Blood, Gut, and Oral Microbiome in Kidney Transplant Recipients

Abstract

Background and Objective: Recent reports describe the existence of a blood microbiome profile not associated with an infection state. Given the high impact that the dysbiotic human microbiome appears to have in chronic kidney disease and, in particular, in the outcome of kidney transplant recipients (KTRs), we aimed to explore the variations and correlations of the gut, oral, and blood microbiome of recipients, 3 months after kidney transplantation. **Materials and Methods:** We conducted a cross-sectional study where the microbiome of stool, saliva, and blood collected from recipients 3 months after kidney transplantation ($N = 6$) was analyzed by polymerase chain reaction (PCR) amplification and sequencing of the V3–V4 hypervariable regions of the 16S rRNA gene using MiSeq Illumina® technology. **Results:** Blood of KTRs harbors a distinct low-abundance microbiome dominated by Proteobacteria and Firmicutes. Gut and oral microbiome of KTRs also present distinct profiles. The existence of a proportion of shared operational taxonomic units among the different body sites is reported, mainly classified as Proteobacteria and Firmicutes. **Conclusions:** This study provides evidence of existence a blood microbiome in KTRs, different from the gut and the oral microbiome profiles, with a small number of operational taxonomic units representing a shared microbiome. The clinical relevance of this observation should be further explored in these patients.

Keywords: *Bloodstream, human microbiome, kidney transplantation, kidney transplant recipients, mobile bacteria*

Susana Sampaio[1,2,3,4], Ricardo Araujo[1,2], Ana Merino-Riba[1,2,5,6,7], Benjamin Lelouvier[8], Florence Servant[8], Janete Quelhas-Santos[1,2,3], Manuel Pestana[1,2,3,4], Benedita Sampaio-Maia[1,2,9]

[1]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, [2]INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Rua Alfredo Allen, 208, 4200- 180 Porto, [3]Faculty of Medicine, University of Porto, [4]Department of Nephrology, São João Hospital Center, EPE, Portugal, [5]Universitat Autònoma de Barcelona, Barcelona, [6]Nephrology Department, Hospital Universitari Germans Trias i Pujol, Badalona, [7]Hospital Universitari Doctor Josep Trueta, Girona, Spain, [8]Vaiomer, Labège, France, [9]Faculty of Dental Medicine, University of Porto, Porto, Portugal

Address for correspondence:
Dr. Ricardo Araujo,
i3S - Instituto de Investigação e Inovação em Saúde,
INEB – Instituto de Engenharia Biomédica (Nephrology and Infectiology R&D Group), Rua Alfredo Allen, 208, 4200-135 Porto, Portugal.
E-mail: ricjparaujo@yahoo.com

Introduction

Disturbances of normal gut microbiome (dysbiosis) may be important in the pathogenesis of chronic kidney disease (CKD).[1] Although complications after kidney transplantation, such as infection and rejection episodes, remain major challenges, few studies have evaluated the human gut microbiome in kidney transplant recipients (KTRs). Changes in gut microbiome composition pre- and posttransplant in KTRs were observed, specifically a decrease in diversity associated with an increase in the relative abundance of Proteobacteria, particularly in members of the Enterobacteriaceae family.[2,3] Besides, it has been demonstrated that the gut microbiota can also influence tacrolimus dosage, and that immunosuppressive therapy may impact on the gut microbiome.[4,5] Moreover, oral bacteriome were found to be altered in KTRs, with an overgrowth of potentially opportunistic pathogens.[2,6] Different

studies have shown the existence of a blood microbiome in healthy patients and in patients with noninfectious diseases.[7-11] Recently, a study has shown that blood microbiome in CKD patients harbors a lower diversity and different taxonomic profiles in comparison to healthy controls.[10] Even if it could be expected that blood microbiome also exists in KTR patients, no information is currently available on the blood microbiome of KTRs, nor its comparison with gut or oral microbiome.

The presence of dysbiosis in KTR may impact on the type and concentration of uremic toxins, systemic inflammation, and the appearance of infections.[12] In fact, previous studies suggested that a specific microbiota profile could be associated with adverse events after kidney transplantation, specifically rejection events and infectious complications (diarrhea and urinary tract infections).[2,3,13-16]

Given the high impact that dysbiosis may have in KTR outcomes, we evelauated the blood microbiome in KTR and its

relationship with the gut and the oral microbiome, 3 months after kidney transplantation.

Materials and Methods

Study design, subjects, and sample collection

This cross-sectional study included adult living donor kidney transplant recipients followed up in the nephrology department of Centro Hospitalar Universitário S. João. Three months after transplantation, the microbiome were evaluated from the stool, saliva, and whole blood samples of six patients. The study protocol was approved by the local ethics committee and is in accordance with the 1964 Helsinki declaration and its later amendments. All recruited patients were asked to give their free written informed consent. The clinical information and infection history were gathered. The blood and saliva samples were collected by trained nurses, and the self-collected stool specimens were brought frozen by the patient within 24 h after collection. All samples were collected in DNA-free sterile containers and were immediately frozen and stored at −80°C.

Specimen processing and microbiome analysis

Genomic DNA was isolated and amplified in a strictly controlled environment at Vaiomer SAS (Labège, France) using a stringent contamination-aware approach.[8,17,18] Polymerase chain reaction (PCR) amplification was performed targeting the V3–V4 hypervariable regions of the bacterial 16S rRNA gene as described previously.[8,17] The 476-base pair amplicon (base on *Escherichia coli* reference genome) was sequenced using the 2 × 300 paired-end Illumina MiSeq kit V3 as recommended by the manufacturer (Illumina, San Diego, CA, USA).

16S rRNA gene sequence data analysis and statistics

The targeted microbiome sequences were analyzed using the bioinformatics pipeline established by Vaiomer based on the Find Rapidly OTUs with Galaxy Solution (FROGS) guidelines for obtaining the operational taxonomic unit (OTU) table.[19] The following filters were applied: 1) the last 10 and 80 bases of R1 and R2 reads, respectively, were removed due to lower quality preventing good read pairing; 2) amplicons with a length <350 nt or >490 nt were removed; 3) amplicons without the two PCR primers were removed (10% mismatches authorized); 4) amplicons with at least one ambiguous nucleotide were removed; 5) OTUs identified as chimera (with VSEARCHv1.9.5)[20] in all samples were removed; 6) OTUs with an abundance lower than 0.005% of the whole dataset were removed; and 7) OTUs with a strong similarity (coverage and identity ≥80%) with the phiX (used as a control for Illumina sequencing runs) were removed. The clustering was produced in two passes of the Swarm algorithm v2.1.6 (the first clustering with an aggregation distance equal to 1 and the second clustering with an aggregation distance equal to 3). OTUs

were produced via single-linkage clustering, and taxonomic assignment was performed by Blast+v2.2.30+ with the databank Silva 128 Parc database. Import, storage, analysis, and graphical display (including Principal Coordinate Analysis [PCoA]) of microbiome census data were done using PhyloSeqv1.14.0. The total sum-normalized OTU table was used for comparison of sample groups based on Bray–Curtis β-diversity. α-Diversity was calculated as the observed number of OTUs and Shannon index. Categorical variables were described through relative frequencies and analyzed by Chi-square independence test or Fisher's exact test when more than one cell had <5 expected counts. $P < 0.05$ was assumed to denote a significant difference. The datasets generated for this study can be found in National Center for Biotechnology Information (NCBI), bioproject PRJNA540316.

Results

CKD etiologies of KTR included autosomal dominant polycystic kidney disease (two patients), IgA nephropathy, obstructive uropathy, focal segmental glomerulosclerosis, and lupus nephritis (one each). Induction therapy included the use of basiliximab in five patients and of antithymocyte globulin in one patient. All patients were under maintenance therapy with prednisone, tacrolimus, and mycophenolate mofetil. Five months after transplant, one patient suffered a cerebrovascular event and mycophenolate was shifted to everolimus. The general clinical characterization of KTR and the infection events are presented in Table 1 and Supplementary Table 1, respectively. All patients presented stable renal function and no rejection episodes up to 2 years after kidney transplantation.

Table 1: Clinical characterization of the KTRs	
	KTRs
Age, years	44.5±13.2
Gender, *n* (%) males	2 (33%)
Dialysis before transplantation, *n* (%)	3 (50%) HD; 2 (33%) PD
Hypertensive patients, *n* (%)	5 (83.3%)
Diabetic patients, *n* (%)	1 (16.7%)
Hypertension	
Systolic	128.2±14.4
Diastolic	81.5±8.3
Glomerular filtration rate, mL/min/1.73 m^2	75.3±17.6
Blood markers	
Hemoglobin, g/dL	13.0±1.9
Albumin, g/L	43.2±1.8
Glucose, mg/dL	97.2±26.7
Urea, mg/dL	47.2±13.9
Creatine, mg/dL	1.0±0.3
Ferritin, ng/mL	320.3±221.2
C-reactive protein, mg/L	5.2±6.9

KTR=kidney transplant recipient; HD=hemodialysis; PD=peritoneal dialysis

Gut, oral, and blood microbiome were analyzed in these patients. A total of 409 OTUs were detected among samples; the following details were observed for the samples' OTUs, which are given as median (range): oral samples with 115 (106–128) OTUs, gut samples with 88 (59–105) OTUs, and blood samples with 50 (42–82) OTUs. The rarefaction curve analysis demonstrated the sufficiency of our read coverage to capture sample diversity [Supplementary Figure 1]. OTUs were clustered with the Swarm algorithm, revealing the relative proportion of different taxonomic phyla in each type of sample and a highly diverse profile at the family level [Figure 1 and Supplementary Figure 2]. The taxonomic profiles in the gut, oral, and blood microbiome were found to be characterized by different phyla [Figure 1]: Bacteroidetes and Firmicutes in the gut microbiome, Proteobacteria, Bacteroidetes, Fusobacteria, and Firmicutes in the oral microbiome, and Proteobacteria, Firmicutes, and Actinobacteria in the blood microbiome. PCoA of β-diversity confirmed the differences in microbial communities among these samples [Supplementary Figure 3]. α-Diversity analysis was conducted across different indexes, and statistical significance was found for the number of observed OTUs and the Chao1 index at most taxonomic levels (phylum, family, genus, OTU) and for Shannon and Simpson at the higher taxonomic levels [see Supplementary Table 2 and Supplementary Figure 4].

Moreover, each patient presented between three and seven different OTUs sharing the three monitored sites. It was found that 3.7% (15 OTUs) of the total OTUs were shared among the gut, oral, and blood samples within the same patient in at least one of the patients. These OTUs were classified mainly as Proteobacteria and Firmicutes, but single representative OTUs among Actinobacteria, Bacteroidetes, and Fusobacteria were also reported [Figure 2]. Among the set of 15 shared OTUs, four OTUs (1%) were present in multiple patients, which were the OTUs classified as *Streptococcus* (two OTUs), *Pseudomonas,* and multi-affiliated Enterobacteriaceae (possibly *Escherichia* or *Enterobacter*). *Lactobacillus* was also found to be shared among multiple samples within some patients, but the OTU was proven to be different among the tested samples, that is, saliva, blood, and feces.

Discussion

We describe the blood microbiome of KTRs and clearly show distinct microbiome profiles among the gut, oral, and blood samples. A small percentage of phylotypes were found to be shared among these different human habitats, unraveling a possible mobile microbiome in stable KTR patients. Interestingly, these phylotypes included possible KTR pathogens such as *Pseudomonas*, *Streptococcus,* and classified Enterobacteriaceae. As previously reported, the gut microbiome was dominated by Bacteroidetes and Firmicutes phyla.[1,2,13] Moreover, Fricke *et al*.[2] showed that the most significant changes in the human microbiome between pre- and posttransplant occurred immediately after transplantation (1 month), justifying the evaluation of KTRs 3 months after kidney transplantation, as conducted in this study. In the oral microbiome, the dominant phyla were Proteobacteria, Bacteroidetes, Fusobacteria, and Firmicutes. This composition disagrees with other studies that evaluated oral swabs in KTRs[2] and dental plaque or saliva in healthy controls,[21,22] where Firmicutes, especially *Streptococcus*, were the most prevalent genus. More similarities were found when we analyzed the most prevalent families in the oral microbiome of the patients: Campylobacteraceae, Fusobacteriaceae, Neisseriaceae, Pasteurellaceae, Porphyromonadaceae, Prevotellaceae, Streptococcaceae. Nonetheless, other studies also reported
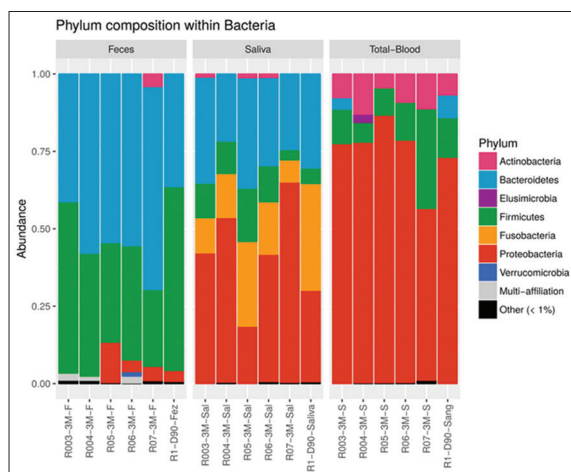

Figure 1: Relative abundance of bacterial taxa at the phylum level in kidney transplant recipients. Taxa are identified by name in the plot for abundance >1%. Taxa are merged into the "Other" category only if they exist in any sample with abundance greater than 0.01%. Taxa are merged into the "Multi-affiliation" category when they can correspond to two or more different taxa
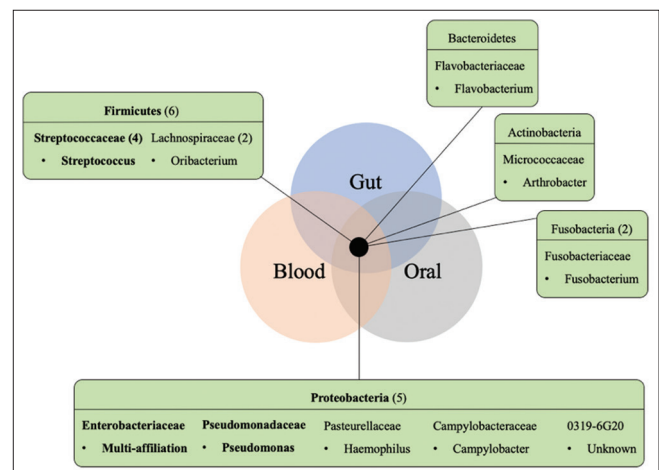

Figure 2: Shared taxa among the gut, oral, and blood samples in kidney transplant recipients. A set of 15 shared OTUs found in the six kidney transplant recipients studied belong to the following represented phyla, with the respective number of OTUs per phyla or family (when higher than 1) given in parentheses. OTU taxonomy (family and respective genus) found in the three habitats of multiple patients are represented in bold. OTU = operational taxonomic unit

slightly different microbiome profiles with lower levels of Firmicutes and a higher proportion of Proteobacteria and Bacteroidetes among solid-organ transplant recipients and Barrett's esophagus patients.[6,23] Fricke et al.[2] reported the blood samples rarely harbored microbial components in transplant recipients. However, presently, multiple studies describe and acknowledge the presence of a distinct blood microbiome in healthy and disease-affected individuals[7,8,11] and even report the detection of viable bacteria.[9]

Our study revealed Proteobacteria, Firmicutes, and Actinobacteria phyla as the dominant phyla in the blood microbiome profile of the KTRs. This is in line with another study that showed higher abundance of Enterobacteriaceae and Pseudomonadaceae families in the blood microbiome of nondiabetic CKD patients in comparison to healthy controls.[10]

Interestingly, a small group of shared OTUs among different human-associated environments (oral, blood, and gut) was reported in this study. Previously, 219 OTUs shared between the urine and gut samples, 193 OTUs shared between the oral and gut samples, and 16 OTUs shared between the urine and oral samples were reported.[2] The present study reports for the first time a shared, and maybe mobile, microbiome in samples collected from three different body sites (gut, blood, and oral). This finding suggests the presence of a systemic microbiome, comprising particular bacteria or bacterial DNA, which could be found in different body habitats and/or are associated with mobility throughout the human body. The full consequence of this phenomenon is still unknown, but it is possibly associated with systemic infections or colonization, and may have an impact in the microbiome, systemic inflammation, and patient blood management. Also, whatever the nature (living bacteria or bacterial DNA) of the taxa found in these three microbiomes, it may represent an interesting source of inflammation or infection risk biomarkers for KTR.

In conclusion, KTRs present a specific blood microbiome profile, different from the gut and oral habitats, but with a low proportion of phylotypes shared among different habitats that can represent a shared microbiome in KTRs and/or an interesting source of biomarkers. The clinical relevance of this observation should be further explored.

Acknowledgements

Financial support and sponsorship

Conflicts of interest

The authors declare that they have no conflicts of interest.

References

1. Sampaio-Maia B, Simoes-Silva L, Pestana M, Araujo R, Soares-Silva IJ. The Role of the gut microbiome on chronic kidney disease. Adv Appl Microbiol 2016;96:65-94.

2. Fricke WF, Maddox C, Song Y, Bromberg JS. Human microbiota characterization in the course of renal transplantation. Am J Transplant 2014;14:416-27.

3. Lee JR, Muthukumar T, Dadhania D, Toussaint NC, Ling L, Pamer E, et al. Gut microbial community structure and complications after kidney transplantation: A pilot study. Transplantation 2014;98:697-705.

4. Lee JR, Muthukumar T, Dadhania D, Taur Y, Jenq RR, Toussaint NC, et al. Gut microbiota and tacrolimus dosing in kidney transplantation. PLoS ONE 2015;10:e0122399.

5. Zaza G, Dalla Gassa A, Felis G, Granata S, Torriani S, Lupo A. Impact of maintenance immunosuppressive therapy on the fecal microbiome of renal transplant recipients: Comparison between an everolimus- and a standard tacrolimus-based regimen. PLoS One 2017;12:e0178228.

6. Diaz PI, Hong BY, Frias-Lopez J, Dupuy AK, Angeloni M, Abusleme L, et al. Transplantation-associated long-term immunosuppression promotes oral colonization by potentially opportunistic pathogens without impacting other members of the salivary bacteriome. Clin Vaccine Immunol 2013;20:920-30.

7. Amar J, Lange C, Payros G, Garret C, Chabo C, Lantieri O, et al. Blood microbiota dysbiosis is associated with the onset of cardiovascular events in a large general population: The D.E.S.I.R. study. PLoS One 2013;8:e54461.

8. Paisse S, Valle C, Servant F, Courtney M, Burcelin R, Amar J, Lelouvier B. Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing. Transfusion 2016;56:1138-47.

9. Schierwagen R, Alvarez-Silva C, Madsen MSA, Kolbe CC, Meyer C, Thomas D, et al. Circulating microbiome in blood of different circulatory compartments. Gut 2019;68:578-80.

10. Shah NB, Allegretti AS, Nigwekar SU, Kalim S, Zhao S, Lelouvier B, et al. Blood microbiome profile in CKD: A pilot study. Clin J Am Soc Nephrol 2019;14:692-701.

11. Whittle E, Leonard MO, Harrison R, Gant TW, Tonge DP. Multi-method characterization of the human circulating microbiome. Front Microbiol 2018;9:3266.

12. Chan S, Hawley CM, Campbell KL, Morrison M, Campbell SB, Isbel NM, et al. Transplant associated infections-The role of the gastrointestinal microbiota and potential therapeutic options. Nephrology (Carlton) 2020;25:5-13.

13. Biehl LM, Cruz Aguilar R, Farowski F, Hahn W, Nowag A, Wisplinghoff H, et al. Fecal microbiota transplantation in a kidney transplant recipient with recurrent urinary tract infection. Infection 2018;46:871-4.

14. Lee JR, Magruder M, Zhang L, Westblade LF, Satlin MJ, Robertson A, et al. Gut microbiota dysbiosis and diarrhea in kidney transplant recipients. Am J Transplant 2019;19:488-500.

15. Burnham P, Dadhania D, Heyang M, Chen F, Westblade LF, Suthanthiran M, et al. Urinary cell-free DNA is a versatile analyte for monitoring infections of the urinary tract. Nat Commun 2018;9:2412.

16. Wu JF, Muthusamy A, Al-Ghalith GA, Knights D, Guo B, Wu B, *et al*. Urinary microbiome associated with chronic allograft dysfunction in kidney transplant recipients. Clin Transplant 2018;32:e13436.

17. Lluch J, Servant F, Paisse S, Valle C, Valiere S, Kuchly C, *et al*. The Characterization of novel tissue microbiota using an optimized 16S metagenomic sequencing pipeline. PLoS One 2015;10:e0142334.

18. Schierwagen R, Alvarez-Silva C, Servant F, Trebicka J, Lelouvier B, Arumugam M. Trust is good, control is better: Technical considerations in blood microbiome analysis. Gut 2020;69:1362-3.

19. Escudie F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, *et al*. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Bioinformatics 2018;34:1287-94.

20. Rognes T, Flouri T, Nichols B, Quince C, Mahe F. VSEARCH: A versatile open source tool for metagenomics. PeerJ 2016;4:e2584.

21. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy "core microbiome" of oral microbial communities. BMC Microbiol 2009;9:259.

22. Gazdeck RK, Fruscione SR, Adami GR, Zhou Y, Cooper LF, Schwartz JL. Diversity of the oral microbiome between dentate and edentulous individuals. Oral Dis 2019;25:911-8.

23. Snider EJ, Compres G, Freedberg DE, Giddins MJ, Khiabanian H, Lightdale CJ, *et al*. Barrett's esophagus is associated with a distinct oral microbiome. Clin Transl Gastroenterol 2018;9:135.

Supplementary Table 1: Infection events with antimicrobial therapy of kidney transplant recipients (KTR) occurring between 6 months before and 2 years after transplantations

Patient	Agent	Local	Time from KTR	Treatment
Patient1	*Staphyococcus aureus*	Peritoneal Catheter exit-site	1.5 before	Sulfamethoxazole
	Escherichia coli	Urine	2 after	Ciprofloxacin
Patient 2	*Herpes simplex vírus 1*	Oral Ulcers	0 after	Valaciclovir
Patient 3	*Herpes simplex vírus 1*	Oesophageal Ulcers	2.5 after	Valaciclovir

Supplementary Table 2: Significance regarding alpha diversity of the stool, saliva, and blood at different taxonomic levels calculated with different indexes

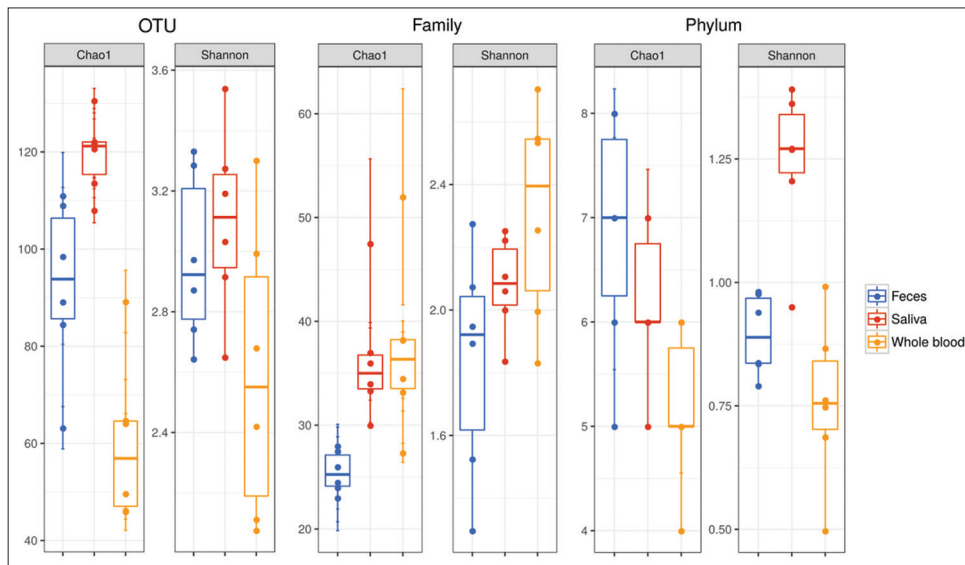| Taxonomic level	Alpha diversity			
	Observed	Chao	Shannon	Simpson
OTU	$P<0.05$	$P<0.05$	ns	ns
Species	$P<0.05$	$P<0.05$	ns	ns
Genus	$P<0.05$	ns	ns	ns
Family	$P<0.05$	$P<0.05$	$P<0.05$	ns
Class	$P<0.05$	ns	$P<0.05$	$P<0.05$
Order	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$
Phylum	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$
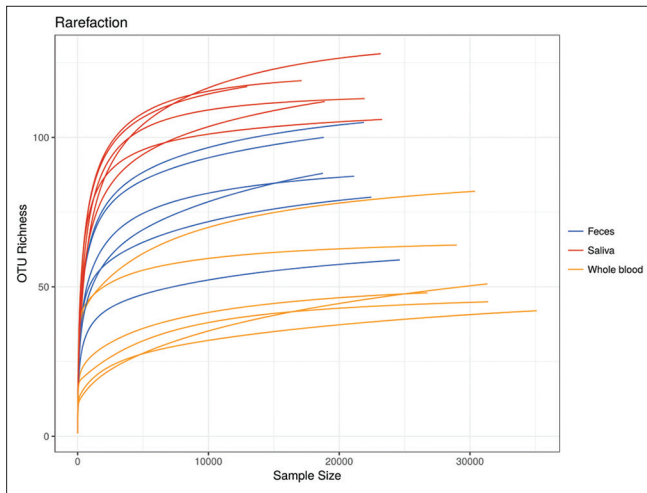
Supplementary Figure 1: The rarefaction curves plotted with all groups cumulatively. The rarefaction analysis curves suggest the sample diversity was captured as expected given the curves reach a plateau
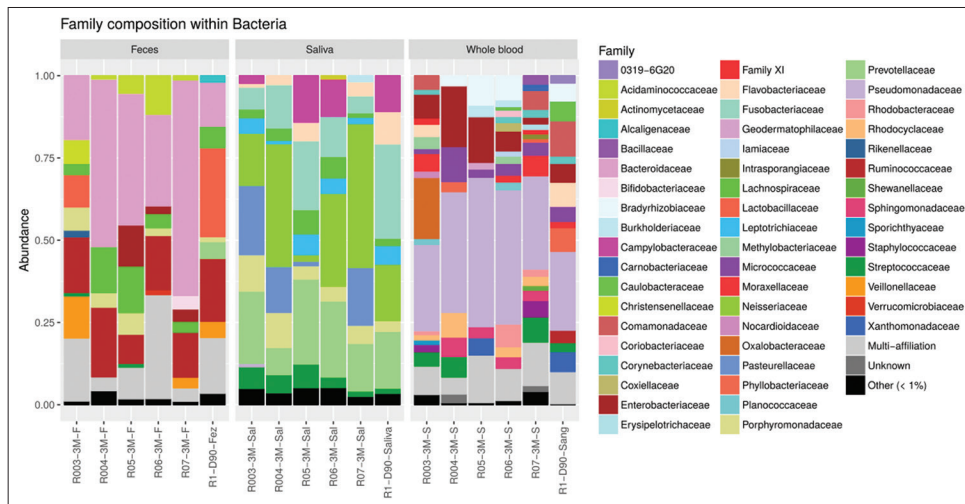
Supplementary Figure 2: Relative proportion taxa for class Family. Taxa are identified by name in the plot for abundance >1%. Taxa are merged into the "Other" category only if it exists in any sample with abundance greater than 0.01%. Taxa are merged into the "Multi-affiliation" category when they can correspond to two or more different taxa
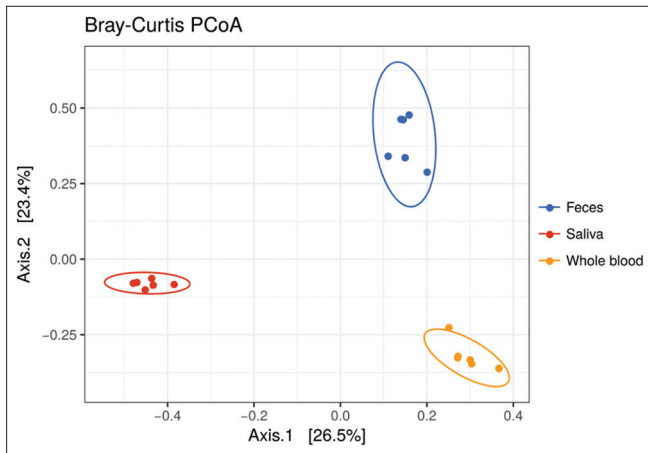
Supplementary Figure 3: Beta-diversity of the stool, saliva, and blood community at the OTU level with Bray-Curtis distance derived from 16S rRNA gene-based sequencing data

Supplementary Figure 4: Alpha diversity of the stool, saliva, and blood at OTU, Family, Phylum taxonomic levels calculated with Chao1 and Shannon indexes