haematologica
Journal of the Ferrata Storti Foundation

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Received: Apr 17, 2024.
Accepted: July 4, 2024.

Citation: Armin Rashidi, Lang Liang, Ted Gooley, Philippe P. Hujoel, Alex Zevin, Marilyn Rothen, Mary K. Hagstrom, Corey Cutler, Stephanie J. Lee, David R. Dean, Hervé Y. Sroussi, and Nathaniel S. Treister. Microbiota signature of oral chronic graft-versus-host disease 6+ years after transplantation.
Haematologica. 2024 July 11. doi: 10.3324/haematol.2024.285650 [Epub ahead of print]

Microbiota signature of oral chronic graft-*versus*-host disease 6+ years after transplantation

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Running title: Oral microbiome in oral chronic GVHD
Keywords: graft-versus-host disease; long-term; microbiome; sequencing

Funding
This work was supported by federal grants from the National Institute of Health awards CA163438 and CA118953, the National Institute of Dental and Craniofacial Research, the National Institutes of Health under award number R01DE028336, the National Center for Advancing Translational Sciences, National Institutes of Health under award number UL1TR002319, and the Genomics & Bioinformatics Shared Resource, RRID:SCR_022606, of the Fred Hutch/University of Washington/Seattle Children's

Cancer Consortium (P30 CA015704). The funders did not have any role in data collection, interpretation, or reporting. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest

All the authors declare that the information included in this clinical report and the research supported by the NIDCR grant acknowledged above were not influenced by commercial or financial relationships that could be construed as a potential conflict of interest. A.R. has received consulting fees from Seres Therapeutics and serves as a member of an Emmes Data and Safety Monitoring Board. S.J.L. has received consulting fees from Mallinckrodt, Equillium, Kadmon, Novartis, Sanofi and Incyte; research funding from AstraZeneca, Pfizer, Sanofi and Syndax, and drug supply from Janssen. She is on clinical trial steering committees for Incyte and Sanofi. She is on the Board of Directors of the National Marrow Donor Program (uncompensated). N.T. is a consultant for Alosa Health, Alira Health, and MuReva Phototherapy and has research support from Thor Photomedicine. C.C. has received consulting fees/honoraria from Sanofi, InhibRx, Cellarity, Astellas, Rigel, Novartis, Incyte; Is on the Scientific Advisory Boards for Cimeio, Oxford Immune Algorithmics, Orca and the Data and Safety Monitoring Boards for Allovir and Angiocrine.

Acknowledgements

We acknowledge the Specimen Processing & Research Cell Bank of FHCC for sample processing and DNA extraction.

Data-sharing statement

The sequencing data reported in this paper are available from NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA1096713. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author (Armin Rashidi, arashidi@fredhutch.org) upon request.

Author contributions

Conceptualization, H.Y.S, N.S.T., and A.R.; Methodology, H.Y.S, N.S.T., D.R.D., and A.R.; Investigation, M.R., M.K.H., D.R.D., H.Y.S., and N.T.T.; Formal Analysis, T.G., P.P.H., and A.R.; Writing – Original Draft, A.R.; Writing – Review & Editing, L.L., T.G., P.P.H., M.R., M.K.H., C.C., S.J.L., D.R.D., H.Y.S., and N.T.T.; Supervision, H.Y.S and N.S.T.

Chronic graft-versus-host disease (cGVHD) affects ~50% of allogeneic hematopoietic cell transplantation (alloHCT) recipients. The mouth is involved in ~60% of patients with cGVHD[1]. Symptoms of oral cGVHD include mucosal sensitivity, dry mouth, and limited mouth opening; late complications include accelerated dental caries, poor nutrition, and an increased risk for oral cancer. Oral cGVHD has deleterious effects on oral health-related quality of life[2]. The oral flora interacts with the local and systemic immune system and this homeostasis is regulated by the saliva[3]. Dry mouth, a common finding in oral cGVHD, may thus lead to microbiota alterations, ultimately resulting in immune dysregulation and cGVHD. We examined whether there is an oral microbiota signature for oral cGVHD several years after alloHCT.

The Chronic Graft-versus-Host Disease Consortium is a NIH-supported collaborative group of 13 academic centers that prospectively followed ~1000 adult alloHCT recipients over 3 years (March 2011 to May 2014)[4]. Long-Term Oral Health Outcomes in the Chronic GVHD Consortium is a prospective cohort nested substudy of patients enrolled in the Consortium study NCT01206309 at the Fred Hutchinson Cancer Center and Dana-Farber Cancer Institute. The study protocol was approved by the central Institutional Review Board (FHCC) and complied with local regulations and the Declaration of Helsinki. All participants provided written informed consent. The objective of the long-term study is to provide a systematic description of the long-term oral health outcomes. At the time of enrollment into the long-term study, typically several years after alloHCT, subjects underwent a baseline oral examination, followed by annual oral examinations for 4 years. Oral examinations, performed by calibrated oral health specialists, characterized oral health status. The diagnosis of oral cGVHD was made per the 2014 NIH diagnostic criteria[5]. The activity of the oral mucosal disease was determined by the NIH Modified Oral Mucosa Rating Scale (OMRS)[6]. An unstimulated salivary sample was collected at each follow-up examination and stored at -80□C.

Saliva pellets collected at the 1-, 2-, and 3-year follow-up timepoints were sequenced. The V3-V4 hypervariable regions of the 16S rRNA gene was sequenced using an Illumina MiSeq platform (2 x 300 paired-end mode). Exact amplicon sequence variants (ASVs) were inferred using DADA2 v1.18.0[7]. Filtering utilized DADA2 default parameters (PHRED score threshold of 2, maximum number of expected errors of 2 for both forward and reverse reads) and truncation lengths of 270 (forward) and 220 (reverse). De-replication, de-noising, merging, and chimera removal were performed using DADA2 default parameters. Taxonomic assignment was performed by the naive Bayesian classifier implemented in DADA2 and the SILVA non-redundant v138.1 training set[8]. Alpha diversity (i.e., within-sample diversity) was quantified by the Shannon index and beta diversity (i.e., between-sample diversity) by the Aitchison distance. Ordination was visualized by principal coordinate analysis and overall compositional differences between the groups were quantified by permutational multivariate analysis of variance (PERMANOVA) using an adonis test with 999 permutations. Differential abundance analysis was performed using ALDEx2[9], with Benjamini-Hochberg corrected P values (i.e., q values)[10]. Within-subject microbiome stability was quantified by the Aitchison distance between microbiota samples of the same subject at different long-term timepoints (e.g., at 1- vs. 2-year follow up visits). A

genus-level microbiota heatmap visualizing the results of unsupervised hierarchical clustering was generated using centered log-ratio abundances and a ward.D function. All available samples were included; therefore, no power or sample size calculation was performed.

Sixty-eight patients and 114 samples were included (**Table 1**). At the time of enrollment in the Long-Term study, 18 patients had moderate/severe oral cGVHD and 50 had no/mild oral cGVHD. Time from alloHCT to enrollment in the Long-Term study was similar between these two groups (median 9 years post-HCT in both groups; range 6-11 in patients with moderate/severe oral cGVHD and 7-11 in patients with no/mild oral cGVHD). In patients with oral cGVHD, the median time between cGVHD diagnosis and enrollment in the Long-Term study was 8 years (range: 1-10). Scores in the 3 domains of the modified OMRS are shown in **Fig. S1a**. Patients with moderate/severe vs. no/mild oral cGVHD provided 31 and 83 samples, respectively, for a sample-to-patient ratio of 1.7 in both groups. Main reasons for missing samples included missed appointments and patient refusal/inability to provide a sample. Patient/sample contribution was balanced between the two centers (33/52 from FHCC; 35/62 from DFCI). The median sequencing depth across all samples was 75,223 reads per sample (range: 13,733-113,014); thus, all samples were retained for analysis. ASVs with a relative abundance of >0.1% in at least 10% of the samples were retained. This preprocessing step yielded a total of 156 unique ASVs mapped to 40 genera, 27 families, and 7 phyla. Although 50 ASVs could be classified down to the level of species, due the limited resolution of short-amplicon sequencing for species-level classification, taxa were collapsed to the genus level.

Taxonomic composition of the samples is shown in **Fig. S2a**. Unsupervised hierarchical clustering suggested a microbiota signature for samples from patients with moderate/severe oral cGVHD (**Fig. 1a**). To account for repeated measures data (up to 3 samples per patient), we first evaluated the contribution of timepoint to microbiome variation. PERMANOVA estimated this contribution to be only 1% ($P = 0.95$), indicating microbiome stability over time. Therefore, we collapsed the samples collected from each patient into a single aggregate by averaging their corresponding taxonomic abundances. Since patients were transplanted at geographically distant centers, we evaluated whether microbiome-relevant factors potentially associated with region (e.g., diet, tobacco use) influenced microbiome composition. This analysis was unremarkable (PERMANOVA $R^2 = 0.02$, $P = 0.11$). Principal coordinates analysis demonstrated significantly different microbiota compositions between patients with moderate/severe vs. no/mild oral cGVHD ($P < 0.001$; **Fig. 1b**). Samples from patients with higher ulcer scores on modified OMRS were characterized by a greater abundance of a genus of the Neisseriaceae family, and those with higher erythema or lichenoid scores by greater abundances of genera *Scardovia*, *Lactobacillus*, *Lacticaseibacillus*, and *Limosilactobacillus* (**Fig. S1b**). Xerostomia severity[11], explained a significant proportion of microbiome variation ($R^2 = 0.11$, $P < 0.001$; **Fig. S2b**). In differential abundance analysis (**Fig. 1c**), *Streptococcus*, *Scardovia*, *Rothia*, *Actinomyces*, *Veillonella* were more abundant in patients with moderate/severe oral cGVHD. One genus (*Prevotella*) was more abundant in patients with no/mild oral cGVHD. Among these taxa,

Streptococcus, *Scardovia*, *Rothia*, *Actinomyces*, and *Veillonella* were significantly and positively correlated with xerostomia index ($q < 0.05$; **Fig. S2c**). Within-sample microbiota diversity was significantly lower in patients with moderate/severe oral cGVHD than those with no/mild disease ($P < 0.001$; **Fig. 1d**).

Finally, we asked whether previous oral cGVHD might have left a lasting effect on the oral microbiome which could be detected even after the resolution of cGVHD. To examine this possibility, we focused on the group of patients with no/mild oral cGVHD and classified them into two groups using Consortium data: those with (N = 33) vs. without (N = 17) previous oral cGVHD. Although principal coordinate analysis suggested some overall compositional differences between the groups (**Fig. 2a**), differential abundance analysis was unremarkable (**Fig. 2b**). Similarly, alpha diversity was not different between the two groups (**Fig. 2c**). Although time was not an explanatory factor in microbiome variation in PERMANOVA, we further investigated the within-individual temporal stability of the microbiome. Using samples at 1-, 2-, and 3-year follow-up timepoints, microbiome stability was not different between patients with moderate/severe vs. no/mild oral cGVHD, and between the two subgroups of patients with no/mild oral cGVHD (**Fig. S3**).

To our knowledge, this is the first report of the oral microbiota several years after alloHCT. Using a relatively large sample size (114 samples from 68 patients) in a longitudinal context, we identified an oral microbiota signature for oral cGVHD 6+ years after transplantation. Oral microbiota hallmarks of moderate/severe oral cGVHD included loss of alpha diversity, expansion of several genera, and shrinkage of one genus. There are two potential explanations for these findings. First, oral microbiota changes observed may have predated oral cGVHD onset, suggesting oral microbiota contribution to cGVHD pathogenesis. The oral microbiota can reshape the structure of the oral epithelial barrier (e.g., keratinization, cellular adhesion, barrier function) and stimulate inflammatory reactions[12]. Alternatively, oral microbiota changes may be a consequence of cGVHD. By providing nutrients for oral microbes and protecting them against colonization by non-oral microbes, saliva has an important role in microbial ecology of the oral cavity. In our analysis, xerostomia severity was associated with several differentially abundant taxa in cGVHD patients, suggesting that their overgrowth was facilitated by reduced saliva production. Many of these taxa (e.g., *Veillonella*, *Actinomyces*, *Rothia*, Streptococcus *salivarius*, *Streptococus parasanguinis*) are associated with overall poor oral health[13].

Only one published study has evaluated the relationship between oral microbiota and oral cGVHD at 1 year or more after HCT[14]. Laheij et al.[14] profiled the oral microbiota from oral rinses in 50 alloHCT recipients. Forty-three and 42 samples were collected at 1- and 1.5-year timepoints, respectively. Short-amplicon sequencing yielded ~17,000 reads on average per sample. Oral cGVHD was not associated with microbiome alpha diversity. The higher sequencing depths in our study provided greater sensitivity and thus enabled identification of differences in diversity and composition between the two groups. One limitation of our study concerns possible antibacterial antibiotic use by the patients. Although patients were generally not expected to be on these antibiotics at the

time of sample collection, data were not consistently available. Nevertheless, a major effect on the oral microbiota seems unlikely as the oral flora is known to be a resilient community[15]. By focusing on a time period of 6+ years after alloHCT, the potential confounding effects of acute GVHD and conditioning-related oral mucositis were minimized. Another limitation concerns the lack of pre-cGVHD samples which makes the direction of potential causality difficult to ascertain. Finally, the potential long-term effect of prior immunosuppressive therapies for cGVHD on the oral microbiota could not be determined because details of such therapies from several years before enrollment into the Long-Term study were not readily available.

In conclusion, we demonstrated an oral microbiota signature for oral cGVHD several years after transplantation, with potential implications for novel, microbiota-directed, preventative and therapeutic strategies. Multi-omics studies may shed light on the pathways involved.

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Table 1: Patient characteristics

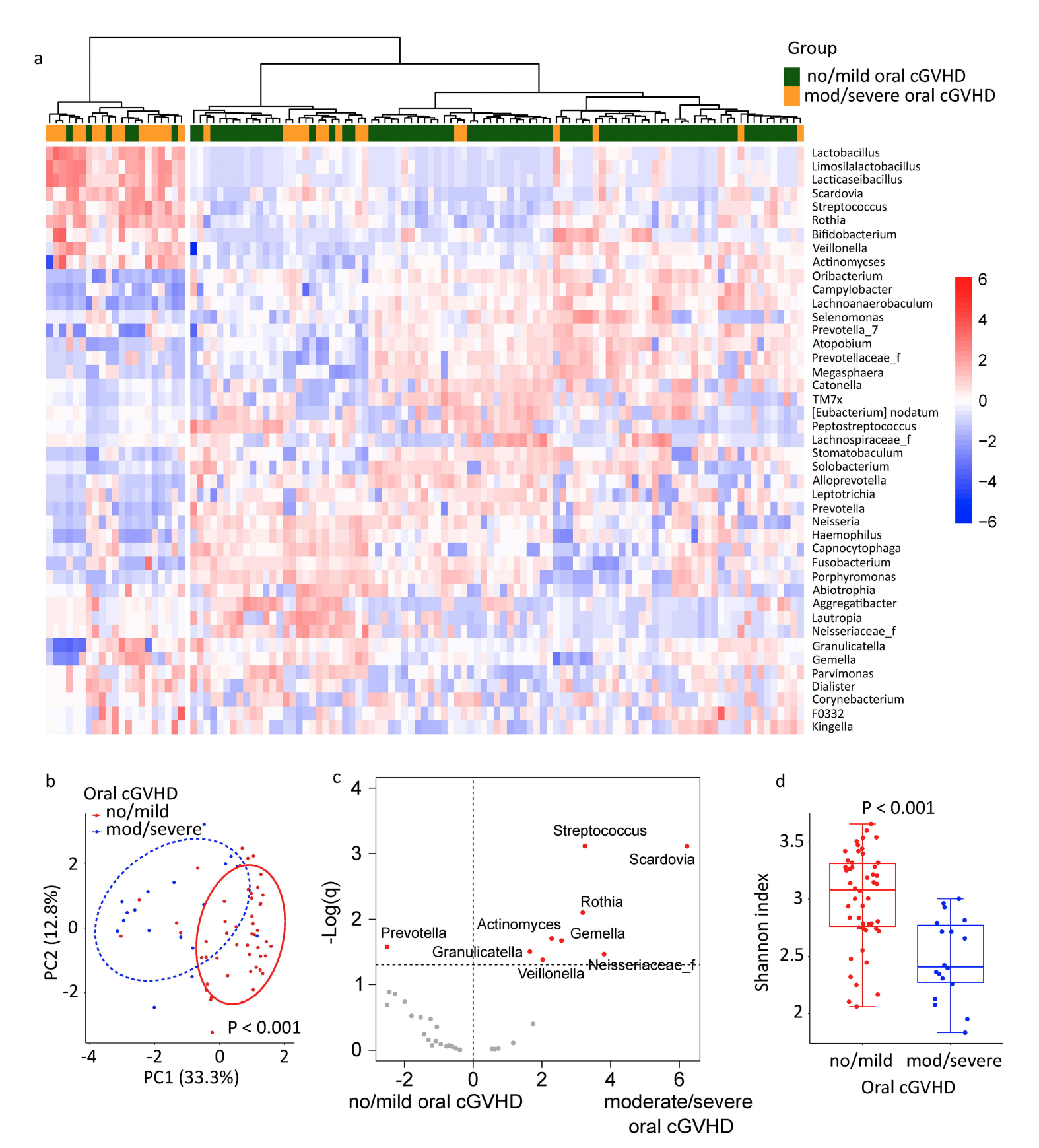
Total, N	68
Center, N (%)	
Dana-Farber Cancer Institute	35 (51)
Fred Hutchinson Cancer Center	33 (49)
Sex, N (%)	
Male	45 (66)
Female	23 (34)
Age at transplantation, yr	
Median (range)	54 (23-70)
Graft source, N (%)	
Peripheral blood	62 (91)
Bone marrow	5 (7)
Cord blood	1 (2)
Donor type, N (%)	
HLA-matched sibling	18 (26)
HLA-matched unrelated donor	44 (65)
Other	6 (9)
Conditioning intensity, N (%)	
Myeloablative	30 (44)
Reduced intensity	38 (56)
Underlying disease, N (%)	
Acute leukemia	23 (34)
MDS/MPN	20 (29)
CLL/NHL	11 (16)
Multiple myeloma	4 (6)
Hodgkin lymphoma	4 (6)
Other	6 (9)

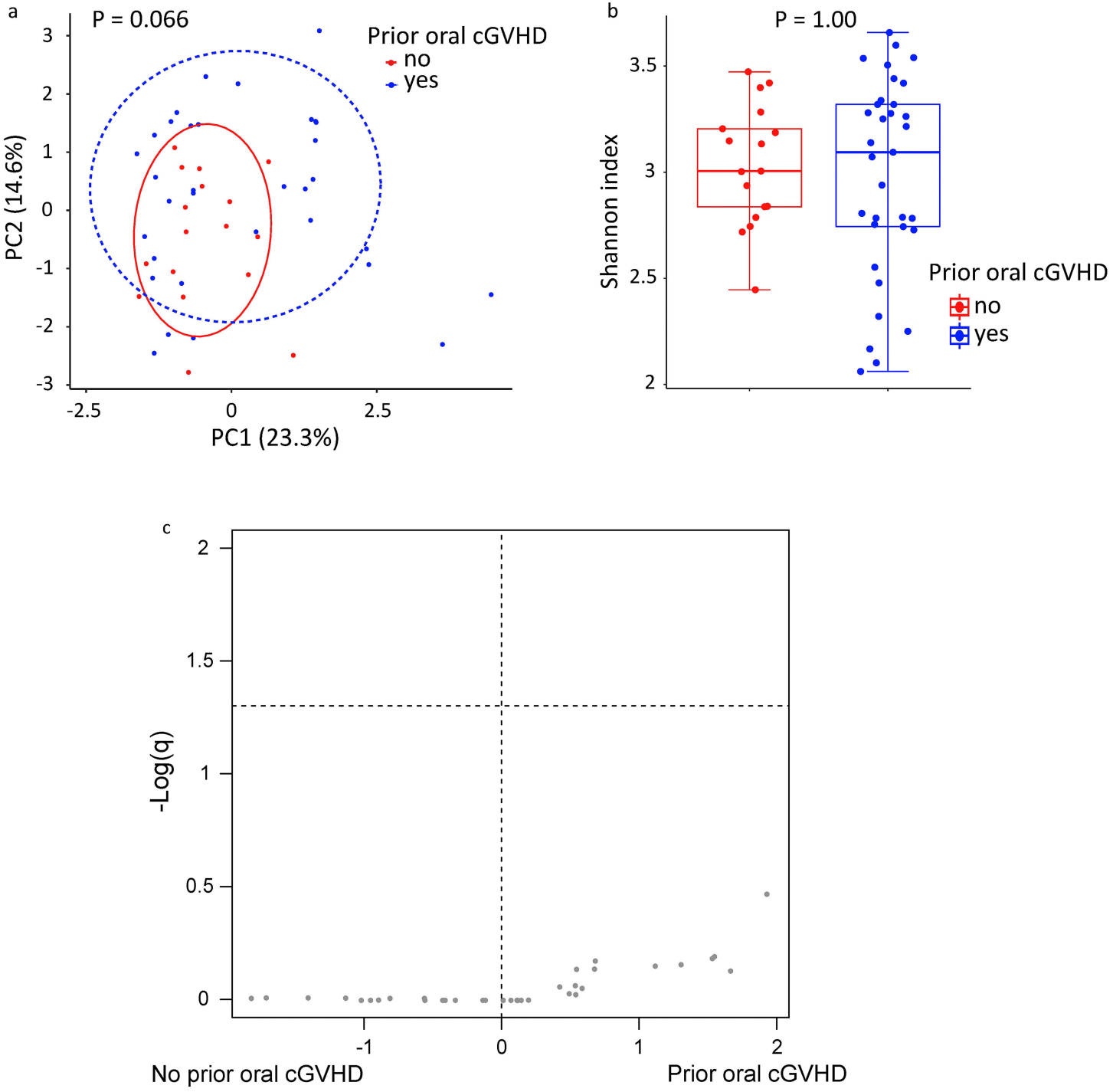
CLL: chronic lymphocytic leukemia; MDS: myelodysplastic syndromes; MPN: myeloproliferative neoplasms; NHL: non-Hodgkin lymphoma

Figure Legends

Figure 1: Microbiota signature for oral chronic GVHD
(**a**) Genus-level microbiota heatmap visualizing the results of unsupervised hierarchical clustering using CLR-transformed taxa abundances and a ward.D function. Each column is a sample, and each row is a species. The blue-red gradient shows species abundances scaled row-wise. cGVHD groups are added along the top border. (**b**) Beta diversity and ordination visualized by principal coordinate analysis. Aitchison distance (using CLR-transformed genus-level abundances) was used to quantify the overall compositional difference between samples. The first two principal coordinates (PC1 and PC2) are shown. Numbers in parentheses indicate percent variation explained by the corresponding axis. P values are from an adonis test with 999 permutations. Each symbol represents a sample. The closer the two samples, the more similar their microbiome composition. 80% ellipses are shown. (**c**) Differential abundance analysis using ALDEx2 comparing patients with moderate/severe vs. no/mild oral cGVHD. Each circle shows one genus. Genera to the right of the dashed vertical line are more abundant in patients with moderate/severe oral cGVHD; those to the left are more abundant in patients with no/mild oral cGVHD. The x axis shows the difference in centered-log ratio abundance between the two groups for each genus. The y axis shows the corrected P values, with circles above the dashed horizontal line representing taxa with statistically significant difference in abundance between the groups. (**d**) Alpha diversity analysis using Shannon's index. Each circle represents a sample. Each box shows median (horizontal middle line) and interquartile range. Whisker lines indicate non-outlier maximum and minimum values. A small jitter is included for better visualization. P values are from a Wilcoxon's test.

Figure 2: Long-term effect of prior oral chronic GVHD on the oral microbiota
The analyses in this Figure were limited to the group of patients with no/mild oral cGVHD. These patients were classified into two groups: those with vs. without previous oral cGVHD. These subgroups were compared. (**a**) Principal coordinate analysis using the same methods as in Fig. 1b. (**b**) Alpha diversity analysis using the same method as in Fig. 1d. (**c**) Differential abundance analysis, using the same methods as in Fig. 1c.


a
Group
no/mild oral cGVHD
mod/severe oral cGVHD
Lactobacillus
Limosilalactobacillus
Lacticaseibacillus
Scardovia
Streptococcus
Rothia
Bifidobacterium
Veillonella
Actinomyces
Oribacterium
Campylobacter
Lachnoanaerobaculum
Selenomonas
Prevotella_7
Atopobium
Prevotellaceae_f
Megasphaera
Catonella
TM7x
[Eubacterium] nodatum
Peptostreptococcus
Lachnospiraceae_f
Stomatobaculum
Solobacterium
Alloprevotella
Leptotrichia
Prevotella
Neisseria
Haemophilus
Capnocytophaga
Fusobacterium
Porphyromonas
Abiotrophia
Aggregatibacter
Lautropia
Neisseriaceae_f
Granulicatella
Gemella
Parvimonas
Dialister
Corynebacterium
F0332
Kingella
6
4
2
0
−2
−4
−6
b
Oral cGVHD
no/mild
mod/severe
P < 0.001
PC2 (12.8%)
PC1 (33.3%)
c
Streptococcus
Scardovia
Rothia
Actinomyces
Gemella
Prevotella
Granulicatella
Veillonella
Neisseriaceae_f
-Log(q)
no/mild oral cGVHD
moderate/severe oral cGVHD
d
P < 0.001
Shannon index
no/mild
mod/severe
Oral cGVHD


a
P = 0.066
Prior oral cGVHD
no
yes
PC2 (14.6%)
PC1 (23.3%)
b
P = 1.00
Shannon index
Prior oral cGVHD
no
yes
c
-Log(q)
No prior oral cGVHD
Prior oral cGVHD

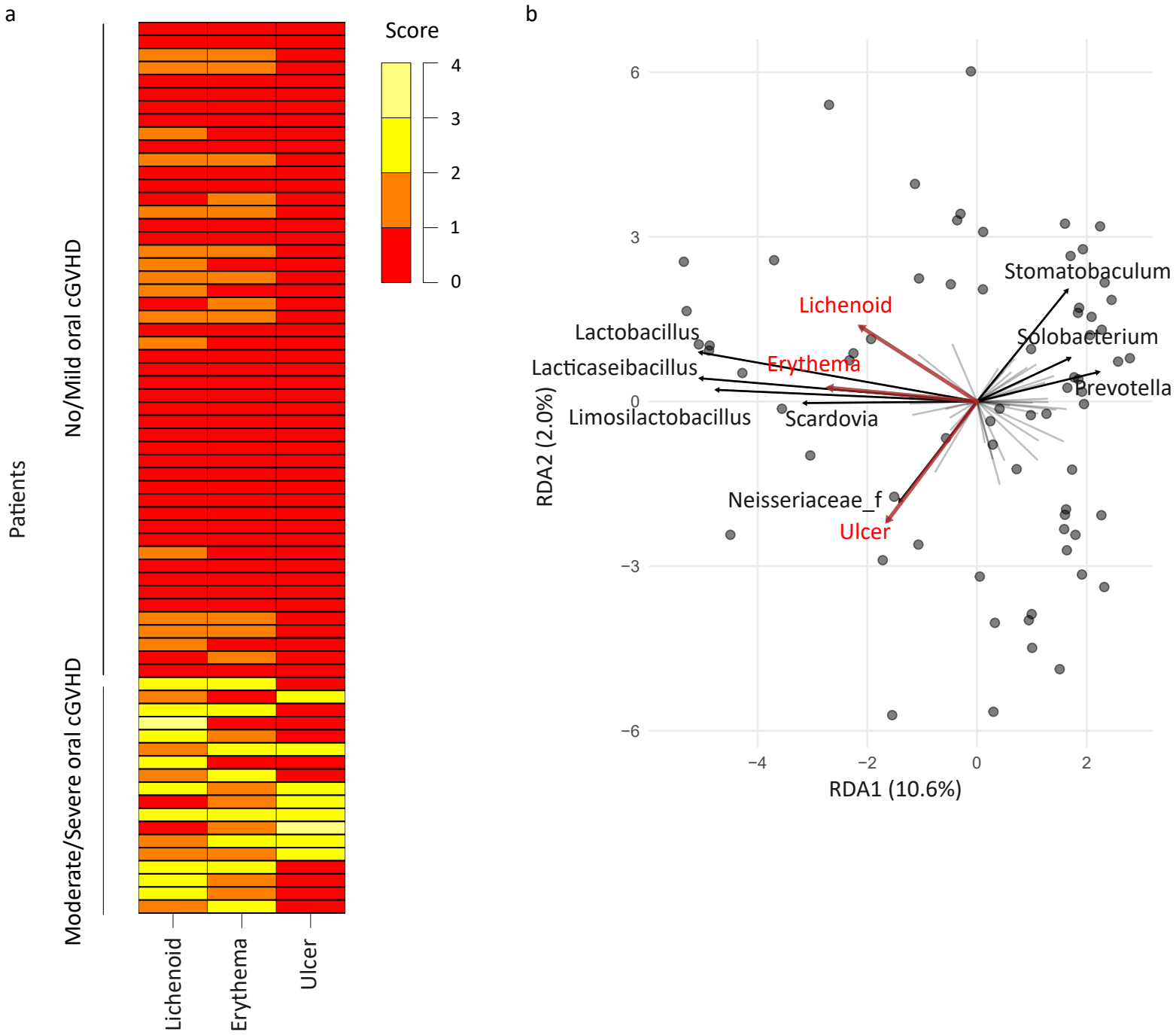


Figure S1: Modified OMRS subscores and their microbiota associations
(**a**) Modified OMRS subscores. The maximum score in each domain during protocol-defined visits for each subject is plotted, with subjects sorted along the y axis based on oral cGVHD status and domains plotted along the x axis. (**b**) Redundancy analysis using modified OMRS subscores in each of the 3 domains. Beta diversity was calculated and ordination visualized similar to Fig. 1b. Taxa with the strongest associations with subscores are shown.

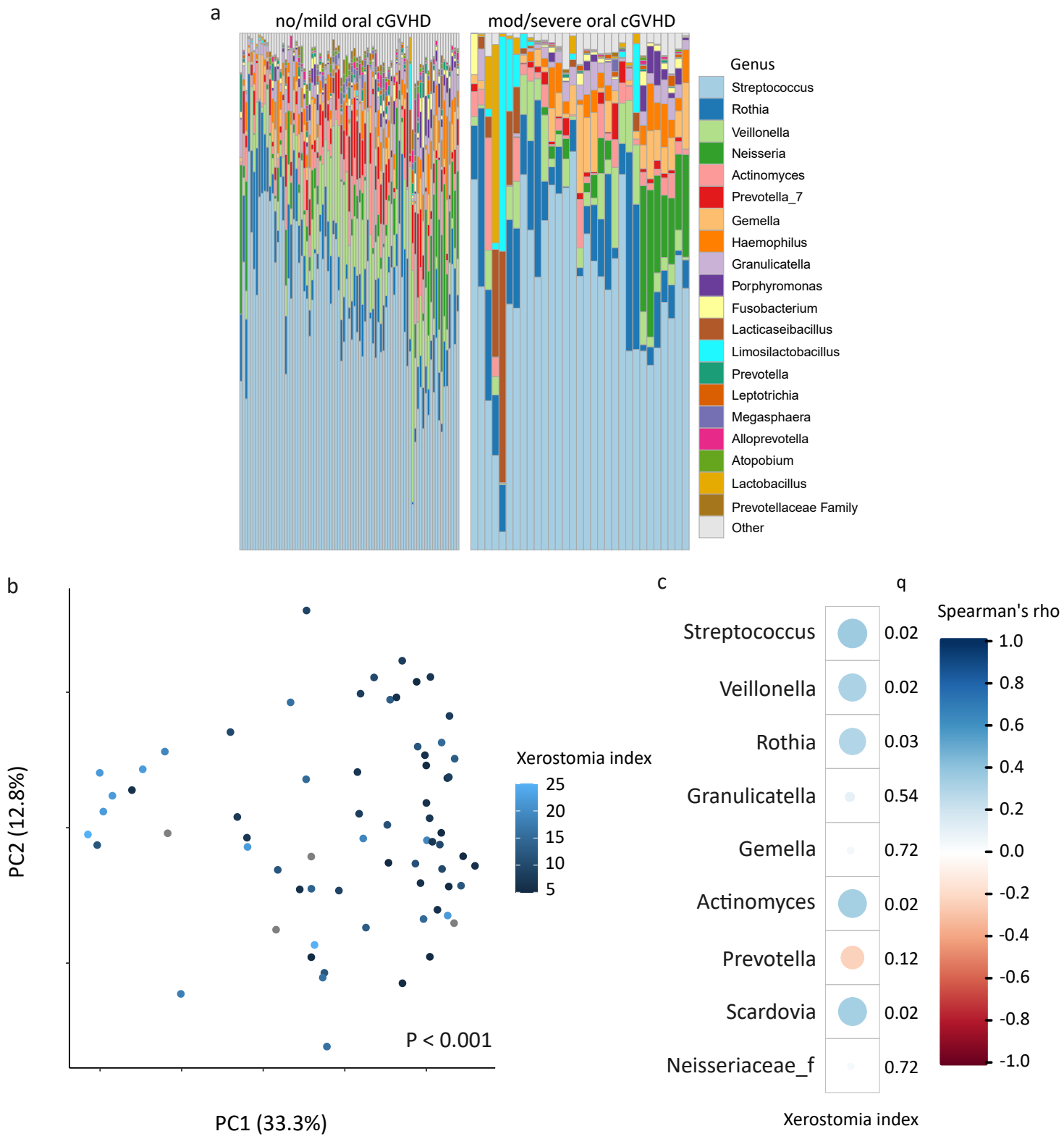


Figure S2: Contribution of xerostomia to microbiota variation

(**a**) The distribution of the 20 most abundant genera in each sample. Less abundant genera were combined in one category. Each column is a sample and its corresponding stacked bars indicate relative abundances of different genera in that sample. (**b**) Beta diversity was calculated and ordination visualized similar to Fig. 1b. Xerostomia index corresponding to the examination at the time of collection of each sample is colored using the gradient shown. The P value is from an adonis test with 999 permutations, reflecting lower scores in patients clustered to the left. (**c**) Correlation plot showing the Spearman's correlation coefficients (color gradient and circle size) and corresponding q values for the correlation between the xerostomia index and significant taxa from differential abundance analysis in Fig. 1c.

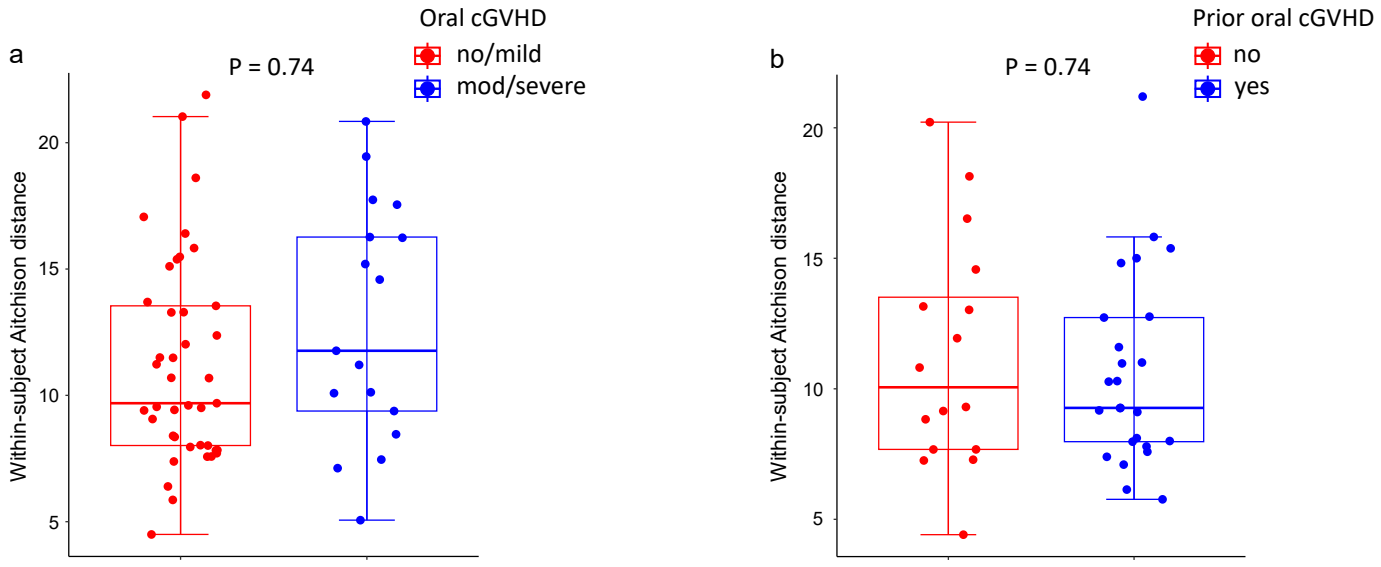


Figure S3: Microbiota stability over time

(**a**) Compositional dissimilarity over time in same-patient microbiota compared between patients with moderate/severe vs. no/mild oral cGVHD. (**b**) Similar comparison between patients with vs. without prior oral cGVHD in the group with current no/mild oral cGVHD. Each box shows median (horizontal middle line) and interquartile range. Whisker lines indicate non-outlier maximum and minimum values. A small jitter is included for better visualization. P values are from a Wilcoxon's test.