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ORIGINAL RESEARCH

Prominence of Microbiota to Predict Fibrous Stenosis in Crohn's Disease

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Purpose: Intestinal fibrous stenosis due to Crohn's disease (CD) is highly prevalent. Although several clinical risk factors for fibrous stenosis have been identified, such as perianal fistulizing disease, small bowel disease location, and deep mucosal ulceration, predicting fibrous stenosis remains challenging. The intestinal microbiota plays a crucial role in the development and progression of CD. However, its role in intestinal fibrous stenosis is poorly understood. Leveraging a single-center cross-sectional study, we aimed to investigate the role of fecal microbiota in CD-associated fibrous stenosis.

Methods: Using metagenomic analysis, we examined the differences in fecal microbiota between CD patients with intestinal fibrous stenosis and those without stenosis. We identified specific microbiota and assessed their predictive accuracy for intestinal fibrous stenosis. Additionally, we explored functional differences in intestinal microbiota between the two groups.

Results: : Our investigation of fecal samples revealed no significant differences in the gut microbiota structure between patients with fibrous stenosis and those without stenosis in CD. However, taxonomically, we found 70 taxa with significantly different abundance ($p < 0.05$) between the two groups. Furthermore, LEfSe analysis indicated that *g_Bacteroides* and *g_Enterocloster* could predict intestinal fibrous stenosis while *p_Actinobacteria, c_Actinomycetia, c_Bacilli, o_Lactobacillales, f_Streptococcaceae* and *g_Streptococcus* could predict CD without stenosis. Functional analysis revealed differential enrichment in five metabolic pathways at the KEGG pathway level in CD patients with fibrous stenosis, including sphingolipid metabolism, lipoic acid metabolism, and biosynthesis of neomycin, kanamycin and gentamicin. In the eggNOG database, we observed differences in four functional categories between the two groups, encompassing cellular process, signaling, and metabolism.

Conclusion: Fecal microbiota significantly impacted intestinal fibrous stenosis in CD. Although there were no significant differences in alpha and beta diversities, fibrous stenosis was associated with changes in microbiota composition and function, suggesting the potential of fecal microbiota in predicting CD-associated fibrous stenosis.

Keywords: Crohn's disease, fibrous stenosis, fecal microbiota, metagenomic analysis

Introduction

Crohn's disease (CD), an inflammatory bowel disease, has seen a significant rise in incidence in China in recent years.[1] Despite this, its etiology remains elusive, and a universally accepted diagnostic criterion for CD is lacking. The clinical manifestation of CD is diverse, ranging from abdominal pain to diarrhea and weight loss, with growth impairment being particularly prevalent among adolescents. Therefore, investigating the pathogenesis of CD and intervening actively in its early stage are paramount.

Among numerous complications associated with CD, intestinal lumen fibrous stenosis stands out as one of the most significant, affecting approximately 50% of patients and predisposing them to other complications such as enterocutaneous fistulas and abscess.[2] Initially, CD-related stenosis is predominantly inflammatory; however, as the disease progresses, fibrous stenosis develops, rendering conventional medications, such as mesalazine, hormones, biologics,

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and small molecule drugs, ineffective.[3] Consequently, 60–70% of luminal fibrous stenosis necessitate endoscopic or surgical intervention, significantly impacting patients' quality of life.[4]

Several lines of evidence suggested that intestinal microbiota contributes to fibrogenesis.[5] For example, *Salmonella typhi* infection in mice led to transmural inflammation and fibrosis.[6] Similarly, injecting peptidoglycan-polysaccharide bacterial component into the intestinal wall of mice induced inflammation and fibrosis, resulting in increased TGFβ1 production.[7] Moreover, animal models failed to develop intestinal fibrosis in the absence of microbiota.[8]

Gut microbial dysbiosis is a primary mechanism in CD development,[9] yet the role of gut microbiota in CD-related fibrous stenosis remains poorly studied. While current research indicated that altered microbiota may contribute to intestinal fibrosis,[10] the specific characteristics of intestinal microbiota in patients with CD-related fibrous stenosis have not been thoroughly investigated. Our study aimed to identify the specific microbiota associated with fibrous stenosis in CD patients, predicting their discriminatory potential by analyzing fecal metagenomes from both stenotic and non-stenotic CD patients. Additionally, we aimed to delineate functional disparities between the microbiota of CD patients without stenosis and with fibrous stenosis.

Materials and Methods

Study Population and Sample Collection

CD patients were recruited from the Department of Gastroenterology, Sichuan Provincial People's Hospital, between April 2020 and November 2023. Patients were included if their clinical, laboratory, radiological, endoscopic, and histological examinations, along with assessment for intestinal fibrous stenosis via Magnetic Resonance Enterography (MRE),[11] confirmed ileal, colonic, or ileocolonic CD. Patients were excluded if they 1) had received antibiotics, probiotics, or prebiotics within the previous 4 weeks, 2) were at pregnant or lactation status, and 3) had other malignant tumors. Disease location and behaviors were categorized using the Montreal classification. After entering the group, the patient receives a special stool collection tube to collect a stool specimen. A small spoon attached to the bottle cap was used to collect fresh stool, which was placed in the stool tube, barcoded, numbered, and then frozen and stored in a −80°C freezer.

DNA Extraction and Library Construction

Total genomic DNA was extracted from fecal samples using the PowerSoil® DNA Isolation kit (Mo Bio Laboratories) according to the manufacturer's instructions. DNA quality and quantity were examined using the Qubit dsDNA HS Assay Kit on a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and electrophoresis on a 1% agarose gel, respectively. Paired-end libraries with an insert size of ~350bp were prepared using a VAHTS Universal Plus DNA Library Prep Kit for Illumina (Vazyme Biotech) and sequenced on an Illumina NovaSeq 6000 platform (Biomarker Technologies Co., Ltd., Beijing, China) in 150-bp paired-end mode to generate two paired FASTQ files. Raw tags were filtered using Trimmomatic (version 0.33) software to obtain high-quality clean tags.

Sequence Quality Control, Genome Assembly, and Metagenomic Sequencing

Metagenomic data were assembled using MEGAHIT (https://github.com/voutcn/megahit), which utilizes succinct de Bruijn graphs. Assembly summary statistics were determined using QUAST software version 2.3. Contigs with a length of 300 bp or more were selected as the final assembly, and these contigs were subsequently used for gene prediction and annotation. Open reading frames (ORFs) from each assembled contig were predicted using MetaGene Mark (http://exon.gatech.edu/meta_gmhmmp.cgi). All predicted genes with a sequence identity of 95% and coverage of 90% were clustered using MMseqs2 software (https://github.com/soedinglab/mmseqs2).

Representative sequences of the non-redundant gene catalog were aligned to the NCBI NR database with an e-value cutoff of 1e-5 using Diamond software for taxonomic annotations. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was conducted using Diamond (version 0.9.29) against the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/keeg/) with an e-value cutoff of 1e-5. Evolutionary Genealogy of Genes: Non-

supervised Orthologous Groups (EggNOG) annotation was conducted using Diamond software (version 0.9.24) with an e-value cutoff of 1e-5.

Statistical Analyses

All statistical analyses were performed using SPSS version 21.0 (IBM Corporation, Armonk, NY, USA). Quantitative data were reported as interquartile range The two-tailed non-parametric Mann–Whitney test or Wilcoxon rank-sum test was used for statistical analyses. Alpha diversity metrics such as Chao1, Ace, Shannon, Simpson and Community evenness were analyzed using the software and database R v3.1.1 (picante, v1.8.2). Beta diversity analyses such as principal component analysis (PCA) were analyzed using python2 (sklearn 0.17.1), principal coordinates analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) were analyzed using the software and database python2 (cogent v1 5.3). Software and database python2 (cogent v1.5.3) was used for unweighted pair-group method with arithmetic mean (UPGMA). Permutational multivariate analysis of variance (PerMANOVA) and analysis of similarities (Anosim) were analyzed using the software and database R v3.1.1 (vegan, v2.3–0). Receiver operating characteristic (ROC) curve analyses were plotted to evaluate the predictive power of selected microbial markers associated with intestinal fibrous stenosis or without stenosis. The linear discriminant analysis effect size (LEfSe) pipeline was employed to identify biomarkers with statistical differences and was performed using software and database python2 (lefse v20171228). All significance tests were two-sided, and differences with $p < 0.05$ were considered statistically significant.

Results

Characteristics of the Study Population and Fecal Metagenomic Sequencing

Sixty patients with an established diagnosis were recruited to the study between April 2020 and November 2023. Fecal samples were obtained from 54 patients, while 4 patients did not provide fecal samples. Patients were evaluated for intestinal fibrous stenosis using MRE[12] (Figure 1). Following quality control of fecal metagenomic sequencing data, one sample was excluded, resulting in a final analysis of 53 samples. The demographic and clinical characteristics of patients are detailed in Table 1. No statistically significant differences were observed between CD patients without stenosis and with fibrous stenosis regarding age, gender, disease duration, and inflammatory markers such as CDAI score, ESR, and CRP. Additionally, after denoising, removal of chimeras, and filtering, the 53 samples yielded a median of 41224699.92 reads with an interquartile range (IQR) of 38465485.00–43,091,074.00.

Alpha Diversity and Beta Diversity Analysis Between CD Patients Without Stenosis and with Fibrous Stenosis

To explore species richness, we computed Chao1 (Figure 2B) and Ace indices (Figure 2A), while for species diversity, we calculated the Pielou_evenness (Figure 2C) and Shannon (Figure 2D) and Simpson indices (Figure 2E). No

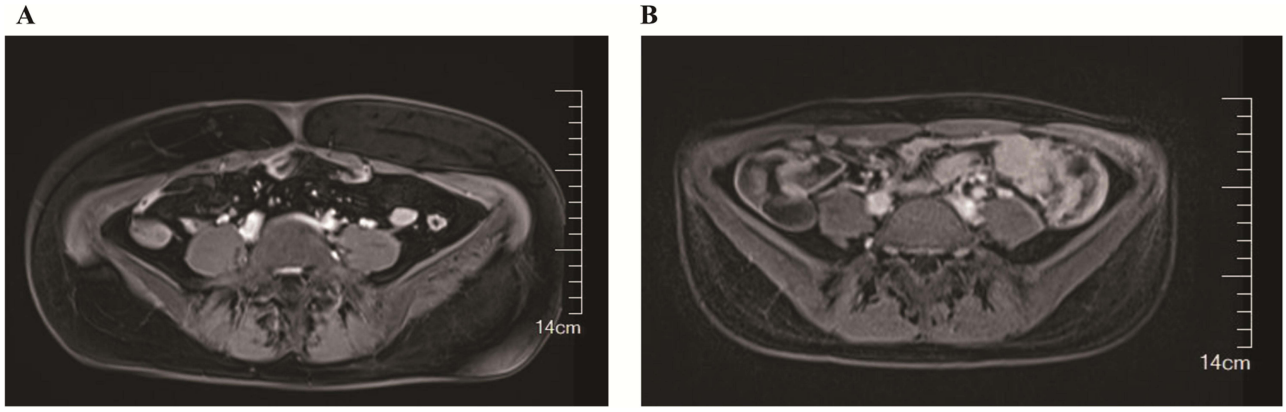

Figure 1 Crohn's disease patients without stenosis or with fibrous stenosis. (**A**) The axial T1WI after contrast enhancement showed the thickened and enhanced intestinal wall with lumen fibrous stenosis. (**B**) The axial T1WI after contrast enhancement showed the thickened and enhanced intestinal wall without lumen stenosis.

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Table 1 Baseline Characteristics of the CD Patients

Characteristics	Fibrous stenosis	Without stenosis	P
Age (y)	39 (32, 46)	34 (29, 40)	0.32
Male/Female (n)	13/11	17/12	0.75
Disease duration (y)	3.7 (2.4, 5.1)	3.6 (2.2, 4.9)	0.72
Montreal classification			
A1/A2/A3 (n)	2/12/10	1/20/8	0.34
L1/L2/L3 (n)	8/5/11	5/13/11	0.14
Perianal lesions	7 (41.2%)	7 (31.8%)	0.68
ESR (mm/h)	45.09 (28.32, 61.08)	56.15 (40.13, 72.17)	0.29
CRP (mg/L)	32.31 (15.21, 49.41)	35.33 (15.83, 54.83)	0.76
CDAI	186.98 (126.12, 247.84)	174.18 (128.40, 219.97)	0.84

Note: Age, disease duration, ESR, CRP, and CDAI were expressed as interquartile range.

significant differences were observed between the two groups across five alpha diversity metrics. Additionally, in examining species composition diversity between the two groups, six beta diversity indices were calculated based on PCA (Figure 3A), PCoA (Figure 3B), NMDS (Figure 3C), UPGMA (Figure 3D), Anosim (Figure 3E), an PerMANOV (Figure 3F). However, no discernible differences were found between the two groups in any of these analysis.

Altered Fecal Microbiota in CD Patients Without Stenosis and with Fibrous Stenosis

To better understand changes in fecal microbiota between CD patients without stenosis and with fibrous stenosis, we conducted the Mann–Whitney *U*-test comparing taxa from the order to species levels. A total of 70 taxa (4 phyla, 6 classes, 15 orders, 15 families, 15 genera, and 15 species) exhibited significant differences in abundance ($p < 0.05$) between the two groups, as detailed in Figure 4.

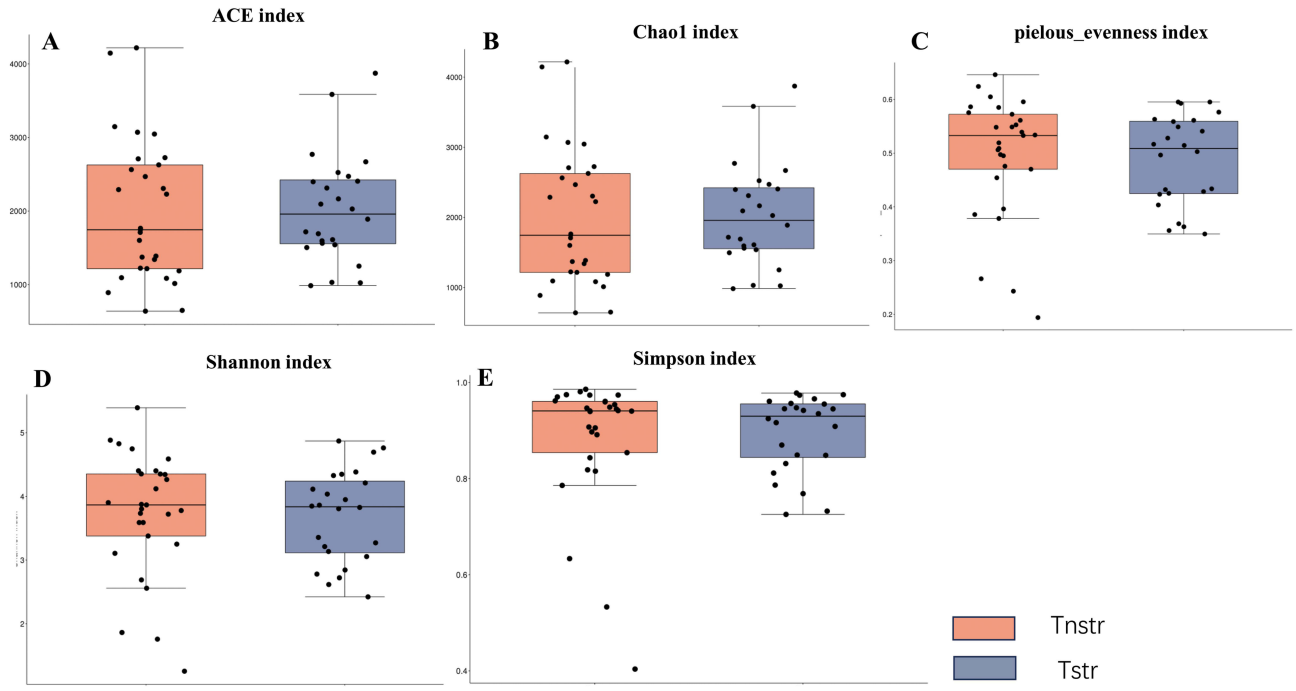

Figure 2 Alpha diversity analysis of fecal microbiota in CD patients without stenosis and with fibrous stenosis. Alpha diversity indices were estimated using the number of observed (**A**) ACE, (**B**) Chao index, (**C**) Pielou_evenness index, (**D**) Shannon index, and (**E**) Simpson index. Tnstr: group without stenosis; Tstr: group with fibrous stenosis.

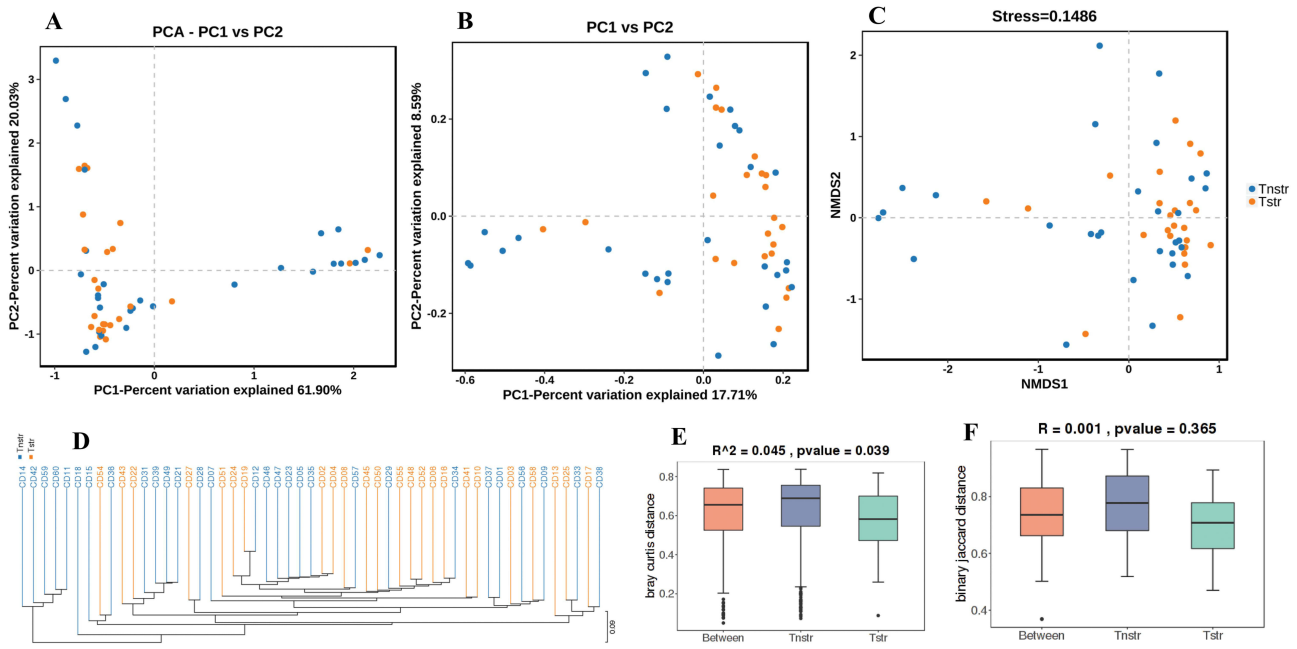

Figure 3 Beta diversity analysis of fecal microbiota in CD patients without stenosis and with fibrous stenosis Beta diversity indices were estimated using the number of observed (**A**) PCA, (**B**) PCoA, (**C**) NMDS, (**D**) UPGMA, (**E**) Anosim, and (**F**) PerMANOVA. Significance was determined by the Mann–Whitney *U*-test, with *P < 0.05. Tnstr: group without stenosis; Tstr: group with fibrous stenosis.

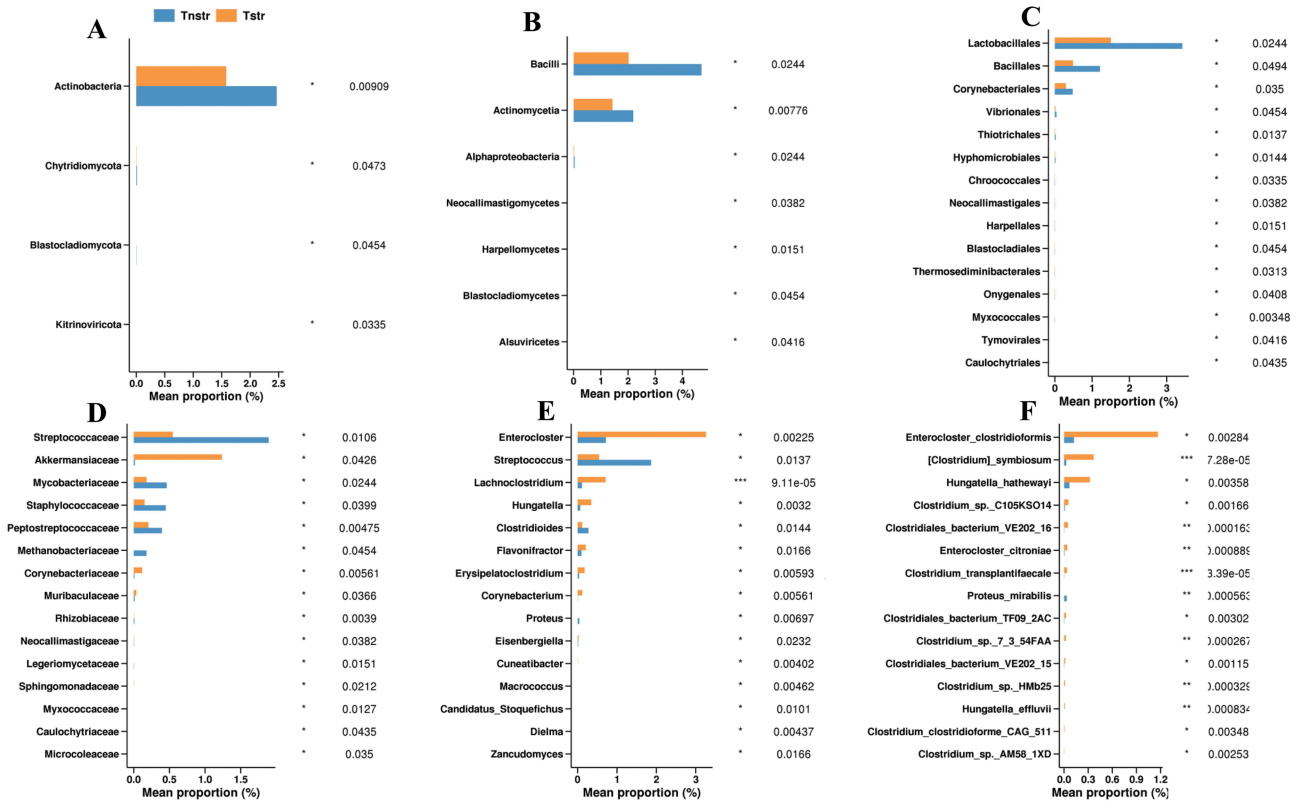

Figure 4 Taxonomic differences in fecal microbiota in CD patients without stenosis and with fibrous stenosis. (**A**) phylum level. (**B**) class level. (**C**) order level. (**D**) family level. (**E**) genus level. (**F**) species level. *P < 0.05, **P < 0.01, ***P < 0.001. Tnstr: group without stenosis; Tstr: group with fibrous stenosis.

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At the phylum level (Figure 4A), *Actinobacteria* (0.025 vs 0.016), *Kitrinoviricota* (4.98E-6 vs 2.39E-6), *Blastocladiomycota* (1.37E-5 vs 33.78E-6) and *Chytridiomycota* (1.2E-4 vs 4.12E-5) exhibited a significant reduction in CD patients with fibrous stenosis compared to those without stenosis. At the class level (Figure 4B), the abundances of *Bacilli* (0.047 vs 0.020), *Actinomycetia* (0.022 vs 0.014), and *Alphaproteobacteria* (3.86E-4 vs 2.49E-4) were significantly increased in CD patients without stenosis. At the order level (Figure 4C), the abundances of *Lactobacillales* (0.034 vs 0.015), *Bacillales* (0.012 vs 0.0048) and *Corynebacteriales* (0.0048 vs 0.0029) were significantly higher in patients without stenosis than in patients with fibrous stenosis. At the family level (Figure 4D), the abundances of *Streptococcaceae* (0.019 vs 0.0055), *Mycobacteriaceae* (0.0046 vs 0.0018), *Staphylococcaceae* (0.0045 vs 0.0015), *Peptostreptococcaceae* (0.0039 vs 0.0020) and *Methanobacteriaceae* (0.0018 vs 1.6E-6) were significantly increased while the abundances of *Akkermansiaceae* (0.00015 vs 0.012) and *Corynebacteriaceae* (8.93E-5 vs 0.0012) were significantly decreased in CD patients without stenosis than in patients with fibrous stenosis. At the genus level (Figure 4E), the abundances of *Streptococcus* (0.0187 vs 0.0054) and *Clostridioides* (0.0028 vs 0.0011) were increased while the abundances of *Enterocloster* (0.0071 vs 0.0326), *Lachnoclostridium* (0.0010 vs 0.00712) and *Hungatella* (0.0007 vs 0.0035) were significantly decreased in CD patients without stenosis than in patients with fibrous stenosis. Remarkably, the abundances of several species within *Clostridiales*, such as *Clostridium_transplantifaecale, Clostridium_symbiosum*, and *Clostridium_sp.*_HMb25, were significantly increased in CD patients with fibrous stenosis than in patients without stenosis (Figure 4F). Briefly, although there were no differences in microbiota diversity between the two groups, significant discrepancies in species composition between the two groups were evident, underscoring their potential implications for disease progression.

Fecal Microbiota as a Predictor of Fibrous Stenosis in CD Patients

Considering the observed alterations in gut microbiota among CD patients without stenosis and with fibrous stenosis, we further conducted LEfSe analyses with a significance threshold of $p < 0.01$ to identify the most significantly enriched taxa between the two groups (Figure 5A and B). Subsequently, *g_Bacteroides* and *g_Enterocloster* were identified to be significantly correlated with fibrous stenosis in CD, while *p_Actinobacteria, c_Actinomycetia, c_Bacilli, o_Lactobacillales, f_Streptococcaceae, g_Streptococcus* were significantly correlated with non-stenosis in CD. These findings suggested the potential utility of these bacteria as promising microbiota biomarkers for predicting fibrous stenosis and non-stenosis in CD patients.

We then assessed the prognostic value of gut microbiota in predicting fibrous stenosis and non-stenosis in CD patients by evaluating the area under the curve (AUC) derived from ROC curve analysis. The use of *g_Bacteroides* or *g_Enterocloster* abundance alone achieved a prediction accuracy of 0.675 (95% CI, 0.524 vs.0.826, P = 0.029) and 0.746 (95% CI, 0.614 vs.0.878, P = 0.002), respectively (Figure 5D). The use of *p_Actinobacteria, c_Actinomycetia, c_Bacilli, o_Lactobacillales, f_Streptococcaceae, g_Streptococcus* abundance alone achieved prediction accuracy of 0.710 (95% CI, 0.562 vs.0.858, P = 0.009), 0.714 (95% CI, 0.572 vs.0.856, P = 0.008), 0.681 (95% CI, 0.536 vs.0.826, P = 0.024), 0.681 (95% CI, 0.536 vs.0.826, P = 0.024), 0.705 (95% CI, 0.564 vs.0.847, P = 0.011), and 0.698 (95% CI, 0.556 vs.0.841, P = 0.014), respectively. Moreover, a combined analysis of the aforementioned microbiota improved the accuracy of predicting non-stenosis in CD to 0.730 (95% CI, 0.593 vs.0.867, P < 0.004) (Figure 5C).

Functional Profiling with KEGG Pathway Enrichment Analysis and eggNOG Gene Annotation

The KEGG database provides systematic metabolic pathways and cellular functions of gene products. Utilizing this database, we investigated metabolic functional differences between CD patients without stenosis and with fibrous stenosis (Figure 6). At level two of KEGG pathways, we observed significant enrichment of two pathways, namely Folding, sorting, degradation, and Transcription, in CD patients without stenosis (p = 0.0416 and p = 0.0202, respectively) compared to those with fibrous stenosis. Moreover, at KEGG pathway level three, we identified five metabolic pathways differentially enriched between CD patients without stenosis and with fibrous stenosis. These

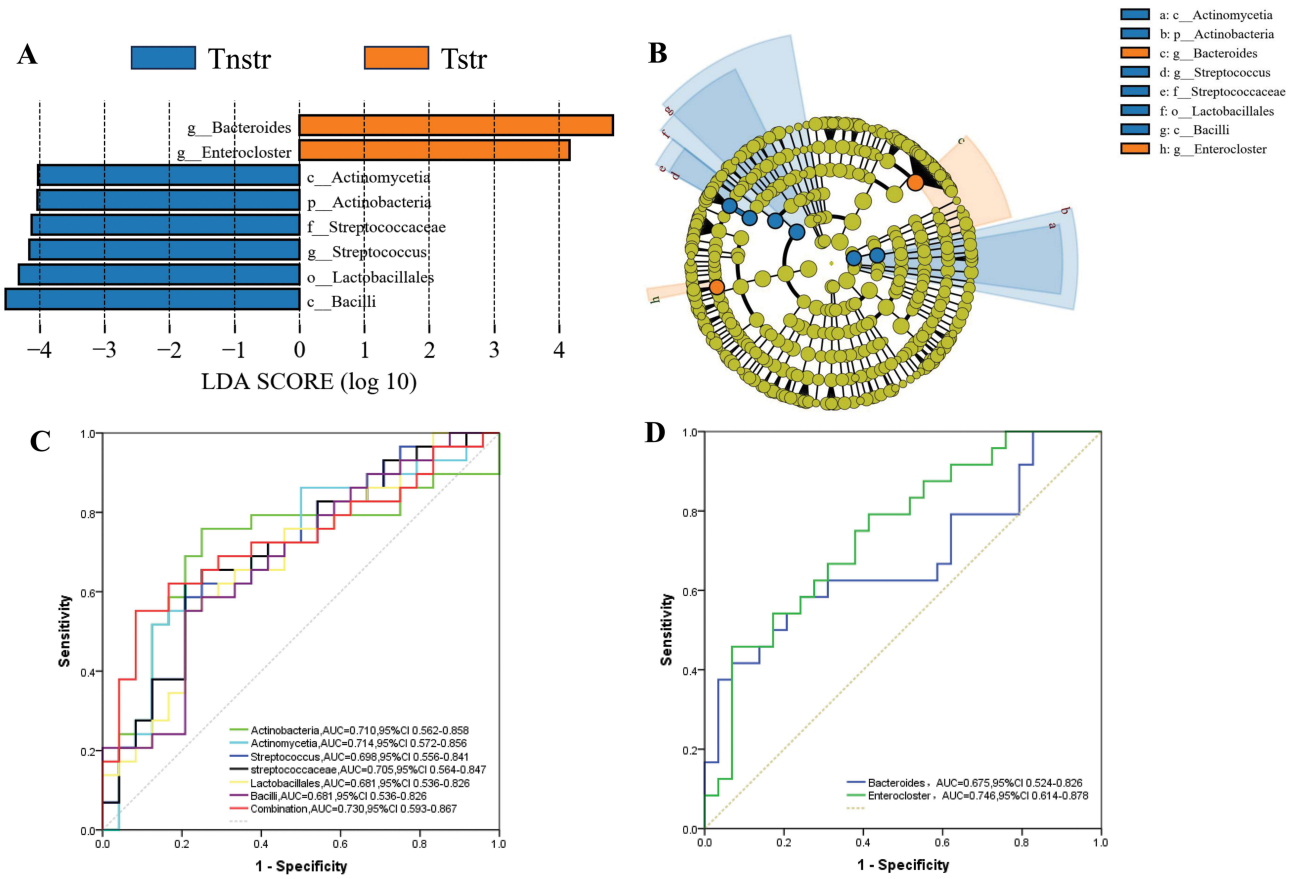

Figure 5 Fecal microbiota as a predictor of fibrous stenosis or non-stenosis in CD. (**A**) Bacterial taxa are differentially represented in patients without stenosis and with fibrous stenosis with a statistical significance indicated by linear discriminant analysis (LDA) score (>2). (**B**) Cladogram of LEfSe analysis illustrating differences in various bacteria between CD patients without stenosis and with fibrous stenosis. Circles from the inside to the outside of the evolutionary branching diagram represent taxonomic levels from kingdom to species. Each small circle at different taxonomic levels represents a taxon at that level, with the circle's diameter proportional to the relative abundance. Taxa with no significant differences are uniformly colored yellow, while those with significant differences are colored red or blue. (**C**) Prediction performance of the non-stenosis model using ROC curves based on fecal microbiota. (**D**) Prediction performance of fibrous stenosis models using ROC curve based on fecal microbiota. Tnstr: group without stenosis; Tstr: group with fibrous stenosis.

pathways are primarily associated with metabolism (sphingolipid metabolism, p = 0.0335; lipoic acid metabolism, p = 0.0416) and biosynthesis (eg, neomycin, kanamycin, and gentamicin biosynthesis, p = 0.014; carbon fixation pathways in prokaryotes, p = 0.0130; penicillin and cephalosporin biosynthesis, p = 0.00035). Notably, the sphingolipid metabolic pathway is associated with fibrosis, while lipoic acid metabolism exhibits anti-inflammatory effects, suggesting their potential roles in the development of intestinal fibrous stenosis in CD patients through bacterial regulation of metabolic pathways.

The EggNOG database was used for identifying and annotating homologous genes to study their evolution and function (Figure 7). Analysis using the eggNOG database revealed differences in four functional categories between the two groups: [R]: General function prediction only (p = 0.0473); [M]: Cell wall/membrane/envelope biogenesis (p = 0.0267), [P]: Inorganic ion transport and metabolism (p = 0.0137), and [T]: Signal transduction mechanisms (p = 0.0561). Apart from [R]: General function prediction, the other three functional categories were higher in CD patients with fibrous stenosis than those without stenosis, representing cellular process, signaling, and metabolism, respectively.

Further correlation analysis of functional genes was conducted based on KEGG pathway_level3 and eggNOG NOG. The top 80 functional genes with the highest abundance were screened, and Spearman algorithm was employed for correlation analysis and statistical testing of abundance changes in each sample.

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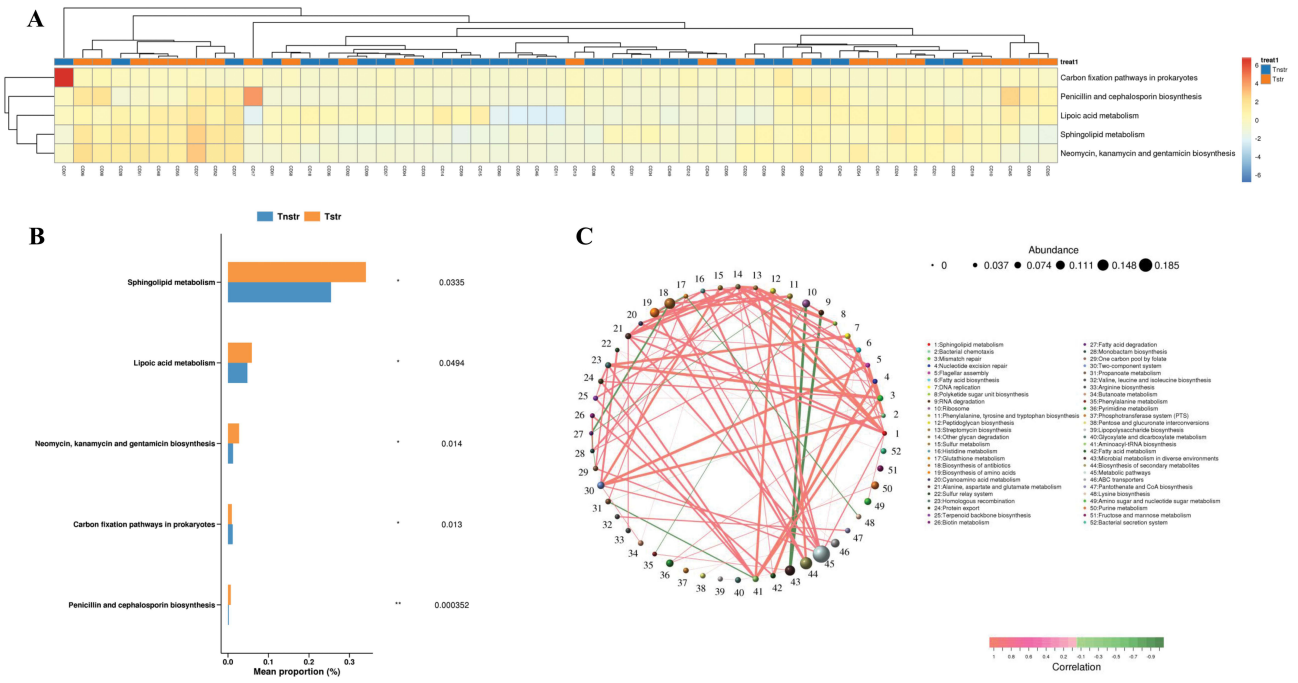

Figure 6 Functional profiling with KEGG pathway enrichment analysis. (**A**) Functional genes were assessed for non-parametric differences with a threshold of $p < 0.05$. The clustering tree on the right depicts the differential functional gene clustering, while the top clustering tree represents sample clustering, with the heatmap in the middle. (**B**) Relative abundance of KEGG pathways that exhibit differential distribution between CD patients without stenosis and with fibrous stenosis ($p < 0.05$). (**C**) Correlation network diagram illustrating correlation analysis based on KEGG pathway_level3. Circles represent functional genes, with the size indicating abundance. Lines denote the correlation between two functional genes, with line thickness representing the strength of correlation, red lines representing positive correlation, and green lines indicating negative correlation. Tnstr: group without stenosis; Tstr: group with fibrous stenosis.

Discussion

Environmental, microbiota, immunological, and genetic factors may contribute to the development of CD.[13] At diagnosis, at least 10% of patients with CD exhibit a stenosis phenotype, such as strictures or fistulae, while the majority present with a purely inflammatory phenotype without complications.[14] Population-based studies have shown that around 20% of patients develop stenosis within 20 years of being diagnosed with CD.[15] However, it is important to note that some studies have used the Montreal or Vienna classification systems based solely on information about patients' symptoms.[16] As a result, these studies might underestimate the true incidence of the stenosis phenotype.

Colonoscopy is one of the important diagnostic methods for CD. However, colonoscopy mainly observes mucosal changes in the intestinal lumen, and sometimes, the inability of colonoscopy to pass through the narrowed intestinal segment hampers its ability to evaluate stenosis. As a result, in clinical practice, we often use cross-sectional imaging analyses, such as ultrasound,[17] computed tomography (CT),[18] or magnetic resonance imaging (MRI),[19] to identify stenosis in the small intestine and colon. However, these tests had some limitations. For example, the activity of the bowel affects the resolution of ultrasound; repeated CT exams cause increased radiation exposure to the patients, which might lead to tumor development; MRI examination takes a long time, and the use of MRI is limited for patients with metal implants in the body, such as pacemakers and metal dentures.

Patients with CD predominantly have inflammatory stenosis in the early stages. When the inflammatory stenosis transforms into fibrous stenosis, patients may experience frequent abdominal pain, vomiting, and ineffective drug therapy. Although multiple clinical, serologic, genetic, and epigenetic factors have been explored to predict patients with fibrous stenosis, their specificity and reliability for predicting the possibility of complicated CD are poor.[20] Thus, they are not recommended for clinical practice.[21] Alterations in the gut microbiota have been associated with the development and progression of CD, and simultaneous alterations in the microbiota and microbiota metabolites might ultimately lead to fibrous stenosis in CD.[22] However, the use of microbiota as a predictive biomarker of fibrous stenosis in CD patients has been poorly explored.

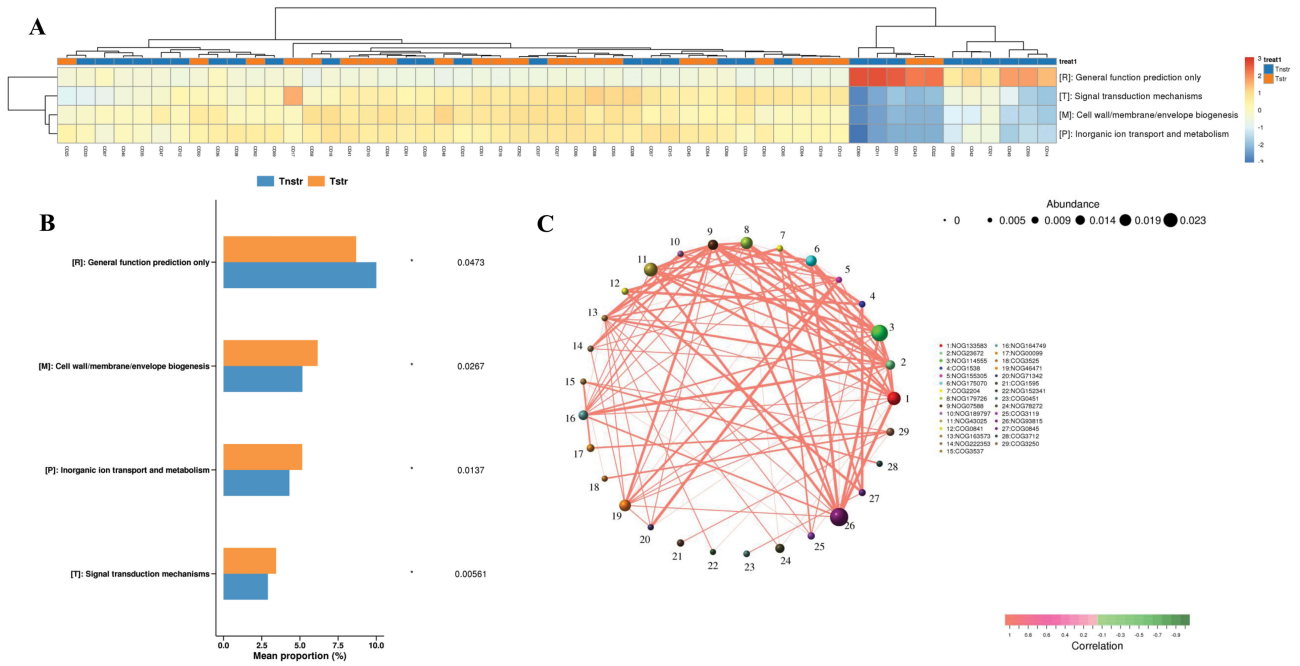

Figure 7 Functional profiling with eggNOG. (**A**) Functional genes were evaluated for non-parametric differences with $p < 0.05$. The right clustering tree depicts the differential clustering of functional genes, while the top clustering tree represents the sample clustering, with the heatmap in the middle. (**B**) Relative abundance of eggNOG categories that exhibited differential distribution between GC patients with fibrous stenosis and without stenosis ($p < 0.05$). (**C**) Correlation network diagram illustrating correlation analysis based on eggNOG. Circles represent functional genes, with their size representing abundance. Lines denote the correlation between two functional genes, with the line thickness representing the strength of correlation. Red lines indicate positive correlations, and green lines represent negative correlations. Tnstr: group without stenosis; Tstr: group with fibrous stenosis.

We analyzed species richness and diversity by alpha and beta diversity, and there were no differences between the fibrous stenosis and non-stenosis groups. Through non-parametric detection of microbiota differences, we found that 70 species differed between the two groups, encompassing 4 phyla, 6 classes, 15 orders, 15 families, 15 genera, and 15 species. Interestingly, several Clostridia were significantly elevated at the species level in CD patients with fibrous stenosis. *Clostridia* are anaerobic or slightly aerobic Gram-positive bacteria. Although most *Clostridia* strains are non-pathogenic, a few are pathogenic and can produce exotoxins and invasive enzymes.[23] The role of *Clostridia* in CD patients with intestinal fibrous stenosis has not been previously studied. Furthermore, LEfSe analysis revealed that the abundance of *g_Bacteroides* and *g_Enterocloster* could be used to predict fibrous stenosis while that of *p_Actinobacteria, c_Actinomycetia, c_Bacilli, o_Lactobacillales, f_Streptococcaceae*, and *g_Streptococcus* could be used to predict non-stenosis. Especially in predicting non-stenosis, the AUC of the combined bacteria reached 73%. These results suggested that microbiota might be a promising predictor of fibrous stenosis and without stenosis in CD patients.

Taxonomically, the composition and relative abundance of the gut microbiota vary considerably. However, its core functions in microbial fitness and adaptation to different niches along the GI tract are similar. Functional changes will affect host-microbe interactions essential for the intestinal mucosa and immune system homeostasis. Thus, functional analysis may be useful for distinguishing fibrous stenosis basis in CD. In our study, certain changes in the metabolic capacity of KEGG pathways and eggNOG were observed in the fecal microbiota of CD patients without stenosis or with fibrous stenosis.

Regarding the KEGG pathway, we found that sphingolipid and lipoic acid metabolism pathways were significantly elevated in CD patients with fibrous stenosis. In the eggNOG database, four functional categories were found to be significantly different between the two groups, such as general function prediction, cell wall/membrane/envelope biogenesis, inorganic ion transport and metabolism, and signal transduction mechanisms. Sphingolipids are plasma membrane components involved in the control of cellular processes such as proliferation, migration, and apoptosis.[24] Increasing evidence suggests that sphingolipids and their signaling pathways play an important role in the development of tissue fibrosis, including pulmonary fibrosis,[25] liver fibrosis,[26] and kidney fibrosis.[27] Our study found that this pathway

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may also be involved in intestinal fibrosis in CD patients. In addition, lipoic acid is traditionally recognized as an effective antioxidant capable of thiol-disulfide exchange. Previous research has found that administrating lipoic acid effectively ameliorated DSS-induced UC in mice by alleviating intestinal cell apoptosis, regulating antioxidant pathways, and inhibiting ferroptosis.[28] The increased metabolism of lipoic acid in the fibrous stenosis group suggested that this metabolic pathway was associated with the alleviation of inflammation and post-inflammatory tissue repair that ultimately led to intestinal fibrous stenosis in CD.

This study has several limitations. First, this was a small single-center cross-sectional study without a validation dataset. Second, all results were solely based on feces-associated microbiota, and no data from the mucosa-associated microbiota was available. Third, further research is needed to investigate the specific mechanisms and pathways by which bacterial communities promote or inhibit fibrous stenosis.

Conclusions

This study revealed a significant association between intestinal fibrous stenosis and changes in intestinal microbiota among CD patients. Moreover, the probability of fibrous stenosis in CD patients can be predicted based on the characteristics of intestinal microbiota. Additional functional investigation also uncovered notable distinctions between patients without stenosis and with fibrous stenosis, underscoring the important roles of intestinal microbiota in the manifestation of fibrous stenosis among CD patients.

Institutional Review Board Statement

The study involving human participants was reviewed and approved by the Institutional Review Board for Clinical Research of Sichuan Provincial People's Hospital (No. 20230215) and registered in the Medical Research Registration Information System (MR-51-23-013534) and China Clinical Trial Registration Center (ChiCTR230069727). Our study complies with the Declaration of Helsinki. All enrolled patients signed an informed consent to participate.

Data Sharing Statement

The data analysis of this study was obtained from the open cloud platform of Beijing BMKGENE Technology Co. (http://international.biocloud.net/). All methods were carried out in accordance with relevant guidelines and regulations. The data used or analysed during the current study are available from the corresponding author on reasonable request.

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Disclosure

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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