**CONFIDENTIAL TO AUTHORS**   
Senior Editor Comments:   
Your manuscript was sent to three outside referees, and their critiques are attached. Overall, while major concerns were identified, the majority of reviewers found your manuscript to be of interest, and we therefore welcome a revised submission. Nevertheless, the reviewers also judged the study to be somewhat limited and narrow, and it will be important to address all of the reviewers' concerns. Reviewer #1 has suggested that the current findings are more incremental than a significant advance, and it would be important to clarify how your findings might impact our understanding of disease states. In addition, the issue of individual-specific differences in sites likely needs to be addressed. Reviewer #3 has pointed out that analysis of only two sites (proximal and distal colon) is somewhat limited, and thus the issue of "patchiness" and whether spatial analysis might provide more insight needs to be addressed. In addition, reviewer #3 has suggested alternative approaches to analyze your data, including Dada2 and an alternative variance stabilization technique for dealing with libraries of different sizes. These and all other concerns will need to be addressed. We look forward to a revised manuscript.   
  
  
Reviewer #1 (Reviewer Comments to the Author):   
  
The study "Spatial variation of the native colon microbiota in healthy adults" by Kaitlin J. Flynn and colleagues investigates the microbial population of the proximal and distal colon mucosa and luminal contents using a 16S rRNA approach. The study is well designed for the question being asked by the investigators and the results are well described. While the study is technically well done, it does not address an important gap and does not provide a significant advance. The primary difference between the current study and previous studies (Am J. Physiol Gastrointest Liver Physiol 2010 Dec; 299(6): G1266-G1275 Nature Reviews Microbiology 14, 20-32 2016), which have described both temporal and radial differences in community structure, is the collection of samples in the absence of colonoscopy preparation. This is indeed important as colonoscopy preparation can affect microbial community. I commend the authors for this approach, but the relevance of the findings is still unclear and how the differences in findings from the current study improve our understanding of the role of specific microbes as compared to previous studies. The singular descriptive finding in the study lacks novelty and appears premature for publication. However, a comparison with samples collected similarly from a disease cohort would help advance the field as it may more accurately reflect microbiota changes and may shed light on previously missed associations.   
  
The authors only do 16S-based compositional analysis, which as expected shows greater inter-individual variation that the temporal changes within an individual, hence looking at OTUs between the two sites from the collection of subjects would be less relevant than individual-specific differences in sites. The ability to classify samples using machine learning though a good bioinformatic exercise does not add to our knowledge as we are already aware of the site from which the samples were collected. It would be more informative to know if functional characteristics of the microbiome, such as with metagenomics or metatranscriptomics, are different across the length of the colon.   
  
The authors report they found certain disease-associated taxa in their analysis which would be expected as the majority of the studies reporting microbiota associations report changes in relative abundance of these bacteria between controls and disease suggesting a quantitative difference; hence just the presence of known disease associated bacteria is not surprising. The authors dedicate two paragraphs reviewing the role of Fusobacterium, which was seen in their samples but the current study does not improve our understanding of the role of Fusobacterium in diseases of the colon. Overall, the discussion is too long and often tangential.   
  
  
  
Reviewer #2 (Reviewer Comments to the Author):   
  
Nice and important study, but the role/presence of biofilms is neglected. Maybe in view of their importance in microbiome-mediated cancer progression (e.g., Li et al. Bacterial Biofilms in Colorectal Cancer Initiation and Progression. Trends Mol Med. 2017 Jan;23(1):18-30), the author should discuss this a bit further and indicate the need for further studies in healthy subjects addressing their absence or presence.   
  
  
Reviewer #3 (Reviewer Comments to the Author):   
  
Flynn et al. characterized the phylogenetic (16S rRNA gene amplicon-based) composition of the microbiota of the proximal and distal colonic mucosa and lumen in 20 healthy subjects in order to understand better the spatial variation of the bacterial communities, motivated by an interest in establishing reference data for comparison with those from disease states affecting the colon. Microbial community biogeography in humans is an important topic for which there is a dearth of good data, especially from studies designed with careful consideration of sampling technique, spatial scale and local physiology. Others have already contributed useful data and findings over the past few decades. This study by Flynn et al. has a number of attractive features, including the use of 'unprepped' subjects (minimal prior disturbance of the gut) (and a reasonable number of subjects at that), simultaneous sampling of mucosa and lumen, and a near-contemporaneous stool sample. Among the interesting findings, the authors provide evidence of community distinctness between proximal and distal colonic mucosa, but not lumens, and greater similarity of stool to distal lumen than of stool to any other sampled site. At the same time, there are some weaknesses to this study, including limited sampling along the longitudinal axis of the colon (only two sites), which leaves unanswered some important questions about spatial patterning and underlying explanatory factors, a week delay between stool collection and other sample collection, and suboptimal choices about data analysis.   
  
1. Given the difference in community structure at the proximal and distal colonic mucosa as reported here, it is disappointing that additional sites were sampled along the longitudinal axis of the colon! The design of the study and the introduction seem to assume that the only geographic issue of interest in the colon is proximal versus distal. But there are both theoretical and anatomic/physiological reasons to postulate that the underlying microbial biogeography is more spatially nuanced and interesting. What about patchiness, as some forms of IBD might predict? What about continuous or discontinuous longitudinal gradients, as the ecology of directional flow systems might suggest? What about circumferential biogeographic patterns, as might be expected from the anatomy of blood supply and lymphatics? What about the cecum, which is the preferred site of some colonic disease? Given that the really tough logistical hurdles had already been overcome (getting healthy consented subjects into the endoscopy suite), it is too bad that a greater degree of spatial/mucosal sampling wasn't undertaken.   
  
2. A 2005 study of microbial biogeography at 6 different mucosal sites along the length of the colon in each of 3 healthy subjects (ref 21 in Flynn et al) yielded data that suggested the possibility of patchiness. The authors might comment upon these findings in this prior study and whether their own study design was suitably organized and powered to have detected a pattern of within-subject patchiness. Here, 'patchiness' should be formally defined and addressed according to biogeographical theory and method.   
  
3. Where exactly in the proximal colon were mucosal and luminal samples obtained? The authors are reasonably precise about the distal location (25 cm of flexible sigmoidoscope), but give no information about how the proximal site was chosen in each subject, and how much variation there might have been in these proximal locations. How far along the pediatric colonoscope??   
  
4. The 20 subjects were said to have had no antibiotic use during the 3 months prior to sample collection. The difficulty of finding subjects with greater periods of abstinence is understood, but because some antibiotics can have effects that last far longer than 3 months, it would be important to provide information on antibiotic use in the last year for each subject, as well as medical history focused on conditions that typically prompt frequent or extended antibiotic use.   
  
5. How were the amounts and consistency of fecal material standardized from sample to sample (especially since consistency is a known important source of variation)?   
  
6. Why were the stool samples collected one week prior to the endoscopic procedures, and not close in time? Stool microbiota structure can vary over the course of a week. How do the authors suggest that this variation be compared to other degrees of within-subject sample variation?   
  
7. Page 6--The identification of OTUs using a % sequence similarity threshold/cutoff is now recognized as importantly flawed (it fails to exclude reads with sequencing errors, and excludes important 'real' reads). This clustering-by-fixed-cutoff approach (used by the authors) is clearly out-performed by what is now a preferred method: 'Dada2' creates a model of sequencing errors and error rates from the raw sequencing data and then tests each read against the null hypothesis for this model, enabling statistical inference of real sequences (Nature Methods 13:581-3, 2016). The much greater 'resolving power' of this method gives more reliable results (especially given the interest of the authors in identifying taxa) and can reveal underlying biology and ecology.   
  
8. Pages 6-7--Rarefaction is problematic, and some would judge to be statistically 'inadmissible' since it requires omission of valid data and undermines the performance of downstream methods (PLoS Comput Biol 10: e1003531, 2014). A preferred approach for dealing with libraries of different sizes is described in this citation, and involves a variance stabilization technique that has been validated and used historically to address this problem with other similar types of data, e.g., RNA-Seq data.   
  
9. Were separate lumen-mucosa and proximal-distal sample comparisons undertaken when controlled for subject, as well as undertaken across subjects? If not, they should be. Given the dominant contribution of individual to inter-sample variation, it would be important then to subtract this source (control for individual), when considering other sources of variation.   
  
10. Page 7--Validation of the findings from the Random Forest model was performed with a leave-one-out approach. This is generally sub-optimal. More robust validation is achieved with the use of separate learning and testing data sets. Ideally, these two data sets are derived from different populations of subjects.   
  
11. Can the authors provide a citation for the statement that proximal mucosa contains the highest oxygen concentrations of the colon (lines 268-9)? Does the anatomy of the colonic blood supply support this statement?   
  
12. Figure 1 is unnecessary, but if included, it certainly doesn't need to be a main figure.   
  
13. There are a number of typos and grammatical errors. For example, in Intro: line 46, missing "in"; line 50, "tumors" can be deleted (since CRC is the subject); line 86, to what does "latter" refer? Also, lines 282-3, "more" seems to be missing in front of "variable".