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HIV-induced immunosuppression is associated with colonization of the proximal gut by environmental bacteria

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Abstract

Objectives—To evaluate the impact of HIV infection on colonization resistance in the proximal gut.

Design—It was a case-control study.

Methods—We contrasted microbiota composition between 8 HIV-1-infected patients and 8 HIV-negative controls to characterize community alteration and detect exogenous bacteria in the esophagus, stomach, and duodenum as well as the mouth using a universal 16S rRNA gene survey and correlated the findings with HIV serostatus and peripheral blood T-cell counts.

Results—HIV infection was associated with an enrichment of *Proteobacteria* ($p=0.020$) and depletion of *Firmicutes* ($p=0.005$) in the proximal gut. In particular, environmental species *Burkholderia fungorum* and *Bradyrhizobium pachyrhizi* colonized the duodenum of HIV patients who had abnormal blood CD4+ T-cell counts but were absent in HIV-negative controls or HIV patients whose CD4 counts were normal. The two species coexisted and exhibited a decreasing trend proximally towards the stomach and esophagus and were virtually absent in the mouth. *B. fungorum* always outnumbered *B. pachyrhizi* in a ratio of approximately 15 to 1 regardless of the body sites ($p<0.0001$, $r^2=0.965$). Their abundance was inversely correlated with CD4 counts ($p=0.004$) but not viral load. Overgrowth of potential opportunistic pathogens e.g. *Prevotella*,

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Competing interests None.

Contributors All authors meet the criteria for authorship. LY performed the laboratory assays, analyzed the data, and wrote the article; MAP obtained funding, collected samples, and proofed the manuscript; GSF was the study statistician; YM provided assistance in bioinformatics analysis; CN extracted DNA from the samples; JAP organized participant recruitment; ZP designed the study, supervised the laboratory work, interpreted the data, and proofed the manuscript.

Fusobacterium, and *Ralstonia* and depletion of beneficial bacteria, e.g. *Lactobacillus* were also observed in HIV patients.

Conclusions—The colonization of the duodenum by environmental bacteria reflects loss of colonization resistance in HIV infection. Their correlation with CD4 counts suggests that compromised immunity could be responsible for the observed invasion by exogenous microbes.

Keywords

Colonization resistance; HIV-1; CD4; duodenum; microbiome; microbiota; dysbiosis; proximal gut; *Burkholderia fungorum*; *Bradyrhizobium pachyrhizi*; *Lactobacillus*; environmental bacteria

Introduction

The human gut hosts trillions of microbes that outnumber the host cells by a factor of 10 and are known collectively as the gut microbiome. Whereas the abundance of individual members can fluctuate within physiological ranges, the membership of gut microbiome is tightly controlled. Breach of this delicate equilibrium can result in opportunistic infections caused by either invasion of exogenous bacteria or outgrowth of certain endogenous bacteria. In contrast, classical infectious diseases often develop following exposure of susceptible hosts to pathogens, such as *Vibrio cholera* in cholera and *Bacillus anthracis* in gastrointestinal anthrax. The mechanism that protects the host from invasion by exogenous bacteria is termed colonization resistance [1]. Endogenous bacteria can directly preclude exogenous bacteria from stable colonization by competing for essential nutrients [2] and mucosal attachment sites [3], and by inhibiting the growth of exogenous bacteria through the secretion of bacteriocin [4]. Besides endogenous bacteria, the host immune system also plays a critical role in maintaining a stable microbiome. Adaptive immunity induced naturally or via vaccination reduces colonization by pathogenic *Streptococcus pneumoniae* by boosting both cellular and humoral immunity [5, 6]. Complex cooperation between commensal bacteria and host innate immunity governs the homeostasis of the gut microbiota [7], as exemplified in a recent study that enterocytes promote colonization resistance against an intestinal opportunistic bacterium, *Enterococcus faecalis* via epithelial IL-22RA1-mediated fucosylation [8].

HIV infection provides an invaluable landscape for studying colonization resistance owing to its profound effect on both adaptive and innate immunity. However, studies have been limited by the technical challenge of exploring the enormously complex microbiome until recently when advances in high-throughput sequencing technology have made it possible to accurately monitor changes in the entire microbiota by examining the presence and abundance of every microbial taxon in the microbiome. This new technology has revealed that the distal gut (colorectal) microbiome, the focus of most studies, is enriched with *Proteobacteria* but depleted in *Firmicutes* and *Bacteroidetes* after HIV or SHIV infections, similar to the profile observed in inflammatory conditions [9–11]. At the genus level, HIV infection is characterized by enrichment of endogenous bacteria with disease-causing potential at the cost of depleting commensal bacteria [12–15]. The dysbiotic distal gut microbiome, although not directly linked to obvious opportunistic infections, is strongly

associated with microbial translocation, immune activation, and viral persistence [9, 10, 13, 16–19].

Clinical manifestations of proximal gut pathology—such as dyspepsia, nausea, vomiting—have long been observed in HIV-1-infected individuals [20, 21]. A range of opportunistic infections different from those in the distal gut have also been recognized [22]. Before the advent of combination antiretroviral therapy, 50–93% of all patients with HIV disease experienced gastrointestinal symptoms over the course of their illness [23, 24]. Furthermore, opportunistic infections still occur, albeit at a lower rate, despite the use of highly active antiretroviral therapy (HAART) and normalized CD4 counts [25–28]. Clinically, our knowledge of opportunistic pathogens in relation to HIV-related proximal gut problems is rather limited, and clinical signs and symptoms alone rarely suggest a specific etiology. Besides the distal gut microbiome, high-throughput sequencing has been used to examine the effects of HIV infection on the microbiomes in the vagina [29–31], lung [32], mouth [33, 34], Semen [35], and anus [36] as well as in normally sterile body sites such as the brain [37] and blood [38, 39], but not in the proximal gut. Unlike its distal counterpart, the status of microbiome in the proximal gut in HIV infection was only examined recently by analysis of gastric juice samples from only two HIV patients [40]. The absence of related studies reflects, in part, the difficulty of obtaining samples from the proximal gut. These can only be obtained using specialized endoscopic procedures. As such, the effect of HIV infection on the proximal gut microbiome is essentially unknown and most of the clinical complaints related to the proximal gut are simply interpreted as resulting from idiopathic HIV enteropathy. Given the lack of knowledge about the pathogens responsible for HIV enteropathy, treatment is limited to supportive measures, such as HAART, nutritional supplementation, and electrolyte replacements [41].

Our working hypothesis is that proximal gut dysbiosis occurs in HIV patients when their CD4 counts drop below the normal level.

Materials and Methods

Patient recruitment

The study was approved by the Institutional Review Board of the New York University School of Medicine (protocol number: 07-927). All participants provided written informed consent. Eight HIV-1 infected individuals and 8 HIV sero-negative controls were recruited from the participants in the NYU HIV Preventive Outreach Program and the NYU Bluestone Center for Clinical Research at the College of Dentistry during the period between 2008 and 2012. The HIV-negative controls were healthy volunteers without GI problems unrelated to HIV infection. HIV-negative controls undertook esophagogastroduodenoscopy in same endoscopy suites with HIV-infected subjects. All subjects including both HIV-infected patients and HIV-negative controls were from New York metropolitan area.

Sample collection

Microbiome sampling was conducted in an endoscopy room. First, the oral cavity was sampled by swabbing the anterior tongue using a sterile Catch-All™ Sample Collection Swab (Epicentre Biotechnologies, Madison, WI) according to manufacturer's instruction. The procedures for sampling the proximal gut were performed during esophagogastroduodenoscopy (EGD) with modifications to minimize cross contamination, similar in principle to the previously described method used to study esophageal microbiome in gastroesophageal reflux disorders [42]. In brief, a sterile disposable cytology brush (Cook Medical, IN) was inserted through the biopsy channel of the endoscope to brush mucosa in the order of the esophagus (2 cm above the squamocolumnar junction), stomach corpus, and duodenum, to minimize contamination from downstream organs. After removal of the cytology brush from each sampling, the biopsy channel was washed with 25 ml sterile water to minimize contamination by upstream organs. As backups, tissue biopsy samples and mucosal wash samples were also obtained.

DNA extraction and PCR amplification

DNA was extracted from the specimens using a modified Qiagen DNA extraction method, as previously described [42]. From the extracted DNA, the V3 and V4 regions of bacterial 16S rRNA genes were amplified using the primer set 347F 5'-GGAGGCAGCAGTRRGAAT and 803R 5'-CTACCRGGGTATCTAATCC, which we previously designed [43]. PCR reactions were carried out as previously described [42] and the amplicons were sequenced on the 454 FLX platform. A few mucosal samples that did not yield sufficient DNA for PCR amplification were replaced with either tissue biopsy samples or mucosal wash samples. Three types of negative controls were included to detect exogenous contamination: (i). Template negative controls (PCR reaction without DNA template) along with the samples from HIV-positive and negative subjects while constructing 16S PCR amplicon libraries; (ii). Specimen-negative controls (use water in lieu of a human specimen) along with the samples from HIV-positive and negative subjects while extracting DNA from the human samples. (ii). Disease-negative controls (HIV-negative) that were run under the same PCR and sequencing condition as the samples from HIV-positive patients.

Bioinformatics analysis

The pre-processed sequences were grouped into operational taxonomic units and were classified to taxonomic levels from the phylum to genus levels using the QIIME pipeline [44] and/or RDP II [45]. Taxa also were grouped based on tinctorial properties of bacteria (Gram-positive or Gram-negative), as previously described [42]. α -diversities in cases and controls were compared by *t*-test with Monte Carlo permutations using `compare_alpha_diversity.py`, a built-in function in the QIIME pipeline. Principal coordinate plots were used to visualize sample separations based on unweighted UniFrac distances matrices [46], with statistical significances calculated by the analysis of similarity (ANOSIM) method in QIIME. To identify bacterial taxa that consistently explained the differences between HIV patients and controls, the sequence data were compared using the linear discriminant analysis (LDA) effect size (LEfSe) method [47].

Statistical analyses

Prevalence of a specific taxon in cases and controls, defined as the number of subjects found to contain the taxon compared with the total number of subjects in a group, was compared by *Pearson's chi-squared* test or *Fisher exact* test. Comparison of median relative abundance of a particular taxon between cases and controls were done by using the nonparametric *Mann-Whitney U* test. Coexistence between taxa was analyzed by *linear regression*. The strength and direction of the linear relationship between CD4+ T cell counts and bacterial abundance was assessed using *nonparametric Spearman's rank correlation coefficients*.

Results

We examined the status of colonization resistance in the proximal guts in eight HIV-1 patients and eight HIV-negative subjects (Supplementary Table 1). For HIV-negative subjects, seronegative status was confirmed using the OraQuick ADVANCE Rapid HIV-1/2 antibody test. None of the HIV-infected patients had received HAART 6 month prior to specimen collection or antibiotics within the previous 8 weeks, except one patient who was on penicillin for syphilis. Samples were collected by brushing the mucosa of the esophagus, stomach, duodenum, and mouth of each subject, with a total of 63 samples acquired after excluding one gastric sample that failed to yield PCR products. The mouth was included as a reference site to contrast changes in the proximal gut. Microbiome analysis was done by universal 16S rRNA PCR coupled with sequencing using the 454 FLX technology, with an average of 5,090 sequence reads yielded per sample. The template-negative control and specimen-negative control did not generate any visible PCR products after 30 cycles of amplification using the universal bacterial 16S rRNA primers.

HIV infection was associated with global alteration of the proximal gut microbiome especially that of the duodenal microbiome

We first assessed α diversity of the proximal gut microbiome and found no significant difference in the diversity between HIV patients and controls in the proximal gut as a whole or in each of the three proximal gut sites (esophagus, stomach or duodenum) in either Shannon diversity index or evenness index ($p>0.05$). β -diversity by principal coordinates analysis using unweighted UniFrac distance metrics showed an overall separation tendency of the samples among the four body sites, including the mouth ($p=0.001$) (Figure 1A) as well as among the three proximal gut sites tested ($p=0.001$) (Figure 1B). Analyses comparing HIV patients and controls showed that differential dispersion of samples was unclear when all four body sites were included in the analysis ($p=0.088$) (Figure 1C), but became significant when the proximal gut was analyzed alone ($p=0.023$) (Figure 1D). In site-specific analyses, separation between HIV patients and controls was significant in the duodenum ($p=0.039$) (Figure 1E) but ambiguous in each of the three other body sites examined ($p>0.05$) (Figure 1F–H). These results suggest that HIV infection mainly affected the duodenal microbiome but the small effect size ($r=0.101$) indicating the necessity for detailed analyses based on individual taxa.

HIV infection abolished colonization resistance to exogenous *Proteobacteria* and altered the endogenous microbiome in the proximal gut

Among the five major phyla in the proximal gut (Figure 2A), HIV infection depleted *Firmicutes* (median relative abundance 43.61% vs. 65.81%, $p=0.005$) accompanied by an enrichment of *Proteobacteria* (11.77% vs. 6.28%, $p=0.020$), without any significant impact on relative abundances of *Bacteroides* ($p=0.848$), *Actinobacteria* ($p=0.469$), and *Fusobacteria* ($p=0.083$) (Figure 2C). In comparison, *Bacteroidetes* was significantly enriched in the mouth (32.05% vs. 20.42%, $p=0.049$) in HIV infection (Figure 2B and D). Using linear discriminant analysis effect size (LEfSe) with LDA score >4 as the cutoff [47], we found that in the proximal gut, HIV infection enriched four genera, including three genera in *Proteobacteria*—*Burkholderia* (0.35% vs. 0%, $p<0.0001$), *Bradyrhizobium* (0.11% vs. 0%, $p<0.0001$), and *Ralstonia* (0.16% vs. 0%, $p=0.001$)—and one genus in *Fusobacteria*—*Fusobacterium* (3.92% vs. 0.94%, $p=0.049$), and depleted one genus in *Firmicutes*—*Lactobacillus* (0% vs. 0.09%, $p=0.003$) (Figure 3A and 3C). HIV infection also enriched *Prevotella* (22.64% vs. 7.85%, $p=0.050$) in the mouth (Figure 3B and D). Of particular interest were *Burkholderia* and *Bradyrhizobium*, which invaded the proximal guts of the majority (5/8) of HIV patients but were absent or very rare (1/7 in the stomach and 0/8 in other sites) in the guts of controls. Detailed analyses demonstrated that *Burkholderia* and *Bradyrhizobium* were represented by *Burkholderia fungorum* and *Bradyrhizobium pachyrrhizi* at the species level, respectively (Supplementary results, Supplementary Figure 1, and Supplementary Table 2).

Burkholderia fungorum and *Bradyrhizobium pachyrrhizi* selectively invaded the three proximal gut sites in HIV-infected patients

Site-specific analyses indicated that the duodenum was the site most altered by HIV infection, with global enrichment of Gram-negative (G⁻) bacteria (60.35% vs. 33.54%, $p=0.050$) and depletion of Gram-positive (G⁺) bacteria (34.72% vs. 62.28%, $p=0.050$) in HIV infection (Supplementary Figure 2A). Furthermore, HIV infection depleted *Firmicutes* (40.07% vs. 67.57%, $p=0.005$) along with its descendant *Lactobacillus* (0.12% vs. 0%, $p=0.010$), accompanied with a trend of enriching *Proteobacteria* (25.01% vs. 5.76%, $p=0.083$) and its descendants *B. fungorum* (8.05% vs. 0%, $p=0.038$) and *B. pachyrrhizi* (0.53% vs. 0%, $p=0.038$) (Supplementary Figure 2B and 2C) (Figure 4A). In comparison, some endogenous *Burkholderiales* were either unchanged (*Delftia*) or suppressed (*Sutterella*, *Tepidimonas*, and *Rubrivivax*) in HIV infection (data not shown). Both *B. fungorum* and *B. pachyrrhizi* colonized 62.5% (5/8) HIV cases but none of the controls. In contrast, *Lactobacillus* colonized 75% (6/8) of the controls but none of the HIV-infected patients. The changes in the duodenum partially extended to the stomach where both *B. fungorum* and *B. pachyrrhizi* colonized the majority (5/8) of HIV patients, albeit at lower abundance than in the duodenum, but were virtually absent (1/7) in controls (Figure 4B). Moreover, *Ralstonia* was enriched in HIV patients (0.79% vs. 0.01%, $p=0.040$) but also found to colonize the controls. In the esophagus, none of the five taxa differed in abundance between HIV patients and controls although *B. fungorum*, *B. pachyrrhizi* and *Ralstonia* were exclusively present in 50% (4/8) of HIV-infected patients but none of the controls (Figure 4C). *Fusobacterium*, although informative of HIV status in the proximal gut (Figure 3C)

was not significantly different between HIV-infected patients and controls when the three proximal gut sites were analyzed separately (Figure 4).

***B. fungorum* coexisted with *B. pachyrrhizi* in HIV infection**

We observed a clear coexistent relationship between *B. fungorum* and *B. pachyrrhizi*, with their concordant presence (n=14) and absence (n=9) observed in nearly all samples (23/24) from the proximal gut in HIV patients. Both species mainly colonized the duodenum with a decreasing abundance along the direction from the duodenum to stomach to esophagus (Figure 5A–C). When present, *B. fungorum* always outnumbered *B. pachyrrhizi* at a ratio of approximately 15:1 ($p<0.0001$, $r^2=0.956$) (Figure 5C). Both species appeared to be mostly confined to the HIV-infected proximal gut since *B. fungorum* was only found in the proximal gut of HIV patients and *B. pachyrrhizi* was detected in one of the seven gastric samples from the controls and one of the eight oral samples in HIV-infected patients (Figure 4). *Lactobacillus* species appeared to have a co-avoidant relationship with *B. fungorum* and *B. pachyrrhizi* in the duodenum, given that both *B. fungorum* and *B. pachyrrhizi* were absent in any of the six *Lactobacillus*-positive samples and conversely *Lactobacillus* were absent in any of the five samples positive for *B. fungorum* and *B. pachyrrhizi* (Figure 4A).

Abundances of *B. fungorum*, *B. pachyrrhizi*, and *Ralstonia* were correlated with reductions of blood CD4+ T cell counts

To explore whether dysbiosis reflected host immune status, we used nonparametric *Spearman's rank correlation coefficients* to correlate the abundance of bacterial taxa with the blood CD4 counts, CD8 counts, CD4/CD8 ratios, and HIV viral loads of hosts. Among the six bacterial taxa indicative of dysbiosis, *B. fungorum* showed the broadest and strongest inverse correlation with CD4 counts in all three proximal gut sites (Supplementary Figure 3A–C). Among the three sites, the duodenum was the primary site affected by HIV infection, with an inverse correlation of *B. fungorum* ($p=0.004$), *B. pachyrrhizi* ($p=0.004$), and *Ralstonia* ($p=0.020$), with CD4 counts (Supplementary Figure 3A). In the esophagus, the abundances of *B. fungorum* ($p=0.010$), *B. pachyrrhizi* ($p=0.010$), and *Ralstonia* ($p=0.010$) were also inversely correlated with CD4 counts, although the effect size was smaller (Supplementary Figure 3C). Qualitatively, patients with normal CD4 counts (n=2) were resistant to colonization by *B. fungorum* and *B. pachyrrhizi*, while those with low CD4 counts ($<500/\text{mm}^3$) (n=6) lost the resistance except for a patient who received antibiotics for syphilis. Interestingly, the patient most enriched with *B. fungorum* (48.4%) and *B. pachyrrhizi* (2.98%) in the duodenum had the lowest CD4 count ($12/\text{mm}^3$). *Lactobacillus* and *Prevotella* showed no correlation with CD4 counts in any of the four body sites analyzed ($p>0.05$) and none of the six taxa analyzed showed any correlation of their abundance in the mouth with CD4 counts (Supplementary Figure 3D). Correlation with the CD4/CD8 ratio appeared to depend on CD4 counts, because the pattern of correlation with CD4/CD8 was similar, albeit narrowed, to that with CD4 (Supplementary Table 3). None of the six taxa showed any correlation of their abundance in any of the four body sites with CD8+ T cell counts or viral loads ($p>0.05$).

Discussion

In this first study specially designed to determine the status of colonization resistance in the proximal gut, we systemically examined mucosal bacterial communities in all three major organs of the proximal gut: the esophagus, stomach, and duodenum. Globally, HIV infection enriched phyla *Proteobacteria* and depleted *Firmicutes*. This type of global changes appears to be a programed reaction as similar changes have also been observed in the distal gut in HIV infection [10, 14, 48, 49]. At the species and genus levels, we found HIV infection abolished colonization resistance to certain exogenous bacteria and weakened the normal control of endogenous bacterial community.

The unique finding in the proximal gut was the consensus loss of colonization resistance to two exogenous bacterial species: *Burkholderia fungorum* and *Bradyrhizobium pachyrhizi* among HIV patients who had low CD4+ T cell counts. Our data indicate that the likelihood that they constitute a significant portion of the endogenous normal microbiome is very low. Given the prior knowledge that the normal bacterial counts in the duodenum are in the order of 100 colony forming units/ml [50], we probably had sampled nearly all bacteria in the proximal gut site with the sequencing depth at approximately 5,090 reads per sample in the present study. If they were endogenous bacteria the high throughput sequencing would have identified their presence in the samples from normal controls.

The sources of these two bacterial species were not identified but our data suggest that the limiting factor that determines their colonization is the condition in the proximal gut rather than exposure to a special source that harbors these bacteria. Based on the prior knowledge, we know that they are commonly present in the environment. In the present study we found that they were unable to establish a detectable colonization in the normal proximal gut but the resistance to their colonization disappeared when the CD4 counts dropped to below the normal limit in HIV infection. The bacteria do not seem related to oral sex as they were not present in the oral cavity as shown in the present study and were not reported to be present in the vagina by previous studies [29–31]. A food or environmental contaminant was unlikely as all subjects including both HIV-infected patients and HIV-negative controls were from New York metropolitan area.

The observed association of *B. fungorum* and *B. pachyrhizi* with HIV infection was consistent with true colonization rather than transitory exposure or contamination because of the following non-random patterns (i). Their pattern of distribution in the four body sites examined exhibited a clear tropism favoring the proximal gut over the mouth; (ii). Within the proximal gut, the both species were more abundant in the duodenum, the most deeply located and least likely to be contaminated organ by an environmental source, with a decreasing gradient towards the esophagus; (iii). In the duodenum, the distribution of these two species among HIV patients were selective, i.e. present in every HIV patients who had abnormal CD4+ T cell counts (not on antibiotic therapy) but absent in all subjects whose CD4 counts were normal; (iv) *B. fungorum* virtually coexisted with *B. pachyrhizi* and maintained a ratio of approximately 15 to 1; (v). The absence of *Burkholderia* and *Bradyrhizobium* in the template-negative control, specimen-negative control, and disease-negative control essentially ruled out the possibility of environmental contamination since

both controls and disease samples would have been equally affected if contamination had occurred.

Little is known about the mechanisms that govern colonization resistance in the proximal gut. However, studies from the distal gut indicate that both endogenous bacteria and host immune system play critical roles. For example, facultative anaerobic *Proteobacteria* are normally suppressed by metabolic exclusion caused by intense competition with obligate anaerobes *Clostridia* and *Bacteroidia* for fermentable carbohydrates in an anaerobic environment [10]. On the other hand, *Proteobacteria* often become a dominant component of the distal gut microbiome during inflammation when nitrate released from the host, unusable by obligate anaerobes, lifts the suppression by serving as an alternative energy source in oxidative phosphorylation for facultative anaerobes [14, 48, 49, 51]. Another mechanism potentially responsible for the shift of bacterial communities from obligate to facultative anaerobes could be the oxygen stress during inflammation from the formation of reactive intermediates, such as reactive oxygen and nitrogen species [52–54]. Infection with HIV causes inflammation of the human duodenum, given that most of the genes upregulated are involved in inflammatory reactions; these include T-cell genes (CD8A, CD5, TNFSF10), interferon-induced genes (RSAD2, OAS2, IFI44, IFITM, HLA-C, HLA-DRB1) and complements [55]. Given that both *B. fungorum* and *B. pachyrhizi* are G[−] obligatory aerobic *Proteobacteria* capable of reducing nitrate [56, 57], their selective overgrowth in HIV infection suggests that the proximal gut is enriched with oxygen and nitrate, consistent with an inflammatory state. *B. fungorum* and *B. pachyrhizi* might home to the proximal gut in reaction to high concentrations of nitrate and oxygen generated in HIV-induced mucosal inflammation. While the nitrate and oxygen hypotheses could in part explain the observed colonization of *B. fungorum* and *B. pachyrhizi*, it remains to be established why these two environmental species were selected over endogenous *Proteobacteria*. Knowledge from their symbiotic relationship with their environmental hosts [56, 57] suggests that mutual selection might be necessary to establish successful colonization.

The clinical significance of *B. fungorum* and *B. pachyrhizi* colonization is unclear. At the genus level, *Burkholderia* is composed of the two large groups, named group A, the plant-beneficial-environmental (PBE) group and group B, the pathogen group [58, 59]. The group A encompasses several plant-associated and saprophytic bacterial species with capability of nitrogen fixation, nodulation, and degradation of aromatic compounds while the group B comprises the *B. cepacia* complex (opportunistic human pathogens), the *B. pseudomallei* subgroup (both human and animal pathogens), and an assemblage of plant pathogenic species. *B. fungorum* belongs to the plant-beneficial-environmental group. *B. fungorum* is well known for its capability of biodegradation of xenobiotics in the environment and main interest in this species has been ecological and commercial potential for bioremediation of polycyclic aromatic hydrocarbons [56, 60]. It is also endosymbiotic with the white-rot fungus *Phanerochaete chrysosporium*, which is a saprophytic fungus capable of organic breakdown of the woody part of dead plants [61]. Besides this study, *B. fungorum* has been isolated from humans in four occasions, all from extra-intestinal sites. The only clinically significant infection reported was bacteremia with *B. fungorum* in a 9-year old girl with septic arthritis [62]. The other three isolates were from the vaginal secretions of a pregnant

woman with candida vaginitis and preterm labor, the cerebrospinal fluid of a 66-year-old woman, and the respiratory secretion of a cystic fibrosis patient, respectively, but the clinical significance could not be determined due to the lack of clinical data [56, 63]. In a bioinformatics analysis, *B. fungorum* was linked the origin of anti-dsDNA antibodies in systemic lupus erythematosus based on epitope mimicry [64]. *B. pachyrhizi* is a symbiotic, root nodulating bacterium inhabiting tuberous root-producing legume *Pachyrhizus*²². There are very few publications about this species, none of which are related to colonization/infection in humans or animals. In the present study, two of the five HIV patients colonized by these two species and one of three uncolonized HIV patients had watery diarrhea. Although both the colonized patients were different from the uncolonized patient by having an additional upper gastrointestinal symptom (nausea and vomiting), available information is inadequate for drawing a conclusion. Their downstream effects on HIV pathogenesis and contributions to clinical diseases need further studies, especially in those with advanced stage of disease.

The limitations pertinent to this study are related to the small sample size and the exploratory nature. We considered the limitation of the small sample size when we analyzed the data. In particular, we tried to minimize the chance of false positive findings by filtering out any differences whose effect size were less than four orders of magnitude (LDA score >4 as the cutoff) when comparing the cases and controls using the linear discriminant analysis (LDA) effect size (LEfSe) method. We also strengthened the study by including the other relevant body sites besides the duodenum in the study, which revealed similar distributions for these two bacterial taxa in the stomach and esophagus. However, it can't be entirely ruled out that the positive findings were by chance in this small study. Our study had a low statistical power typical of a small study that was insufficient for detection of all effects that are truly present (false negativity or type-II errors) unless the effect sizes were large. The small sample size also limited our capability of stratifying factors other than HIV infection (such as gender, race, and HIV progression) for their contribution to difference observed between cases and controls. Thus, a large-scale study is needed to reach more comprehensive conclusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Global differences of microbiome among body sites and between HIV-infected patients and controls, by principal coordinate analysis with unweighted UniFrac distances matrices, with p values calculated by ANOSIM. Comparisons were made among the four body sites (A, duodenum in green, stomach in orange, esophagus in purple, and mouth in blue) and among the three proximal gut sites (B, duodenum, stomach, and esophagus). Comparisons were also made between HIV-infected patients (red) and controls (green) in all four body sites (C), proximal gut (D), duodenum (E), stomach (F), esophagus (G), or mouth (H).

Figure 2.

Major phyla and their differences in relative abundance between HIV-infected patients and controls in the proximal gut and mouth. Phylum level compositions of the proximal gut (A) and mouth (B) microbiomes in HIV-infected patients and controls are shown in stacked bars (mean relative abundance). Detailed comparisons of each major phylum between HIV-infected patients and controls are shown for the proximal gut (C) and the mouth (D) (median relative abundance). Statistical significant differences were calculated based on the relative abundances using *Mann–Whitney U* test.

Figure 3. Genus level differences in relative abundance between HIV-infected patients and controls in the proximal gut and mouth. Differential representation of bacterial lineages identified by LefSe between HIV-infected patients and controls in the proximal gut (A) and mouth (B) are shown in the cladograms (LDA scores >4). Circles represent phylogenetic levels from phylum to genus inside out. Each circle's diameter is proportional to the taxon's abundance, red for HIV-infected patients and green for controls. Histograms of enriched bacterial taxa in HIV-infected patients are displayed in red and in controls in green. Detailed comparisons of each genus between HIV-infected patients and controls are shown for the proximal gut (C) and the mouth (D). P values were calculated using *Mann–Whitney U* test.

Figure 4.

Distribution of differential taxa between HIV-infected patients and controls in the proximal gut. The 5 taxa informative of HIV infection in the proximal gut were further analyzed at each of the three specific sites of the proximal gut including the duodenum (A), stomach (B), and esophagus (C). P values were calculated using *Mann-Whitney U* test.

Figure 5.

Coexistence between *B. fungorum* and *B. pachyrhizi* in HIV infection. Both *B. fungorum* (A) and *B. pachyrhizi* (B) were most abundant in the duodenum (green circles) and exhibited a decreasing trend proximally towards the stomach (orange circles) and esophagus (purple circles) and were virtually absent in the mouth (grey circles). P values for statistical difference among the four body sites was calculated using a nonparametric, *Friedman 2-way ANOVA by ranks*. Post-hoc comparisons were performed with pairwise Friedman test. Coexistence between the two species was analyzed by linear regression (C).