Rectal swabs in critically-ill patients provide discordant representations of the gut microbiome compared to stool samples: a brief methodologic report.

Katherine Fair, MDc[1]; Daniel G. Dunlap, MD[1,2]; Adam Fitch, MS[2]; Alison Morris, MD, MS[1,2,3]; Bryan J. McVerry, MD[1,2]; Georgios D. Kitsios, MD, PhD[1,2]

[1]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, Pittsburgh, PA, USA; [2]Center for Medicine and the Microbiome, University of Pittsburgh; [3]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

Corresponding Author:

Georgios D. Kitsios, MD, PhD

Assistant Professor of Medicine

Division of Pulmonary, Allergy and Critical Care Medicine

University of Pittsburgh Medical Center

Address: UPMC Montefiore Hospital, NW628, 3459 Fifth Avenue, Pittsburgh, PA 15213

Email: kitsiosg@upmc.edu

Abstract

The role of the gut microbiome in critical illness is being actively investigated, but the optimal sampling methods for sequencing studies of gut microbiota remain unknown. Stool samples are generally considered gold-standard but are not practical to obtain in the intensive care unit (ICU), and thus, rectal swabs are often used. However, the reliability of rectal swabs for gut microbiome profiling has not been established in this clinical setting. In this study, we compared 16S rRNA gene sequencing results between rectal swab and stool samples collected at three timepoints in mechanically-ventilated critically-ill adults. Rectal swabs comprised 89% of samples collected at the baseline timepoint, but stool samples became more available at later time-points. Significant differences in alpha and beta-diversity between rectal swabs and stool samples were observed ($p<0.003$), but these differences were primarily due to baseline samples. Higher relative abundance of Actinobacteria phyla (typically skin microbes) was present in rectal swabs compared to stool samples ($p<0.02$), a difference that was attenuated overtime. The progressive similarity of rectal swabs and stool samples likely results from increasing stool coating of the rectal vault and direct soiling of the rectal swabs taken at later time points. Therefore, inferences about the role of the gut microbiome in critical illness should be drawn cautiously and take into account the actual type and timing of samples analyzed.

Statement of Importance

Rectal swabs are considered reliable alternatives to stool samples for gut microbiome profiling in outpatients or healthy adults, but their reliability in critically-ill patients has not been defined. Because stool sampling is not practical or often feasible in the intensive care unit, we performed a detailed comparison of gut microbial sequencing profiles between rectal swabs vs. stool samples in a longitudinal cohort of critically-ill patients. We identified systematic differences in

gut microbial profiles between rectal swabs and stool samples, and highlighted that timing of rectal swabbing had a significant impact on sequencing results. Our methodological findings should inform future investigations of the role of the gut microbiome in critical illness.

Introduction

Gut microbial dysbiosis is a plausible contributor to the onset, evolution and outcome of critical illness, but the mechanisms involved have not been fully elucidated (Kitsios et al. JCC 2017; Dickson Lancet RM 2015). Fecal microbial communities in critically-ill adults have lower diversity and distinct taxonomic signatures compared to fecal samples from healthy individuals (McDonald et al. mSphere 2016). Specific compositional features such as the ratio of Bacteriodetes to Firmicutes phyla (B/F ratio) have been associated with in-hospital mortality (Ojima et al. Dig Dis Sci 2016). Thus, defining the pathogenetic disruptions of gut communities during critical illness may help identify new targets for intervention (Kitsios et al. JCC 2017; Dickson Lancet RM 2015).

Sampling gut microbiota for sequencing analyses can be challenging in the Intensive Care Unit (ICU). Critically-ill patients frequently experience constipation or ileus (Nassar et al. JCC 2009)., while early enteral nutrition is not often provided. Thus, critically-ill patients may not have any bowel movements, especially early in their ICU course. Furthermore, standard decontamination practices in ICU care (Donskey et al. AJIC 2016) often result in stool disposal before samples are collected. For these reasons, rectal swabs represent an attractive, non-invasive method for sampling gut microbiota, which is routinely used in clinical practice for Vancomycin-Resistant *Enteroccocus* colonization screening.

Rectal swabs have demonstrated reliability as stool sample alternatives in ambulatory patients (Bassis et al. BMC Microbiol 2017), but data in critical illness are limited. Recent work from Bansal et al. (mSphere 2018) in nine critically-ill patients showed compositional discrepancies between rectal swabs and stool samples when rectal swabs were not visibly soiled by stool. To further examine for systematic differences in gut microbial profiles captured by stool vs. rectal swab samples, we obtained data from a larger cohort of 106 patients admitted to the medical ICU at a tertiary academic center.

In this observational cohort study, we prospectively enrolled consecutive mechanically-ventilated patients with acute respiratory failure from any cause. We collected rectal swabs and/or stool samples at three time intervals starting at the time of intubation and continuing up to 10 days if the patient remained in the ICU (defined as baseline [days 0-2], middle [days 3-6] and late intervals of follow-up [days 7-10]). Rectal swabs were collected as per routine clinical practices (i.e. placing the patient in a lateral position, entering the tip of the swab in the rectal canal and rotating gently for 5 seconds), unless clinical reasons precluded movement of the patient (e.g. severe hemodynamic or respiratory instability). Stool samples were collected when available, either by taking a small sample from an expelled bowel movement (before cleaning of the patient and disposal of stool), or through a fecal management system (rectal tube) placed for management of diarrhea and liquid stool collection. We extracted bacterial DNA and performed 16S rRNA gene sequencing (V4 subunit) on Illumina MiSeq with standard protocols as previously described (Kitsios et al. Frontiers Microbiology 2018). Sequencing data were analyzed for alpha and beta diversity and taxonomic composition with the R software.

We enrolled 106 patients comprising a total of 175 samples (137 rectal swabs and 38 stool samples). Stool samples were available from 24 (23%) patients during the study period, and 10

68 patients had both sample types available. Patients with and without stool samples available were
69 similar in terms of baseline demographics (age, gender, BMI) (Figure 1A). As expected, patients
70 with stool samples had higher incidence of recorded diarrhea ($p<0.009$), but also higher severity
71 of illness by Sequential Organ Failure Assessment (SOFA) scores ($p<0.001$) and duration of ICU
72 stay and mechanical ventilation ($p<0.001$). Sample type availability varied by follow-up interval:
73 rectal swabs constituted the majority of samples at the baseline interval (89%), but stool samples
74 were progressively available for larger proportions of patients (58% of patients at late interval,
75 Figure 1B).

76 *Results*

77 Rectal swabs and stool samples overall produced similar number (N) of 16S rRNA gene sequences
78 (median 3886 vs. 3805 reads, respectively, $p=0.73$) suggesting successful recovery of bacterial
79 DNA signal by both types of samples. Stool samples demonstrated lower alpha diversity (median
80 Shannon [interquartile range–IQR] = 2.4[1.7]) compared to rectal swabs (3.1[1.3], $p<0.003$)
81 (Figure 2A). Stratifying samples by follow-up interval revealed that baseline samples accounted
82 for most of the observed differences in alpha diversity between rectal swabs and stool samples,
83 while samples collected later had similar alpha diversity (Figure 2B).

84 Rectal swabs and stool samples were also systematically different by beta diversity metrics (Bray-
85 Curtis dissimilarity index), as visualized via principal coordinate analysis

86 (Figure 2C). In subgroup analysis by follow-up interval, a statistically significant temporal effect
87 on beta diversity was found only for rectal swabs ($p=0.001$), but not stool samples. Rectal swabs
88 collected later in the course were more similar to stool samples. To assess for the relative impact
89 of patient identity vs. sample type on beta-diversity, we performed a multivariate permutational

90 analysis of variance for patients with both stool and rectal swabs available at different intervals. In
91 this analysis, the sample type (rectal swab vs. stool) variable significantly accounted for more
92 inter-sample variance ($p<0.001$) than the patient identity variable.

93 Analyzing the taxonomic composition at the phyla level, we noted that rectal swabs had higher
94 relative abundance of Actinobacteria (a commensal skin microbe) compared to stool samples
95 ($p=0.02$), but the Actinobacteria relative abundance declined over time in rectal swabs ($p=0.03$ for
96 repeated measures analysis of variance, Figure 3). At the genus level, stool samples had higher
97 relative abundance of *Akkermansia*, *Bacteroides* and *Enterococcus* taxa, which are considered
98 typical members of the gut microbiome of critically-ill patients (all p-values <0.004). Examination
99 of the taxonomic composition at the genus level for 10 patients who had both sample types
100 available at different follow-up intervals showed marked discordance between rectal swabs vs.
101 stool samples (Supplemental Figure).

102 *Discussion*

103 Our analyses in a large cohort of critically-ill patients highlight significant differences between the
104 more accessible rectal swabs and the harder to obtain, but effectively gold-standard, stool samples.
105 In this study, stool sample availability captured distinct patient characteristics, perhaps because
106 morbid critically-ill patients with longer ICU stays had higher likelihood of stool passage and
107 collection during the study follow-up period.

108 Systematic differences in alpha and beta diversity by sample type were largely attributable to the
109 baseline samples acquired close to ICU admission and endotracheal intubation. At this early
110 timepoint, 89% of the available samples were rectal swabs and most patients were not receiving

enteral nutrition (58%). Stool presence in the rectal vault may have been limited, resulting in "unsoiled" swabbing of the rectal mucosa and peri-rectal skin, which were then represented by disproportionate abundance of skin bacteria (i.e. Actinobacteria phyla) than what would be expected for gut profiles. The temporal convergence of microbial profiles between rectal swabs and stool samples observed in our study suggests that progressive recovery of gut motility during ICU course and stool presence in the rectal vault may have improved the reliability of "soiled" rectal swab sampling, although we did not qualitatively score the macroscopic appearance of swabs as in the study by Bansal et al. (mSphere 2018).

Our results call for caution in the design of gut microbiome studies in critically-ill patients. Despite their availability, rectal swabs may introduce bias by contaminating skin flora, especially when conducted early in the course of critical illness. Consequently, analyses of gut microbiome studies need to take into account sample types and timing of sample acquisition, because rectal swabs and stool samples are not interchangeable for the purpose of microbial sequencing profiling, as they are considered in other populations. Accurate and reproducible delineation of the role of the gut microbiome in critical illness will require stringent sampling protocols, clinical variable recording and longitudinal assessments.

Funding support: National Institutes of Health [K23 HL139987 (GDK); U01 HL098962 (AM); P01 HL114453 (BJM); R01 HL097376 (BJM); K24 HL123342 (AM)]

127 **Data Availability Statement:**

128 Raw sequences used for this project have been deposited and are publicly available at

129 http://www.ncbi.nlm.nih.gov/bioproject/516701

130 Taxa tables, metadata and R statistical code required for the conduct of the analyses described

131 here in have been deposited and are publicly available at https://github.com/MicrobiomeALIR/

References

Bansal S, Nguyen JP, Leligdowicz A, Zhang Y, Kain KC, Ricciuto DR & Coburn B. Rectal and naris swabs: practical and informative samples for analyzing the microbiota of critically ill patients. mSphere 2018; 3(3); e00219-18. doi:10.1128/mSphere.00219-18.

Bassis CM, Moore NM, Lolans K, Seekatz AM, Weinstein RA, Young VB & CDC Prevention Epicenter Program. Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. BMC Microbiol 2017; 17(1): 78. doi:10.1186/s12866-017-0983-9.

Budding AE, Grasman ME, Eck A, Bogaards JA, Vandenbroucke-Grauls CM, van Bodegraven AA & Savelkoul PH. Rectal swabs for analysis of the intestinal microbiota. PLoS One 2014; 9(7): e101344. doi:10.1371/journal.pone.0101344.

McDonald D, Ackerman G, Khaukive L, Baird C, Heyland D, Kozar R, Lemieux M, Derenski K, Kind J, Vis-Kampen C, Knight R, Wischmeyer P. Extreme Dysbiosis of the Microbiome in Critical Illness. mSphere 2016; 1(4): e00199-00116. doi:10.1128/mSphere00199-16.

Donskey CJ & Deshpande A. Effect of chlorhexidine bathing in preventing infections and reducing skin burden and environmental contamination: A review of the literature. Am J Infect Control 2016; 44: 5 Suppl :e17-21. doi: 10.1016/j.ajic.2016.02.024.

Dickson RP. The microbiome and critical illness. Lancet Respir Med 2016; 4(1); 59-72. doi:10.1016/S2213-2600(15)00427-0.

Grice EA & Segre JA. The skin microbiome. Nat Rev Microbiol 2011; 9(4): 244-253. doi:10.1038/nrmicro2537.

Kitsios GD, Morowitz MJ, Dickson RP, Huffnagle GB, McVerry BJ, & Morris A. Dysbiosis in the intensive care unit: Microbiome science coming to the bedside. J Crit Care 2017; 38; 84-91. doi:10.1016/j.jcrc.2016.09.029.

Nassar AP Jr, da Silva FM, de Cleva R. Constipation in intensive care unit: incidence and risk factors. J Crit Care 2009; 24:630.e9-12. doi: 10.1016/j.jcrc.2009.03.007.

Ojima M, Motooka D, Shimizu K, Gotoh K, Shintani A, Yoshiya K, et al. Metagenomic analysis reveals dynamic changes of whole gut microbiota in the acute phase of intensive care unit patients. Dig Dis Sci 2016; 61: 1628–34. doi: 10.1007/ s10620-015-4011-3.

Zhang NL, Li TZ, Zheng K, Mou DL, Liang LC, Zhang T, He QS. Use of Rectal Swab Samples for Analysis of the Intestinal Microbiome in Children. Chin Med J 2018;131;492. doi:10.4103/0366-6999.225065.

Figure 1: Cohort characteristics and sample type availability overtime. A. Table with baseline characteristics and clinical outcomes of patients with rectal swabs only vs. patients with stool samples available. P-values are from non-parametric tests for continuous or categorical variables as indicated (highlighted in bold when significant $p<0.05$). B. Stacked bar-graph of number of rectal swabs vs. stool samples at each time interval (purple for rectal swabs and brown for stool samples). The proportion of stool samples available at each time interval is shown in white.

A. Baseline characteristics and outcomes

Variable	Patients with rectal swabs only	Patients with stool samples available	p value
N	82	24	
Age, median (IQR)	56.2 (20.4)	60.2 (21.4)	0.56
BMI, median (IQR)	30.3 (9.5)	30.6 (10.2)	0.97
Female, N (%)	36 (43.9)	11 (45.8)	1
Worst PaO2:FIO2 ratio, median (IQR)	166 (99)	132 (109)	0.3
pHa median, (IQR)	7.37 (0.10)	7.37 (0.04)	0.96
WBC median, (IQR)	13.2 (5.7)	10.3 (10.0)	0.1
Lowest MAP, median (IQR)	64 (18)	56 (8)	**0.01**
SOFA score*, median (IQR)	6 (4)	8 (3)	**<0.001**
LIPS score, median (IQR)	11.0 (2.5)	12.0 (2.1)	**0.03**
ICU LOS, days, median (IQR)	7 (6)	14 (9)	**<0.001**
Duration of mechanical ventilation, days, median (IQR)	5.5 (4.8)	9.0 (8.0)	**<0.001**
30-day mortality, N (%)	21 (22)	3 (12)	0.47

Abbreviations: IQR: interquartile range; BMI: body mass index; LIPS: lung injury prediction score; SOFA: sequential organ failure assessment; PaO_2: partial pressure of arterial oxygen; FiO_2: Fractional inhaled concentration of oxygen; WBC: white blood cell count; MAP: mean arterial pressure; ICU: intensive care unit; LOS: length of stay. * SOFA score calculation does not include the neurologic component of SOFA score because all patients were intubated and receiving sedative medications, impairing our ability to perform assessment of the Glasgow Coma Scale in a consistent and reproducible fashion.

B. Sample availability overtime

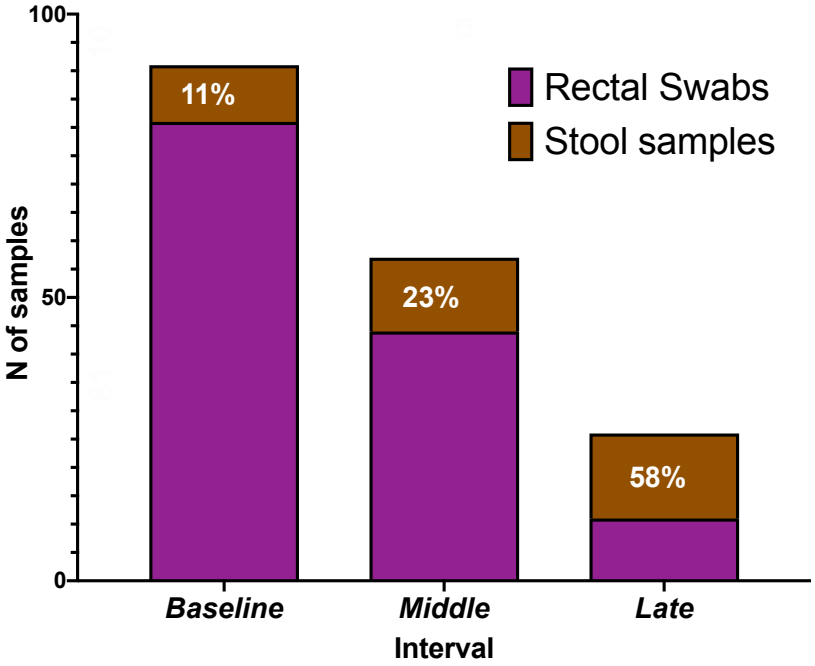


Figure 2 – Alpha and beta-diversity comparisons show markedly different representations of the gut microbiome by sample type. Panel A – Alpha diversity analyses for all samples available showed that rectal swabs had higher Shannon index than stool samples ($p<0.003$). Panel B – Alpha diversity analyses by sample type and follow-up interval showed that the higher alpha diversity (Shannon index) observed in rectal swabs compared to stool samples was significant only at the baseline timepoint by a Wilcoxon rank sum test and not in subsequent follow-up intervals. Panel B – Beta Diversity analyses: Principal coordinates analyses of Bray-Curtis dissimilarity indices between rectal swabs and stool samples. Greater distance between samples indicates greater dissimilarity. In the middle panel, all available samples are stratified by sample type, showing significant differences between rectal swabs and stool samples (permutation analysis of variance $p=0.001$). In the left and right panels, subgroup analyses conducted separately for rectal swabs and stool samples highlight that temporal variability was significant for rectal swabs only (different follow-up intervals shown in variations of purple), but not for stool samples (different follow-up intervals shown in variations of brown).

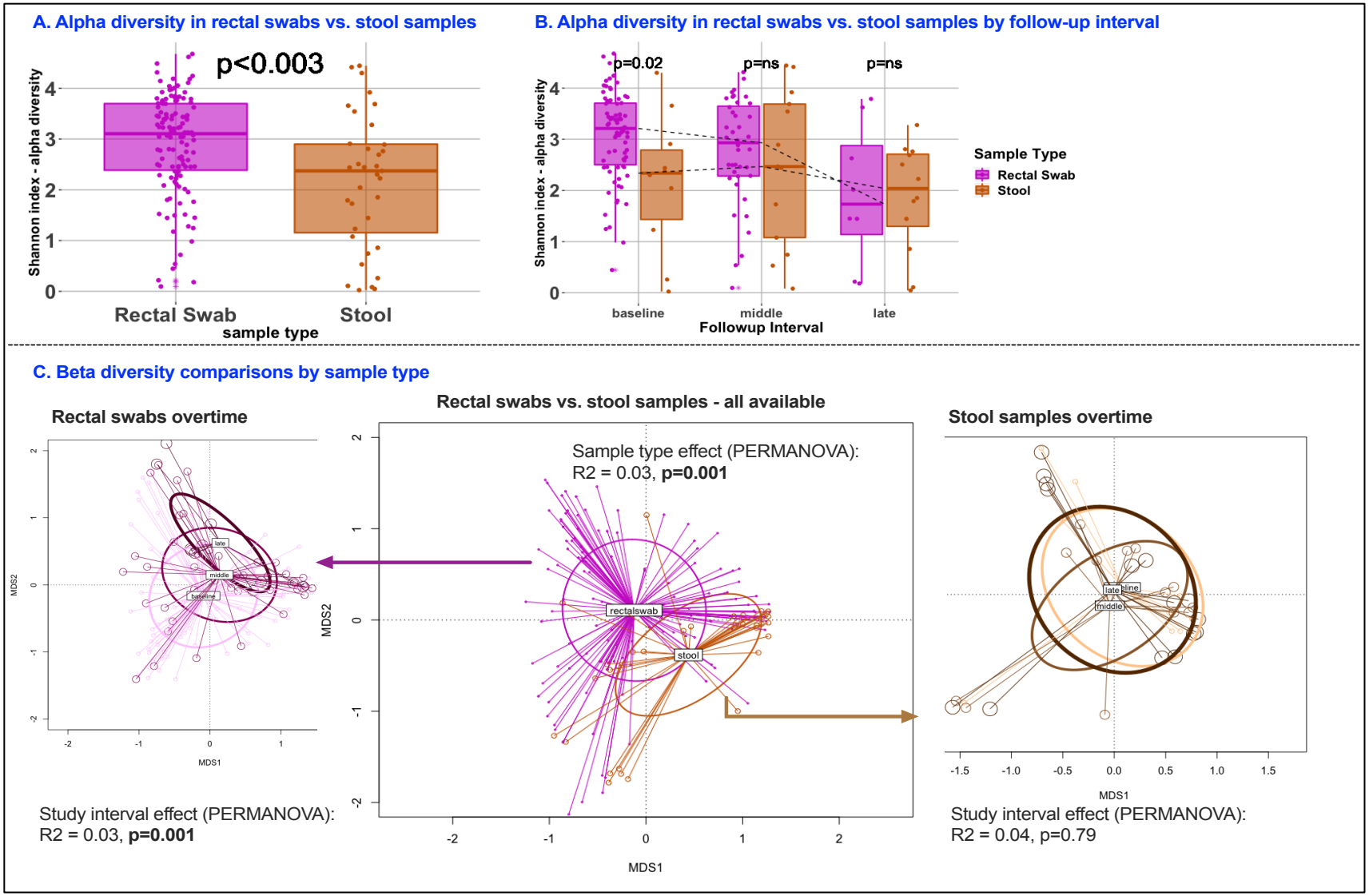
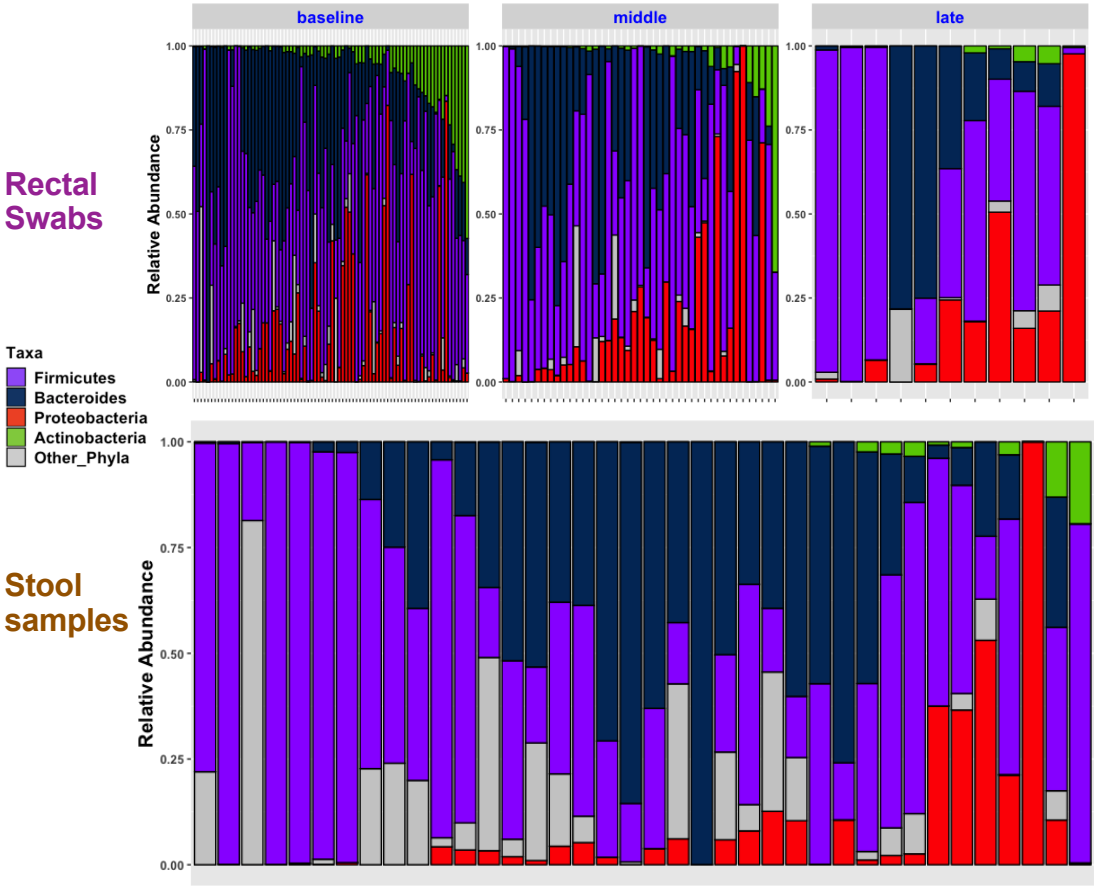


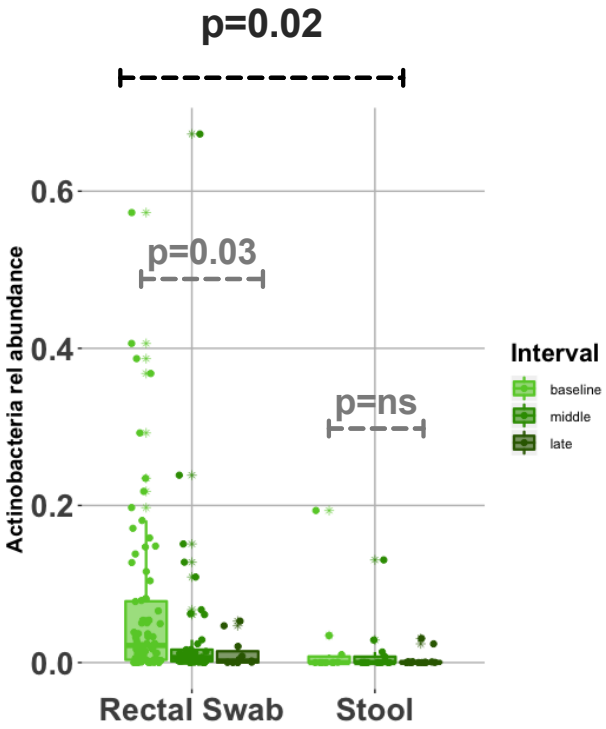
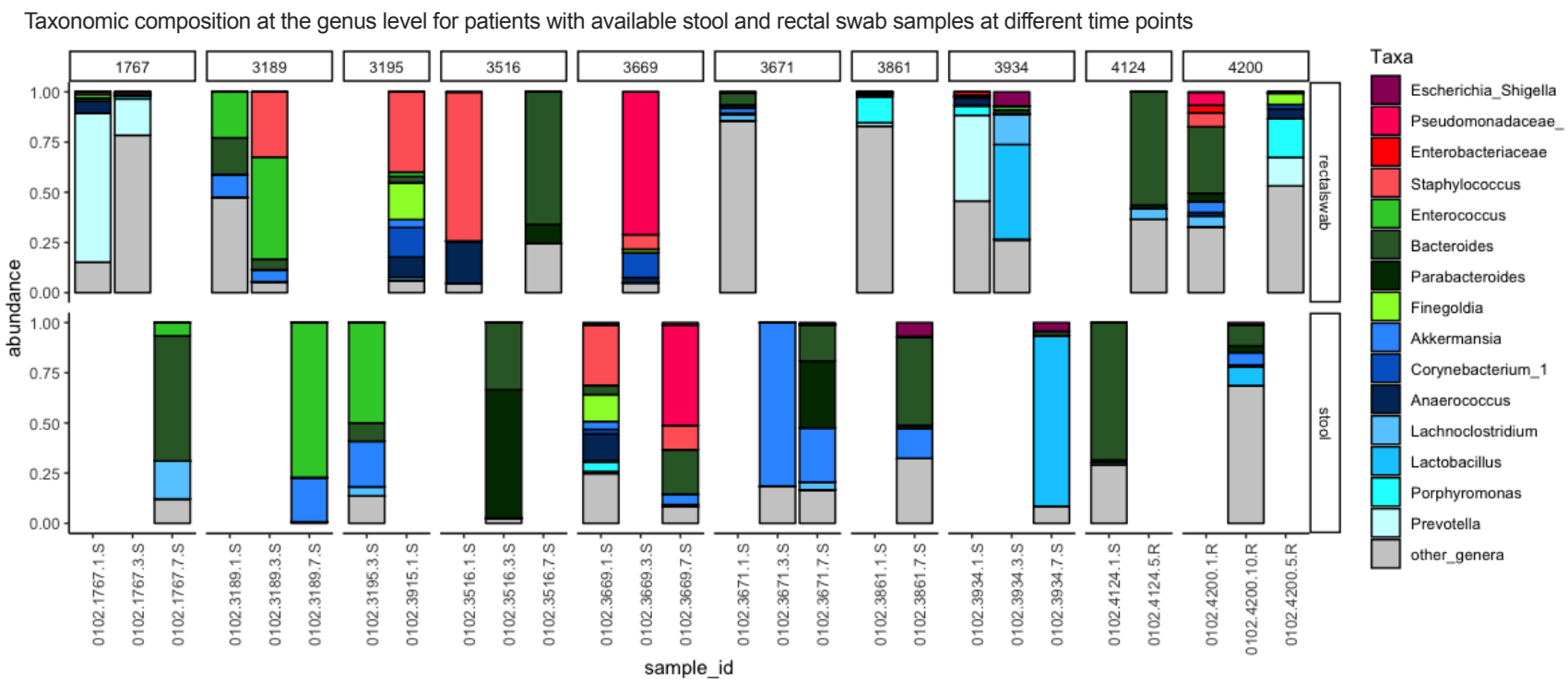
Figure 3: Taxonomic composition comparisons at the phyla level. Panel A: Taxonomic composition of individual samples stratified

by sample type and follow-up interval at the phyla level. Rectal swabs at the baseline interval had higher relative abundance of Actinobacteria phyla (shown in green) that declined over time. Panel B: Significant differences in relative abundance of Actinobacteria phyla between rectal swabs and stool samples (p=0.02), mainly attributable to baseline interval samples. Comparisons of repeated measures analysis of variance for Actinonobacteria relative abundance in rectal swabs showed significant decline over time (p=0.03).

A. Taxonomic composition for rectal swabs (by follow-up interval) and stool samples



B. Relative abundance of Actinobacteria phyla in rectal swabs vs. stool samples, by follow-up interval



Supplemental figure: **Taxonomic composition bar plot at the genus level for 10 patients with both stool samples and rectal swabs available at different time points.** Rectal swabs are shown in the top row and stool samples in the bottom row, with each patient unique 4-digit ID highlighting samples belonging to the same patient. The relative abundances for the 15 most common taxa are shown with different colors, and the added relative abundance of all other component taxa is shown in gray ("other genera"). Marked discordance between rectal swabs and stool sample taxonomic composition is demonstrated.