

Supplementary Material

| | | |
|---|------------------------------|--------|
| 1 | | |
| 2 | | |
| 3 | Supplementary Methods | Page 2 |
| 4 | Supplementary Tables | Page 6 |
| 5 | Supplementary Figure Legends | Page 7 |
| 6 | Supplementary References | Page 8 |
| 7 | | |
| 8 | | |

9 **Supplementary Methods:**

10 Overview of the Study Design

11
12
13 For the current study, we first included participants with confirmed coronavirus disease 2019
14 (COVID-19) enrolled as part of a randomized controlled trial (RCT) examining the effect of
15 several types of nasal irrigations on COVID-19-related outcomes. The detailed methods for this
16 RCT have been previously reported (1-3). Inclusion criteria included age ≥ 18 years, a qualitative
17 PCR test positive for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) performed
18 at Vanderbilt University Medical Center (VUMC) or one of its affiliated centers, planned self-
19 quarantine after being diagnosed with COVID-19, and residence within a 30-mile radius of the
20 main VUMC campus in Nashville, Tennessee. Exclusion criteria included current use of nasal
21 saline irrigations or other nasal medications (such as nasal steroids), inability to perform nasal
22 irrigations or to collect upper respiratory tract (URT) samples in a separate house bathroom or
23 away from household contacts, and the need for hospitalization related to COVID-19. Thus, only
24 participants with symptomatic, mild-to-moderate COVID-19 (based on criteria from the World
25 Health Organization (4)) were included in the RCT. Eligible participants were contacted and
26 enrolled in the RCT within 24 hours of the initial diagnosis of COVID-19. Only participants who
27 were not randomized to one of the RCT's intervention arms –thus, were not assigned to one of
28 the nasal irrigations being tested– were included in the current study (n=24).

29
30 In parallel to conducting the aforementioned RCT, we also enrolled asymptomatic participants
31 within the VUMC community (including employees, students, and faculty, among others) to
32 serve as controls (n=24). Inclusion criteria included age ≥ 18 years and absence of COVID-19-
33 related symptoms (e.g., runny nose, cough, or fever). Exclusion criteria included current use of
34 nasal saline irrigations or other nasal medications (such as nasal steroids) and current or prior
35 diagnosis of COVID-19. The asymptomatic participants were not related to those with COVID-
36 19 or lived in the same household.

37
38 Following adequate training, all of the above participants (n=48) were asked to collect a mid-
39 turbinate swab on the day of enrollment (day 1) using a self-collection kit (FLOQSwabs, Copan
40 Diagnostics Inc.). Those with COVID-19 were also asked to collect serial samples using the
41 same method on follow-up days 3, 5, 7, 10, 14, and 21. This last day of follow-up (21 days) was
42 decided based on the presumed duration of SARS-CoV-2 transmission and the recommended
43 period of isolation for adults with COVID-19 at the time when the aforementioned RCT was
44 being conducted (5). The collection of all samples included in the current study occurred in
45 Spring-Summer of 2020. Each adult provided informed consent for his/her participation. The
46 VUMC Institutional Review Board and Biosafety Committee approved this study.

47 SARS-CoV-2 Testing by Quantitative Reverse Transcription PCR

48
49 To measure viral load in participants with COVID-19 and rule out asymptomatic infection in
50 controls, quantitative reverse transcription PCR in all mid-turbinate swabs collected at
51 enrollment was performed. Total RNA was extracted from samples using a phenol-chloroform-
52 based method. Samples were placed in Red 1.5 mL RINO[®] screw-cap tubes (NextAdvance) pre-
53 filled with RNase-free zirconium oxide beads and QIAzol Lysis Reagent (Qiagen) was added.
54 Then, samples were homogenized in a Bullet Blender 24 Gold (NextAdvance) for 3 minutes at

55 maximum speed. Once samples were homogenized, genomic DNA was eliminated with gDNA
56 Eliminator columns (Qiagen), and RNA was purified using the RNeasy Mini Plus Kit (Qiagen)
57 following the manufacturers' protocols. RNA quality was measured using an Agilent 2100
58 Bioanalyzer (Agilent Technologies). The United States Centers for Disease Control and
59 Prevention primers and probes designed for the detection of SARS-CoV-2 (2019-nCoV) were
60 purchased from Integrated DNA Technologies (6). The SARS-CoV-2 nucleocapsid gene regions
61 1 and 2 were both targeted for the detection of SARS-CoV-2. RNase P was also examined as a
62 measure of RNA quality and quantity. RT-qPCR was performed using SuperScript III One-Step
63 RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) on a Bio-Rad CFX96 Touch
64 Real-Time PCR Detection System (Bio-Rad) as per manufacturer's instructions. Plasmid
65 controls for SARS-CoV-2 nucleocapsid genes and RNase P were also ordered from Integrated
66 DNA Technologies at a concentration of 66,666 copies/reaction. No-template-controls and an
67 extraction negative control were used as negative controls. Reactions were prepared using 12.5
68 µl SuperScript III Master Mix (ThermoFisher), 1 µl each 400nm forward and reverse primer, 1 µl
69 400nM FAM-labelled probe, 1 µl Platinum Taq polymerase, 3 µl of template RNA, and 7.25 µl
70 PCR Certified Water (Teknova). RNA was reverse transcribed at 50°C for 15 minutes, and PCR
71 conditions were run at 95°C denaturation step for 2 minutes, followed by 40 cycles of 95°C for
72 15 seconds and 55°C for 30 seconds. To measure SARS-CoV-2 viral load, the cycle threshold
73 values were captured, analyzed, and calculated by the CFX Maestro software (Bio-Rad).

74

75 Characterization of the URT Microbiota

76

77 The methods used to characterize the URT microbiota have been previously described in detail
78 (3, 7-10). First, bacterial DNA from the mid-turbinate swabs using the DNeasy PowerSoil kit
79 (Qiagen) was extracted. Dual-indexed universal primers appended with Illumina-compatible
80 adapters were used to amplify the 16S ribosomal RNA (rRNA) gene. Primer sequences were
81 515F: 5'-AATGATACGGCGACCACCGAGATCTACAC NNNNNNNN TATGGTAATT **GT**
82 **GTGCCAGCMGCCGCGGTAA**-3' and 806R: 5'-CAAGCAGAAGACGGCATAACGAGAT
83 NNNNNNNN AGTCAGTCAG CC **GGACTACHVGGGTWTCTAAT**-3', where the string of
84 8 N's represents the indices, and the bolded nucleotides represent the sites that bind to 16S rRNA
85 gene target sequence (7). For this, amplicons targeting the V4 region of the 16S rRNA gene were
86 generated by combining 7 µl of template, 12.5 µl MyTaq HS Mix (Bioline), 0.75 µl DMSO
87 (Sigma), 3 µl PCR Certified Water (Teknova), and 1 µl of each 10 µM primer. DNA was
88 denatured at 95°C for 2 min, followed by 30 cycles of 95°C for 20 seconds, 55°C for 15 seconds,
89 and 72°C for 5 minutes, and a final extension at 72°C for 10 minutes. Each amplified sample was
90 run on a 1% agarose gel to confirm reaction success. Amplicons were cleaned and normalized
91 with the SequalPrep Normalization Kit (ThermoFisher). Normalized amplicons were pooled and
92 cleaned with 1X AMPure XP beads (Beckman Coulter). The pool was then sequenced on an
93 Illumina MiSeq platform with 2x250 base pair reads. Two ZymoBIOMICS Microbial
94 Community DNA Standard (Zymo) mock community controls were processed concurrently with
95 participant samples. Negative controls (7 extraction and 15 PCR negative controls) were also
96 amplified and sequenced concurrently with participant samples. Following the sequencing
97 procedure, both mock community controls had a community composition very similar to what
98 was expected. In addition, only a small fraction of 16S rRNA gene sequences were found in the
99 negative controls compared to participant samples, and the bacterial sequences recovered had
100 little overlap with participant samples.

101
102 Next, the 16S rRNA gene sequences were processed using the R package *dada2* by applying its
103 standard operating procedure (available at: <https://benjjneb.github.io/dada2/tutorial.html>) (11).
104 To this end, sequences were grouped into amplicon sequence variants (ASVs), and taxonomy
105 was assigned using the SILVA reference database version 132 (12). Low-quality sequences,
106 chimeras, and non-bacterial sequences were discarded as part of the *dada2* pipeline. To remove
107 any suspected contaminants that were found in the negative controls, the remaining sequences
108 were processed using the R package *decontam* (13) using the “prevalence” method, with default
109 parameters. The full taxonomy of contaminant ASVs identified by *decontam* are listed in **Table**
110 **S1**. Those ASVs that were present in >1 sample were then retained and samples with <1,000
111 sequences (n=33) were discarded using the R package *phyloseq* (14, 15). Last, the relative
112 abundances of individual ASVs were calculated using simple proportions.

113
114 The raw 16S ribosomal RNA data discussed in this publication have been deposited in NCBI's
115 Sequence Read Archive and are accessible through accession number PRJNA726992 (16).

116 Statistical Analyses

117
118 *General Approach:* Statistical analyses were conducted at the ASV level in R version 3.1.10
119 (17). To compare the URT microbiota between groups in a comprehensive manner, different
120 types of statistical analyses that are frequently employed in microbial ecology were used,
121 including α -diversity (richness \pm evenness), β -diversity (overall composition), and differential
122 abundance (individual taxa) analyses. For α - and β -diversity analyses only, the processed dataset
123 was rarefied to the lowest library size of all samples (n=1,154). This rarefaction process was
124 repeated multiple times (n=400) and the results were averaged. Common α -diversity (observed
125 species, Shannon, and inverse Simpson indices) and β -diversity (Bray-Curtis [based on taxa
126 abundance] and Jaccard [based on taxa presence *vs.* absence] indices) metrics were calculated.
127 For differential abundance analyses, a non-rarefied dataset was used and, to minimize the impact
128 of rare taxa, only ASVs with a relative abundance across all samples >0.01% were included. If a
129 particular ASV lacked species classification, then its identity at the next higher available
130 taxonomic level (e.g., genus or family) was used. Initial steps were conducted using the R
131 package *phyloseq* (14). Final comparisons were conducted using several R packages, which are
132 described below. All statistical models included age, sex, and the presence of at least one
133 comorbidity (i.e., obesity, diabetes, hypertension, lung disease, or heart disease, coded as yes *vs.*
134 no or not reported) as covariates. Age was centered and scaled prior to statistical analyses. When
135 appropriate, *p*-values were controlled for multiple testing using the Benjamini-Hochberg
136 procedure and the resulting *q*-values are reported (18). Statistical significance was defined as a *p*-
137 or *q*-value <0.05. Figures were created using the R packages *ggplot2*, *ggalluvial*, and
138 *ComplexHeatmap* (19). Minor aesthetic edits to figures (e.g., paneling, text insertion, or label
139 formatting) were made with Inkscape version 1.0.1 (available at: <https://inkscape.org/>).
140 Following statistical analyses, we attempted to identify the corresponding species of unclassified
141 ASVs that were found to be differentially abundant between groups using the Basic Local
142 Alignment Search Tool (BLAST) database and based on the highest expected value (E value)
143 and percent identity (20).

144
145

146 *Comparisons between SARS-CoV-2-infected and -uninfected participants:* First, the URT
147 microbiota of SARS-CoV-2-infected participants at each of the 7 study time points to that of
148 controls at enrollment was compared. The comparisons of α -diversity metrics were conducted
149 using linear regression. To compare β -diversity metrics, non-metric-multidimensional scaling
150 (NMDS) and permutational multivariate analysis of variance (PERMANOVA) was used as
151 implemented in the *metamds* (with 999 iterations) and *adonis2* (with 999 permutations and the
152 by = “margin” argument) functions of the R package *vegan*, respectively (21, 22). Differential
153 abundance analyses were performed using the R package *DESeq2* (23). For the different types of
154 statistical analyses, the Benjamini-Hochberg procedure was used to control for multiple testing
155 (18). In the case of α - and β -diversity analyses, this was done separately for each index by
156 including all 7 *p*-values obtained from the individual comparisons in the false-discovery rate
157 calculations. Because *DESeq2* already implements the Benjamini-Hochberg procedure to control
158 for multiple testing when performing analyses and to avoid overcorrection, no further correction
159 for multiple testing was performed in differential abundance analyses. However, based on pre-
160 established criteria, ASVs were considered differentially abundant only if, in addition to
161 statistical significance, they 1) had an absolute fold change ≥ 2 (equivalent to an absolute fold
162 change ≥ 1 in the \log_2 scale), and 2) were differentially abundant in ≥ 3 of the 7 individual
163 comparisons.

164
165 *Longitudinal effect of SARS-CoV-2 viral load on the URT microbiota:* Among participants with
166 COVID-19, the URT microbiota was then compared between those with and without high viral
167 load over time. High viral load was defined as a cycle threshold value for the detection of the
168 coronavirus nucleocapsid gene region 1 below the median of all samples collected on day 1 from
169 SARS-CoV-2-infected participants. The comparisons of α -diversity metrics were conducted
170 using generalized estimating equations (GEE) as implemented in the R package *geepack*
171 (clustering by participant, using an exchangeable correlation structure, and with robust standard
172 errors) (24). To compare β -diversity metrics, NMDS and PERMANOVA were used as
173 implemented in the *metamds* (with 999 iterations) and *adonis2* (with 999 permutations, the by =
174 “margin” argument, and restricted permutations by participant) functions of the R package
175 *vegan*, respectively (21, 22). Differential abundance analyses were performed using the
176 *permuspliner* function of the R package *SplinectomeR* (25). For this, we used the relative
177 abundances of individual ASVs, restricted the statistical analyses to the top 25 most predominant
178 ASVs in SARS-CoV-2-infected participants (which together represented 90.33% of all reads),
179 and controlled for multiple testing with the Benjamini-Hochberg procedure (18). Because
180 *SplinectomeR* does not allow for covariate adjustment, the association of SARS-CoV-2 viral load
181 with the relative abundance of ASVs found to be significant in *SplinectomeR* were then tested
182 using GEE as described above while adjusting for potential confounders. The statistical models
183 for α -diversity and differential abundance analyses using GEE also included the study time point
184 and a multiplicative interaction between SARS-CoV-2 viral load and the study time point as
185 additional covariates.

186 **Supplementary Tables:**

187

188 **Table S1:** Full taxonomy of all ASVs removed by the R *decontam* package (see attached .csv
189 file).

190

191 **Supplementary Figure Legends:**

192

193 **Figure S1:** Differential abundance of taxa of the upper respiratory tract microbiota between
194 SARS-CoV-2-uninfected and -infected participants. The figure shows box plots of *DESeq2*
195 normalized counts (y-axes) of selected ASVs by group (x-axes). Only ASVs that were
196 differentially abundant between groups using *DESeq2* models are shown (see text and Figure 4).
197 For SARS-CoV-2-infected participants, the y-axes represent the mean *DESeq2* normalized
198 counts across all follow-up days. *Definition of abbreviations:* ASV = Amplicon sequence
199 variant, SARS-CoV-2 = Severe acute respiratory syndrome coronavirus-2.

200

201

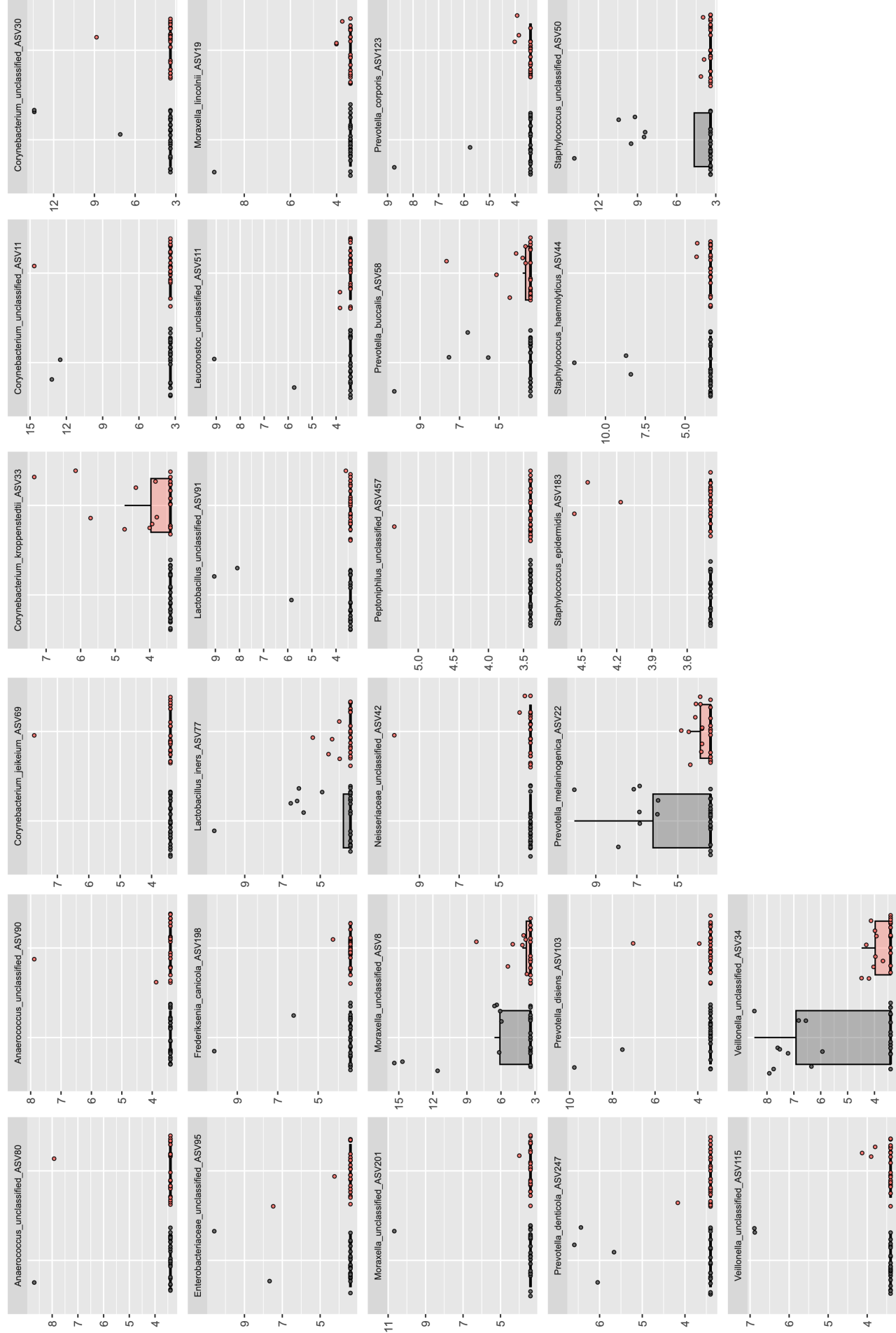
202 **Supplementary References:**

203

- 204 1. Kimura KS, Freeman MH, Wessinger BC, Gupta V, Sheng Q, Huang LC, et al. Interim
205 Analysis of an Open-label Randomized Controlled Trial Evaluating Nasal Irrigations in Non-
206 hospitalized Patients with COVID-19. *Int Forum Allergy Rhinol.* 2020.
- 207 2. Esther CR, Jr., Kimura KS, Mikami Y, Edwards CE, Das SR, Freeman MH, et al.
208 Pharmacokinetic-based failure of a detergent virucidal for SARS-COV-2 nasal infections: A
209 preclinical study and randomized controlled trial. *Int Forum Allergy Rhinol.* 2022.
- 210 3. Rosas-Salazar C, Kimura KS, Shilts MH, Strickland BA, Freeman MH, Wessinger BC, et
211 al. SARS-CoV-2 infection and viral load are associated with the upper respiratory tract
212 microbiome. *J Allergy Clin Immunol.* 2021;147(4):1226-33 e2.
- 213 4. World Health Organization. Clinical management of COVID-19: interim guidance, 27
214 May 2020. World Health Organization; 2020.
- 215 5. World Health Organization. Criteria for releasing COVID-19 patients from isolation:
216 scientific brief, 17 June 2020. World Health Organization; 2020.
- 217 6. Centers for Disease Control and Prevention. CDC 2019-Novel Coronavirus (2019-nCoV)
218 Real-Time RT-PCR Diagnostic Panel 2020 October 01, 2020. Available from:
219 <https://www.fda.gov/media/134922/download>.
- 220 7. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-
221 index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the
222 MiSeq Illumina sequencing platform. *Appl Environ Microbiol.* 2013;79(17):5112-20.
- 223 8. Singh K, Gobert AP, Coburn LA, Barry DP, Allaman M, Asim M, et al. Dietary Arginine
224 Regulates Severity of Experimental Colitis and Affects the Colonic Microbiome. *Front Cell
225 Infect Microbiol.* 2019;9:66.
- 226 9. Hiremath G, Shilts MH, Boone HH, Correa H, Acra S, Tovchigrechko A, et al. The
227 Salivary Microbiome Is Altered in Children With Eosinophilic Esophagitis and Correlates With
228 Disease Activity. *Clin Transl Gastroenterol.* 2019;10(6):e00039.
- 229 10. Shilts MH, Rosas-Salazar C, Strickland BA, Kimura KS, Asad M, Sehanobish E, et al.
230 Severe COVID-19 Is Associated With an Altered Upper Respiratory Tract Microbiome. *Front
231 Cell Infect Microbiol.* 2021;11:781968.
- 232 11. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2:
233 High-resolution sample inference from Illumina amplicon data. *Nat Methods.* 2016;13(7):581-3.
- 234 12. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a
235 comprehensive online resource for quality checked and aligned ribosomal RNA sequence data
236 compatible with ARB. *Nucleic Acids Res.* 2007;35(21):7188-96.
- 237 13. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical
238 identification and removal of contaminant sequences in marker-gene and metagenomics data.
239 *Microbiome.* 2018;6(1):226.
- 240 14. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis
241 and graphics of microbiome census data. *PloS one.* 2013;8(4):e61217.
- 242 15. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, et al. Normalization and
243 microbial differential abundance strategies depend upon data characteristics. *Microbiome.*
244 2017;5(1):27.
- 245 16. Leinonen R, Sugawara H, Shumway M, Collaboration INSD. The sequence read archive.
246 *Nucleic Acids Res.* 2010;39(suppl_1):D19-D21.

- 247 17. R Development Core Team. R: A language and environment for statistical computing.
248 Vienna, Austria: R Foundation for Statistical Computing; 2006. Available from: [http://www.R-](http://www.R-project.org/)
249 [project.org/](http://www.R-project.org/).
- 250 18. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - a Practical and
251 Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-
252 Methodological. 1995;57(1):289-300.
- 253 19. Wickham H. ggplot2: elegant graphics for data analysis: springer; 2016.
- 254 20. McGinnis S, Madden TL. BLAST: at the core of a powerful and diverse set of sequence
255 analysis tools. Nucleic Acids Res. 2004;32(Web Server issue):W20-5.
- 256 21. Anderson MJ. A new method for non-parametric multivariate analysis of variance.
257 Austral Ecology. 2001;26(1):32-46.
- 258 22. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan:
259 Community Ecology Package 2014 [updated 2014. Available from: [http://CRAN.R-](http://CRAN.R-project.org/package=vegan)
260 [project.org/package=vegan](http://CRAN.R-project.org/package=vegan).
- 261 23. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
262 RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
- 263 24. Højsgaard S, Halekoh U, Yan J. The R Package geepack for Generalized Estimating
264 Equations. Journal of Statistical Software. 2005;15(2):1 - 11.
- 265 25. Shields-Cutler RR, Al-Ghalith GA, Yassour M, Knights D. SplinctomeR Enables Group
266 Comparisons in Longitudinal Microbiome Studies. Frontiers in microbiology. 2018;9:785.
267

DESeq2 normalized counts



SARS-CoV-2

Uninfected

Infected

