for subsequent mechanistic investigation (Fig. 4(f)). Metascape platform-based Gene Ontology (GO) enrichment analysis revealed BA's predominant involvement in biological processes, cellular components, and molecular functions, including leukocyte migration regulation, cell-cell junctions, and IL-6 production regulation, respectively (Fig. 4(g)). KEGG pathway analysis demonstrated significant enrichment in IL-6/STAT3 signaling, with both IL-6 and STAT3 participating in thirty of the top 20 pathways (Fig. 4(h) and Fig. S11 in the ESM). Notably, the IL-6/STAT3 axis emerged as a critical mediator, given its established role in CD-associated mucosal inflammation and immune dysregulation. KEGG enrichment analysis also revealed the involvement of signaling pathways associated with ROS regulation, including the PI3K-Akt and HIF-1 signaling pathway. Moreover, structural validation of target engagement molecular docking simulations revealed favorable binding between BA and STAT3

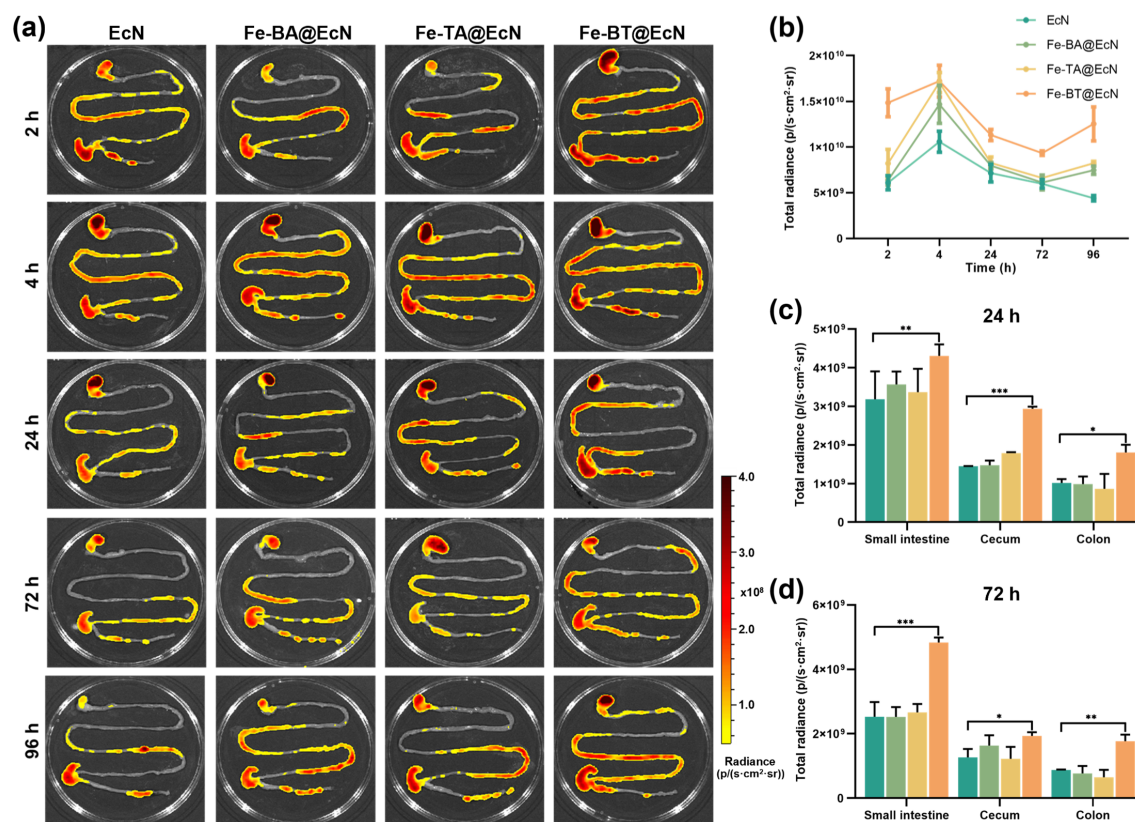


Figure 3 (a) *Ex vivo* fluorescence images of EcN, Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN in the gastrointestinal tract after oral administration. (b) The corresponding total fluorescence intensity at 2, 4, 24, 72 and 96 h. (c) and (d) The total fluorescence intensity of EcN, Fe-BA@EcN, Fe-TA@EcN, and Fe-BT@EcN in the small intestine, cecum, and colon at 24 and 72 h. $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and n.s.: no significance.

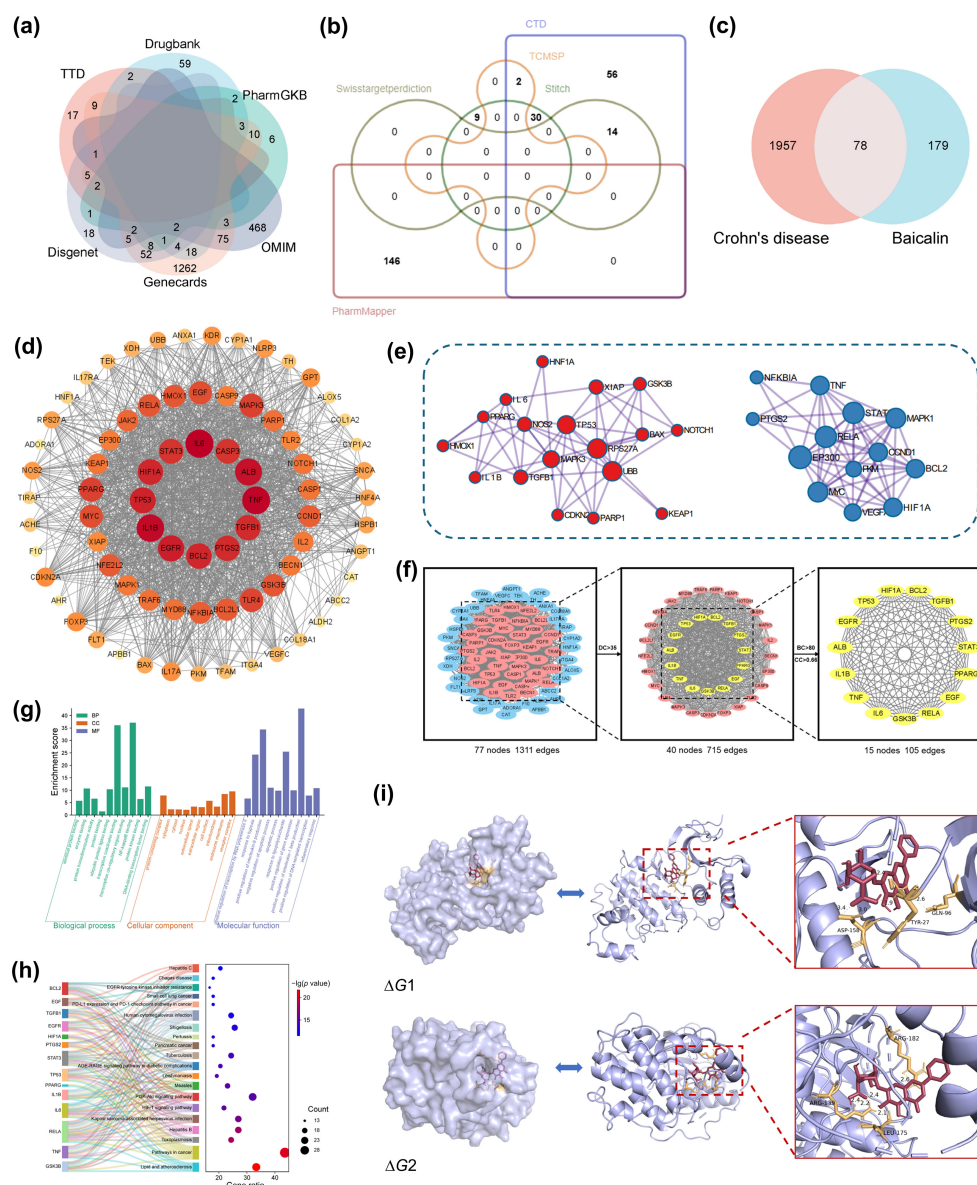


Figure 4 (a) and (b) Venn diagram of BA and CD targets. (c) Venn diagram of common targets of BA-CD. (d) PPI network interaction diagram. (e) Screening key targets by CytoNCA plugin. (f) Screening of core targets. (g) and (h) GO enrichment and KEGG enrichment analysis of common targets of BA-CD. (i) Molecular docking visualization of BA with STAT3.

($\Delta G1 = -7.9$ kcal/mol, $\Delta G2 = -7.7$ kcal/mol), with key interactions observed at the SH2 domain (Fig. 4(i)). The stable binding network suggested plausible inhibition of STAT3 phosphorylation, potentially disrupting its nuclear translocation and downstream pro-inflammatory signaling.

3.4 Intracellular anti-oxidative stress of BA and ROS regulation by STAT3 signaling

To evaluate the antioxidant efficacy of BA, the cell viability assay was conducted on IEC-6 cells to determine the optimal concentration of 5 μ M for BA administration (Fig. 5(a)). As indicated by the ROS fluorescent probe, DCFH-DA, the addition of exogenous H_2O_2 significantly increased intracellular ROS levels, while treatment with BA markedly reduced ROS levels (Figs. 5(b) and 5(c)). Furthermore, the cells were treated with H_2O_2 , $FeSO_4 + H_2O_2$, and xanthine + XO to mimic the elevated oxidative stress

levels induced by H_2O_2 , $\cdot OH$, and $O_2\cdot$, followed by incubation with Fe-BT@EcN and staining with calcein-AM/propidium iodide (PI). As shown in Fig. 5(d), the treatment with H_2O_2 , $FeSO_4 + H_2O_2$, and xanthine + XO resulted in intense red fluorescence in cells, indicating widespread oxidative damage. However, the supplementation with Fe-BT@EcN specifically alleviated oxidative stress to preserve cellular viability, as evidenced by intracellular green fluorescence. These results proved that BA could effectively protect epithelial cells from oxidative damage. As the activation of p-STAT3 promotes the production of inflammatory cytokines, such as IL-6 and TNF- α , to exacerbate the inflammatory response, we explored the interaction between BA and the STAT3 expression (Figs. 5(e)–5(h)). LPS increased the p-STAT3 level in cells, while the treatment with ROS scavenger, N-acetylcysteine (NAC), and BA significantly reversed the trend. These results suggested that the action mechanism of BA involved eliminating ROS and suppressing p-STAT3 activation, which could reduce the

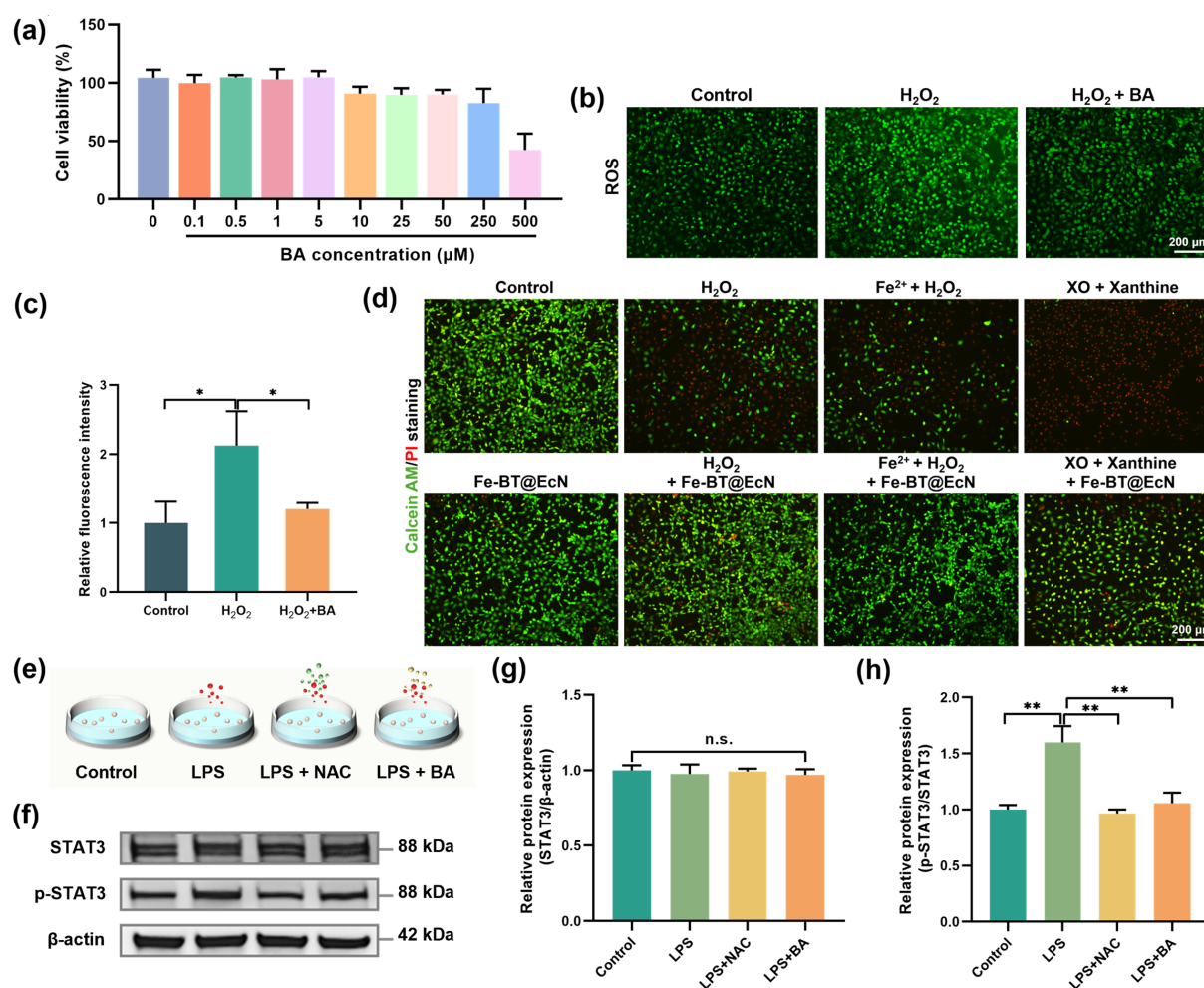


Figure 5 (a) The cell viability of IEC-6 cells under different BA concentrations. (b) and (c) The fluorescence images and relative fluorescence intensity of IEC-6 cells upon treating with H₂O₂ or H₂O₂ + BA and staining with DCFH-DA dye. (d) Calcein-AM/PI staining images of IEC-6 cells treated with H₂O₂, Fe²⁺ + H₂O₂, and XO + Xanthine in the absence or presence of Fe-BT@EcN. (e)–(h) The expression patterns of STAT3 and p-STAT3 proteins in IEC-6 cells after incubation with LPS, LPS + NAC, and LPS + BA by western blot. $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.: no significance.

production of inflammatory cytokines and alleviate persistent inflammatory responses.

3.5 Synergistic therapy of Fe-BT@EcN against TNBS-induced CD

2,4,6-Trinitrobenzenesulfonic acid (TNBS) triggers a Th1-type immune response by binding to intestinal proteins through the hapten effect, resembling the T-cell polarization observed in CD patients [46]. This leads to upregulation of pro-inflammatory cytokines (TNF- α , IL-6, IL-12, and IFN- γ) and recruitment of neutrophils/macrophages, driving transmural inflammation. Then, TNBS disrupts epithelial tight junctions via ROS overproduction, increasing the risk of bacterial translocation. In synergy with the microbiota dysregulation, it forms a vicious cycle of inflammation and barrier damage, ultimately mimicking the core pathological features of CD.

To establish the CD model, the mice were presensitized with 1 wt.% TNBS and raised for 7 days, followed by treatment with a 2.5 wt.% TNBS enema on day 8 (Fig. 6(a)). The body weight of mice significantly decreased and the immunofluorescence staining of colon tissue revealed a remarkable increase of CD4⁺ expression in the TNBS-treated group, thereby confirming the successful

induction of CD (Fig. 6(b) and Fig. S12 in the ESM). However, the administration of Fe-BA@EcN, Fe-TA@EcN, Fe-BT@EcN, and BA could recover the body weight, with Fe-BT@EcN exhibiting the superior effect (Fig. 6(c)). Moreover, Fe-BT@EcN significantly reduced the disease activity index, extended the colon length, and mitigated the spleen hypertrophy of CD mice, demonstrating the effective alleviation of CD symptoms (Figs. 6(d)–6(g), and Figs. S13 and S14 in the ESM). Subsequently, the histological change was evaluated by hematoxylin-eosin (H&E) staining of the colon tissue. Tissue inflammation, lesion depth, crypt injury, and lesion area were used to calculate the histological score. As shown in Figs. 6(h)–6(j), the TNBS-treated mice exhibited the highest degree of inflammatory cell infiltration and crypt damage in the colon tissue, while the Fe-BA@EcN, Fe-TA@EcN, Fe-BT@EcN, and i.g. BA groups showed significantly reduced features. Compared to other groups, Fe-BT@EcN treated mice had the lowest histological grading. Meanwhile, the proportion of myeloperoxidase (MPO) positive cells decreased from 47.3% to 31.3%, as indicated by the relative quantification after Fe-BT@EcN treatment.

To further assess the inflammatory level, the colons were subjected to immunohistochemical staining of interleukin-6 (IL-6). In contrast to the TNBS group, Fe-BT@EcN treatment led to a 16.9% reduction in IL-6 expression levels, as indicated by the

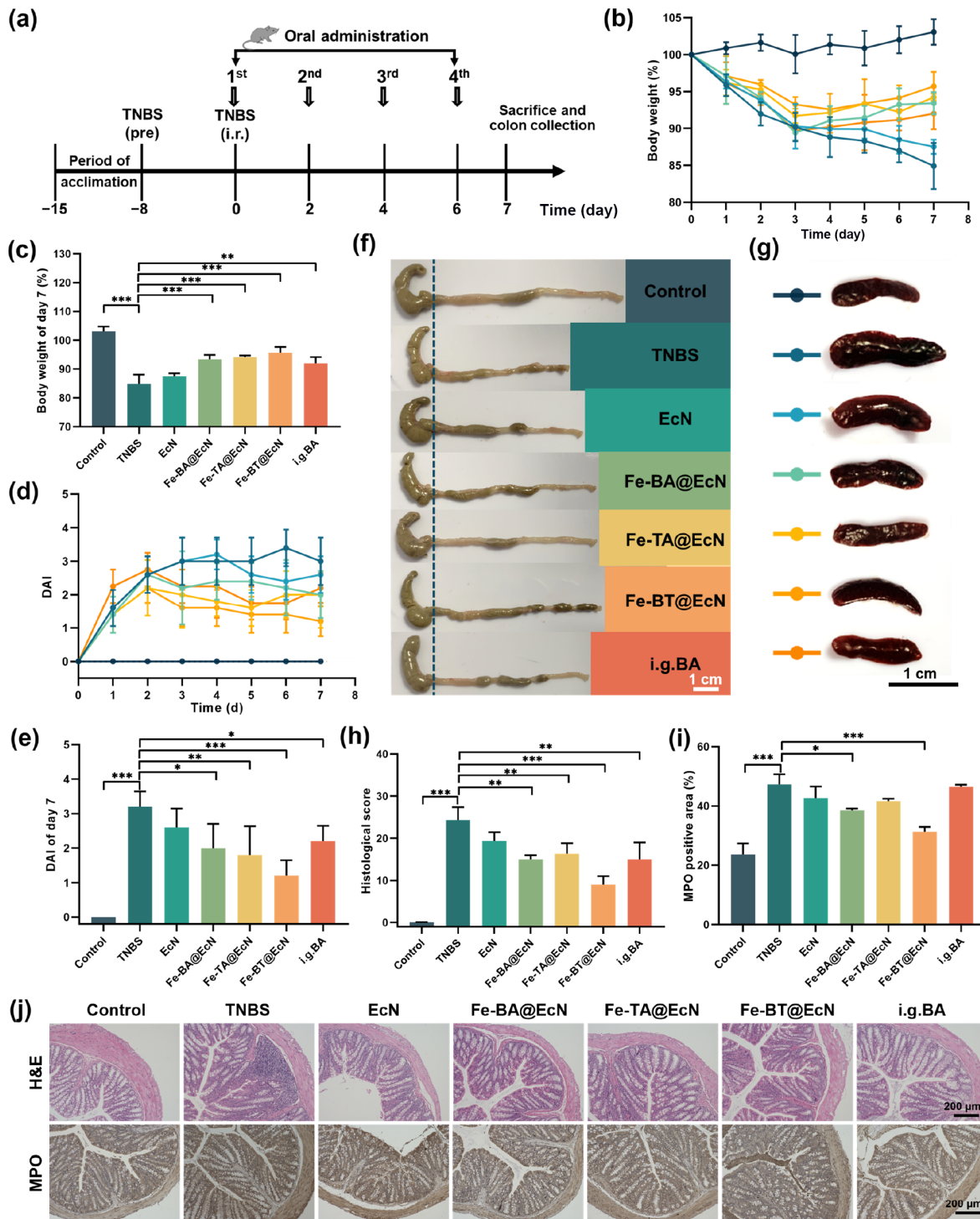


Figure 6 (a) Experimental scheme for CD treatment after oral administration of Fe-BT@EcN. (b) Body weight of mice during the treatment period. (c) The body weight of mice on day 7. (d) DAI index during the treatment period. (e) DAI index on day 7. (f) and (g) Photographs of colons and spleens of treated mice. (h) Images of H&E staining and MPO immunohistochemical staining of colon tissues. (i) and (j) The quantitative histological score and the MPO positive area. $n = 5$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and n.s.: no significance.

relative fluorescence intensity (Figs. 7(a) and 7(b)). To reveal the expression level of p-STAT3, the potential therapeutic targets identified through molecular docking, the immunofluorescence staining of colon tissues were performed. As depicted in Figs. 7(c) and 7(d), the level of p-STAT3 was markedly elevated by 45.5% in the TNBS group as compared with the control group, while it was significantly reduced by 32.4% following Fe-BT@EcN treatment.

These results proved Fe-BT@EcN could significantly reduce inflammation levels in CD-bearing mice.

The intestinal epithelial barrier is usually impaired in CD, which could lead to disease progression. As the primary tight junction-associated proteins, Zonula Occludens-1 (ZO-1) and Occludin play a crucial role in maintaining the structure and function of the intestinal epithelium and the integrity of the intestinal barrier. The

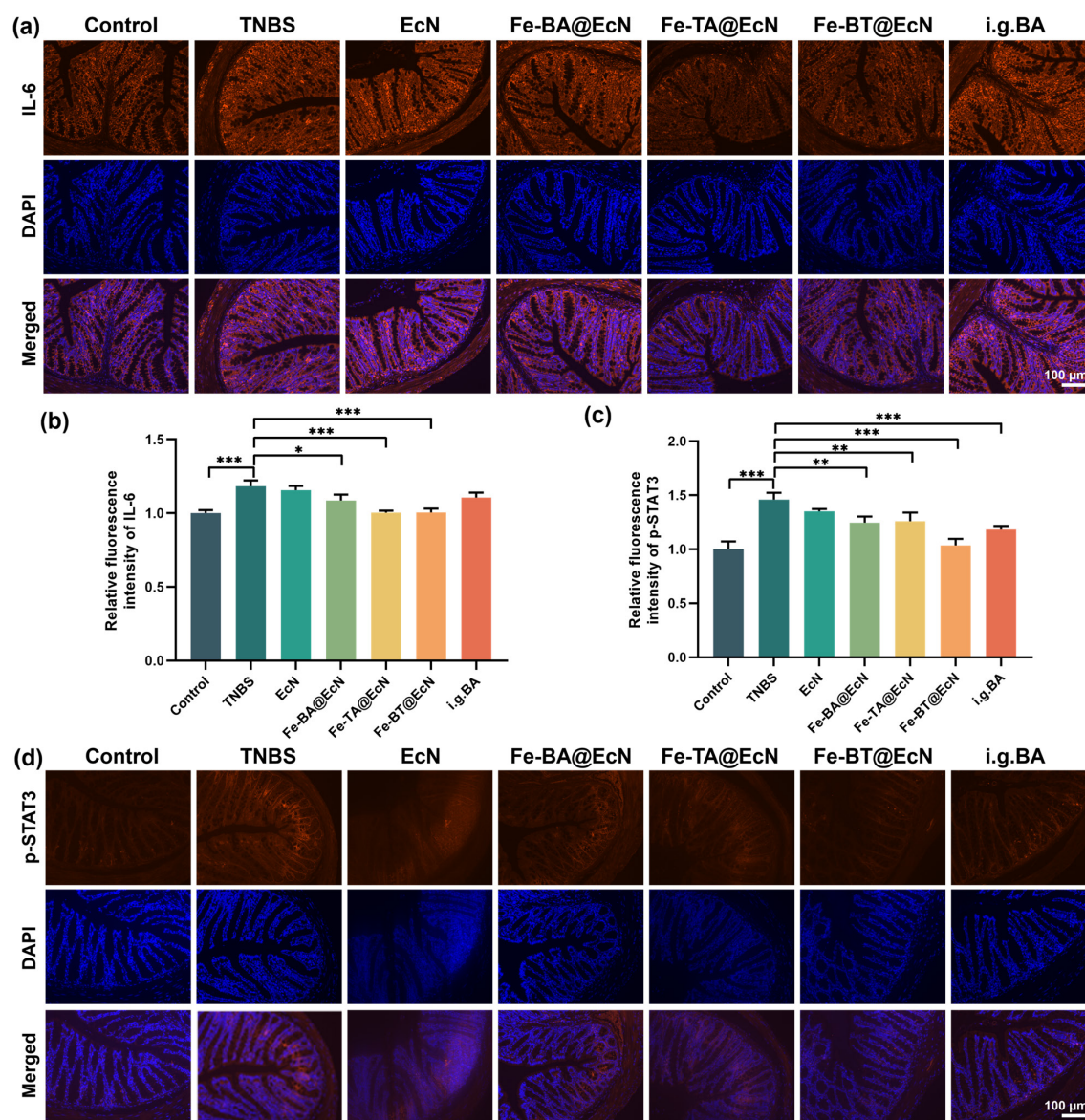


Figure 7 (a) and (b) Immunofluorescence images and the relative fluorescence intensity of IL-6 in colon tissues. (c) and (d) Immunofluorescence images and the relative fluorescence intensity of p-STAT3 in colon tissues. $n = 5$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and n.s.: no significance.

expression of ZO-1 and Occludin in colon tissue was evaluated using immunofluorescence staining, and the results indicated that TNBS treatment significantly reduced their expression levels by 32.2% and 28.1% (Fig. 8(a)). Upon treatment with native EcN, no significant alterations were noted, indicative of its limited therapeutic efficacy. Compared to the TNBS group, Fe-BA@EcN, Fe-BT@EcN, and i.g.BA clearly reversed this trend, with the Fe-BT@EcN group showing the most favorable results with ZO-1 and Occludin expression increased by 23.3% and 25.7% (Figs. 8(b) and 8(c)). These results indicated that Fe-BT@EcN was able to enhance the tight junction-associated protein levels to restore the intestinal barrier.

3.6 Regulatory effect on the gut microbiota

The modulation of the gut microbiota was confirmed through the analysis of fecal microbiota from mice by 16S rRNA gene sequencing after subjection to various treatments. Compared to the TNBS group, the Fe-TA@EcN and Fe-BT@EcN groups distinctly

increased the observed operational taxonomic units (OTUs) by 223 and 251, and the Shannon diversity index by 1.47 and 1.42, indicating enhanced intestinal flora abundance and distribution evenness (Figs. 9(a) and 9(b)). Meanwhile, the ACE index indicated Fe-BT@EcN remarkably increased species richness and homogeneity (Fig. 9(c)). VA Venn diagram based on OTU abundance was utilized to illustrate the richness and similarity of microbiota composition among the groups. Compared to the control group, the species richness of the intestinal flora decreased in the TNBS group, while a significant increase was observed in the Fe-BT@EcN group (Fig. 9(d)). Further analysis of the relative abundance of microbiota was conducted at the phylum and genus levels. Compared with the TNBS group, the phylum-level analysis revealed that Fe-BT@EcN administration significantly increased the relative abundance of Firmicutes, the majority of which are recognized as beneficial bacteria contributing to gut microbiota homeostasis (Fig. 9(e)). At the genus level, a notable decrease in *Lachnospiraceae_NK4A136*, *norank_f_Ruminococcaceae*, and

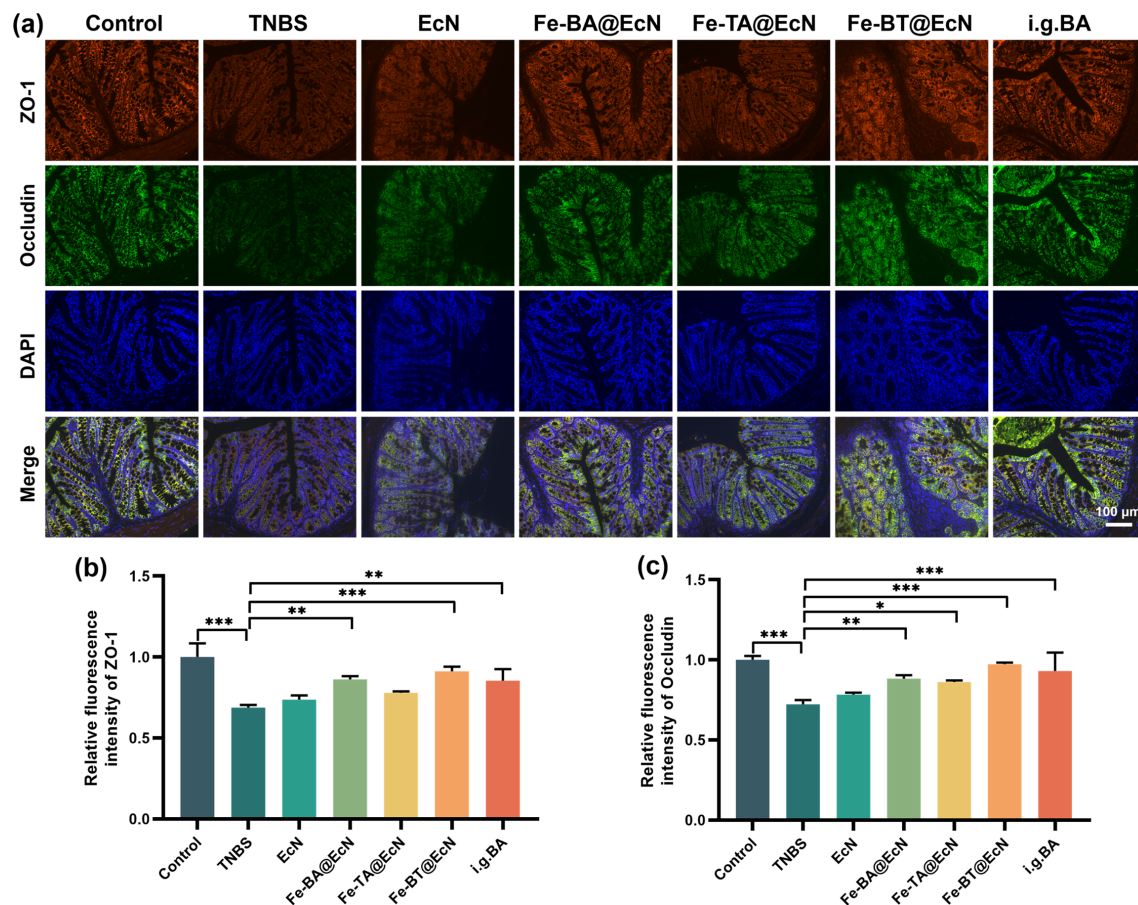


Figure 8 (a) Immunofluorescence images of ZO-1 and Occludin in colon tissues. (b) and (c) The relative fluorescence intensity of ZO-1 and Occludin. $n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.: no significance.

Roseburia, classified as butyrate-producing and anti-inflammatory bacteria, was observed in the TNBS group (Figs. 9(f) and 9(g)). However, after oral administration of Fe-BT@EcN, a significant increase in their relative abundance was detected (Fig. S15 in the ESM). Furthermore, *Coriobacteriaceae* UCG-002 and *norank_f_Erysipelotrichaceae*, which are harmful bacteria that typically aggravate intestinal inflammation and epithelial permeability, showed a significant increase in abundance in the TNBS group but were notably reduced after the treatment with Fe-BT@EcN (Figs. 9(h) and 9(i)). Overall, Fe-BT@EcN revealed EcN-mediated modulation of the intestinal microbiota homeostasis by enhancing the relative abundance of beneficial bacterial populations while reducing the colonization of detrimental bacterial communities, thereby effectively alleviating CD symptoms.

4 Conclusions

In summary, baicalin nanoengineered probiotics Fe-BT@EcN were developed for CD treatment through the metal-phenolic coordination interaction. The nanoencapsulation endowed probiotics with remarkable tolerance against gastrointestinal microenvironment as well as enhanced intestinal adhesion and colonization. Notably, Fe-BT@EcN effectively scavenged ROS and inhibited the IL-6/STAT3 signaling pathway, thereby protecting epithelial cells from oxidative damage. In a murine TNBS-induced CD model, Fe-BT@EcN demonstrated synergistic therapeutic efficacy with significantly recovered body weight, reduced DAI

score, and elongated colonic length, mainly attributed to the multimodal mechanisms of mitigating the intestinal inflammation, repairing the epithelial barrier, and balancing the gut microbiota. This rationally designed strategy is applicable to produce multifunctional oral microecological formulations for combined CD therapy through integrating various probiotic strains and natural bioactive compounds.

Electronic Supplementary Material: Supplementary material (Figs. S1–S12) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94908355>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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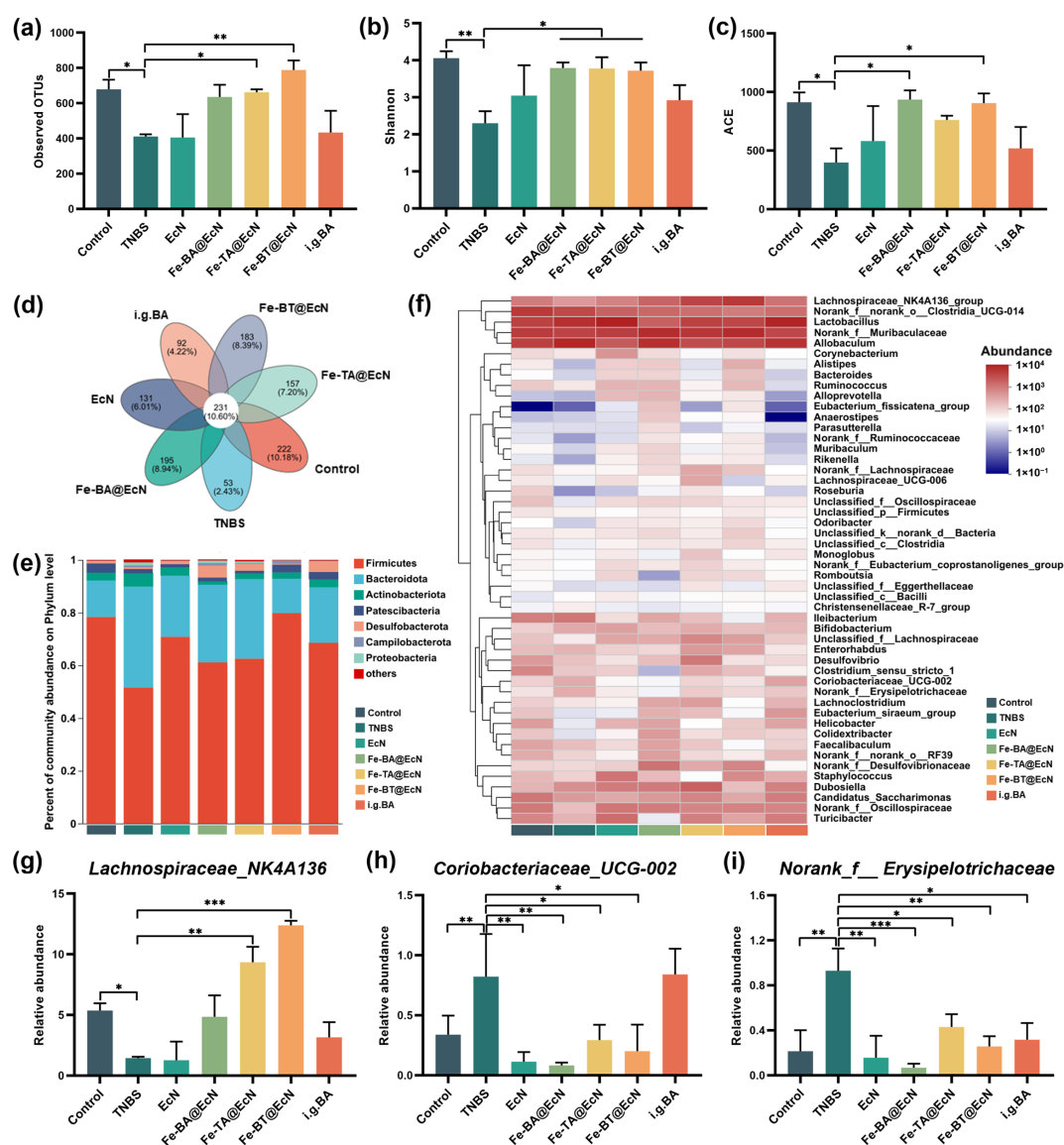


Figure 9 (a) Observed OTUs, (b) Shannon, (c) ACE diversity index, and (d) Venn diagram of intestinal flora after different treatments. (e) and (f) Microbiota analysis at the phylum and genus taxonomic levels. (g)–(i) Relative abundance of *Lachnospiraceae_NK4A136*, *Coriobacteriaceae_UCG-002*, and *norank_f_Erysipelotrichaceae*. $n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.: no significance.

Declaration of competing interest

All the contributing authors reports no conflict of interests in this work.

Author contribution statement

J. R. W.: Methodology, investigation, data curation, writing – original draft. X. Z.: Methodology, data curation. P. Y. C.: Methodology. P. D. P.: Methodology. C. F. N.: Methodology. L. F. Y.: Methodology. L. Y.: Methodology. P. R. W.: Methodology. T. F.: Conceptualization, supervision, investigation, writing – review & editing. P. L.: Conceptualization, supervision, writing – review & editing. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All animal experimental procedures adhered to the animal experimentation guidelines of Northwestern Polytechnical University and were ratified by the University's Animal Ethics Committee (ethical code: 202301033).

Use of AI statement

None.

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