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Disturbances of the Perioperative Microbiome Across Multiple Body Sites in Patients Undergoing Pancreaticoduodenectomy

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Abstract

Objective: The goals of this study were to characterize bacterial communities within fecal samples, pancreatic fluid, bile, and jejunal contents from patients undergoing pancreaticoduodenectomy (PD) and to identify associations between microbiome profiles and clinical variables.

Methods: Fluid was collected from the pancreas, common bile duct, and proximal jejunum from 50 PD patients. Postoperative fecal samples were also collected. The microbial burden within samples was quantified with droplet digital polymerase chain reaction. Bacterial 16S ribosomal RNA gene sequences were amplified, sequenced, and analyzed. Data from fecal samples were compared with publicly available data obtained from volunteers.

Results: Droplet digital polymerase chain reaction confirmed the presence of bacteria in all sample types, including pancreatic fluid. Relative to samples from the American Gut Project, fecal samples from PD patients were enriched with *Klebsiella* and *Bacteroides* and were depleted of anaerobic taxa (eg, *Roseburia* and *Faecalibacterium*). Similar patterns were observed within PD pancreas, bile, and jejunal samples. Postoperative fecal samples

from patients with a pancreatic fistula contained increased abundance of *Klebsiella* and decreased abundance of commensal anaerobes, for example, *Ruminococcus*.

Conclusions: This study confirms the presence of altered bacterial populations within samples from PD patients. Future research must validate these findings and may evaluate targeted microbiome modifications to improve outcomes in PD patients.

Key Words: bile, Klebsiella, microbiome, microbiota, pancreas, pancreatic fistula, pancreaticoduodenectomy

Pancreaticoduodenectomy (PD), the standard surgical treatment for patients with pancreatic ductal adenocarcinoma (PDA) of the pancreatic head, is a morbid procedure with complications exceeding 50% even in modern series from high-volume centers.[1] An unexplored possibility is that the microbiota (either within the gut or in the pancreas) impacts postoperative outcomes. Such a possibility is consistent with compelling recent research demonstrating that the microbiota impacts responsiveness to chemotherapy and risk of anastomotic leak after colon surgery.[2,3] It is possible that patient survival and pancreatic fistula formation after PD are both linked to the status of the microbiome.

Historically, the pancreas was viewed as sterile, but recent studies suggest this may not be true.[4] It has been clearly shown that enteric or biliary microbes can colonize the inflamed pancreas in experimental models.[5] Similarly, it has been shown that the pancreatic duct or stents within the duct in the setting of chronic pancreatitis contain a rich biofilm containing bacteria.[6,7] The bile duct has also been considered to be generally sterile,[6] but a number of studies have demonstrated the presence of bacteria in the biliary system in the setting of bile duct obstruction.[6,8,9] However, little is known about the presence of microbes within the pancreas or bile duct in the setting of cancer or how these microbes might affect treatment outcomes.

The goal of this study was to characterize the microbiota of the pancreas, the bile duct, the jejunum, and fecal samples in patients undergoing PD. Human fecal samples have been heavily studied, but only rarely has the microbiota been assessed in the perioperative period, when it is likely to be impacted by antibiotics, lack of enteral nutrition, and exposure to nosocomial pathogens. In the case of PD patients, the microbiota may be further impacted by biliary stent placement and/or chemotherapy. If it can be shown that dysbiosis affects patient outcomes for PD patients, then future strategies to improve postoperative outcomes may include simple interventions to modify the microbiota, for example, dietary modification or fecal transplantation.

Materials and Methods

Sample Collection

Fifty patients at the University of Pittsburgh Medical Center were recruited for study participation (January 2014 to December 2014) prior to an electively scheduled PD. All patients received a magnesium citrate bowel prep regimen and broad-spectrum prophylactic antibiotics (most commonly cefoxitin) prior to surgery and for 0 to 2 days postoperatively. During the PD procedure, sterile swabs (BBL CultureSwab EZ; Becton Dickinson, Franklin Lakes, NJ) were used to sample fluid from the common bile duct, the main pancreatic duct, and the proximal jejunum at the site of the pancreatic anastomosis. The first postoperative fecal sample from each subject was also collected in a sterile container. All samples were stored at −80°C until batch processing. The study was conducted with institutional approval from the University of Pittsburgh (institutional review board no. 13070219).

DNA Extraction

Microbial DNA was extracted from all swabs and fecal samples using the MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc, Carlsbad, Calif). For fecal samples, the samples were added directly into bead tubes and incubated at 65°C for 10 minutes followed by 95°C for 10 minutes. After addition of 60 μL of Solution C1, the bead tubes were then shaken horizontally on a lab mixer for 10 minutes at maximum speed using a MO BIO vortex adaptor. All remaining steps followed the manufacturer's protocol. For swab samples, the swab head was cut off directly into bead tubes containing 60 μL of Solution C1 and then incubated at 65°C for 10 minutes. Tubes were then shaken horizontally on a lab mixer for 3 minutes at maximum speed using a MO BIO vortex adaptor. All remaining steps followed the manufacturer's protocol. To quantify bacterial load, droplet digital polymerase chain reaction (PCR) was performed as previously described.[10]

16S rRNA Amplicon PCR and Sequencing

Polymerase chain reaction amplification of the small subunit ribosomal RNA gene (16S rRNA) was performed in triplicate 25-μL reactions. Amplicons were produced utilizing primers adapted for the Illumina MiSeq (San Diego, Calif). Amplicons target the V4 region, and primers utilized either the Illumina adaptor, primer pad, and linker (forward primer) or Illumina adaptor, Golay barcode, primer pad, and linker (reverse primer) followed by a sequence targeting a conserved region of the bacterial 16S rRNA gene as described by Caporaso et al.[11] The only deviation from the protocol was that PCR was run for 30 cycles. Individual PCR amplicons were purified, quantified, and pooled in equimolar ratios, and the library pool was gel purified prior to submission for sequencing on the Illumina MiSeq at the University of Illinois' Roy J. Carver Biotechnology Center High-Throughput Sequencing and Genotyping Unit.

Sequence Analysis

Sequencing reads were demultiplexed and quality filtered using QIIME (v. 1.9), then UPARSE was used to cluster reads into operational taxonomic units (OTUs) using an identity cutoff of 0.97, remove chimeric sequences, and generate a table of OTUs. QIIME was used to assign taxonomic classifications to OTUS, using the RDP classifier trained on the most recent Greengenes database (v. gg_13_8). Unassigned OTUs were treated as potential human contaminants, and OTUs classified as streptophytes were assumed to result from plant pollen were removed as well. Finally, samples with read counts of less than 400 were excluded from all downstream analysis with the exception of comparison to controls. A second OTU table was generated including samples from the American Gut (AG) Project (http://americangut.org) using the same parameters, but with shorter read lengths. This data set was used for α and β diversity comparisons and for taxonomic comparisons between sites. The AG samples were filtered according to the following parameters: age (18+ years), antibiotics (none in prior 6 months), quinolone (no), country (United States or Canada), pregnancy (no), body mass index (18–30 kg/m^2), skin condition (none), nitroimidazole (no), smoking frequency (nonsmoker), and alcohol frequency (nondaily). Following filtering for read depth, 831 adult fecal samples from the AG Project remained in our data set.

α Diversity was calculated using the Chao1 metric, on a data set rarefied to a depth of 2000 reads per sample. Comparisons of diversity between sites were performed using the QIIME compare_alpha_diversity.py python script on 10 replicates of rarefied data sets, and using the Monte Carlo permutations. β Diversity distances were estimated using QIIME using the weighted UniFrac method, and principal coordinate analysis (PCoA) plots were generated using QIIME. The R vegan library was used to perform PERMANOVA comparisons between PD fecal samples and AG fecal samples on a distance matrix limited to fecal samples. Taxonomic summary tables were generated at all ranks using QIIME, and LEfSe was used to predict taxonomic biomarkers both between sites and between clinical conditions using correction for multiple independent comparisons. Biomarker taxa were further limited to those present at levels higher than 0.01 on average within the group of samples under consideration.

To evaluate whether laboratory contamination contributed to our sequencing results, extraction controls without clinical samples and "no template" PCR controls were analyzed with each set of samples. As expected, environmental contaminants were commonly observed. *Pseudomonas* was the most abundant contaminant. Among the contaminants were also species of Enterobacteriaceae, although at much lower levels than present in clinical samples. A heat map and Hclust clustering of all samples by taxonomic composition placed nearly all controls in a single cluster (see Supplemental Digital Content 1, Fig. S1, http://links.lww.com/MPA/A534) distinct from clusters containing clinical samples. Furthermore, the abundance

of *Pseudomonas* or *Enterobacteriaceae* did not correlate with the abundance of these taxa within corresponding negative controls.

RESULTS

Patient Cohort

Clinical information for all study subjects is shown in Table S1 (Supplemental Digital Content 2, http://links.lww.com/MPA/A535). The median age of the study population was 66 years. Of the 50 patients, 11 underwent an open PD procedure, and 39 underwent robotic-assisted procedures. Surgical pathology confirmed a cancer diagnosis in all but 5 study subjects. Eight patients (16%) developed a postoperative pancreatic fistula, and 8 patients (16%) developed surgical site infections requiring treatment. Survival at 1 year was 84%.

We collected 40 postoperative fecal samples and 149 total swabs (pancreatic fluid, bile, or jejunal contents) from the 50 study subjects. Many samples could not be analyzed because of insufficient DNA yield or inadequate number of sequencing reads after preparation of 16S rRNA amplicons. The final number of samples included in this analysis (see Table S2 in Supplemental Digital Content 2, http://links.lww.com/MPA/A535) is 36 fecal samples and 22, 26, and 17 pancreatic duct, bile duct, and jejunal samples, respectively.

Quantitative PCR Demonstrates Presence of Bacterial DNA in Intraoperative Pancreatic and Biliary Samples

We performed droplet digital PCR with a universal bacterial primer to detect bacterial DNA in representative sets of samples from 6 patients. These patients were selected because samples from each body site passed quality filtering, and thus, it was possible to make comparisons across body sites. As expected, fecal samples contained far more bacterial DNA than other environments, and there was a statistical significance across sample types (Kruskal-Wallis test $P = 1.328e-09$) (Fig. 1). Post hoc Mann-Whitney U tests with Bonferroni correction showed all sample types including pancreatic duct fluid contained more bacterial DNA than negative controls used for reference ($P < 0.05$). The microbial burden within swabs of bile and jejunal contents varied significantly among patients. Interestingly, the density of bacterial DNA per swab was not significantly different among pancreas, bile, and jejunal samples. These results document that pancreatic duct fluid in PD patients contains microbial populations similar in density to that seen in the jejunum and biliary tree.
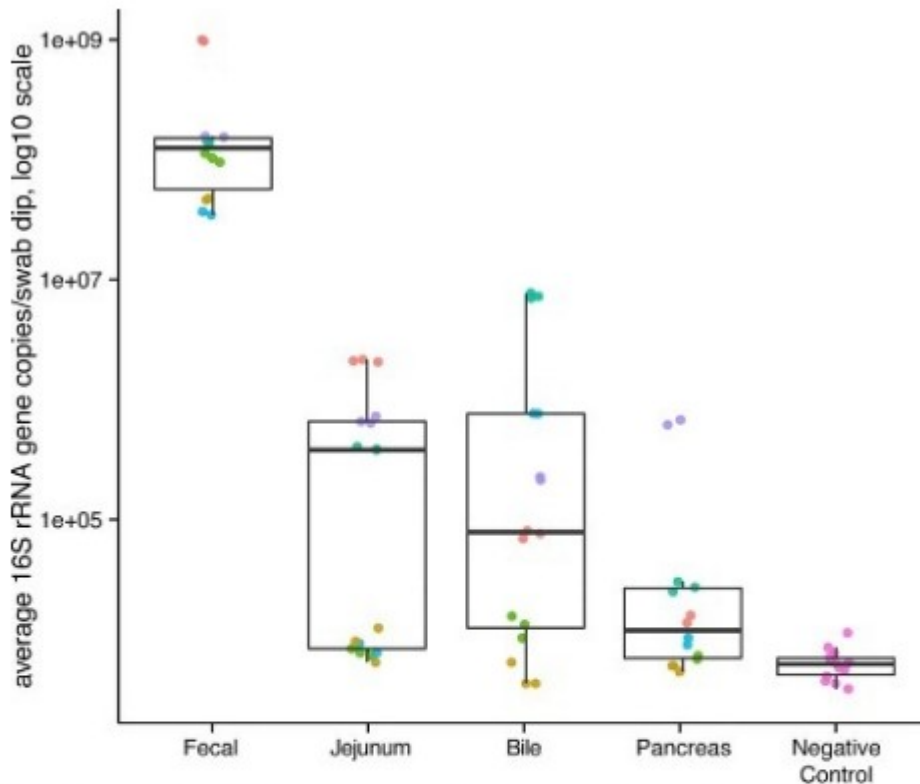
FIGURE 1. Bacterial load per sample assessed via droplet digital PCR counts. A highly conserved region of the bacterial 16S rRNA gene was targeted for biomass quantification via droplet digital PCR. Dots represent counts from technical replicates, which are plotted behind a standard box plot. Samples were analyzed from 6 patients with complete sample sets, that is, patients for whom quality DNA sequencing reads were obtained from all body sites (fecal, pancreas, bile, and jejunal samples). Different colors represent samples from different patients.

Microbial Diversity Across Body Sites

Alpha diversity is a measure of how many taxonomic groups are present in a sample and how evenly the abundance of these groups is distributed.[12] Using data from the AG Project (http://americangut.org), we found that α diversity within PD fecal samples (Fig. 2) was significantly lower than samples from participants in the AG Project (Chao1 metric, nonparametric test, t stat -11.35, $P = 0.01$). Similarly, α diversity was significantly lower in pancreas, biliary, and jejunal samples in comparison to PD and AG fecal samples ($P \leq 0.01$). No significant difference in diversity was observed between the intestinal, bile, and pancreatic groups ($P > 0.05$).
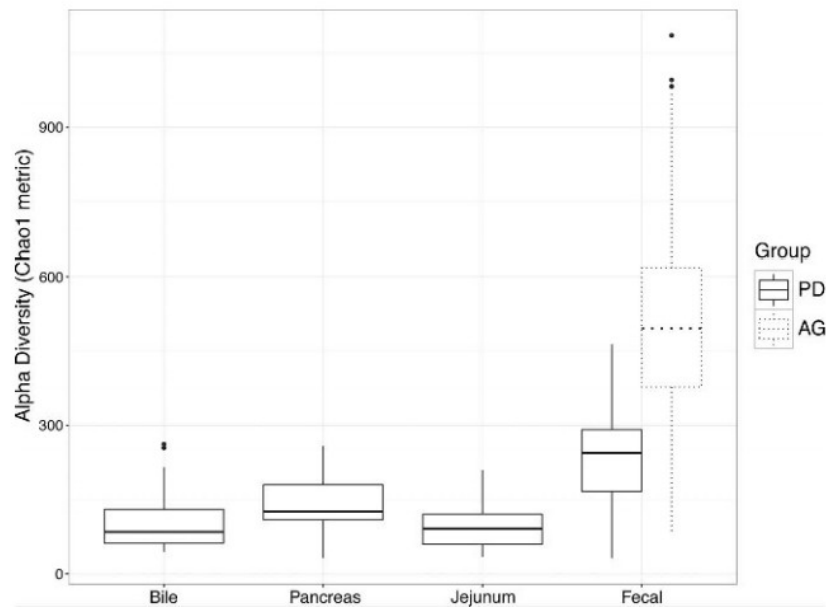
FIGURE 2. α Diversity comparisons of microbial communities within samples from PD patients and adult citizen scientists participating in the AG Project. Shown are the Chao1 indicators for each sample group. Diversity is highest in fecal samples from both PD patients and AG participants. Lower diversity is observed in the pancreas, bile, and jejunum samples.

To compare the composition of microbial communities, we calculated weighted UniFrac distances, an accepted measure of β diversity.[13] Community composition differed significantly between fecal samples from AG participants and PD patients, although with a very small effect size (PERMANOVA, $P < 0.05$, pseudo-F = 16.9, R^2 = 0.02). Principal coordinate analysis produced a cluster of PD fecal samples that grouped together apart from the pancreas, bile, and intestinal samples (Fig. 3A). Community membership within pancreas, bile, and jejunum samples overlapped significantly (Fig. 3B).
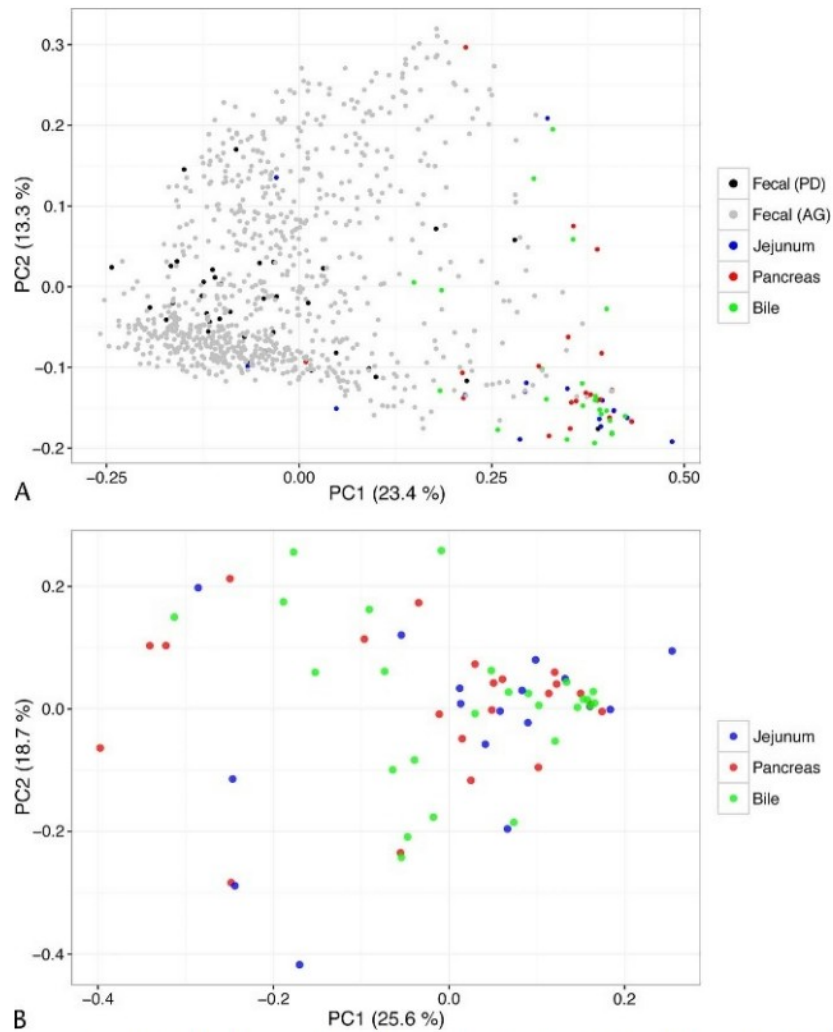
FIGURE 3. β Diversity comparisons of microbial communities within samples from PD patients and AG participants. Displayed are principal component analyses of weighted UniFrac distances between (A) all samples analyzed, including fecal samples from AG participants, and (B) intraoperative samples from the pancreas, bile, and jejunum of PD patients. Axis labels indicate the proportion of variance explained by each principal coordinate axis. In A, fecal samples from AG participants and fecal samples from PD patients cluster separately within PCoA space (PERMANOVA, $P < 0.001$, $R = 0.02$). As shown in B, samples from the pancreas, bile, and jejunum overlap significantly within PCoA space rather than clustering according to body site.

Taxonomic Features of Bacterial Communities in Fecal, Pancreas, Bile, and Jejunal Samples

As seen in other studies, fecal samples from PD patients and healthy volunteers were generally dominated by the phyla Bacteroidetes and Firmicutes (Fig. 4 and Table S3 in Supplemental Digital Content 2, http://links.lww.com/MPA/A535). However, the PD fecal samples were markedly enriched with sequences from the genera *Klebsiella*, *Bacteroides*, and *Parabacteroides*. The PD samples were significantly depleted of sequences from many commensal anaerobic taxa important for intestinal health. At the taxonomic level of genus, we found that PD fecal samples were depleted of *Roseburia*, *Blautia*, and *Faecalibacterium*, which are anaerobic taxa associated with production of short-chain fatty acids via

fermentation. Samples from healthy volunteers contained a higher abundance of *Escherichia* and *Pseudomonas* than did PD patients.
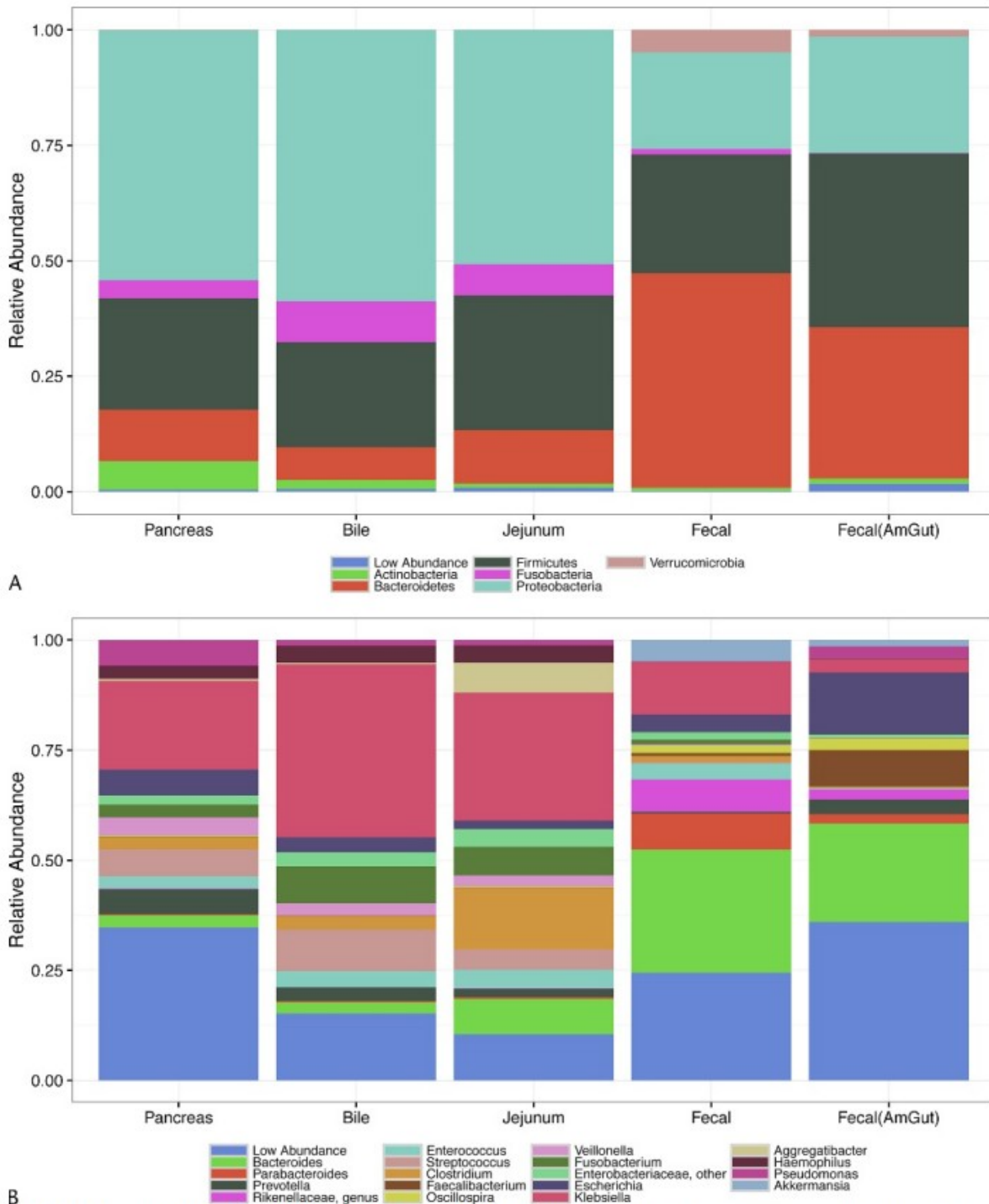
FIGURE 4. Mean relative abundances of bacterial taxa in samples from PD patients and AG participants. Taxonomic assignments are shown at the level of (A) phylum and (B) genus. Note the expansion of *Klebsiella* (phylum Proteobacteria) in all PD samples relative to fecal samples from AG citizen scientists. A complete list of taxa found to be enriched or depleted within PD samples is provided in Supplemental Table 4, http://links.lww.com/MPA/A535.

The observed changes within PD fecal samples could be a manifestation of perioperative antibiotic exposure and bowel rest. These samples were collected after a median of 5 days following surgery (Table S2 in Supplemental Digital Content 2, http://links.lww.com/MPA/A535), and the abundance of *Klebsiella* in the postoperative fecal samples generally correlated with postoperative antibiotic exposure (see Fig. S2 in Supplemental Digital Content 3, http://links.lww.com/MPA/A536). However, we also found that intraoperative pancreas, bile, and jejunal samples were enriched with the same taxa, notably *Klebsiella*, suggesting that these taxa were present at the time of surgery. Taken together, these findings support the conclusion that pathogenic taxa such as *Klebsiella* were present at the time of surgery and later grew in abundance in the postoperative period in some patients. This growth was possibly related to postoperative antibiotic exposure. Importantly, we found that negative controls (containing no patient sample) also contained a low abundance of *Klebsiella*. As noted, however, detailed analysis of negative controls (see Fig. S1, Supplemental Digital Content 1, http://links.lww.com/MPA/A534) suggested that contamination was not the source of these taxa within patient samples.

By comparing PD samples across multiple body sites (Fig. 4 and Table S3 in Supplemental Digital Content 2, http://links.lww.com/MPA/A535), we found that bile samples were enriched with sequences from the genera *Fusobacterium* and *Streptococcus*, and pancreas samples were enriched with the pathogen *Acinetobacter*. Fecal samples were enriched with *Ruminococcus*, *Akkermansia*, *Bacteroides*, and *Parabacteroides*. There were no taxa that were specifically enriched or depleted in the jejunal samples.

Associating Microbiota Profiles With Clinical Features

To better understand relationships between clinical variables and the microbiome, we examined associations between microbiome data and 4 specific patient variables of interest: preoperative biliary stent placement, neoadjuvant chemotherapy, postoperative pancreatic leak, and death at 1 year. Each of these univariate comparisons was performed with LEfSe.[14] We found that pancreatic leak was strongly associated with increased abundance of *Klebsiella* and a decreased abundance of the commonly observed commensals *Parabacteroides* and *Ruminococcus* in postoperative fecal samples (Fig. 5 and Table S4 in Supplemental Digital Content 2, http://links.lww.com/MPA/A535). Pancreatic leak was also associated with decreased abundance of the commensal anaerobe *Bifidobacterium* within pancreatic fluid (Figs. 5A, B). In addition, we found that preoperative stent placement for biliary obstruction was associated with increased abundance of *Acinetobacter* and *Sphingobium* in pancreatic samples and decreased abundance of *Haemophilus* within bile (see Table S4 in Supplemental Digital Content 2, http://links.lww.com/MPA/A535). Neoadjuvant therapy was associated with decreased *Bifidobacterium* in pancreatic fluid, increased *Bacteroides* and *Megasphaera* within bile, and

increased *Clostridium* and *Enterococcus* within fecal samples. Interestingly, death at 1 year was associated with decreased *Klebsiella* within fecal samples.
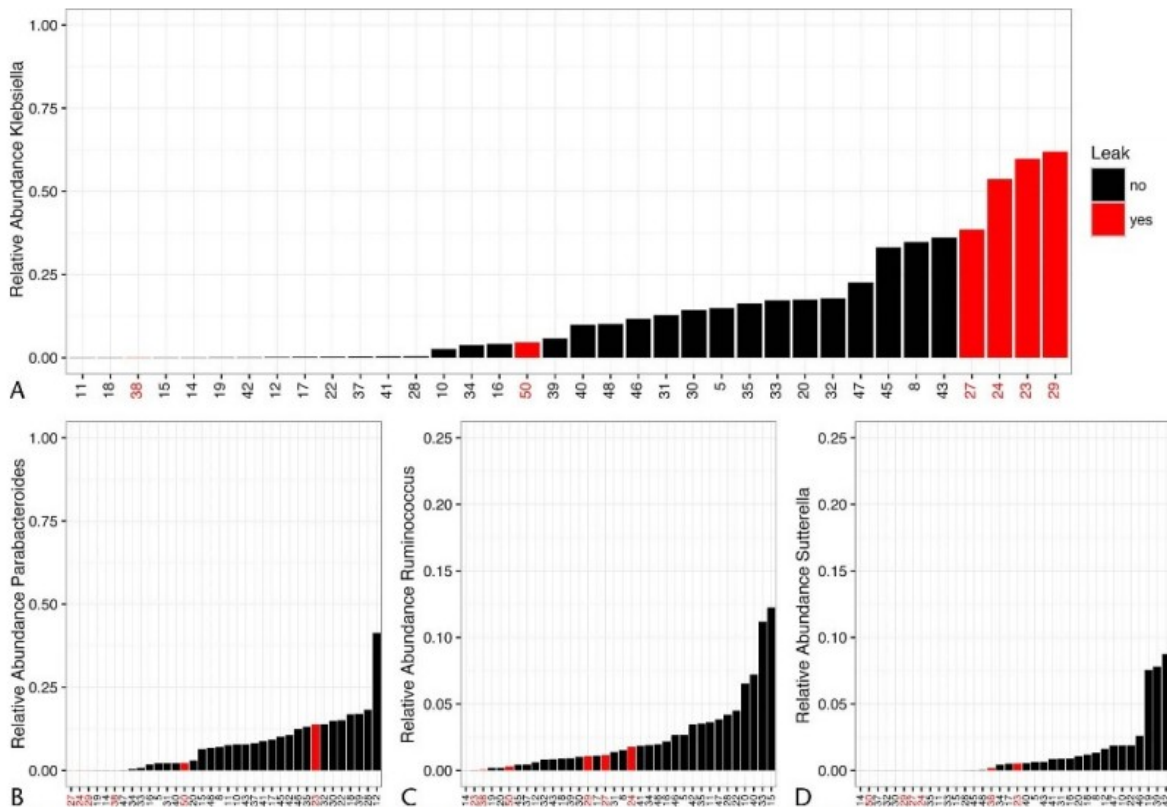

FIGURE 5. Abundance of bacterial taxa in fecal samples from PDA patients with and without postoperative pancreatic fistula. Shown is the abundance of *Klebsiella* (A), *Parabacteroides* (B), *Ruminococcus* (C), and *Sutterella* (D) in fecal samples 42 PDA patients without fistula, and 8 PDA patients with fistula. For both genera, the mean relative abundance among patients with and without fistula was significantly different ($P < 0.05$).

DISCUSSION

The convergence of personalized medicine and awareness about the human microbiome has naturally led to efforts to identify microbial factors associated with clinical outcomes.[15] Important studies have shown that configuration of the microbiota can be linked to tumor responsiveness during chemotherapy,[3,16] survival after stem cell transplantation,[17] and weight loss after Roux-en-Y gastric bypass.[18] In line with these concepts, we theorized that an individual's short- and long-term outcomes after PD may be similarly affected by the status of the microbiota. In particular, the notion that the microbiota contributes to the pathogenesis of pancreatic fistula represents a novel way of thinking about an old problem. Some evidence for this concept was provided in a recent article demonstrating the frequent presence of bacteria in fluid from peritoneal drains of patients who later developed postoperative pancreatic fistula.[19]

The most striking finding from this study was an unusually high abundance of the gram-negative pathogen *Klebsiella* in samples from PD patients,

particularly among fecal samples from PD patients who proceeded to develop a pancreatic fistula. Interestingly, an abundance of *Klebsiella pneumoniae* in fecal samples has been identified as a putative triggering factor involved in the pathogenesis of Crohn disease and ankylosing spondylitis.[20] The mechanism linking *Klebsiella* with autoimmunity involves molecular mimicry, whereby patients harboring *Klebsiella* generate cross-reactive antibodies against HLA antigens and collagen molecules.[21] We speculate that leakage from a pancreaticojejunal anastomosis could involve microbiota-induced generation of cross-reactive antibodies that contribute to collagen degradation. Such a mechanism would be analogous to a process recently described in anastomotic leakage after colon surgery.[2] Notably, we also observed that fecal samples from patients with a leak harbored very little if any *Parabacteroides* and *Ruminococcus*, commensal anaerobes with anti-inflammatory properties.[22] We propose that adverse events after PD may reflect a high abundance of proinflammatory microbes as well as a corresponding absence of anti-inflammatory commensals. Prior studies have indicated that alterations of the gut microbiota can be strongly associated with disturbances of systemic immunity and host metabolism.[23,24]

Surprisingly, we found highly similar microbial communities within the pancreas, bile, and jejunum of PD patients. This may be explained by bacterial spread during a preoperative procedure (namely, endoscopic retrograde cholangiopancreatography) or reflux from the proximal gastrointestinal tract into the diseased biliary tree and pancreas. At each of these locations, we observed a preponderance of *Klebsiella*. *Klebsiella* species (most commonly *K. pneumonia* and *Klebsiella oxytoca*) are ubiquitous gram-negative organisms that cause nosocomial infections in humans[25] and commonly harbor genes for antibiotic resistance.[26] Several prior studies of the microbiology of the jejunum,[18] biliary tree,[8,27-31] and pancreas[7] also identified an abundance of *Klebsiella* in patients with gallstone disease, cholangitis, and cancer. This pattern of results suggests that conditions within the pancreas and biliary tree are favorable for colonization by *Klebsiella* species, particularly in the setting of disease. Many pancreas, bile, and jejunal samples in this study were also enriched with Fusobacteria. Species from the phylum Fusobacteria (particularly *Fusobacterium nucleatum*) are commonly observed in the oral cavity of individuals with periodontal disease, and recent mechanistic evidence has linked *F. nucleatum* to carcinogenesis.[32] Thus, as suggested by epidemiologic links between the oral microbiota and pancreatic cancer development,[33,34] it may be that *Fusobacterium* contributes to pancreatic cancer pathogenesis in some patients.

We observed substantive differences between the microbiota identified in postoperative fecal samples from PD patients and well-established profiles of the microbes within fecal samples from participants in the AG Project. This result may reflect the simple fact that PD samples were collected after

patients had received broad-spectrum antibiotics and had been unable to eat regular food for several days. However, it may also suggest that pancreatic cancer patients harbor distinct patterns of colonic microbiota, as has been reported for colorectal cancer patients (most notably, an increased abundance of *F. nucleatum*).[35,36] Thus, the microbial composition of postoperative fecal samples may have predictive value in identifying patients at high risk of pancreatic cancer. Future studies will be required to tease out these possibilities. Regardless, it is clear that the postoperative fecal microbiota deviates sharply from the patterns of microbes generally regarded as normal in healthy individuals. Notably, there is a depletion of strict anaerobes (*Bacteroides*, *Roseburia*, and *Faecalibacterium*) that are known to contribute to overall health partly by exerting systemic anti-inflammatory effects. In addition, there is an apparent expansion of some taxa from the phylum Proteobacteria, and it is well recognized that a bloom of Proteobacteria in the gut represents an unstable configuration of the gut microbiota.[37]

In conclusion, we have shown that clinically relevant populations of bacteria exist in pancreas, bile, and jejunal contents of patients undergoing PD, and we have shown that the gut microbiome is highly abnormal in the postoperative period. Although the human microbiome is generally resilient in healthy volunteers,[38] it is not yet known
whether microbiome abnormalities in hospitalized patients are transient or permanent. A limitation of the current study was the large number of swabs that did not generate 16S rRNA gene sequencing data—potential explanations for these sequencing failures include low microbial biomass within those specimens, variation in the amount of fluid captured on individual swabs and the possible presence of inhibitors that interfered with PCR reactions. Further investigation with a larger sample size should make it possible to evaluate these possibilities and improve experimental protocols. It will also be possible to formally test the hypothesis that differences in these communities impact surgical complications such as PF formation. If this were true, then there may be a role for preoperative and postoperative testing of the microbiome. Discovery of abnormal microbiome profiles may lead to interventions to specifically modify or "rescue" the microbiome on a personalized basis.

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