Alterations of the Subgingival Microbiota in Pediatric Crohn’s Disease Studied Longitudinally in Discovery and Validation Cohorts

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Background: Oral manifestations are common in Crohn’s disease (CD). Here we characterized the subgingival microbiota in pediatric patients with CD initiating therapy and after 8 weeks to identify microbial community features associated with CD and therapy.

Methods: Pediatric patients with CD were recruited from The Children’s Hospital of Pennsylvania. Healthy control subjects were recruited from primary care or orthopedics clinic. Subgingival plaque samples were collected at initiation of therapy and after 8 weeks. Treatment exposures included 5-ASAs, immunomodulators, steroids, and infliximab. The microbiota was characterized by 16S rRNA gene sequencing. The study was repeated in separate discovery (35 CD, 43 healthy) and validation cohorts (43 CD, 31 healthy).

Results: Most subjects in both cohorts demonstrated clinical response after 8 weeks of therapy (discovery cohort 88%, validation cohort 79%). At week 0, both antibiotic exposure and disease state were associated with differences in bacterial community composition. Seventeen genera were identified in the discovery cohort as candidate biomarkers, of which 11 were confirmed in the validation cohort. Capnocytophaga, Rothia, and TM7 were more abundant in CD relative to healthy controls. Other bacteria were reduced in abundance with antibiotic exposure among CD subjects. CD-associated genera were not enriched compared with healthy controls after 8 weeks of therapy.

Conclusions: Subgingival microbial community structure differed with CD and antibiotic use. Results in the discovery cohort were replicated in a separate validation cohort. Several potentially pathogenic bacterial lineages were associated with CD but were not diminished in abundance by antibiotic treatment, suggesting targets for additional surveillance.

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Interactions between the host genome and environmental factors, notably the gut microbiota, are implicated in the pathogenesis of inflammatory bowel diseases (IBD). Multiple recent studies have demonstrated associations between the composition of the intestinal microbiota and IBD, but the microbiota of the oral mucosa in Crohn’s disease (CD) is less well characterized. Oral manifestations are observed in 5% to 80% of pediatric patients with CD.1–3 Pediatric patients with oral CD frequently develop manifestations before the onset of gastrointestinal symptoms, allowing earlier diagnosis.2 All areas of the oral cavity can be involved in CD, with the most commonly affected being the buccal, gingival, and labial mucosa. Manifestations include prototypical gingival lesions, aphthous lesions, edema of the mucosa, gingivitis, and periodontitis. Gingival CD, a common oral finding, is described

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as cobblestone-like gingival alterations and significant thickening of the tissue. These are similar to the intestinal findings of active CD, often associated with isolated edema of the mucosa and aphthous lesions. As with intestinal CD, oral lesions frequently respond to therapy with antibiotics such as metronidazole. Thus, we sought to determine if alterations of the subgingival microbiota are associated with CD and antibiotic therapy.

The structure of the oral microbiota has been previously associated with the development of periodontitis, gingivitis, and dental caries. The epithelial layer in the subgingival crevice is the initial interface between periodontopathic organisms and the host. The presence of bacteria in the subgingival environment can lead to the development of a biofilm, leading to an innate immune response associated with the production of cytokines and antimicrobial peptides, reminiscent of responses at the mucosal interface in the gut.

Two previous studies evaluated the oral microbiota and CD, showing decreased diversity in patients with CD compared with healthy controls and bacterial infections associated with chronic periodontitis. However, the reproducibility of these studies was not assessed, the effects of antibiotic use were not probed in detail, and longitudinal changes were not investigated.

In this study, we compared the subgingival oral microbiota in longitudinal samples from pediatric patients with CD and healthy controls to determine associations with the disease, relative disease activity, and specific CD phenotypes. We then confirmed our findings with a second validation cohort. Our data demonstrate that CD and antibiotic exposure are each associated with separate components of the oral microbiota.

**MATERIALS AND METHODS**

**Study Subjects**

The study was approved by The Children’s Hospital of Philadelphia Institutional Review Board (Protocol IRB 11-008043). Patients with CD and healthy controls were recruited from The Children’s Hospital of Philadelphia between July 2011 and April 2013. Subjects aged between 2 and 21 years were eligible for inclusion, a range spanning from early childhood to late adolescence as defined in NICHD pediatric terminology. Patients with CD were recruited from the gastroenterology inpatient and outpatient service. Healthy control subjects were recruited from primary care or orthopedics clinic. Subjects with a known history of medical or systemic disease, including medical conditions likely to influence nutrient intake, intestinal transit, or bowel health were excluded from the healthy control group. Demographic, dietary, and phenotypic data were collected on each subject enrolled. Subjects were asked to continue their routine, and all subjects had nothing to eat or drink for 30 minutes before oral sampling. Mouthwash use was recorded at the time of sampling. Sampling occurred once at the start of the study (week 0) and again following 8 weeks of therapy (week 8). The entire study was repeated using independent discovery and validation cohorts.

Disease activity was measured using the Pediatric Crohn’s Disease Activity Index (PCDAI) and fecal calprotectin (FCP). The PCDAI was measured at baseline and week 8. Moderate disease activity was defined as PCDAI ≥ 20. Clinical response was defined as a reduction in PCDAI from week 0 to week 8 by ≥12.5 points or PCDAI ≤10 at week 8. FCP concentration was measured (Genova Diagnostics) at week 0 and week 8 as a marker of intestinal mucosal inflammation in a subset of patients. For this study, moderate disease activity was defined as an FCP level of ≥250 μg/g. Response was defined as greater than 30% reduction in FCP level between week 0 and week 8. If FCP was missing at either week, an analysis for FCP response was not performed.

**Subgingival Sampling**

Subgingival plaque samples were collected at week 0 and week 8. The subgingival area was selected for sequencing because bacterial populations embedded in mucus and biofilms in this area are likely to be in more direct contact with the host immune system, compared with the saliva or other oral locations. Samples were collected from the subgingival space by means of a sterile microbrush and stored at −80°C. The oral mucosa was examined at the time of sampling for the presence of gingival disease and ulceration. No patient had evidence of periodontitis or gingival disease.

**Sample Processing and DNA Sequencing**

DNA was isolated from the microbrush using the PSP Spin Stool DNA Plus Kit (STRATEC Molecular, Berlin, Germany). The microbrush tips were cut directly into bead tubes containing 300 μL stool stabilizer, and bead beaten for 1 minute. For liquid samples, 1.4 mL was centrifuged at 13,400g for 10 minutes. Tubes were incubated at 70°C for 10 minutes. DNA was then extracted per manufacturer’s instructions and stored at −20°C. We amplified bacterial 16S rRNA genes using barcoded V1V2 primers and AccuPrime Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA). Products were pooled, purified, and pyrosequenced on a 454 Life Sciences FLX instrument (Roche Diagnostics Corp., Branford, CT).

Sequence data were processed using the QIME analytical pipeline (version 1.8). To assign reads to samples, we required an exact match to the sample-specific barcode and the PCR primer sequence. Reads were discarded if more than 1 base call was undetermined (N) or the expected PCR primer sequence was not found at the end of the read. Reads were assigned to operational taxonomic units (OTUs) in the Green genes database (version 13_8) at a threshold of 97% similarity using UCLUST (version 1.2.24q). Reads not matching to a 16S sequence in the reference database were removed from the analysis (2% of input).

Sequence data were deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRP057992 (discovery cohort data) and SRP058007 (validation cohort data).
RESULTS

Clinical Characteristics

The discovery cohort comprised 35 patients with CD and 46 healthy control subjects. The age range was 6 to 17 years in the CD group and 5 to 20 years in the healthy control group. The median age was 13 in both groups. Disease location in the CD group was predominately ileocolonic (80% of patients). The duration of disease ranged from 1 month to 6 years. Disease activity was moderate or severe at week 0 in 83% (PCDAI) and 82% (FCP) of patients with CD. Medications at week 0 included corticosteroids, immunomodulators (6-MP, methotrexate), enteral therapy, antibiotics, and 5-ASAs. Most patients in this cohort were recruited at week 0 of initiation of infliximab therapy. At week 8, the 30 patients scheduled to receive infliximab had completed the induction regimen and received 3 doses. At week 8, 88% and 82% of all patients with CD demonstrated response in PCDAI and FCP, respectively, reflecting improvement in disease status.

For the validation cohort, 44 patients with CD and 31 healthy control subjects were recruited. The age ranged from 2 to 18 years in the CD group and 3 to 21 years in the healthy control group. The median age was 13 years for both groups. The duration of disease ranged from 1 week to 6 months. Disease activity was quantified primarily by PCDAI and was moderate or severe in 83% of patients with CD at week 0. FCP levels were available for only 15 patients in the validation cohort, and no patient was measured at both week 0 and week 8. Medications at week 0 included 5-ASAs, antibiotics, immunomodulators, corticosteroids, and nutritional therapy. Patients had no previous biological therapy (including infliximab) exposure at least 6 months before initiation of therapy and again after 8 weeks. Patients with CD were sampled before initiation of therapy and again after 8 weeks. CD subjects currently receiving antibiotics (CD Abx+) are summarized separately from other patients with CD (CD Abx−). Healthy control subjects (Control Abx−) were sampled at the same time interval and were not exposed to antibiotics during the study or 2 months previously. The study was repeated using separate (A) discovery and (B) validation cohorts.

Sequences from subgingival brushing samples. Patients with CD were sampled before initiation of therapy and again after 8 weeks. CD subjects currently receiving antibiotics (CD Abx+) are summarized separately from other patients with CD (CD Abx−). Healthy control subjects (Control Abx−) were sampled at the same time interval and were not exposed to antibiotics during the study or 2 months previously. The study was repeated using separate (A) discovery and (B) validation cohorts.

Several characteristics of the discovery cohort differed from those of the validation cohort (see Table, Supplemental Digital Content 1, http://links.lww.com/IBD/B56, which lists details for both study cohorts). The validation cohort contained substantially fewer patients initiating therapy with infliximab relative to the discovery cohort (13/43 versus 30/35 patients, respectively). The proportion of patients with moderate or severe disease activity was lower in the validation cohort. Despite these differences, the 2 cohorts are representative of clinical response across a wide range of ages and therapies.

Sequencing Results

A total of 322,000 reads were obtained for samples in the discovery cohort, and 304,000 for the validation cohort. Samples having fewer than 200 reads were removed from the analysis. The median number of reads per sample was 1506 for the discovery cohort and 1377 for the validation cohort. Taxonomic assignment of sequence reads revealed a bacterial community composition similar to that reported previously for subgingival plaque (see Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/B57, which contains heatmaps of bacterial taxa observed). Five phyla were prominent in the data set: Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, and Proteobacteria (Fig. 1).
A Combined Model of Bacterial Community Composition Reveals Independent Signatures of CD and Antibiotic Use

We first compared the subgingival microbial community structure in CD with that of healthy controls at week 0. Figure 2 presents ordinations of pairwise UniFrac distances for the discovery and validation cohorts. To assess the magnitude and significance of differences in UniFrac distance between groups, we built a combined model that fit the effects of antibiotic use and disease state.

We found that current antibiotic use produced substantial alterations in the bacterial community structure of the discovery cohort (unweighted UniFrac $P < 0.001$, weighted UniFrac $P = 0.02$). This observation was confirmed in data from the validation cohort (unweighted UniFrac $P < 0.001$, weighted UniFrac $P < 0.001$).

Patients in the discovery cohort showed differences associated with CD in both unweighted ($P = 0.025$) and weighted UniFrac distance ($P = 0.036$), although the size of the effect was roughly half that of antibiotic use. For the validation cohort, we observed a difference in unweighted UniFrac distance associated with disease state ($P = 0.018$), but only a trend in the weighted analysis ($P = 0.09$). The estimated effect size for unweighted UniFrac distance was similar in the discovery and validation cohorts ($R^2 = 0.027$ and 0.025, respectively).

We next sought to determine the specific bacterial lineages responsible for the differences in community composition. Using a Kruskal–Wallis test, we identified 17 candidate genera in the discovery cohort that contributed to the difference in the subgingival microbiota between healthy controls, CD without current antibiotics, and CD with current antibiotics (Fig. 3A). We also found that the abundance of the TM7 phylum differed between the 3 groups. Because TM7 appeared in low abundance (about 1% of total) and because the phylum contained no genera in the Greengenes taxonomy, we chose to include it in the list of candidate genera.

Eleven of the 17 candidate genera were confirmed in the validation cohort (Fig. 3B). We again used the Kruskal–Wallis test to detect differences between healthy controls, CD subjects not on antibiotics, and CD subjects receiving antibiotics. The confirmed genera were *Alloprevotella* ($P = 0.003$),

![Figure 2](image_url)

**FIGURE 2.** Analysis of bacterial community composition in the discovery and validation cohorts. Communities were compared to determine differential membership using unweighted UniFrac distance (A and B) or community proportional abundance using weighted UniFrac distance (C and D). Samples were ordinated using principal coordinate analysis. Open triangles represent the group centroid position for healthy control subjects (blue), patients with CD not on antibiotics (red), and patients with CD on antibiotics (green). The PERMANOVA test was used to assess association of sample–sample distance with disease state (CD) and current antibiotic usage (Abx).
Campylobacter ($P = 0.02$), Capnocytophaga ($P = 0.01$), Catonella ($P = 0.02$), Fusobacterium ($P = 0.003$), Porphyromonas ($P = 0.003$), Prevotella ($P = 0.006$), Rothia ($P = 0.003$), Selenomonas ($P = 0.01$), TM7 ($P = 0.02$), and Veillonella ($P = 0.005$).

In the validation cohort, we found that Capnocytophaga ($P = 0.001$), Rothia ($P = 0.001$), and TM7 ($P = 0.004$) were significantly different between healthy controls and CD subjects not on antibiotics (Mann–Whitney test; see Table, Supplemental Digital Content 3, http://links.lww.com/IBD/B58, which lists test results for all comparisons at week 0). All were increased in the CD group relative to healthy controls. We performed the same comparisons on the discovery cohort data and obtained identical results. Thus, we observed reproducible differences in the subgingival microbiota associated with pediatric CD.

We returned to the validation cohort to make follow-up comparisons of genus abundance between subjects in the CD group receiving antibiotics and CD subjects not on antibiotics. Patients on antibiotics had a decreased proportion of Alloprevotella ($P = 0.002$), Campylobacter ($P = 0.005$), Catonella ($P = 0.02$), Fusobacterium ($P < 0.001$), Porphyromonas ($P = 0.003$), Prevotella ($P = 0.002$), Selenomonas ($P = 0.003$), and Veillonella ($P = 0.005$). The antibiotics used were Ciprofloxacin, Flagyl, and Vancomycin, which have broad specificities that include the above lineages. The taxonomic alterations associated with antibiotic use here resemble some of those previously described in the oral microbiota following therapy for periodontitis.7,8 The antibiotic-associated differences in genus abundance were also observed in the discovery cohort. Thus, we conclude that antibiotics affected a separate set of genera, apart from those associated with CD.

We wondered whether the changes associated with antibiotic use might be attributable to loss of species within the identified genera. Rarefaction analysis (Fig. 4) showed that community richness was lower in CD subjects treated with antibiotics relative to healthy controls and obtained identical results. Thus, we observed reproducible differences in the subgingival microbiota associated with pediatric CD.

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![Figure 3](https://example.com/fig3.png)
Disease Activity Is Not Associated with Alteration of the Subgingival Microbiota

We then analyzed samples to determine whether community structure or specific taxa correlated with the level of disease activity. At week 0 in the discovery cohort, 83% of subjects had active disease, based on PCDAI value. We observed no association between community composition and PCDAI value (unweighted UniFrac \( P = 0.46 \), weighted UniFrac \( P = 0.46 \)). FCP level was likewise not correlated with pairwise UniFrac distance in the discovery cohort (unweighted UniFrac \( P = 0.55 \), weighted UniFrac \( P = 0.26 \)). No differences were found in particular subgingival bacterial lineages correlating with measures of disease activity in the discovery cohort.

In the validation cohort at week 0, 49% of subjects had active disease based on PCDAI value. As in the discovery cohort, bacterial community composition was not associated with PCDAI (unweighted UniFrac \( P = 0.18 \), weighted UniFrac \( P = 0.26 \)). Evidently, disease activity at week 0 was not strongly correlated with subgingival community structure.

Differences in Microbial Community Structure Associated with CD Resolve After 8 Weeks of Therapy

We next asked whether the subgingival microbiota changed following effective treatment. Bacterial community composition was analyzed by comparing pairwise UniFrac distances in the set of samples obtained at week 8 (see Fig., Supplemental Digital Content 4, http://links.lww.com/IBD/B59). Differences between CD and healthy control samples observed at baseline were not detected at week 8 (unweighted UniFrac \( P = 0.19 \), weighted UniFrac \( P = 0.28 \) for discovery cohort; unweighted UniFrac \( P = 0.18 \), weighted UniFrac \( P = 0.28 \) for validation). Conversely, differences due to antibiotic use persisted at week 8 (\( P \leq 0.001 \), weighted and unweighted UniFrac in both cohorts).

Analysis of confirmed genera from week 0 reinforced the conclusions from the community-level analysis (see Table, Supplemental Digital Content 5, http://links.lww.com/IBD/B60, and Fig., Supplemental Digital Content 6, http://links.lww.com/IBD/B61). Four genera that were reduced with antibiotic use at week 0 also decreased at week 8 for both cohorts: Alloprevotella (discovery cohort \( P = 0.03 \), validation cohort \( P = 0.04 \)), Fusobacterium (\( P = 0.02 \) and \( P < 0.001 \)), Porphyromonas (\( P = 0.01 \) and \( P = 0.001 \)), and Prevotella (\( P = 0.007 \) and \( P = 0.009 \)). Other antibiotic-associated genera were not found to be different in both cohorts. The taxa associated with disease state at week 0 did not differ between healthy controls and CD subjects in either the discovery or validation cohort at week 8 (\( P = 0.2 \) and \( P = 0.5 \), Rothia \( P = 0.3 \) and \( P = 0.2 \), TM7 \( P = 0.4 \) and \( P = 0.5 \) in the discovery and validation cohorts, respectively). Thus, we conclude that 8 weeks of therapy was sufficient to resolve...
observed differences in the subgingival microbiota associated with CD.

A Machine Learning Approach Identifies a Characteristic Signature of CD in Pediatric Subgingival Samples

Given that a distinctive signal was seen in week 0 samples associated with CD, we asked whether we could identify a predictive microbial signature potentially of use in molecular diagnosis. We used the random forest method to create a predictive classifier that sorted CD subjects from healthy controls based solely on data from the discovery cohort.24 Because of the relatively strong antibiotics effect, we excluded CD subjects taking antibiotics at week 0 from the random forest analysis. Using the discovery cohort data, we were able to make a classifier that sorted CD samples from healthy control samples with 27% error. The classifier was then applied to the validation cohort (Fig. 5A), where 83% of the healthy samples were classified correctly. However, only 15 of the 34 total CD samples were correctly identified, indicating that random forest classification is relatively specific but not sensitive at a probability threshold of 0.5.

Classification sensitivity can be improved if more false positives are acceptable. To evaluate the performance of the classifier at all threshold values, we constructed a receiver operating characteristic curve (Fig. 5B). The area under the curve was 0.8, meaning that 80% of the time a randomly selected CD sample will have a higher probability for CD assignment than a randomly selected healthy sample.

The random forest classifier placed high importance on OTUs from *Capnocytophaga*, *Rothia*, and TM7, taxa identified as differentially abundant between the CD and healthy groups at week 0. Figure 5C lists the 20 most important OTUs as determined by a mean decrease in accuracy for the classifier (out-of-bag estimate). Two *Capnocytophaga* OTUs were ranked highest in importance for the classifier, the first of which was found to be increased more than 10-fold in the CD group. Other OTUs had a modest difference between groups but were used collectively by the classifier to improve accuracy. Thus, the random forest signature provides a molecular marker for discriminating CD and healthy controls.

**DISCUSSION**

Here we report that the composition of the subgingival oral microbiota in active CD differs from healthy control subjects. The CD signature was seen in the absence of gingival disease, and was replicated in a validation cohort, demonstrating the reproducibility of these findings. The CD signature was not present in samples obtained after 8 weeks of the study, suggesting that the CD community structure was altered in association with successful therapy.

We identified 3 taxa that were enriched in the CD group at the initial time point: *Capnocytophaga*, *Rothia*, and TM7. Although these taxa are part of the normal subgingival flora, they have each been implicated in the disease and provide a basis for further investigation as environmental mediators of CD pathogenesis. We found that 8 weeks of therapy were sufficient to return these taxa to healthy levels. These 3 taxa were not among those affected by antibiotic use.

*Capnocytophaga*, a facultative anaerobic Gram-negative bacillus, is part of the commensal oral flora, but is enriched in subgingival plaque relative to other oral surfaces.25 It is an opportunistic pathogen, and is associated with periodontal disease, particularly in immunocompromised patients.26,27 It is most often detected in the setting of mucosal ulcerations, gingivitis, and bleeding gums. It has been shown to lead to systemic infection in immunocompromised patients, particularly children, presenting...
with oral ulcers and decreased granulocytes. Additionally, it has been detected in higher abundance in patients with type 1 diabetes mellitus.

The genus Rothia is also a member of the commensal subgingival flora. Although early studies found it to be enriched in periodontitis subjects, it has been associated with periodontal health in several deep sequencing studies. Rothia species may have important interactions with the host immune system that give rise to a role in inflammatory diseases such as CD. The type species, Rothia dentocariosa, was previously shown to increase TNF-α production in vitro, and thus may act as an agent for increased inflammation in the oral cavity. More importantly, Rothia may be an opportunistic pathogen in subjects with impaired immune function. Recently, R. dentocariosa was implicated in a case of bacteremia in a subject receiving infliximab, the same therapy used in this study.

The TM7 division was previously associated with CD in samples of colonic mucosa. The diversity of TM7 organisms in colonic biopsies, many showing >99% 16S similarity to oral clones, was increased in a previous study of adult subjects with CD. In the same study, the diversity of TM7 bacteria was not increased in subjects with ulcerative colitis, indicating an involvement with CD specifically. In the oral cavity, a number of studies have found associations between TM7 bacteria and periodontitis. Because the organisms have never been grown in culture, mechanisms for involvement in inflammatory disease remain unclear. However, assembly of a TM7 genome from metagenomic data revealed some potential virulence factors in 1 study.

We found that Alloprevotella, Fusobacterium, Porphyromonas, and Prevotella were consistently decreased in the antibiotic-exposed CD group, compared with CD subjects not using antibiotics. The measurement was repeated at 2 time points in each of the 2 independent study cohorts. Previous studies have documented similar microbial changes following antibiotic therapy in periodontitis. Species from all 4 genera, Alloprevotella, Fusobacterium, Porphyromonas, and Prevotella, were previously reported to be less prevalent in subjects responding to antibiotic therapy for periodontitis. Here, we report parallel changes in the patients with IBD exposed to antibiotics. The same study reported increased prevalence of the CD-associated genera identified here, Capnocytophaga and Rothia. Although these groups were not increased in association with antibiotic use in our data, the published data suggest the possibility that antibiotic exposure promoted colonization by these CD-associated organisms.

In summary, we document extensive alterations of the subgingival microbiota associated with CD and antibiotic use. These findings and those of others provide a tractable model for studies of microbiota interactions at the subgingival mucosal surface, where sampling is simpler than for intestinal sites. This accessibility was leveraged here to allow acquisition of separate discovery and validation cohorts, providing much more secure conclusions than is possible in studies of single cohorts in isolation. The lineages consistently enriched in abundance provide specific candidates for new rounds of studies of mechanisms of disease and antibiotic therapies. The random forest analysis provides a diagnostic signature, allowing potential discrimination of CD and healthy controls based on convenient oral sampling. In future work, it will be of interest to determine whether such signatures can be sharpened and used to partition patients into groups that are relatively more responsive to specific IBD therapies.

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