Bacterial Microbiome of Foreskin Tissue, Impact of Short-course PrEP, and Relation to Host Inflammation

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Abstract

HIV pre-exposure prophylaxis (PrEP) is effective at preventing HIV acquisition, but its impact on the microbiome of the penis has not been described. As part of the CHAPS randomized clinical trial, we sequenced a segment of the bacterial 16S rRNA gene from foreskin tissue collected during surgical penile circumcision of 144 adolescents in South Africa and Uganda after randomization to placebo, emtricitabine with tenofovir disoproxil fumarate, or emtricitabine with tenofovir alafenamide. We found a large proportion of *Corynebacterium* in addition to other anaerobic species. *Cutibacterium acnes* was more abundant among participants from South Africa than Uganda, though this made no difference in surgical recovery. We did not find a difference in bacterial populations by treatment received nor bacterial taxa that were differentially abundant between participants who received placebo versus active drug. Using RNAseq libraries from the same participants, we found generally negative correlations between the relative abundance of bacterial taxa and the expression of genes downstream of the innate response to bacteria and regulation of the inflammatory response. When participants were divided into two clusters based on bacterial community composition, these clusters were also distinguished by high and low bacterial diversity. Random forest classification showed higher expression of *NFATC3* and *SELENOS* and lower expression of *STAP1* and *NLRP6* in the higher diversity group compared to the lower. Our results show no difference in the tissue microbiome of the foreskin in the different PrEP arms versus placebo. Bacterial taxa were largely inversely correlated with gene expression, consistent with non-inflammatory colonization.

Importance

We found that a short course of oral medicine to prevent HIV was not associated with a difference in the bacterial community of the foreskin of the penis. Previous studies found increased inflammation with certain anaerobic bacteria from swabs taken under the foreskin, but we found that higher relative abundances of the bacteria were correlated with lower

51 expression of inflammatory genes. Understanding the relationship between bacteria and
52 inflammation in the penis will help us to understand how interventions like penile circumcision
53 reduce the risk of acquiring sexually transmitted infections such as HIV.
54

55 # Introduction

56 HIV remains a significant global health challenge despite substantial clinical, public health, and
57 basic science research efforts. While condom use (1, 2), pre-exposure prophylaxis (PrEP) (3), and
58 medical penile circumcision (4) are effective at reducing HIV incidence in men, the contribution
59 of the penile microbiome to these mechanisms has not been fully explored. Previous work has
60 shown a predominance of anaerobic species in the microbiota of swabs taken from the coronal
61 sulcus or urethra (5-15) and have reported associations between species such as *Prevotella* and
62 increased mucosal inflammation, HIV target cell density and risk of HIV acquisition (8, 16).
63 Following circumcision, the surface microbiota shifts to be dominated by more aerobic species
64 as found on other skin surfaces (10, 17, 18). Thus far, no data exist as to the microbiota of the
65 foreskin itself, and its relation to tissue inflammation.

66 Antiretrovirals (ARVs) are used for both treatment and prevention of HIV, and limited data
67 have shown a complex relationship with bacteria. When applied as topical vaginal pre-exposure
68 prophylaxis, *L. crispatus* was shown to endocytose Tenofovir (TFV) then either actively
69 metabolize or release it back into the environment (19). Similarly, *Gardnerella vaginalis* and other
70 anaerobes have been shown to metabolize TFV (20) or block its entry into cells by secretion of
71 adenine (19). Antiretrovirals may also theoretically alter bacteriophage populations which can
72 dramatically reshape the bacterial component of the microbiome which they infect (21). At the
73 rectal mucosa, small studies have investigated the effects of oral emtricitabine (FTC) with
74 tenofovir disoproxil fumarate (TDF) on the bacterial microbiome in men who have sex with
75 men (MSM) and transwomen (22-24) but with varying results.

76 In a randomized, open-label trial of 1-2 doses of FTC with TDF versus with tenofovir
77 alafenamide (TAF) prior to circumcision, we characterized the bacterial microbiome and
78 inflammatory gene expression of foreskin tissue removed during circumcision, hypothesizing
79 that short courses of PrEP, as utilized in a dose-finding trial, would not result in significant

80 changes to the bacterial microbiome of the foreskin, but that there would be a relationship
81 between the microbiota and inflammatory gene expression.

82 Materials and Methods

83 Cohort and Specimen Collection

84 The Combined HIV Adolescent PrEP and Prevention Study (CHAPS) was a randomized
85 controlled trial that enrolled 144 men living without HIV aged 13-24 years between 2019 and
86 2021 from the Chris Hani Baragwanath Academic Hospital in Soweto, South Africa (n=72) and
87 the Entebbe Regional Referral Hospital in Entebbe, Uganda (n=72). Inclusion criteria were male
88 sex at birth, hemoglobin > 9 g/dL, weight > 35 kg, two successive negative rapid HIV antibody
89 tests, and clinical eligibility for surgical circumcision. Exclusion criteria were conditions
90 precluding circumcision or receipt of the study medications. Participants were randomized to
91 placebo versus FTC with either TDF or TAF for 1-2 days prior to surgical penile circumcision to
92 investigate ARV dosing for on-demand PrEP (25).

93 All participants underwent a physical exam at study entry and completed survey instruments
94 including sexual history at the randomization visit. At the circumcision visit, they provided
95 midstream urine for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhea* (GC) testing via nucleic
96 acid amplification testing (NAAT) prior to surgery. If an asymptomatic sexually transmitted
97 infection was diagnosed, antibiotic treatment was prescribed at the post-operative visit.

98 Penile circumcision was performed using the dorsal slit method and the removed prepuce was
99 placed immediately in cold Dulbecco's Modified Eagle Medium and shipped on ice within 1
100 hour (median 30 minutes) to the local laboratories in Uganda (Medical Research
101 Council/Uganda Virus Research Institute) and South Africa (Perinatal HIV Research Unit in
102 Johannesburg) for processing. Smaller, 5–7 mm^2-sized sections were stored dry at -80° C until
103 the samples were transported on dry ice to the Seattle Children's Research Institute, U.S.A for
104 microbiome studies and to the Karolinska Institutet, Sweden for transcriptome analyses.

105 ## Ethics and Human Subjects

106 Ethical clearance to conduct the CHAPS trial was obtained from the South African Health
107 Products Regulatory Authority (20181004), the Uganda Virus Research Institute research and
108 ethics committee (GC/127/18/12/680), Uganda National Council of Science and Technology
109 (HS 2534), Uganda National Drug Authority (618/NDA/DPS/09/2019), and the London School
110 of Hygiene and Tropical Medicine research ethics committee (Ref:17403). Informed written
111 consent was collected from all participants. The Swedish Ethics Review Authority approved the
112 transcriptome studies of the collected specimens at the Karolinska Institutet (2020-00941). The
113 ethics approval for the microbiome analysis was granted by the Seattle Children's Institutional
114 Review Board (STUDY00003430).

115 ## Specimen Processing

116 *16S rRNA Analysis*

117 At the time of analysis, vials were thawed and approximately 25 mg of tissue was dissected and
118 processed by a customized PowerSoil Pro protocol (Supplement S1) for extraction of DNA
119 using a QIAcube instrument. A negative extraction control consisting of solution CD1 without
120 specimen was also included. Specimens from each collection site were extracted on separate
121 plates. The resulting total DNA was diluted 1:4 to reduce PCR inhibition.

122 The *16S* rRNA gene V3-V4 region was amplified using 319F/806R universal primers for 20
123 cycles of PCR as previously described (26) for each specimen along with a negative PCR control
124 reaction consisting of mastermix without DNA template for each replicate and evenly and
125 staggered genomic DNA from mock bacterial libraries (BEI Resources) as positive sequencing
126 controls. The amplified products were purified using Agencourt AMPure XP beads (Beckman
127 Coulter) and submitted to an additional 10 rounds of PCR with indexing primers (Illumina).
128 The resulting libraries were pooled by volume with specimens at 100x the positive controls. The
129 resulting library was purified using a MinElute PCR purification column (Qiagen), followed by

130 the QiaQuick gel extraction kit (Qiagen). The cleaned library was quantitated using qPCR
131 (NEBNext Library Quant Kit for Illumina), then pooled with PhiX, denatured, and loaded onto
132 a MiSeq instrument (Illumina) with a v3 2x300 flow cell following the manufacturer's protocol.

133 Sequences were de-multiplexed using Illumina's BaseSpace workflow. Primers and adapters
134 were removed by cutadapt 2.7 (27). Sequences were further trimmed for quality, then filtered
135 and merged using dada2 1.22.0 (28) to generate amplicon sequence variants (ASVs). Taxa were
136 annotated using the Silva 138.1 database (29) with additional genital-associated species (30)
137 using a 100% identity threshold. The phyloseq 1.40.0 (31) and vegan 2.6-2 (32) R packages were
138 used to manipulate ASV tables and calculate diversity measures. ASV sequences were aligned
139 using ssu-align 0.1.1 (33), and a maximum likelihood phylogeny was generated using PhyML
140 3.3.20220408 (34) with a GTR substitution model. Contaminating sequences were identified by
141 their presence in negative controls for the extraction and PCR amplification or mock community
142 using decontam 1.16.0 (35) and microfiltR (36). After decontamination, specimens with fewer
143 than 25-fold as many reads than extraction and PCR controls were excluded. For differential
144 abundance analysis, decontaminated ASVs were filtered with prevalence >= 10% and relative
145 abundance threshold of $1x10^{-4}$ before combining counts for all ASVs classified as the same
146 species. ALDEx2 1.28.1 (37), ANCOM-BC 1.6.2 (38), and DESeq2 1.36.0 (39) (using the poscounts
147 factors estimation) were used for differential abundance testing to overcome the documented
148 limited power and accuracy of these tools when used individually on 16S data sets which
149 contain a high proportion of zero counts (40, 41).

150 *RNAseq of Foreskin Tissue*

151 Foreskin samples were disrupted and homogenized using a Tissuelyzer (Qiagen) and total RNA
152 isolated using the RNeasy Kit (Qiagen) according to manufacturer's instructions. RNA was
153 subjected to quality control with Agilent Bioanalyzer (Agilent). To construct libraries suitable
154 for Illumina sequencing, the Illumina stranded mRNA prep ligation sample preparation
155 protocol was used with starting concentration of 200 ng total RNA. The protocol includes

156 mRNA isolation, cDNA synthesis, ligation of adapters and amplification of indexed libraries.
157 The yield and quality of the amplified libraries were analysed using Qubit by (Thermo Fisher)
158 and the Agilent Tapestation (Agilent). The indexed cDNA libraries were normalized and
159 combined, and the pools were sequenced on the Illumina Novaseq 6000 S4 flowcell to generate
160 150 bp paired-end reads.

161 Sample demultiplexing was performed using bcl2fastq 2.20.0 (Illumina), and quality and
162 adapter trimming of reads was performed using Cutadapt 2.8 (27). Sample quality was assessed
163 using FastQC 0.11.8 (Babraham Bioinformatics) and MultiQC 1.7 (42). Reads were aligned to the
164 Ensembl GRCh38 reference genome using STAR 2.6.1d (43). Counts for each gene were
165 obtained using featureCounts 1.5.1 (44).

166 The Gene Ontology (GO) term "inflammatory response" (GO:0006954) selected 860 putative
167 inflammatory genes which were filtered to only those with at least two copies detected in at
168 least 90% of specimens. RNA read counts were normalized then transformed by centered log
169 ratio (CLR). The 16S ASVs were filtered and combined as described for the differential
170 abundance analysis and also CLR-transformed. We calculated the correlation between the gene
171 counts and bacterial relative abundances, then filtered for $r > 0.4$ and Benjamani-Hochberg-
172 adjusted p-value < 0.05. The resulting genes were manually inspected for their most relevant
173 GO annotation and grouped according to their immunological function and pro- or anti-
174 inflammatory nature (Supplemental table S1).

175 Normalized gene counts were used to perform random forest feature selection as implemented
176 in the Boruta R package 7.0.0 (45). Only the importance measures of statistically significant
177 ($p<0.01$) features were reported.

178 Statistical Analyses

179 All statistical analyses were performed in R version 4. Alpha diversity comparisons were
180 evaluated using the Wilcoxon rank sum test. Beta diversity was compared using Permutational

181 Multivariate of Variance (PERMANOVA) using the adonis2 function of the vegan R package.
182 The relationship between treatment arm and CST was assessed using multinomial logistic
183 regression. RNAseq and 16S taxa correlations were calculated using Pearson coefficient. A
184 significance threshold of $\alpha = 0.05$ was used for the differential abundance hypothesis testing.

185 **Data Availability**

186 The 16S raw reads and RNASeq libraries will be deposited in the National Center for
187 Biotechnology Information Short Read Archive and European Bioinformatics Institute
188 (respectively) upon publication. R code to reproduce the analysis is available at
189 https://github.com/bmaust/CHAPS/. The completed STORMS (Strengthening The
190 Organizing and Reporting of Microbiome Studies) checklist (46) for this project is located at
191 https://doi.org/10.5281/zenodo.7080176.

192 # Results

193 *Clinical STI testing*

194 No participants reported STI symptoms, and no physical exams revealed urethral discharge or
195 other genital abnormality. None of the participants had clinical balanoposthitis or evidence of
196 macroscopic inflammation. No GC infections were diagnosed, but NAAT for seven participants
197 was positive for CT: five from Uganda and two from South Africa.

198 *Microbiome 16S sequencing*

199 After filtering and contamination removal, 137 specimens from the 144 enrolled participants
200 had sufficient bacterial DNA reads to proceed with analysis. The identified bacterial taxa
201 include a variety of skin-associated Gram-positive and genital-associated anaerobic species in
202 addition to Gram-negative enterics (Figure 1). *Corynebacterium* was the most prevalent and
203 abundant genus, appearing in 132 (97%) of specimens at median relative abundance of 34%
204 (range: 0.14% to 98%). Anaerobic species were extremely common, including bacteria that are

205 commonly found in bacterial vaginosis, an inflammatory dysbiosis of the vagina, including
206 *Prevotella*, *Anaerococcus*, *Finegoldia* and *Porphyromonas*. There were no ASVs in the *Chlamydiaceae*
207 family which includes *C. trachomatis*.

208 Unsupervised partition around medioids clustering separated the unweighted Unifrac
209 distances into two community structure types (CST) with distinct community structure
210 (PERMANOVA $R^2 = 0.25$ with $p < 0.001$) and alpha diversity ($p=2.39x10^{-19}$) (Figure 2). Two
211 clusters maximized the silhouette score with acceptable within sum of squares and gap
212 statistics. CST1 was highly diverse with a median Shannon index of 3.05. CST2 was dominated
213 by *Corynebacterium tuberculostearicum* and *Finegoldia magna* and, with median relative
214 abundances of 21% and 9.6%, respectively. CST2 also had a significantly lower median Shannon
215 index of 1.68 (Wilcoxon unpaired exact, $p = 2.39x10^{-19}$). Though *F. magna* and *C.*
216 *tuberculostearicum* were also highly abundant in CST1 (median relative abundances of 4.6% and
217 2.5%), they shared high relative abundance with *Anaerococcus, Campylobacter, Fenollaria,*
218 *Finegoldia*, *Ezakiella*, *Mobiluncus*, and *Peptinophilis* species without a clear dominant taxon.

219 *Microbiome differences by study site*

220 The 137 participants with 16S data included 69 (50.3%) individuals from South Africa and 68
221 (49.7%) from Uganda. We compared microbiota between the two study sites and found no
222 differences in within-participant alpha diversity (Shannon entropy) or between-participant beta
223 diversity (unweighted Unifrac distance) (Figure 3, A and B). Differential abundance testing
224 identified *Cutibacterium acnes* as significantly higher in participants from South Africa compared
225 to those from Uganda by ALDEx2 with a CLR difference of 3.3 between sites (Wilcoxon rank
226 test with Benjamani-Hochberg correction p=0.0184). ANCOM-BC identified the same ASV with
227 a significant q-value (0.0079), but the log_2fold change of 1.87 failed to meet the effect size
228 threshold. DESeq2 did not identify any taxa as significantly differentially abundant. (Figure 3,
229 C-E).

230 *Microbiome differences by treatment arm*

231 We performed similar analyses for the bacterial populations in participants who received active
232 drug versus placebo. The participants with 16S data were equally distributed among treatment
233 arms with FTC-TAF and FTC-TDF (n=59 and 62, respectively, χ^2 p=0.437). All 16 participants
234 who received placebo had sufficient sequences for analysis. We found no differences in alpha or
235 beta diversity (Figure 4, A and B) between placebo and FTC-TAF or FTC-TDF regimens.
236 Combining both treatment groups and comparing to placebo, no species were significantly
237 differentially abundant by any of the three tools (Figure 4, C-D). An un-annotated *Dialister*
238 species was identified as statistically significant by ANCOM-BC but did not meet the effect size
239 threshold. The treatment arm was not a significant predictor of the CSTs identified above
240 (p=0.32 for placebo, p=0.99 for FTC/TAF, p=0.88 for FTC/TDF).

241 *Inflammatory genes and bacterial taxa*

242 Forty inflammatory genes showed significant correlation with 31 bacterial species (Table 1). Six
243 genes had insufficient evidence for inflammatory function and were excluded. The remaining
244 34 genes were primarily pro-inflammatory with negative correlation to bacterial species (Figure
245 5) without difference by environmental niche. IL-15 was the most frequently correlated gene,
246 with significant negative correlations to seven bacterial taxa not typically associated with
247 invasive infection: *Brevibacterium luteolum*, *Corynebacterium urealyticum*, *Dietzia timorensis*, and
248 unannotated species in the *Cutibacterium, Corynebacterium, Dietzia,* and *Nosocomiicoccus* genera.
249 The majority of the bacterial taxa significantly correlated with other genes were gram-positive
250 organisms which frequently colonize the skin. The CLR-transformed relative abundance of
251 *Corynebacterium massilliense*, in particular, was associated with significantly lower expression of
252 genes involved in regulation of inflammatory responses and neutrophil chemotaxis and
253 activation. Anaerobes also found in the oral cavity such as *Parvimonas* and *Porphyromonas* were
254 also correlated with primarily lower expression of inflammatory genes. However, the oral
255 anaerobe *Rothia amarae* was associated with higher expression of the regulatory factor *GHSR*

256 and an unclassified species also in the *Rothia* genus was associated with higher expression of the
257 pro-inflammatory gene *REG3G*. Species in three genera canonically associated with BV,
258 *Atopobium, Prevotella,* and *Sneathia,* were correlated with lower expression of inflammatory
259 genes, but one unannotated *Prevotella* ASV was negatively correlated with *ZFP36*, an immune
260 regulatory gene.

261 We performed a similar analysis, grouping bacterial ASVs at the genus level (Supplemental
262 table S2). Eight genes showed correlation with nine bacterial genera. As expected, all the
263 correlated genes and bacterial genera were also identified in the species-level analysis.

264 We conducted a separate query of associations between inflammatory genes and the CSTs
265 described in Figure 2 A distinguished by high and low diversity bacterial populations. In the
266 random forest classification, four features achieved statistical significance. Nuclear Factor of
267 Activated T cells 3 (*NFATC3*) and Selenoprotein S (*SELENOS*) showed higher expression in the
268 highly diverse CST1 relative to CST2, while Signal Transducing Adapter Family Member 1
269 (*STAP1*) and Nod-like Receptor Pyrin domain-containing 6 (*NLRP6*) showed lower expression
270 (Figure 6).

271 Discussion

272 Our study is the first of which we are aware to analyze the tissue-level microbiome of the
273 foreskin. Consistent with previous reports using penile swabs (5-18, 47), the bacteria we
274 identified are predominated by taxa commonly colonizing the skin (chiefly *Corynebacteria* spp)
275 in addition to anaerobic bacteria such as *Prevotella, Dialister, Murdochiella, Peptoniphilus,* and
276 *Negativicoccus*. These species have been associated with increased inflammation and HIV
277 acquisition in uncircumcised men (8) and bacterial vaginosis in women (48). In the foreskin, we
278 describe two major CSTs, one significantly more diverse than the other. While we found no
279 effect of one or two doses of antiretroviral exposure on the foreskin bacterial microbiome,

280 further studies of more prolonged ARV use may better elucidate whether there is an effect on
281 bacterial or viral communities of the penis.

282 The overall composition of the bacterial community did not appear to differ by study site;
283 however, we did find *Cutibacterium acnes* to be significantly more abundant in South African
284 than Ugandan participants. *C. acnes* is typically resident in the deep dermis in association with
285 sebaceous glands and hair follicles (49), which our study sampled by digesting full-thickness
286 specimens rather than resuspending skin swabs. While its contribution to its namesake acne
287 vulgaris is debated, it is otherwise non-pathogenic in immunocompetent hosts without artificial
288 material (49). It is frequently identified in surgical cultures, with unclear significance to sterility,
289 likely due to its resistance to surgical sterilization techniques and transection of deep dermal
290 structures during surgery (50). The differential abundance we observed may have been caused
291 by different surgical preparation at the two sites or by an actual difference in bacterial
292 populations. As there were no surgical complications observed during the study, the difference
293 does not appear to have clinical significance.

294 We did not identify bacterial species that were differentially abundant between participants
295 receiving placebo compared to emtricitabine with either of the two forms of tenofovir. While
296 vaginal-associated species such as *Lactobacillus* and *Gardnerella* take up tenofovir from their
297 environment (19, 20), a longer exposure than our study may be required to see a potential effect
298 on bacterial growth or to alter bacteriophage activity.

299 Lack of concordance between the clinical STI testing with NAAT and our 16S results is not
300 surprising, as *C. trachomatis* is an obligate intracellular pathogen targeting the urethral
301 epithelium and upper genital tract including the prostate and seminal vesicles but not the glans
302 or foreskin of the penis (51). Our lack of detection in foreskin tissue is consistent with the
303 previously described cellular tropisms and emphasizes the need for screening via the
304 appropriate site to evaluate infection.

305 Our inflammatory gene analysis primarily identified an inverse relationship between
306 expression of genes associated with response to bacteria and skin commensals such as
307 *Corynebacterium* and *Cutibacterium*, consistent with non-inflammatory colonization. IL-15, the
308 most commonly correlated gene, is a pleotropic cytokine secreted by a narrow range of cell
309 types, in foreskin tissue including epithelial cells, fibroblasts, Langerhans cells, and monocytes
310 (52). It has broad immunostimulatory function, promoting NK cell differentiation and survival
311 (53), inflammatory cytokine production by macrophages (54) and dendritic cells (55), neutrophil
312 activation, survival, and phagocytosis (56), germinal center B cell proliferation (57), $CD8^+$ T cell
313 survival (58), and it is required for development of skin-resident memory $CD8^+$ T cells (59). Its
314 lower expression correlating with higher relative abundances of non-pathogenic bacteria is
315 consistent with reduced inflammatory signaling corresponding to increased bacterial growth.

316 When examining gene expression between the participant groups with higher and lower
317 diversity bacterial communities, we did not confirm previous findings in the penis (8, 10, 16)
318 and vagina (60) that high bacterial diversity, particularly with anaerobic taxa, is associated with
319 increased inflammation. While we frequently identified anaerobic taxa, individual species
320 showed lower prevalence than in studies using surface swabs. This may have reduced our
321 power to detect an inflammatory association. Another explanation could be in the structure of
322 our experiment: rather than measuring secreted cytokines, we processed the entire tissue
323 specimen for bulk RNAseq, which likely included many cells not directly interacting with
324 bacteria or immune cells. Alternatively, the pre-procedure sterilization or the surgery itself may
325 have preferentially removed inflammatory species. The sterilization or collection could also
326 have altered host-bacterial interactions, though the rapid timeframe makes this explanation
327 seem less likely. Lastly, the bacterial strains in our cohort may employ different metabolic or
328 virulence strategies that render them less inflammatory. Unfortunately, our experiment was not
329 structured to investigate this possibility.

330 In the vagina, high diversity communities are highly inflammatory (60) and associated with
331 higher risk of adverse outcomes including HIV acquisition (61, 62) and preterm birth (63). The
332 foreskin microbiome had two obvious CSTs, one highly diverse CST1 and a less diverse CST2.
333 We hypothesized that the more diverse CST1 would be associated with higher levels of genes
334 related to inflammation, as in the vagina. However, when comparing gene expression between
335 CST1 versus CST2 specimens, random forest feature selection identified four genes, not
336 including canonical acute innate antibacterial response pathways. While named for its key
337 activity in T-Cell Receptor (TCR) signaling, NFAT family members play a broad role in cell
338 differentiation in the immune system and beyond (64), including in B cells (65), Toll-like
339 receptor (TLR) signaling in monocytes (66), and proliferation in perivascular tissue (67) and
340 keratinocytes (68). Higher expression of NFATC3 in CST1 specimens may represent increased
341 signaling from any of these cell types. SELENOS is up-regulated by cytokines such as IL-1β and
342 TNF-α via NF-κB and in turns acts to suppress cytokine secretion in macrophages (69). Its
343 higher expression in CST1 samples is consistent with increased suppression of chronic
344 inflammation. STAP1 has the best evidence for signaling downstream of the B-Cell Receptor
345 (BCR) (70), and lower expression could represent a response to sustained signaling (although
346 this was not supported by the expected changes in other genes). NLRP6 is both an inducer and
347 a component of the inflammasome, binds directly to the lipopolysaccharide of gram negative or
348 lipoteichoic acid of gram positive bacterial cell membranes, and plays both a pro- and anti-
349 inflammatory role in tissues such as liver, kidney, and intestine (71). Given these contrasting
350 roles, the effects of its lower expression are difficult to predict with available data. None of the
351 identified genes plays a first-line role in regulating bacterial sensing or inflammatory response.

352 In summary, we report on the first tissue-level examination of the bacterial microbiome of the
353 foreskin, without apparent effect of brief antiretroviral drug exposure. Correlating bacterial
354 species with RNAseq data revealed largely negative correlations with genes involved in the
355 inflammatory response, consistent with maintenance of immune tolerance.

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Tables

Taxon (Genus species)	Gene Symbol	Pearson corr	adj p
(Uknown *Muribaculaceae* family)	ADAMTS12	-0.404	5.90E-04
(Uknown *Muribaculaceae* family)	TUSC2	-0.497	2.63E-06
Aliicoccus (unclassified sp)	IL15	-0.557	2.41E-08
Anaerococcus lactolyticus	ODAM	0.479	8.41E-06
Arcanobacterium (unclassified sp)	CXCL9	-0.415	3.31E-04
Atopobium (unclassified sp)	JAM3	-0.420	2.64E-04
Brevibacillus (unclassified sp)	IL1RN	-0.401	6.89E-04
Brevibacterium luteolum	IL15	-0.563	1.48E-08
Campylobacter (unclassified sp)	CCL11	0.492	3.68E-06
Campylobacter (unclassified sp)	ITGB2	-0.465	2.03E-05
Campylobacter (unclassified sp)	RIPK2	-0.461	2.55E-05
Corynebacterium confusum	FPR3	-0.402	6.46E-04
Corynebacterium confusum	MS4A2	-0.439	9.72E-05
Corynebacterium confusum	SMPDL3B	-0.543	7.86E-08
Corynebacterium coyleae	APOA2	0.542	8.40E-08
Corynebacterium genitalium	BDKRB2	-0.474	1.14E-05
Corynebacterium genitalium	POLB	-0.434	1.23E-04
Corynebacterium genitalium	PPBP	0.414	3.52E-04
Corynebacterium massiliense	CAMK1D	-0.405	5.56E-04
Corynebacterium massiliense	DAB2IP	-0.595	7.04E-10
Corynebacterium massiliense	KDM6B	-0.482	6.95E-06
Corynebacterium massiliense	LILRB4	-0.419	2.83E-04
Corynebacterium massiliense	PLCG2	-0.412	4.01E-04
Corynebacterium massiliense	PRCP	-0.469	1.51E-05
Corynebacterium massiliense	RELA	-0.454	3.88E-05
Corynebacterium massiliense	SPATA2	-0.556	2.43E-08
Corynebacterium riegelii	APOA2	0.516	6.69E-07
Corynebacterium urealyticum	IL15	-0.607	2.22E-10
Cutibacterium (unclassified sp)	IL15	-0.650	1.75E-12
Cutibacterium (unclassified sp)	S1PR3	-0.407	5.03E-04
Cutibacterium (unclassified sp)	TLR1	-0.402	6.55E-04
Cutibacterium acnes	GPRC5B	-0.405	5.46E-04
Dermabacter vaginalis	FOS	-0.401	6.84E-04
Dietzia (unclassified sp)	IL15	-0.583	2.48E-09
Dietzia timorensis	IL15	-0.470	1.49E-05
Enhydrobacter (unclassified sp)	HDAC4	-0.423	2.21E-04
Lactobacillus iners	BDKRB2	-0.440	8.68E-05
Lactobacillus iners	POLB	-0.428	1.72E-04
Lactobacillus iners	PSTPIP1	-0.423	2.21E-04
Mycoplasma spermatophilum	IL9	0.432	1.36E-04
Nosocomiicoccus (unclassified sp)	IL15	-0.424	2.11E-04
Nosocomiicoccus (unclassified sp)	SMPDL3B	-0.489	4.44E-06
Parvimonas (unclassified sp)	RIPK2	-0.430	1.44E-04
Parvimonas micra	CCL18	-0.409	4.56E-04
Porphyromonas somerae	C4A	-0.409	4.52E-04
Prevotella bergensis	CXCR6	-0.430	4.52E-04
Prevotella_7 (unclassified sp)	ZFP36	-0.464	1.51E-04
Rothia (unclassified sp)	REG3G	0.534	2.18E-05
Rothia amarae	EGFR	-0.468	1.62E-07
Rothia amarae	GHSR	0.480	1.66E-05
Sneathia amnii	XCR1	-0.419	7.99E-06

Table 1 Bacterial taxa and human gene correlations

Figures

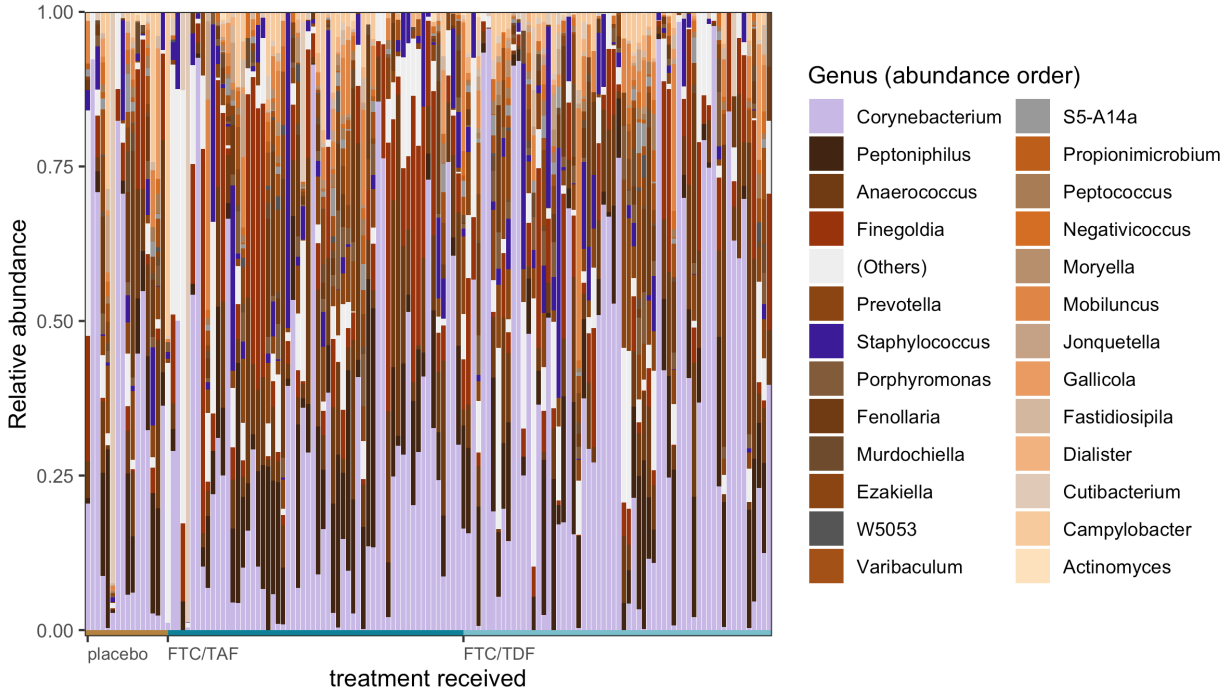


Figure 1 Top 25 most abundant bacterial genera across participants by study treatment received. (anaerobic taxa in earth tones, aerobes in purples, uncultured or unidentified species in grays)

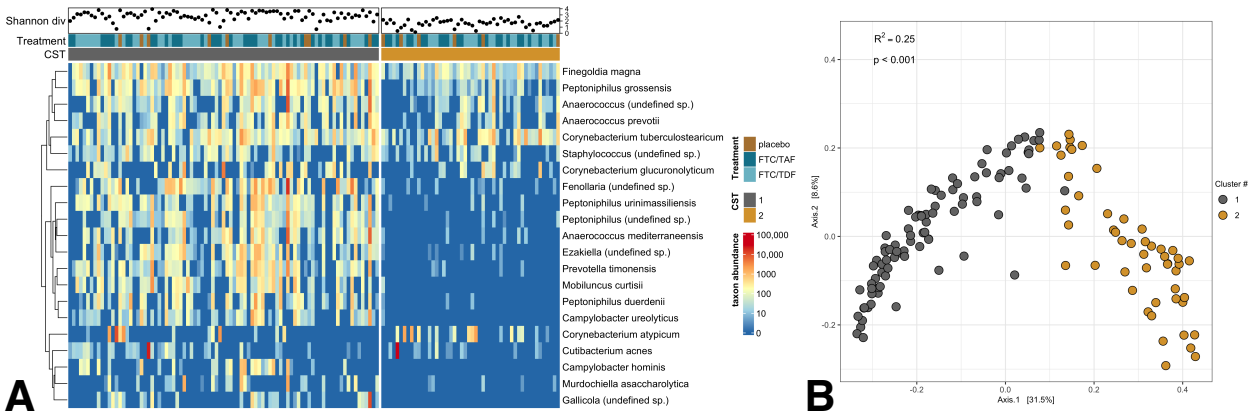
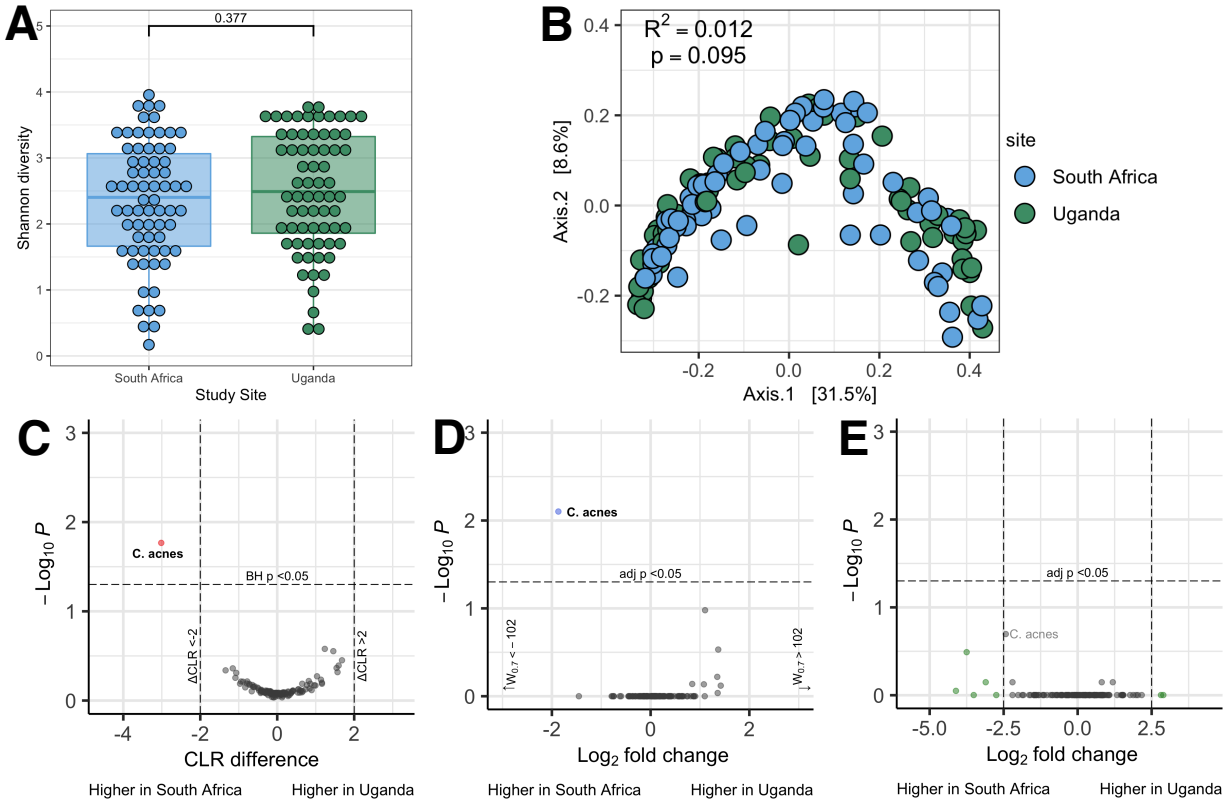


Figure 2 (A) Heatmap of top 25 bacterial species by CST, (B) PCoA ordination of bacterial beta diversity by unweighted Unifrac distance, colored by CST



577

578 Figure 3 Comparisons by study site: alpha (A) and beta (B) diversity; differential abundance

579 testing with ALDEx2 (C), ANCOM-BC (D), and DESeq2 (E)

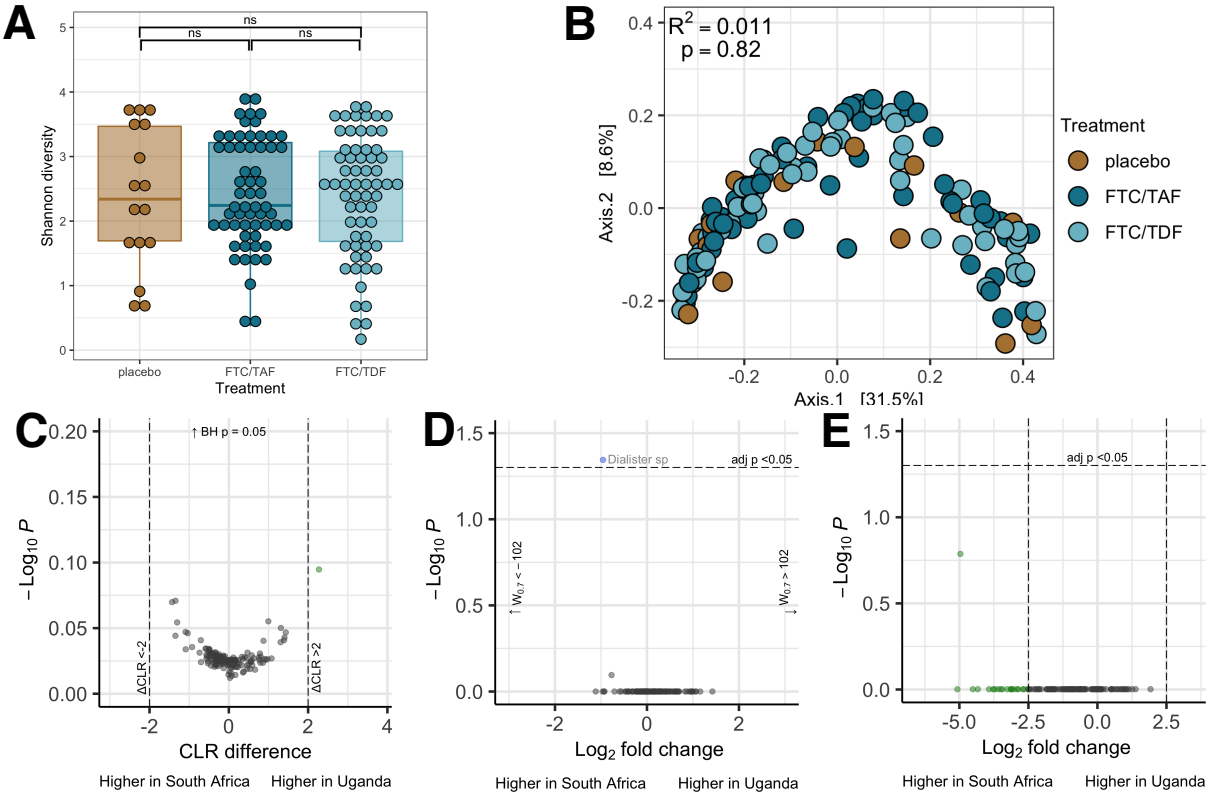
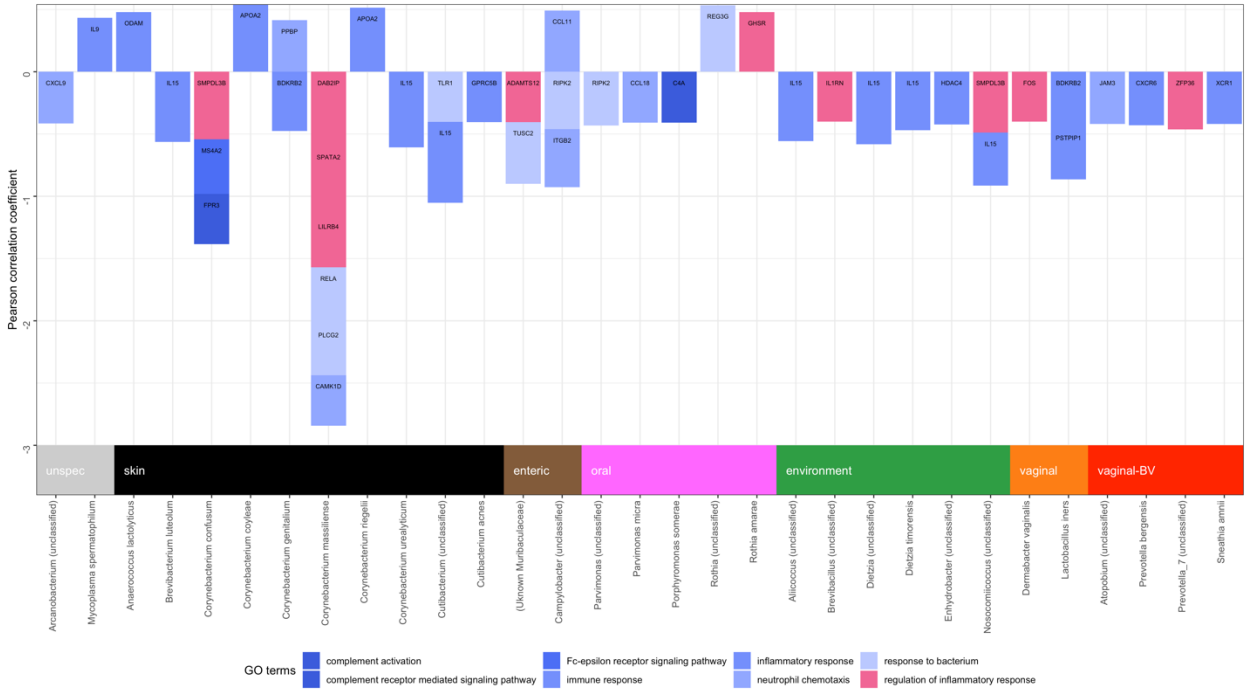


Figure 4 Analysis by treatment arm: alpha (A) and beta (B) diversity by drug received; differential abundance testing with ALDEx2 (C), ANCOM-BC (D), and DESeq2 (E)



585

586 Figure 5 Bacterial taxa and inflammatory gene associations by immune function

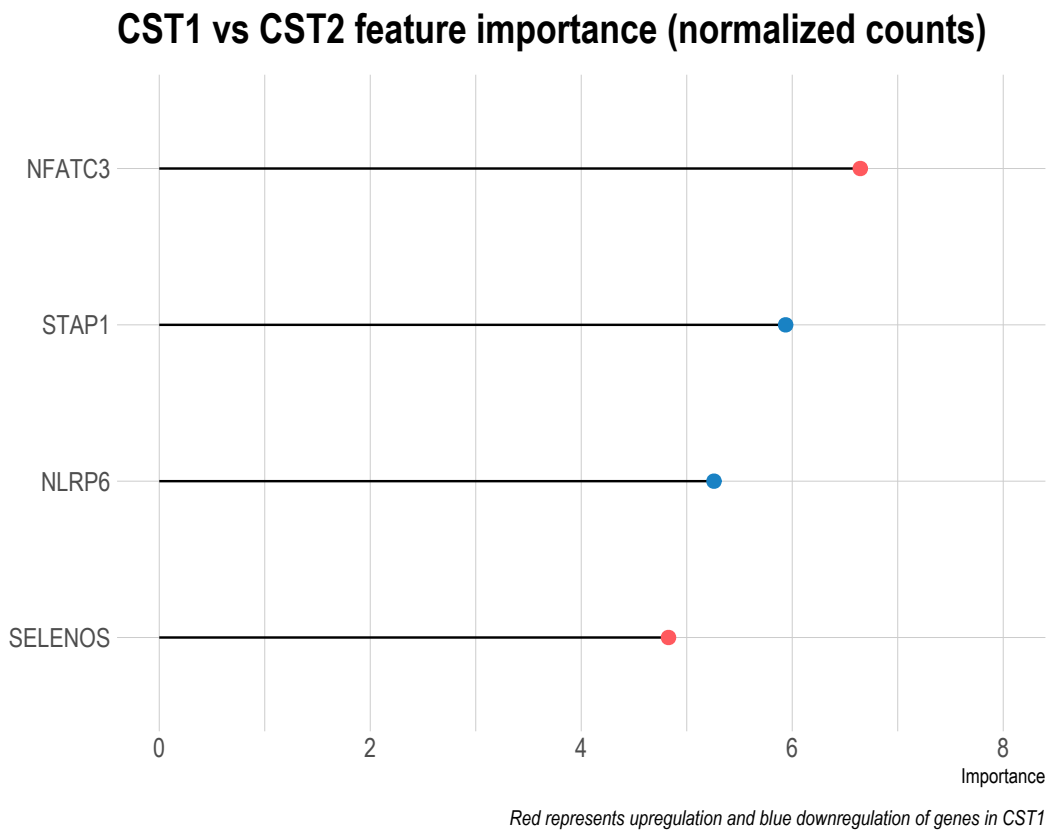


Figure 6 Random Forest Feature Importance comparing RNAseq results between CST 1 and CST 2

Supplemental items

Supplemental Table S1: Gene categorization

Supplemental Table S2: Bacterial genera and human gene correlations

Supplement S1: Foreskin Tissue DNA Extraction Workflow