MOLECULAR ANALYSIS REVEALS UNIQUE MICROBIOME IN ILEAL POUCH

DURING POUCHITIS COMPARED TO HEALTHY POUCHES IN ULCERATIVE

COLITIS AND FAMILIAL ADENOMATOUS POLYPOSIS

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TITLE: Molecular Analysis Reveals Unique Microbiome in Ileal
Pouch During Pouchitis Compared to Healthy Pouches in
Ulcerative Colitis and Familial Adenomatous Polyposis

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ABSTRACT

Molecular Analysis Reveals Unique Microbiome in Ileal Pouch During Pouchitis
Compared to Healthy Pouches in Ulcerative Colitis and Familial Adenomatous Polyposis

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In severe cases of ulcerative colitis (UC) unresponsive to current treatment options, patients require a complete proctocolectomy, or surgical removal of the colon. Ileal pouch anal anastomosis (IPAA) has become the preferred surgical technique for patients who require surgery, as this method restores rectal function. This procedure is also used to treat colorectal cancers such as adenocarcinoma and familial adenomatous polyposis (FAP). The surgery involves an abdominal colectomy with the construction of an ileal pouch created from folded tissue recovered from the ileal portion of the small intestine. Up to 50% of patients who require IPAA surgery experience an episode of pouchitis, a non-specific inflammation of the constructed ileal pouch with unknown etiology. Several hypotheses have been proposed regarding the pathogenesis of pouchitis. Current theories include bacterial overgrowth due to fecal stasis, microbial imbalance (dysbiosis), immune alteration, genetic susceptibility, metaplasia, ischemic complications of surgery, a recurrence of UC, or even a novel form of inflammatory bowel disease. The efficacy of antibiotics and probiotics in treating pouchitis and maintaining remission underscores the importance of gut microbiota in the development of this condition. In the study, we aimed to characterize the intestinal bacterial communities that inhabit IPAA pouches of both UC and FAP patients, in an effort to investigate the hypothesis that bacterial dysbiosis is involved in the pathogenesis of pouchitis. Mucosal biopsy and stool samples were analyzed from patients with UC and pouchitis (UCP), healthy UC controls (HUC) and healthy pouches with a background of FAP (FAP). Samples were examined through analysis of terminal restriction fragment length polymorphisms (TRF) and DNA sequencing. The data presented here demonstrate that a microbial imbalance exists in pouchitis, as bacterial communities in pouchitis differ significantly from healthy UC pouches and pouches constructed for FAP. Both methods identified potential groups of organisms that may play a role in the development of pouchitis, including decreases in protective *Lactobacillus* and *Bacteroides* and increases in mucin-degrading *Clostridium* and *Akkermansia*. A better understanding of the factors driving the pathogenesis of pouchitis will not only benefit patients with this disease, but also lead to a better understanding of the complex

relationship that exists between the human host and the diverse community of organisms that inhabit the gastrointestinal tract.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure

1.1 INTRODUCTION

Ulcerative colitis (UC) is a chronic but intermittent inflammatory bowel disease (IBD) characterized by continuous ulcers, or open sores, in the mucosal layer lining the large intestine [1]. This disease has a prevalence of 246 per 100,000 in the United States, equating to approximately 700,000 Americans suffering from symptoms including abdominal pain and cramping, bloody diarrhea, and fever [2]. The symptoms range in severity and can be localized to the rectum or involve the entire colon, but unlike Crohn's disease, UC is restricted to the large intestine [1]. The etiology of this condition is unknown, but studies indicate that both genetic and environmental factors are important. Hypotheses regarding the origin of UC include an autoimmune response to an antigen, a dysfunctional immune response to commensal organisms, or an infection with a pathogenic microbe [1].

Treatment for UC includes anti-inflammatory drugs and immunomodulators, which effectively suppress the disease in most cases [1]. Approximately 20-30% of patients, however, do not respond effectively to drug therapy and instead require surgical intervention [3]. Unlike Crohn's disease, ulcerative colitis can be cured, in some cases, by removal of the large intestines. The restorative proctocolectomy with ileal pouch anal anastomosis (IPAA) has become the preferred surgical procedure used to treat patients with severe UC [4]. This method involves an abdominal colectomy with the creation of a pouch that is connected to the anus. The pouch is constructed from loops of folded tissue recovered from the ileal portion of the small intestine. The alternative surgical procedure involves the connection of the small intestine directly through the abdominal wall. IPAA surgery is favored because the rectal muscular cuff and anal sphincter are left intact, eliminating the need for a collection bag.

Although IPAA surgery has functional advantages over previously used surgical techniques, a significant number of patients exhibit post-surgical complications. The

1

most common is pouchitis, a nonspecific inflammatory condition that occurs in the reconstructed ileal pouch. Approximately one-half of all UC patients who receive IPAA surgery will develop pouchitis during their lifetime, a syndrome involving watery, frequent diarrhea accompanied by urgency, incontinence, abdominal cramping, and fever [5-6]. Although the majority of pouchitis patients respond to antibiotics, the condition will frequently return and becomes recurrent in approximately10-15% of patients [5, 7].

Several hypotheses have been proposed regarding the pathogenesis of pouchitis. Current theories include the following: bacterial overgrowth due to fecal stasis, bacterial dysbiosis (imbalance), immune alteration, genetic susceptibility, metaplasia, ischemic complications of surgery, a recurrence of UC, or even a novel form of IBD [8]. Multiple lines of evidence point to a pivotal role for bacteria in pouchitis. First of all, inflammation of the mucosa has been shown to be localized to areas with the highest concentration of bacteria [9]. In addition, sonicated flora from pouchitis patients induces activation of *ex vivo* mononuclear cells while sonicates from healthy patients did not [10]. Furthermore, symptoms associated with pouchitis are often effectively treated with antibiotics [11-12]. Four week treatment of metronidazole with ciprofloxacin has been shown to be highly effective in patients with recurrent or refractory pouchitis [13]. Metronidazole accumulates in anaerobes and produces toxic free radicals, while ciprofloxacin is a broad spectrum antibiotic that inhibits DNA gyrase, halting bacterial replication. These two antibiotics have been shown to work best synergistically [7, 14].

Additional evidence supporting the involvement of intestinal microflora in pouchitis is the efficacy demonstrated by probiotic VSL#3 in maintaining remission after recovering from an episode of pouchitis [7, 15]. VSL#3, marketed as "The Living Shield", is s mixture of eight different strains of bacteria, including *Bifidobacteria, Lactobacillus,* and *Streptococcus*. A once daily high dose administration of VSL#3 has been shown to be effective in maintaining antibiotic introduced remission in patients experiencing

recurrent pouchitis or pouchitis refractory to therapy [7]. Patients treated with VSL#3 are shown to have lower levels of mucosal mRNA expression of proinflammatory cytokines IL-1β, IL-8, and IFNγ [16]. VSL#3 has also demonstrated efficacy in treating ulcerative colitis [17]. Single-strain probiotic *Lactobacillus rhamnosus* GG has also been tested in cases of pouchitis, but effects were limited [18].

This clinical evidence has prompted much investigation into the relationship between pouchitis and intestinal bacteria. The results of these studies, however, are mixed, and no study has been able to identify a single pathogenic organism or toxin responsible for pouchitis. Multiple groups have used culture-based techniques that indicate a decrease in the ratio of anaerobic to aerobic bacteria in pouchitis [19-21]. Several studies cite an increase in sulfate reducing bacteria in the pouches of UC patients and implicate the production of hydrogen sulfide gas in the development of pouchitis [22-23]. Other studies have implicated increases in *Clostridia* or Fusobacter and decreases in *Lactobacilli*, *Bifidobacteria* or *Streptococci* [24-26]. Yet other studies indicate no difference in microbiological communities between inflamed and healthy pouches [22, 27-29].

Part of the reason for the inconsistent findings between studies is the high subjectivity in the classification of pouchitis. Prior to 1994, the criteria used in the diagnosis of pouchitis varied widely between clinicians. A classification system has since been developed in order to objectively quantify the condition of pouchitis, using a diagnostic scoring system known as the pouchitis disease activity index (PDAI) [30]. The PDAI score is determined using three principal components: symptoms (0-6pts), endoscopy (0-6pts), and histology (2-6pts). The clinical section of the PDAI rates symptoms such as stool frequency, rectal bleeding, fecal urgency or abdominal cramping, and fever. The level of inflammation is described during endoscopy through observation of factors such as edema, granularity, and ulceration. The histological

section of the score considers levels of polymorphic nuclear leukocyte infiltration and ulcerations per low-power field. A combined score greater than seven qualifies as pouchitis. The use of the PDAI scoring system has led to more consistent classification of pouchitis patients.

Another probable reason for the discrepancies in findings between studies is the variation in the types of control samples used for comparison to pouchitis. It is difficult to compare ileal pouch flora from pouchitis patients directly to ileal flora of healthy patients, due to inherent challenges in collecting samples from the small intestines. The majority of studies compare inflamed UC pouches to healthy UC pouches or to ileostomy. The problem with this comparison is that it does not detect differences that may be due to the underlying condition of ulcerative colitis, since all samples have a background of the disease. In the study presented here, patients who receive IPAA surgery for UC are compared to patients who underwent the same surgery for familial adenomatous polyposis (FAP). FAP is a heritable condition in which hundreds to thousands of polyps form in the mucosal surface lining the intestine. If not treated, these polyps almost inevitably transform into malignancies, therefore surgery is employed in FAP cases to eliminate the risk of colon cancer. Patients who undergo IPAA surgery due to familial adenomatous polyposis (FAP) are ten times less likely to experience symptoms of pouchitis [31-32].

In addition to subjectivity in classification and variation in the types of samples compared, a third reason for inconstant findings within pouch microbiota studies is high variability in the experimental techniques (compounded by high intersubject variability). Available methodologies for studying gut microbiota have changed dramatically over the past twenty years. Original culture based techniques were problematic with gut samples, due to the high numbers of anaerobes and the difficulty in growing fastidious organisms or those with mutualistic dependence on other species. It is estimated that only about

20% of organisms can be cultured from the gut. Molecular techniques for community fingerprinting are now more commonly used and are typically based on variability within conserved regions of the 16S ribosomal RNA gene. The techniques are similar in that all start with PCR using primers for sequences in this gene that are conserved in all prokaryotes. The methods differ in how the fragments are separated. Density Gradient Gel Electrophoresis (DGGE) uses chemical gradients of urea and formamide to separate fragments and temperature gradient gel electrophoresis (TGGE) is based on sequence dependent differences in the temperature at which double stranded DNA melts. In Terminal Restriction Fragment Length Polymorphism (TRFLP or TRF) one end of each PCR-amplified fragment is fluorescently labeled and the fragments are digested with restriction enzymes and separated using capillary gel electrophoresis on an automated sequence analyzer. TRF data have the advantage of being simply and rapidly produced, providing more information than DGGE or TGGE. Data are easily converted into a form that can be analyzed using a variety of statistical approaches and databases are available for comparison. Ideally, TRF is used for monitoring bacterial community structure over time. The resolution of TRF is, however, limited, as fragment sizes overlap in most cases, making absolute identification impossible. As more restriction enzymes are used, however, more information is available to differentiate species of bacteria. In addition to analysis using TRF profiles, PCR amplicons derived from this study were also sent for DNA sequencing. Sequences of 16S ribosomal RNA genes provide more power to precisely identify the community composition in inflamed pouches versus healthy controls.

Both colonic biopsies and stool samples were obtained from IPAA patients enrolled in this study. The concurrent analysis of both mucosal and luminal samples is a strength of this study compared to earlier studies that generally assessed only stool samples. Analysis of mucosa is important since it has been shown that microorganisms

interacting with the mucosa are significantly different than those found in fecal samples, and the interaction between the organisms is thought to be integral to the development of pouchitis [25, 33]. Only two other pouchitis studies compared mucosal and fecal flora [20, 25].

The aim of this study was to characterize the intestinal bacteria that inhabit IPAA pouches of both UC and FAP patients to investigate the hypothesis that an imbalance in bacterial populations is involved in the pathogenesis of pouchitis. Mucosa and stool samples were analyzed from patients with UC and pouchitis, healthy UC controls and FAP controls. Samples were examined using both TRF and DNA sequencing. The data presented here demonstrate that bacterial dysbiosis exists in pouchitis and the bacterial communities in pouchitis differ significantly from healthy UC pouches as well as from pouches constructed for FAP. Both methods identified potential groups of organisms that may play a role in the development of pouchitis. A better understanding of the factors driving the pathogenesis of pouchitis will not only benefit patients with this disease, but also lead to a better understanding of the complex relationships that exist between the human host and the diverse community of organisms that inhabit the gastrointestinal tract.

1.2 METHODS

Inclusion Criteria

Patients who have received care for a restorative proctocolectomy with IPAA at Massachusetts General Hospital were invited to participate in this study. Patients were enrolled in this study only if IPAA surgery was performed more than three months prior to enrollment. Patients were divided into three cohorts: those with UC and pouchitis (UCP); those with UC but no pouchitis (healthy UC pouch, HUC); and those with a history of FAP without pouchitis (healthy FAP pouch, FAP). Subjects enrolled as case subjects were those experiencing symptoms of at least three episodes of pouchitis in the

6

past year. Pouch inflammation was documented by histological and endoscopic investigation and a PDAI score was assigned. PDAI scores greater than seven qualified the patient into the pouchitis (UCP) group. HUC control subjects included patients who had never experienced an episode of pouchitis or who had received a PDAI score less than seven.

Exclusion Criteria

Subjects taking probiotics, non-steroidal anti-inflammatory drugs, and immunosuppressants were excluded from the study, as well as any subject who had received topical immunosuppressant rectal therapy in the previous two weeks. Subjects chronically taking antibiotics were allowed in the study if the antibiotics were administered more than three months prior to sample collection and if the patient's symptoms persisted with antibiotic use. Subjects with any clinical evidence of Crohn's disease or any other immunological or hematological illness deemed significant to this study were excluded. Nineteen test subjects ultimately enrolled in the study and provided informed consent. The Massachusetts General Hospital and California Polytechnic State University human subject committees each approved the protocol and written consent was obtained from each individual.

Specimen collection

The pouch of each patient was examined to determine PDAI score. Each enrolled patient underwent a flexible sigmoidscopy performed by one of the study physicians. Patients received a bowel preparation one day in advance with phospho-soda. Both stool and mucosal biopsy specimens were obtained from each test subject. One ml stool samples were suctioned into three vials containing 10% glycerol/90% sterile water and were immediately placed in dry ice. Biopsies were removed from the most inflamed area within the pouch, at least four centimeters from the anus. The biopsies were washed in 500 mL of saline and placed into a vial containing 10%

glycerol/90% sterile water were immediately placed in dry ice. Both types of samples were frozen at -80°C within 30 minutes.

DNA extractions

DNA was isolated using MoBio's Power Soil DNA extraction kit, according to the manufacturer's protocol (MoBio Laboratories, Carlsbad, CA). Triplicate 0.1 g samples were used for the extraction of stool samples, while the entire biopsy sample was used for the isolation of DNA from tissue samples. The 16S subunit bacterial ribosomal RNA gene was amplified from both isolates using the forward primer 8DF (5'-AGA GTT TGT TCM TGG CTC AG-3') and the reverse primer 536-K2R (5'-GTA TTA CCG CGG CTG CTG G-3'). The forward primer was fluorescently labeled with a phosphamide dye. 50 μl reactions were assembled using 1 μl of undiluted DNA, 5 μl of 10X buffer, 3 μl of 10 mM dNTPs, 2 μl of 20mg mL^{-1} BSA, 7 μl of 25 mM MgCl$_2$, 1 μl of each primer, and 0.3 μl of 5 UL^{-1} TaqGold (Applied Biosystems, Foster City, CA). Reaction temperatures and times were as follows: 95°C for 10 min followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 10 min. The quality of the extractions and PCR reactions were confirmed using gel electrophoresis. PCR triplicates were combined during a PCR cleanup performed using MoBio's PCR Cleanup Kit following the manufacturer's protocol (MoBio Laboratories, Carlsbad, CA). PCR products were quantified using a FLX800 microplate fluorescence reader tuned to the labeling dye (Bio-Tek Instruments; Winooski,VT).

Digestion and Resolution of TRF patterns

Enzyme digests were independently performed using cleaned-up PCR product and the restriction endonucleases HaeIII, HpaII, and AluI (New England Biolabs; Beverly, MA). Each 40 μl digest included 75 ng DNA, 1 U enzyme, and 4 μl buffer. Samples were digested for 4 h at 37°C and inactivated for 20 min at either 65°C (HpaII and AluI) or 80°C (HaeIII). Resulting fragments were ethanol precipitated and

8

resuspended in 20 µl formamide and 0.25 µl CEQ 600 base pair standard. Terminal restriction fragments were separated using capillary gel electrophoresis and profiles were obtained using a Beckman Coulter CEQ8000X DNA analysis system.

Terminal restriction fragment lengths (in nucleotides) and relative peak areas (relative abundance) were exported from the CEQ8000 into Excel (Microsoft, Seattle, WA). The area under each peak was normalized for loading and expressed in parts per million to standardize the data for comparison. Peaks with an area less than 10,000 ppm (<1.0% of the total) were excluded from the analysis to reduce excess noise. Data from three independent restriction digests were performed to provide better resolution (reduce incidence of distinct sequences with equivalent TRF lengths) and thus help tentatively identify groups when performing database comparisons between fragment lengths and *in silico* restriction enzyme digests of 16S rRNA gene sequences in GenBank (NCBI, Bethesda, MD).

Data analysis

TRF data sets were transformed by taking the square root of the area under each peak to de-emphasize large TRF peaks while still accounting for relative abundance. Transformed data were compared using Bray-Curtis similarity, multidimensional scaling, and analysis of similarity (Primer, London, UK; Microsoft Excel, Seattle, WA). TRF fragments were subjected to ANOSIM analysis to determine the TRF fragments that differ most between groups. These fragments were compared to available GenBank sequences to tentatively identify potential bacterial populations associated with bacterial dysbiosis. The database for TRF matching was created by obtaining all 16S rRNA gene sequences from GenBank. These sequences were processed using *in silico* PCR and restriction enzyme digests. Observed TRF peaks were compared to the predicted lengths based on the database, allowing for a difference of one base pair between the lengths of observed and predicted fragments.

Statistics

Transformed data were compared between groups using Bray-Curtis similarity, multidimensional scaling, and analysis of similarity (ANOSIM) (Primer E, Plymouth, UK). Bray-Curtis was used to determine overall level of similarity between groups. ANOSIM was used to identify the factors that made the groups different. ANOVA was used to compare relative abundances of specific TRF elements between groups.

16S Ribosomal DNA-based DNA Sequencing

Four fecal samples from each of the UCP and FAP groups were selected and pooled for sequencing analysis. Samples were chosen based on greatest intersubject similarity, using Bray-Curtis similarity indices. Extracts from selected samples were subjected to PCR with the same primers used in TRF, without the fluorescent label. Resulting fragments were pooled and sent to the Broad Institute in Cambridge Massachusetts for sequencing.

Samples were cloned into pCR2.1-TOPO vectors and sequenced on an ABI3730 DNA sequencer. Resulting sequences were trimmed using LUCY, a tool that examines raw DNA sequence data for quality assurance [34]. Read pairs from each clone were assembled using an alignment-assisted assembly method implemented at the Broad Institute. 16S rRNA gene sequences obtained from Greengenes provided the alignment template used as a reference sequence for the read pairs [35]. The reference sequence with the greatest number of k-mers matching with the aggregate k-mer set of both forward and reverse reads was selected. The forward and reverse reads were aligned to the core reference sequence using basic local alignment search tool (NCBI, Bethesda, MD) [36]. Aligned forward and reverse reads were then assembled based on base quality scores. High quality reads longer than 1100 nucleotides were used for classification. Sequences were classified according to comparisons with the Ribosomal Database Project sequence collection [37-38].

1.3 RESULTS

Patient Characteristics

Nineteen patients were enrolled in the study. The samples were divided into groups based on the reason for IPAA surgery (ulcerative colitis vs. familial adenomatous polyposis) and the PDAI score (healthy pouch vs. pouchitis) (Table 1). Twelve patients received surgery for ulcerative colitis, and of these 9 had pouchitis (UCP) and 3 had healthy pouches. Seven patients were enrolled that received IPAA surgery for familial adenomatous polyposis. There were no significant differences between patient demographics (age and gender) or disease characteristics (duration of ulcerative colitis and pouch). PDAI scores were significantly higher in the pouchitis group, and each biopsy in this group were shown to display histologic evidence of active pouchitis as part of the PDAI score (defined by presence of polymorphonuclear infiltrates and ulcerations). Several patients enrolled in the UCP group were receiving chronic antibiotic therapy. Two patients were currently taking antibiotics, one for eight years and one for one year, while still having episodes of pouchitis. Three other patients had received short courses of antibiotics during the past year, but not during the three month time span prior to enrolling in the study.

TABLE 1. Patient Demographic and Disease Characteristics

	UCP (*n*=9)	FAP (*n*=7)	HUC (*n*=3)	*P*-value
Mean age in years (range)	32.2 (22-61)	45.5 (29-55)	39.3 (26-55)	0.15
Gender	M = 2 (22%)	M = 4 (57%)	M = 1 (33%)	0.48
	F = 7 (78%)	F = 3 (43%)	F = 2 (67%)	
Mean duration of pouch in years (range)	5.9 (1.5-13)	8.7 (1.4-21)	12.3 (2-20)	0.51
Mean duration of UC in years (range)	8.8 (2.5-14)	n/a	14.7 (3-25)	0.31
Mean PDAI score (range)	8.8 (7-12)	0	1 (1)	< 0.001*

*Statistically significant. UCP, UC pouchitis; FAP, familial adenomatous polyposis; HUC, healthy UC.

Terminal Restriction Fragment Analysis

The total number of peaks provides a measure of overall community diversity. There were significant differences in the total number of TRF peaks in the mucosal biospsies compared to stool (average total peaks, mucosa=64.8, feces=41.3, $p < 0.001$). (Figure 1). Fecal and mucosal microbial fingerprints were significantly more similar within samples from a single subject compared to between subjects ($p = 0.027$). Compared to stool samples, mucosal samples taken as a whole revealed significantly greater intersubject percentage similarity ($p < 0.001$). The total number of fecal TRF peaks, a marker of overall diversity, was lower in pouchitis compared to stool from healthy subjects without proctocolectomy (42.3 in UCP, 64.0 in normal stool, $p < 0.001$). TRFs used for this analysis were collected from healthy seniors at California Polytechnic State University as part of an independent study. However, HUC and FAP groups also showed significantly lower microbial diversity in the stool samples compared to healthy subjects without proctocolectomy (HUC=35.3, $p < 0.05$; FAP=41.4, $p < 0.005$).
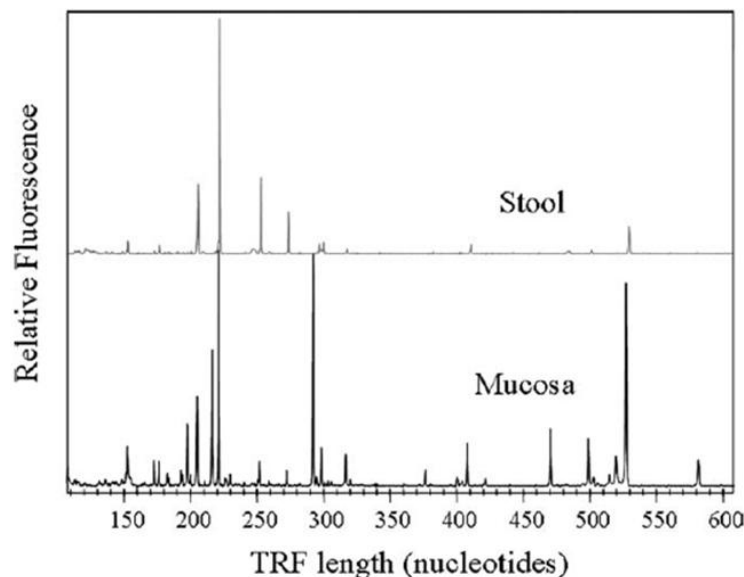

Figure 1. Representative TRF profiles. These two patterns were generated from the stool and mucosa of a single patient. The mucosa had a higher degree of community diversity, based on the total number of peaks.

Overall, there was a higher degree of similarity within TRF profiles from the FAP group (37.78%) compared to the UCP group, where microbial profiles were more variable overall (20.11%). TRF profiles were significantly different between UCP and FAP groups when evaluating mucosal and stool separately, or when combined ($p<0.05$, Figure 2). Profiles were also distinct between UC patients with and without pouchitis ($p<0.05$). No significant differences were observed in patients taking antibiotic therapy.

The analysis of similarity (ANOSIM) analysis from the three different restriction digests were compared to find sets of peaks that were most different between groups in each digest. This analysis revealed a set of TRFs matching *Lactobacillus* and *Streptococcus* (Hae 264-5, Hpa 97-9, Alu 76,532) were present at higher relative abundance in both mucosal and fecal samples from FAP patients compared to UCP (ratio 5:1 in mucosa, 3:1 in stool). A second set of TRF peaks (Hae 272-4, Hpa 222-3, Alu 440) matching *Clostridium, Eubacterium* and *Roseburia* genera were present at five time the relative abundance in stool from UCP patients compared to FAP patients. Fecal samples from HUC pouches also had fewer peaks matching *Clostridium, Eubacterium* and *Roseburia* compared to UCP (ratio 1:15). Profiles from healthy UC pouches had fewer *Escherichia, Streptococcus*, and various sulfur-oxidizing bacteria (Hpa 496, Hae 205, Alu 74) at a ratio of 1:2 compared to UCP. Mucosal samples from healthy UC pouches also showed less *Escherichia, Streptococcus*, and various sulfur-oxidizing bacteria compared to UCP (ratio 1:2). There were no groups that were increased in healthy UC pouches that were statistically significant.

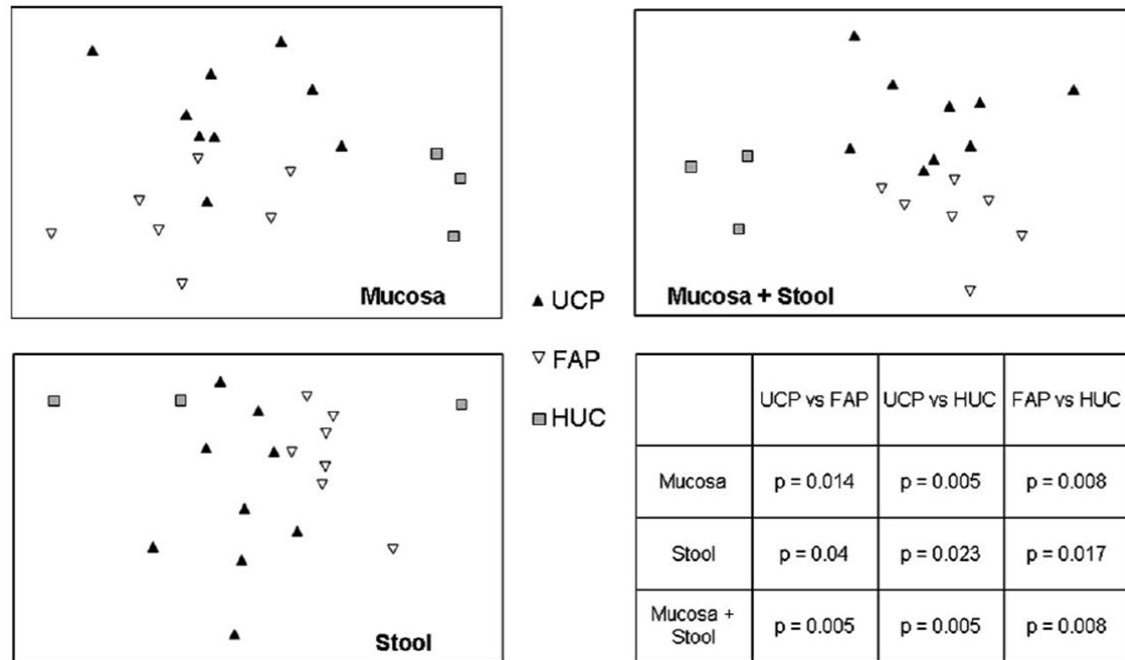|	UCP vs FAP	UCP vs HUC	FAP vs HUC
Mucosa	p = 0.014	p = 0.005	p = 0.008
Stool	p = 0.04	p = 0.023	p = 0.017
Mucosa + Stool	p = 0.005	p = 0.005	p = 0.008

Figure 2. Multi-dimensional scaling (MDS) analysis. This analysis is used to compare TRF profiles between groups. Each symbol represents the profile of one subject and the distance between symbols is representative of overall similarity. Significant differences in microbial communities were observed between each experimental group.

DNA Sequencing

Pooled fragments from the UCP and FAP group were sent for cloning and sequencing at the Broad Institute. For each of the samples, 2304 clones were processed in total. In the UCP pooled sample, 712 sequences were identified at the genus level, and for the FAP samples, 1015 were identified. The groups were first compared at the phylum level. These data showed increased Firmicutes and Verrucomicrobia in the UCP group and more Bacteroidetes in the FAP group (p<0.001)(Table 2). Sequences between groups were also compared at the genus level. Table 3 shows the classification of the sequences that were different between experimental groups. Like the TRF data, the sequencing data revealed more *Clostridia*, namely *Roseburia* in the UCP group and more *Escherichia* in the FAP group.

TABLE 2. Comparison of DNA Sequencing Results by Phylum

	Percent of Identifiable DNA Clones (Absolute Number)		
	UCP	FAP	P-value
Bacteroidetes	20% (144)	71% (716)	< 0.001
Firmicutes	52% (379)	19% (216)	< 0.001
Fusobacteria	1% (4)	<1% (4)	NS
Proteobacteria	5% (30)	5% (44)	NS
Verrucomicrobia	22% (155)	3% (35)	< 0.001
Actinobacteria	<1% (0)	<1% (1)	NS

NS, not significant; UCP, UC pouchitis; FAP, familial adenomatous polyposis.

TABLE 3. Identifiable 16S rDNA Sequences in Fecal Samples of the UCP Subgroup Compared to the FAP Subgroup

Phylum	Class	Order	Family	Genus	Absolute Number of Identifiable Sequences		Portion of Total Identifiable Sequences		P-value
					UCP	FAP	UCP	FAP	
Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides *	99	678	0.14	0.67	< 0.001
			Porphyromonadaceae	Parabacteroides *	0	21	0.00	0.02	< 0.001
			Prevotellaceae	Prevotella *	45	11	0.06	0.01	< 0.001
			Rikenellaceae	Alistipes	0	6	0.00	0.01	NS
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0	1	0.00	0.00	NS
		Lactobacillales	Lactobacillaceae	Pediococcus	1	0	0.00	0.00	NS
			Streptococcaceae	Streptococcus	2	3	0.00	0.00	NS
	Clostridia	Clostridiales	Clostridiaceae	Clostridium *	64	9	0.09	0.01	< 0.001
			IncertaeSedisXI	Finegoldia	0	2	0.00	0.00	NS
			IncertaeSedisXIII	Anaerovorax	0	1	0.00	0.00	NS
			Lachnospiraceae	Dorea	0	2	0.00	0.00	NS
				Lachnospira *	12	0	0.02	0.00	< 0.001
				LachnospiraceaeIncertaeSedis *	200	102	0.28	0.10	< 0.001
				Roseburia *	60	1	0.08	0.00	< 0.001
			Peptostreptococcaceae	PeptostreptococcaceaeIncertaeSedis	7	3	0.01	0.00	NS
			Ruminococcaceae	Anaerotruncus	1	0	0.00	0.00	NS
				Faecalibacterium *	0	62	0.00	0.06	< 0.001
				Papillibacter	0	2	0.00	0.00	NS
				RuminococcaceaeIncertaeSedis	3	0	0.00	0.00	NS
				Subdoligranulum	5	11	0.01	0.01	NS
			Veillonellaceae	Acidaminococcus *	0	10	0.00	0.01	0.01
				Megasphaera	1	1	0.00	0.00	NS
				Veillonella *	23	5	0.03	0.00	< 0.001
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	ErysipelotrichaceaeIncertaeSedis	0	1	0.00	0.00	NS
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia *	155	35	0.22	0.03	< 0.001
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	Fusobacterium	4	4	0.01	0.00	NS
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	0	6	0.00	0.01	NS
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Campylobacter	1	0	0.00	0.00	NS
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter *	5	0	0.01	0.00	0.01
				Escherichia *	0	24	0.00	0.02	< 0.001
				Proteus	4	0	0.01	0.00	NS
				Providencia	1	0	0.00	0.00	NS
				Shigella	8	7	0.01	0.01	NS
		Pasteurellales	Pasteurellaceae	Haemophilus	11	7	0.02	0.01	NS
				Total identifiable sequences	712	1015			

1.4 Conclusions/Discussion

The precise role of intestinal bacteria in the pathogenesis of pouchitis remains unclear. This investigation, however, has revealed the existence of distinct pouch environments in patients who have pouchitis and those with healthy pouches in UC and FAP, using TRF and DNA sequencing techniques based on sequence variability in the gene for 16S rRNA. The pouch microbial environment, including both mucosal and luminal communities, is significantly altered in patients with UC-associated pouchitis compared to patients who received IPAA surgery in the setting of FAP. These findings demonstrate that bacterial dysbiosis exists in pouchitis and thus underscore the importance of the relationship between microbiota colonizing the gastrointestinal tract and the health of the host. This study was limited by the small number of healthy ulcerative colitis patients enrolled and the necessity of pooling fragments prior to DNA sequencing.

Multiple studies have indicated reductions in community diversity in pouchitis [27, 29], yet other studies demonstrate no change in diversity [39]. In TRF, diversity is represented by total number of peaks. The data collected here indicates that there is less community diversity in stool samples after IPAA surgery, indicated by fewer overall peaks in stool samples from all enrolled patients compared to stool from healthy subjects with intact colons. The finding is expected based on the fact that the colon supports a much greater bacterial load [40]. Between different experimental groups, however, there were not significant differences in total numbers of peaks between each of the three groups. The profiles of mucosal biopsies was shown to harbor greater numbers of total peaks than the fecal samples, providing supporting evidence for the idea that the bacteria found in stool comprise a subset of bacteria found in at the mucosal surface [33] (Figure 2).

The bacterial community profiles were shown to be significantly different between all three experimental groups. This finding suggests that pouchitis is related to underlying factors in ulcerative colitis, since the communities found in healthy UC pouches was different from healthy FAP pouches. Although symptoms of UC are localized to the large intestine, underlying genetic and immune alterations (i.e. NOD2 polymorphisms or increased cytokine production) could lead to a similar pathology in the small intestines post-surgery. This finding is supported by clinical evidence that UC pouches are known to be 10X more susceptible to pouchitis than FAP pouches [32].

Both TRF and sequencing data indicate *decreased* abundance of protective *Lactobacillus* and *Bacteroides* in diseased pouches. Both species have well-demonstrated protective effects for the host. *Lactobacillus* is a known probiotic organism that protects the gut through competitive inhibition of other colonizing microbes. Certain species of *Lactobacillus* have been shown to reduce inflammation in the mouse model of inflammatory bowel syndrome, likely by modulating cytokine function. *Lactobacillus* have been shown to increase occludin and claudin production, upregulating tight junction formation and helping to maintain epithelial barrier function in the host. Several other studies have indicated decreased *Lactobacillus* in pouchitis [24, 26]. Bacteroidetes, specifically genus *Bacteroides*, typically comprise a large portion of the gastrointestinal tract. These mutualistic bacteria also provide competitive inhibition of pathogenic species, likely gaining a competitive advantage through the ability to process complex polysaccharides. Studies investigating the effects of probiotics on microbial flora report increases in *Bacteroides* after probiotic therapy [41], and this effect may help explain the efficacy demonstrated by probiotic therapy in pouchitis. In general, the importance of protective species is underscored by the efficacy of probiotic species in maintaining remission in pouchitis.

Both TRF and sequencing data indicate *increased* abundance of Phylum Firmicutes in diseased pouches, including genus *Clostridium* and *Roseburia*. Clostridia are resistant spore-forming anaerobes and multiple members of this class are known human pathogens. *Clostridium difficile* is a common opportunistic pathogen that is known to increase in abundance after antibiotic treatment. *C.difficile* infections are common in pouchitis, with or without antibiotic treatment [42]. Several species of *Clostridium* are mucolytic and capable of breaking down the protective mucus layer in the host's intestinal lumen, potentially leaving the underlying epithelium more prone to invasion. Mucins provide an important energy source for bacteria as well as important barrier for the human host. In a healthy host, there exists a balance between the production of mucus by goblet cells and the breakdown of mucins by resident bacteria. An overabundance of *Clostridium* could disrupt this balance, due to enhanced glycosidase activity. Several other studies have demonstrated increases in Clostridia in pouchitis, including *Clostridium perfringens* [11, 24]. Members of genus *Roseburia* were also increased in diseased pouches. *Roseburia* are common flagellated butyrate producing bacteria. Bacterial flagellin has been shown to be a dominant antigen in Crohn's disease and, furthermore, serum IgG from patients with CD and mice with colitis both reacted specifically to flagellin from *Roseburia* [43]. Another group that was shown to be increased in diseased pouches was *Akkermansia* in Phylum Verrucomicrobia. This genus, like *Clostridia*, is known for mucin degradation. The loss of the protective mucus layer allows for direct contact between the intestinal epithelium and commensal organisms and may perpetuate inflammation.

These data, along with many previous studies, fail to pinpoint one specific group of bacteria associated with pouchitis, and therefore do not support the hypothesis that a single pathogenic organism leads to inflammation in pouchitis. Evidence in a shift of predominant microbial communities, however, was apparent. These findings provide

support for the hypothesis that pouchitis involves alterations in the homeostasis of commensal organisms. Increased abundance of colitogenic bacterial species, such as *Clostridia* and *Akkermansia* identified in this study, may initiate inflammation by disrupting barrier function or destroying the mucosal surface of the host gastrointestinal tract.

It is, however, difficult to separate cause from consequence. Changes in microbial structure could initiate inflammation, but changes in immune function could drive changes in microbial communities. There is mounting evidence that links immune alterations to pouchitis, and it is hypothesized that a loss of tolerance to commensal organisms leads to inflammation. Ferrante *et al* retrospectively investigated serum antibody production and pattern recognition receptor polymorphisms in a large cohort of IPAA patients and found associations between pouchitis and outer-membrane porin antibodies and toll-like receptor 1 polymorphisms, implying immune dysfunction [44]. Another study demonstrated increased proliferation of immature plasma cells near ulcers and inflamed mucosa of pouchitis, suggesting an immune defect in B cell development [45]. Increases have been demonstrated in levels of activated mucosal CD4+ cells and multiple proinflammatory cytokines, including IFNγ, IL-1β, IL-6, IL-8, and TNFα [46-47]. High proinflammatory cytokine production promotes inflammation and can lead to intestinal epithelial destruction and crypt hyperplasia. Genetic susceptibility loci have also been identified that likely contribute to immune dysfunction. A recent study indicates that nucleotide oligomerization domain-2 (NOD2) mutations are found at higher frequencies in patients with severe pouchitis [48].

Although it is difficult to separate changes in community makeup and immune function in terms of cause and consequence, the importance of the relationship between commensal microorganisms and the human host are becoming increasingly clear. Host-microbe relationships in the gut are the focus of a rapidly growing field of research aimed

at understanding how the two interact. Much advancement has been made in understanding the role of bacteria in human health. Germ-free mice were the first to demonstrate the critical role of bacteria in the development and maturation of the immune system, as mice raised in a sterile environment have altered development of both gut mucosal and peripheral immune systems, with a lack of expansion of CD4+ T-cells and a lack of lymphoid structure [49]. Stimulation by bacterial products is necessary and sufficient to initiate the maturation process [50].

In addition to being instrumental in the initial development of the immune system, bacteria also help maintain the health of the adult host. Some species of bacteria actively stimulate the host to produce anti-microbial peptides and proteins involved in tight junction formation, contributing to the maintenance of host epithelial integrity [40]. It is well established that bacteria help us digest complex polysaccharides, but only recently has it been appreciated that bacteria play a role in how we extract nutrients from food and in the uptake of lipids and dietary fiber. The composition of the gut microflora has recently been implicated in metabolic disorders such as obesity and diabetes [40]. As more and more data are compiled describing the composition of human intestinal microflora, enterotypes, or robust clusters of certain species, are emerging. Certain species drive the different enterotypes, based more on microbial functions (i.e. metabolism) than host factors (i.e. age or gender) [51]. It is hypothesized that persons with different microbial enterotypes respond differently to diet and drug intake.

Collectively, research on host-microbe interactions in the gut has demonstrated that there is a complex interplay in mucosal immunology between three systems that determine gastrointestinal health: host genetics, bacterial community structure, and immune function. Each system can influence the other systems and the wrong combinations of aberrant factors lead to mucosal pathogenesis. The pathogenesis of pouchitis, like gut health in general, is likely to be multi-factorial, involving overlapping

21

factors and creating a spectrum of disorders rather than a single disease. Much work is left to be done to uncover the complex host-microbe relationships and interactions between microbes and the host. Future studies should aim to concurrently study microbiota while also investigating immune function through cytokine and antibody production, or attempt to link pouchitis with genetic polymorphisms in large patient cohorts. Knowledge gained from future research efforts in this area will have wide applications to improving human health and well-being, not only including patients suffering from pouchitis after IPAA surgery. The complexity and diversity of life in our intestines and the relationship between the host and resident bacterial community pose a significant challenge to researchers, but the knowledge gained from these studies has the potential to dramatically increase our understanding of human health.

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