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ORIGINAL ARTICLE

The oral microbiome of patients undergoing treatment for severe aplastic anemia: a pilot study

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Abstract
The microbiome, an intriguing component of the human body, composed of trillions of microorganisms, has prompted scientific exploration to identify and understand its function and role in health and disease. As associations between microbiome composition, disease, and symptoms accumulate, the future of medicine hinges upon a comprehensive knowledge of these microorganisms for patient care. The oral microbiome may provide valuable and efficient insight for predicting future changes in disease status, infection, or treatment course. The main aim of this pilot study was to characterize the oral microbiome in patients with severe aplastic anemia (SAA) during their therapeutic course. SAA is a hematologic disease characterized by bone marrow failure which if untreated is fatal. Treatment includes either hematopoietic stem cell transplantation (HSCT) or immunosuppressive therapy (IST). In this study, we examined the oral microbiome composition of 24 patients admitted to the National Institutes of Health (NIH) Clinical Center for experimental SAA treatment. Tongue brushings were collected to assess the effects of treatment on the oral microbiome. Twenty patients received standard IST (equine antithymocyte globulin and cyclosporine) plus eltrombopag. Four patients underwent HSCT. Oral specimens were obtained at three time points during treatment and clinical follow-up. Using a novel approach to 16S rRNA gene sequence analysis encompassing seven hypervariable regions, results demonstrated a predictable decrease in microbial diversity over time among the transplant patients. Linear discriminant analysis or LefSe reported a total of 14 statistically significant taxa ($p < 0.05$) across time points in the HSCT patients. One-way plots of relative abundance for two bacterial species (*Haemophilus parainfluenzae* and *Rothia mucilaginosa*) in the HSCT group, show the differences in abundance between time points. Only one bacterial species (*Prevotella histicola*) was noted in the IST group with a p value of 0.065. The patients receiving immunosuppressive therapy did not exhibit a clear change in diversity over time; however, patient-specific changes were noted. In addition, we compared our findings to tongue dorsum samples from healthy participants in the Human Microbiome Project (HMP) database and found among HSCT patients, approximately 35% of bacterial identifiers ($N = 229$) were unique to this study population and were not present in tongue dorsum specimens obtained from the HMP. Among IST-treated patients, 45% ($N = 351$) were unique to these patients and not identified by the HMP. Although antibiotic use may have likely influenced bacterial composition and diversity, some literature suggests a decreased

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impact of antimicrobials on the oral microbiome as compared to their effect on the gut microbiome. Future studies with larger sample sizes that focus on the oral microbiome and the effects of antibiotics in an immunosuppressed patient population may help establish these potential associations.

Keywords Oral microbiome · Tongue brushings · Immunosuppression and hematopoietic stem cell transplant

Background

Severe aplastic anemia (SAA)—a rare, life-threatening, heterogeneous hematologic disease—results from bone marrow failure [1]. Hallmarks of the disorder include loss of hematopoietic stem cells with hypocellular bone marrow resulting in pancytopenia [1, 2]. The disease largely afflicts children and young adults with almost half of cases occurring during the first three decades of life [3]. The clinical definition requires at least two of the following three conditions in peripheral blood: (1) an absolute neutrophil count of less than 500 per microliter, (2) an absolute reticulocyte count of less than 60,000 per microliter (automated analysis), and (3) a platelet count of less than 20,000 per microliter [4].

Historically, the pathophysiology of the disorder has been categorized as autoimmune—principally because of the disease response to immunosuppressive agents [1, 5–7]. Successful, curative, or ameliorative treatment regimens for the majority of patients include either hematopoietic stem cell transplantation (HSCT) or immunosuppressive therapy (IST) [6, 8]. Factors including age, patient performance status, cost, disease severity, and human leukocyte antigen (HLA)-matched donor availability influence a managing physician's treatment decision [8, 9].

HSCT from a HLA-matched donor frequently provides excellent survival rates typically in the range of 80% for patients younger than 50 years of age [10]. However, bone marrow transplantation is not without challenge; in the best case, only about 30% of patients find an HLA-matched sibling [11]. In addition, complications after HSCT include systemic bacterial and fungal infection, graft-versus-host disease, graft failure [12, 13], and pulmonary problems [14].

IST versus HSCT is the preferred treatment course with equine anti-thymocyte globulin (hATG) and cyclosporine A (CSA) for older adults who are susceptible to higher transplant-associated toxicities and younger patients who lack suitable donors [6]. Nevertheless, in recent studies, IST with hATG and CSA have shown a hematologic response rate as high as 60–70% [6, 15]. Recent studies adding the thrombopoietin receptor agonist, eltrombopag, have reported higher response rates and improved outcomes [5].

A strong link between immunity and the gut microbiome has been identified [16]. More specifically, changes in the gut microbiome related to immune modulations have been identified in allogeneic stem cell transplantation [17, 18]. In addition, a broad range of evidence supports a far-reaching impact of microbiome perturbations on a host of conditions from obesity and diabetes [19], cardiovascular disease [20], psoriasis [21], colitis [22], periodontitis [23], irritable bowel syndrome [24], and many others. The immunopathophysiology of SAA combined with treatment-induced immune system modulation and the obligate need for antimicrobial use in patients who are commonly neutropenic and develop opportunistic infections may reveal a concomitant shift in oral microbial community composition as microorganisms respond to changing microenvironments. Specifically, the oral microbiome because of its location, its varied microbial niches, and ease of sampling creates a valuable focus for future research. With the completion of recent large-scale studies such as Human Microbiome Project (HMP) [25, 26] and the compilation of the Human Oral Microbiome Database (HOMD) [27], researchers are well-positioned to deeply and thoroughly examine how microorganisms contribute to human health and disease.

In this pilot study, we detail oral microbiome findings from 24 immunocompromised patients receiving either HSCT or IST and present distinct microbial communities among individual patients and treatment groups. The purpose of this study is twofold: (1) to compare longitudinally the oral microbiome in patients with SAA during their therapeutic course and (2) to determine if patients receiving differing treatments for SAA display a different composition of oral microorganisms.

Methods

Study criteria

Research participants who were diagnosed with SAA and eligible to be admitted for clinical trial treatment were screened for possible participation in this oral microbiome protocol. Patients met inclusion criteria if they were greater than or equal to 18 years of age and if they were scheduled to receive either HSCT or IST treatment. Informed consent was obtained from participants when they were seen in clinic or in the hospital after admission. Patients who were edentulous or had significant tooth loss were excluded from study participation. In addition, patients were followed for 1 year after treatment to determine if they developed respiratory complications from treatment and required intubation.

Oral assessment and clinical samples

Oral assessment scores, demographics, laboratory values, and medications (steroids and antibiotics) were collected (Fig. 1). The clinical acute graft-vs-host disease (GVHD) measure was assessed at each time point [28]. This measure is used to grade GVHD with a clinical grade of 1–4. A patient who exhibited no signs or symptoms of GVHD was given a score of zero. The Decayed Missing and Filled Teeth (DMFT) score was collected by a dentist at study entry during a protocol-included dental screening visit [29]. A periodontal risk score involving a periodontal probing exam was initially attempted but was discontinued from the study as a result of many participants' low platelet counts [30]. Two oral assessment scores were collected at each time point: the Becks Modified Oral Assessment Score (BMOAS) and the Mucosal Plaque Score (MPS). The BMOAS measures five characteristics of the oral cavity-lips, gingiva/mucosa, tongue, teeth, and saliva [31]. This modified measure ranges from a normal of 5 to 20. The higher the number, the greater the injury or dysfunction. The MPS examines mucosa and plaque [32]. The MPS ranges from a normal of 2 to 8. Again, the higher the number, the greater the injury or dysfunction. In addition, the World Health Organization (WHO) Mucositis Grading Scale was collected at each time point [33]. The scores range from 0 or none to grade 4 mucositis where the patient cannot tolerate any oral intake.

Descriptive statistics were used to compare demographics, smoking, and length of stay (Table 1). Oral assessment scores (BMOAS and MPS) were re-coded and compared using Cochran's Q test for related categories.

Since clinical treatment and follow-up appointments differ for patients receiving HSCT and IST, oral tongue brushing sampling time points did not coincide (Fig. 1). Nevertheless, three samples were collected with one occurring at baseline and two subsequent time points following treatment in each group. In IST patients, baseline specimens were collected before treatment began and the second and third specimens were collected at the 3- and 6-month scheduled follow-up clinic appointments, respectively. In HSCT patients, the first sample was obtained before transplant conditioning treatment. The second specimen was collected at engraftment, defined as two absolute neutrophil counts of greater than 500 per microliter for at least 2 days. The final specimen collection occurred at the participant's scheduled clinic visit approximately 100 days following transplant. If the patient developed respiratory complications within the 1-year post-treatment requiring intubation, additional specimens would be collected. It was planned to gender and age match any patients who developed respiratory complications with healthy controls.

All oral samples were collected at least 2 h after eating, drinking, or any type of oral care. Two aliquots were collected per patient during each collection time point. The tongue was swabbed by brushing back and forth for approximately 10–20 s with a sterile cytology brush. The specimens were refrigerated after collection. The brush handle was cut with sterile scissors, and the brush was suspended in a 2-mL microfuge tube containing 1 mL of sterile phosphate-buffered saline (PBS, pH = 7.4). Tubes were vortexed for approximately 1 min to dislodge particles from the cytobrush. Cytobrush tips were then centrifuged at 13,000 rpm at 4 °C for 5 min. If a distinct pellet did not form, the tubes were re-centrifuged using the same conditions. Using sterile forceps, cytobrush tips and excess supernatant were discarded, leaving behind an intact pellet. The oral specimens were stored at − 80 °C until DNA extraction.

DNA extraction

DNA extraction was carried out using the MoBio BiOstatic Bacteremia DNA Isolation Kit (MoBio, now known as QIAamp BiOstic Bacteremia DNA Kit, Qiagen, Carlsbad, CA) according to the manufacturer's instructions. In addition, AMPure XP beads (Beckman Coulter, Indianapolis, IN) were used for PCR purification.

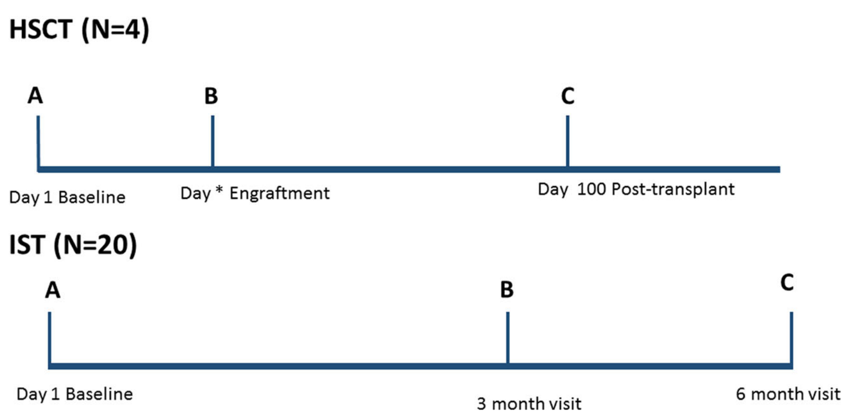


Fig. 1 Study schema. Study schema of three oral specimen collection periods by treatment regimen. Hematopoietic stem cell transplantation (HSCT) participants were sampled at baseline (A—prior to transplantation), at engraftment (B—following transplantation), and at 100 days post-transplant (C). Immunosuppressive therapy (IST) participants were sampled at baseline (A—prior to treatment) and at two time points following treatment—at (B) 3 months and at (C) 6 months following treatment initiation. *Day of engraftment for the HSCT patients was approximately 2–3 weeks post-transplant

16S rRNA gene sequencing

The Ion Torrent Personal Genome Machine (Ion Torrent ThermoFisher Scientific, Waltham, MA) 16S next-generation sequencing platform was used for all sequencing. The 16S gene was amplified using the Ion 16S™ Metagenomics Kit (Ion Torrent, ThermoFisher Scientific, Waltham, MA), capable of amplifying seven hypervariable regions of the 16S rRNA gene. Two separate PCR reactions were performed in accordance with the manufacturer's instructions. DNA libraries were constructed with 50 ng of pooled amplicons using Ion Plus Fragment Library Kit™ and Ion XpressBarcode Adapters, 1-16™ (Life Technologies, ThermoFisher Scientific, Waltham, MA). After the library preparation, the final quality check was performed with Bioanalyzer 2100 prior to template preparation. The Ion 316 Chip Kit v2 was used with the 400-bp kit. The Torrent Suite version 4.4.2 (Life Technologies, ThermoFisher Scientific, Waltham, MA) was used for base calling and run demultiplexing using default parameters. FASTQ files were obtained using FileExporter version 4.4.0 (Life Technologies, ThermoFisher Scientific, Waltham, MA).

Data pre-processing

Demultiplexed raw sequence reads were processed according to previously published methods up to the point where the reads were separated into their respective targeted regions. Briefly, reads were filtered to remove those with fewer than 200 bp and with greater than 1 total expected errors for all bases in the read after truncation (filtering method used: fastq_filter with -fastq_maxee E argument set to 1). Since reads were already demultiplexed, read identifications were edited so that they corresponded to their sample names and individual sample FASTA files were combined into one to be further processed through the pipeline. This last step mimics multiplexed reads and is specific to the analytical pipeline workflow. From this set, reads were subjected to align.seqs in Mothur and steps were followed as previously described to separate reads into their respective forward and reverse targeted regions [34]. Once reads were separated into their respective targeted hypervariable regions, they were submitted for operational taxonomic unit (OTU) picking using the k-mer analysis pipeline described below. The majority of results presented in this paper focus on the forward reads from the V2 region of the 16S rRNA gene. Results from additional regions may be provided upon request.

K-mer analysis pipeline at Forsyth Institute

Demultiplexed forward-only reads were mapped against a database of human oral (tongue brushings) microbial 16S strain-specific sequences (HOMD v14.3) using a k-mer analysis pipeline version 1.0 developed by Forsyth Institute, Cambridge, MA. The k-mer pipeline used is an extension to the Simrank algorithm [35]. Briefly, the k-mer analysis pipeline determines the number of times each possible motif of nucleotides of 11 length occurs in each reference 16S sequence. This constitutes the "k-mer profile" of the 16S sequence. Then, the query sequences were sequence profiled in a similar fashion and the query profiles were compared against the reference profiles. A query whose profile overlaps the profile of a single reference sequence at 80% or more was classified with that label. Query sequences that mapped equally well to more than one reference profile were further resolved to the species, if possible, or the genus level. Query sequences that could not be resolved to a single genus were classified as "undefined." This approach yielded a set of organisms at the strain, species, and genus level, as well as one "undefined" category. Bacterial taxa described at these levels will be referred to as unique bacterial identifiers. For each sample, all sequences mapping to these categories were counted and used as a quasi-OTU table for subsequent analysis. The 16S data published by the HMP consortium was selected as a positive control for the analysis since it provided many samples taken from a healthy patient population. The trimmed and demultiplexed 16S sequences from tongue dorsum samples were downloaded from https://www.hmpdacc.org/hmp/HM16STR/ and filtered by V region [36]. The k-mer tool was used to generate k-mer profiles, which were then compared to the HOMD reference database as described above. In the HMP, not all samples were sequenced at all V regions. The data was kept separate because the Ion Torrent data was also split up by V region.

LefSe analysis on SAA samples

The microbial communities of two conditions were compared in a pairwise fashion and potential microbial biomarker species indicative of one or the other condition were identified using the linear discriminant analysis effect size (LEfSe) tool [37]. First, the OTU tables generated as described above were filtered to remove potential noise caused by low-abundance taxa by including taxa that are present in 1% of the samples and at an abundance of at least 5%. Then, the LefSe analysis was performed with values of $\alpha < 0.1$ for the Kruskal–Wallis test and LDA score > 2.0 to determine discriminative features.

Data interpretation and visualization

OTU tables with counts from each region per methods above were visualized using the JMP statistical discovery software (version 10, SAS Headquarters, Cary, NC). The relative abundance within a sample was calculated for all samples, and relative abundance bar chart plots were generated for both the HSCT and IST patients. To further investigate the difference between the time points for all samples, bacterial taxa with a count of 1 or greater were summed over for each sample. This number was deemed the total OTU count. Total OTU counts by the three sampling time points were plotted in a one-way plot to visualize the total number of OTUs for each sample at each time point. The Shannon diversity index was calculated for each sample in order to investigate sample alpha diversity at each time point.

HMP comparison to SAA samples

Bacterial identifiers from the k-mer tool were merged from all three HMP tables (9 V regions: V1–3, V3–5, and V6–9). Bacterial identifiers with a read count of 1 or greater were deemed as present on any of the 9 regions analyzed from the HMP data. A bacterial identifier was deemed as present in the SAA data if any bacterial identifier was present across any samples for any region (V2, V3, V4, V67, or V8) and had at least a read count of 1. In order to compare across the HMP and the SAA data, the bacterial identifiers found to be present on either study were merged and Venn diagrams were generated to investigate overlaps.

Patient antibiotic use and visualization

Patient antibiotic use throughout the study was investigated. A horizontal bar graph was generated for both the IST and HSCT groups in order to visualize the different antibiotics used and the duration of use. For calculation of antibiotic use, baseline was defined as day 0 (day of study enrollment). The second and third time points for the HSCT group was calculated as (1) the total number of days between baseline and time point B (engraftment) and (2) the total number of days between engraftment and time point C. Time point C is 100 days post-transplant. For the IST group, (1) the second time point was calculated as the total number of days between baseline and 3 months and (2) the third time point was calculated as the total number of days between 3-month clinic visit and 6-month clinic visit. The timeline continues throughout the remainder of the study (180 days for IST patients and 100 days post-transplant for the HSCT patients).

Results

Sample

This study was approved by the National Heart Lung and Blood Institute's (NHLBI) intramural investigational review board (NCT01900119). The first patient was consented in December 2013, and the last patient was consented in March 2015. Study participants were followed for a year after treatment to potentially capture additional samples from patients who developed severe respiratory symptoms that required intubation. However, only one patient required intubation.

Thirty-one eligible patients were approached for study participation. Of the 31, 7 patients interviewed declined to participate, stating they were uninterested in participating. Twenty-four patients with SAA signed the consent and participated in the study. Twenty patients received standard IST and eltrombopag on an institutional protocol (NCT01623167). Standard immunosuppressive therapy included cyclosporine and hATG. All IST patients received prednisone to prevent the complications of the hATG. Four who had failed prior immunosuppressive treatment, received HSCT on institutional transplant protocols (Table 1). All the IST patients received prophylactic antivirals and antifungals. For the patients who received IST, inhaled pentamidine or atovaquone was administered for pneumocystis pneumonia prophylaxis. The HSCT patients received antiviral, antifungal, and either sulfamthoxazole/trimethoprim (Bactrim) or pentamidine for pneumocystis pneumonia prophylaxis. One HSCT patient received total body irradiation in preparation for the transplant.

The majority of study participants were white and non-Hispanic (Table 1). Of the four transplant patients, two self-reported as Hispanic and one self-identified as Asian. Approximately 45% of patients were between 20 and 29 years of age (Table 1). Few of the participants smoked; only two endorsed smoking at time of study participation (Table 1). There were no patients that had a previous transplant. All patients spent at least 5 days as inpatients during their treatment (Table 1). The HSCT patients spent a significantly longer time in the hospital as compared to patients undergoing IST (Table 1). The absolute neutrophil count ranged from 0.02 to 6.15 (1000/μL) throughout the study. The mean score at baseline was the lowest at 0.53 (SD = 0.54) (1000/μL).

The majority of IST patients were consented either a day proceeding or the day of treatment initiation; baseline specimens were collected prior to the patient receiving either immunosuppression or conditioning for transplant treatment. However, in patients receiving HSCT, baseline specimen collection coincided with their pre-treatment clinic visit. On average, baseline specimens from HSCT patients were collected 40 days prior to their date of transplant (range 89 days; 9–98 days).

Besides the oral assessment scores (Table 1), the oral environment was evaluated by a dentist. The exam consisted of an oral exam and collecting the DMFT. The mean DMFT score was 8.45 (SD = 6.8) with values of 0 (no decayed missing, filled teeth) to 28 (all teeth were decayed, missing or filled). The median score was 7.0. Clinically, the oral assessment mean scores between time points varied but very slightly (Table 1). The oral scores for the BMOAS and the MPS did not significantly change over the three time points as calculated using the Cochran's Q test [Cochran's Q test = df (2) =

Table 1 Demographic features of study participants

Variable	$N = 24$
Treatment group	
IST	20, 83.3%
HSCT	4, 16.7%
Age	20–67 years old
20–29 years old	11, 45.8%
30–39	3, 12.5%
40–49	5, 20.8%
50–59	3, 12.5%
60–67	2, 8.3%
Gender (male)	12, 50%
Race	
White	17, 71%
Black or AA	2, 8.3%
Asian	3, 12.5%
Other	1, 4.2%
Unknown	1, 4.2%
Ethnicity	
Hispanic	5, 21%
Smoking/tobacco use	
Never smoked or used tobacco	14, 58.3%
Smoking now or use of tobacco	2, 8.3%
History of smoking or tobacco use	8, 33.3%
Hospital length of stay (days)	Mean (SD)
IST	16.3 (16.5)
HSCT	80.7 (27.40)
Oral assessment scores	Mean (SD)
DMFT	8.45 (6.8)
BMOAS (5–20)	Mean (SD)
Time point	
A	5.25 (0.74)
B	5.70 (1.08)
C	5.33 (0.64)
MPS (2–8)	Mean (SD)
Time point	
A	2.12 (0.33)
B	2.46 (0.78)
C	2.33 (0.64)

Table 1 describes demographic data of the sample. Oral assessment scores are listed. DMFT decayed missing and filled teeth score

IST immunosuppressive therapy, *HSCT* hematopoietic stem cell transplantation, *BMOAS* Becks Modified Oral Assessment Score, *MPS* Mucosal Plaque Score, *AA* African American

6.00, $p = 0.54$ (exact) and for MPS Cochran's Q test = $df(2) = 4.750$, $p = 0.114$ (exact)]. Only one transplant patient developed mild mucositis and at the 100-day time point a grade 1 GVHD. Some of the patients used chlorhexidine rinses as part of their oral care, but the 2-h exclusion before specimen collection included any type of oral care. One patient had a cleaning procedure, and another patient had a carious tooth extraction, but these procedures were performed days after the initial oral specimens were collected and months before the second specimen collection. Both these patients received immunosuppression so that the second specimen collection was at 3 months from baseline. Two of the transplant patients had buccal biopsies for evaluation of chronic GVHD performed before the 100 day sample (second sampling time point). One IST patient with adequate platelets had a periodontal examination after the baseline specimen collection.

Sequencing results

Sequencing was accomplished as previously described using the Ion 16S™ Metagenomics Kit (Life Technologies, ThermoFisher, Waltham, MA). A total of 75 samples were sequenced. The average number of reads across all samples was 204, 914. Seventy specimens were included in the analysis. Five specimens were not included. The patient who was intubated had three additional specimens collected—two oral tongue brushings and one tracheal aspirate and since no other patients required intubation, these specimens were not included. Two other oral specimens had very low quantity of DNA after amplification. Both of these two post-treatment oral samples were from the same patient who received intensive broad spectrum antibiotic treatment. A total of 267 bacterial identifiers were identified in the 60 immunosuppressive samples as compared to 190 bacterial identifiers classified in the 10 transplant samples. A table of bacterial identifiers identified by the six other hypervariable regions is presented in Supplemental Table 1. An additional column in this table cites the overlapping bacteria identified from these SAA patients and healthy participants in the HMP (https://www.hmpdacc.org/hmp/HM16STR) [36]. Among HSCT patients, approximately 35% of identified bacterial taxa ($N = 229$) were unique to this study population and were not present in tongue dorsum specimens obtained from the HMP. Among IST patients, 45% ($N = 351$) were unique to these patients and not identified by the HMP (Fig. 2). One-way plots showing the number of bacterial identifiers in each specimen at each time point among patients receiving either HSCT or IST are shown in Fig. 3. The mean number of bacterial identifiers (center green line in mean diamonds) displays a downward trend from baseline to 3 months and then an increase back toward baseline at the 6-month time point among the IST patients. In contrast, the average number of bacterial identifiers in the HSCT patients shows a more dramatic decrease between baseline and engraftment. Pairwise significance testing did not indicate a significant difference between time points A vs B or B vs C. To quantitate these findings, the Shannon diversity index (SDI) was calculated and displayed in a one-way plot for each patient for each treatment group (Figs. 4 and 5). Figure 4 displays the four transplant

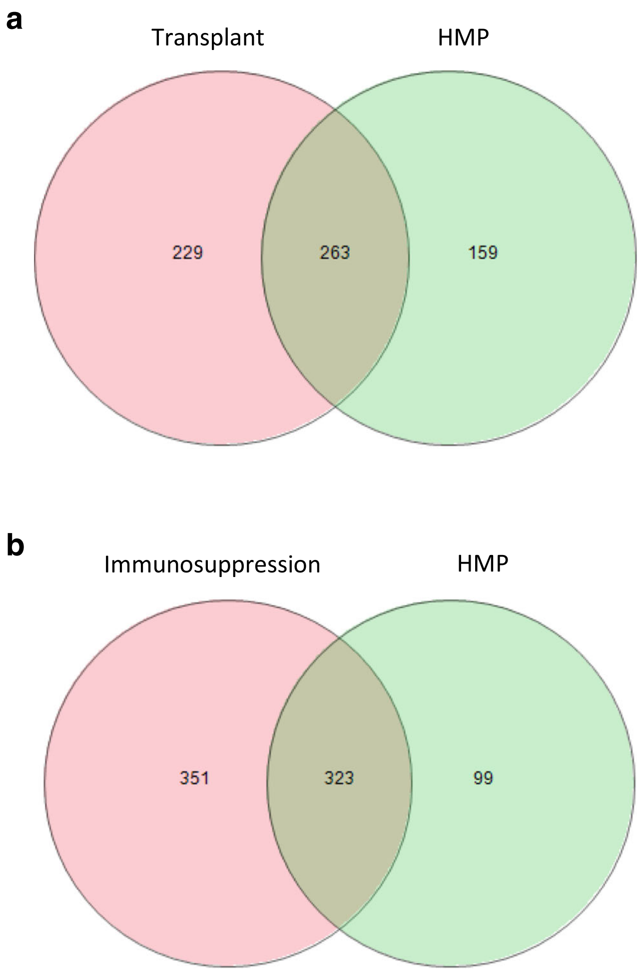


Fig. 2 Transplant and immunosuppression patients' comparison of unique and shared bacterial taxa with the Human Microbiome Project Patients with severe aplastic anemia who received either **(a)** stem cell transplantation or **(b)** immunosuppressive therapy and healthy volunteers who completed the Human Microbiome Project. Bacterial taxa are identified using two sets of primers, identifying the hypervariable regions, primer set 1, V2, V4, V8 and primer set 2, V3,V6-V7, V9 among severe aplastic anemia participants. Tongue dorsum samples from the HMP bacterial taxa are characterized through hypervariable regions. A count of one or greater in any hypervariable region represents bacterial presence. Among HSCT patients, approximately 35% ($N = 229$) of identified bacteria were unique to this study population and were not present in tongue dorsum specimens obtained from the HMP. Among IST patients, 45% ($N = 351$) were unique to these patients and not identified by the HMP

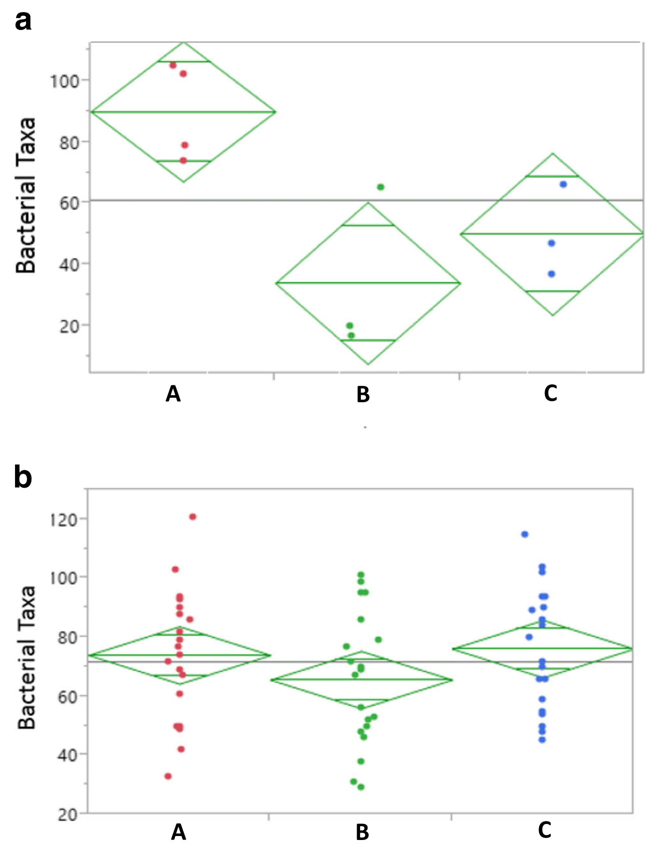


Fig. 3 One-way plot of the number of bacterial taxa identified in hypervariable region V2 over three time points (**a**) hematopoietic stem cell transplantation or (**b**) immunosuppressive therapy. One-way plots show the three oral sampling time points on the *x*-axis and total bacteria taxa identified on the *y*-axis. Kite diagrams show means (mid-lines) and upper and lower confidence limits for each sampling point

patients' data and compares their antibiotic use data with the SDI. Because antibiotic use can be a confounding factor in microbiome studies, patient antibiotic use was examined. The four HSCT patients received a total of 17 different antibiotics with a mean number of 8.5 antibiotics per patient. The average SDI of HSCT patients changed between baseline (time point A) and engraftment (time point B), with an average SDI of 3 at baseline to 1.4 at engraftment. At 100 days post-transplant (time point C), the SDI increased to 2.3.

In contrast, the IST group (Fig. 5) did not exhibit a decreasing or increasing trend and alternatively displayed relatively stable SDIs at the three time points, namely baseline, 3 months and 6 months (2.7, 2.6, and 2.8, respectively). Three of the 20 IST patients received no antibiotics throughout their treatment period. Despite these patients receiving no antibiotics, their diversity change over time did not differ from those who received antibiotics nor could any pattern be found among the three patients. The 17 remaining IST patients received a combined total of 16 different antibiotics with a mean of 2.8 antibiotics per patient. In an attempt to group the IST patients and identify trends, Fig. 5b was generated and shows four groups. Group 1, the largest group ($n = 8$), shows a similar pattern as the HSCT patients with SDI decreasing at the second time point then increasing back to pre-treatment ranges at the third time point. Group 2 has only two patients who demonstrate a decreasing trend from baseline to the third time point. Group 3 has the exact opposite trend increasing from baseline to the third time point. Finally, group 4, the second largest group, shows increasing SDI from baseline to second time point and

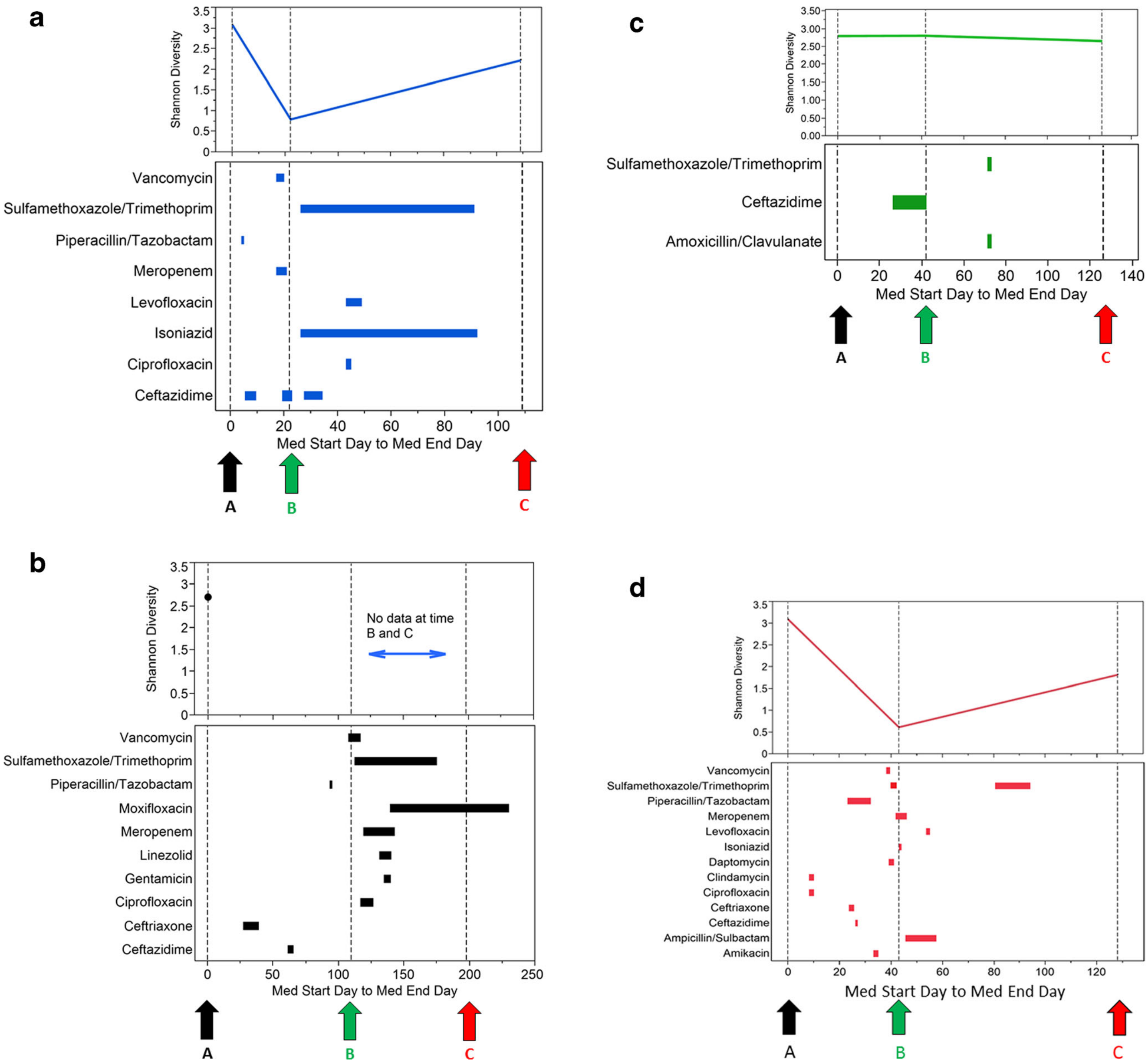


Fig. 4 Duration of antibiotic use and days at specimen collection compared to diversity for four HSCT patients. Shannon diversity index and antibiotic administration by patient (**a**–**d**) receiving hematopoietic stem cell transplantation. Vertical dotted lines indicate sampling time points. Colored horizontal lines indicate the type and duration of each study participant's antibiotic regimen. The black arrow signifies the baseline sample, green arrow signifies sampling time point B, and red arrow signifies sampling time point C for all four patients

then at the third time point decreasing diversity. The three IST patients who received no antibiotics were in three different groups.

Figure 6 demonstrates the type and duration of antibiotics administered during the protocol window among the IST patients. Patients in both groups were administered most of their antibiotics between baseline and the first post-treatment specimen (engraftment for HSCT or 3-month visit for IST). The HSCT patients received more antibiotics past this time point than did the IST. In addition to antibiotics, all IST patients received anti-thymocyte globulin, prednisone, cyclosporine, and eltrombopag, in accordance with the SAA treatment protocol. The HSCT group also received parent protocol-driven conditioning and immunosuppressive agents which included anti-thymocyte globulin, prednisone, and cyclosporine or tacrolimus. While these patients received many different antibiotics throughout the course of the study, correlations between Shannon diversity index and the duration of antibiotic therapy was investigated. Not surprisingly, some correlations were seen within the two treatment groups, suggesting that

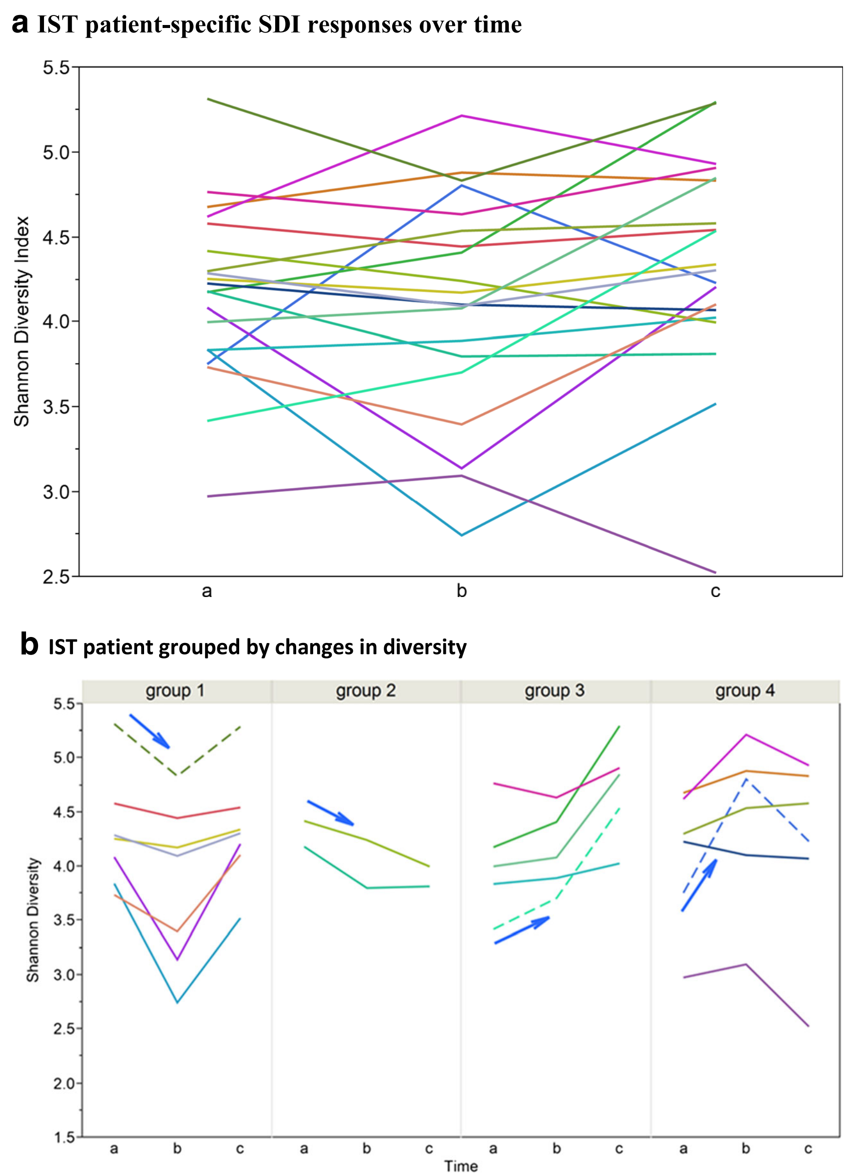


Fig. 5 Shannon diversity index (SDI) for each sample at each time point for immunosuppressive therapy (**a**). Separate lines represent a different patient (**b**) shows patient-specific responses grouped by changes in SDI over time points. a = baseline, b = 3-month clinic visit, and c = 6-month clinic visit. Groups 1 and 2 show those patients whose diversity decreased from baseline to 3 months post-treatment. Groups 3 and 4 shows those patients whose diversity increased from baseline to 3 months post-treatment. Blue arrows indicate SDI change from time points A and B for each group. Three dotted lines (groups 1, 3, and 4) indicate the three patients that did not receive antibiotics through the course of the study

antibiotic use may cause changes in the diversity of the oral microbiome. More studies need to be conducted to further investigate this finding.

Figure 7 shows relative abundance plots for HSCT patients and IST patients. Twenty patient samples at each specimen collection point are shown among the IST patients. The figure shows only those bacterial identifiers with an abundance of greater than or equal to 1% or greater abundance for HSCT and 5% or greater abundance for IST, although all bacterial identifiers are depicted in the plot. In the IST group, the three most abundant bacterial identifiers across all three time points include *Haemophilus parainfluenzae*, *Prevotella melaninogenica*, and *Neisseria* spp. representing on average at least 10% or greater abundance. In the HSCT group, the three most abundant bacterial identifiers across all time points include *Haemophilus parainfluenzae*, *P. melaninogenica*, and *Rothia mucilaginosa* representing on average at least 11% or greater abundance.

The results of the LefSe analyses reported 14 statistically significant taxa ($p < 0.05$) after running three different time point comparisons (i.e., A vs B, B vs. C, and A vs. C) in the HSCT patients (Fig. 8). Two of the taxa (*Haemophilus parainfluenzae* and *Rothia mucilaginosa*) were chosen to be shown in a one-way plots of relative abundance. These plots illustrate differences in abundance between A vs B and B vs C (Fig. 8a). Only one species, *Prevotella histicola*, was noted from the LefSe analysis in the IST group with a p value of 0.065 (Fig. 8b).

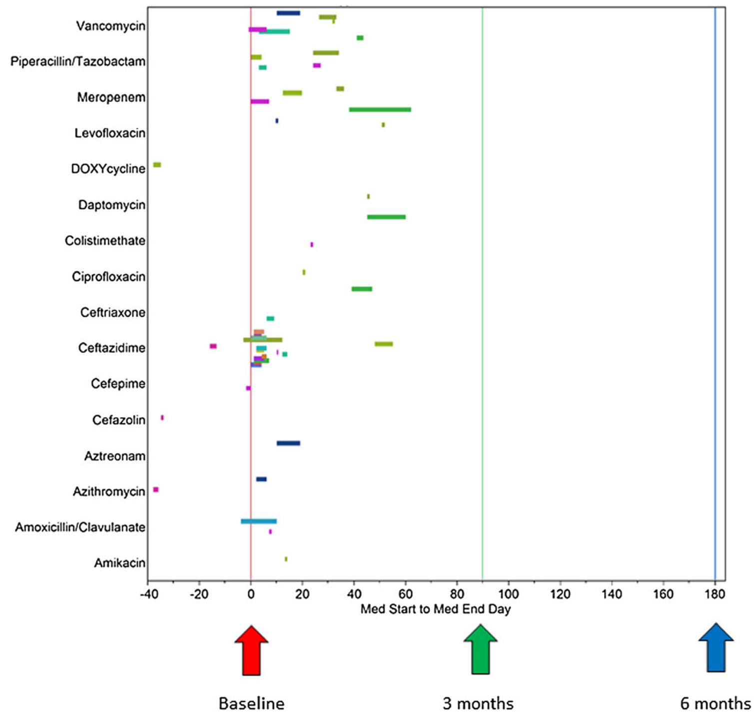


Fig. 6 Antibiotic administration by patient throughout for 17 immunosuppressive therapy patients. Vertical lines indicate sampling time points. Colored horizontal lines correlate to the type and duration of each study participant's antibiotic regimen. The vertical lines refer to sampling time points, i.e., the baseline (red line), 3 months (green line), and 6 months (blue line). The color-coded horizontal lines refer to each patient's specific antibiotic regimen. There were five IST patients who received antibiotics for numerous reasons prior to consent

Discussion

To our knowledge, this is the first study of the longitudinal analysis of the oral microbiome among patients receiving experimental treatment for severe aplastic anemia. Although much of the current microbiome research emphasizes on examination of the gut microbiome, in this study, the oral microbiome was chosen for its location of the oral cavity to the aerodigestive tract and ease of sampling. The oral microbiome may provide particularly valuable and efficient insight for predicting future changes in disease status, infection, or treatment course in this immunosuppressed population.

Untreated SAA patients or normal volunteers to serve as a control group were not specifically selected as a part of this study design; however, data comparisons from healthy volunteers from the HMP study were carried out. In comparing our data with the tongue brushing specimens contained in the HMP database, we found many congruent bacterial identifiers. There were 323 shared between the HMP and the IST patients and 263 shared between the HMP and the HSCT patients. Notably, we characterized 351 taxa unique to this immunosuppressed population, i.e., not identified in the healthy volunteers of the HMP study. This finding is not surprising when one considers some important differences between the two datasets, and we hypothesized that our sample should differ from the HMP study for numerous reasons. One main reason for this difference can be attributed to the medications aplastic anemia patients received, i.e., immunosuppressive medications and antibiotics. Both of these medications could severely change a patient's microbiome. Another possible explanation is the SAA samples were based on a time course where a patient's microbiome was investigated throughout treatment whereas the majority of the HMP were collected at one time point. Finally, while the same bioinformatics pipeline was applied to both datasets, the sequencing files arose from two separate sequencing platforms and there could be inherent bias just from the platform itself. There remains much to be learned about whether there is a "core oral microbiome" and this idea is still a very controversial topic of research interest [38, 39]. In a large time-series study in ten normal volunteers, Hall and others again emphasize that the oral microbiome have "both stable and variable components" p.5 [40]. These authors stressed the need to examine the variable components of the oral microbiome in health and disease. A larger sample of SAA patients with additional specimen collection time points and an age- and gender-matched normal volunteer group would be an optimal design for a future project to assist in answering these questions.

A few studies have analyzed the gut microbiome during allogeneic stem cell transplant [18, 41, 42]. Some investigators have studied the microbiome in relation to the immunosuppressive therapy in renal transplantation patients [43, 44]. Each of these studies measured bacterial diversity as an outcome, and Taur et al. linked decreased microbial diversity to increased mortality in allogeneic stem cell transplantation [18]. A more recent paper measured microbial diversity before allogeneic transplant and did not find an association between transplant outcomes (i.e., survival or relapse) and gut microbial diversity [41]. There are many reviews and animal studies correlating the gut microbiome to immunity [45–47]. However, with the exception of kidney transplant literature cited above, we found no information on immunosuppression

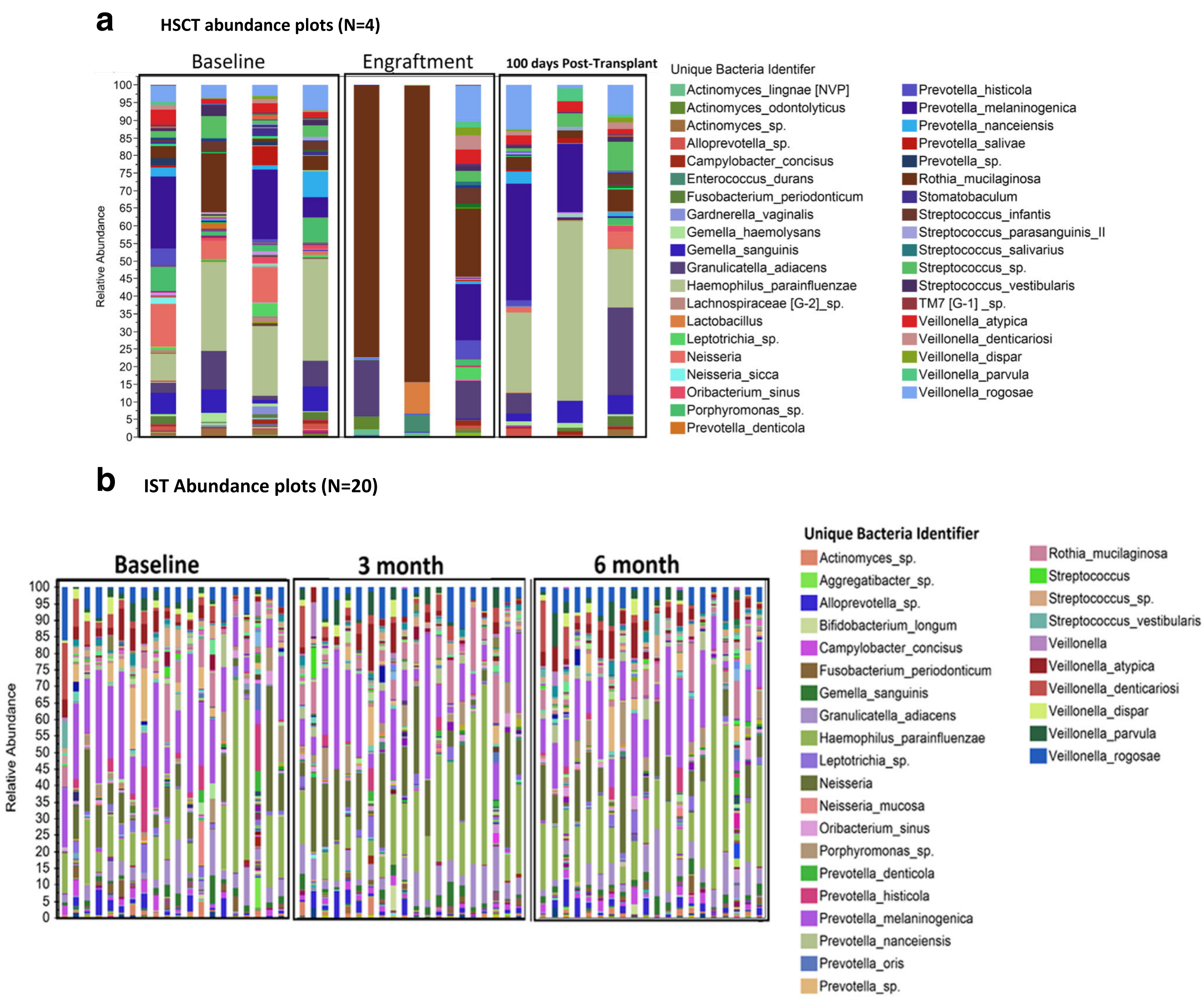


Fig. 7 Relative abundance plots by sampling time point for participants receiving (**a**) hematopoietic stem cell transplantation or (**b**) immunosuppressive therapy. Each stacked bar chart was constructed with data from V2 and represents a single study participant's sample. Legends report bacteria identified in transplant patients and immunosuppressive patients at an abundance level of 1% or greater (Fig.7a) or 5% or greater (Fig. 7b), respectively. Although there were four transplant patients consented, the bacterial DNA from engraftment and 100 days failed to sequence in one patient

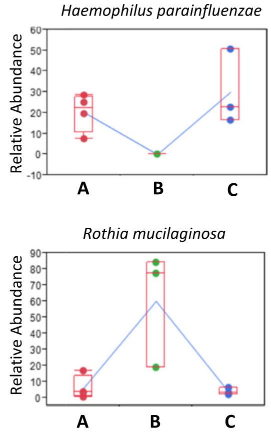
regimens given for SAA treatment and their subsequent effects on the microbiome. It is important to note that many of the immunosuppressive therapeutics used for the treatment of SAA including cyclosporine, ATG, and steroids are also used in patients receiving solid organ transplant.

In this paper, we demonstrated that the tongue microbiome could be changed as a result of the effects of SAA treatment. Changes in the diversity of the oral microbiome are particularly evident among HSCT patients (Fig. 4; $n = 3$) and the group 1 of the IST patients (Fig. 5b; $n = 8$). In the IST patients, this change in diversity is not consistent across patients, although diversity does change within patients throughout the course of treatment.

It is difficult to assess if the DMFT scores received by participants in this study were within the normal range of the US population. First, the most current DMFT national scores are from 2004 [29]. Second, the mean reported DMFT increases with age. The participants' scores range from 0 to 28 with a mean of 8.45 and a median of 7. The reported mean scores from the national statistics range from 6.16 (20–34 age group) to 18.49 (65–75 age group). The DMFT in this study, as a measure of oral health that changes slowly over time, is within these parameters. Two measures (BMOAS and MPS) recorded normal scores across the majority of the study time points (BMOAS 75% and MPS 76%). This finding demonstrated that the general oral environment changed very little during the study. We could not rule out periodontitis since participants could not be probed because of low platelet counts.

a **LeFSe HSCT results** Transplant

Bacterium	A vs. B	B vs. C	A vs. C
Haemophilus parainfluenzae	0.049	0.05	—
Rothia mucilaginosa	0.049	0.05	—
Actinomyces lignae NVP	0.049	—	—
Gemella sanguinis	0.03	0.05	—
Oribacterium sinus	0.046	—	—
Prevotella denticola	0.036	—	0.046
Gemella haemolysans	0.036	0.04	—
Neisseria	0.037	—	—
Fusobacterium periodonticum	0.04	—	—
Leptotrichia sp.	—	—	0.05
Stomatobaculum	0.037	—	0.046
*TM7_G_1__sp_*	0.04	—	—
Prevotella_nanceiensis	0.049	—	—
*Porphyromonas_sp_*	0.049	—	—



b **LefSe IST results** **Immunosuppression**

Bacterium	A vs. B	B vs. C	A vs. C
Prevotella histicola	0.065	—	—

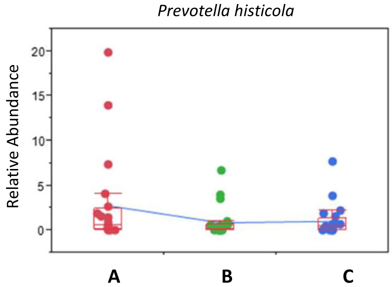


Fig. 8 Statistically significant bacteria as determined by LefSe analysis for (**a**) hematopoietic stem cell transplantation. Only one bacterial species is identified for the (**b**) immunosuppressive therapy group. For each significant organism, *p* values are shown across time point comparisons. An absent *p* value indicates not statistically significant or marginally statistically significant difference between the two sampling time points. One-way abundance plots shows two bacteria for HSCT and one marginally significantly different bacteria for IST

The oral cavity has multiple commensal sites which are not exclusive of each other. Saliva washes all of the oral surfaces and contributes differing microbial communities to the many distinctive sites in the oral cavity. Although all oral sites do not have the same bacterial species, there is evidence that oral samples collected by either tongue brushings or saliva share many of the same species [25]. Other studies have also examined the tongue and salivary microbiomes, comparing the congruence of the two [48, 49]. A recent study examined the salivary microbiome of 20 patients who underwent solid organ transplantation compared to 19 non-immunocompromised controls [50]. In this similar patient population, researchers commented on an increased prevalence of potential extraoral pathogens such as *Klebsiella pneumoniae* and *Enterococcus faecalis* among those solid organ transplants. However, the most common and abundant bacterial taxa were apparently not affected by immunosuppression [50].

We noted two bacterial species, namely *Rothia mucilaginosa* and *Haemophilus parainfluenzae*, as statistically significant between time points among HSCT participants (Fig. 8). Interestingly, *Rothia mucilaginosa* was identified in the expanded publication about the HMP as being a site-specific species [25]. Relative abundance plots (Fig. 7) depict the marked change in relative abundance of these species between sampling time points. In both species, relative abundance decreased from baseline to engraftment and increased back to baseline levels between engraftment and 100 days post-transplant. These longitudinal data demonstrate the variability of the oral microbiome; as microorganisms respond to changing microenvironments and occupy new niches, abundance of certain taxa expand while others decline.

Although this study was not designed to test the effects of antibiotics on the oral microbiome, we sought to analyze these data for trends. It was difficult to correlate antibiotic use with changes in diversity or abundance of particular organisms since specimen collection was not synchronized with antibiotic courses. Differential diversity changes occurred between the two treatment groups—a finding at least partially explained through antibiotic use trends. However, definitive conclusions regarding the influence of antibiotics on oral microbiome diversity remains challenging as these patients received a combination of antibiotics, antifungals, antivirals, and immunosuppressive agents that may preclude a distinct antibiotic effect. Furthermore, in this susceptible population of immunocompromised and neutropenic patients, a low threshold exists for clinical antibiotic necessity. As stated in the guidelines for fever and neutropenia, immediate broad spectrum antibiotic treatment should be initiated "swiftly and broadly" (p.427) [51]. Using next-generation sequencing, current literature has not ascertained the duration of an antibiotic effect and few studies comment on particular antibiotics and microbial diversity. One study correlated the length of an antibiotic regimen with temporal changes in the oral microbiome [52]. Other recent studies mention that microbial changes vary by antibiotic type as well as the initial state of the microbiome [53, 54]. Some literature has even suggested the oral microbiome as more resistant to antibiotic perturbations than the gut microbiome [55]. Certainly, there are many factors to consider in discerning an antibiotic effect on the oral microbiome of these immunosuppressed patients, including their prior treatment and comorbidities. Future projects that synchronize specimen collection with administration of particular antibiotics are necessary to begin to address and gain insight into this complex research question.

Several important limitations of this study should be considered. The sample size was small, but severe aplastic anemia is a rare disease and this was a pilot study. Although the study was designed to compare an equal number of HSCT and IST patients, study participants were recruited from the National Heart, Lung and Blood Institute treatment protocols. Over the 2-year enrollment period, more patients received IST rather than HSCT, resulting in 20 enrolled microbiome IST patients and only 4 HSCT participants. Despite the smaller than planned sample size, this study began the groundbreaking work of identifying issues and establishing a framework to analyze a complex clinical database. Furthermore, specimen collection occurred at clinically scheduled follow-up appointments and, therefore, specimen collection was not matched between treatment groups (Fig. 1). After the initial hospitalization for either HSCT or IST, these patients were discharged and seeing their own private physician where they lived. It is possible that during this time they were given antibiotics that were not reported to the research team.

Conclusion

The tongue microbiome demonstrates changes throughout both hematopoietic stem cell transplantation treatment and immunosuppressive therapy with eltrombopag in patients with SAA. Although tongue microbial results from this patient population were compared to bacterial identifiers in HMP database, there were many taxa that were different. This pilot study has begun the work to establish a framework for analysis of complex populations. The clinical relevance of the oral microbiome remains an area for future work. Future studies are needed to further analyze and identify valuable key biomarker bacterial taxa that allow for identifying these differences and for evaluating, assessing, and predicting clinical outcomes.

Author contributions Nancy Ames—Principal investigator, designed and conceived the study. Collected the majority of specimens. Contributed to the analysis. Wrote the paper.

Jennifer Barb—Assisted with the design of the study. Analyzed and interpreted all bacterial sequence data. Wrote major sections of the paper.

Alexandra Ranucci—Assisted with the design of the study. Processed all specimens. Assisted in the analysis of the sequence data. Contributed to the paper.

Sarah Mudra—Contributed to the analysis and edited and wrote major section of the final manuscript.

Richard Childs—Assisted with the analysis. Suggested adding significant component to the analysis. Contributed to writing the paper and interpreting data.

Hyung-Suk Kim—Assisted with the design of the study. Processed all specimens and bacterial sequencing.

Bruce Paster—Interpretation of sequence data and critical assessment and editing of final manuscript.

Lina Faller—Interpretation of sequence data and critical assessment and editing of final manuscript.

Danelle Townsley—Contributed to the design and made substantive changes to the final manuscript.

Ann Cashion—Contributed to the design and made substantive changes to the final manuscript.

Gwenyth R. Wallen—Scientific review of the original study design and study implementation, co-author revisions to the draft manuscript.

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Compliance with ethical standards The authors declare that they have no conflict of interest.

Ethical approval All participants provided informed consent before any study procedures were performed.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the NIH.

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