Alteration of Fungal Microbiota After 5-ASA Treatment in UC Patients

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The effect of treatment regimens on fungal microbiota is unclear in ulcerative colitis (UC) patients. Here, we aimed to clarify the effect of 5-aminosalicylic acid (5-ASA) treatment on gut fungal microbiota in UC patients. Fifty-seven UC patients, including 20 treatment-naïve and 37 5-ASA-treated, were recruited into an exploration study. We compared the gut fungal profiles of these 2 groups of patients using *ITS1-2 rDNA* sequencing. Ten out of 20 treatment-naïve UC patients were followed up and enrolled for a validation study and underwent a 5-ASA treatment. We assessed the longitudinal differences of fungal microbiota in these patients before and after 5-ASA treatment. Results acquired from the validation study were accordant to those from the exploration study. Ascomycota was the dominant phylum in both noninflamed and inflamed mucosae. At the phylum level, Ascomycota decreased in inflamed mucosae before 5-ASA treatment. At the genus level, pathogens such as *Scytalidium*, *Morchella*, and *Paecilomyces* increased, and *Humicola* and *Wickerhamomyces* decreased in inflamed mucosae. After 5-ASA treatment, Ascomycota and *Wickerhamomyces* increased and *Scytalidium*, *Fusarium*, *Morchella*, and *Paecilomyces* decreased in both noninflamed and inflamed mucosae. Additionally, the balanced bacteria–fungi correlation was interrupted in inflamed mucosae, and 5-ASA treatment altered group-specific fungal microbiota and restored bacteria–fungi correlation in UC patients. Our results demonstrated that fungal diversity and composition were altered and the bacteria–fungi correlation was restored in inflamed mucosae after 5-ASA treatment.

Key Words: ulcerative colitis, 5-aminosalicylic acid, mucosal microbiota, fungal dysbiosis

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a lifelong illness. Although the pathogenesis of IBD remains ambiguous, it is comprehensively accepted that the interruption of the host immunity–microbiota interaction plays a pivotal role in the process of IBD development.[1, 2] Nowadays, bacterial dysbiosis is frequently reported in IBD pathogenesis. Apart from bacteria, fungal microbiota is an indispensable part of human gut microbiota.

In healthy adult subjects, fungal microbiome is constituted by Ascomycota (34% ± 14%), Basidiomycota (44% ± 14%), and a small content of other phyla (22% ± 19%); in addition, *Saccharomyces*, *Debaryomyces*, *Penicillium*, *Candida*, *Malassezia*, *Alternaria*, and *Fusarium* constituted the main part of fungal microbiome at the genus level.[3] Although different fungal entities presented in different recruited healthy subjects, additional studies also confirmed that *Saccharomyces*, *Malassezia*, and *Sporobolomyces* were the dominant fungal genera in healthy adults and pediatric subjects.[3–7]

It is reported that fungal dysbiosis is related to IBD development, which is highlighted by the increased abundance of Basidiomycota and decreased abundance of Ascomycota.[3, 5, 8] In addition, compared with healthy subjects, skewed fungal microbiota was observed, with a decreased proportion of *Saccharomyces cerevisiae* and an increased proportion of *Candida albicans*.[5] Antibody directed against *Saccharomyces cerevisiae* mannan (anti-*Saccharomyces cerevisiae* antibody [ASCA]) was reported to be associated with CD, and it was used as an advisable effect for diagnosing CD pathogenesis. Gene expression associated with antifungal immunity was shown to be involved in IBD, such as Card9 and Dectin-1. Lacking the genes involved in sensing fungal stimulation, the host had a fungal overcolonization and was susceptible to experimental colitis.[1, 2]

Of note, the effect of treatment strategy on the bacterial microbiota has been rarely studied in IBD patients. Zheng reported the modulation of sulfasalazine (SASP) on bacterial composition and function in experimental colitis. In his study, SASP restored 2,4,6-trinitrobenzenesulfonic acid (TNBS)–induced bacterial dysbiosis, increased short-chain fatty acid (SCFAs)–producing bacteria, and decreased Proteobacteria abundance.[9] Apart from SASP, 5-aminosalicylic acid (5-ASA), another anti-inflammatory modulator, is the primary therapeutic regimen for controlling the inflammatory status of IBD patients.[10, 11] Although it was reported that 5-ASA altered fecal

Received for publications May 9, 2019; Editorial Decision August 17, 2019.

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Supported by: This study was supported by the Capital Characteristic Clinic Project (Z161100000516161), Beijing Municipal Science and Technology Project (Z171100000417022), and National Natural Science Foundation of China (81670499).

Conflicts of interest: None.

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doi: 10.1093/ibd/izz207
Published online 21 November 2019

bacterial microbiota in patients with irritable bowel syndrome (IBS),[12] the effect of 5-ASA on mucosal fungal microbiota in UC patients is still unclear. Therefore, we aimed to clarify the effect of 5-ASA treatment on the fungal microbiota in this study.

METHODS

Study Subjects and Biopsy Collection

This study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital. All UC patients were enrolled from Peking University People's Hospital from January 2015 to January 2017. The diagnosis of UC was established according to the World Gastroenterology Organization Global Guidelines.[13] Fifty-seven UC patients in the active stage were enrolled for an exploration study, including treatment-naïve (n = 20) and 5-ASA-treated (n = 37) groups. Only patients undergoing regimens for at least 1 month were assigned to the 5-ASA-treated group. Furthermore, half of the treatment-naïve patients from the exploration study were followed up. These 10 patients underwent a 5-ASA treatment for about 6 months and showed a decrease of the Mayo Clinic score, but not a complete clinical remission. All of them were recruited into a validation study to confirm the data acquired from the exploration study (Table 1; Supplementary Table 1). All recruited patients were asked to avoid using probiotics and antibiotics at least 2 weeks before sampling. After that, for each patient, inflamed mucosae and adjacent noninflamed mucosae were obtained endoscopically and stored at −80°C after freezing in liquid nitrogen until DNA was extracted.

DNA Extraction

Microbial genomic DNA was extracted from biopsy samples using the QIAamp DNA Stool Mini Kit protocol (Qiagen, German) according to the manufacturer's instructions with minor modifications. Briefly, each biopsy sample was re-suspended in 200 μL of PBS with 80 μL of enzyme solution (22.5 mg of lysozyme powders; Sigma, USA) and 40 units of mutanolysine dissolved in 80 μL of 10-mM Tris-HCl/1 mM EDTA (Sigma, USA). After 40 minutes of incubation at 37°C, 2 zirconium beads (0.1 cm) were added, and the mixtures were homogenized in a Mini-Bead Beater (FastPrep, USA).[14] The following steps were performed according to the manufacturer's instructions.

Fungal ITS1-2 rDNA Amplification and Sequencing

After DNA extraction, fungal ITS genes were amplified using ITS1-2-specific barcoded primers, according to methods described by Luan et al.[15] Briefly, the ITS1-2 region was amplified using the following primers, 5'-GGAAGTAAAAGTCGTAACAAGG-3' (forward) and 5'-GCTGCGTTCTTCATCGATGC-3' (reverse). For amplification of the ITS gene, a 25-μL-volume system containing 0.2 μL of Kapa 2G Robust HotStar DNA polymerase (Kapabiosystem, USA), 5 μL of GC buffer, 0.5 μM of each primer, 0.5 μL of dNTP, 2 μL of genomic DNA, and 16.3 μL of ddH_2O was used. The reaction was held at 95°C for 3 minutes, followed by 35 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 1 minute, and an elongation at 72°C for 2 minutes in an ABI thermocycler (Applied Biosystems 2720, USA). The PCR products of the ITS1-2 genes were purified and quantified. After quantification using an ND-1000, version 3.3.0, spectrophotometer (NanoDrop, USA), paired-end sequencing (2 × 125 bp) in the exploration study was performed on an Illumina HiSeq 2500 sequencer in 2 lanes at the Center for Molecular Immunology of the Chinese Academy of Sciences (Beijing, P.R. China). To enhance the sequence quality, we performed paired-end sequencing (2 × 250 bp) in our validation study. Additionally, bacterial abundance was generated from 16S rRNA sequencing, as mentioned in a previous report.[16]

TABLE 1. Demographic and Clinical Data of UC Patients in the Exploration Study

Treatment		Treatment-Naïve	5-ASA-Treated	*P*
No.		20	37	–
Sex	Male/female, No.	12/8	9/28	0.032*
Age	Mean ± SD, y	48 ± 14	47 ± 16	0.796
Mayo Clinic score (endoscopic)	Mean ± SD	2.3 ± 0.6	2.1 ± 0.7	0.516
	Normal or inactive (0), No. (%)	0 (0)	0 (0)	–
	Mild (1), No. (%)	1 (5)	6 (16)	–
	Moderate (2), No. (%)	13 (65)	20 (54)	–
	Severe (3), No. (%)	6 (30)	11 (30)	–
Montreal classification	E1, No. (%)	4 (20)	10 (27)	–
	E2, No. (%)	7 (35)	11 (30)	–
	E3, No. (%)	9 (45)	16 (43)	–

Fungal ITS1-2 rDNA Sequences Analysis

The Illumina reads were sorted into different samples according to their barcodes. Both Vsearch, version 2.8.1, and Usearch, version 10 bit 32, were used in the process of sequencing analysis. Two-side reads were merged, and reads with low frequency (<10 in the exploration study and <8 in the validation study) were removed; high-quality reads (~1500) were used for further analysis. The exact sequence variants (ESVs) method was performed to filter chimeras with Unoise3.[17, 18] Operational taxonomic units (OTUs) were aligned using the Vsearch and taxonomically classified using the UNITE ITS database (utax_reference_dataset_22.08.2016).[18] We used the linear discriminant analysis (LDA) effect size (LEfSe) method to identify species that showed statistically significant differential abundance among groups.[17] We characterized fungal alpha diversity in the community by calculating the Shannon, richness, and Simpson diversity indexes. In addition, we analyzed fungal beta diversity using the principal coordinate analysis (PCoA) with weighted UniFrac distance.[19] In the validation study, the top 100 fungal OTUs were picked for building a phylogenetic tree with mafft and fastTree software,[20, 21] and then visualization was performed using the Interactive Tree Of Life (iTOL) website.[22] Based on OTUs, an Upsetplot was drawn for analysis of group-specific bacterial microbiota using the UpsetR package. Furthermore, to identify some fungal species indicators, all identified fungal genera were analyzed using the indicspecies package. The EdgeR package was used for fungal gene differential analysis, and group-to-group differential analysis data are displayed as a Manhattan plot.

Analysis of Microbial Interaction Pattern

To analyze microbial interaction patterns in diverse treatment strategies, pairwise bacterial and fungal abundance at the genus level was used for determining correlation using Spearman's method. The data of bacterial abundance were acquired from our previous report.[16] Correlation coefficients were calculated using the *psych* package of R software 3.5.1. Cytoscape 3.4.0 was launched to visualize the patterns of microbial interaction networks. Only significant correlations ($P \leq 0.05$ after false discovery rate correlation) were displayed.

Data Availability Statement

The ITS sequences generated in the present study are available through the NCBI Sequence Read Archive (accession number SRP136683), and 16S sequences used in this study are available through accession number SRP136321.

Statistical Analysis

The Adonis test was used for statistical analysis of the beta diversity. R software 3.5.1 with EdgeR was used for 2-group comparative analysis, and false discovery rate (FDR) was used to adjust *P* values. Analysis of variance using the least significant difference test was performed for multigroup comparisons in analysis of continuous variables. Only significant correlations ($P \leq 0.05$) are displayed.

Ethical Consideration

This study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital (Document No. 2016PHB024-01). All adult subjects provided written informed consent, and a parent or guardian of each child participant provided written informed consent on the child's behalf.

RESULTS

General Information on Recruited People

Fifty-seven active UC patients, including treatment-naïve (n = 20) and 5-ASA-treated (n = 37), were enrolled for the exploration study (Table 1). There was a significant sex-dependent difference in these 2 groups of patients (60% and 24% male in the treatment-naïve and 5-ASA-treated groups, respectively; $P = 0.032$). There was no significant difference in mean age between the treatment-naïve and 5-ASA-treated groups ($P = 0.796$). To correct the difference from sampling location and severity, about equal percentages of patients in Mayo endoscopic score and Montreal classification were enrolled in these 2 groups.

Of the 20 treatment-naïve patients, 10 out were followed up and enrolled for another independent validation study (Table 2). Mucosal samples were collected before and after 5-ASA treatment. The mean age of this group of patients was 33 ± 14 years. To avoid sex-based bias, about equal percentages of patients were enrolled in the validation study. The mean therapy time was 6 months. The total Mayo Clinic score was significantly decreased after 5-ASA treatment ($P = 0.002$), which reflected the anti-inflammatory efficiency of 5-ASA.

Alteration of Fungal Diversity in UC Patients After 5-ASA Treatment

Difference of fungal diversity between treatment-naïve and 5-ASA-treated UC patients in the exploration study

We compared the alpha diversity of 20 treatment-naïve and 37 5-ASA-treated UC patients, including Shannon's effective, richness, and Simpson's effective (Fig. 1A–C). There was higher richness but lower evenness in fungal diversity in the inflamed mucosae (UI) of treatment-naïve UC patients when compared with noninflamed mucosae (UN). Analysis of beta diversity with PCoA showed a separated clustering in the UN and UI groups (Adonis test, $P = 0.0002$) (Fig. 1D). We further analyzed the fungal content in the inflamed mucosae of treatment-naïve and 5-ASA-treated UC patients (UI and AI).

TABLE 2. Demographic and Clinical Data of UC Patients in the Validation Study

5-ASA Treatment		Before	After	*P*
No.			10	–
Sex	Male/female, No.		4/6	–
Age	Mean ± SD, y		33 ± 14	–
Median therapy time	Months		6	–
Mayo Clinic score (total)	Mean ± SD	9.2 ± 2.5	5.9 ± 1.8	0.002**
	Normal or inactive (0–2), No. (%)	0 (0)	0 (0)	–
	Mild (3–5), No. (%)	2 (20)	4 (40)	–
	Moderate (6–10), No. (%)	4 (40)	6 (60)	–
	Severe (11–12), No. (%)	4 (40)	0 (0)	–
Montreal classification	E1, No. (%)	5 (50)		–
	E2, No. (%)	2 (20)		–
	E3, No. (%)	3 (30)		–

Before, UC patients before 5-ASA treatment; After, UC patients after 5-ASA treatment.

Compared with the UI group, there was a higher Shannon's effective in the AI group ($P = 0.010$). Additionally, the fungal beta diversity showed a treatment-specific clustering in the inflamed mucosae of all samples (Adonis test, $P = 0.008$) (Fig. 1D).

Alteration of fungal diversity after 5-ASA treatment of UC patients in the validation study

We further analyzed the fungal diversity of 10 UC patients in the validation study. Fungal richness was higher ($P = 0.022$), but evenness was lower (although not significantly so) in inflamed mucosae (preI) than noninflamed mucosae (preN) before 5-ASA treatment (Fig. 1E–G). Analysis of beta diversity showed a distinct clustering of samples in the preN and preI groups (Adonis test, $P = 0.011$) (Fig. 1H). These data suggest a difference in fungal composition in the noninflamed and inflamed mucosae of UC patients before treatment. Compared with the preI group, richness in inflamed mucosae decreased, but evenness increased (although not significantly so) in the postI group after 5-ASA treatment (Fig. 1D, E). Accordingly, PCoA data showed no significant difference in beta diversity in the postI and preI groups. Notably, after 5-ASA treatment, there was no distinct clustering of samples in the postN and postI groups (Fig. 1F), which reflected a similarity of fungal composition in noninflamed and inflamed mucosae after 5-ASA treatment.

Alteration of Fungal Composition in UC Patients After 5-ASA Treatment

Difference of fungal composition between treatment-naïve and 5-ASA-treated UC patients in the exploration study

In agreement with previous studies,[3, 5] Ascomycota was the main fungal microbiota in identified OTUs (Fig. 2A). Before 5-ASA treatment, at the phylum level, there was a lower abundance of Ascomycota in the UI group than the UN group. At the genus level, there were higher abundances of *Scytalidium*, *Sporidiobolus*, *Vanrija*, and *Verticillium* in the UI group than the UN group (Fig. 2B).

To investigate the effect of 5-ASA treatment on fungal microbiota, we analyzed fungal composition in inflamed mucosa. We compared fungal microbiota in the UI and AI groups at the phylum and genus levels. We found that the abundance of Ascomycota was lower at the phylum level and that of *Mortierella, Scytalidium*, and *Verticillium* was higher at the genus level in the AI group than the UI group (Fig. 2A, B).

Alteration of fungal composition after 5-ASA treatment of UC patients in the validation study

In the validation study, Ascomycota was also the dominant phylum. We found that the abundance of Ascomycota markedly decreased and Zygomycota increased in the preI group compared with the preN group, which was in accordance with previous studies (Fig. 2C). At the genus level, *Wickerhamomyces* was the dominant genus (Fig. 2E). Compared with the preN group, *Aspergillus*, *Morchella*, *Mortierella*, *Paecilomyces*, and *Scytalidium* increased remarkably, but *Wickerhamomyces* decreased in the preI group (Fig. 2D, F).

We also estimated the 5-ASA treatment effect on fungal microbiota. Compared with inflamed mucosae before 5-ASA treatment (preI), the abundance of Ascomycota was markedly increased, and Zygomycota decreased at the phylum level in inflamed mucosae after 5-ASA treatment (postI) (Fig. 2C). At the genus level, *Wickerhamomyces* notably increased and *Scytalidium, Fusarium, Sporobolomyces*, *Paecilomyces*, *Morchella*, and *Mortierella* decreased significantly after 5-ASA treatment in the postI group compared with the preI group (Fig. 2D, F).

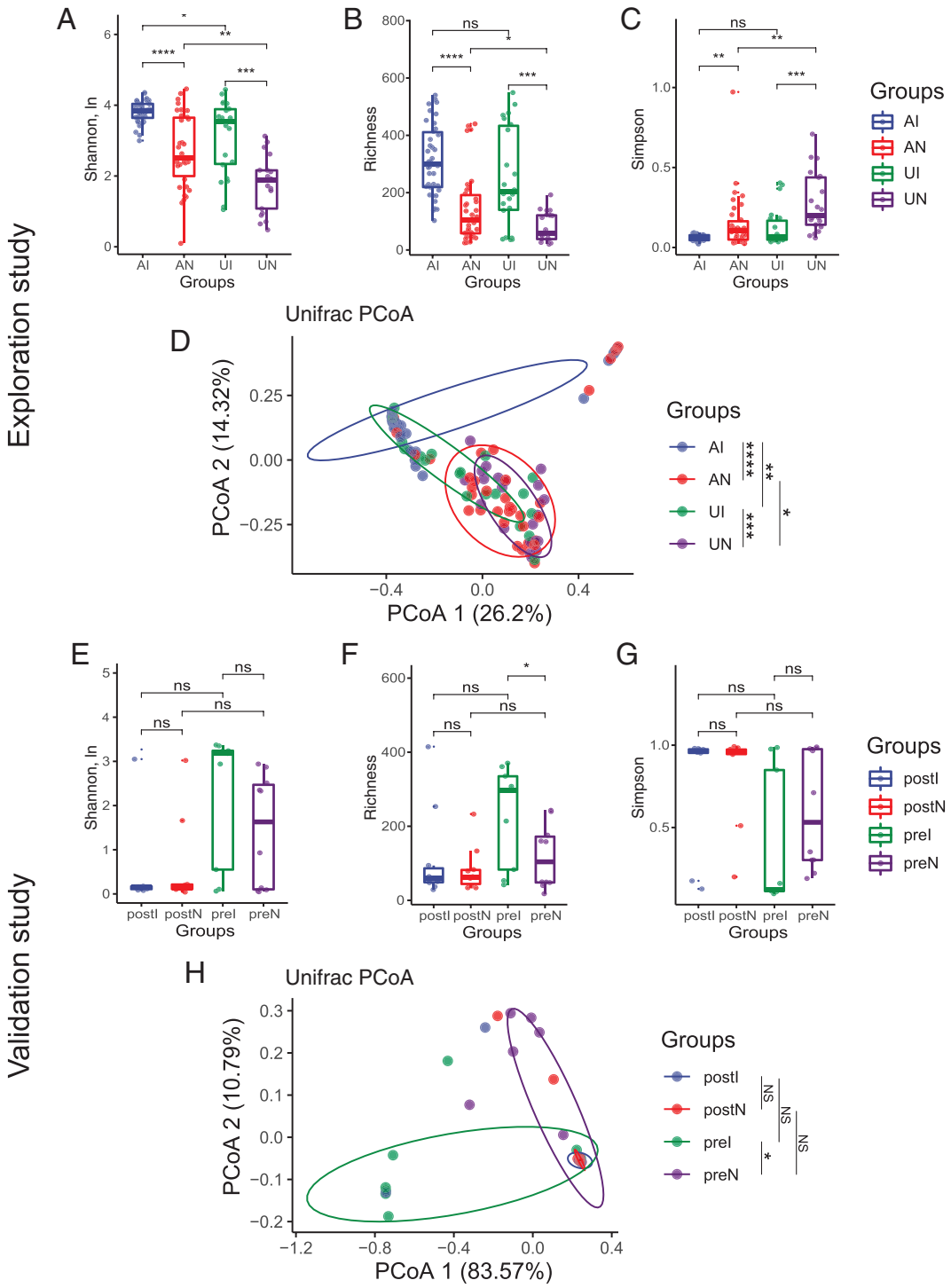

FIGURE 1. Fungal diversity in the exploration and validation studies. The fungal alpha diversity in the exploration study ((A) Shannon's effective dealt with natural logarithm ln; (B) richness index; (C) Simpson's effective). D, PCoA of the fungal beta diversity based on Unifrac distance in the exploration study. Abbreviations: AI, inflamed mucosae of 5-ASA-treated UC patients; AN, noninflamed mucosae of 5-ASA-treated UC patients; UI, inflamed mucosae of treatment-naïve UC patients; UN, noninflamed mucosae of treatment-naïve UC patients. Fungal alpha diversity in the validation study ((E) Shannon's effective dealt with natural logarithm ln; (F) richness index; (G) Simpson's effective). H, PCoA of the fungal beta diversity based on Unifrac distance in the validation study. Abbreviations: ns, nonsignificant; postI, inflamed mucosa of UC patients after 5-ASA treatment; postN, noninflamed mucosa of UC patients after 5-ASA treatment; preI, inflamed mucosa of UC patients before 5-ASA treatment; preN, noninflamed mucosa of UC patients before 5-ASA treatment. *$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$; ****$P \le 0.0001$.

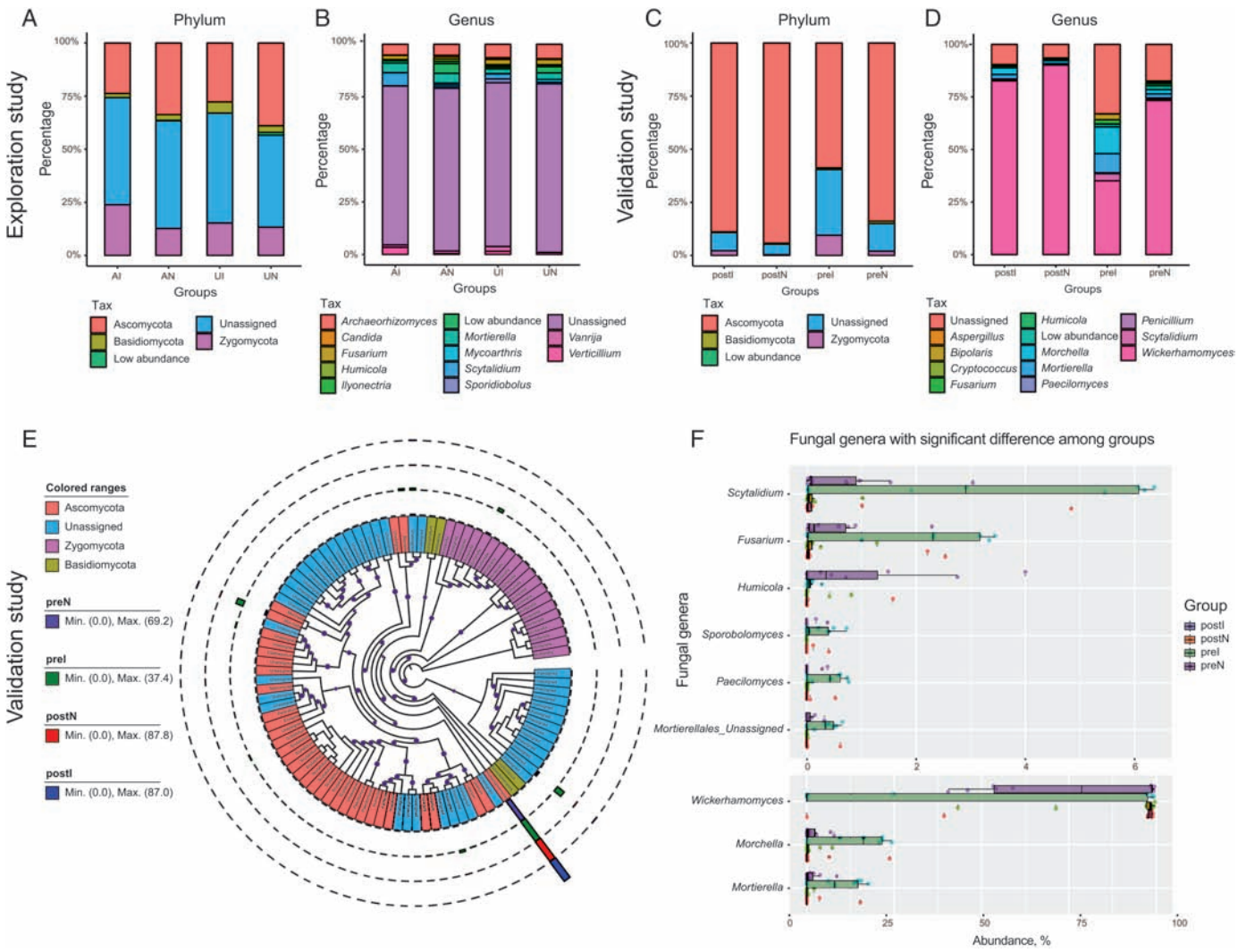

FIGURE 2. Fungal composition in the exploration and validation studies. Fungal composition at the phylum (A) and the genus (B) levels in the exploration study. Abbreviations: AI, inflamed mucosa of 5-ASA-treated UC patients; AN, noninflamed mucosa of 5-ASA-treated UC patients; UI, inflamed mucosa of treatment-naïve UC patients; UN, noninflamed mucosa of treatment-naïve UC patients. Fungal composition at the phylum (C) and the genus (D) levels in the validation study. E, Fungal phylogenetic tree. Top 100 fungal OTUs were picked to build this tree. Visualization was performed using the Interactive Tree of Life website (https://itol.embl.de/). The color of the inner circle represents the specific fungal phylum. The 4 outer circles represent 4 different groups, and the bar height displays the abundance of specific OTUs. F, Fungal genera with significant differences among groups in the validation study. Abbreviations: postI, inflamed mucosae of UC patients after 5-ASA treatment; postN, noninflamed mucosae of UC patients after 5-ASA treatment; preI, inflamed mucosae of UC patients before 5-ASA treatment; preN, noninflamed mucosae of UC patients before 5-ASA treatment.

Group-Specific Microbiota and Differential Fungal Genus in the Validation Study

First, we analyzed group-specific fungal microbiota in the validation study. There were 1344 OTUs identified in our validation study. One hundred ninety-six OTUs were shared by all groups, but different groups had different fungal microbiota. Two hundred eighty-one OTUs only existed in noninflamed mucosae (preN and postN), and 617 OTUs exclusively colonized in inflamed mucosae (preI and postI). Five hundred fifteen OTUs only existed in intestinal mucosae before 5-ASA treatment (preN and preI). Two hundred ninety-one OTUs, which were not colonized in the preN and preI groups, were colonized in intestinal mucosae after 5-ASA treatment (postN and postI). These data suggest that there were more fungal microbiota present in inflamed mucosae than noninflamed mucosae (preI vs preN, postI vs postN), additionally, after 5-ASA treatment, fungal microbiota was less colonized in both noninflamed and inflamed mucosae (postN vs preN, postI vs preI) (Fig. 3A).

We further analyzed the group-specific fungal genera in the 4 groups and identified the indicator species for each

group (Fig. 3B). We found that the following fungal genera, such as *Udeniomyces*, *Sarocladium*, *Rhizopus*, *Paecilomyces*, *Myrothecium*, *Lentinula*, and *Laetisaria*, were enriched in preI group. *Paecilomyces* tended to be a better indicative genus for inflamed mucosa before treatment. Notably, few indicative fungal genera were identified in the postN and postI groups.

Additionally, to investigate the fungal characterization in inflamed mucosae before treatment, we compared fungal genera in the preI and preN groups. We found that *Bipolaris* was significantly enriched in the preI group. *Humicola*, *Blumerla*, *Cryptococcus*, *Aureobasidium*, *Eutypella*, and *Schizophyllum* were significantly depleted in the preI group (Fig. 3C).

To explore the effect of 5-ASA treatment on fungal colonization in inflamed mucosae, we compared fungal genera in inflamed mucosae before and after 5-ASA treatment (postI and preI). We found that *Chaetothyriales*, *Chaetomium, Latorua*, *Aureobasidium*, and *Cryptococcus* were enriched but *Bipolaris* was depleted in the postI group (Fig. 3D). These depleted fungal genera in the preI group were enriched in inflamed mucosae after 5-ASA treatment (postI). We also compared the postI group with the postN group to display the difference between inflamed and noninflamed mucosae after 5-ASA treatment (Fig. 3E). A few differential fungal genera were found between the postI and postN groups, which indicated that a similar fungal colonization in inflamed and noninflamed mucosae after 5-ASA treatment occurred.

Microbial Correlation Patterns of UC Patients in the Validation Study

The microbial interaction (eg, bacteria–fungi interaction) was found to be important for keeping microbial homeostasis.[5, 23] To investigate microbial correlation networks in the validation study, we selected the top 50 bacterial genera and top 20 fungal genera for analysis depending on microbial abundance.

Before 5-ASA treatment, although the microbial correlation pattern was weak (a few lines between bacterial and fungal genera) in the preN group, there were still a few bacteria–fungi correlations. In these correlations, there were negative correlations among a small number of Firmicutes bacteria (including *Enterococcus* and *Lactococcus*) and *Escherichia-Shigella* (Fig. 4). *Propionibacterium*, a genus of bacteria for synthesis of propionic acid, was negatively correlated to *Escherichia-Shigella*, *Roseburia*, *Bacteroides*, and *Subdoligranulum*. In fungal microbiota, *Wickerhamomyces* was negatively correlated to *Morchella*, *Penicillium*, *Fusarium*, and *Anaerostipes*. Of note, although there were more microbial correlations, the bacteria–fungi correlation pattern was interrupted in the preI group, and few correlations could be observed between bacterial and fungal genera.

We further analyzed the microbial correlation patterns in mucosae after 5-ASA treatment. We found that there was a tense bacteria–fungi correlation pattern in noninflamed mucosae after 5-ASA treatment (post-N). In this pattern, *Wickerhamomyces* exerted potent negative correlation with some Firmicutes and Bacteroidetes. In addition, *Enterococcus* and *Lactococcus* were negatively correlated with Ascomycota. After 5-ASA treatment, the bacteria–fungi correlation pattern was re-built in the inflamed mucosae (postI). *Propionibacterium* was also extensively correlated with Firmicutes and Proteobacteria. Additionally, *Enterococcus* and *Lactococcus* negatively interacted with *Klebsiella*, *Escherichia-Shigella*, *Parabacteroides*, *Bacteroides*, and *Prevotella*. These data indicated that 5-ASA affected microbial correlation patterns, and to some extent, disrupted bacteria–fungi correlation in inflamed mucosae was re-established after 5-ASA treatment.

DISCUSSION

Microbial dysbiosis is one of the mechanisms for IBD pathogenesis. The disturbance of the host–microbiota interaction has been reported to induce inflammation in IBD, which is mediated by the host immune system.[24, 25] Re-establishment of gut microbial and immunological homeostasis tends to be an advisable treatment strategy for IBD. Currently, 5-ASA is the primary regimen for regulating inflammatory status in IBD treatment. Nevertheless, whether 5-ASA treatment affects the microbiological characteristics is rarely reported. Our previous study showed that 5-ASA treatment altered mucosal bacterial microbiota, especially in inflamed mucosae.[16] In this study, we further investigated the impact of 5-ASA treatment on fungal microbiota in mucosa samples of UC patients.

It was pointed out that the location of mucosal sampling sites influenced microbial composition.[26–29] To avoid the influence of sampling location, we enrolled an almost equal percentage of patients with each type (E1, proctitis; E2, left-sided; or E3, extensive) according to the Montreal classification to correct this effect. Furthermore, a previous study also reported a sex-based effect on mucosa-associated bacteria in the human colon.[29] We completed 2 independent studies (the exploration study and the validation study) in our research. We found that there was a significant sex-based difference in our exploration study. To avoid the sex-based bias, we further confirmed the results obtained from the exploration study in our validation study.

We first analyzed fungal microbiota in the exploration study and then performed a confirmation in the validation study. In our exploration study, there were ~50% reads that could not be identified in the database. We inferred that the length of the paired-end sequence was too short (2 × 125 bp) for alignment. We thus enhanced our sequencing length (2 × 250 bp); as a result, only ~5% of reads were unidentified in our validation study. Based on these results, we mainly analyzed fungal diversity in our exploration study. Apart from these unidentified OTUs, fungal microbiota was constituted by Ascomycota in the exploration study, in accordance with previous studies.[3, 5, 8]

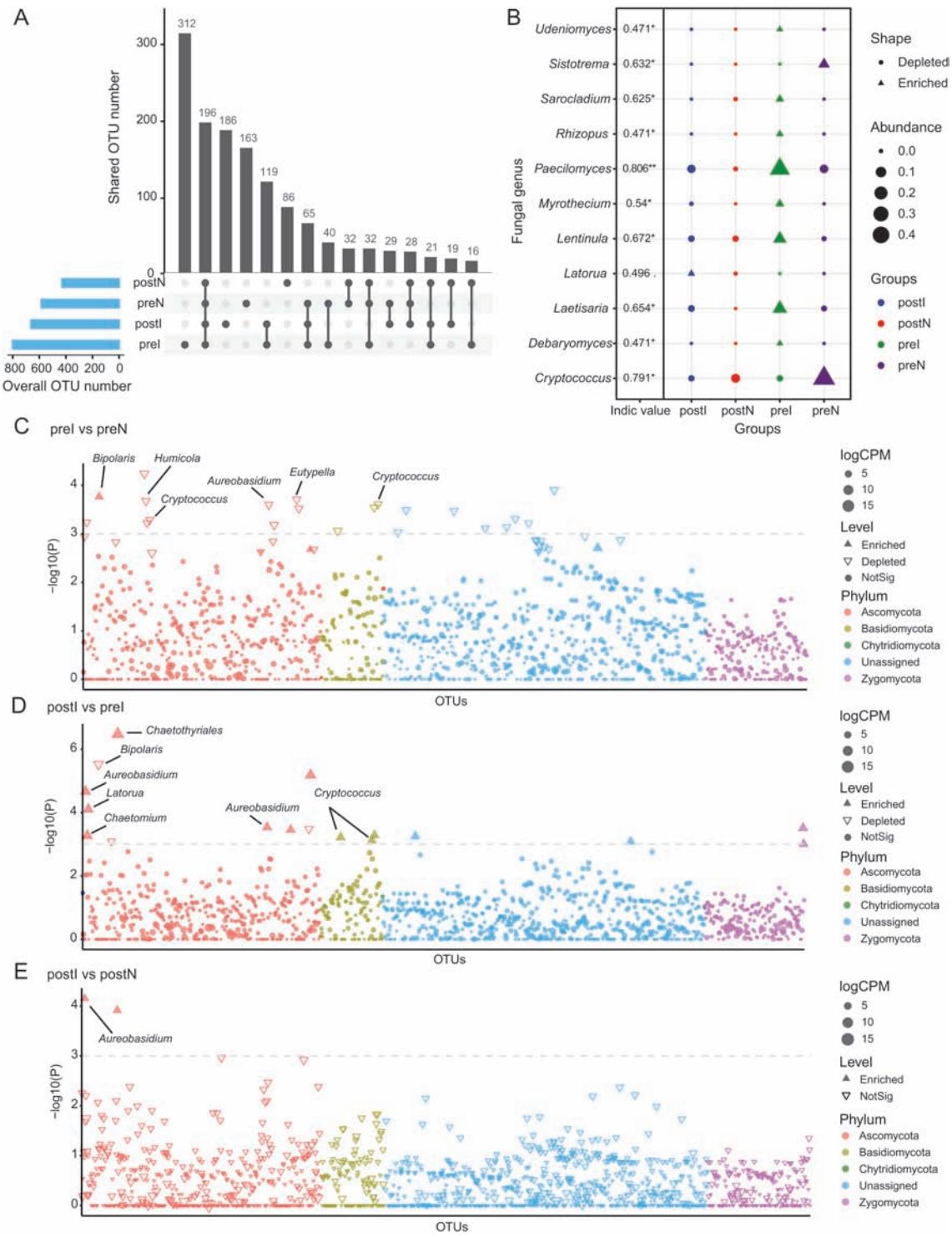

FIGURE 3. Group-specific fungal microbiota in the validation study. A, The upset plot shows the OTU count in each group. B, Indicator species in each group. Fungal abundances at the genus level were analyzed using the indicspecies package. The shape of the point represents OTUs enriched or depleted in the group, and point size represents the abundance of OTUs. Comparative analysis of OTU abundance between 2 groups ((C) preI vs preN; (D) postI vs preI; (E) postI vs postN). The EdgeR package was used for comparative analysis. The difference between the 2 groups is shown as a Manhattan diagram. Point shape indicates OTUs enriched, depleted, or not significant in the former group compared with the latter. Point size indicates the abundance of OTUs. Abbreviations: CPM, count per million.

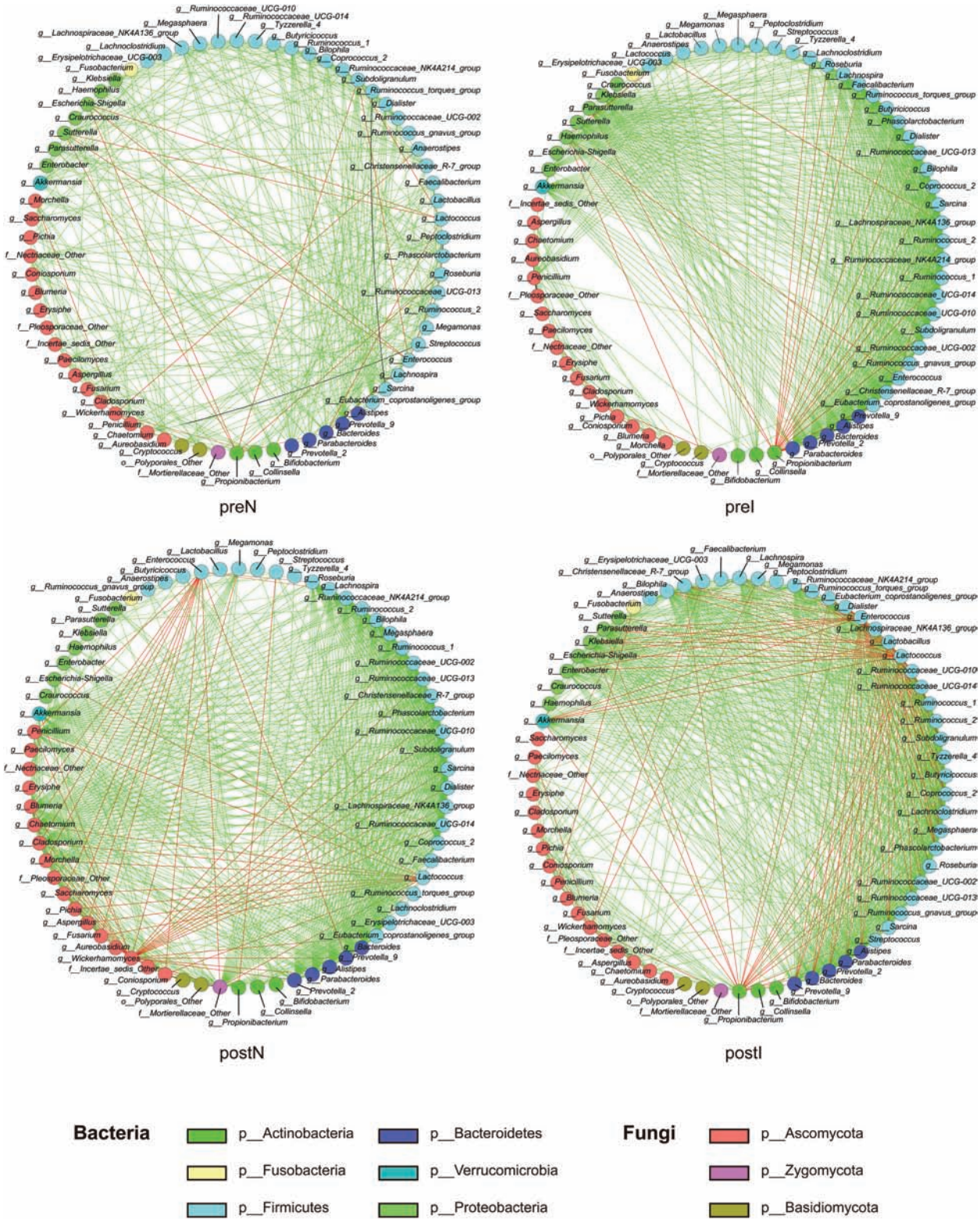

FIGURE 4. Microbial interaction patterns of UC patients in the validation study. The abundance of bacteria (top 50) and fungi (top 20) was analyzed using Spearman's test. Only significant correlations ($P \leq 0.05$) are displayed with an edge. The edge colors indicate positive (green) or negative (red) correlations, which depended on Spearman's correlation coefficient. The nodes represent microbial genera—the colors of which represent microbial phyla. Abbreviations: postI, inflamed mucosae of UC patients after 5-ASA treatment; postN, noninflamed mucosae of UC patients after 5-ASA treatment; preI, inflamed mucosae of UC patients before 5-ASA treatment; preN, noninflamed mucosae of UC patients before 5-ASA treatment.

In our exploration study, we found higher fungal richness and lower evenness in the inflamed mucosae of treatment-naïve UC patients. There was higher fungal richness in the inflamed mucosae of 5-ASA-treated UC patients. Additionally, beta diversity showed a mucosal type–specific clustering in treatment-naïve UC patients. We confirmed these results in our validation study. Previous studies have reported that 5-ASA treatment also affects bacterial diversity in UC and IBS patients.[12, 16] Data acquired in this study indicated that 5-ASA treatment altered fungal diversity and also impacted fungal composition in UC patients.

We further analyzed data of fungal composition in the mucosae of UC patients. Ascomycota was the dominant phylum in fungal microbiota, in accordance with earlier reports.[3, 5] Notably, we found that the fungal phylum Zygomycota constituted ~10% of fungal microbiota in the inflamed mucosae of untreated UC patients; nevertheless, few previous studies have studied this phylum.[3, 5, 6] Our data highlight the important role of fungi from the Zygomycota phylum, which suggests that more attention should be paid to this phylum in inducing human disease.[30] We further performed the comparative analysis of fungal composition at the genus level in different groups, which was based on the results of the validation study.

Wickerhamomyces was reported to be abundantly increased in UC patients and animals with dextran sulfate sodium (DSS)–induced colitis.[4, 27] In our study, we also found that *Wickerhamomyces* was the main part of fungal microbiota in UC patients, whereas it was significantly decreased in inflamed mucosae. Analysis of microbial interaction patterns showed that several fungal genera were negatively correlated to *Wickerhamomyces*, which reflected an abundant increase of these fungi in inflamed mucosae. *Candida*, *Saccharomyces*, and *Penicillium* were common fungal pathogens in IBD patients, especially in CD patients,[3, 5, 8] whereas in our study the abundance of these 3 fungi was too low to be detected. Previous studies reported that *Candida*, *Saccharomyces*, and *Penicillium* were identified only in stools, but not in gastrointestinal biopsies,[31, 32] which indicated a low abundance of these fungi in the mucosae. Additionally, 5-ASA treatment increased *Wickerhamomyces* in both noninflamed and inflamed mucosae, which indicated a suppressive competence to some other fungal pathogens.

Analysis of group-specific fungal microbiota also displayed an effect of 5-ASA treatment on fungal composition. *Paecilomyces*, a fungal pathogen found in food, was reported to induce infections in immunocompromised and immunocompetent patients.[33] We found in our study that *Paecilomyces* was highly enriched in inflamed mucosae before treatment. Besides, fungal pathogens such as *Sarocladium*, *Rhizopus*, *Myrothecium*, and *Lentinula* were also enriched in inflamed mucosae.[34–36] 5-ASA treatment decreased the colonization of these fungal pathogens in inflamed mucosae, which indicates that 5-ASA treatment decreased pathogenic fungi colonization in UC patients, especially in inflamed mucosae.

Coexisting within the gastrointestinal tract, microbiota such as bacteria and fungi interact directly or indirectly with each other.[23, 37] As reported, yeast mannose is a viable food source for *Bacteroides thetaiotaomicron*.[25] We herein investigated the microbial correlation pattern in the mucosae of UC patients and found that an interrupted bacteria–fungi correlation existed in inflamed mucosae. 5-ASA treatment increased the intensity of microbial correlations in both noninflamed and inflamed mucosae. These data suggest that 5-ASA treatment altered the microbial correlation patterns in UC patients.

Zheng reported that sulfasalazine (SASP) influenced bacterial composition and function in TNBS-induced colitis.[9] Nevertheless, with a sulfonamide group in its chemical structure, SASP exerting anti-inflammatory function is partly associated with its antimicrobial effect. Being different than SASP, the anti-inflammatory effect of 5-ASA is mainly mediated by inhibiting TNF-α-regulated IκB degradation and NF-κB activation.[38] The previous study also expressed that the proposed anti-inflammatory and bacteriological effects of 5-ASA were well aligned with factors implicated in IBS and UC pathogenesis.[12, 16] The interaction between the immune system and microbiota is essential for healthy immune defense of the host.[39] Conversely, the interruption of the host immunity–microbiota interaction plays an essential role in inducing inflammation in IBD, which is mediated by the host immune system.[24, 40–42] Based on the interaction between gut microbiota and the host immune system, we infer that 5-ASA's impact on gut microbiota in IBD patients is associated with its anti-inflammatory effect; however, this needs further investigation.

In conclusion, we observed an increased pathogenic fungi colonization and an interrupted bacteria–fungi correlation in the inflamed mucosae of treatment-naïve UC patients. Fungal diversity and composition altered after 5-ASA treatment, a change characterized by decreasing fungal pathogen colonization and restoration of the bacteria–fungi correlation in inflamed mucosae.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

ACKNOWLEDGMENTS

The authors thank Zhu Baoli, Lv Na, and Liu Fei from the Institute of Microbiology, Chinese Academy of Sciences, for their warmhearted assistance in microbial genomics sequencing.

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