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The rectal mucosal but not fecal microbiota detects subclinical ulcerative colitis

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ABSTRACT

Ulcerative colitis (UC), a subtype of inflammatory bowel disease, is characterized by repetitive remission and relapse. Gut microbiome is critically involved in pathogenesis of UC. The shifts in microbiome profile during disease remission remain under-investigated. Recent studies revealed that UC pathogenesis is likely to originate in the mucosal barrier. Therefore, we investigated the effectiveness of mucosal tissue microbiomes to differentiate patients with subclinical UC from healthy individuals. The microbiomes of cecal and rectal biopsies and feces were characterized from 13 healthy individuals and 45 patients with subclinical UC. Total genomic DNA was extracted from the samples, and their microbial communities determined using next-generation sequencing. We found that changes in relative abundance of subclinical UC were marked by a decrease in Proteobacteria and an increase in Bacteroidetes phyla in microbiome derived from rectal tissues but not cecal tissue nor feces. Only in the microbiome of rectal tissue had significantly higher community richness and evenness in subclinical UC patients than controls. Twenty-seven operational taxonomic units were enriched in subclinical UC cohort with majority of the taxa from the Firmicutes phylum. Inference of putative microbial functional pathways from rectal biopsy microbiome suggested a differential increase in interleukin-17 signaling and T-helper cell differentiation pathways. Rectal biopsy tissue was suggested to be more suitable than fecal samples for microbiome assays to distinguish patients with subclinical UC from healthy adults. Assessment of the rectal biopsy microbiome may offer clinical insight into UC disease progression and predict relapse of the diseases.

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Introduction

Ulcerative colitis (UC), a subtype of inflammatory bowel disease (IBD), is a debilitating illness that can cause morbidity and mortality if left unmanaged. The disease is commonly associated with a westernized lifestyle, and the majority of IBD cases are reported in Northern Europe and North America.[1] However, UC incidence has risen in the Asia-Pacific region over the past two decades, and this has been associated with rapid urbanization.[2] Southeast Asian countries are already densely populated and continue to expand, giving UC the potential to impact a substantial demographic of their people.[3]

The impact of UC extends beyond the initial onset. It has been reported that remission patients have a relapse rate of 38%–76%.[4] Early diagnosis of relapse for early treatment is important for positive clinical outcomes. However, early prediction of disease relapse remains a challenge even by using the conventional approaches, which include endoscopic and histological examinations, radiology, and serological profiling.[5] The emergence of high-throughput amplicon sequencing opened a new field of research, with potential implications for diagnosing UC. Characterization of the fecal microbiome has been the focal point for most UC microbiome studies, as fecal samples are non-invasive and easy to collect. However, the fecal microbiome is not sensitive enough to detect gut dysbiosis in the early stages of the disease.[6] A comprehensive study by Schirmer et al. have documented temporal changes of gut microbiome in children,[7] but little is known about how microbiomes shift in the early or remission

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stages of UC for adults. Mucosal biopsy was first documented to effectively diagnose UC in the early 1960s.[8] An increasing body of evidence suggests that UC pathogenesis starts in the colonic mucus barrier.[9,10] Accordingly, we hypothesized that the mucosal microbiome is a good candidate for establishing a baseline microbial profile for dysbiosis comparison with subclinical UC patients. We thus set forth to determine whether mucosal tissue microbiomes can effectively diagnose subclinical UC.

Results

Characterization of fecal- and mucosa-associated microbiomes in subclinical UC patients

Cecal and rectal biopsies and fecal samples were collected from 58 volunteers —45 UC remission patients with subclinical symptoms (Mayo Score ≤ 5) and 13 healthy individuals scheduled for routine health examination (hereinafter "control"). Clinical parameters of the patients are outlined in Table 1. In total, microbiomes of 174 samples were characterized by sequencing the V3-V4 region of 16S rRNA. A total of 8,964,585 sequence reads from all samples were assigned to 768 different operational taxonomic units (OTUs). On average, each sample had 27,083 reads, which were clustered into 92 OTUs, with 96.2% successfully assigned at the genus level. Rarefaction analysis of samples suggested that the majority of the microbial diversity was captured around 15,000 reads (Figure S1). Samples with fewer than 15,000 reads were omitted, resulting in a total of 134 samples used in the final analysis (n = 40, 36, and 58 for cecal biopsy, rectal biopsy, and fecal, respectively). The three major gut bacterial phyla – Proteobacteria, Bacteroidetes, and Firmicutes – contributed on average 16.7%–35.0%, 42.5%–55.4%, and 25.0%–29.0% of relative abundance across all 134 samples, respectively. Large inter-individual variations were observed (Figure 1(a)). Compared to control, mucosa-associated microbiome (MAM) in subclinical UC patients exhibited lower relative abundance in Proteobacteria and higher in Bacteroidetes and Firmicutes. In contrast, the relative abundances of Proteobacteria and Bacteroidetes in the fecal-associated microbiome (FAM) shifted in the opposite direction to that of MAM (Figure 1(b)). However, only major phyla of rectal MAM were found significantly different between subclinical UC and control groups (ANOVA; p = 0.000, 0.000, 0.030 for Proteobacteria, Bacteroidetes, and Firmicutes, respectively).

Table 1. Patient clinical parameters.

	Subclinical UC (n = 45)	Healthy (n = 13)
Age (yr, mean ± SD)	46.9 ± 10.8	46.5 ± 13.3
Gender		
Male	31	6
Female	14	7
Mayo Score		
0–2	30	NA
Cecal endoscopic grade (2/1/0)	1/3/26	NA
Rectal endoscopic grade (2/1/0)	3/20/7	NA
3–5	15	NA
Cecal endoscopic grade (2/1/0)	1/1/13	NA
Rectal endoscopic grade (2/1)	13/2	NA
Therapy (with/without)		
ASA Rx	38/7	NA
Sulfasalazine	2/43	NA
Steroid	6/37	NA
Diet control (with/without)	30/15	0/13
Clinical complications[a]		
Mayo Score > 2	19	NA
Severe relapse/Admission	0	NA
Mortality	0	NA
Samples with sufficient reads[b]		
Cecum biopsy	32	8
Rectal biopsy	27	9
Feces	45	13

n: case numbers; UC: ulcerative colitis; ASA Rx: aminosalicylic acid prescriptions.
[a]Monitoring period of 4 years; [b]Samples containing a minimum of 15,000 reads

Patients with subclinical UC exhibit significant different MAM and FAM

Relationships between sample types and cohorts were examined by identifying shared OTU using samples originated from the same patients (n = 6 and 23 triplets in control and subclinical UC, respectively). The majority of microbiomes were represented by 139 OTUs (Figure 2(a)) shared across all samples regardless of site and cohort (87%–97.5% relative abundance; Figure 2(b)). Of these OTUs, significant difference in major phyla relative abundance between mucosal samples of control and subclinical UC cohort (Figure 2(b), ANOVA; Bacteroidetes p = 0.005; Firmicutes p = 0.047, Proteobacteria p = 0.000 for rectal samples). We observed some OTUs in subclinical UC cohort exhibited a different dynamic (relative abundance of cecal – rectal – fecal) to controls (OTU1, OTU7, OTU8, and

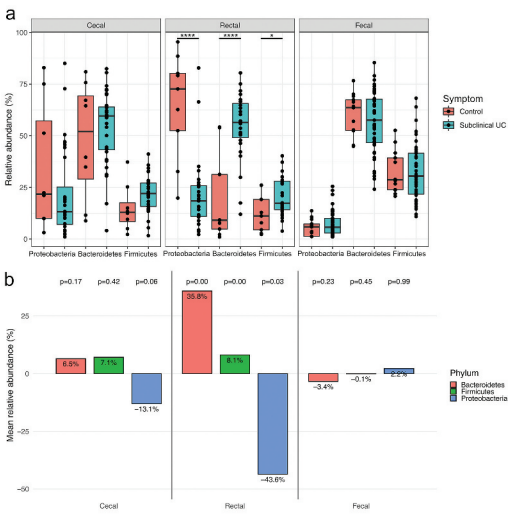

Figure 1. The relative abundances of the three major phyla in tissue and fecal microbiomes. (a) Percentage relative abundance of the three major phyla of biopsy and fecal samples between the control and subclinical UC cohorts. Boxplots show the interquartile ranges and medium relative abundances. Dots represent sample outliers. Significant differences between cohorts are labeled with asterisks (* $p < 0.05$; **** $p < 0.0001$). (b) Percentage change in mean relative abundance in the subclinical UC cohort relative to the control.

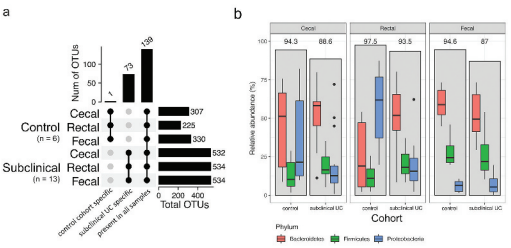

Figure 2. (a) Shared OTUs in different cohorts for patient with triplet samples. Total number of OTUs for each cohort are shown by bar plot on the right. Top panel bar plot represents the number of OTUs found among the cohorts, which is indicated by the connected dots. (b) Relative abundance of the 139 shared OTU in the 29 patients. Number on the top of bar chart indicate the total percentage abundance represented by the shared OTUs. Box plot within the bar chart showed the relative abundance of major phyla represented by these OTUs.

OTU13; Figure S2). In other cases where the abundance change dynamics were similar between the two cohorts, the variation in abundance level among each sample type in subclinical UC was greater compared to the control cohort (OTU10, OTU11, and OTU15; Figure S2). Additionally, we identified one and 73 specific OTUs found only in all control and subclinical cohort, respectively (Figure 2(a)). These OTUs exhibited 0.0018%–0.016% and 0.9%–3.2% relative abundance in their respective cohort.

Rectal mucosa-associated microbiome differentiates subclinical UC patients from controls

The community richness (Chao1) and evenness (Shannon) analyses indicated that subclinical UC showed a significantly higher richness in rectal MAM than the controls (p value = 0.009, Figure 3), which were observed in neither cecal MAM nor fecal microbiome. Shannon indices also showed higher diversity in rectal MAM, albeit without statistical significance (p value = 0.13). Unexpectedly, the community richness of FAM was similar in both cohorts. Notably, previous studies on gut inflammation have suggested that UC is usually associated with a decrease in gut microbial diversity, which were based on fecal microbiota only.[11–13]

Data in the beta diversity ordination separated along Axis1 and explained 47.7% of the variation among all samples. The microbial community profiles were not clustered based on sample type, as MAM overlapped with FAM (Figure 4(a)). However, PERMANOVA analysis suggested significant differences between sample type and disease status (p value = 0.03, FAM vs. MAM; p value = 0.001, control vs. subclinical UC). Ordination analysis for each sample type revealed significant differences in community profile of subclinical UC and control in rectal MAM (adjusted p value = 0.015, Figure 4(b)), whereas the diversity was indistinguishable for FAM and cecal MAM in our study (adjusted p value = 1.0). Inter-individual variation was minimized by comparing sequencing results from patients with all three sample types (n = 29, Figure 4(c)). The clustering pattern in Figure 4(c) was similar to that of Figure 4(b). The cecal MAM had better, but not significant, separation between the control and subclinical UC (p value = 0.405, Figure 4(c)). In conclusion, our findings

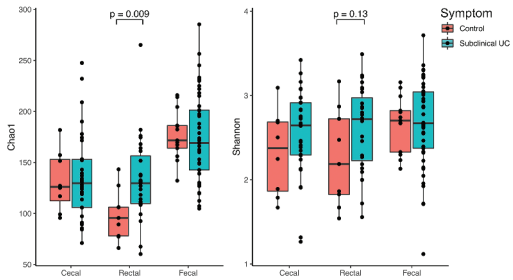

Figure 3. Shannon and Chao1 diversity indices of all sequenced samples. Alpha diversity variation of different disease statuses within sample type. Dots represent sample outliers. p values were determined using ANOVA and post-hoc test Tukey's HSD.

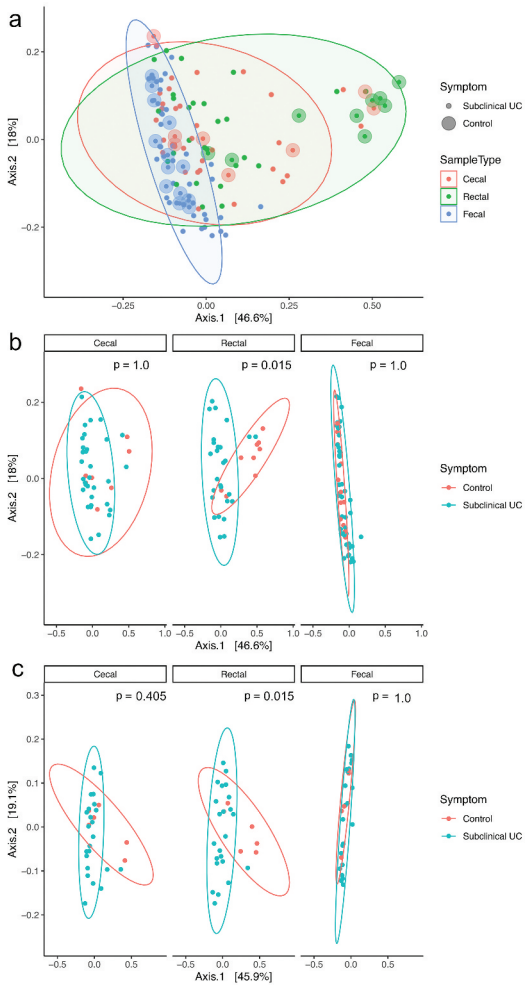

Figure 4. Ordination of samples using principal coordinate analysis with weighted UniFrac distance matrix. (a) The plot contains all sample points; the color and size of data points indicate different sample types and patient cohorts, respectively. (b) Sample plot as (a), but separated by sample types. (c) Ordination plot containing data points where all three sample types were collected from the same patients.

indicated that rectal MAM was the best indicator of changes in microbial community between control and subclinical UC patients.

OTUs associated with UC dysbiosis in rectal biopsy

We searched for the microbial biomarkers that were potentially able to diagnose and predict subclinical UC dysbiosis. A total of 27 OTUs were found to be differentially abundant in the rectal MAM of subclinical UC patients ($n = 27$) compared to control ($n = 9$) (Table 2). Otu1-*Bacteroides* and Otu7-*Prevotella* showed significant increases in mean relative abundance (Figure 5; Otu1: 7.1% vs. 18.4% adjusted p value = 0.031, Otu7: 2.0% vs. 15.2%, adjusted p value = 0.031; control vs. subclinical UC) and contributed to the huge rise in Bacteroidetes observed in Figure 1. Significant changes in abundance were not found for fecal nor cecal samples in the same patients (Figure 5). *Clostridium XIVa* had been suggested to act as a beneficial microbe to protect the intestinal lumen.[14] In our study, the Otu149-*Clostridium XIVa* is one of the 27 differentially abundant OTUs in Table 2. However, high abundance of Otu149 is correlated with rectal MAM of subclinical UC cohort instead of the control (Figure S3).

The subclinical UC patient were continuously monitored for four years. During the monitoring period, none of them had an overt recurrence (Mayo score >5), but twelve had at least one episode with Mayo score > 2. These patients with mild clinical recurrence were categorized as the subgroup of high risk ($n = 15$) of recurrence compared to those without recurrence (low risk, $n = 12$). We identified only one differentially abundant OTU of *Megasphera* genus (OTU22) between high and low risk of recurrence for UC (low-risk cohort 0.024 ± 0.082%; high-risk cohort 0.274 ± 0.724%; adjusted $p = 0.001$).

Functional prediction of KEGG pathway associated with UC

Gut microbial functional pathways were predicted by inferring KEGG orthologues from OTUs present in each sample. The results were filtered for orthologues with at least a two-fold difference in relative abundance and p value < 0.05. A total of 38 differentially-abundant KEGG terms were identified (Figure 6). Homeostasis luminal short-chain fatty acid level was shown to be critical for healthy gut epithelial barrier function.[15] In the control cohort, the number of pathways related to the degradation of bile acid precursor and sterol compounds were founded to be more (ko00984-steroid, ko00623-toluene, and ko00930-caprolactam degradation). This indirectly suggests a below-normal bile acid biosynthesis in the subclinical UC cohort. The absorption of dietary lipids, much aided by bile acid, allows short-chain fatty acid biosynthesis to occur. On the other hand, the type of pathways increased in subclinical UC cohort suggested signs

Table 2. Significantly abundant OTUs in the rectal MAM of subclinical UC patients.

	Taxonomic information						Rectal control*n* = 9		Rectal subclinical*n* = 27		Subclinical vs control	
OTU	Domain	Phylum	Class	Order	Family	Genus	Mean	SD	Mean	SD	Adjusted p	Abundance in subclincal
Otu1	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	7.169	9.424	18.362	16.532	0.031	Up
Otu7	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	2.025	3.015	15.197	20.78	0.031	Up
Otu13	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	1.138	1.873	4.324	3.448	0.045	Up
Otu21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	0.322	0.449	1.225	2.024	0.047	Up
Otu25	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	0.211	0.291	1.204	1.499	0.005	Up
Otu24	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	0.059	0.176	0.942	2.793	0.012	Up
Otu26	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae	0.064	0.072	0.648	1.156	0.015	Up
Otu57	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	0.227	0.68	0.535	1.939	0	Up
Otu110	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Barnesiella	0	0	0.337	1.039	0	Up
Otu54	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	0	0.001	0.208	0.598	0.013	Up
Otu74	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Flavonifractor	0.008	0.023	0.169	0.246	0.003	Up
Otu76	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Sutterellaceae	Parasutterella	0.003	0.007	0.147	0.325	0.025	Up
Otu123	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Sedis	0.007	0.013	0.144	0.401	0.021	Up
Otu104	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae	0.003	0.009	0.101	0.152	0.022	Up
Otu203	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae	0.001	0.004	0.097	0.193	0.024	Up
Otu79	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcaceae	0	0	0.097	0.192	0.018	Up
Otu153	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	0.008	0.011	0.087	0.167	0.018	Up
Otu112	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae	0.004	0.011	0.078	0.214	0.031	Up
Otu84	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter	0	0.001	0.067	0.159	0.037	Up
Otu121	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	0	0	0.056	0.168	0	Up
Otu140	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	XIVb	0	0	0.053	0.133	0	Up
Otu132	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae	0.001	0.002	0.05	0.127	0.045	Up
Otu116	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	IV	0.001	0.001	0.044	0.047	0.005	Up
Otu2	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	31.259	22.071	7.701	8.787	0.019	Down
Otu33	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	3.013	2.728	0.726	0.912	0.047	Down
Otu42	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0.119	0.18	0.03	0.127	0.004	Down
Otu257	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia	0.084	0.149	0.001	0.003	0.004	Down

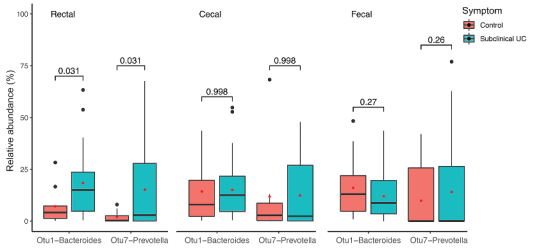

Figure 5. Relative abundances of OTU1 and OTU7 in each sample type between subclinical UC patients and healthy controls.

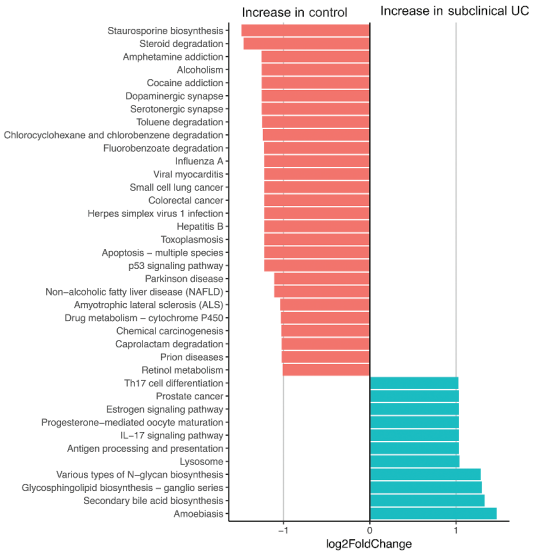

Figure 6. Comparison of the KEGG terms between the rectal MAM in control and subclinical UC cohorts. KEGG orthologues were inferred from OTU abundance. The fold changes are referred to the subclinical UC cohort.

of inflammation. Proinflammatory cytokines and interleukins can be regulated by lysosome (ko04142), which showed a two-fold increase. The increase in the number of pathways related to antigen processing and presentation (ko04612), Th17 cell differentiation (ko04659), and IL-17 signaling (ko04657) further demonstrate signs of dysbiosis in subclinical UC. These pathways promote the upregulation of innate immune responses, which usually result in host inflammation.

Discussion

In this study, we characterized and compared the microbiome profiles of cecal and rectal mucosal biopsies and fecal samples between subclinical UC patients and healthy adults. Notably, rectal mucosal biopsy, but not cecal mucosal biopsy or stool samples, demonstrated significant inter-cohort differences in microbiome throughout our analysis, with an apparent shift in phylum abundances and community profiles. Our findings strongly suggest that rectal MAM predicts subclinical UC status better than conventional fecal microbiota. Studies on UC remission after fecal transplant treatment emphasized tracking changes in patients' newly introduced microbiome.[16,17] We believe that providing a detailed profile of the rectal mucosal microbiomes of UC patients in subclinical state allows for a more accurate diagnosis and recurrence prediction than that of fecal microbiome.

A recent study revealed that the strength of the gut mucus barrier is vital in early UC pathogenesis.[9] This coincides with our observation that the relative abundance of subclinical UC mucosal tissue differed significantly but not in the fecal microbiome. However, our results differed to previous studies that mucosal samples in IBD were associated with increased Proteobacteria abundance.[11,18–20] Previous studies have demonstrated that the gut microbiomes are inherently different among patients with different ethnicities, residencies, and ages,[21] suggesting that microbiome shift studies of active UC[22,23] or IBD should establish a baseline variation for their participants to yield biological meaning from the analysis. We speculate that the phenotype of our subclinical UC cohort added to this complexity, and acknowledge a larger pool of control would warrant a more robust abundance baseline to validate our findings.

A striking result of our study was that the microbiomes of the rectal mucosa were more diverse in the subclinical UC cohort than the control. The Chao1 index of rectal MAM in the subclinical UC cohort was higher compared to the control (Figure 3; left panel). This suggests that, in a healthy individual, MAM and FAM are highly differentiated, which might be attributed to their environments supporting a localized niche group of microbes. Mild inflammation altered the epithelial gut environment, thus further supporting microbes with different growth niches. However, the perturbed niche does not eliminate the resident population, as the majority of OTUs were shared and still dominant in the same sample types (Figure 2(b)). Shift in microbial profiles in subclinical cohort were attributed to the increased relative abundance of the rare species. This finding may also explain the increase in the alpha diversity

indices of MAM in the UC cohort (Figure 3; right panel).

A large portion of the variation in the microbiome was reported to originate from inter-individual variation.[10] When determining disease status, we minimized patient-patient variation by analyzing both fecal and mucosal biopsies from the same patient. Beta diversity analysis indicated that the microbial profiles of cecal MAM and FAM cannot separate the control and subclinical UC patients. Statistical differences were detected only in the rectal MAM.

The relative abundance of *Blautia, Ruminoccocaceae* and *Lachnospiraceae* were increased in our subclinical UC cohort (Table 2). Such observations were opposite to Schirmer et al. for their decreased relative abundance in mild and moderate UC pediatric patients.[7] One of the possible explanation in the discrepancies may be the age group differences of patients, as microbiome of children have been shown to be different from adults.[24–26] Further analysis of differentially abundant taxa at the genus level revealed two OTUs (*Bacteroides spp, Prevotella spp.*) were associated with dysbiosis of subclinical UC, consistent with previous report[19] and attributed to inflammatory responses.[27,28] Similarly, the multi-omics study by Lloyd-Price et al. indicated that the presence of many *Bacteroides* and *Prevotella* species were highly correlated with UC dysbiosis.[10] An increase in proinflammatory *Prevotella spp*. was correlated with the differential increase in Th17 cell differentiation and IL-17 signaling pathways, suggesting that these two OTUs play a role in UC pathogenesis. Accordingly, we propose that rectal MAM is a more suitable indicator for UC disease state, even at the preclinical or subclinical state.

No OTUs were found to correlate with both subclinical UC dysbiosis and recurrence. OTU22-*Megasphaera* is the only OTU showed to have differential increase in high risk for UC relapse. Relatively low abundance of *Megasphaera* has been previous reported in fecal samples,[29,30] but not necessarily correspond to a differential increase in UC dysbiosis. Clinical significance of this taxa in UC dysbiosis warrants further investigation.

The differential analysis of putative KEGG orthologues in sequenced samples revealed immune system pathways associated with the subclinical UC. Th17 cell differentiation (ko04659) and IL-17 signaling pathways (ko04657) have been implicated in *Prevotella*-mediated chronic inflammation of the gut mucosa.[27] The increase in the IL-17 pathway has been shown to promote angiogenin and phospholipase A2 expression in innate immune defense. This promotes the growth of *Prevotella spp*. in the colon and suppresses growth of the beneficial *Clostridium XIVa*.[14,31] While the increase in *Prevotella* found in subclinical UC patients coincides with previous findings, the associated decrease in *C. XIVa* was not detected. Our data indicated a differential increase for Otu149-*C. XIVa* in the rectal MAM of subclinical UC (Figure S3). A possible explanation is that inflammation, even at subclinical status, creates a niche that differentiates the colonization of microbiome, which cannot be detected in feces. Secondary bile acid biosynthesis pathways (ko00121) were also shown to increase in the subclinical UC cohort. Heinken et al. showed that metabolism and the biosynthesis of secondary bile acid require cooperation among multiple microbial communities.[32] Bile acids are known regulators for gut microbiome,[33] these findings suggest a feedback regulatory mechanism involved in response to the changes in gut environment and warrant further investigations.

In summary, UC usually runs a complex but unpredictable clinical course. Our findings provided insights into FAM and MAM profiles at the subclinical stage of UC. Rectal MAM was demonstrated to be a better indicator than FAM to distinguish subclinical UC from those at sustained remission and healthy individuals. Putative OTUs implicated in subclinical UC dysbiosis and UC recurrence were also identified. We propose that these findings are a foothold for further developing a method to monitor and early detect the relapse of UC in patients at remission. Further studies to delineate the multifaceted relationship between the changes in rectal MAM and host immune responses are warranted.

Material and methods

Study population and sample collection

Fifty-eight patients (45 subclinical UC patients and 13 healthy subjects) were recruited from the outpatient clinics of Chang-Gung Memorial Hospital (Linkou, Taiwan) from 2014 to 2015. The subclinical UC cohort

consisted of patients that sustained remission with subclinical symptoms. The study was approved by the Institutional Review Board (IRB), and written consent to participate in the study was obtained from patients. The control group consisted of healthy adults scheduled for routine health check and have opted for a colonoscopy examination. UC was diagnosed based on standard clinical, endoscopic, radiological, and histological criteria.[34] None of the 45 patients with UC were at an active disease stage, nor had any had infectious diarrhea relapses or used antibiotics within three months of the sample collection. Cecal and rectal endoscopic examination revealed low inflammation grade for all subclinical UC patients. UC activity was evaluated using the Mayo score.[35] Fresh fecal samples were collected and stored at −80°C less than 1 hour before DNA extraction. For mucosa biopsy sampling, tissue samples were taken from the cecum and rectum during the colonoscopy. Colonic cleansing was performed before the colonoscopy. Biopsy samples were immediately suspended in an 1.5 ml tube with RNAlater™, solution (Cat#: R0901, Merck, Darmstadt, Germany) and flash-frozen with liquid nitrogen. Biopsy samples were stored at −80°C until use.

DNA extraction

The experimental protocol was adapted from the Human Microbiome Project.[36] Total genomic DNA was extracted using DNeasy PowerSoil kit (Cat #: 12888, QIAGEN, Hilden, Germany) with slight modifications. During sample pre-processing, the bead solution was added to the frozen stool sample in a 15 ml Falcon tube (2.0 ml/g frozen stool; 1.8 ml/biopsy). The mixture was vortexed vigorously for 30 s, then incubated at 65°C for 10 min and 95°C for 10 min using a water bath. Large particles were pelleted by centrifugation at 1,500 g × for 5 min, and 900 µl of supernatant was transferred to the PowerSoil bead tube. The rest of the protocol is as detailed by the manufacturer, with the following exceptions in reference to the user manual: (1) In Step 3, sample homogenization was performed using PowerLyzer®24 Homogenizer (Cat #: 13155, QIAGEN, Hilden, Germany) set to 4,200 rpm for 45 s. (2) In Step 13, 1040 µl of Solution C4 was added. (3) In Step 16, the spin column was washed twice with 500 µl of solution C5 before elution.

Amplicon library construction for sequencing

Amplicon sequencing libraries were prepared as previously described.[37] Amplicons were visualized by running 2 µl of the product on 2.0% (w/v) agarose gel to confirm that a product was generated. Sample normalization was performed using SequalPrep Normalization Plate Kit, 96-well (Cat #: A1051001, ThermoFisher, Waltham, Massachusetts, U.S.). Normalized amplicon products were pooled at equal volumes. The pooled DNA library was concentrated using an equal volume of Agencourt AMPure XP beads (Cat #: A63880, Beckman Coulter, Pasadena, California, U.S.). Sequencing was performed by the NGS High Throughput Genomics Core in Biodiversity Research Center, Academia Sinica, Taiwan. Sequencing of the 16S amplicon was carried out using Illumina MiSeq with paired-end 2 × 250 bp chemistry.

Sequencing data processing pipeline and statistical analysis

Fastq files were pre-processed with the UParse v10.0.240 pipeline.[38] Paired reads were merged into single sequences and quality filtered; unique sequences were determined and clustered into OTUs at 97% sequence similarity. SINTAX algorithm was used for taxonomic assignment.[39] Processed data were imported into R environment (v3.5.0) in the form of an OTU table. Amplicon sequencing data were analyzed using Phyloseq (v1.26.0) and DESeq2 (v1.22.1). The microbial community distance between samples was calculated using weighted UniFrac distances, and samples ordinated using principal coordinate analysis (PCoA). Statistical tests for categorical data against alpha diversity indices were performed using ANOVA; post-hoc analysis was performed using Tukey's HSD test. Variables with a skewed sample size were not assessed (e.g., 2/43; with/without sulfasalazine treatment). Statistical significance of beta diversity between samples was tested using the *adonis* function in R. Dispersion of the data points was analyzed using the *betadisper* function in R. Differentially abundant OTUs or functional pathways between the two parameters were determined at a 95% confidence level using the Wald test from the DESeq2 package. Functional pathways in the samples were predicted using Piphillin,[40] with the Kyoto Encyclopedia of Genes and Genomes (KEGG) as the reference database (version Oct 2018).

Abbreviations

FAM	Fecal-associated microbiome
IBD	Inflammatory bowel disease
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAM	Mucosal-associated microbiome
OTU	Operational taxonomic unit
UC	Ulcerative colitis

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Disclosure of potential conflicts of interest

The authors of this manuscript declared that they have no conflict of interests.

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