Mechanism Study of Prussian Blue Nanozymes in the Treatment of Crohn's Disease Based on Metagenomic Analysis

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Abstract: Crohn's Disease (CD) is a type of inflammatory bowel disease (IBD) with an unknown etiology, characterized by gastrointestinal immune-related inflammation. Currently, there is no specific drug for treating IBD, and existing treatments only alleviate symptoms with significant side effects that vary among individuals. Numerous studies have shown a close relationship between gut microbiota and Crohn's disease. However, due to the unclear pathogenesis of Crohn's disease, microbial-based therapies have not been widely adopted. In this study, we used a TNBS (2, 4, 6-trinitrobenzene sulfonic acid)-induced rat model of Crohn's disease to treat the model group with Prussian Blue Nanozymes (PB). Based on metagenomic analysis, we examined the diversity, community changes, and species differences in gut microbiota before and after treatment, identifying 20 disease-related species, 16 PB treatment-related species, and significant community changes. This provides insights for further understanding the pathogenesis of Crohn's disease and the therapeutic mechanisms of Prussian Blue Nanozymes.

1 Introduction

Inflammatory Bowel Disease (IBD) encompasses a range of chronic and recurrent gastrointestinal conditions with unclear causes, mainly comprising Crohn's Disease (CD) and Ulcerative Colitis (UC). While UC is confined to the colon, CD can impact any section of the gastrointestinal tract. Beyond inflammation, CD often induces fibrosis, leading to strictures in the ileal wall [1]. Typical manifestations of CD are abdominal pain, diarrhea, and weight loss, frequently alongside complications such as fistulas, perianal issues, and abdominal masses. CD patients face a significantly elevated risk, 90-100 times higher, of developing ileal and colorectal cancers compared to the general population, and are also more susceptible to conditions like anal squamous cell carcinoma, Hodgkin's lymphoma, and non-Hodgkin's lymphoma [2].

Currently, there is no specific treatment for IBD, and therapies are limited to symptom management. Treatments for CD vary based on disease severity and include aminosalicylates, corticosteroids, and immunomodulators [3]. However, due to individual variability, some patients do not respond to the first two treatments, and immunomodulators may take weeks to months to show efficacy, with a high recurrence rate. Biologic therapies targeting specific pathways, such as TNF-α, JAK family inhibitors, and IL-12/IL-23 inhibitors, have been approved by the FDA for IBD treatment [4]. However, these biologics often come with adverse effects or lack efficacy in about one-third of patients, highlighting the need for more effective and safer treatments.

Increasing evidence suggests a strong link between gut microbiota and Crohn's disease. For instance, significant changes in gut microbiota have been observed in CD patients, with the presence of Pseudomonas-like bacteria in affected tissues. Pathogenic bacteria like Escherichia coli are enriched in the gut, while beneficial bacteria that stimulate anti-inflammatory factors and regulate gut homeostasis are significantly reduced in IBD patients. Additionally, changes in microbial metabolites, such as bile acid metabolism and tryptophan metabolism, have been noted in CD patients. Although these findings do not fully elucidate the relationship between gut microbiota and CD, they provide new avenues for treatment.

Microbial-based therapies for intestinal inflammation aim to restore gut microbiota balance by increasing microbial diversity, targeting microbial pathways, and modulating microbial metabolites [5]. This study uses a chemically induced IBD rat model to analyze fecal metagenomic sequencing data, examining changes in gut microbiota before and after treatment with Prussian Blue Nanozymes (PB). The goal is to understand the dynamic changes in gut microbiota during treatment, identify potential biomarkers, and explore new therapeutic targets for Crohn's disease.

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2 Materials and Methods

2.1 TNBS-induced Crohn's Disease Model

Chemically induced IBD models in rats are created by damaging intestinal epithelial cells with chemical agents, triggering immune responses or directly activating immune pathways in gut cells. Different chemicals induce different immune pathways, leading to varied IBD symptoms. In this study, we used the TNBS-induced CD model, where TNBS, a hapten, is administered rectally with ethanol to induce colitis in susceptible rat strains [6]. Ethanol increases mucosal permeability, allowing TNBS to penetrate the intestinal wall and trigger a Th1-mediated immune response, characterized by CD4+ T cell, neutrophil, and macrophage infiltration. This results in intestinal wall thickening, ulceration, muscle layer destruction, and granuloma formation, mimicking human CD pathology.

Before establishing the CD model, we selected Sprague-Dawley (SD) rats of the same sex and genetic background to minimize variability. Rats were pre-sensitized by applying 1% TNBS to the skin, and those with less than 95% of their initial body weight after eight days were excluded. The remaining rats were administered TNBS-ethanol solution rectally weekly for four weeks, with fecal samples collected in the third and fourth weeks.

2.2 Prussian Blue Nanozyme Treatment

Prussian Blue Nanozymes (PB) are coordination-based polymers characterized by a cubic crystalline framework, demonstrating both peroxidase-like and catalase-like enzymatic properties. These nanozymes efficiently neutralize reactive oxygen species (ROS), thereby mitigating oxidative stress and preventing damage to intestinal tissues. PB nanoparticles play a crucial role in regulating the intestinal immune environment by suppressing the release of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, while simultaneously enhancing the production of anti-inflammatory cytokines like IL-10. Additionally, they contribute to the protection of intestinal epithelial cells and the restoration of the gut barrier by upregulating the expression of tight junction proteins. Known for their exceptional biocompatibility and minimal toxicity, PB has received FDA approval for use in the treatment of poisoning caused by radioactive cesium and thallium [7].

In this study, half of the TNBS-induced CD model rats were randomly selected for PB treatment. PB was dissolved in phosphate-buffered saline (PBS) at 5 mg/mL and administered orally at 2 mg/kg. Treatment was conducted concurrently with the final TNBS injection.

2.3 HE Staining

Hematoxylin and Eosin (HE) staining is a common histological method for visualizing cell nuclei and cytoplasm. Hematoxylin stains nuclei blue-purple, while eosin stains cytoplasm and extracellular matrix pink. This method is widely used in pathology to observe inflammation, tumors, and other intestinal tissue changes [8]. In this study, intestinal tissue samples from rats were sectioned, HE-stained, and examined under a microscope to assess modeling and treatment efficacy.

2.4 Metagenomic Sequencing

Metagenomic sequencing is a high-throughput technique for analyzing microbial communities in environmental samples without prior cultivation. It is widely used to study complex microbial ecosystems, such as gut microbiota and soil microbiomes. The process involves sample collection, DNA extraction, library preparation, high-throughput sequencing, data processing, and functional and taxonomic analysis. In this study, fecal samples were sequenced using next-generation sequencing to obtain microbial community information for further analysis.

2.5 Sequencing Data Analysis

After obtaining metagenomic sequencing data, we used FastQC for quality assessment and kneaddata to remove low-quality reads and adapter sequences. Host genome contamination was removed by aligning reads to the host genome. Quality-controlled reads were annotated using MetaPhlAn2 to generate species abundance tables.

The Alpha diversity indices, including the Shannon index, Simpson index, and Chao1 index, were calculated for the samples using QIIME2. The differences in Alpha diversity between different groups were compared using the Kruskal-Wallis test, and the results were visualized using boxplots. Beta diversity was calculated using Bray-Curtis and Jaccard distances, and the differences between samples were visualized through Principal Coordinate Analysis (PCoA). This allowed for the assessment of whether there were significant differences in microbial community distribution among the sample groups.

Depending on the grouping of samples, different differential analysis methods were employed to identify differentially abundant microbial taxa and visualize the results using heatmaps. For comparisons between two groups, such as pre-treatment fecal samples, a t-test was performed on species abundance, and significantly different species ($p < 0.05$) were identified, with p-values adjusted for false discovery rate (FDR) to control the false positive rate. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) was conducted on species abundance to identify significantly different species ($p < 0.05$), followed by pairwise comparisons between groups. The LEfSe (Linear Discriminant Analysis Effect Size) tool was used to screen for differentially abundant species between groups, with thresholds set at an LDA score ($LDA > 3.0$) and a p-value ($p < 0.05$) [9]. This allowed for the identification of significantly different species or functional features, which were visualized using LEfSe-generated histograms and cladograms to highlight the distribution of differential species.

3 Results

3.1 Intestinal Tissue Staining Results

Histological examination of intestinal tissue sections from each sample revealed that the control group (Figure 1A-C) exhibited a higher number of intact goblet cells and a well-organized arrangement of intestinal glands. In contrast, the model group (Figure 1D-F) displayed a significant reduction in goblet cells, accompanied by an increased presence of inflammatory cells and erythrocytes, indicating extensive inflammatory and hemorrhagic regions within the intestinal tract. Additionally, the intestinal glands in the model group showed signs of atrophy and structural damage, demonstrating that the administration of TNBS solution induced severe intestinal inflammation in the rat model.

In the samples treated with Prussian Blue Nanozymes (Figure 1G-I), although some degree of cellular damage and inflammatory response persisted compared to the control group, a notable increase in goblet cells was observed relative to the model group. Additionally, the number of inflammatory cells and erythrocytes was significantly reduced, and glandular atrophy was alleviated. These findings validate that Prussian Blue Nanozymes exert a therapeutic and mitigating effect on inflammatory bowel disease in the rat model.

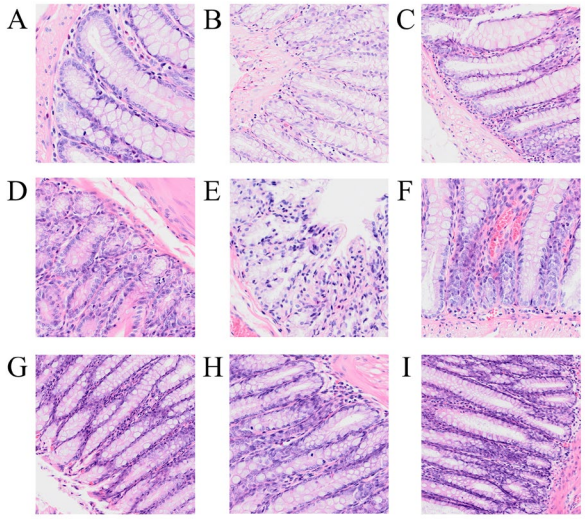

Figure 1. HE staining results. **A-C**: HE staining results of the control group; **D-F**: HE staining results of the model group; **G-I**: HE staining results of the treatment group.

3.2 Diversity and Differential Analysis of Fecal Microbial in the Crohn's Disease Model

Fecal samples were collected from rats after three injections and divided into control and model groups. Following metagenomic sequencing, the data underwent preprocessing and host removal, and were analyzed using MetaPhlAn2 to generate species abundance tables. Both alpha and beta diversity analyses were performed. As shown in Figure 2A, the alpha diversity boxplot, visualized using the Shannon index, revealed that the control group exhibited higher species richness and more even distribution compared to the model group, suggesting that TNBS-induced Crohn's disease may lead to a reduction in gut microbial diversity. The beta diversity results (Figure 2B) indicated a significant increase in inter-group variability in the model group, demonstrating instability. Moreover, significant changes in microbial communities were observed between the control and model groups, indicating that the gut microbiota in the disease model tended to be less stable compared to the control group, which may be one of the pathogenic manifestations of Crohn's disease.

To identify differentially abundant species between the control and model groups and further explore potential biomarkers for Crohn's disease, LEfSe analysis was performed. The thresholds were set at an LDA score (LDA > 3.0) and a p-value ($p < 0.05$) to screen for significantly different species. As shown in Figure 3AB, a total of 20 species were identified, all of which were significantly more abundant in the control group. Among these were Ligilactobacillus_murinus, a species within the Lactobacillus genus, recognized as a potential probiotic. It helps maintain gut microbiota equilibrium by generating lactic acid and other metabolites, which suppress pathogenic bacteria and strengthen the intestinal barrier. Additionally, Romboutsia_ilealis, a member of the Clostridia class, has the ability to break down dietary fibers, resulting in the production of short-chain fatty acids (SCFAs).[10], such as acetate and butyrate, which help maintain intestinal barrier function, modulate immune responses, and provide energy. Additionally, an increase in the abundance of Romboutsia_ilealis has been associated with the alleviation of intestinal inflammation [11]. The notable decrease in the levels of these beneficial microbial species within the model group implies their potential role as biomarkers or therapeutic targets for Crohn's disease. However, no species with significantly increased abundance were observed in the model group. To further investigate changes in the microbial community, additional differential analysis methods were employed.

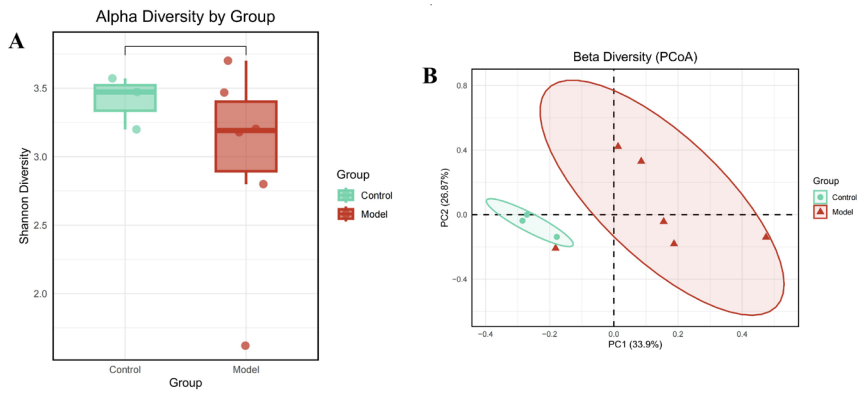

Figure 2. Alpha Diversity and Beta Diversity

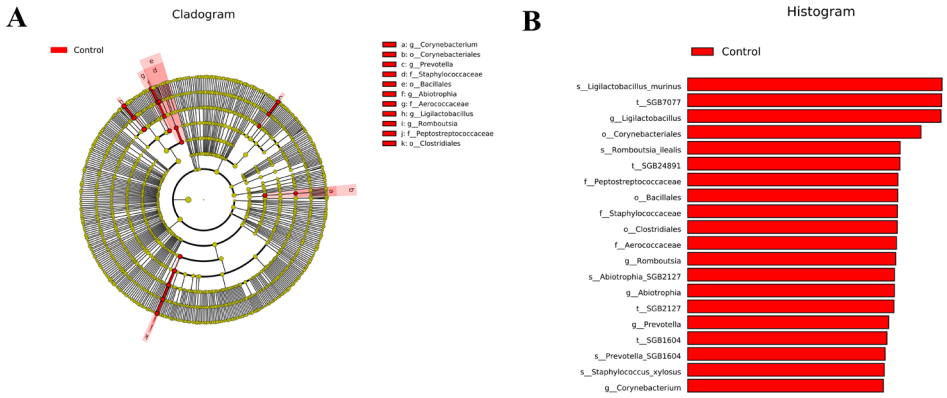

Figure 3. LEfSe Differential Analysis. **A**: Cladogram; **B**: LDA Score Distribution Histogram

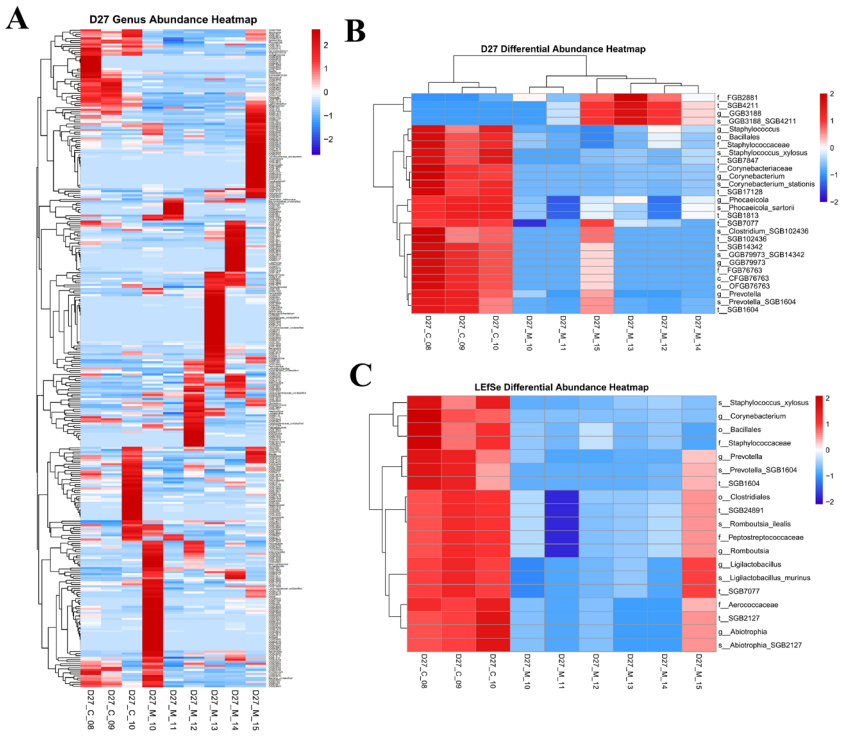

Figure 4. Species Abundance Heatmap. **A**: Genus-level species abundance heatmap; **B:** Heatmap of significantly different species identified by t-test ($p < 0.05$); **C:** Heatmap of significantly different species identified by LEfSe analysis (LDA > 3.0, $p < 0.05$)

The significantly different species identified through LEfSe differential analysis were extracted and visualized in a heatmap, as shown in Figure 4C. This confirmed that the 20 species were expressed at lower levels in the model group. To comprehensively analyze the distribution of species abundance, the species abundance

table was clustered at the genus level, and the results were visualized in a heatmap (Figure 4A). It was observed that each sample in the model group exhibited different significantly expressed species, with little overlap among them. Further t-tests were conducted, screening for significantly different species with a threshold of $p < 0.05$. A total of 28 differentially abundant species were identified, among which four species showed significantly lower expression in the control group compared to the model group (Figure 4B). However, in nearly half of the samples, these four species were not highly expressed. Integrating the results of the differential analysis and the genus-level heatmap, it is evident that the abundance of certain beneficial bacteria was significantly reduced in the model group compared to the control group, with notable variability among samples. This supports the earlier findings from beta diversity analysis, which demonstrated inter-group instability. These results may provide insights for microbial-based therapeutic strategies for Crohn's disease.

3.3 Diversity and Differential Analysis of Fecal Microbial After Treatment with Prussian Blue Nanozymes

After three weeks of model establishment, three rats from the model group were randomly selected as the treatment group and administered Prussian Blue Nanozymes. Both the model group and the treatment group continued to receive TNBS solution injections according to the predetermined schedule. The treatment group underwent oral gavage with Prussian Blue Nanozyme solution before and after the fourth injection. Fecal samples were collected after the final injection and subjected to metagenomic sequencing. The sequencing data underwent preprocessing and host removal, followed by MetaPhlAn2 analysis to generate species abundance tables for the control group, model group, and treatment group. Diversity and differential analyses were performed on the fecal samples from the control group, model group, treatment group, and pre-treatment samples to further investigate the therapeutic effects and mechanisms of Prussian Blue Nanozymes on Crohn's disease.

Alpha and beta diversity analyses were conducted on the metagenomic sequencing data from three pre-treatment fecal samples and the post-treatment fecal samples. The alpha diversity visualization results (Figure 5A) indicated that the post-treatment fecal samples exhibited higher microbial diversity, characterized by increased species richness and more even distribution. The beta diversity visualization results (Figure 5B) revealed significant changes in the microbial community distribution in the post-treatment fecal samples, with a notable reduction in inter-group variability. These findings, from a microbial perspective, validate the therapeutic efficacy of Prussian Blue Nanozymes in the treatment of Crohn's disease.

To intuitively understand the changes in microbial composition before and after treatment, the abundance profiles were clustered at the phylum level, resulting in 12 phyla. The phylum-level abundances were then clustered by sample and visualized using a stacked bar chart (Figure 6). The results showed a significant decrease in the proportion of Firmicutes, while Actinobacteria and Proteobacteria significantly increased. Notably, Actinobacteria are known for their antibiotic-producing capabilities, with many members (e.g., Streptomyces) being important producers of antibiotics such as streptomycin, tetracycline, and erythromycin. Proteobacteria play a crucial role in nitrogen cycling [12]. The stacked bar chart at the phylum level distinctly shows a rise in the abundance of beneficial bacteria within the gut microbiota following treatment, accompanied by a notable improvement in microbial diversity. These observations indicate that Prussian Blue Nanozymes could play a role in fostering the growth of beneficial bacteria and enhancing the overall diversity of gut microbial communities.

To further investigate the mechanism of Prussian Blue Nanozymes in gut microbiota, LEfSe differential analysis was performed on the species abundance tables of fecal microbiota before and after treatment. The thresholds were set at an LDA score ($LDA > 3.0$) and a p-value ($p < 0.05$) to screen for significantly different species. As shown in Figure 7AB, a total of 16 species were identified, with the majority being significantly more abundant after treatment. The two species that were significantly more abundant before treatment were unclassified and unrecorded, potentially providing a basis for future therapeutic targets in Crohn's disease.

In the post-treatment samples, species from the order Coriobacteriales, which belong to the phylum Actinobacteria, play a significant role in the gut microbiota and are closely tied to host metabolic health. Research suggests that variations in their abundance could be linked to conditions like obesity, type 2 diabetes, and inflammatory bowel disease [13]. Similarly, bacteria from the Atopobiaceae family, also part of the Actinobacteria phylum, are capable of fermenting dietary fibers to generate short-chain fatty acids (SCFAs). This process may help maintain gut microecological equilibrium by competitively inhibiting pathogens and modulating intestinal pH levels [14]. Studies have shown that the presence of Atopobiaceae bacteria is associated with intestinal inflammation and metabolic disorders, including obesity and type 2 diabetes. These results highlight the beneficial impact of Prussian Blue Nanozymes on fostering the growth of advantageous gut bacteria, potentially alleviating disease symptoms by supporting their proliferation and survival.

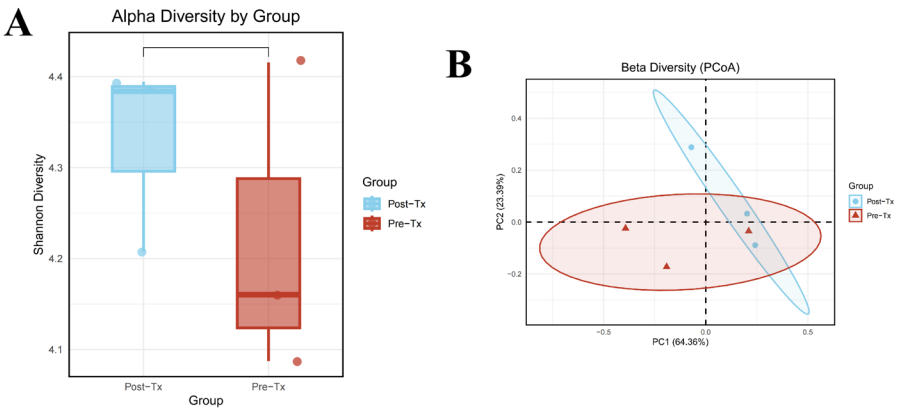

Figure 5. Alpha and Beta Diversity Before and After Treatment

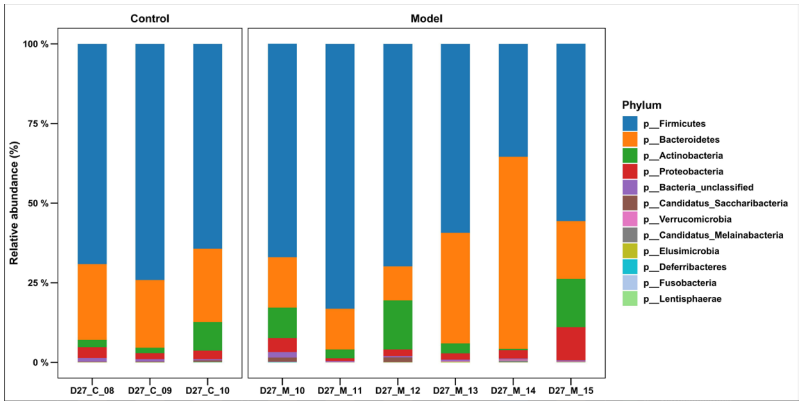

Figure 6. Phylum-Level Stacked Bar Chart

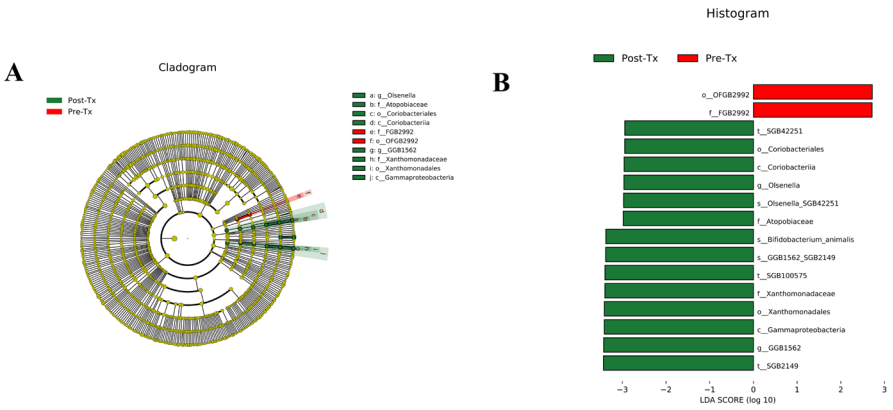

Figure 7. LEfSe Differential Analysis Before and After Treatment. **A**: Cladogram; **B**: LDA Score Distribution Histogra**m**.

4 Discussion

Crohn's disease is a chronic inflammatory gastrointestinal disorder that was initially prevalent primarily in European and North American countries. However, in recent years, the incidence rates in these high-prevalence regions have stabilized, while in low-prevalence areas such as South America and Asia, including China, the incidence of Crohn's disease has been steadily increasing. As of 2020, the prevalence rate in China has reached 2.29 cases per 100,000 people, with an average incidence rate of 1.21 cases per 100,000 people. The pathogenesis of Crohn's disease remains unclear, but it is likely caused by complex interactions among genetic susceptibility, environmental factors, and alterations in gut microbiota, leading to dysregulation of innate and adaptive immune responses. Current treatments for Crohn's disease are limited to symptom management based on disease severity, and existing therapies such as immunomodulators and targeted treatments vary in efficacy among individuals. Some patients may not respond to treatment for up to three months or may not respond at all. However, an increasing body of research has demonstrated a strong association between gut microbiota and Crohn's disease.

Microbial-based therapies, such as probiotic supplementation and fecal microbiota transplantation, have shown minimal harm to the human body and hold promise as new approaches for elucidating the pathogenic mechanisms and achieving a cure for Crohn's disease. In this study, based on metagenomic analysis, we validated changes in fecal microbiota in a rat model. Following treatment with Prussian Blue Nanozymes, we visualized the abundance of gut microbiota and conducted differential analysis, identifying significantly different species. These findings provide insights for further research into the pathogenesis and treatment of Crohn's disease.。

TNBS solution, as a classic chemical inducer, demonstrates high efficiency and reliability in constructing a rat model of Crohn's disease. Its mechanism primarily involves inducing immune and inflammatory responses in the intestinal mucosa, mimicking the pathological features of Crohn's disease. Experimental results indicate that TNBS solution successfully established a rat model exhibiting typical characteristics of Crohn's disease, including weight loss, diarrhea, and significant changes in intestinal tissue as observed in HE staining. In the HE staining results, intestinal tissue sections from the disease model showed notable alterations in goblet cells, inflammatory cells, blood cells, and glandular atrophy. Subsequent metagenomic analysis identified beneficial bacteria that were significantly more abundant in the control group, such as Ligilactobacillus_murinus (genus Lactobacillus) and Romboutsia_ilealis (class Clostridia), further validating the feasibility of using TNBS to establish a rat model of Crohn's disease.

Prussian Blue Nanozymes (Prussian Blue, PB), as a nanomaterial with excellent antioxidant and anti-inflammatory properties, have shown significant potential in the treatment of Crohn's disease. The mechanisms through which they operate encompass: (1) Antioxidant properties: PB reduces oxidative stress-related harm to intestinal tissues by neutralizing reactive oxygen species (ROS) in the gut; (2) Anti-inflammatory properties: PB regulates immune responses in the intestines by suppressing the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β; and (3) Protection of the intestinal barrier: PB enhances the expression of tight junction proteins, thereby restoring the integrity of the intestinal barrier. In this study, Prussian Blue Nanozymes markedly reduced inflammation in the Crohn's disease model and enhanced the outcomes of HE staining in intestinal tissue samples. From the perspective of microbial species abundance and community changes, the proportion of beneficial bacteria in the gut microbiota increased after treatment, accompanied by a significant enhancement in microbial diversity. Furthermore, the analysis of significantly expressed species revealed an increase in beneficial bacteria such as those belonging to the order Coriobacteriales and the family Atopobiaceae within the phylum Actinobacteria. These findings indicate that Prussian Blue Nanozymes restore the balance of gut microbiota, demonstrating their role in modulating gut microbes during the treatment of Crohn's disease. This also provides a theoretical foundation for further research on Prussian Blue-based therapies for Crohn's disease.

Acknowledgments

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References

1. Imhann F, Vila A V, Bonder M J, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease[J]. Gut, 2018, 67(1):108-119.
2. Vaghiri S, Alipouriani A, Knoefel W. T, Kessler H, & Prassas D. Extended mesenteric resection reduces the rate of surgical recurrence in Crohn’s disease: a systematic review and meta-analysis[J]. International Journal of Colorectal Disease, 2025, 40(1), 51.
3. Cushing K, Higgins P D R. Management of Crohn disease: a review[J]. Jama, 2021, 325(1): 69-80.
4. Lu Q, Yang M, Liang Y, et al. Immunology of inflammatory bowel disease: molecular mechanisms and therapeutics[J]. Journal of inflammation research, 2022, 15: 1825-1844.
5. Lloyd-Price J, Arze C, Ananthakrishnan A N, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases[J]. Nature, 2019, 569(7758): 655-662.
6. Fu W, Xu L, Chen Z, et al. Recent advances on emerging nanomaterials for diagnosis and treatment of inflammatory bowel disease[J]. Journal of Controlled Release, 2023, 363: 149-179.
7. Gao W, Wang Y, Zheng Y, et al. Prussian blue nanoparticle: from a Photothermal Conversion Agent and a drug delivery system, to a Bioactive Drug[J]. Accounts of Materials Research, 2024, 5(6): 687-698.
8. Feng S, Cao X, Zheng W, et al. In-situ formed Prussian blue nanoparticles supported by porous biochar as highly efficient removal of cesium ions[J]. Journal of Environmental Chemical Engineering, 2022, 10(3):107972.
9. Nam N N, Do H D K, Loan Trinh K T, et al. Metagenomics: An effective approach for exploring microbial diversity and functions[J]. Foods, 2023, 12(11):2140.
10. Jackson D J, Cerveau N, Posnien N. De novo assembly of transcriptomes and differential gene expression analysis using short-read data from emerging model organisms–a brief guide[J]. Frontiers in Zoology, 2024, 21(1): 17.
11. Franzosa E A, McIver L J, Rahnavard G, et al.

Species-level functional profiling of metagenomes and metatranscriptomes[J]. Nature methods, 2018, 15(11):962-968.

12. Lande R. Statistics and partitioning of species diversity, and similarity among multiple communities[J]. Oikos, 1996: 5-13.
13. Liu H, Li J, Singh B K. Harnessing co-evolutionary interactions between plants and Streptomyces to combat drought stress[J]. Nature Plants, 2024, 10(8): 1159-1171.
14. Gao Q, Lu S, Wang Y, et al. Bacterial DNA methyltransferase: A key to the epigenetic world with lessons learned from proteobacteria[J]. Frontiers in microbiology, 2023, 14: 1129437.