

Dysbiosis of Gut Fungal Microbiota is Associated With Mucosal Inflammation in Crohn's Disease

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Goals: We aim to characterize the fungal microbiota in the intestinal mucosa and feces in patients with Crohn's disease (CD).

Background: Fungi represent a diverse microbial community in the human intestine and might play a role in the pathogenesis of CD; however, little is known about the structure and composition of the fungal microbiota especially adhering to the intestinal mucosa in CD patient.

Study: Nineteen patients with active CD and 7 healthy individuals were recruited in this study. The mucosa-associated and fecal fungal microbiotas in CD patients were analyzed using culture-independent community fingerprint techniques.

Results: The fungal richness and diversity were significantly elevated in the inflamed mucosa compared with the noninflamed mucosa. The predominant fungal composition in the inflamed mucosa was strikingly altered, mainly characterized by expansion in the proportions of *Candida* spp., *Gibberella moniliformis*, *Alternaria brassicicola*, and *Cryptococcus neoformans*. The fecal fungal community was perturbed in CD patients as accompanied by increased fungal diversity and prevalence in *Candida albicans*, *Aspergillus clavatus*, and *C. neoformans*. The species richness and diversity of the mucosal fungal community were associated with the expression of TNF- α , IFN- γ , or IL-10 ($P < 0.05$). The diversity of the fecal fungal microbiota positively correlated with serum C-reactive protein and CD activity index ($P < 0.05$).

Conclusions: This study first demonstrates that the fungal microbiota in the inflamed mucosa is distinguishable from that of the noninflamed area. Shifts of gut fungal microbiota composition may be associated with mucosal inflammation and disease activity of CD. Our data would provide novel insights into understanding the potential of gut fungal microbiota in the pathogenesis of CD.

Key Words: Crohn's disease, fungal microbiota, mucosal inflammation, denaturing gradient gel electrophoresis, DNA sequencing
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Crohn's disease (CD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract. Although its etiology is not fully understood, it has been well established that the gut bacterial microbiota plays an important role in the initiation and perpetuation of the intestinal inflammation in CD.^{1–6} The development of mucosal inflammation in CD is critically dependent on the presence of the complex intestinal microbiota.^{7,8} Recent studies have primarily focused on enteric bacteria in the pathogenesis of CD; gut fungal microbiota may also be important in determining disease susceptibility in CD.

Fungi represent a dynamic and ecologically diverse microbial community and are considered an important element of the human flora.^{9–12} There is a dynamic balance between the fungal and bacterial microbiota, and fungal microbiotic diversity is inversely related to bacterial diversity in the gastrointestinal tract.¹³ Recently, research has addressed concerns on the importance of fungi presented in the lumen of the gut and the interaction between commensal fungi and intestinal inflammation.^{14–16} However, there have been a very limited number of studies in it in CD patients. The fungal microbiota could be of substantial and underappreciated importance in the maintenance of CD. Alteration in the diversity of fungal microbiota and the precise fungal species involved in CD remains unknown.

CD is a disorder of mucosal inflammation and the mucosal bacteria microbiota seems to be of peculiar relevance for the disease.^{17–19} The mucosa-associated bacterial microbiota has a critical role in the initiation and perpetuation of CD. Chronic intestinal inflammation in CD may be closely involved in the compositional change of mucosal bacterial microbiota. The fungal microbiota adhering to the mucosa may have specific responses to the disturbance of local immune state in the gut. The composition of the mucosal microbiota is significantly different from feces.^{20–22} Therefore, it is a critical implication for characterizing the structure and diversity of fungal microbiota in the intestinal mucosa in CD. The involvement of gut fungal microbiota in intestinal inflammation in CD needs to be elucidated.

In the present study, we have applied 18S rDNA-based molecular fingerprinting techniques to characterize the commensal fungal composition in the ileal mucosa and feces in CD patients. Our findings indicate that there are significant differences in the fungal community diversity and composition between the inflamed and noninflamed mucosa. The fecal fungal microbiota is disturbed in CD

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patients in comparison with the healthy controls. We present a potential association between gut fungal microbiota and mucosal inflammation or disease activity of CD. The data would contribute to improve our knowledge concerning the gut fungal microbiota composition and its possible roles in CD.

MATERIALS AND METHODS

Subjects and Sample Collection

Nineteen patients with active CD and 7 healthy individuals were recruited in this study. The median (range) ages were 41.3 (18 to 67) years for the CD group and 38.5 (22 to 62) years for the controls. The diagnosis of CD was made in accordance with established clinical, endoscopic, radiologic, and histologic criteria.²³ Disease activity was assessed by the Crohn's disease activity index (CDAI)²⁴ and all patients were in an active phase of the disease (CDAI > 150). Serum C-reactive protein (CRP) levels were measured on AU680 Analyzers (Beckman Coulter Inc., Brea, CA) at sampling day. Clinical data of the patients, including sex, age, CDAI, serum CRP, and concurrent medical therapy, are shown in Table 1. Exclusion criteria included the use of the following treatments: anti-TNF- α agents in the preceding 12 weeks; antibiotics, probiotics, or prebiotics in the preceding 8 weeks; rectal preparations during the preceding 2 weeks; and anti-inflammatory drugs during the preceding week. All the healthy subjects were in good health and had no history of either gastrointestinal or metabolic disease or previous surgery. These individuals were genetically unrelated and lived on a Chinese diet. No volunteer had received antibiotics, probiotics, prebiotics, or any other medical treatment potentially influencing gut microbiota within the preceding 8 weeks before sampling. The study was approved by the Administrative Panel for Medical Research on Human Subjects of Jinling Hospital. Written informed consent was obtained from each participating individual before study enrollment.

The ileal mucosal specimens including the inflamed and noninflamed areas were surgically obtained from 7 patients with active CD, all of which were routinely administered with prophylactic antibiotics within 1 hour before surgical incision. Immediately after collection, the surgical samples were agitated in normal saline. The solution was then removed and replaced with fresh solution, and the process was repeated 3 times to remove the luminal contents and other potential contaminations. Aliquots of

each sample were placed into sterile cryotubes and frozen in liquid nitrogen before DNA extraction. The feces were freshly obtained from 12 patients at acute phase and 7 healthy subjects using sterile containers. The samples were immediately homogenized with a sterile spatula in a biosafety cabinet. Aliquots (220 mg) of the samples were transferred to sterile eppendorf tubes and frozen at -80°C until DNA analysis.

Histopathologic Analysis

For routine histologic analyses, intestinal segments from the inflamed and noninflamed sites were fixed in 10% neutral buffered formalin, processed for paraffin embedding, and stained with hematoxylin and eosin (H&E). The slides were independently interpreted by 2 gastrointestinal pathologists. The histologic changes in mucosal specimens from CD patients were evaluated as described previously.²⁵

DNA Extraction

DNA extraction of the mucosal tissues was carried out by QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total DNA of fecal samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). All the samples were processed in duplicate to evaluate the reproducibility of the DNA extraction. The extracted DNA was subjected to 1% agarose gel for assessment of DNA quality and was quantified using a Beckman DU800 spectrophotometer.

PCR Amplification

The partial fragment of fungal 18S rRNA gene was amplified by a nested PCR protocol. The duplicate DNA of each sample was independently used as templates for PCR amplification. The first round of PCR amplification was carried out with primers NS1 and FR1, and the second round with the primer set EF390/GC-FR1.²⁶ PCR was performed in a final volume of 50 μL , consisting of 5 μL 10 \times PCR buffer, 1.8 mM MgCl₂, 200 μM dNTPs, 2.5 U of Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA), 0.3 μM of each primer, and 25 ng DNA template. PCR cycling was performed with an ABI 2720 thermocycler (Applied Biosystems, Foster City, CA). In the first step, the PCR program consisted of an initial denaturation at 94 $^{\circ}\text{C}$ for 5 minutes, and then a total of 25 cycles were performed at 94 $^{\circ}\text{C}$ for 30 seconds, annealing at 45 $^{\circ}\text{C}$ for 30 seconds, and extension at 72 $^{\circ}\text{C}$ for 1.5 minutes and a final extension at 72 $^{\circ}\text{C}$ for 7 minutes. The PCR products from the first cycling step were used as DNA template (diluted at 1:100) for the second round of

TABLE 1. Clinical Data of the Patients With Crohn's Disease and Healthy Subjects

| Characteristics | CD Patients (n = 19) | Healthy Subjects (n = 7) |
|--|----------------------------|--------------------------|
| Male/female | 11/8 | 4/3 |
| Median age (range) (y) | 41.3 (18-67) | 38.5 (22-62) |
| Disease site (%) | | |
| Ileocolonic | 15 (78.9) | |
| Colonic | 4 (20.1) | |
| CDAI scores (range) | 214.3 \pm 35.7 (158-285) | |
| C-reactive protein levels (range) (mg/L) | 37.8 \pm 27.9 (8-97) | |
| Concomitant medications (%) | | |
| AZA/6-MP | 11 (57.9) | |
| Mesalazine | 8 (42.1) | |
| Methotrexate | 2 (10.5) | |
| None | 2 (10.5) | |

amplification, in which the reaction mixture contained the same constituents as the first one. In the second step, the annealing temperature rose to 48°C and the extension time declined to 30 seconds. PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

Denaturing Gel Gradient Electrophoresis (DGGE)

DGGE was performed using the D-Code universal mutation detection system (Bio-Rad, Hercules, CA). A volume of 20 μ L of each PCR product was loaded onto 8% (wt/vol) acrylamide gel and electrophoresed in 1 \times Tris-acetate-EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) (TAE). The gel had a denaturing gradient ranging from 25% to 52.5%.²⁶ Electrophoresis was carried out at 60°C with a constant voltage of 120 V for 7.5 hours. After electrophoresis, the gel was stained using SYBR Green I nucleic acid stain (Invitrogen) (10,000-fold dilution in 1 \times TAE) and photographed under UV transillumination with the ChemiDOC XRS instrument (Bio-Rad).

Comparative Analyses of DGGE Profiles

Cluster analysis of the DGGE profiles was performed with QuantityOne software (version 4.2; BioRad), as described previously.²⁶ After background subtraction, the lanes were normalized to compensate for differences in the migration distance of the DNA in each lane. The bands in each lane were detected automatically to create matching profiles. Moreover, the matching profiles were then used to construct a dendrogram using unweighted pair group method with arithmetic average. Dendrograms generated using this method was applied for the analysis of clustering patterns between different lanes. The intensity of fragments was expressed as a proportion (%) of the sum of all fragments in the same lane of the gel.²⁷ Species richness and diversity of fungal community were calculated as number of bands and as weighted diversity scores according to Shannon and Weaver, respectively.^{28,29} Principal component analysis (PCA) plots were generated on the basis of the relative abundance of DGGE bands using the multivariate statistics software, Canoco (version 4.5; Microcomputer Power, Ithaca, NY).

Sequence Analysis of DGGE Band

To identify the fungal species corresponding to the bands, separated fragments were excised from the gels using a sterile scalpel and incubated in Tris-EDTA buffer overnight at 4°C. Before sequencing, the eluted DNA was reamplified using the primer set EF390/GC-FR1 and checked by electrophoresis in denaturing gels, as described previously.²⁶ Subsequently, the reamplified fragments were cloned onto the pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5- α cells (Promega). Plasmid DNA was isolated from positive cells and was sequenced using primers T7 and SP6 on an ABI PRISM 3730 sequencing system (Applied Biosystems).

Phylogenetic Analysis

The retrieved sequences were manually aligned and compared with known 18S rDNA sequences from the National Center for Biotechnology Information databases. Moreover, search was conducted in GenBank using BLAST to determine the closest known relatives of the partial 18S rDNA sequences obtained. To analyze the phylogeny of the sequences, alignments were made with sequences from these closely related species using the Clustal X software.³⁰ The phylogenetic trees were

calculated by neighbor-joining using the algorithm of Saitou and Nei,³¹ and MEGA 4 software was used to construct phylogenetic trees.³²

Flow Cytometric Analysis of T-Cell Subpopulation

Mucosal lymphocytes were separated from the ileal specimens as described previously.³³ Purified single-cell suspensions were stained with monoclonal antibodies against CD3, CD4, or CD8- α labeled by allophycocyanin, fluorescein isothiocyanate, or phycoerythrin (BD Pharmingen, San Diego, CA). Appropriate isotype-matched monoclonal antibodies were used as negative controls. Flow cytometric analysis was performed on FACSCalibur cytometer (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software.

Immunofluorescent Staining

The mucosal specimens from inflamed and non-inflamed regions were embedded in optimum cutting temperature compound (Tissue Tek, Sakura, Japan) and sectioned with a cryostat at 5 μ m. After fixation with cold acetone, the cryosections were blocked for 10 minutes at room temperature with phosphate-buffered saline containing 3% bovine serum albumin. The sections were incubated with monoclonal antibodies against TNF- α , IFN- γ , or IL-10 (Abcam, Cambridge, UK) (1:150 dilution) at 4°C overnight, and then incubated with secondary goat anti-mouse antibody conjugated to Alexa 633 (Molecular Probes, Eugene, OR) for 1 hour. Slides were viewed using a laser scanning confocal microscope (LEICA TCS SP2, Heidelberg, Germany). The mean fluorescent intensity of each image was quantified by NIH Image-Pro Plus 6.0 analyzer software.

Statistical Analysis

Continuous variable was reported as mean \pm SD and categorical variables as frequency or percentages. The statistical significance of differences was analyzed by the Student *t* test for quantitative data and the χ^2 test for categorical data with the SPSS 16.0 software (SPSS Inc, Chicago, IL). For detection of correlation between 2 variances, we performed linear regression analysis using the Pearson test. A *P*-value of <0.05 was considered significant.

RESULTS

Histopathologic Characteristics

The inflamed mucosa showed typical inflammatory changes, including crypt dilation, goblet cell depletion, mixed cell infiltration, involving mainly mononuclear cells and lymphocytes, and injury with ulceration (Fig. S1, Supplemental Digital Content 1, <http://links.lww.com/JCG/A104>). The infiltration of inflammatory cells toward the lamina propria and loss of the intestinal epithelial cells was observed in the inflamed mucosa. The epithelium in the noninflamed mucosa was regular and had normal architectures. Only a few inflammatory cells were present in the noninflamed mucosa.

Variations of the Structure and Diversity in Mucosa-associated Fungal Microbiota

As shown in Figure 1, the dominant fungal composition of each patient varied significantly. Some specific bands appeared more frequently in the profiles in the

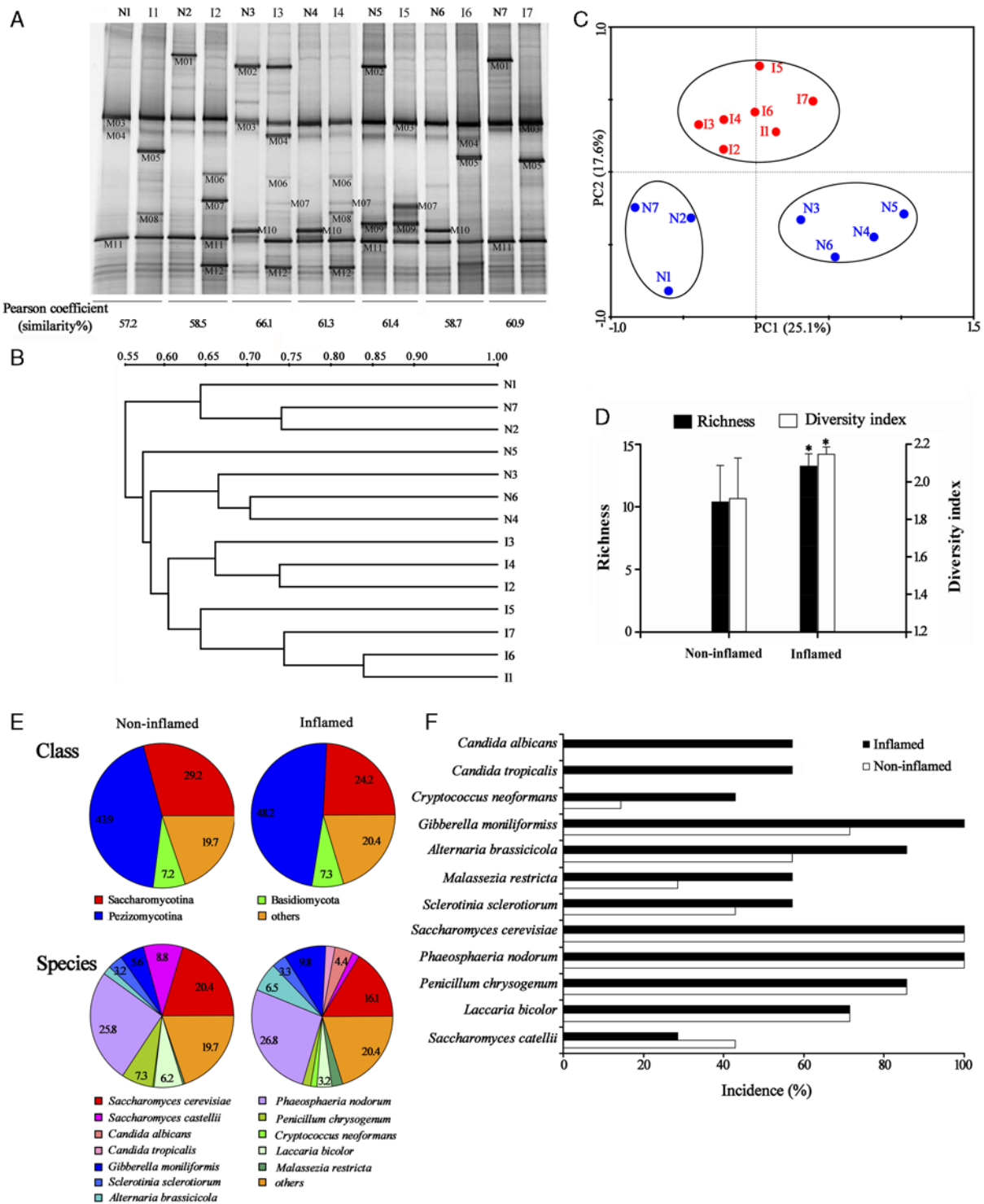


FIGURE 1. Comparison of the fungal communities in the inflamed and noninflamed mucosa. **A**, Molecular fingerprinting of the mucosa-associated fungal populations. I indicates inflamed ileal mucosa; N, noninflamed specimen. The figures represent the number of the patients. The similarity coefficient of the fungal microbiota between the inflamed and noninflamed mucosa is shown in the down regions of the panel. **B**, Clustering analysis of mucosal DGGE profiles from CD patients. The similarities between mucosal specimens are shown in the dendrogram. **C**, PCA plot of the mucosal fungal flora in CD patients. The plot demonstrated the difference of fungal community composition in the inflamed and noninflamed regions. The percentage of variation explained by each principal component is shown in brackets. **D**, Biodiversity of mucosal fungal populations from the inflamed and noninflamed ileum. The mean values and SD (bars) are shown, * $P < 0.05$. **E**, Pie charts showing proportion of the predominant fungi in each class (top) and species (bottom) for the noninflamed and inflamed mucosa. **F**, The frequencies of fungal species presented in the noninflamed and inflamed regions.

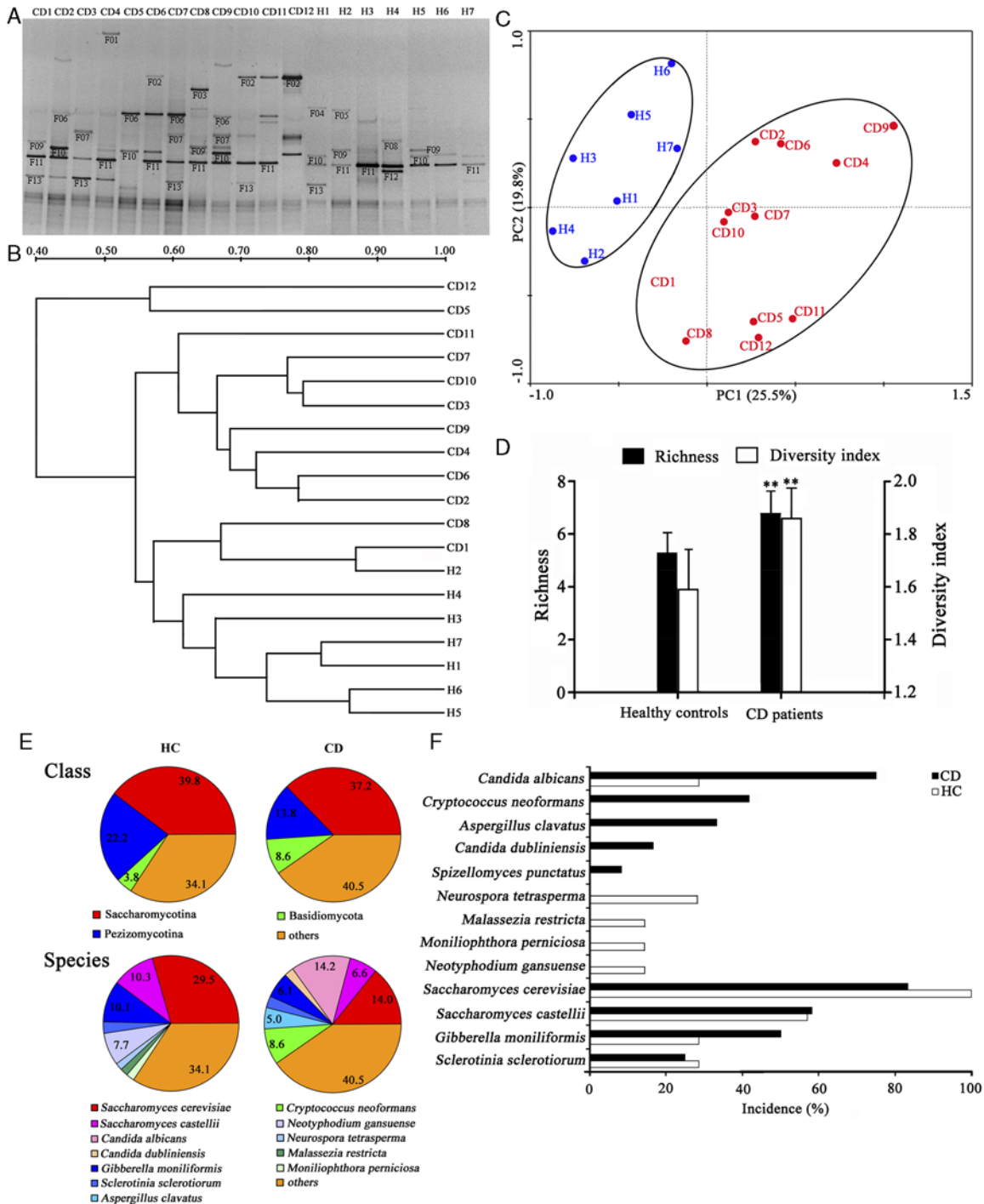


FIGURE 2. The variability of intestinal fungal communities as determined by DGGE analysis of samples from the feces. **A**, Representative DGGE profiles of the fecal samples from CD patients and healthy controls. **B**, Dendrogram generated from the fungal community fingerprints of the feces, which was conducted with cluster analysis by the unweighted pair group method using arithmetic averages. **C**, Principal component analysis of the DGGE data for the fecal fungal community compositions from CD patients and controls. Each circle is representative of a single sample and shaded according to the relative abundance of DGGE bands. The plot shows different fungal community composition in the feces from the patients and controls. The percentage of variation explained by each principal component is shown in brackets. **D**, The diversity of fungal communities revealed by the numbers of DGGE bands and Shannon-Wiener diversity indices. For each group, the mean value and SD (bars) are shown, $**P < 0.01$. **E**, Visualization of taxonomic levels. Pie charts showing proportion of fecal predominant fungi in each class (top) and species (bottom) for the controls and the CD. **F**, The incidences of fungal species presented in the fecal samples from the controls and CD patients. CD indicates Crohn's disease; H, healthy control.

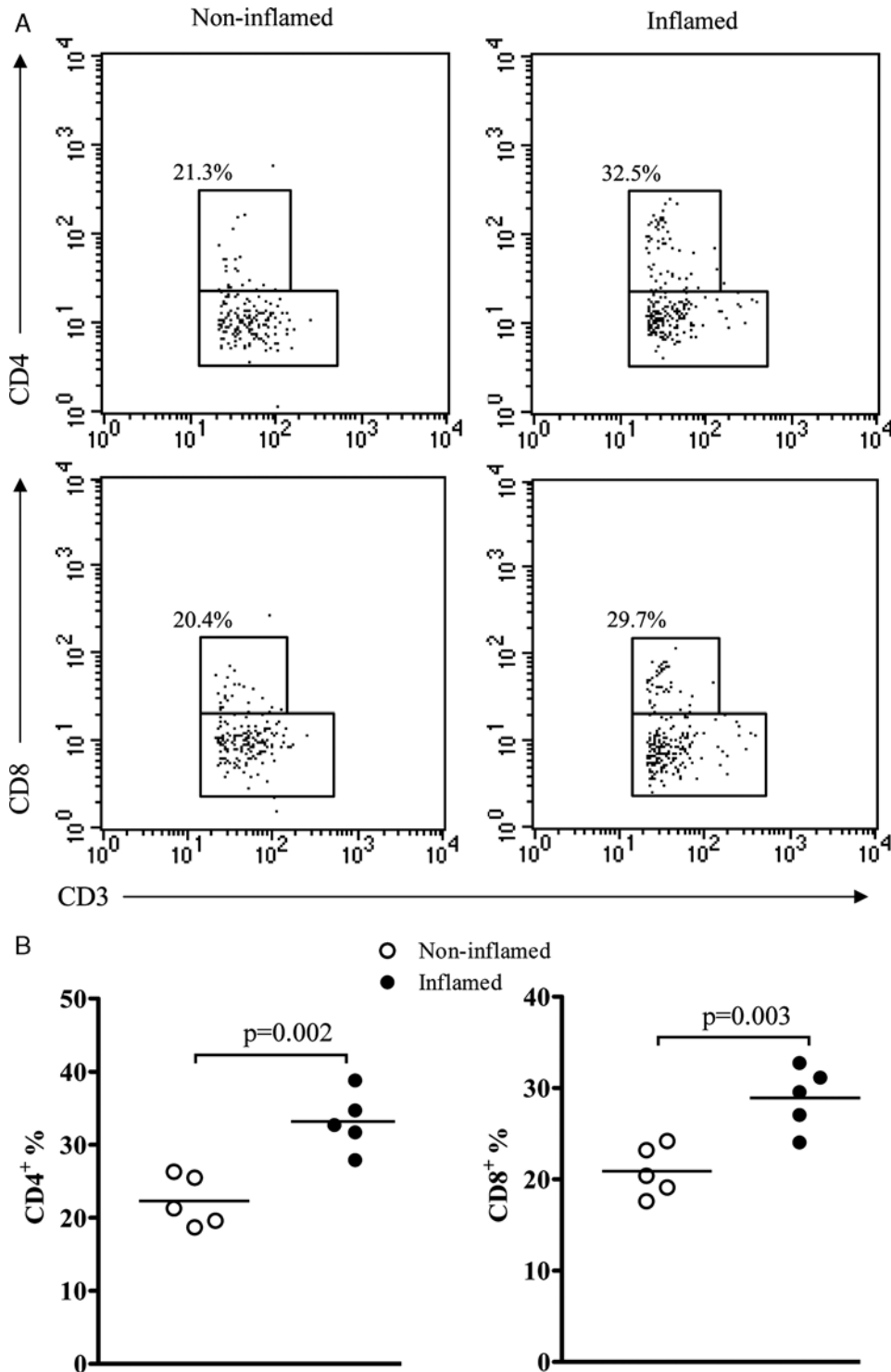


FIGURE 3. Analysis of T-cell subsets in the intestinal mucosal tissues. A, Representative histograms of flow cytometry showing dynamics of percentage of CD4⁺CD3⁺ and CD8⁺CD3⁺. B, The proportions of CD4⁺CD3⁺ and CD8⁺CD3⁺ in the inflamed and noninflamed mucosa. Data are presented as mean ± SD.

inflamed specimens. The Pearson similarity coefficient between the inflamed and noninflamed mucosa ranged from 57.2% to 66.1% (Fig. 1A). The dendrogram showed that the inflamed mucosal samples were clustered together

and noninflamed mucosa generated several subclusters (Fig. 1B). The PCA displayed that the inflamed group appeared close and clustered together, but the noninflamed groups were dispersed and far from the inflamed data sets

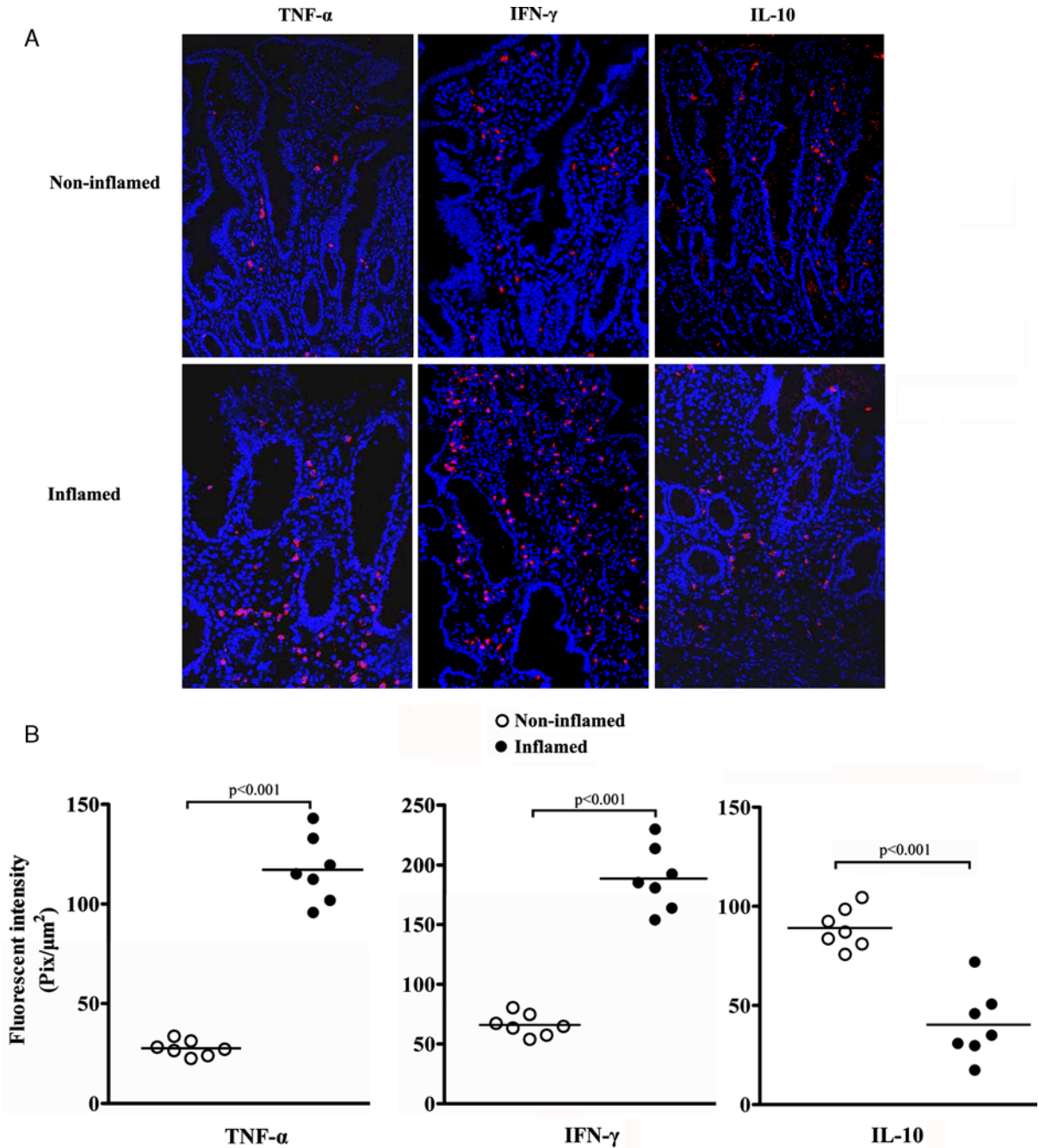


FIGURE 4. Expression of cytokines in ileal mucosa by immunofluorescent staining. A, Representative images of cytokine expression in the inflamed and noninflamed mucosa. Longitudinal visualization of TNF- α , IFN- γ , and IL-10 production (red) was performed by confocal laser scanning microscopy. Nuclei were stained with DAPI (blue). B, The intensities of TNF- α , IFN- γ , and IL-10 expression per HPF (magnification, $\times 200$) were measured by Image-Pro Plus 6.0. Data are presented as mean \pm SD.

(Fig. 1C). Species richness of the fungal community was higher in the inflamed mucosa than in the noninflamed area (13.29 ± 0.95 vs. 10.43 ± 2.88 , $P < 0.05$) (Fig. 1D). Shannon-Wiener diversity analysis indicated increased fungal diversity in the inflamed mucosa (2.15 ± 0.016 vs. 1.91 ± 0.21 , $P < 0.05$). The findings suggested that the fungal microbiota structure and composition in the inflamed mucosa were significantly distinct from that of the noninflamed sites in CD patients.

Shifts of Fungal Species in Inflamed Mucosa

In an attempt to elucidate specific changes in mucosal fungal microbiota under chronic intestinal inflammation in CD, the predominant fungal species was determined by DNA sequence analysis. Twelve fungal phyla were identified in the mucosal samples, which belonged to 3 major fungal taxa: the classes Saccharomycotina, Pezizomycotina, and Basidiomycota (Fig. S2, Supplemental Digital Content 2, <http://links.lww.com/JCG/A105>; Table S1, Supplemental

Digital Content 6, <http://links.lww.com/JCG/A109>). At class level, a marked expansion in Pezizomycotina and a decrease in Saccharomycotina were observed in the inflamed mucosa (Fig. 1E). *Candida albicans* (M05) and *C. tropicalis* (M06) were detected abundantly in the inflamed regions, whereas they were absent in the noninflamed mucosa ($P < 0.05$). *Gibberella moniliformis* (M12), *Alternaria brassicicola* (M04), and *Cryptococcus neoformans* (M07) were more enriched in the inflamed mucosa than in the noninflamed areas (Fig. 2B). In contrast, *Saccharomyces cerevisiae* (M11) and *S. castellii* (M10) were less present in the inflamed mucosa (Fig. 1F).

Fecal Fungal Microbiota in CD Patients

The variations in the composition and diversity of fecal fungal microbiota in CD patients were also characterized. DGGE band patterns of fecal fungal microbiotas in CD patients were obviously different from those of healthy controls (Fig. 2A). Hierarchical analysis on the basis of Pearson similarity coefficient showed significant variances in the banding patterns from the CD and healthy samples, as revealed by obviously separated clusters (Fig. 2B). Except for 1 healthy subject (H2), the profiles from the healthy controls were clustered together with similarity indices ranging from 62% to 86%. The similarity coefficient between the CD and healthy groups was $< 60\%$. As shown in the scatter plot obtained from PCA, CD and healthy groups were assigned into 2 separated clusters (Fig. 2C). According to both PC1 and PC2, which accounted for 45.3% of the variance, the great differences in the fungal community composition between CD and healthy individuals were identified. In addition, the number of recognizable DGGE bands was higher in the profiles of CD patients than that of the healthy controls (6.80 ± 0.83 vs. 5.29 ± 0.76 , $P < 0.01$) (Fig. 2D). The Shannon-Wiener diversity index was 1.86 ± 0.11 for the patients and 1.59 ± 0.15 for the healthy controls ($P < 0.01$).

The closest fungal relatives corresponding to excised bands in the fecal samples were further determined (Table S2, Supplemental Digital Content 7, <http://links.lww.com/JCG/A110>). Three fungal classes, including Pezizomycotina, Saccharomycotina, and Basidiomycota, were predominant in the fecal fungal microbiota (Fig. S3, Supplemental Digital Content 3, <http://links.lww.com/JCG/A106>). The mean proportion of the Pezizomycotina class was 13.8% in the fecal fungal microbiota of CD patients, which was lower than that in healthy controls (22.2%) (Fig. 2E). The relative abundance of the Basidiomycota class was markedly increased in the CD patients ($P < 0.05$) (Fig. 2E). *S. cerevisiae* (F11) displayed a decreased proportion in CD patients ($P < 0.01$). *Aspergillus clavatus* (F02) and *C. neoformans* (F07) appeared to be over-represented in a subset of CD patients, whereas they were absent in healthy controls (Fig. 2E). *C. albicans* (F06) was prevalently detected in CD patients (75.0% vs. 28.6%, $P < 0.01$) (Fig. 2F). *Moniliophthora perniciosa* (F04), *Neurospora tetrasperma* (F05), *Malassezia restricta* (F08), and *Neotyphodium gansuense* (F12) were occasionally present in healthy individuals (Fig. 2F).

Potential Association of Intestinal Fungal Microbiota With Mucosal Inflammation and Disease Activity

The percentage of CD4⁺ T cells was higher in the inflamed mucosa than in the noninflamed mucosa

(32.96 ± 4.01 vs. 22.28 ± 3.45 , $P < 0.01$) (Fig. 3). Similarly, the proportion of CD8⁺ T cells in the inflamed mucosa was markedly increased compared with that of the noninflamed area (29.06 ± 3.44 vs. 20.90 ± 2.76 , $P < 0.01$). The expressions of TNF- α and IFN- γ were significantly increased in the inflamed regions, whereas IL-10 was decreased compared with the noninflamed mucosa (Fig. 4). The species richness of mucosal fungal microbiota was positively correlated with the expression intensities of TNF- α and IFN- γ in intestinal mucosa, respectively ($r = 0.537$, $P = 0.048$; $r = 0.614$, $P = 0.020$) (Figs. S4A, S4C, Supplemental Digital Content 4, <http://links.lww.com/JCG/A107>). The Shannon-Wiener diversity indices of mucosa-associated fungal microbiota were also positively associated with the expression levels of TNF- α and IFN- γ ($r = 0.595$, $P = 0.025$; $r = 0.671$, $P = 0.009$) (Figs. S4B, S4D, Supplemental Digital Content 4, <http://links.lww.com/JCG/A107>). The richness and diversity indices of the mucosal fungal community showed negative correlations with the levels of IL-10 expression in intestinal mucosa, respectively ($r = -0.765$, $P = 0.001$; $r = -0.813$, $P = 0.004$) (Figs. S4E, S4F, Supplemental Digital Content 4, <http://links.lww.com/JCG/A107>).

We further evaluated the correlation of gut fungal microbiota with serum marker of mucosal inflammation and disease activity. In the patients, serum levels of CRP were strikingly higher than the upper limitation (8 mg/L) (Table 1), providing evidence of intestinal inflammation. Interestingly, significantly positive correlation was observed between the diversity indices and serum CRP concentrations in the patient ($r = 0.6870$, $P = 0.0137$), whereas weak correlation was observed between the species richness and serum CRP (Figs. S5A, S5B, Supplemental Digital Content 5, <http://links.lww.com/JCG/A108>). We also examined whether increased fungal diversity was associated with the disease activity. The species richness in fecal fungal microbiota showed positive correlation with CDAI in CD patients ($r = 0.6841$, $P = 0.0141$) (Fig. S5C, Supplemental Digital Content 5, <http://links.lww.com/JCG/A108>). The diversity indices were also correlated positively with CDAI ($r = 0.8917$, $P < 0.001$) (Fig. S5D, Supplemental Digital Content 5, <http://links.lww.com/JCG/A108>).

DISCUSSION

In this study, we focus on defining the characteristics of gut fungal microbiota especially adhering to the inflamed mucosa in CD patients. Our findings demonstrate that the fungal microbiota in the inflamed mucosa is strikingly different from that of the noninflamed regions, as characterized by elevated fungal diversity and the expansion in *Candida* spp., *G. moniliformis*, *A. brassicicola*, and *C. neoformans*. The dysbiosis of fecal fungal microbiota is developed in CD patients, with increased biodiversity and prevalence, in particular *C. albicans*, *A. clavatus*, and *C. neoformans*. Interestingly, we found that the gut fungal community diversity is correlative with mucosal inflammation and the disease activity of CD patients. Our data initially indicate that the perturbations of mucosal fungal microbiota might be involved in aberrant intestinal inflammation, which would provide a novel insight into the host-fungus interaction in CD.

The complexity of the microbial community in the gastrointestinal tract makes it difficult to determine the structure and composition of gut microbiota, mainly owing

to methodological limitations. Until recently, the intestinal fungal microecosystem and its diversity are poorly defined. Here we utilize 18S-rDNA-based molecular techniques to characterize the changes of gut fungal microbiota composition in CD patients. Our results reveal significant differences in fungal diversity and composition between inflamed and noninflamed mucosal sites in the patients (Fig. 1). The fungal community diversity is observed to be significantly elevated in the inflamed mucosa (Fig. 1D). The data suggest that increased fungal colonization in inflamed mucosa might be involved in intestinal inflammation in CD. The commensal bacteria comprise an abundant and diverse population and play a key role in the maintenance of intestinal microbial homeostasis through restricting overgrowth of enteric fungi.^{13,21} Our previous study has demonstrated that intestinal bacterial diversity is reduced in CD patients.³⁴ These findings support the concept that intestinal fungal diversity is inversely related to bacterial diversity.¹³ Fungi constitute a small fraction (10^6 g^{-1} of feces) of gut microbiota relative to the bacterial community (10^{12} g^{-1} of feces); therefore, the expansion of fungal diversity presented here is most likely a consequence of bacterial microbiota imbalance in CD. In intensive care units, fungal overgrowth is a typical complication of gut bacterial dysbiosis after antibiotic or immunosuppressive therapy.^{26,35} Alterations of fungal colonization in the mucosal flora may represent an imbalance in the complex microbial ecosystem in CD.

Mucosa-associated bacterial microbiota has been reported being perturbed after intestinal inflammation; however, very little is known about how the fungal microbiota responds to mucosal inflammation in CD. Here we provide clear evidence for the compositional shifts of the fungal microbiota in the inflamed mucosa in CD patients. The opportunistic fungal pathogens, such as *C. albicans* and *C. tropicalis*, are abundantly observed in the inflamed mucosa, whereas they are absent in the noninflamed area (Fig. 1E). *Candida* species scarcely colonize in the gastrointestinal mucosa in healthy subjects.³⁶ The data observed here suggest that *Candida* species, especially *C. albicans*, might be a potential candidate for the diagnosis of mucosal inflammation in CD. In addition, *G. moniliformis*, *A. brassicicola*, and *C. neoformans* are prevalently present in the inflamed mucosa (Fig. 1F). The overgrowth of these fungal species at the inflamed sites probably results from diminished competition of the commensal bacteria in intestinal mucosa. It is also obscure whether the entire fungal microbiota or individual pathogens are primarily responsible for the induction of inflammation in CD.

Previous studies have demonstrated that fecal/luminal microbiota differs significantly from mucosa-associated microbiota.^{20–22} The mucosa-associated microbiota may directly interact with the host.^{13,17–19} The compositional and functional differences between luminal and mucosa-associated microbiota highlight the importance of characterizing enteric microorganisms in both niches while investigating the role of the microbiota in intestinal diseases. Fecal samples could be easily obtained and more often applied to investigate the changes of gut microbiota in humans. Therefore, the fecal samples are obtained from CD patients and used for the characterization of gut fungal microbiota composition. We show that DGGE banding patterns of the fecal samples from CD patients appear to be different from healthy samples, indicating the alterations of gut fungal community structure in the patients. The fungal

richness and diversity are significantly increased in CD patients (Fig. 2D), consistent with previous findings.³⁷ A different spectrum of fecal fungal microbiota is found in CD patients. Some specific fungal species including *C. albicans*, *A. clavatus*, and *C. neoformans* show an inconsistent distribution, with a higher prevalence in CD patients compared with the controls. A high percentage of *C. albicans* is identified in the patients, which is in line with the previous findings in CD.³⁷ *A. clavatus* and *C. neoformans* are also detected in CD patients. These 2 fungal species are abundantly presented in response to intestinal inflammation in intestinal infectious diseases and immunocompromised patients.^{26,38} This study has identified the signatures of the intestinal fungal microbiota in CD patients, which would contribute to further clarify the exact role of the fungal colonization in the pathophysiology of CD.

Commensal enteric bacteria have been found to exacerbate the adaptive immune system in CD, leading to an immunologic imbalance characterized by an excessive production of proinflammatory cytokines in the genetically predisposed individuals.^{3,6} In contrast to the enteric bacterial community, the role of fungal colonization and their diversity in the pathophysiology of CD is not precisely defined. We therefore evaluate the possible relationship between the fungal microbiota and mucosa inflammation. Our results indicate that CD4⁺ and CD8⁺ T cells are activated in the inflamed mucosa (Fig. 3). T lymphocytes constitute the predominant cell type and dominate chronic intestinal inflammation in inflammatory bowel disease.³⁹ The activation of immune cell populations in intestinal mucosa is accompanied by the production of inflammatory mediators.⁴⁰ As expected, the production of TNF- α and IFN- γ is strikingly increased in the inflamed mucosa, whereas IL-10 showed an opposite trend (Fig. 4). Changes in the fungal community diversity correlate with the production of mucosal cytokines including TNF- α , IFN- γ , and IL-10 in CD (Fig. S4, Supplemental Digital Content 4, <http://links.lww.com/JCG/A107>). Historically, inflammatory cytokines, such as IFN- γ , are considered to provide protection against fungal infections, whereas immunosuppressive cytokines (IL-10, IL-4) contribute to increase susceptibility of infections to various fungal pathogens.⁴¹ It has been suggested that the adaptive immune system has co-evolved with commensal fungi. Distinct phenotypes of CD4⁺ T cells have protective or nonprotective functions against fungi.^{15,41} Through the production of the signature cytokine IFN- γ , Th-1 cells are instrumental in the optimal activation of phagocytes at the sites of infection.⁴¹ The failure of T cells to deliver activating signals to effector phagocytes may predispose patients to overwhelming fungal infections. In this study, the activation of CD4⁺ T cell and release of IFN- γ at inflamed sites might be a host adaptive immune response against opportunistic fungi invaded into the intestinal mucosa. The data presented here reveal a possible relationship between the gut fungal microbiota and mucosal immune cell; however, it is still unclear whether the change in gut fungal community is a causative factor for inducing intestinal inflammation or a consequence of mucosal inflammatory responses in CD. It would be of critical importance to further explore the causal relation for in-depth understanding of the pathogenesis of CD.

Measures of activity in CD are of critical importance for enrolling homogenous groups of patients and evaluating therapeutic efficacy in clinical trials. In the past 3

decades, CDAI has been used extensively to evaluate the response of the patient to treatment in clinical practices.^{24,42} Serum CRP is a laboratory marker commonly used to reflect the inflammatory response in the patient. Recent studies have reported that serum CRP seems to be highly useful in predicting mucosal inflammation.^{43,44} In this study, we evaluate the activity of CD using CDAI score in combination with serum CRP, and also detect the possible association between gut fungal microbiota and the activity markers. Our findings indicate that gut fungal diversity are positively associated with serum CRP and CDAI scores in the patients (Fig. S5, Supplemental Digital Content 5, <http://links.lww.com/JCG/A108>), giving the possibility of gut fungal microbiota in predicting disease activity and mucosa in CD.

Intestinal balance is sustained by a constant crosstalk between the intestinal microbiota and the host.^{45,46} An alteration in gut bacterial microbiota composition can disrupt the mutual relationship of these 2 partners, leading to chronic bowel disorders. Perturbation of intestinal homeostasis is thus believed to play a pivotal role in the pathogenesis of CD.⁶⁻⁸ It is not surprising that gut fungal community, as an important component of bowel flora, also contributes to microbial immune homeostasis. For instance, *C. albicans* can promote immune tolerance, whereas overgrowth of this fungus results in mucosal candidiasis and gut inflammation.^{15,16} Therefore, the fungal microbiota might be actively involved in the balance between inflammation and tolerance at mucosal surfaces in CD. The changes in the host-fungus interplay might be one of the mechanisms underlying CD. In addition, our results highlight the presence of unprecedented intestinal fungal inhabitants, such as *C. albicans*, *A. clavatus*, and *C. neoformans*; however, their functional roles are as yet unknown in CD. It would be an important implication to distinguish which fungal species may be a cause required for CD, or a sign resulting from this disease.

The pathogenesis of CD is still unknown. The currently accepted hypothesis is that genetic susceptibility and environmental factors cooperate to trigger the chronic inflammation in the disease process. Recent evidence implicating the gut bacterial microbiome in CD development has generated interest in its characterization.⁴⁷⁻⁴⁹ The involvement of fungal communities in the etiology of CD remains extremely unclear. Here we characterize the changes of fungal microbiota in intestinal mucosa and feces in CD patients using culture-independent techniques. To the best of our knowledge, this is the first study to determine the differences of fungal composition and diversity between inflamed and noninflamed mucosal area in CD patients. In addition, we identify the prevalence of potentially pathogenic fungi that might be specifically involved as a signature of CD. Our findings would improve our knowledge on the nature of gut fungal compositional change in CD and might facilitate better understanding on the pathogenesis of the disease. Unlike the enteric bacterial community, our understanding of the commensal fungal microbiota and its roles remains very limited. Further, the information on host-fungus interaction and the dynamic of bacteria-fungus in the intestine is less available. Therefore, our analysis could not conclude about the causal relationship of the fungal microbiota dysbiosis and intestinal inflammation in CD.

In conclusion, we present the first report to characterize the fungal microbiota in the inflamed mucosa in

comparison with the noninflamed region, and provide more comprehensive view thus far into the intestinal microbiome of patients with CD. Our findings demonstrate that the fungal microbiota composition in the inflamed mucosa is distinct from the noninflamed areas, characterized by increased diversity and prevalence of potentially pathogenic fungi. This study provides primary data on the potential association between the alterations of gut fungal populations and intestinal inflammation in CD. On the basis of these data, we speculate that the changes in gut fungal microbiota in CD might be a consequence of enteric bacterial microbiota dysbiosis, intestinal inflammation, or both. Better understanding of the relationship between the host and fungal microbiota would allow us to elucidate the involvement of the commensal fungi in CD pathogenesis and to translate this knowledge into new diagnostic strategies for CD.

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