# Bacterial Mucosa-associated Microbiome in Inflamed and Proximal Noninflamed Ileum of Patients With Crohn's Disease

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**Background:** Microbiota is most likely essential in the pathogenesis of Crohn's disease (CD). Fecal diversion after ileocecal resection (ICR) protects against CD recurrence, whereas infusion of fecal content triggers inflammation. After ICR, the majority of patients experience endoscopic recurrence in the neoterminal ileum, and the ileal microbiome is of particular interest. We have assessed the mucosa-associated microbiome in the inflamed and noninflamed ileum in patients with CD.

**Methods:** Mucosa-associated microbiome was assessed by 16S rRNA sequencing of biopsies sampled 5 and 15 cm orally of the ileocecal valve or ileocolic anastomosis.

**Results:** Fifty-one CD patients and forty healthy controls (HCs) were included in the study. Twenty CD patients had terminal ileitis, with endoscopic inflammation at 5 cm, normal mucosa at 15 cm, and no history of upper CD involvement. Crohn's disease patients (n = 51) had lower alpha diversity and separated clearly from HC on beta diversity plots. Twenty-three bacterial taxa were differentially represented in CD patients vs HC; among these, *Tyzzerella 4* was profoundly overrepresented in CD. The microbiome in the inflamed and proximal noninflamed ileal mucosa did not differ according to alpha diversity or beta diversity. Additionally, no bacterial taxa were differentially represented.

**Conclusions:** The microbiome is similar in the inflamed and proximal noninflamed ileal mucosa within the same patients. Our results support the concept of CD-specific microbiota alterations and demonstrate that neither ileal sublocation nor endoscopic inflammation influence the mucosa-associated microbiome.

**Key Words:** Crohn's disease, mucosal microbiota, microbiome.

## INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) characterized by transmural inflammation of the gastrointestinal (GI) tract. It may involve the entire GI tract from the oral cavity to the perianal area. Approximately 75% percent of patients have small bowel involvement, usually in the distal ileum and 25%–30% of all patients have ileitis exclusively.[1, 2] The etiology of CD is not known; however, an abnormal immune reaction toward environmental factors, including gut microbiota, in genetically predisposed individuals is the most widely accepted hypothesis. During their lifetime, 75%–80% of CD patients will require surgical intervention,[1, 3] most commonly ileocecal resection (ICR).[2, 4] Furthermore, 75%–80% of patients experience endoscopic recurrence of disease, usually in the neoterminal ileum, proximal to the surgical anastomosis.[1–3, 5]

Patients with CD have an altered gut microbiome composition compared with healthy controls, characterized by decreased bacterial diversity and alterations in bacterial composition, including increased abundances of potentially harmful bacteria and decreased abundances of protective bacteria.[6–9] More specifically, the altered mucosal microbiome in the ileal mucosa of CD patients is characterized by increased abundances of Proteobacteria and Fusobacteria phyla, in addition to Enterobacteriaceae, Veillonellaceae, Gemellaceae, and Fusobacteriaceae families, in combination with decreased abundance of Firmicutes phylum and Lachnospiraceae, Bifidobacteriaceae, and Erysipelotrichaeceae families.[7, 10–14]

Previous studies have found large differences between the bacterial composition in fecal samples and in mucosal biopsies from the colon.[14, 15] Because microbes found in fecal samples may be derived from any part of the GI tract, fecal samples are not ideal for studying microbial changes within the small bowel.

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Investigating the ileal microbiome may be crucial to understand etiologic aspects of CD, for instance, why fecal diversion after ileocecal resection prevents downstream CD recurrence, whereas reestablishment of bowel continuity or infusion of intestinal content is associated with recurrence of disease.[16, 17] Analyses of ileal mucosal microbiome both at the time of ICR and postoperatively have identified characteristics of the microbiome that are associated with postoperative recurrence.[18–20] Sokol et al[20] found that increased abundances of Gammaproteobacteria, *Corynebacterium*, and *Ruminococcus gnavus* and reduced abundance of *Ruminoclostidium 6* at the time of ileocecal resection were predictive of disease recurrence. Additionally, a recent analysis of 2 separate cohorts found that reductions in a specific cluster of bacteria in postoperative CD patients was associated with increased risk of disease recurrence.[6] Postoperative recurrence of disease most often occurs in the surgical anastomosis and immediately proximal to the anastomosis,[1, 3, 5] and analyses of the mucosal microbiome in the inflamed and proximal noninflamed ileum could increase our understanding of the microbial role in disease recurrence.

In the current study, we assessed the ileal bacterial microbiome of adult CD patients and compared the bacterial mucosa-associated microbiome in the inflamed ileum with the proximal noninflamed mucosa within the same patients, which to the best of our knowledge, has not been performed previously.

## MATERIALS AND METHODS

### Patients and Control Subjects

Patients were recruited from the Department of Gastroenterology, St. Olav's Hospital, Trondheim, Norway, between 2017 and 2019. Patients with Norwegian ethnicity who were 18 to 70 years old and referred to an endoscopic examination involving the small intestine were invited to participate if they were eligible. Inclusion criteria included an established diagnosis of CD based on clinical, endoscopic, and histological criteria or a clinical suspicion of CD that was confirmed after both endoscopic and histologic evaluation. The Montreal classification was used to describe CD characteristics.[21] Age- and sex-matched patients referred to ileocolonoscopy due to rectal bleeding or screening for disease were included as healthy controls (HCs) if the endoscopy and histologic evaluation of biopsies were normal. Exclusion criteria included use of antibacterial or antifungal treatment for the past 2 months or a diagnosis of either diabetes mellitus, liver diseases including primary sclerosing cholangitis and primary biliary cirrhosis, or celiac disease. Additional exclusion criteria for the HC group were history of gastrointestinal surgery, gastrointestinal polyps, cancer, diverticulitis, or irritable bowel disease fulfilling ROME IV criteria.[22]

### Endoscopic Procedure

Ileocolonoscopy was performed using either Olympus Exera II GIF HQ190 or PH190L or enteroscope SIF-Q180 (Olympus Europa GmbH, Hamburg, Germany). A total of 4 ileal pinch biopsies were collected from each study participant. An overview of the study design is provided in Figure 1. In CD patients, 2 biopsies were sampled from the inflamed area (approximately 5 cm orally of the ileocecal valve or ileocolic anastomosis) and 2 from normal appearing mucosa (approximately 15 cm from the ileocecal valve or anastomosis). In CD patients with active inflammation where the proximal limit of inflammation could not be reached by the endoscope, in CD patients in remission and in the HC group, biopsies were sampled 5 cm and 15 cm proximal to the ileocecal valve or anastomosis. In the CD patients with an ileal stenosis preventing further intubation of the ileum, 5-cm samples were collected exclusively. Degree of endoscopic ileal inflammation was evaluated according to Rutgeerts score;[23] inflammation was defined as Rutgeerts score ≥1. One pair of mucosal pinch biopsies sampled at 5 and 15 cm from the ileocecal valve were put on formalin for histological grading of inflammation, and the remaining pair were put directly on liquid $N_2$ and stored on $N_2$ until subsequent DNA isolation and sequencing of the mucosal bacterial microbiome.

### Histological Evaluation of Biopsies

Formalin-fixed biopsies were stained with hematoxylin and eosin (H&E) and evaluated blindly by an experienced pathologist and scored according to Global Histologic Disease Activity Score (GHAS) and Robarts score.[17, 24, 25] No validated histological scoring index for evaluation of disease activity in CD exists. Due to the focality of CD and poor correlation between histological activity and other measurements of disease activity, it is claimed that the significance of histologic disease activity is uncertain.[25, 26] However, blinded histological evaluation of all biopsies ensured classification of a histological, normal appearing ileal mucosa in biopsies from HCs.

### Microbial Analyses

DNA was isolated from the mucosal biopsies using DNeasy Powersoil kit (Qiagen, Hilden, Germany) according to manufacturer's protocol with the following adjustments: steps 3 and 4 (vortexing) were replaced, and instead the provided PowerBead tubes were vortexed using Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm × 3 rounds of 40 seconds (step 3). After bead beating, 20 µL of Proteinase K 20 mg/mL was added, and samples were incubated at 65°C for 30 minutes according to Qiagen's recommendations before centrifugation (step 5). The isolated DNA was quality tested using NanoDrop (Thermo Fisher Scientific, MA) and Qubit (Thermo Fisher Scientific). Then 16S metagenomic

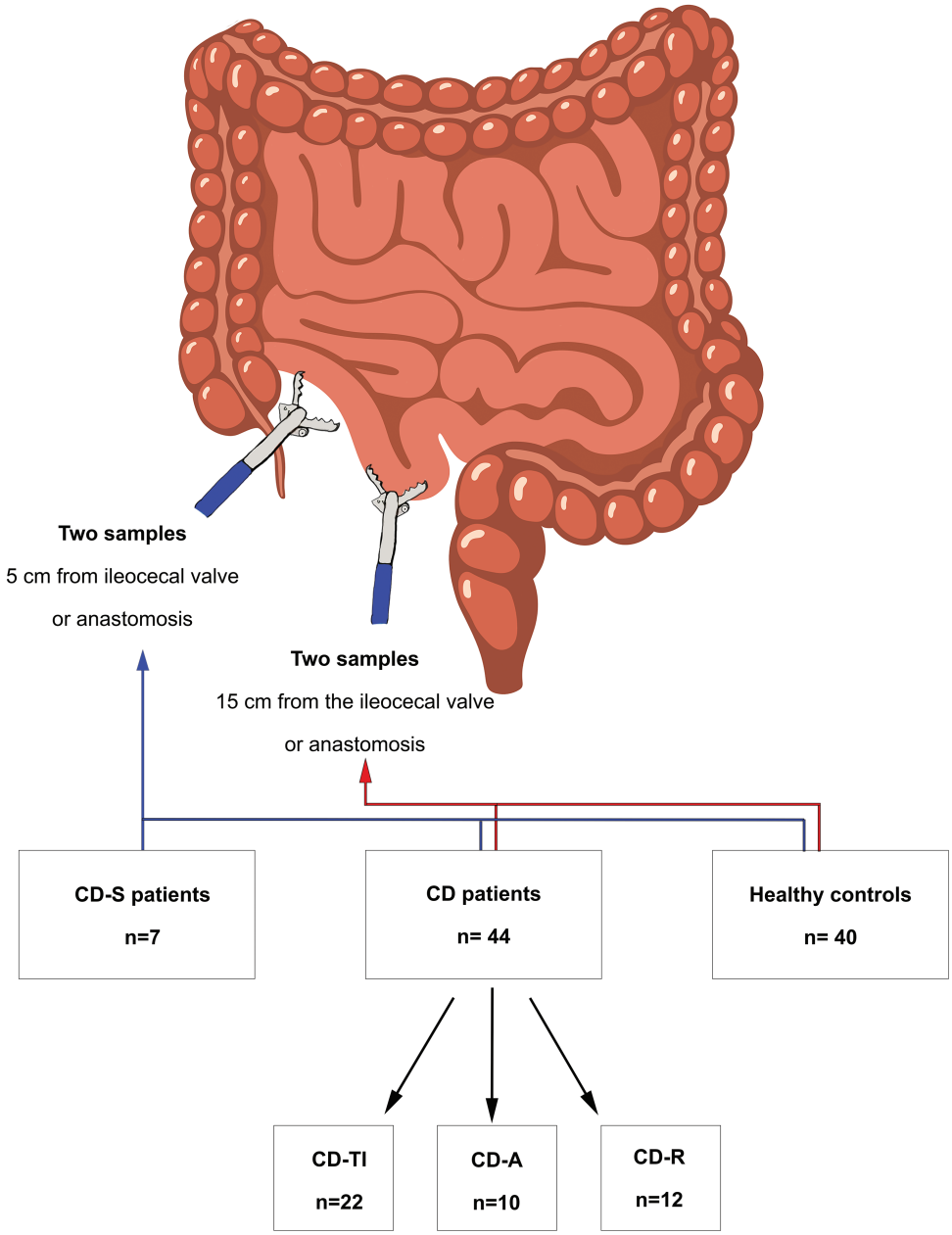


FIGURE 1. Illustration of study design with mucosal pinch biopsy location and number from the study participants. Fifty-one Crohn's disease patients including 7 CD patients with ileal stenosis (CD-S), 22 CD patients with terminal ileitis (CD-TI) with endoscopic inflamed mucosa at 5-cm location (Rutgeerts score ≥1) and normal endoscopic appearing mucosa at 15-cm location, 10 CD patients with active disease (CD-A) with endoscopic inflamed mucosa at both 5-cm and 15-cm location, and 12 CD patients in remission (CD-R) with endoscopic normal appearing mucosa at both 5-cm and 15-cm location and 40 healthy controls (HC) were included in the cohort. Two mucosal pinch biopsies were collected on each location in the 44 CD patients and HCs, in total 4 mucosal pinch biopsies per study participant. Two mucosal biopsies were collected in the 7 CD-S patients at 5-cm location because of ileal stenosis mucosal pinch biopsies could not be collected at 15-cm location.

sequencing libraries were prepared according to the "16S Illumina Demonstrated Library Prep Guide,"[27] with minor adjustments. In brief, 22.5 ng genomic DNA (extracted from biopsies samples) was used as a template for polymerase chain reaction (PCR) amplification (25 cycles) of the 16S V3 and V4 regions. The 16S ribosomal RNA gene PCR primers were based on sequences first published by Klindworth.[28] Illumina adaptor compatible overhang nucleotide sequences were added to the gene/locus specific sequences (16S Amplicon PCR Forward Primer = 5' TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCTA CGGGNGGCWGCAG and 16S Amplicon PCR Reverse

Primer = 5' GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGGACTACHVGGGTATCTAATCC), resulting in a PCR product of the expected size of approximately 550 bp. The PCR products were then cleaned using AMPure XP beads to purify 16S V3 and V4 amplicons from free primers and primer-dimer products. In a second PCR amplification step (8 cycles), dual indices and Illumina sequencing adaptors were added by using the Nextera XT indexing kit (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. A second PCR clean-up step was performed using AMPure XP beads before validation of the library by a LabChip GX DNA high sensitivity assay (PerkinElmer, Inc., Waltham, MA). Libraries were normalized and pooled to 10 pM and subjected to clustering on 1 MiSeq v3 flowcell. Finally, paired end read sequencing was performed for 2X300 cycles on a MiSeq instrument (Illumina Inc.) according to the manufacturer's instructions. Base calling was done on the MiSeq instrument by RTA v1.18.54. FASTQ files were generated using bcl2fastq2 conversion software v2.17 (Illumina Inc.).

## Statistics

IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY) was used to conduct the statistical analysis apart from the sequencing data. Demographic and clinical characteristics are presented as percentages (n) for categorical variables, median (interquartile range [IQR]) for skewed distributed variables, and mean value (SD) for normally distributed variables; $\times^2$ test, Mann-Whitney *U* test, or independent *t* test were used for comparing CD patients with HCs. A *P* value <0.05 was considered statistically significant.

Sequencing data were processed using QIIME II pipeline and denoised using DADA2. Data generated by the QIIME II pipeline were imported into the R software environment version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) using the phyloseq package and subsequently filtered to include only operational taxonomic units from the bacteria kingdom and excluding operational taxonomic units classified as mitochondria, chloroplast or cyanobacteria/chloroplast. Sequences were classified taxonomically using Silva (release 132) reference database. Alpha diversity was assessed by Shannon entropy and beta diversity by Bray-Curtis dissimilarity index. The count tables merged at a given taxonomic rank were imported into the DESeq2 R package to estimate differential expression. *P* values were estimated using a Wald test and adjusted for multiple testing by Benjamini-Hochberg false discovery rate correction. For all statistical analysis, adjusted *P* values <0.05 were considered statistically significant.

## Ethical Considerations

The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference, 2016/2164). All study participants provided written informed consent.

# RESULTS

## Patient Characteristics

A total of 51 CD patients and 40 HCs were included in the study. Demographic and clinical characteristics of all study participants are presented in Table 1. Overview of study participants is provided in Figure 1. Seven of the 51 CD patients had a stenosis preventing intubation 15 cm into the ileum (CD-S). For the remaining 44 CD patients, biopsy specimens were sampled from both 5 and 15 cm proximal to the ileocecal valve or anastomosis; 22 of these had terminal ileitis (CD-TI) with endoscopic inflammation at 5 cm and normal appearing mucosa at 15 cm; 10 had endoscopic active inflammation both at 5 and 15 cm (CD-A); and 12 were in endoscopic remission with normal appearing mucosa at 5 and 15 cm (CD-R). Crohn's disease–specific characteristics are presented in Table 2. The majority of CD patients had previously undergone ileocecal resection (n = 32), 63.6% and 57.1% in the CD and CD-S groups, respectively. A total of 190 pinch biopsies were sampled from all CD patients; of these, 95 biopsy specimens underwent histologic evaluation, and the remaining 95 underwent 16S rRNA sequencing for analysis of the bacterial microbiome (Supplementary Table 1).

We found 76% agreement between endoscopic and histologic characterization of inflammation. In the majority of cases where endoscopic and histologic conclusion differed, endoscopy disclosed inflammation, but histology was described as normal, plausibly due to the focality of CD.

## Mucosa-associated Bacterial Microbiota in CD Patients vs HC

Crohn's disease patients (n = 51) had lower alpha diversity compared with HC ($P = 2.4 \times 10^{-7}$; Fig. 2A). There was also a clear separation between CD patients and HCs on nonmetric multidimensional scaling (NMDS) plots of Bray-Curtis dissimilarity, reflecting differences in bacterial microbiome composition (Fig. 2B). Differential expression analysis identifying taxa differentially expressed between CD patients and HCs showed that CD significantly increased abundances of Proteobacteria phylum ($P = 1.2 \times 10^{-12}$) and Enterobacteriaceae family ($P = 9.9 \times 10^{-7}$) compared with HCs. At genus level, CD patients had higher abundances of *Tyzzerella 4* (27-fold [log2]; $P = 4.1 \times 10^{-68}$) and *Escherichia shigella* and lower abundances of *Ruminiclostridium 5, Ruminiclostridium 6, Eisenbergiella* and *Fecalibacterium*. In total, 7 bacterial families and 15 bacterial genera were significantly differently expressed between CD and HC (Fig. 2C). The increased abundance of *Tyzzerella 4* in CD patients was remarkable; this genus was further identified as *Tyzzerella sp. Marseille-P3062*. However, 16S rRNA sequencing is not the

**TABLE 1.** Demographic and Clinical Characteristics of Crohn's Disease Patients and Healthy Controls

| | CD | HC | P[a] |
|---|---|---|---|
| Number of patients, n | 51 | 40 | |
| Male gender, n (%) | 26 (51%) | 19 (47.5%) | 0.785 |
| Age, years, mean (SD) | 41.5 (14.2) | 36.6 (12.9) | 0.94 |
| BMI, mean (SD) | 25.9 (4.7) | 26.6 (4.7) | 0.502 |
| Acid reflux medication, n (%) | | | 0.839 |
| PPI | 5 (9.8%) | 2 (5%) | |
| $H_2$ blockers | 1 (2.0%) | 0 | |
| PPI on demand | 1 (2.0%) | 0 | |
| $H_2$ blockers on demand | 1 (2.0%) | 1 (2.5%) | |
| Smoking | | | 0.560 |
| Never smoker | 25 (49%) | 25 (62.5%) | |
| Active smoker | 6 (11.8%) | 5 (12.5%) | |
| Snuff | 12 (23.5%) | 8 (20%) | |
| Ex-smoker | 8 (15.7%) | 2 (5%) | |
| Laboratory values | | | |
| Hb (g/dL), mean (SD) | 13.9 (1.4) | 14.5 (1.7) | 0.09 |
| Leukocytes (x$10^9$/L), median (IQR) | 6.7 (2.0) | 6.5 (2.3) | 0.261 |
| CRP (mg/L), median (IQR) | <5 (5) | <5 (0) | **0.006** |

[a]Comparing CD with HC using Mann-Whitney *U* test for skewly distributed continuous variables, independent *t* test for normal distributed continuous variables, and Fisher exact test for categorical variables.

preferred method for analyzing bacteria on species level. The microbiome community composition shift within individual patients is visualized in Supplementary Figure 1.

## Mucosa-associated Microbiota at Different Locations in the Ileum

Alpha diversity did not differ between 5-cm and 15-cm biopsy samples within CD patients ($P$ = 0.83; Fig. 3A). Similarly, bacterial composition did not differ between 5 cm and 15 cm within CD patients (Fig. 3B). Differential expression analysis did not identify any bacterial phyla, families, or genera that differed in expression at 5 cm and 15 cm in the ileum in CD patients. When CD and HC samples were pooled together, there was no separation between 5 cm and 15 cm regarding beta diversity, nor within the HC or CD groups (Fig. 3C). We also performed differential expression analysis comparing abundances of bacterial taxa at 5 cm and 15 cm within the HC samples. We found the Peptostreptococcaceae family to be overrepresented at 15 cm vs 5 cm ($P$ = 0.02); however, this was the only taxa on phylum, family, genus and species level that was differentially expressed between 5 cm and 15 cm in the ileum of HC.

## Mucosal Microbiome in the Inflamed and Proximal Noninflamed Ileum in CD Patients

To separate the effects of localized inflammation itself from a potentially disseminated alteration in mucosa-adjacent bacteria, we compared the microbiome in inflamed ileal mucosal microbiome at 5 cm and the orally noninflamed mucosal microbiome 15 cm from the ileocecal valve or anastomosis in CD patients. Twenty-two CD patients had terminal ileitis with inflammation at 5 cm and endoscopic normal appearing mucosa at 15 cm (CD-TI); however, 2 patients had a history of concomitant upper gastrointestinal CD and were excluded from these particular subanalyses. There was no difference in alpha diversity between 5-cm samples and 15-cm samples from the 20 CD-TI patients ($P$ = 0.88; Fig. 4A). Similarly, there was no separation between 5-cm and 15-cm samples according to beta diversity (Fig. 4B), reflecting a similar bacterial microbiome composition in inflamed and proximal noninflamed ileal mucosa. Differential expression analysis provided similar results; no taxa (phylum, family, genus, or species level) were differentially represented between inflamed 5 cm vs noninflamed 15 cm ileal location. However, the 20 CD-TI patients had significantly lower alpha diversity ($P$ = 0.0013) than HCs (Fig. 4C), and beta diversity analysis demonstrated a clear separation between CD-TI patients and HCs (Fig. 4D).

## Effects of Inflammation on Mucosa-Associated Microbiome

To further explore if microbiome differences found in CD patients vs HC were associated with inflammation, we compared the microbiome in CD biopsies from locations characterized as inflamed at endoscopy (n = 49) and

**TABLE 2.** Crohn's Disease Characteristics, Current and Previous Medical Treatment, and Surgical History of CD Patients

| | **Crohn's disease (5 + 15 cm samples)** | **Crohn's disease with stenosis (CD-S, only 5 cm sample)** |
|---|---|---|
| Number of patients, n | 44 | 7 |
| Disease duration, years (median (IQR)) | 10.0 (19.8) | 8.0 (13.0) |
| Subclassification of patients,[a] n (%) | | |
| CD-TI[b] (inflamed 5 cm, normal 15 cm) | 22 (50.0%) | 0 |
| CD-A[c] (inflamed 5 cm and 15 cm) | 10 (22.7%) | 0 |
| CD-R[d] (noninflamed 5 cm and 15 cm) | 12 (27.3%) | 0 |
| CD-S[e] (ileal stenosis 5 cm) | 0 | 7 (100%) |
| Montreal location, n (%) | | |
| Terminal ileum (L1) | 23 (52.3%) | 1 (14.3%) |
| Ileocolonic (L3) | 16 (36.4%) | 4 (57.1%) |
| Ileocolonic + Upper GI (L3 + L4) | 5 (11.4%) | 2 (28.6%) |
| Montreal behaviour, n (%) | | |
| Nonstricturing, nonpenetrating (B1) | 8 (18.2%) | 0 |
| Nonstricturing, nonpenetrating + perianal (B1p) | 2 (4.5%) | 1 (14.3%) |
| Stricturing (B2) | 15 (34.1%) | 3 (42.9%) |
| Stricturing + perianal (B2p) | 6 (13.6%) | 2 (28.6%) |
| Penetrating (B3) | 11 (25%) | 1 (14.3%) |
| Penetrating + perianal (B3p) | 2 (4.5%) | 0 |
| Montreal age (age at diagnosis), n (%) | | |
| 16 years or younger (A1) | 12 (27.3%) | 1 (14.3%) |
| 17–40 years (A2) | 22 (50%) | 5 (71.4%) |
| Over 40 years (A3) | 10 (22.7%) | 1 (14.3%) |
| CD-medication, n (%) | [f] | [g] |
| No medical therapy for CD | 18 (40.9%) | 1 (14.3) |
| Budesonide | 7 (15.9%) | 4 (57.2%) |
| Prednisolone | 4 (9.1%) | 0 |
| 5-ASA | 3 (6.8%) | 1 (14.3%) |
| Azathioprine | 6 (13.6%) | 0 |
| Methotrexate | 3 (6.8%) | 0 |
| Adalimumab | 4 (9.1%) | 2 (28.6%) |
| Infliximab | 7 (15.9%) | 1 (14.3%) |
| Vedolizumab | 1 (2.3%) | 1 (14.3%) |
| Treatment naïve, n (%) | 6 (13.6%) | 0 |
| TNFα naïve, n (%) | 23 (52.3%) | 2 (28.6%) |
| Rutgeerts score, n (%) | | |
| i0 | 12 (27.3%) | 0 |
| i1 | 12 (27.3%) | 0 |
| i2 | 5 (11.4%) | 0 |
| i3 | 6 (13.6%) | 0 |
| i4 | 9 (20.5%) | 7 (100%) |
| Ileocecal resection | 28 (63.6%) | 4 (57.1%) |

[a]Based on endoscopic evaluation of inflammation
[b]CD-TI; Crohn's disease patients with terminal ileitis
[c]CD-A; Crohn's disease patients with endoscopic active inflammation
[d]CD-R; Crohn's disease patients in endoscopic remission
[e]CD-S; Crohn's disease patients with ileal stenosis
[f]Comedication: n = 8 (18.2%) used 2 CD medications, n = 1 (2.3%) used 3 CD medications
[g]Comedication: n = 3 (42.9%) used 2 CD medications, n = 0 used 3 CD medications

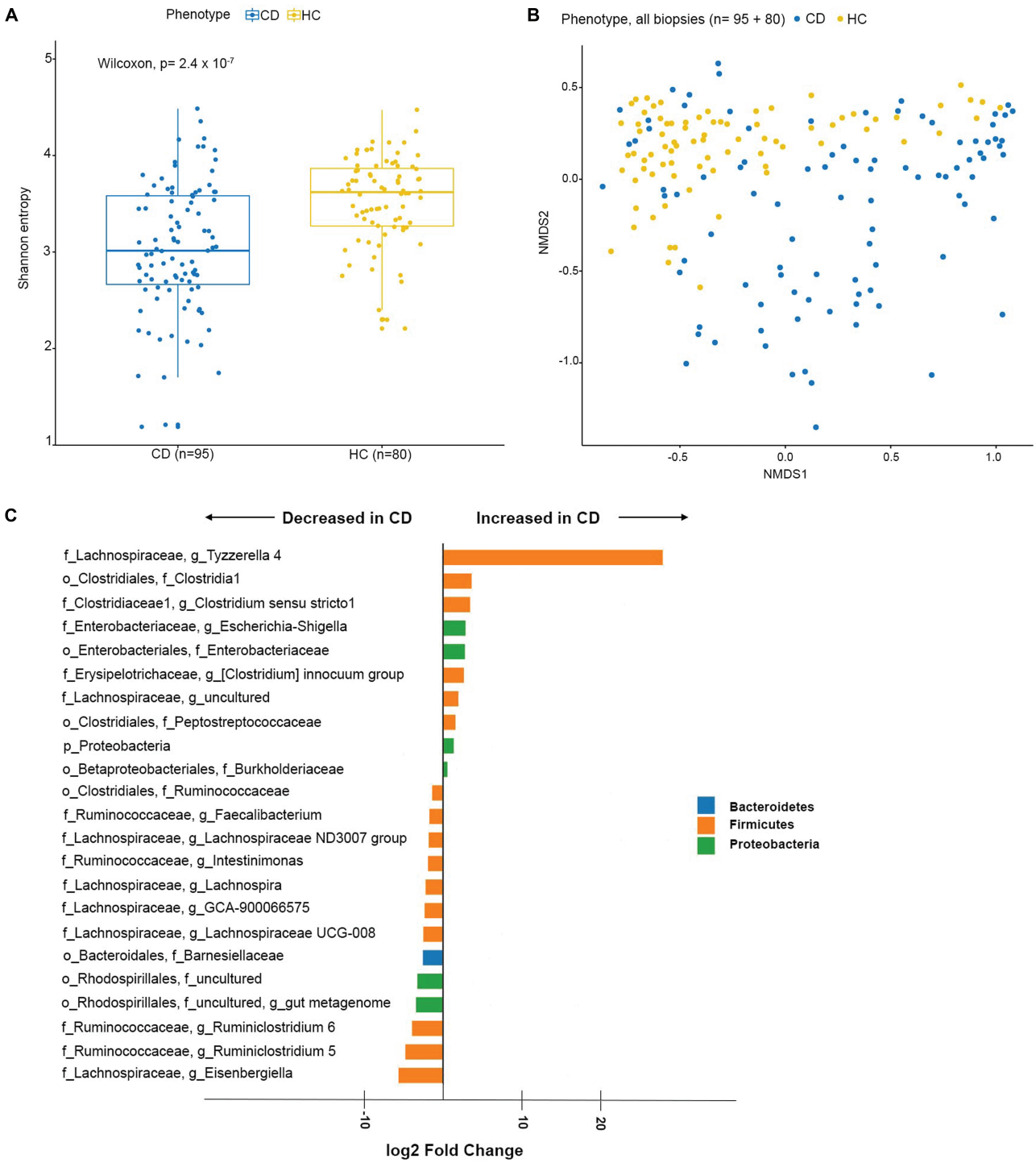


FIGURE 2. Ileal mucosa-associated bacterial microbiome in 51 Crohn's disease patients and 40 healthy controls, mucosal biopsies sampled 5 and 15 cm proximal from the ileocecal valve or anastomosis, respectively (except for 7 CD patients with stenosis where only 5 cm sample was obtained). In total, 95 biopsy specimens from 51 CD patients and 80 biopsy specimens from 40 HCs. A, Alpha diversity illustrated by Shannon entropy index in CD patients vs HC compared with Wilcoxon test. B, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot, each sample colored according to phenotype (CD and HC). C, Bacterial taxa significantly differentially represented (adjusted $P < 0.05$) in patients with CD vs HC, illustrated by log2 fold change.

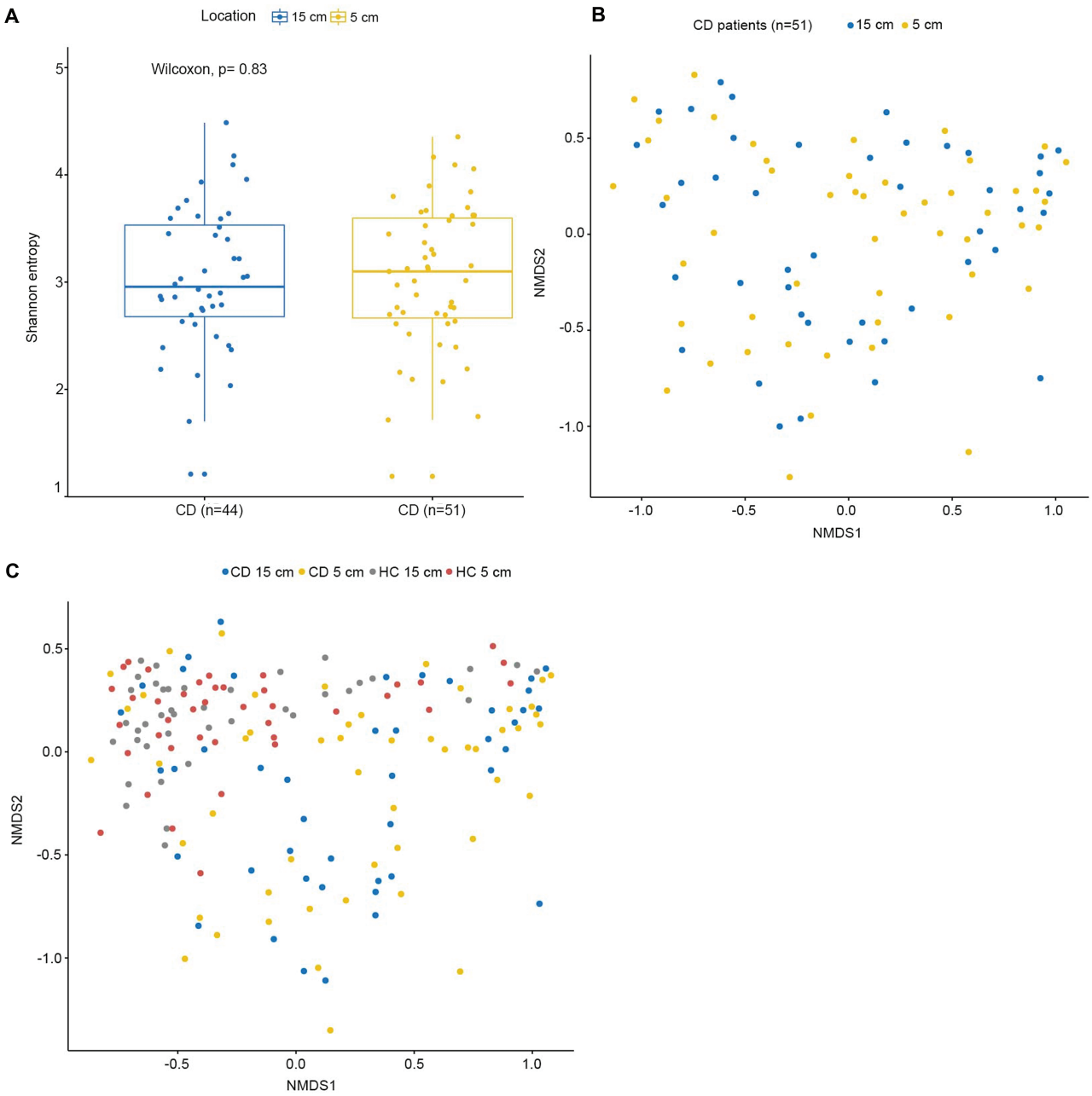


FIGURE 3. Ileal mucosa-associated bacterial microbiome at 5-cm and 15-cm location (proximal from the ileocecal valve or anastomosis respectively) in 51 Crohn's disease patients; biopsies not collected from 7 CD patients at 15-cm location due to ileal stenosis. A, Alpha diversity, illustrated by Shannon entropy index, at 5-cm and 15-cm location compared with Wilcoxon test. B, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot; each sample colored according to mucosal pinch biopsy location; 5 and 15 cm. C, Beta diversity in 51 CD patients and 40 healthy controls, illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot; each sample colored according to phenotype and location; CD 15 cm, CD 5 cm, HC 15 cm, and HC 5 cm.

histology (n = 32), with biopsies from locations that were noninflamed at endoscopy (n = 46) and with normal histology (n = 63). There was no significant difference in alpha diversity between biopsies from endoscopically inflamed vs noninflamed locations ($P = 0.54$; Fig. 5A). However, biopsies from locations with histological inflammation had significantly lower alpha diversity than locations where biopsies were normal ($P = 0.03$; Fig. 5B). There were no differences in beta diversity between inflamed and noninflamed locations, according to neither endoscopic nor histologic evaluation

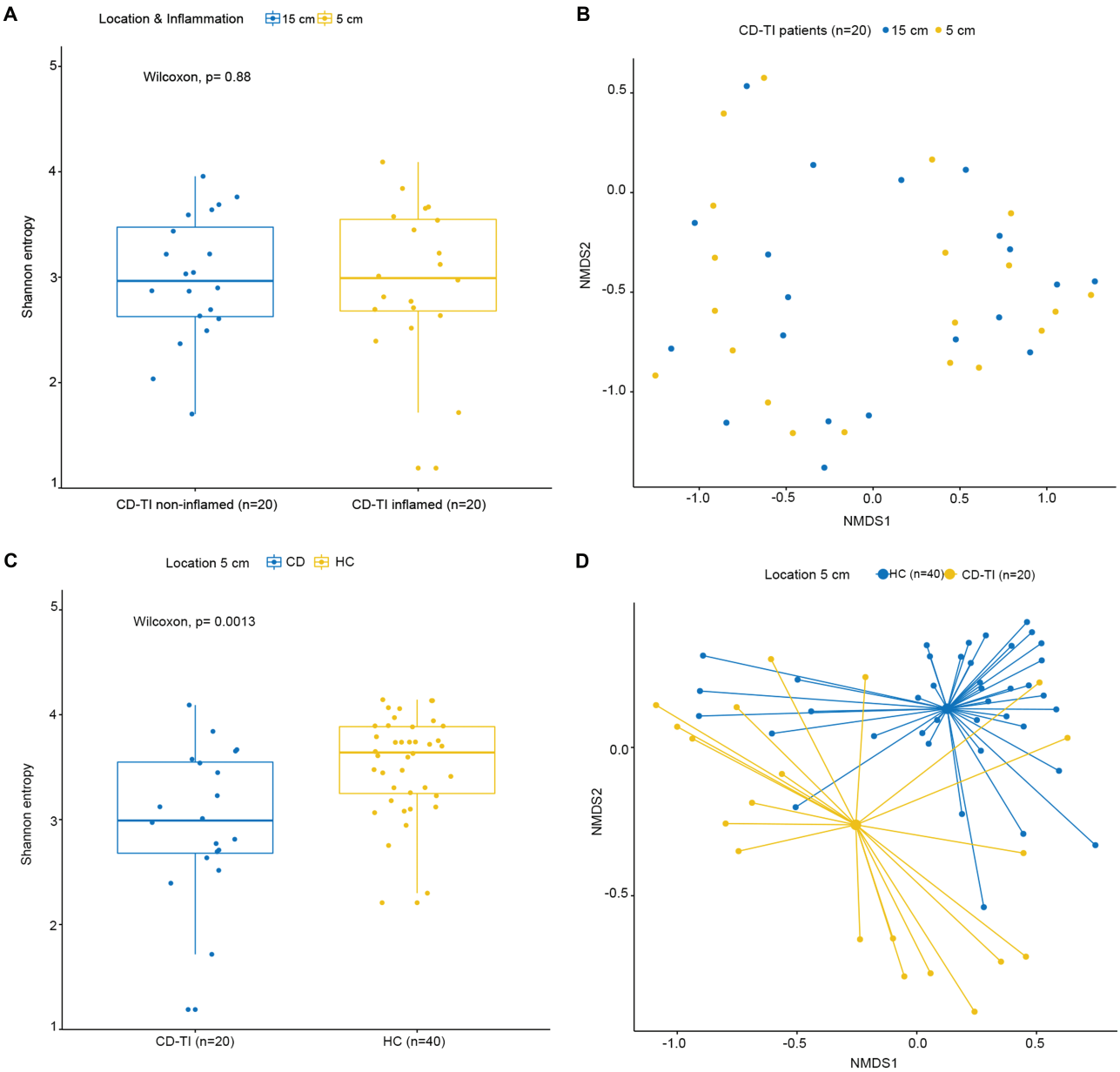


FIGURE 4. Ileal mucosa-associated bacterial microbiome in 20 Crohn's disease patients with terminal ileitis with endoscopic inflamed mucosa at 5 cm proximal from the ileocecal valve or anastomosis and normal endoscopic appearing mucosa at 15 cm proximal from the ileocecal valve or anastomosis. A, Alpha diversity, illustrated by Shannon entropy index according to location, compared with Wilcoxon test. B, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot, each sample colored according to mucosal pinch biopsy location; 5 and 15 cm. C, Alpha diversity, illustrated by Shannon entropy index, at 5-cm location in 20 CD-TI patients vs 40 healthy controls compared with Wilcoxon test. D, Beta diversity, illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling (NMDS) plot, in 5-cm samples from CD-TI patients and HCs; each sample colored according to phenotype; HC and CD-TI.

of inflammation (Fig. 5C and D). Similarly, differential expression analysis did not find any bacterial taxa on phylum, family, or genus level to be differentially expressed between CD patients with endoscopic inflammation and endoscopic remission.

## The Microbiome in Patients with Ileal Stenosis (CD-S)

Alpha diversity in CD-S patients was similar to that of other CD subgroups (Supplementary Fig. 2); however, on beta diversity NMDS plots, CD-S patients clustered furthest

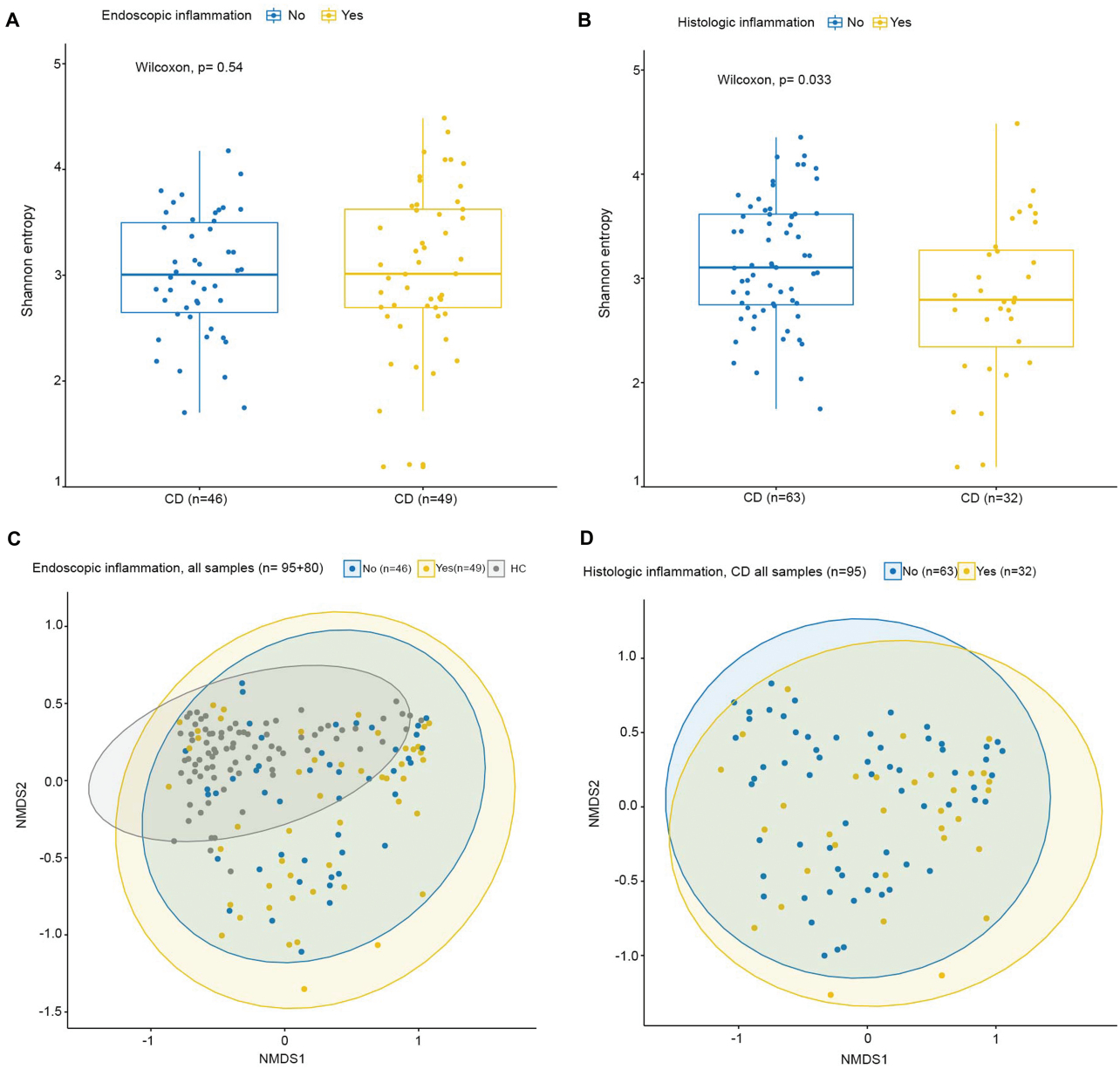


FIGURE 5. Ileal mucosa-associated bacterial microbiome in 95 mucosal pinch biopsy specimens (sampled from 5 and 15 cm proximal from the ileocecal valve or anastomosis) from 51 Crohn's disease patients according to endoscopic inflammation (Rutgeerts score ≥1) and histologic inflammation (GHAS and Robarts score ≥1) at biopsy sample location. A, Alpha diversity, illustrated by Shannon entropy index, according to endoscopic inflammation compared with Wilcoxon test. B, Alpha diversity, illustrated by Shannon entropy index, according to histologic inflammation compared with Wilcoxon test. C, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot in 95 mucosal pinch biopsies from 51 CD patients and 80 mucosal pinch biopsies from 40 healthy controls; each sample colored according to endoscopic inflammation (yes or no) or HC. D, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot in 95 mucosal pinch biopsies from 51 CD patients; each sample colored according to histologic inflammation (yes or no).

away from HCs compared with patients with terminal ileitis (CD-TI) or in remission (CD-R; Fig. 6). In a differential expression analysis identifying taxa that were differentially expressed between CD-TI patients and CD-S patients, we found a trend toward higher abundances of Akkermaniaceae family in CD-TI compared with CD-S patients ($P$ = 0.098). The same trend was found for Akkermansia genus, but neither was statistically significant. At species level, three species were significantly overrepresented in CD-TI patients in comparison with CD-S patients: *Bacteroides massiliensis B84634,* unidentified species of *Sutterella*, unidentified species of *Akkermansia*.

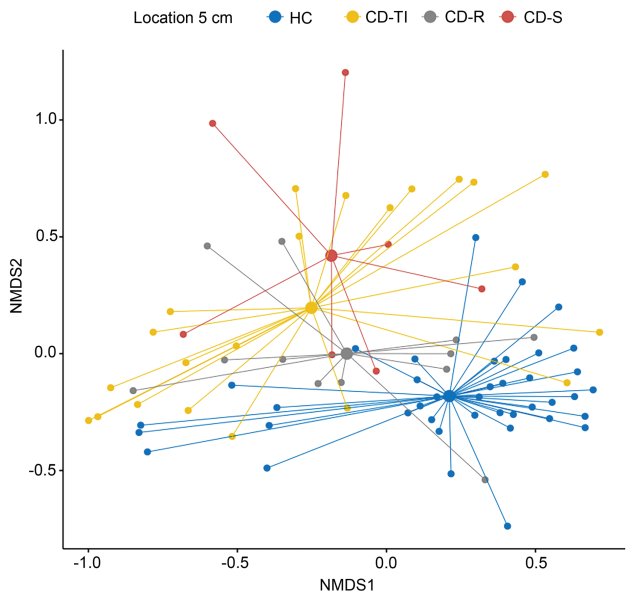


FIGURE 6. Ileal mucosa-associated bacterial microbiome composition in 40 healthy controls, 20 Crohn's disease patients with terminal ileitis and endoscopic inflammation, 12 CD patients in remission and endoscopic normal appearing mucosa, and in 7 CD patients with ileal stenosis. Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot; each biopsy sample colored according to phenotype; HC, CD-TI, CD-R and CD-S. Each study participant represented with 1 mucosal pinch biopsy sampled 5 cm proximal to the ileocecal valve or anastomosis, respectively.

## Effects of ICR on Ileal Mucosa-Associated Microbiome

In our cohort, 62.7% of CD patients had previously undergone ICR (n = 32). An overview of CD patients' surgical history is provided in Supplementary Table 2. When we analyzed samples according to ICR, we found lower alpha diversity in the ICR group ($P = 0.021$) compared with CD patients who had not undergone ICR (Supplementary Fig. 3A). However, bacterial composition did not seem to differ according to ICR status (Supplementary Fig. 3B). Furthermore, bacterial composition at 5 cm and 15 cm proximal to the anastomosis did not differ in patients having undergone ICR (Supplementary Fig. 3C). We performed a differential expression analysis comparing the microbiome of ICR patients in remission (n = 6) with ICR patients with disease recurrence (endoscopic inflammation, n = 26) and found increased abundances of *Parasutterella* genus to be associated with disease recurrence ($P = 6.8 \times 10^{-18}$).

## DISCUSSION

This is the first study to assess the mucosa-associated microbiota in the inflamed and proximal noninflamed ileum within the same patients. We did not find differences in alpha diversity or beta diversity when comparing inflamed with proximal noninflamed locations within the same patients. Furthermore, no bacterial taxa were differentially expressed in the inflamed vs proximally noninflamed mucosa. Our findings suggest that the altered ileal mucosa-associated microbiota in CD patients is present across locations and independent of inflammation itself at the biopsy location. In consistence, the beta diversity in mucosal biopsies from CD and ulcerative colitis (UC) patients pooled according to inflammation status, and intestinal location did not seem influenced by these factors.[29] Our findings are also supported by analyses of ileal biopsies from pediatric treatment-naïve IBD patients, where dysbiosis seemed to exist in absence of inflammation.[10] The present findings of an altered microbiome in CD patients also proximal to the upper border of inflammation suggests that the ileal mucosa-associated microbiota is altered regardless of inflammation status and location and contributes to delineate the role of bacteria in CD pathogenesis.

Crohn's disease–specific alterations in ileal mucosa–associated microbiota were confirmed in our cohort; CD patients had lower alpha diversity and separated clearly from HCs on beta diversity plots. Furthermore, we identified 23 bacterial taxa that were differentially represented in CD patients vs HCs. In accordance with previous reports, Proteobacteria phylum and Enterobacteriaceae family were increased, and Ruminococcaceae family and several genera from the Lachnospiraceae family were depleted in CD patients.[7, 10, 12, 13] Interestingly, we found *Tyzzerella 4* to be profoundly overrepresented in CD patients. At species level, this genus was identified as *Tyzzerella sp. Marseille-P3062*. Previously, *Tyzzerella 4* has been reported to be increased in a cohort of UC patients from China.[8] The literature describing *Tyzzerella 4* is very limited, but previous reports have found this genus to be increased in patients with a high-risk profile of cardiovascular disease and associated with an increased lifetime risk of cardiovascular disease.[30] *Tyzzerella 4* is also overrepresented in patients with a diet that was characterized as unhealthy by a healthy eating index (HEI).[31] Interestingly, numerous recent studies have identified IBD as a risk factor of cardiovascular disease.[32] Hypothetically, increased abundances of *Tyzzerella 4* could mediate this risk, but further research on CD patients with and without cardiovascular disease in addition to dietary patterns could clarify this. Two of the most decreased taxa in CD patients were the genera *Ruminiclostridium 5* and *Ruminiclostridium 6*. *Ruminiclostridium* has been described to be depleted in the mucosa of newly diagnosed and treatment-naïve CD patients,[33] and increased abundances of *Ruminiclostridium 6* seem protective with respect to endoscopic recurrence of CD after ICR.[20]

In analysis of all CD patients in the cohort, we confirmed that ileal sublocation did not impact microbiome diversity or composition, neither within CD patients nor within HCs. When we assessed the bacterial microbiome according to inflammatory variables, we found that biopsy samples evaluated as histologically inflamed had a lower alpha diversity in comparison with samples from CD patients that were histologically

normal, although alpha diversity in endoscopically inflamed biopsies vs endoscopically normal tissue was similar. Biopsies that were histologically inflamed were, in the majority of cases, also evaluated as endoscopically inflamed. This could suggest that alpha diversity is reduced in patients with severely inflamed ileal mucosa but not in modestly inflamed ileal mucosa, supported by Sokol et al[20] who found alpha diversity only to be reduced in patients with Rutgeerts score i2–i4 and not in patients with Rutgeerts score i0–i1. Ileal mucosa-associated microbiome composition did not differ between inflamed and noninflamed locations, neither according to histological nor endoscopic inflammation status.

Crohn's disease phenotype subgroups separated on beta diversity plots, and there was a trend toward reduced abundance of Akkermaniaceae family in CD-S patients compared with CD-TI patients. Although our cohort only contained seven patients with ileal stenosis (CD-TI), we assessed if there were differences in the mucosa-associated microbiota between CD-S patients and patients with terminal ileitis without stenosis (CD-TI). Our findings indicate that *Bacteroides massiliensis B84634* and unidentified species of *Sutterella* and *Akkermansia* are underrepresented in patients with stricturing CD. Previous research has found the abundance of *Akkermanisa muciniphila* to be correlated with time in remission in UC patients and increased in HC and UC patients in long-term remission.[34] Similarly, *Sutterella* abundance has been found to be inversely correlated to ileal-pouch inflammation.[35] Specific microbiome alterations in stricturing CD have been reported previously. In a prospective pediatric cohort study assessing the ileal mucosa-associated microbiome in patients before treatment, increased abundances of *Ruminococcus* was associated with development of stricturing disease.[36] Unique microbial profile in stricturing CD was also found in 2 data sets from separate cohorts.[37] In conclusion, our results suggest that patients with stenosing CD have a more profound loss of presumed beneficial bacteria compared with CD patients with inflammation but without stenosis. Although the current study has a cross-sectional design that prevents separation of primary and secondary alterations in microbial signatures, it is possible that stenosing disease behavior may be caused by specific bacteria and their products. Previous reports have found specific microbiome characteristics both at the time of ICR and postoperatively to be associated with postoperative recurrence.[6, 18–20] We found increased abundances of *Parasutterella* to be associated with disease recurrence after ICR in our cohort. *Parasutterella* genus belongs to the Gammaproteobacteria class (according to SILVA database but classified as a betaproteobacteria in GenBank, for example). Increased abundances of Gammaproteobacteria have previously been identified as a part of the microbial signature for postoperative recurrence.[20] However, it should be noted that in our patients, ICR was performed months to years ahead of study sampling.

Our study has some limitations. We have assessed the ileal mucosa-associated bacterial microbiome in a heterogeneous population of CD patients with longstanding disease, and the majority of patients had undergone ICR. Although we did not find that ICR impacted the ileal bacterial composition, it would be desirable to repeat the analyses on a cohort of newly diagnosed treatment-naïve patients. Even so, pediatric studies on treatment-naïve CD patients have found similar alterations in the mucosa-associated microbiota,[10, 14] arguing against medical treatment and disease duration as major contributors to the observed microbiome alterations in CD patients.

## CONCLUSION

In conclusion, this study demonstrates that the ileal mucosa-associated microbiota alterations in CD patients do not seem affected by inflammatory status or sublocation in the terminal ileum. The abundance of *Tyzzerella 4* is profoundly increased in CD patients, and depleted abundances of presumed favorable bacteria are found in CD patients with ileal stenosis.

## SUPPLEMENTARY DATA

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

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