Persistent Diarrhea in Patients With Crohn's Disease After Mucosal Healing Is Associated With Lower Diversity of the Intestinal Microbiome and Increased Dysbiosis

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BACKGROUND & AIMS: **In patients with inflammatory bowel diseases (IBDs), symptoms do not always associate with the severity of endoscopic inflammation and can persist after mucosal healing. We investigated whether symptoms in patients with successfully treated IBD are related to the composition of the intestinal microbiome.**

METHODS: **We analyzed 590 tissue biopsy specimens from 215 patients with IBD and 48 healthy individuals (controls). We obtained mucosal biopsy specimens from 2 colon sites (ascending and rectosigmoid) and from the terminal ileum along with clinical data. Bacterial DNA was extracted from the biopsy specimens and the V4 region of 16s ribosomal RNA sequenced by Miseq and processed using the QIIME v1.9 pipeline.**

RESULTS: **Mucosal biopsy specimens from patients with Crohn's disease (CD) who achieved mucosal healing (Mayo scores of 0–1 or segmental endoscopic severity CD scores of 0–5) had lower Chao1 diversity than biopsy specimens from patients with ulcerative colitis (UC) or unclassified IBD (IBD-U), or controls. After endoscopic evidence of improvement in patients with UC or IBD-U, diversity of the tissue-associated microbiota did not differ significantly from that of controls. Colon biopsy specimens from patients with CD had lower microbial diversity, before and after healing (segmental endoscopic severity CD scores, 0–2), than colon biopsy specimens from controls ($P < .002$). In patients with CD who achieved mucosal healing, residual clinical activity (CD activity index scores >150; $P = .03$) and persistent diarrhea were associated with reduced microbial diversity ($P = .01$). Continued diarrhea was associated with a trend toward dysbiosis, based on the microbial dysbiosis index ($P = .059$). In patients with UC or IBD-U with moderate to severe inflammation, increasing severity of diarrhea was associated with reduced microbial diversity ($P = .03$).**

CONCLUSIONS: **In an analysis of biopsy specimens from patients with IBD and controls, we found that despite endoscopic evidence of improvement or remission, α-diversity of the tissue-associated intestinal microbiome remained lower in patients with CD than in controls. This observation, along with the reduced Chao1 diversity and greater dysbiosis in intestinal microbiota of patients with residual symptoms of IBD, indicates that microbiome composition could be associated with persistent diarrhea.**

Keywords: Outcome; Response to Therapy; Microbe; Prognostic Factor.

Abbreviations used in this paper: CD, Crohn's disease; CDAI, Crohn's disease activity index; HC, healthy control; IBD, inflammatory bowel disease; IBD-U, inflammatory bowel disease unclassified; IBS, irritable bowel syndrome; IBS-D, diarrhea-predominant irritable bowel syndrome; MD index, Microbial Dysbiosis index; OTU, operational taxonomic unit; SES, segmental endoscopic severity.

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External environmental factors, the intestinal microbiome, inherited genetic risk, and dysregulation of the innate and adaptive immune responses participate in a complex interplay, influencing disease development and prognosis in inflammatory bowel disease (IBD), and dysbiosis has been described in both single-time-point and dynamic longitudinal studies of IBD.[1–3] It is unclear if an increased abundance of pathobionts in the presence of active inflammation impacts the severity of patient symptoms and plays a role in the conundrum of unexplained residual clinical activity in patients with treated IBD. Several hypotheses have been proposed to explain the persistence of clinical symptoms in patients who have had objectively successful treatment. Given the link between dysbiosis and diarrhea-predominant irritable bowel syndrome (IBS-D),[4] we sought evidence of an association between alterations in the tissue-associated microbiome and specific symptoms in IBD patients.

We analyzed 590 tissue biopsy specimens taken from 215 recruited patients with colonic and ileal Crohn's disease (CD), ulcerative colitis, or IBD-unclassified (IBD-U), and 48 healthy controls (HCs) and have characterized the tissue-associated microbiome at 2 colon sites (ascending and rectosigmoid) and at the terminal ileum. In biopsy samples, analysis of the microbiome by disease location is valuable because the detected bacteria are in direct contact with the host tissue. Analyzing features of tissue-associated microbiome diversity and composition may differentiate between IBD phenotypes both in active and endoscopically quiescent disease, determining the relationship between patient-reported symptoms and the intestinal microbiome.

What You Need to Know

Background

In patients with inflammatory bowel diseases (IBDs), symptoms do not always associate with the severity of endoscopic inflammation and can persist after mucosal healing.

Findings

In an analysis of biopsy specimens from patients with IBD and controls, we found that despite patients' endoscopic improvement and remission, α-diversity of intestinal microbes remained lower in patients with Crohn's disease than in controls.

Implications for patient care

Continued alterations in intestinal microbiomes of patients with IBD after treatment might contribute to persistent diarrhea.

Materials and Methods

Patient Selection and Recruitment

Patients with confirmed IBD and asymptomatic HCs who were attending for colorectal cancer screening were recruited at colonoscopy. Study activities were conducted with ethical approval. Biopsy specimens were taken from the sigmoid, ascending colon, and terminal ileum when technically possible, and snap-frozen in liquid nitrogen. Endoscopic scores were determined by the endoscopist (IBD specialists and advanced IBD fellows) using Mayo scores (UC/IBD-U), and in CD the segmental endoscopic severity (SES-CD) score was used.[5,6] The severity of endoscopic disease was graded on a 4-point scale (Table 1). The ordinal score reflects the segment of maximal inflammation (ie, severe terminal ileal disease with normal colon). Patients with a maximum Mayo score of 1 or a SES-CD score of 0 to 5 were considered to have mucosal healing/endoscopic improvement. Endoscopic remission was defined as a Mayo score of 0 or a SES-CD score of 0 to 2.

Clinical and demographic data were recorded and use of antibiotics at endoscopy was a criterion for exclusion. Clinical activity was defined as a partial Mayo score (pMayo) of 2 or higher in UC/IBD-U or a CD Activity Index score greater than 150.[7–9] Stool frequency, abdominal pain, use of antidiarrheal medications, nocturnal diarrhea, and rectal bleeding were recorded at endoscopy. Daily diarrhea was classified as 2 loose stools more than normal or more than 3 loose stools daily, and residual diarrhea reflects daily diarrhea despite mucosal healing.

Microbiome Analysis

Total microbial DNA was extracted from biopsy specimens in 2 batches using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), as per the manufacturer's protocol, with an additional bead beating step to ensure adequate cell lysis. Bead beating was performed

Table 1. Endoscopic Score Ranges Used to Generate a Continuous 4-Point Scale of Endoscopic Inflammation for Microbiome Analysis

Ulcerative colitis/IBD unclassified	Mayo score
Endoscopic score of 0	0
Endoscopic score of 1	1
Endoscopic score of 2	2
Endoscopic score of 3	3
Crohn's disease	**SES-CD score**
Endoscopic score of 0	0–2
Endoscopic score of 1	3–5
Endoscopic score of 2	6–15
Endoscopic score of 3	>15

CD, Crohn's disease; IBD, inflammatory bowel disease; segmental endoscopic severity.

using both 5-mm stainless steel beads to disrupt tissue (69989; Qiagen) and glass beads (Mo-Bio, Mississauga, Ontario, Canada) to disrupt bacterial cells, in conjunction with the FastPrep tissue homogenizer (MP Biomedicals, Santa Ana, CA). Additional enzymatic lysis was conducted through the addition of proteinase K (as per the Qiagen protocol) and incubation of samples at 65°C.

Amplicon sequencing of the V4 hypervariable region of 16s ribosomal RNA bacterial DNA was completed using primers 515F/806R[10] on an Illumina MiSeq platform (Illumina, San Diego, CA). Paired-end sequences were processed using the QIIME 1.9 pipeline using an algorithm of closed-reference operational taxonomic unit (OTU) picking, which included reference-based chimera searching.[11] OTUs were assigned using the Greengenes database 13_8.[11,12] α-diversity was calculated using Chao1 and the Shannon index after a rarefaction depth of 8500 reads per sample. Associations between outcomes and taxa were performed for each taxonomic level from phylum to genus. Association testing corrected for covariates including smoking status, sex, disease location, and activity. The Microbial Dysbiosis (MD) index is a unique framework for assessment of ileal dysbiosis in CD based on log abundance of specific taxa and was applied to quantify dysbiosis.[2]

Statistical Analysis

Shannon and Chao1 α diversity indices were compared using the t test, and Bray–Curtis β-diversity using Adonis within QIIME software. The Deseq2 model[13] was used while accounting for sex, age, and inflammation. A logistic regression was applied for binary traits adjusting for age, sex, inflammation, and the total number of reads.

The correlation between MD index scores and endoscopic inflammation was calculated using the nonparametric Spearman correlation coefficient.[2] Taxa associations were considered significant when the corrected q value was less than 5%, corresponding to a raw P value less than 2.1×10^{-4} (considering 235 taxa from Phylum down to genus). Paired analysis of sigmoid and ascending colon tissue was performed in UC patients using the corrected paired t test.

Results

Cohort Description

We enrolled 215 patients with IBD (n = 114 UC/IBD-U, n = 101 CD) and 48 HCs (Table 2). Patients with UC (n = 99) and IBD-U (n = 15) were considered together given phenotypic similarities.

Seven patients on antibiotics at endoscopy were excluded, leaving 590 samples with complete metadata. Sixty-four percent of the CD cohort and 68% of UC/IBD-U patients had endoscopic improvement or remission (Mayo score, 0–1; or SES-CD score, 0–5) (Figure 1).

Table 2. Data Represent Patients Included in This Study With Relevant Clinical and Demographic Data as Well as Samples Available From Each of the 3 Ileal and Colon Biopsy Sites

Variable	uc/IBD-U (%) (n = 114)	CD (%) (n = 101)	HC (%) (n = 48)
Male	64 (56%)	52 (51%)	30 (62.5%)
Median age, *y* (range)	36 (18–72)	28 (17–56)	56 (31–71)
Median age at diagnosis, *y* (range)	25 (11–59)	20 (5–57)	
Smoker, current	10 (9%)	9 (9%)	3 (6%)
White, self-described ethnicity	90 (79%)	91 (90%)	47 (98%)
Montreal class			
A1	24 (21%)	35 (35%)	–
A2	69 (61%)	57 (58%)	–
A3	16 (14%)	9 (7%)	–
Unknown	5	2	–
B1	–	21 (21%)	–
B2	–	20 (20%)	–
B3	–	60 (60%)	–
Perianal	–	28 (28%)	–
L/E1	13 (11%)	15 (15%)	–
L/E2	32 (28%)	22 (22%)	–
L/E3	69 (61%)	64 (64%)	–
Medications			
Methotrexate	0	11 (11%)	–
Azathioprine/6-MP	13 (11%)	20 (20%)	–
Anti-TNF biologic	20 (17.5%)	33 (33%)	–
Anti-integrin biologic	1 (1%)	1 (1%)	–
Steroids (oral)	11 (10%)	2 (2%)	–
Surgery, intra-abdominal	–	20 (20%)	–
Extraintestinal manifestations	19 (16.5%)	20 (19.8%)	–

CD, Crohn's disease; HC, healthy control; IBD-U, inflammatory bowel disease unclassified; HC, healthy control; 6-MP, 6 mercaptopurine; TNF, tumor necrosis factor; UC, ulcerative colitis.

In the Absence of Endoscopic Inflammation, the Tissue-Derived Microbiota Is Less Diverse in Crohn's Disease Patients Compared With Healthy Controls

Lower α-diversity metrics in patients with CD have been described previously.[3,14,15] Here, when compared with UC/IBD-U or HCs, patients with CD had reduced α-diversity in both sigmoid ($P = .01$ and $P = 9.4 \times 10^{-6}$ for CD vs UC/IBD-U and CD vs HCs, respectively) and terminal ileum mucosa ($P = .03$ and $P = .001$ for CD vs UC/IBD-U and CD vs HCs, respectively) (Supplementary Figure 1). Similarly, Bray–Curtis β-diversity in sigmoid mucosal samples was lower in CD patients when compared with either UC/IBD-U or HCs ($P < .001$ in CD vs UC/IBD-U, $P < .001$ in CD vs HCs) (Supplementary Figure 2*A*). The MD index has been validated in terminal ileum samples, but in our cohort, both terminal ileal and sigmoid colon inflamed samples showed increased

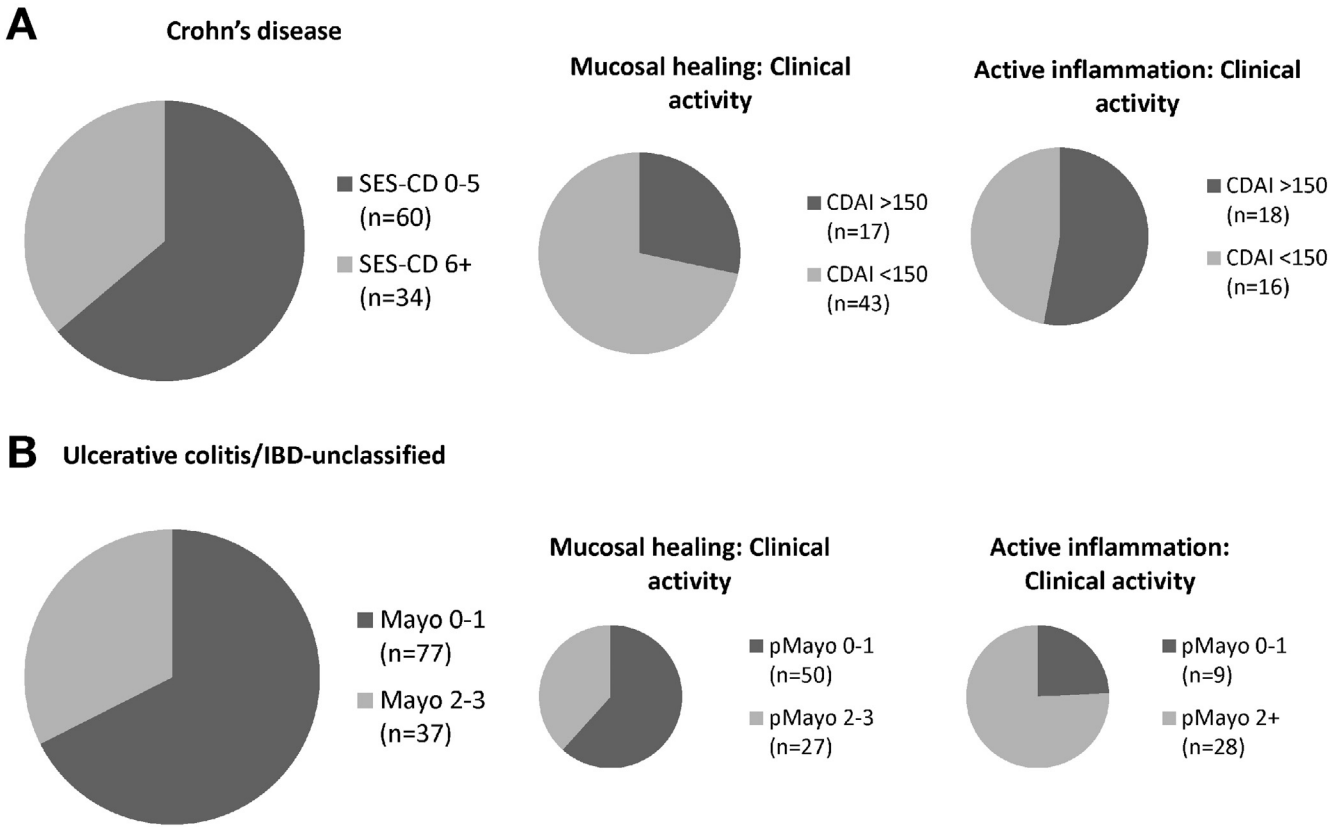

Figure 1. Proportion of inflammatory bowel disease (IBD) patients with active disease. (*A*) Mucosal healing (MH) in 64% of patients with Crohn's disease (CD) (n = 60 of 94). Twenty-eight percent of patients with MH and 53% with moderate-severe inflammation had a Crohn's disease activity index (CDAI) of greater than 150. (*B*) A similar proportion (68%) of patients with ulcerative colitis (UC) or UC/IBD-U (n = 77 of 114) had MH. Approximately one third of patients with mucosal healing had a pMayo score 2 or greater. Despite moderate–severe inflammation in 37 patients, 28% had partial Mayo (pMayo) scores of 0 to 1. SES, segmental endoscopic severity. pMayo, partial Mayo.

dysbiosis in CD compared with UC/IBD-U or HCs ($.01 \leq P \leq .03$) (Supplementary Figure 3, Supplementary Data File 1).

To determine whether microbiome composition is associated with clinical symptoms, we identified patients in endoscopic remission (Mayo score of 0 or SES-CD score of 0–2). In sigmoid colon samples, patients with CD (n = 32) showed reduced Chao1 α-diversity when compared with UC/IBD-U (n = 38; $P = .03$) or HCs (n = 48; $P = 4 \times 10^{-4}$) (Figure 2*A*). Biopsy-specific scoring of inflammation diversity was not mirrored by lower Chao1 α-diversity or increased dysbiosis using the MD index ($P = .24$, rho = .12 in CD; $P = .06$, rho = .2 in UC/IBD-U) (Supplementary Figure 4).

Increased Relative Abundance of Fusobacteria and Proteobacteria and Other Taxa Variations Are Associated With Crohn's Disease Patients With Mucosal Healing

We analyzed individual taxa at 3 sites in remission. Patients with CD had increased relative abundance of Fusobacteria at the phylum and lower taxonomic ranks in the terminal ileum and sigmoid colon ($P < 7 \times 10^{-5}$), and increased Proteobacteria genera in ileal samples ($P < 1 \times 10^{-4}$) compared with UC/IBD-U and HCs. Two *Prevotella* species were more abundant in UC/IBD-U than in CD patients at the terminal ileum ($P \leq 1 \times 10^{-4}$) (Supplementary Data File 2). In terminal ileum, *Fusobacteriaceae cetobacterium* abundance ($P = 2.7 \times 10^{-5}$) was increased in CD vs HCs, while a lower abundance of Bacteroidetes genera including *Akkermansia muciniphila* ($P = 2 \times 10^{-4}$) (Supplementary Data File 2) was observed. Comparing CD sigmoid samples with HCs, genera from Fusobacteria and Actinobacteria phyla have increased relative abundance in CD, as did *Dorea* from the Firmicutes phylum ($6.9 \times 10^{-10} \leq P \leq 2 \times 10^{-5}$). In Mayo score 0 UC/IBD-U there was an increased prevalence of *Lactobacillus* at the terminal ileum compared with HCs ($P = 9.7 \times 10^{-4}$) (Supplementary Data File 2).

UC/IBD-U patients with a Mayo score of 0 to 1 had an increased relative abundance of mucin-residing *A muciniphila* among UC/IBD-U ($1 \times 10^{-4} \leq P \leq 2 \times 10^{-4}$) (Supplementary Data File 3).

Paired analysis of sigmoid and ascending colon samples in patients with quiescent left-sided disease in endoscopic remission identified no differentially abundant taxa in previously diseased mucosa when compared with the historically unaffected right colon (Supplementary Data File 4).

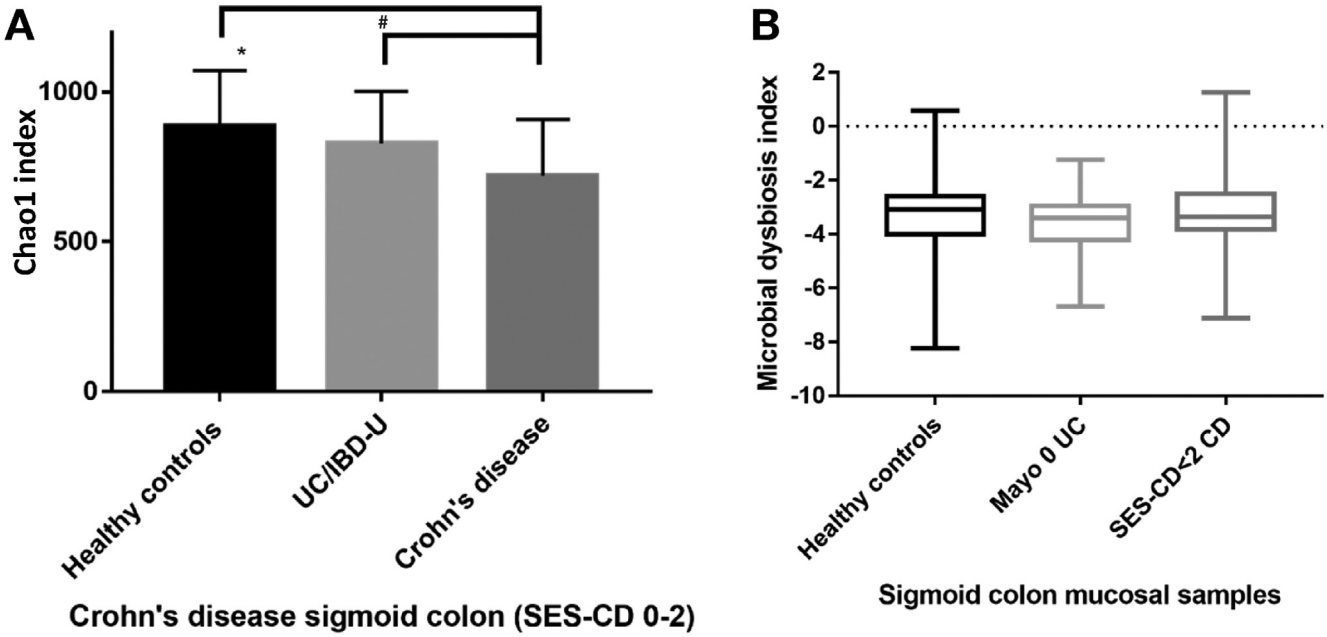

Figure 2. α-diversity analysis and the microbial dysbiosis (MD) index scores of sigmoid mucosa from patients with endoscopic remission (Mayo score, 0 in ulcerative colitis/inflammatory bowel disease unclassified [UC/IBD-U], n = 27; and segmental endoscopic severity [SES]–Crohn's disease [CD] score, 0–2 in CD, n = 26). (*A*) Chao1 α-diversity of sigmoid mucosa. *CD vs healthy controls (HCs) (P = .001) and $^{\#}$CD vs UC/IBD-U (P = .002). Data show mean observations after rarefaction at 8500 sequences. (*B*) Comparison of MD index of sigmoid colon between IBD (CD, n = 26; UC, n = 38) and controls (n = 48; CD vs HCs, P = .4; CD vs UC, P = .48; and UC vs HC, P = .75).

Persistent Clinical Activity and Daily Diarrhea in the Absence of Endoscopic Activity in Crohn's Disease Is Associated With Reduced Diversity

After mucosal healing, 28% of CD (n = 17 of 60) and 35% of UC/IBD-U patients (n = 28 of 77) met criteria indicating active clinical disease (Crohn's disease activity index [CDAI] >150 or partial Mayo score >1). There were no associations between individual taxa abundance and specific symptoms or clinical activity in CD (Supplementary Data File 5) or UC/IBD-U (Supplementary Data File 6). In CD patients with endoscopic improvement, we noted Bray–Curtis β-diversity dissimilarity in patients with a CDAI greater than 150 compared with patients in clinical remission (P = .01, r = 0.24) (Supplementary Figure 2*B*). Furthermore, α-diversity was lower in patients with a CDAI greater than 150 (n = 17 of 43; P < .03) (Figure 3*A*). When α-diversity was analyzed according to the presence of rectal bleeding, nocturnal diarrhea, daily diarrhea, or pain, only daily diarrhea in endoscopically improved CD was associated with a trend toward lower α-diversity (P = .06) (Figure 3*B*). In endoscopic remission (SES-CD score, 0–2), the association between reduced diversity and daily diarrhea (n = 8 of 26) was significant (P = .01) (Figure 3*C*), with no relationship between histologic activity and residual diarrhea (P = .70) (Figure 3*D*). We noted a trend toward dysbiosis of sigmoid samples in patients with residual diarrhea (P = .059) (Figure 3*E*). Daily diarrhea was associated with the presence of *Lactobacillus* (q = .04) (Figure 3*F*).

In Moderate to Severe Ulcerative Colitis/ Inflammatory Bowel Disease Unclassified, Increasing Clinical Activity Is Associated With Reduced Diversity

Despite active disease, 24% with a Mayo score of 2 to 3 and 47% of CD patients with a SES-CD score greater than 6 were in clinical remission (pMayo, 0–1; or CDAI, <150) (Figure 1). Neither diversity nor dysbiosis in moderate–severe endoscopic inflammation were associated with clinical activity or specific symptoms in CD (Supplementary Data File 7). α-diversity was lower in patients with daily diarrhea in moderate–severe UC/IBD-U in sigmoid colon samples (P = .03) (Figure 4*A*), accompanied by a higher MD index (P = .03) (Figure 4*B*). Daily stool frequency correlated negatively with an abundance of Firmicutes *Anaerostipes* (q = 0.037; rho = -0.61) and 2 unidentified members of the *Christensenellaceae* family, 1 in ileum (q = 0.04; rho = -0.6) and the other in sigmoid colon (q = 0.046; rho = -0.40) (Supplementary Data File 8). A relative abundance of a genus from the phylum Proteobacteria, *Luteimonas*, also correlated with increasing diarrhea in ileal samples in UC/IBD-U (q = 0.047; rho = 0.6) (Supplementary Data File 8).

Discussion

In CD patients in endoscopic remission, the mucosa-associated microbiome has less species richness and

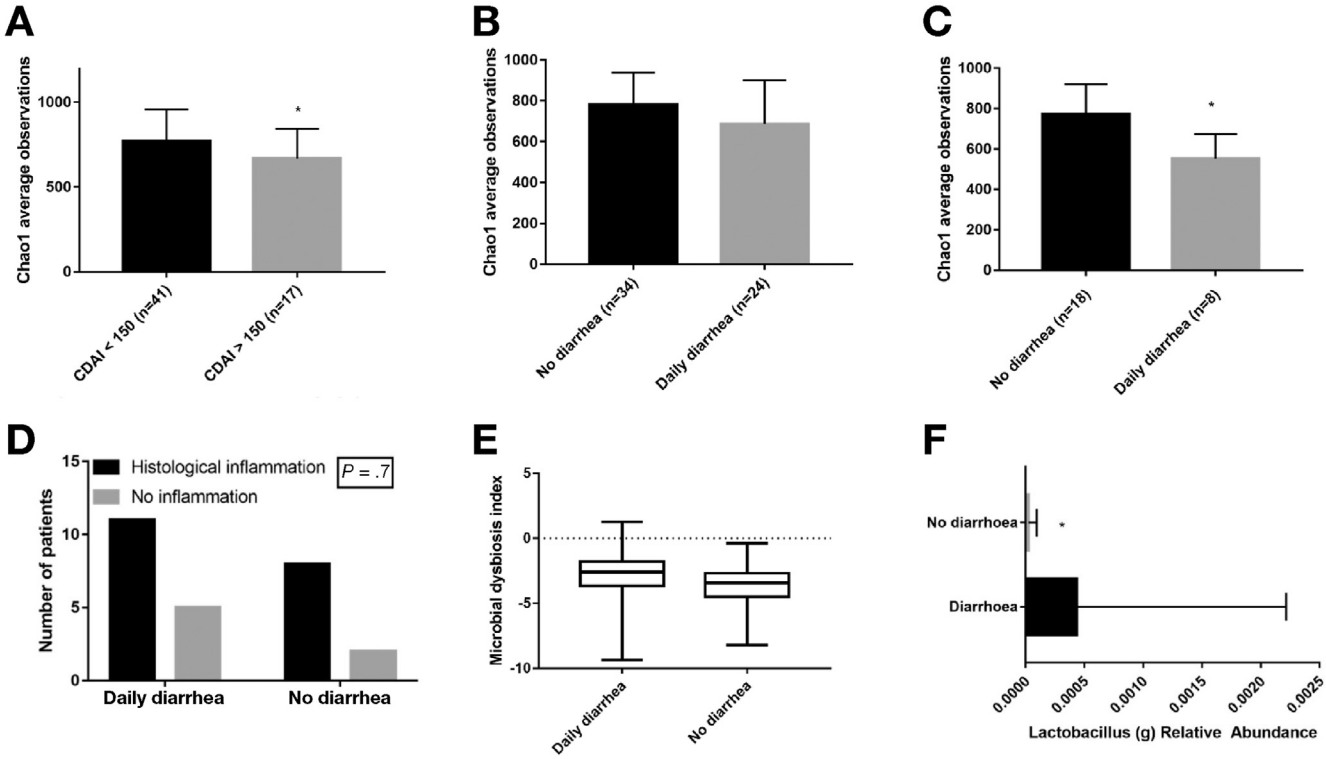

Figure 3. α-diversity analysis and microbial dysbiosis (MD) index scores of sigmoid samples in Crohn's disease (CD) after mucosal healing. (*A*) Chao1 α-diversity $\pm$ SEM in CD patients with mucosal healing (segmental endoscopic severity [SES]-CD, 0–5), comparing a Crohn's disease activity index (CDAI) score of greater than 150 with clinical remission ($P = .03$). Data show mean observations after rarefaction at 8500 sequences. (*B*) Comparison of Chao1 diversity according to the presence or absence of residual diarrhea in patients with SES-CD score of 0 to 5 ($P = .06$) and (*C*) SES-CD score of 0 to 2 ($P = .01$). (*D*) Proportion of patients with histologic inflammation according to the presence of diarrhea. (*E*) MD index in CD patients with mucosal healing and daily diarrhea ($P = .059$). (*F*) Increased *Lactobacillus* abundance in the presence/absence of taxa analysis in daily diarrhea ($q = 0.04$).

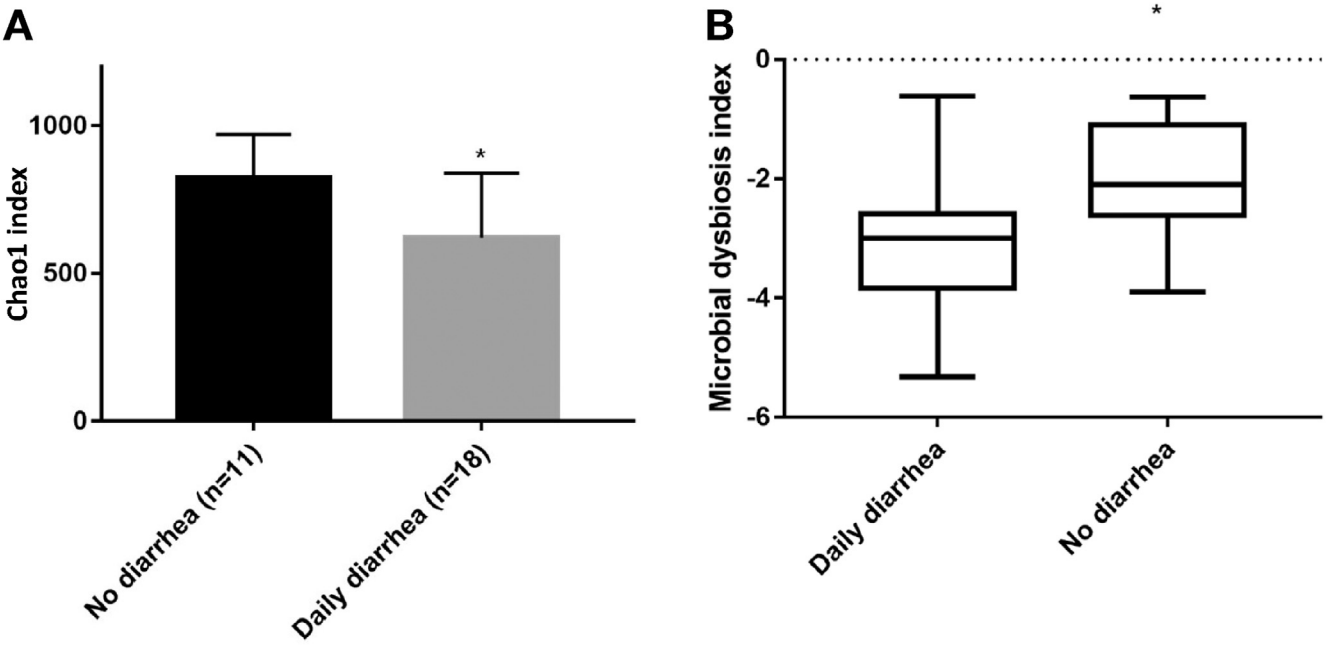

Figure 4. α-diversity and microbial dysbiosis (MD) index from sigmoid mucosa in moderate–severe ulcerative colitis/inflammatory bowel disease unclassified (UC/IBD-U). (*A*) Chao1 diversity in disease with a Mayo score of 2 to 3. Data represent the comparison of Chao1 diversity according to daily diarrhea (diarrhea, $n = 18$; no diarrhea, $n = 11$; $P = .03$). Mean observations after rarefaction at 8500 sequences. (*B*) MD index in moderate–severe UC/IBD-U reporting daily diarrhea (diarrhea, $n = 18$; no diarrhea, $n = 11$; $P = .03$).

greater dysbiosis than UC/IBD-U or controls. Furthermore, in CD with endoscopic improvement, patients with persistent clinical symptoms have dissimilar β-diversity and reduced Chao1 α-diversity than those in remission. Echoing a previous study,[1] we found no relationship between persistent symptoms and histologic activity, previous treatments, or flares over a 12-month follow-up period. The detailed reporting of clinical activity, endoscopic inflammation, and extensive metadata leaves this cohort well placed to compare the microbiome between IBD phenotypes in the presence and absence of inflammation.

Despite variable sites of disease activity, differences in α-diversity and MD index scores were evident in ileum and rectosigmoid samples (Supplementary Figures 1 and 3). *Fusobacterium* abundance was increased in both colon and TI samples in CD relative to UC/IBD-U, but other differential taxa at 1 location were not replicated at the other sites. This suggests that, even in active inflammation, an abundance of tissue-associated taxa may differ depending on the sampling site. We noted increased relative abundance of *A muciniphila* in ileal CD and in UC/IBD-U patients with endoscopic improvement relative to those with moderate–severe inflammation. This bacterium effects epithelial response and goblet cell function, influencing the gut protective mucus layer, which is compromised in UC[16,17] and was predictive of endoscopic remission in IBD.[18]

There was no relationship between either α-diversity or specific taxa abundance and the severity of inflammation at the biopsy site (Supplementary Figure 4). In our analysis, the lack of correlation between either diversity or taxa abundance and endoscopic inflammation supports the hypothesis that the microbiome may predict IBD phenotype through analysis of community structure[19] or specific OTU combinations, rather than individual species abundance. However, dysbiosis and diversity indices may have the potential to differentiate between phenotypes. The MD index was lower in CD compared with UC/IBD-U and control patients in this population. This dysbiosis score has not been validated for use in IBD phenotypes other than CD, but we propose that this may have the potential to differentiate between IBD phenotypes in active inflammation. Chao1 α-diversity in sigmoid samples was persistently lower in CD relative to UC/IBD-U and HCs, even in patients with healed colons (Mayo score, 0; SES-CD score, 0–2) (Figure 2). In addition, paired analysis of ascending and sigmoid colon samples in patients with quiescent left-sided UC/IBD-U showed no differentially abundant taxa. These findings suggest that the microbial community is different long after successful treatment in CD, whereas healed UC/IBD-U patients cannot be distinguished from controls. This may reflect the effects of pervasive bowel damage in CD.

Noninvasive clinical activity indices do not reliably identify patients who have achieved endoscopic remission, with 19% to 39% of these patients reporting persistent symptoms.[20–22] We hypothesized that there may be a relationship between microbiome composition and persistent symptoms. In CD patients with endoscopic improvement, both residual clinical activity (CDAI, $>$150), and patients who reported daily diarrhea had significantly reduced α-diversity and greater dysbiosis (MD index) compared with patients in clinical remission (Figure 3). In those reporting diarrhea, we found an association with *Lactobacillus*, an aero-tolerant Firmicute, often reported in greater abundance in active CD, with more than 180 species having variable strain-specific effects on gut health.[23] This genus generally is bile salt–resistant, in part owing to bile salt hydrolase capacity, and CD is associated with bile salt–mediated diarrhea.[24] Furthermore, the microbiota plays a role in bile acid metabolism through deconjugation and dehydroxylation of primary bile acids.[25] Select *Lactobacillus* species can thrive in a bile salt–rich environment[26] owing to resistance mechanisms preventing protein misfolding and facilitating bile salt efflux.[27] It is possible that greater dysbiosis in our CD patients with residual diarrhea may be related to altered bile salt metabolism.

Another explanation for residual symptoms is an irritable bowel syndrome (IBS) overlap. The IBD in the South Eastern Norway study group found that 27% of patients in remission met criteria for co-existing IBS.[28] In IBS-D, community-level dysbiosis and reduced α-diversity are recognized.[29,30] The presence of loose stool itself has been associated with altered fecal species richness and community structure.[31–33] The extent to which microbiome community composition in our CD cohort is a cause or consequence of residual diarrhea is unclear. Depletion of genera from the *Christensenellaceae* family, as in our cohort (Supplementary Data File 8), has been reported in IBS-D.[34] Although microbiome composition and dynamic stability differs in IBS and IBD, the association between altered diversity and daily diarrhea in patients without IBD suggests that the microbiome could play a role in residual symptoms in CD.

Although there was no association between residual symptoms and dysbiosis in quiescent UC/IBD-U, in moderate–severe endoscopic disease (Mayo score, 2–3), lower α-diversity was associated with higher clinical activity, daily diarrhea, and increasing stool frequency (Figure 4). This was associated with a reduced relative abundance of butyrate-producing genera and an increased relative abundance of *Luteimonas*, a Proteobacterium. Similar changes in the microbiome have been reported in intestinal inflammation from a variety of etiologies.[35–37] The association between lower diversity, greater dysbiosis, and increasing diarrhea in our inflamed UC/IBD-U cohort may point to a relationship between the microbiome and clinical symptoms, given the lack of correlation between diversity and site-specific Mayo scores here. The extent to which this is merely a consequence of ongoing intestinal inflammation is unclear.

In healed colon and ileal samples, CD patients have lower α-diversity than UC/IBD-U or HCs, suggesting long-term microbiota changes in CD in remission (Figure 2). Residual clinical activity and daily diarrhea were associated with lower α-diversity of the tissue-associated microbiome (Figure 3). This study was strengthened by detailed phenotyping, facilitating analysis of the tissue-associated microbiome relative to both validated clinical activity scores and specific symptoms. Furthermore, site-specific microbiome variability was considered, with sampling of 3 distinct sites. Limitations included the small sample size in groups with endoscopic remission, the potential influence of colonoscopy preparation on the gut microbiome, and the lack of longitudinal data, which would go some way to evaluate microbiome stability. Furthermore, visceral hypersensitivity data and depression as predictors for persistent symptoms or IBD/IBS overlap are lacking. Fecal calprotectin was not widely available for patients at recruitment and may be useful to confirm remission. Our findings associate persistent clinical symptoms after mucosal healing with the gut microbiome, particularly lower diversity and species richness. The potential to control symptoms with a microbiome-based strategy should be explored further in prospective studies and may represent a novel therapeutic approach for these patients.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2020.03.044.

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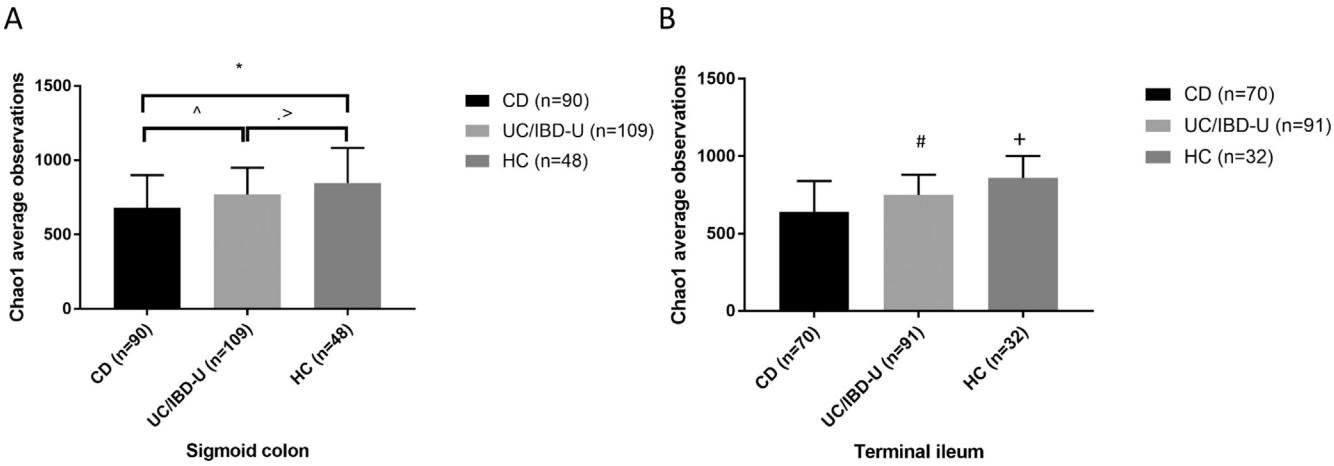
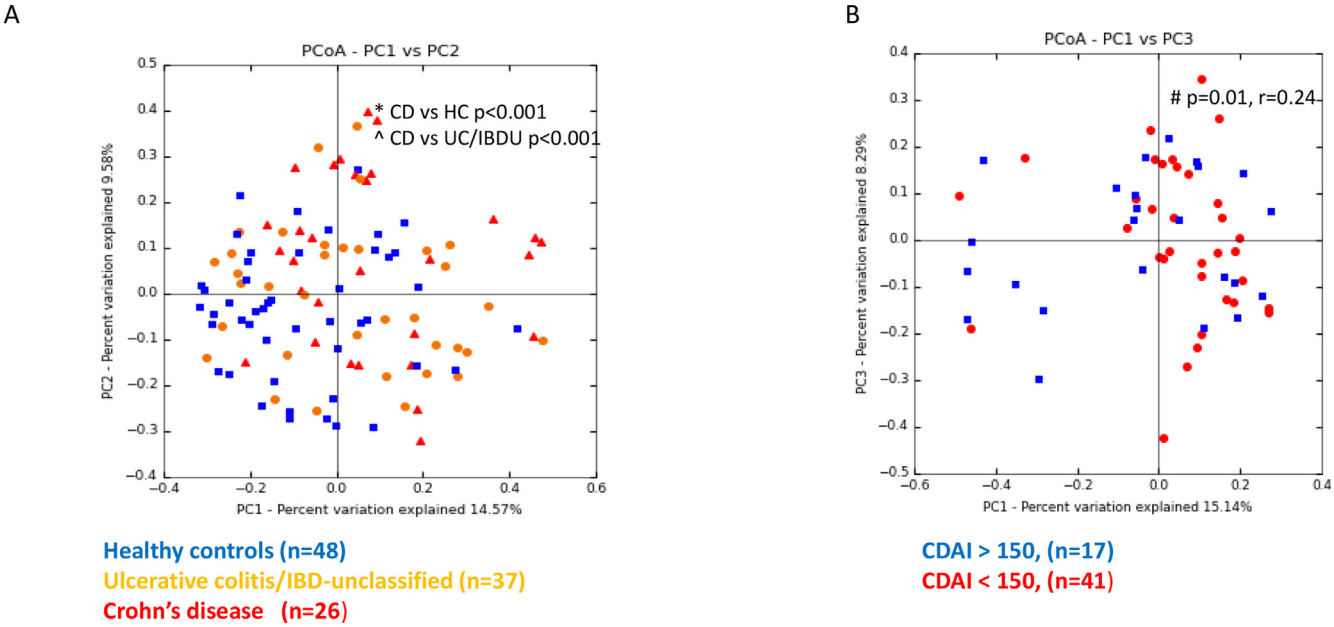
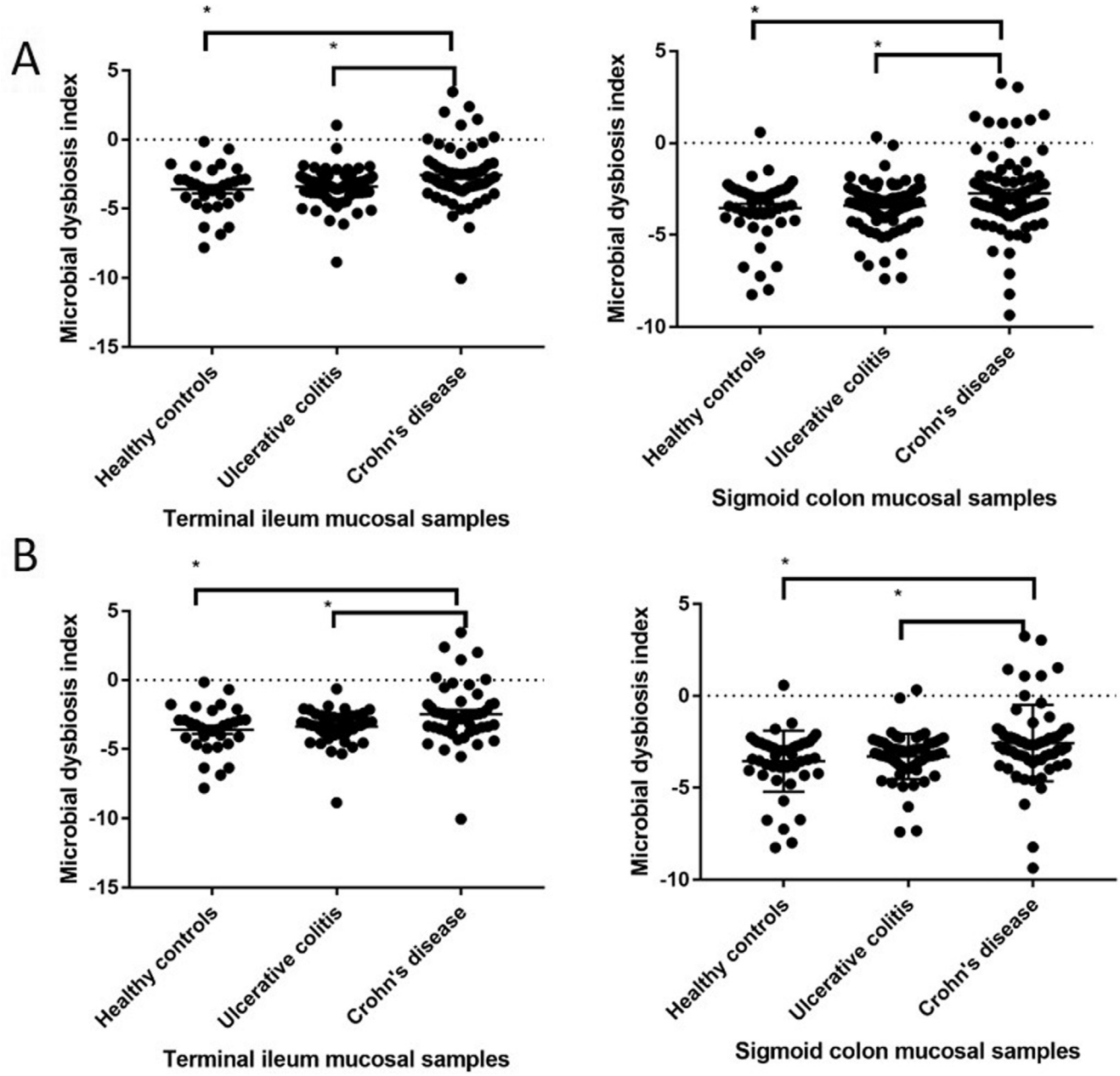


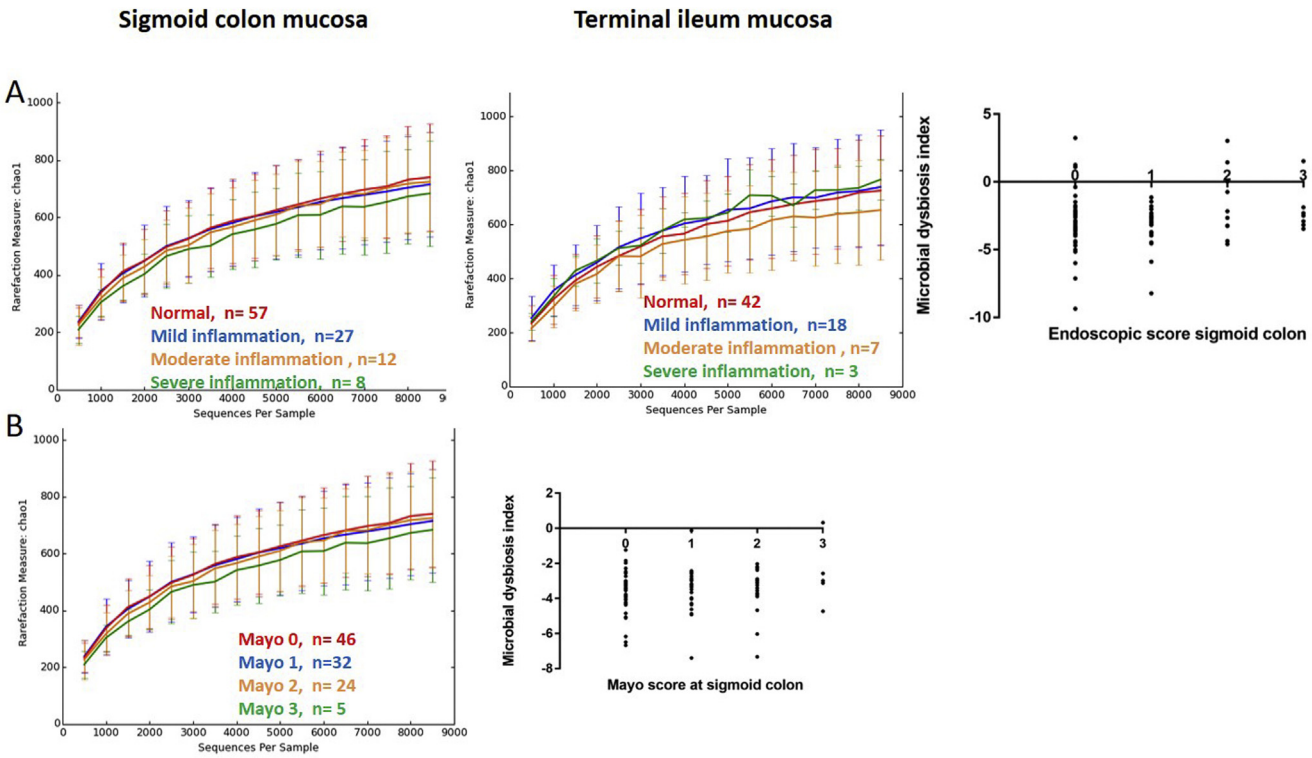
Supplementary Figure 1. α-diversity metrics in patients stratified according to IBD phenotype. (*A*) Chao1 α-diversity in sigmoid samples of patients with CD, UC/IBD-U, and healthy controls. *CD vs HC, $P = .04$. ^UC/IBD-U vs CD, $P = .02$. >UC/IBD-U vs HC, $P < .0001$. (*B*) Chao1 α-diversity in terminal ileum samples. *$P = .03$, CD vs UC/IBD-U. #$P = .03$, CD vs UC/IBD-U.$^{+}P = .001$ CD vs UC/IBD-U. CD, Crohn's disease; HC, healthy control; IBD-U, inflammatory bowel disease unclassified; UC, ulcerative colitis.



Supplementary Figure 2. β-diversity of sigmoid colon biopsy specimens in HCs, CD, and UC/IBD-U. (*A*) Bray–Curtis β-diversity analysis indicating population dissimilarity between HC (n = 48), UC/IBD-U (n = 37), and CD (n = 26). *CD vs HC, $P < .001$ and #CD vs UC/IBD-U, $P < .001$. (*B*) Bray–Curtis dissimilarity between patients with Crohn's disease activity index (CDAI) greater than 150 (n = 17) compared with CDAI less than 150 (n = 41). *$P = .01$, #$P = .01$. r = 0.24. CD, Crohn's disease; HC, healthy control; IBD-U, inflammatory bowel disease unclassified; PC, principal coordinate; PCoA, principal coordinates analysis; UC, ulcerative colitis.



Supplementary Figure 3. Microbial dysbiosis (MD) index scores of the mucosa-associated microbiome calculated in terminal ileum and sigmoid colon samples and compared in patients with UC/IBD-U, CD, and HCs. (*A*) All patients with available samples from the sigmoid colon (HC vs CD, $P = .04$; UC vs CD, $P = .01$) and terminal ileum (HC vs CD, $P = .02$; UC vs CD, $P = .004$). (*B*) Data represent patients with inflammation (Mayo score, >1 in UC, n = 48; and SES-CD, >5 in CD, n = 50). MD index scores were calculated from tissue-associated microbiome of inflamed sigmoid colon samples (HC vs CD, $P = .01$; UC vs CD, $P = .03$) and terminal ileum (HC vs CD, $P = .01$; UC vs CD, $P = .02$). *Statistical differences ($P < .05$) across groups. CD, Crohn's disease; HC, healthy control; IBD-U, inflammatory bowel disease unclassified; UC, ulcerative colitis.



Supplementary Figure 4. Comparison of Chao1 diversity and microbial dysbiosis (MD) index according to severity of endoscopic inflammation. (*A*) Chao1 index of species richness at sigmoid and terminal ileum biopsy sites in CD patients with data representing 4 groups categorized by severity of inflammation at that biopsy segment (0, normal; 1, mild; 2, moderate; 3, severe). Chao1 metrics were analyzed with individual comparisons between groups with no statistical significance ($0.89 < P < 1$). There was no correlation between the MD index and severity at the biopsy site ($P = .24$, rho $= 0.12$). (*B*) Chao1 diversity index at the sigmoid colon in ulcerative colitis, categorizing patients according to Mayo scores. Chao1 metrics were compared between groups with no statistical significance ($0.5 < P < 1$). There was no correlation between the MD index and severity ($P = .06$, rho $= 0.2$).