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Diversity of Duodenal and Rectal Microbiota in Biopsy Tissues and Luminal Contents in Healthy Volunteers

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The diverse microbial communities that colonize distinct segments of the gastrointestinal tract are intimately related to aspects of physiology and the pathology of human health. However, most recent studies have focused on the rectal or fecal microbiota, and the microbial signature of the duodenum is poorly studied. In this study, we compared the microbiota in duodenal and rectal samples to illustrate the characteristic microbial signatures of the duodenum in healthy adults. Nine healthy volunteers donated biopsies and luminal contents from the duodenum and rectum. To determine the composition and diversity of the microbiota, 454-pyrosequencing of bacterial 16S rRNA was performed and multiple bioinformatics analyses were applied. The α-diversity and phylogenetic diversity of the microbiota in the duodenal samples were higher than those of the rectal samples. There was higher biodiversity among the microbiota isolated from rectal biopsies than feces. Proteobacteria were more highly represented in the duodenum than in the rectum, both in the biopsies and in the luminal contents from the healthy volunteers (38.7% versus 12.5%, 33.2% versus 5.0%, respectively). *Acinetobacter* and *Prevotella* were dominant in the duodenum, whereas *Bacteroides* and *Prevotella* were dominant in the rectum. Additionally, the percentage of OTUs shared in biopsy groups was far higher than in the luminal group (43.0% versus 26.8%) and a greater number of genera was shared among the biopsies than the luminal contents. Duodenal samples demonstrated greater biological diversity and possessed a unique microbial signature compared with the rectum. The mucosa-associated microbiota was more relatively conserved than luminal samples.

Keywords: Microbiota, duodenum, rectum, biopsy, mucus, feces

Introduction

Approximately 10^{14} bacteria live in the human gastrointestinal (GI) tract [2], which plays an essential role in human health and disease. Certain bacteria have been found to be associated with diseases such as inflammatory bowel disease [14], irritable bowel syndrome (IBS) [7], colorectal carcinoma [32], and even systemic diseases such as diabetes [5], obesity [24], and psychological disorders [6]. Most studies have targeted the microbiota of the large intestine and the feces because these samples are easily collected. Conversely, samples from the duodenum are difficult to access and knowledge of the microbiota in the duodenum is limited [39]. The duodenal microbiota is complex because the duodenum is located at a strategic crossroads between the acid-secreting stomach and the nutrient-absorbing jejunum and ileum [28]. They may participate in multiple processes and functions of the upper GI tract and thus exert a profound impact on various aspects of host physiology [13]. The duodenal microbiota was closely associated with various diseases such as small intestinal bacterial overgrowth (SIBO) and celiac disease [38]. SIBO has close relationships with obesity [22], cirrhosis [15], and IBS [38]. However, a fundamental problem of SIBO is the

lack of a universally accepted and applied gold standard for its diagnosis [27]. One potential method is the application of molecular microbiological methods to characterize the small intestine microbiome, which may truly permit discrimination between what is normal and what is abnormal. However, the microbial signature of the duodenum in healthy persons must first be characterized.

Although several studies [12, 17, 30, 31, 37] included normal controls and some information about the duodenal microbiota were obtained, the methods to study the microbiota that were applied in these studies, such as denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH), microarray analysis, real-time quantitative PCR (qPCR), and terminal restriction fragment length polymorphism (T-RFLP), are relatively limited. These culture-independent molecular techniques may target the dominant members of microbial communities and omit information for relatively less-abundant microbes [35]. However, recent advances in sequencing technology, such as the 454-pyrosequencing approach, which is based on the production of light from luciferase for the detection of individual nucleotides added to nascent DNA, address these limitations and provide sufficient microbial information to illustrate characteristic microbial signatures in a single sample [23].

In the present study, we obtained microbiota samples from healthy adult mucosal biopsies and luminal contents and applied high-throughput 16S rRNA gene sequencing to illustrate the characteristic microbial signature of the duodena of healthy adults. We believe that our data can provide information for the further investigation of the duodenal flora in basic and clinical studies.

Materials and Methods

Study Subjects and Sampling

The nine volunteers in this study were 21 to 51 years of age (6 males and 3 females) and were healthy with no gastrointestinal symptoms or known diseases. Their body mass indices ranged from 19 to 24, and they had not used antibiotics or drunk beverages containing probiotics for the three months prior to sample collection (for detailed information, see Table S1). This study was approved by the Institutional Ethical Review committee of Huazhong University of Science and Technology, and all individuals provided written informed consent prior to sample collection.

Duodenal luminal content (mucus) for sequencing was obtained endoscopically following a procedure described previously [29]. Briefly, a sterile catheter that was 230 cm long and 2.5 mm in width (Olympus, Japan) was introduced through the working channel of the endoscope after the endoscope had reached the distal duodenum (location approximately 5 to 10 cm below the major duodenal papilla) and was advanced to the duodenal wall. A sterile 50 ml syringe was applied to suction 1 ml of intestinal fluid, which was transferred immediately to a sterile Eppendorf tube (Eppendorf, Germany).

Fresh feces was collected immediately from the subjects; we sampled the upper layer of feces to avoid contamination and all subjects underwent a colonoscopy without a laxative preparation. A total of 1 g of feces was stored in a 1.5 ml Eppendorf tube and frozen at −80°C for sequencing.

Duodenum biopsy samples were collected in a sterile 1.5 ml Eppendorf tube. The location of the mucosal biopsies in the duodenum was approximately 5 to 10 cm below the major duodenal papilla. Four pieces (approximately 18 mg) of mucosal tissue were harvested by biopsy. Three were stored in 0.05 M potassium phosphate buffer (pH 7.0) and the final piece was stored in phosphate-buffered saline (containing, per liter, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 (pH 7.2)) with 4% paraformaldehyde. The biopsy forceps were sterilized before each biopsy was performed. The rectal biopsy samples were obtained from the rectum (10 cm above the anus) and had no visible feces on the surface of the mucosa. The procedure was identical to the procedure for biopsies from the duodenum. Tissue samples from the duodenum and rectum were ground in a sterile homogenizer in 1 ml of bacterial preservation solution (2.0 g of peptone, 9.0 g of NaCl, 0.3 g of L-cysteine, and 1,000 ml of distilled water (pH 7.2)) and were frozen at −80°C for sequencing.

DNA Isolation

Total bacterial DNA was extracted from biopsies and the luminal contents of the intestine according to the manufacturer's instructions using the FastDNA SPIN Kit (Tiangen, Beijing, China). PCR amplification of bacterial 16S genes (V1−V3 regions) was performed using universal primers as described previously [42] (forward: 5′-AGAGTTTGATCCTGGCTCAG-3′; reverse: 5′-TTACCGCGGCTGCTGGCAC-3′) and fusion primers (forward: 5′-454adapter-mid-AGAGTTTGATCCTGGCTCAG-3′; reverse: 5′-454adapter-TTACCGCGGCTGCTGGCAC-3′). PCR mixtures contained 16.375 µl of distilled water, 2.50 µl of 10× buffer, 2.5 mM dNTPs, 10 µM of each primer, 0.125 µl of Takara Pyrobest polymerase (Takara Biotechnology Co., Ltd, Japan), and 2 µl of DNA template in a final volume of 25 µl. The PCR was performed as follows: denaturation at 94°C for 4 min, followed by 27 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were separated by electrophoresis through a 1.5% agarose gel in 1× TAE and purified from the gel using the Qiagen QIAquick Gel Extraction Kit (Qiagen Gmbh, Germany). The product pool was analyzed using a 454/Roche GS FLX at Personal Biotechnology Co., Ltd. (Shanghai, China). The sequence reads were separated based on sequence length for the 36 different intestinal samples.

Operational Taxonomic Unit-Based Sequence Analysis

The analysis of sequence reads was carried out using the QIIME pipeline as described in a previous study [7]: sequences with lengths less than 200 nt or greater than 1,000 nt, with a mean quality score of less than 25, with ambiguous bases greater than 1, with homopolymer lengths greater than 6, or with maximum primer mismatches greater than 0 were eliminated. Similar sequences were clustered with a threshold of 97% sequence identity into operational taxonomic units (OTUs) and the reads were identified as taxonomies using the Ribosomal Database Project classifier.

Community Analyses

The number of observed OTUs was applied to determine the species richness for each community. Rarefaction curves of observed and Chao1 estimated species richness were plotted using the QIIME pipeline [7]. Venn diagrams were generated to represent the unique species and percentages (%) of overlapping species in samples. Shannon's diversity and the rank abundance curve were used to compare the diversity of microbiota between different groups. Commonness and rarity of species were demonstrated by species abundance distributions annotated with phylum- and genus-level taxonomies. The less stringent method of Benjamini and Hochberg was used to assess bacterial identity at the species and genus levels. Principle coordinate analysis (PCoA) was performed on the communities with the most abundant bacterial OTUs to determine variation of the bacterial communities between samples from the duodenum and rectum.

Results

Overview of Pyrosequencing Data in Samples from the Duodenum and Rectum

A total of 354,563 high-quality sequences were obtained from the biopsies and luminal contents from the duodenum and rectum, with an average of 11,119 (±1,640 SD) reads for each duodenal biopsy sample, 11,321 (±2,281 SD) for each duodenal luminal sample, 9,321 (±2,217 SD) for each rectal biopsy sample, and 7,633 (±1,070 SD) for each rectal luminal sample. All pyrosequencing reads were subjected to OTUs and different numbers of OTUs were obtained from different group samples as shown in Table 1. A rarefaction analysis was carried out to determine whether all the OTUs present in the datasets had been sufficiently recovered in our study. Each rarefaction curve showed a similar pattern, reaching a plateau and a saturation phase, which verified that most of the species present in each sample from four groups were observed (Fig. 1). Good's coverage index was used to estimate the completeness of each sample *via* a probability calculation based on a randomly selected amplicon sequence. Chao1 and ACE were used to assess the abundance of OTUs, and relative data are shown in Table S2.

Higher Microbial Community Diversity in the Duodenum than in the Rectum

To estimate the diversity of the microbial communities in the biopsies and the luminal contents of the duodenum and rectum, analyses of α-diversity, which is represented by Shannon's diversity and phylogenetic diversity, were applied. The data revealed that Shannon's diversity indices of the microbiota in duodenal biopsies (4.50 ± 0.33) and mucus (4.38 ± 0.27) were significantly higher than in rectal biopsies (3.95 ± 0.47) and feces (3.39 ± 0.51) (all $p < 0.01$). The α-diversity values of the two duodenal groups did not differ significantly from each other ($p > 0.05$); however, the α-diversity of the rectal biopsies was higher than that of feces ($p < 0.01$) (Fig. 2A). Similarly, the data for phylogenetic diversity revealed that the microbiota of the duodenum was more diverse than that of the rectum; no difference in diversity was observed between duodenal biopsies and mucus, although a higher diversity was found in rectal biopsies than in feces (Fig. 2B). To further confirm that the diversity of the duodenal microbiota was higher than that of the rectum, rank abundance curve analysis (which is based on the analysis of the relative OTU abundance of each sample, and the diversity of different samples was shown by comparing the length of the curve) of all duodenal and rectal samples was performed. Again, the

Table 1. Overview of pyrosequencing data in samples from the duodenum and rectum.

	Duodenum	Rectum
Total reads	201,965	152,598
Total OTUs in biopsy group	2,049	1,525
Total OTUs luminal group	1,669	1,172
Total phyla	22	17
Total genera	381	230
Genera in the biopsy group	322	229
Genera in the luminal (mucus/feces) group	291	67

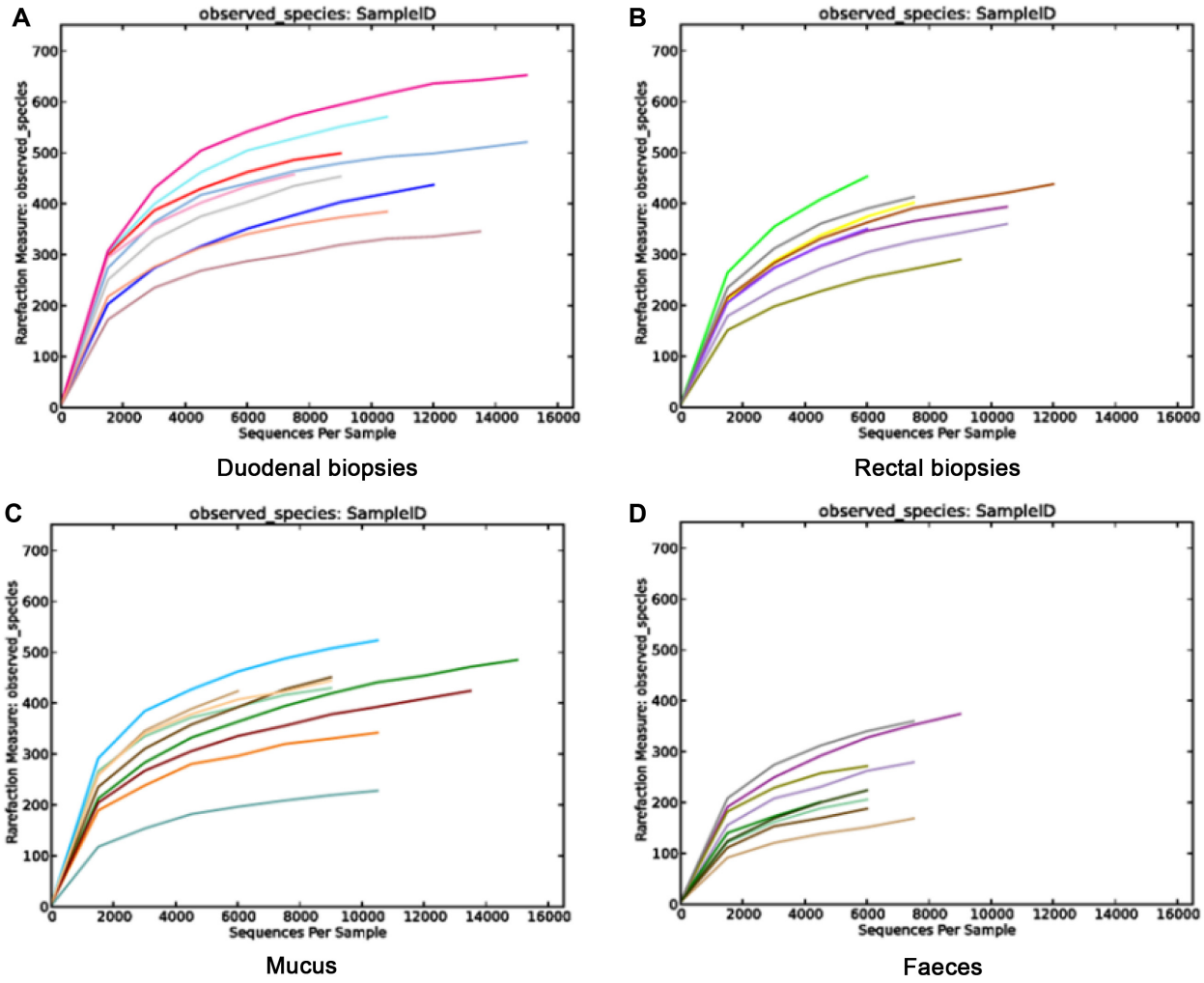

Fig. 1. Rarefaction curves for each sample from four groups calculated at the species level.
(**A**) Duodenal biopsies. (**B**) Rectal biopsies. (**C**) Mucus. (**D**) Faeces. Rarefaction curves were obtained by plotting the number of observed OTUs against the number of cloned sequences. If the curves reach or nearly reach a plateau, this indicates that most of the species present in all samples have been observed.

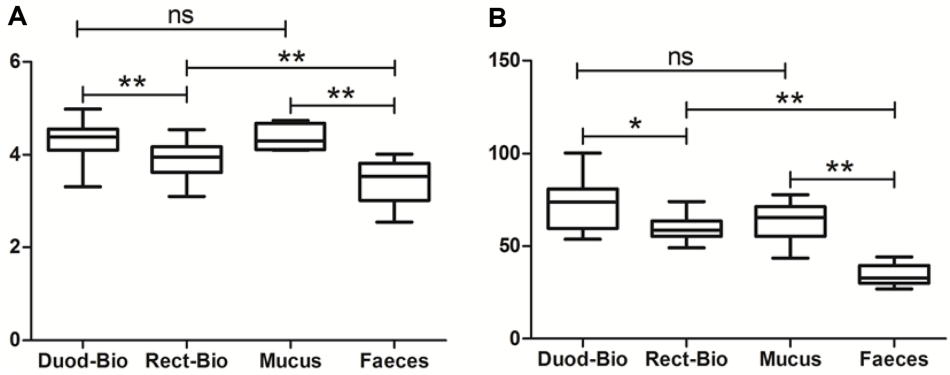

Fig. 2. Higher diversity of the microbiota in the duodenum compared with the rectum.
Comparison of microbiota indices across the four cohorts (Duod-Bio indicates duodenal biopsies, Rect-Bio indicates rectal biopsies). (**A**) Shannon index. (**B**) Phylogenetic diversity (Mann-Whitney tests were performed for each pairwise comparison.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Kruskal-Wallis p-values refer to tests performed across all groups.)

same results were obtained, and the data are shown in Fig. S1.

Differential Microbial Composition in the Duodenum and Rectum

PCoA, which is based on the unweighted UniFrac distances of 16S rRNA sequences, was applied to cluster the microbial populations of samples from the duodenum and rectum. The microbial community of each biopsy sample was separated according to gut location, and the maximum variations were 19.85% (PC1) and 4.96% (PC3) (Fig. 3A), while each luminal content sample was also separated according to gut location and maximum variations and were 19.90% (PC1) and 4.56% (PC3) (Fig. 3B). These data demonstrated that microbial communities of the duodenum were distinct from those of the rectum, both in biopsies and luminal contents.

Differential Composition of Bacteria at the Phylum and Genus Levels

All sequences were identified and subjected to different taxonomic levels (usually at the phylum and genus levels). Twenty-two phyla and 381 genera were detected in the duodenal samples, whereas 17 phyla and 230 genera were detected in rectal samples (Table 1). As reported previously [26, 33], five phyla, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria, commonly encountered in the human intestine specifically, were prevalent in all samples from the duodenum and rectum. However, the proportion of predominant phyla varied between duodenal and rectal samples (Fig. 4). For the duodenum samples, Firmicutes and Proteobacteria phyla predominated and accounted for an average of 70.3% (Fig. 4A); however, Firmicutes and Bacteroidetes phyla predominated and accounted for an average of 84.2% in rectal samples (Fig. 4B). The proportions of Proteobacteria phyla in biopsies and luminal contents of the duodenum and rectum were 38.7% versus 12.5% and 33.2% versus 5.0%, respectively, which suggested that Proteobacteria phyla was more prevalent in the duodenum samples (both $p < 0.01$). Additionally, some rare phyla were only detected in duodenal samples, such as Deferribacteres, OP10, Spirochaetes, SR1, Tenericutes, and Thermotogae, whereas Nitrospira was only found in the rectum (see Table S3). The presence of more types of bacterial phyla in the duodenum than in rectum also suggested higher microbial community diversity in the duodenum than in the rectum.

At the genus level, dominant microbes (at a ratio of more than 1%) were investigated in the duodenum and rectum. Dominant microbes in the duodenal samples differed between the biopsy and mucus samples. The most frequently represented genera in biopsy samples from the duodenum

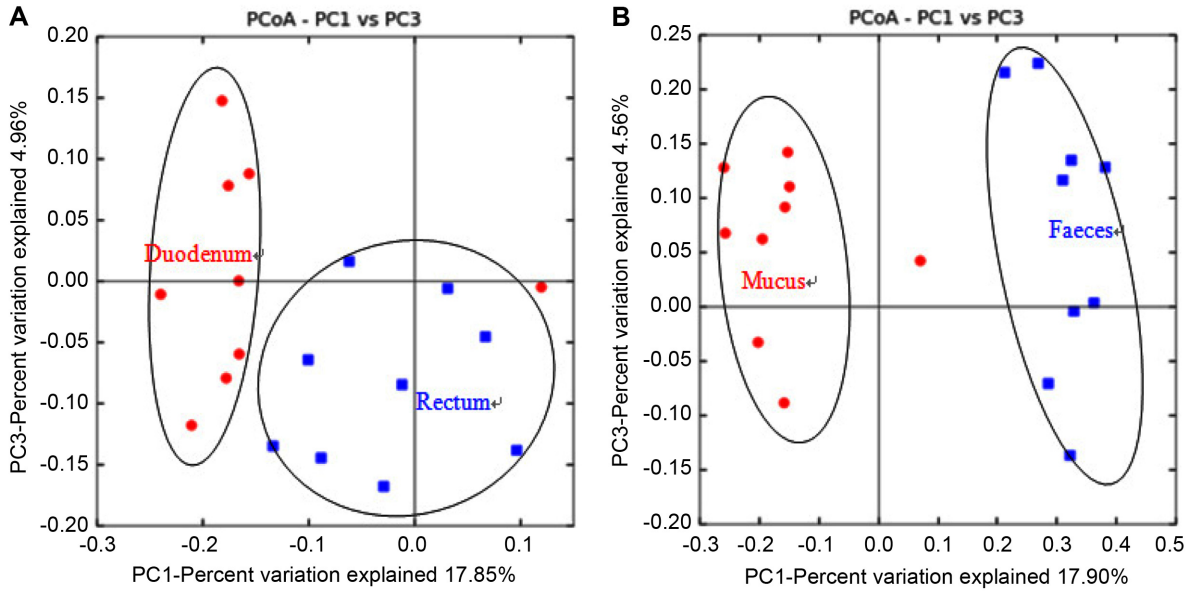

Fig. 3. Differences in microbial communities between duodenal and rectal samples as shown by PCoA based on unweighted UniFrac distances.

(**A**) Plots indicating microbial composition in duodenal (red) and rectal (blue) biopsy samples and luminal samples. (**B**) Plots indicating microbial composition in mucus (red) and feces.

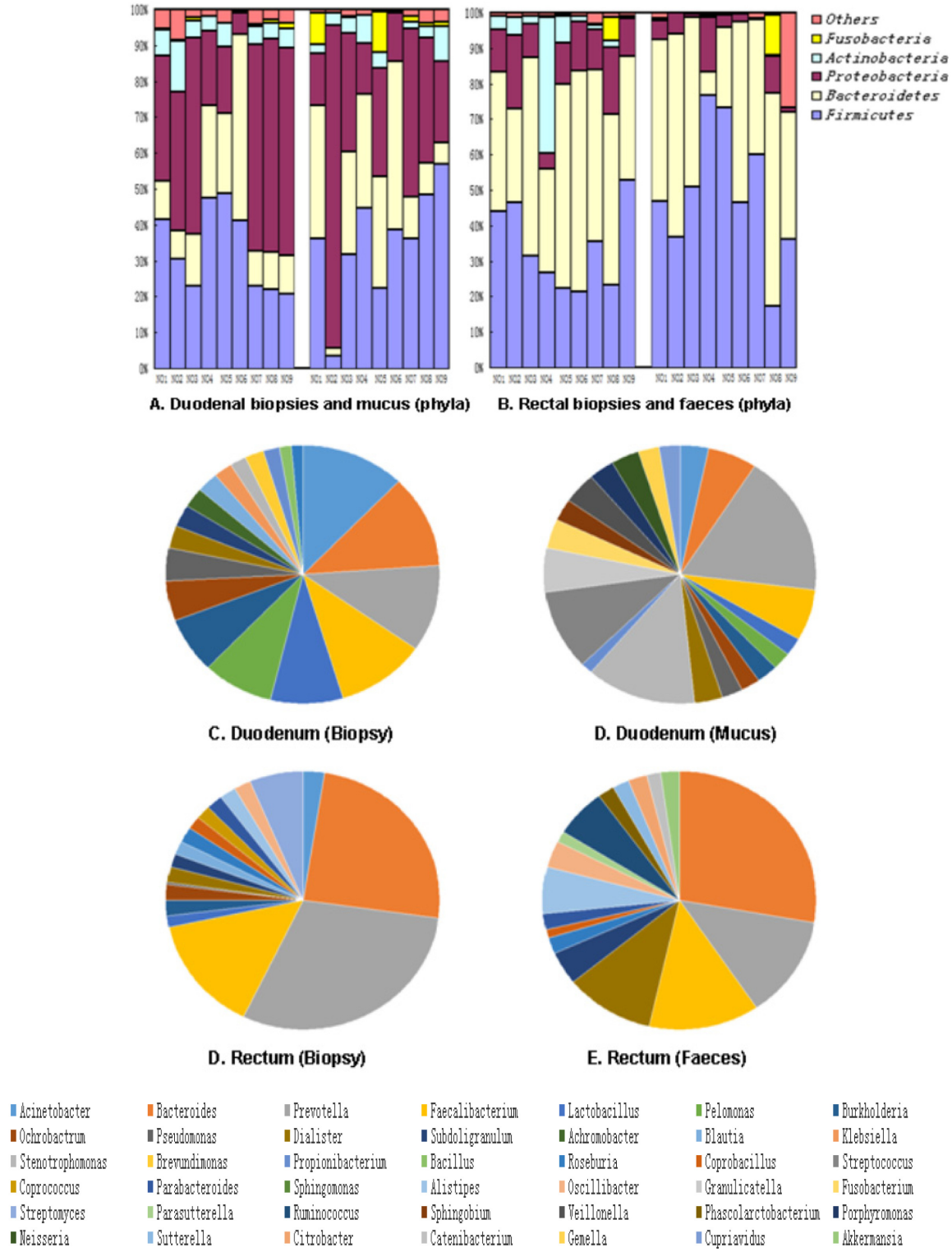

Fig. 4. Composition and relative abundance of dominant microbes at the phylum and genus levels for individual samples. (**A**) and (**B**) Composition and relative abundance of five dominant microbes at the phylum level in duodenal and rectal samples are shown. (**C**–**F**) Composition and relative abundance of dominant microbes at the genus level in biopsies and luminal content samples from the duodenum (**C**, **D**) and rectum (**E**, **F**). NO1–NO9 refer to the individual donors.

were *Acinetobacter*, *Bacteroides*, and *Prevotella* (accounting for 12.2%, 11.7%, and 10.8%, respectively), and the dominant genera in the mucus were *Prevotella*, *Stenotrophomonas*, and *Streptococcus* (the latter two genera are aerobic) (accounting for 17.8%, 12.9%, and 10.0%, respectively) (Figs. 4C and 4D). However, for the rectum, known anaerobic microorganisms such as *Prevotella*, *Bacteroides*, and *Faecalibacterium* were predominant in biopsy samples (accounting for 30.0%, 24.8%, and 14.6%, respectively) and feces [33] (accounting for 12.7%, 27.8%, and 13.1%, respectively) (Figs. 4E and 4F). Furthermore, an interesting phenomenon was observed, in that there were 36 genera shared among duodenal biopsies from all 9 subjects and 27 genera shared among mucus samples, as well as 29 genera among rectal biopsies and 8 among faeces (Table S4). Compared with these bacteria, 10 genera, including *Pseudomonas*, *Sphingomonas*, *Fusobacterium*, and others (see Table S4), were more prevalent in the duodenum. These data further indicated that microbes of the duodenum were unique compared with the rectum. Moreover, microbial communities in biopsies were less variable and more conserved than those in luminal contents within an individual.

OTU Overlap across the Duodenum and Rectum within an Individual

The overlap of OTU clusters between duodenal and rectal samples was calculated, and Venn diagrams were used to demonstrate the numbers of shared OTUs (Fig. 5). The number of OTUs shared between duodenal and rectal biopsies was 1,075 (Fig. 5A), and 600 OTUs were shared by mucus and feces (Fig. 5B). The percentage of shared OTUs between duodenal and rectal biopsies (45.3%) was higher than between mucus and feces (26.8%), which also implied that the mucosa-associated microbiota was relatively more conservative compared with luminal microbiota.

Discussion

In this study, we collected two different types of samples (biopsies and luminal contents) to examine the differences in the composition and diversity of mucosa-associated and luminal microbiotas in the duodenum and rectum. *Via* a high-throughput 454-pyrosequencing technique, we were able to determine the diversity of microbiotas, comparing between the duodenum and rectum. Additionally, analysis at the phylum, genus, and species levels using a variety of statistical approaches provided a comprehensive examination of differences in the duodenum and rectum. Our results indicate a greater diversity of microbes in the duodenum both for the mucosa-associated and luminal microbiota compared with the rectum. The microbial community composition in the duodenum and rectum was unique to each location. Additionally, the mucosa-associated microbiota was less variable and more conserved than the luminal microbiota within an individual.

A previous study showed that the total amounts of culturable bacteria in rectal content (feces) were far greater than in duodenal content [11]. Consequently, people might assume a higher diversity in the rectum compared with the duodenum. However, with the 454-pyrosequencing technique and multiple analyses of the diversity of the microbiota, including α-diversity, phylogenetic diversity [10], and rank abundance curve analysis, we found that the diversity of the microbiota is far greater in the duodenum regardless of whether it is the mucosa-associated or luminal microbiota. This finding conflicts with that of Di Cagno *et al.* [12], who compared duodenal mucosa-associated bacteria and fecal bacteria using DGGE analyses and found that PCR-DGGE profiles of fecal samples were richer than duodenal biopsies. However, as Taverniti and Guglielmetti [35] summarized, this method may target the dominant members of microbial

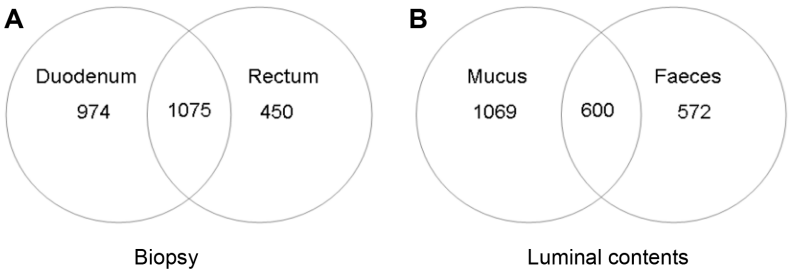

Fig. 5. Venn diagrams demonstrating 97% OTU cluster overlap within mucosal biopsies and mucus/feces samples taken from the duodenum and rectum.

OTU numbers from each group were clustered in a subset. The total numbers of unique and shared OTUs from the nine individuals were clustered and compared for samples taken from the duodenum and the rectum as biopsies (**A**) and luminal contents (mucus and feces) (**B**).

communities and result in missing information for relatively less abundant microbes. Additionally, it might be inaccurate to use different types of sample for comparison. Another study [33] performed 16S rRNA gene sequencing and the authors found that the phylogenetic diversity in duodenal biopsies appeared to be higher than that of colon and stool, but there was no statistical significance given as only four samples were involved. Indeed, our study of nine samples confirmed this observation and found the same result for the luminal contents. Additionally, a higher diversity of mucosa-associated microbiota compared with luminal microbiota (feces) was observed in the rectum, but no similar tendency was observed for the duodenal mucosa-associated and luminal microbiota, which also indicated the greater complexity of the microbiota in the duodenum. An illustrative explanation to understand why this happened is given as follows: the microbiota in the human intestine can be likened to a long-distance marathon-running "athlete" who came from a "nationwide district" (such as the mouth, which has the highest diversity in GI tract [33], food [34], water [18], stomach [38], and even the gallbladder [16]) and underwent harsh selection (primarily from gastric acid and the host immune system [3]) and arrived at the first station, the duodenum, and then started a competition. Given the extent of the intestinal distance and arrival into the complex environment, fewer bacteria survived and arrived in the large intestine, which experiences slower transit of intestinal content and thus confers an advantage for bacterial development. Therefore, a lower diversity but larger numbers of the microbial community are formed in the large intestine.

We identified specific microbes belonging to either the duodenum or rectum at the phylum and genus levels. Composition analysis showed that the most abundant phyla identified in the duodenal and rectal samples were Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria, which are in accordance with previous studies [8, 26]. However, the biggest difference between the duodenum and rectum was that the Proteobacteria, which includes a wide variety of pathogen genera [4], was more highly represented in the mucosa and luminal contents of the duodenum than the rectum in healthy individuals, which was similar to the results of Cheng *et al.* [9]. The role of these decreased numbers of Proteobacteria might be investigated in the future. Additionally, some rare phyla found only in the duodenum, such as Spirochaetes and Thermotogae, were recently found to be potential butyrate producers [36] and might be beneficial for enterocytes. SR1 participates in fermentation [40] and Deferribacteres is phylogenetically proximal to the Proteobacteria [20] and might be related to a high proportion of Proteobacteria in the duodenum. In summary, more studies to investigate the roles of these rare duodenal phyla in human health must be performed in the future.

The compositions of mucosa-associated and luminal microbiotas in the duodenum and rectum were different at the genus level. The dominant bacterial genus in duodenum mucosal biopsy samples was *Acinetobacter*, which includes a group of strictly aerobic species such as *A. baumannii* that is related to nosocomial infections [25]. The ability to detect sequences of potentially harmful organisms such as *Acinetobacter* might have potential diagnostic value and possible prophylactic applications. Although the proportion of *Prevotella* was highest, two aerobic microbes, *Stenotrophomonas* and *Streptococcus,* were highly prevalent in mucus. The microenvironments were suitable for aerobes, and our sequencing data verified that more aerobes dwelled in the duodenum, which was consistent with previous culture results [10]. Two known anaerobic microorganisms, *Prevotella* and *Bacteroides*, were dominant in the rectal mucosa and feces, and these two genera were used to identify different gut enterotypes [1, 21]. However, according to our data, the proportion of *Prevotella* and *Bacteroides* was different in the mucosa and feces, and the application of different samples would result in different human enterotypes. Our data also revealed a greater number of shared OTUs in mucosal biopsies than in luminal contents, indicating that mucosa-associated microbiotas of the duodenum and rectum were more conserved than luminal microbiota, which was similar to the findings of Zhang *et al.* [41], who found that mucosal microbial components at higher taxonomic levels tended to be more stabilized along the intestine. Moreover, the numbers of genera identified in all individuals might demonstrate inter-individual variation, and we found greater numbers of the same genera in mucosal biopsies compared with luminal contents, both in the duodenum and rectum; therefore, we hypothesized that the mucosa-associated microbiota was less variable than the luminal microbiota. Recently, some studies have suggested that it is necessary to rethink the enterotypes for large variation within an individual [19]. The mucosa-associated microbiota might be a candidate for the classification of enterotypes.

Our data indicated positive statistical trends and provide novel information regarding the diversity and composition of the microbiota in the duodenum and rectum, regardless of the small sample size in our study. Duodenum samples showed greater biological diversity compared with rectal samples, although fewer amounts of total bacteria were

obtained from the duodenum than the rectum. Potentially harmful organisms such as Proteobacteria and rare phyla might have greater potential value and possible applications to explore. The characteristic composition of the microbiota from individuals in good health may be useful for understanding microbial variation in health and disease, and more studies must be conducted in the future.

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