The cervicovaginal microbiome of pregnant people living with HIV on antiretroviral therapy in the Democratic Republic of Congo: A Pilot Study and Global Meta-analysis.

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Abstract (253words)

Recent studies are revealing that a suboptimal cervicovaginal microbiome (CVMB), including enrichment of anaerobic bacteria associated with multiple female genital disorders, and adverse pregnancy and birth outcomes in pregnant people. Problematically, however, the majority of the available data to date are biased towards highly developed, Global North countries, leaving underrepresented populations like the Democratic Republic of Congo (DRC) poorly characterised. Here, we investigate the CVMB from a cohort of 82 pregnant people living with HIV (PLWH) on antiretroviral therapy (ART) from the DRC. Specifically, we explore the associations between the CVMB via 16S rRNA gene sequencing and maternal peripheral immune factors. Additionally, we compare the CVMB of PLWH-ART from DRC to publicly available CVMB data (5 studies, 1861 samples) in a meta-analysis to

elucidate the impact of HIV on the CVMB. Combined, these analyses revealed differences in community structure and predicted function of the microbiota between PLWH-ART and pregnant people without HIV (PWoH). Taxonomically, the CVMB of DRC PLWH-ART were enriched for *Lactobacillus iners*-dominated CVMBs (53%) or a diverse, polymicrobial CVMB, i.e., bacterial vaginosis (BV) (43%). Functional predictions made from these taxa suggested that protein-coupled receptors, amino sugar and nucleotide sugar metabolism, fatty acid metabolism, and polycyclic aromatic hydrocarbon degradation pathways were differentially abundant between communities. Correlation with host plasma immune factors revealed putative links between some CVMB metrics (e.g., alpha diversity and species abundance) that have been linked to adverse pregnancy and birth outcomes.

Importance: HIV remains prevalent in sub-Saharan Africa, where it has been linked to adverse birth outcomes. . Suboptimal CVMBs have shown similar links. This pilot study fills critical gaps in understanding how HIV interacts with the pregnant CVMB in populations underrepresented in microbiome research, like the Democratic Republic of Congo. We identified maternal systemic immune factors associated with suboptimal CVMBs that have been linked to poor birth outcomes. In a global meta-analysis, we found significant taxonomic and functional difference in the CVMBs between pregnant people living with and without HIV, revealing potential biomarkers that for increased risks for adverse birth outcomes. These findings provide crucial insights into CVMB features that may influence pregnancy health in pregnant people living with HIV, guiding future research and tailored interventions to support safer pregnancies in the DRC and similar populations.

61 # Introduction (480words)

62 Human immunodeficiency virus (HIV) is transmitted through sexual contact, blood,
63 needles, or from mother to infant and it attacks immune cells and leads to chronic systemic
64 infection. In July 2024, UNAIDS estimated that women in sub-Saharan Africa account for
65 approximately 62% of HIV infections (1). Several studies have established that pregnant
66 PLWH on ART (PLWH-ART) have a higher risk of adverse pregnancy and birth outcomes, such
67 as low birth weight (2–4) and preterm birth (5–7). This association with adverse birth
68 outcomes also extends to the cervicovaginal microbiome (CVMB), a community of microbes
69 residing in the vagina, which when perturbed towards dysbiosis and bacterial vaginosis (BV)
70 has been associated with preterm birth (8,9) and factors that can lead to preterm birth like
71 premature rupture of membranes (10,11), chorioamnionitis (12,13), and preeclampsia (14).

72 Currently, the CVMB is characterized using a community state type (CST) framework
73 which can be utilized to assess the compounding impacts of the CVMB and/or HIV infection
74 on pregnancy and birth outcomes. Under this framework, high diversity, non-optimal CVMB,
75 clinically referred to as BV, are classified as CST IV, and *Lactobacillus*-dominated, optimal
76 CVMBs are classified as CSTs I, II, III, and V (15). CST III is the transitional state type that
77 oscillates between being optimal and non-optimal (16). In sub-Saharan Africa, a high
78 percentage of women, particularly those of African ancestry, have CST IV and CST III CVMBs,
79 which are associated with a higher rate of HIV acquisition (17,18) and adverse pregnancy
80 and birth outcomes (19). Therefore, there is a need to elucidate the role of how HIV-
81 associated changes to the CVMB may modulate maternal and infant health outcomes.

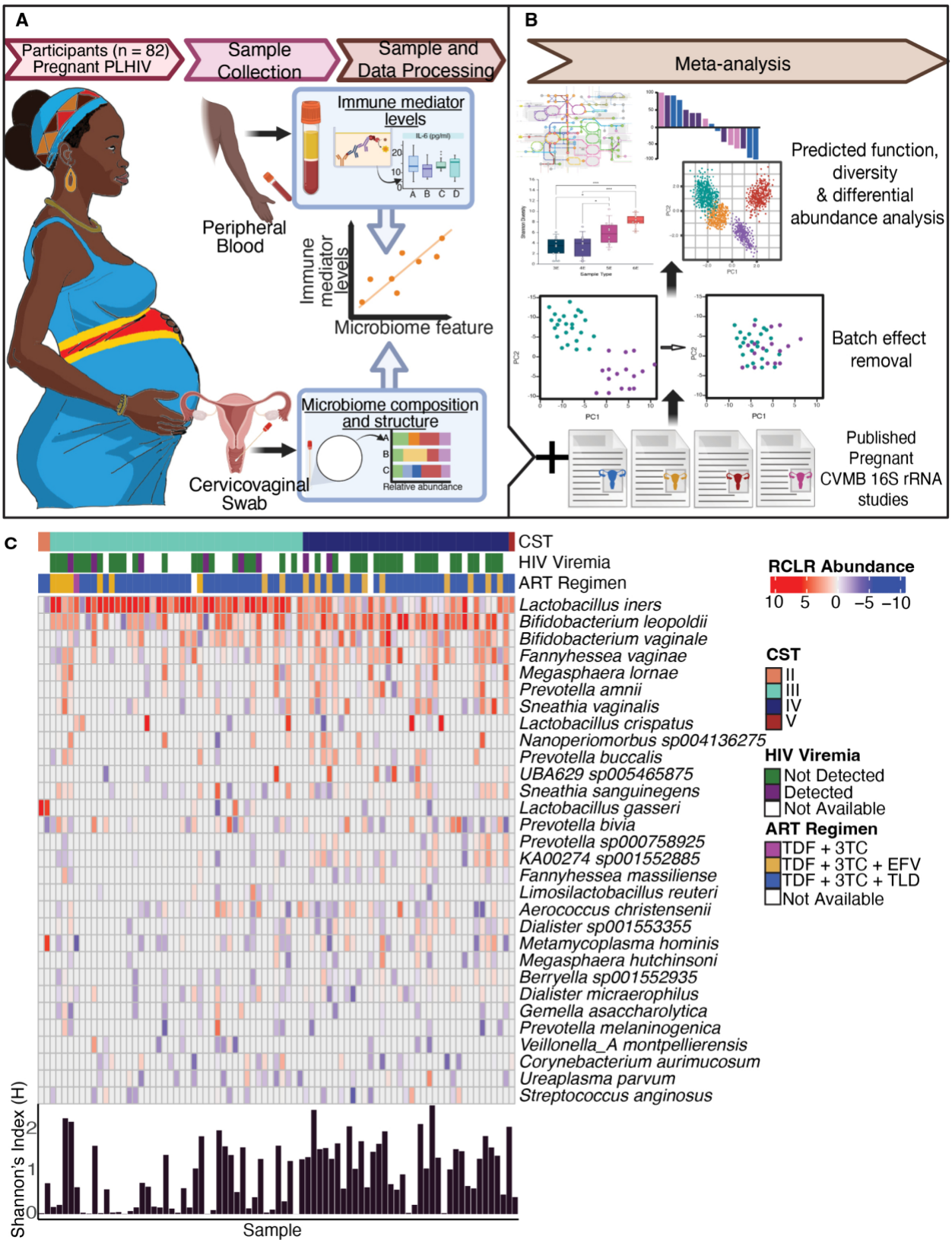
82 The impact of HIV on the CVMB has yielded conflicting results. Some studies report
83 changes in composition, and therefore CST, in the CVMB of PLWH-ART (20,21) while others
84 show no significant differences when compared to people without HIV (PWoH) (22,23). In
85 contrast to these conflicting results, there are some more consistent findings, including that
86 CST IV and HIV-associated CVMBs are linked to increased genital inflammation marked by
87 increased levels of proinflammatory cytokines and chemokines (24,25). In pregnant PLWH-
88 ART such phenotypes are associated with adverse birth and pregnancy outcomes like
89 preterm birth (26–28). Similarly, systemic inflammation PLWH-ART has been well
90 documented (29,30) and is also linked to adverse health outcomes like cardiovascular
91 disease (31,32).

92 To date, no study has investigated the potential relationship between the CVMB and the
93 soluble systemic immune factors in pregnant PLWH-ART. Here, we present an exploratory
94 study of the CVMB of pregnant PLWH-ART from Kinshasa, Democratic Republic of Congo
95 and to identify CVMB - associated circulating immune factors. Furthermore, we
96 contextualize the results from this with other CVMB datasets that include pregnant people
97 in a meta-analysis, providing a more global perspective on the interactions between the
98 CVMB and PLWH-ART. Finally, we discuss how these findings relate to pregnancy and birth
99 outcomes in PLWH-ART.

Results and discussion (3941 words)

CQI-PMTCT study population, sampling strategy, data generation and meta-analysis

In this pilot study, we sought to generate 16S rRNA gene sequencing data to investigate the CVMB of PLWH-ART in the CQI-PMTCT cohort and its association to the systemic immune system. To that end, we collected cervicovaginal swabs and peripheral blood from a subset of 82 pregnant PLWH-ART living in Kinshasa, DRC to generate 16S rRNA gene data and plasma immune factor levels, respectively (see **Methods, Fig. 1A**). Participant characteristics, stratified by CST, including participant age, gestational age, ART regimen, gravidity, socioeconomic status, and adverse birth outcomes are summarized in **Table 1.** The CQI-PMTCT cohort included only PLWH-ART participants, and since pregnant PWoH groups were lacking for comparison, the conclusions we can make from these analyses are limited. Hence, we also conducted a meta-analysis where we integrated CVMB data from the CQI-PMTCT cohort with other published and publicly available pregnant CVMB datasets to investigate the differences in the CVMB between PLWH-ART and PWoH (**Fig. 1B**).


A
Participants (n = 82) Pregnant PLHIV
Sample Collection
Sample and Data Processing
Immune mediator levels
IL-6 (pg/ml)
A B C D
Peripheral Blood
Immune mediator levels
Microbiome feature
Microbiome composition and structure
Relative abundance
Cervicovaginal Swab
B
Meta-analysis
Predicted function, diversity & differential abundance analysis
Batch effect removal
Published Pregnant CVMB 16S rRNA studies
C
CST
HIV Viremia
ART Regimen
RCLR Abundance
10 5 0 −5 −10
Lactobacillus iners
Bifidobacterium leopoldii
Bifidobacterium vaginale
Fannyhessea vaginae
Megasphaera lornae
Prevotella amnii
Sneathia vaginalis
Lactobacillus crispatus
Nanoperiomorbus sp004136275
Prevotella buccalis
UBA629 sp005465875
Sneathia sanguinegens
Lactobacillus gasseri
Prevotella bivia
Prevotella sp000758925
KA00274 sp001552885
Fannyhessea massiliense
Limosilactobacillus reuteri
Aerococcus christensenii
Dialister sp001553355
Metamycoplasma hominis
Megasphaera hutchinsoni
Berryella sp001552935
Dialister micraerophilus
Gemella asaccharolytica
Prevotella melaninogenica
Veillonella_A montpelliersis
Corynebacterium aurimucosum
Ureaplasma parvum
Streptococcus anginosus
CST
II
III
IV
V
HIV Viremia
Not Detected
Detected
Not Available
ART Regimen
TDF + 3TC
TDF + 3TC + EFV
TDF + 3TC + TLD
Not Available
Shannon's Index (H)
0
1
2
Sample

116

117 **Fig 1. Study schematic and CVMB composition of the CQI-PMTCT cohort A)** Study
118 schematic of the CQI-PMTCT study. Peripheral blood and cervicovaginal swabs were
119 collected from PLWH-ART in the Democratic Republic of Congo and processed in
120 Columbus, OH. **B)** CVMB data from the CQI-PMTCT cohort were combined with other
121 publicly available pregnant CVMB data in a meta-analysis **C)** Heatmap of RCLR abundances
122 of top 30 most abundant microbial taxa from 81 women. CSTs were determined using
123 VALENCIA. HIV viremia, ART regimen, and per sample Shannon Index values are shown.
124
125 **Table 1**: Participant demographics at enrolment stratified by community state type[a]

	No. of participant (%)[b] with characteristic		
Characteristic	**All (n = 81)**	**CST III (n = 43)**	**CST IV[d] (n= 35)**
Median age in years (IQR[c])	32 (28-35)	33 (30-34)	30 (26-35)
HIV-1 Viral load			
Detected (> 40 copies/mL)	8 (10)	6 (14)	2 (6)
Not Detected (≤ 40 copies/mL)	42 (52)	20 (47)	19 (54)
Missing	31 (38)	17 (40)	12 (34)
Gestational age (weeks)			
Second Trimester (13-26)	28 (35)	14 (33)	13 (37)
Third Trimester (27+)	32 (40)	19 (44)	11 (31)
Missing	21 (26)	10 (23)	11 (31)

ART regimen[e]			
TDF + 3TC	1 (1)	1 (2)	0 (0)
TDF + 3TC + EFV	19 (23)	9 (21)	10 (29)
TDF + 3TC + TLD	59 (73)	32 (74)	24 (69)
Missing	2 (2)	1 (2)	1 (3)
Gravidity			
Primigravida	13 (16)	5 (12)	8 (23)
Multigravida	68 (84)	38 (88)	27 (77)
Adverse birth outcome (preterm birth, stillbirth, low birth weight)			
Yes	22 (27)	12 (28)	8 (23)
No	56 (67)	26 (60)	26 (74)
Missing	3 (6)	5 (12)	1 (3)
SES quartile[f]			
1(lowest)	1 (1)	0 (0)	1 (3)
2	15 (19)	9 (21)	6 (17)
3	37 (46)	20 (47)	14 (40)
4 (highest)	28 (35)	14 (33)	14 (40)

126 [a]CST, community state type

127 [b]Percentages rounded off to the nearest whole number.

128 [c] IQR, interquartile range.

129 [d]CST IV combined subtypes A (n=4), B (n=6) and C (n =25). Statistics of three participants in
130 CST II (n=2) and CST V (n=1) are not reported.
131 [e]ART, antiretroviral therapy; 3TC, lamivudine; TDF, tenofovir disoproxil fumarate; EFV,
132 efavirenz; TLD, tenofovir/lamivudine/dolutegravir
133 [f]SES, socioeconomic status
134

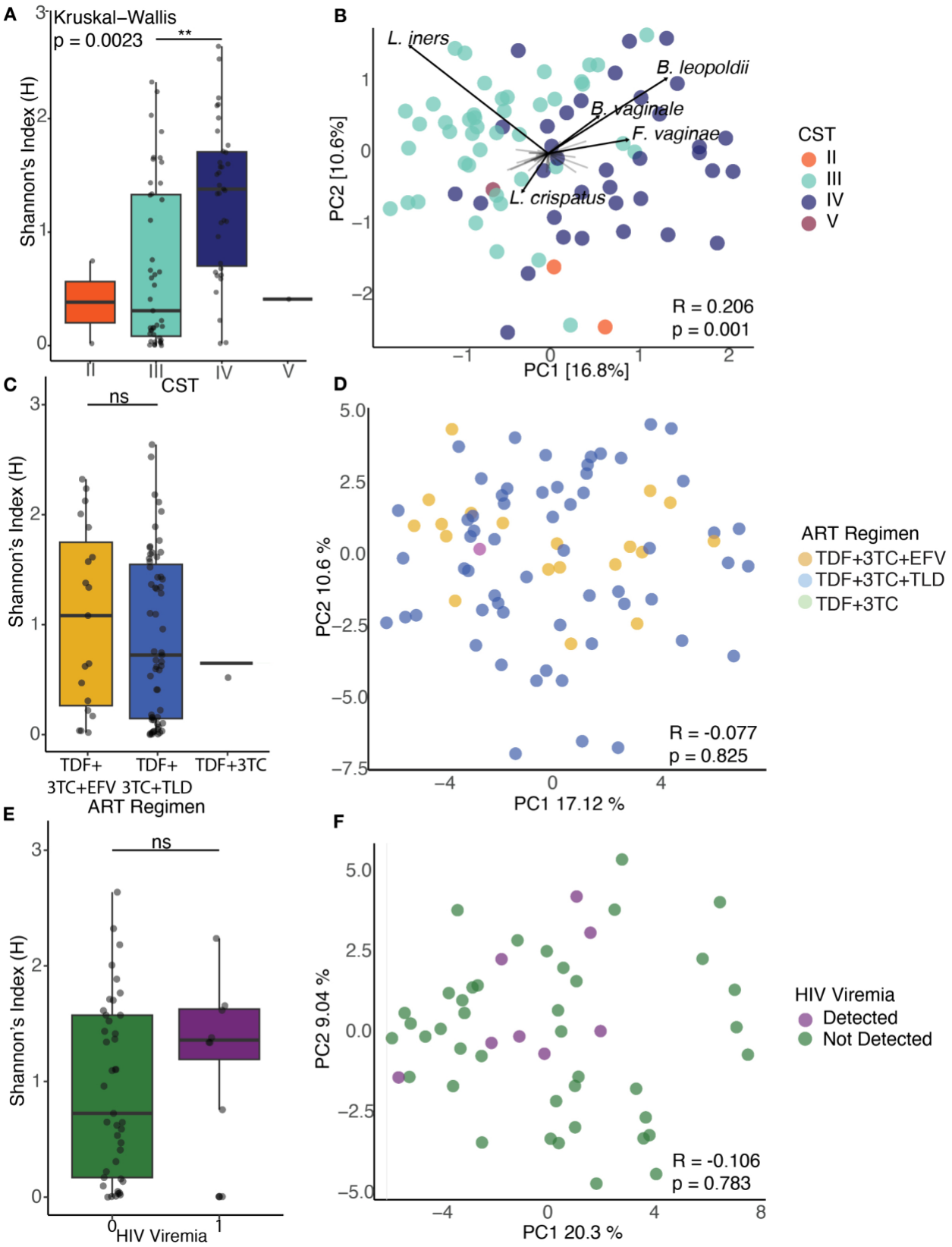
135 In the CQI-PMTCT cohort, CST III and CST IV types are
136 predominant in the CVMB of pregnant PLWH-ART in the DRC.

137 To date, no study has investigated the CVMB of people in the DRC. We generated 16S
138 rRNA gene sequencing data and survey the CVMB of pregnant PLWH-ART. Focusing on the
139 top 30 most abundant species for CVMB analyses revealed 53% of the participants (n = 43)
140 were dominated by *Lactobacillus iners,* while another 43% of the participants (n=35) were
141 dominated by a consortia of polymicrobial anaerobic bacteria (i.e., *Bifidobacterium*
142 *leopoldii , Bifidobacterium vaginae, Fannyhesea vaginae, Prevotella spp*. and *UBA629*
143 *sp0054587)* (**Fig. 1C**). These findings classify the CVMBs of PLWH-ART to CST III (transitional
144 CST) or CST IV (the non-optimal CST), respectively.
145 Evaluating diversity across these datasets revealed the following. First, within
146 sample diversity showed significant differences between identified CSTs (p = 0.0023,
147 Kruskal-Wallis) (**Fig. 2A**). This agrees with the paradigm that CST IV CVMB are more diverse
148 compared to *Lactobacillus*-dominated CSTs (15). Second, alpha diversity demonstrated
149 that samples cluster by CST (ANOSIM, R = 0.206, p = 0.001, **Fig. 2B**), with *L. iners* driving the

150 highest variation in the CST III cluster and *B. vaginale, B. leopoldii and F vaginae,* are driving

151 the variation in the CST IV cluster.

152



153

154 **Fig 2. Ecological statistics of the CVMB in the CQI-PMTCT cohort. A).** Shannon entropy

155 diversity stratified by CSTs (Kruskal-Wallis p =0.00234). **B)** PCA biplot of Robust Aitchison
156 distances coloured by CSTs (ANOSIM, R = 0.206,p = 0.001). The loadings of the top 5 most
157 abundant species across all samples are labelled to primary highlight drivers of variation
158 amongst CST clusters with black arrows to indicate the magnitude and directionality. **C)**
159 Shannon indices aggregated by ART regimen (Kruskal-Wallis p=0.695). **D)** PCA of Robust
160 Aitchison distances colored by ART regimen for 79 participants (ANOSIM R =-0.077, p =
161 0.825). **E)** Aggregated Shannon entropy values of HIV viremia for 50 participants (Wilcox test
162 p = 0.2853). **F)** PCA of robust Aitchison distances colored by detected viral load (ANOSIM R=
163 -0.106, p = 0.783).
164 Within each boxplot, the horizontal lines denote median values and the boxes extend from
165 the 25th to the 75th percentile. The vertical lines in each box plot show the values with 1.5
166 IQR. **p<0.01, ns>0.05
167
168 These results are consistent with other studies that show sub-Saharan African
169 women are more likely to harbor a high risk, non-optimal CST IV CVMB (17,33,34).
170 Additionally, other studies with similar cohorts in geography and size have shown that
171 pregnant PWH have CVMBs largely dominated by *Gardnerella spp*. and *L. iners* (21,28).
172 Incidentally, *L. iners* is the common *Lactobacilli* in CVMBs of sub-Saharan African people
173 and people across the globe (15,33), however it is not the most optimal *Lactobacillus*
174 compared to *L. crispatus* or *L. jensenii*.
175 Despite its high prevalence in the global CVMB, the role of *L. iners* in vaginal health
176 and in pregnancy and birth outcomes remains ambiguous. For example, in pregnant PLWH-

177 ART, *L. iners*-dominated CVMBs were associated with risk of preterm birth (21,28), however,
178 in pregnant PWoH, *L. iners* was associated with lower risk of preterm birth (35,36). These
179 studies underscore the complexity of *L. iners* role in vaginal health and pregnancy
180 outcomes. *L. iners* can exist with other 'non-optimal' CST IV bacteria, is less stable in the
181 vaginal microbiome and so can easily allow the transition to non-*Lactobacillus* dominated,
182 CST IV communities (i.e. BV) (37). Additionally, *L. iners* has been shown to have pathogenic
183 potential; for example, it produces the less protective L-lactic acid isomer (38–40), and has
184 highly expressed homologs of cholesterol-dependent lysins, pore forming exotoxins, which
185 have been found in other pathogenic bacteria and not in other *Lactobacilli* (41–43). However,
186 its presence even in healthy, asymptomatic people, along with its existence as a variety of
187 strains, demonstrates that it is also a vaginal symbiont and is able to persist in the CVMB
188 despite other more beneficial or protective vaginal *Lactobacilli* (44,45). Further studies are
189 needed to clarify the enigmatic roles of *L. iners* in vaginal health and pregnancy outcomes
190 using strain resolved metagenomics.

191 The other two most abundant bacteria in the CQI-PMTCT cohort, *B. leopoldii* and *B.*
192 *vaginale* (both formerly *G. vaginalis* until a recent amendment (46)), have been shown to be
193 in high abundance in sub-Saharan populations (21,28), even in healthy, asymptomatic
194 CVMBs (47–49). These CST IV bacteria have been associated with adverse pregnancy and
195 birth outcomes like preterm birth (8,50) and fetal death (51–53). Overall, our results are in
196 accordance with other studies showing the CVMBs of sub-Saharan African PLWH-ART have
197 non-*Lactobacillus* dominated (CSTs IV) or *L.iners*-dominated (CST III) CVMBs, and may

198 contribute to higher rates of HIV and sexually transmitted infections (54,55) as well as
199 preterm birth (20,21).
200

201 CVMB structure is not associated with HIV viremia and ART
202 regimen in the CQI-PMTCT cohort.

203 Next, we sought to investigate clinical characteristics that might influence the CVMB
204 in the CQI-PMTCT. We focused particularly on type of ART regimen and HIV viremia.
205 Currently, there are inconclusive results on whether ART impacts the CVMB (56–58). The
206 same is true for HIV viremia, particularly in chronic HIV infection. (21,22,59). Therefore, we
207 asked whether the type of ART regimen or HIV viremia in PLWH-ART had influence in the
208 diversity and structure of the CVMB. We hypothesized that type of ART will impact the CVMB
209 and that participants with detectable HIV viremia (n = 8, **Table 1**) will have more diverse
210 CVMB, with communities that were distinct from those with un-detectable HIV viremia (n
211 =42, **Table 1**).
212 Contrary to our hypotheses, we detected no significant differences in alpha diversity
213 at the species level between ART regimen (Kruskal Wallis test p = 0.695, **Fig. 2C)** and no
214 differences in CVMB structure between types of ART regimen (ANOSIM R= -0.077, p = 0.825,
215 **Fig. 2D).** We also did not see differences in alpha diversity between detected and
216 undetected HIV viremia (Wilcoxon Test, p =0.285, **Fig. 2E**) and in beta diversity (ANOSIM R=
217 -0.106, p = 0.783, **Fig. 2F**).

218 Studies with similar cohorts in size and geographic location have shown that ART use
219 did not impact the CVMB of Ugandan PLWH-ART (56). However, CVMBs have been
220 associated with varying concentrations of ART in the female genital tract and plasma
221 meaning there is a possibility that long term ART use could have some influence in the CVMB
222 (60). Currently, it is known that topical ARTs, like PreP and tenofovir, are metabolized by CST
223 IV cervicovaginal bacteria thereby reducing their efficacy (61–63) but these ARTs do not
224 seem to alter the CVMB composition and structure (56–58). While further research might be
225 necessary to investigate the link between ART use and CVMB composition, our results
226 contribute to the growing knowledge of the impact, or lack thereof, between ART use and the
227 CVMB (64).

228 Studies have shown before that there are no differences in vaginal communities due
229 to HIV viremia or status (22,59). Conversely, other 16S rRNA gene studies have shown that
230 HIV-associated CVMBs have a lower abundance of *Lactobacillus* species but there was no
231 conclusion on how CVMB structure (i.e. beta diversity) is different (55,65,66). Curiously,
232 there is inconclusive evidence on how HIV influences CVMB composition. On one hand,
233 cervicovaginal bacteria have been shown to interact with the HIV virus or host cells to
234 increase risk of contracting HIV. For example, CST IV bacteria like *G. vaginalis* induce a local
235 inflammation response, reduce cervicovaginal epithelial cell barrier and integrity hence
236 allowing bacterial and viral pathogens, like HIV, to infect the host (17,67,68). A recent study
237 also showed that bacterial lectins specific to O-glycans, bind to the HIV-1 virion and
238 envelope glycoproteins to increase HIV infectivity and resistance to antibodies (69).
239 However, the mechanisms by which HIV directly impact bacterial physiology and

240 metabolism remain undefined and unexplored. It is possible that the HIV viral load in the
241 vagina is sufficiently suppressed for there to be a detectable interaction between the virus
242 and vaginal bacteria (70,71), however studies on this subject are highly limited. The
243 ambiguity of the impact of chronic and systemic viral sexually transmitted infections like HIV
244 on the CVMB remains to be fully explored however, based on our results for the CQI-PMTCT
245 cohort, we can tentatively conclude and recapitulate the findings of studies that state HIV
246 viremia does not influence the CVMB (22,23,59). The high percentage of people with
247 undetectable HIV viremia is a testament to the effectiveness of ART use in this cohort.
248

249 Meta-analysis reveals that CVMB are distinct between PLWH-
250 ART and PWoH globally.

251 The CQI-PMTCT study discussed above has limitations including lack of a pregnant
252 PWoH group for comparison. Hence, to better understand the role of HIV on the CVMB, we
253 integrated this study with five other publicly available pregnant CVMB datasets. To reduce
254 batch effects, data from studies were selected to be as similar to the CQI-PMTCT CVMB data
255 as possible (see **Methods** and **Fig. S1**).

256 After adjusting for study specific batch effects using PLSDA (see **Methods**), we found
257 that within sample (alpha) species diversity was significantly higher in samples from PLWH-
258 ART compared to PWoH (**Fig. 3A**) Additionally, CVMB community structure was significantly
259 different between pregnant PLWH-ART and pregnant PWoH CVMBs (ANOSIM R= 0.518, p
260 =0.001, **Fig. 3B**). When stratified by CST, within samples species diversity was highest in CST

IV samples, which is suboptimal in the CVMB (**Fig. 3C)** and CVMB community structure was different by CSTs (ANOSIM R= 0.399, p =0.001, **Fig. 3D**). All this indicates that there are different bacterial communities between the PLWH-ART and PWoH groups.

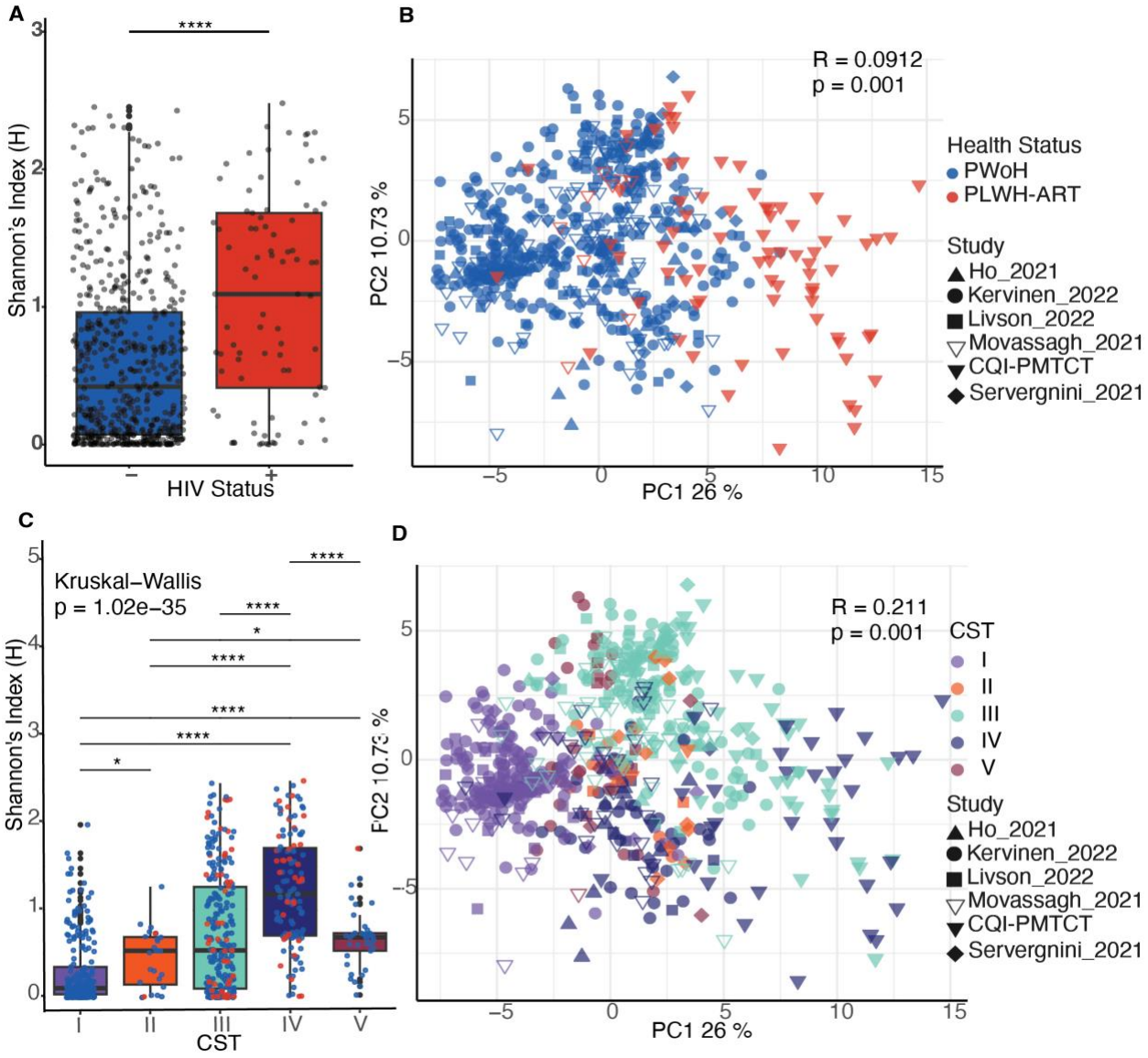
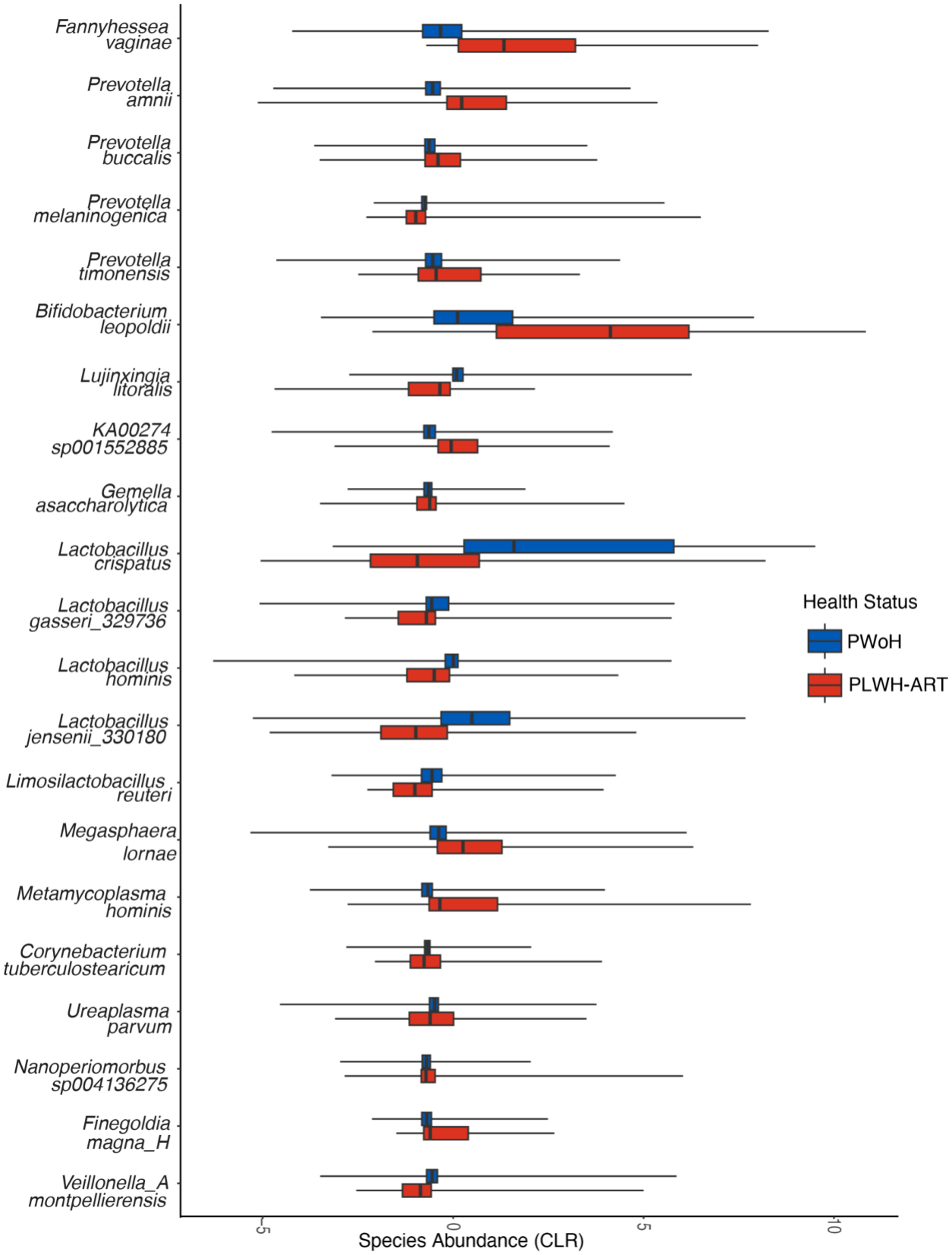


Fig 3. Ecological statistics of the integrated datasets. A) Shannon Entropy values between CVMBs of PWoH and PLWH-ART separated by study. **B)** PCA of Aitchison Distances showing different bacterial communities by health status (ANOSIM R= 0.518, p= 0.001). **C)** Shannon

269 Entropy values stratified by CST (Kruskal-Wallis p = 1.023e-35). **D)** PCA of Aitchison
270 Distances showing different bacterial communities by CST (ANOSIM R=0.399, p= 0.001).
271 *p<0.05, ****p<10^{-4}
272
273 To further investigate this claim, we used differential abundance analyses (see
274 **Methods**) to identify bacterial species that were differentially enriched between the two
275 groups. We found that 21 cervicovaginal bacterial species from 14 families were
276 differentially abundant between the PLWH-ART and PWoH groups (**Fig. 4**). *Lactobacilli spp.,*
277 namely beneficial bacteria such as *L. crispatus, L. gasseri, L. hominis* and *L. jensenii* and
278 *Limosilactobacilus reuteri* were more abundant in the CVMB of PWoH. In contrast, the
279 PLWH-ART group had high abundance of CST IV, non-optimal, BV-associated bacteria
280 compared to the PWoH group. For example, species in the *Bacteroidaceae* family,
281 particularly *P. amnni* and *P.timonensis spp.* were more abundant in the PLWH-ART group.
282 Other CST IV associated bacteria that were more abundant in the PLWH-ART group were *B.*
283 *leopoldii, M. lornae, KA00274 sp001552885* (AKA *Amygdalobacter nucleatus*), *F.magna, F.*
284 *vaginae, M. hominis* and *C. tuberculostearicum* (**Fig. 4**).
285



286

287 **Fig 4.** Boxplots showing differentially expressed species in pregnant PLWH-ART and

288 pregnant PWoH as determined using three differential abundance analysis methods (see
289 **Methods)**. Within each box, the vertical lines denote median values, and the boxes extend
290 from the 25th to the 75th percentile. The horizontal lines in each box plot show the values with
291 1.5 IQR.
292
293 Other studies have found *Lactobacillus spp.* to be more dominant in PLWH not on ART
294 compared to those on ART, regardless of pregnancy state (55,65,66). This is corroborated by
295 the CST IV, non-optimal bacteria being more differentially abundant in PLWH-ART group
296 compared to the PWoH. It is possible that this differential abundance in (non)-
297 *Lactobacillus*-dominated CVMBs between PLWH-ART and PWoH existed prior to and during
298 HIV acquisition, as such differences are associated with risk of HIV acquisition (72). For that
299 reason, these communities may have remained stable in their respective states since.
300 Indeed, one study determined that in 79.7% of CVMBs from two longitudinal cohorts (16,73),
301 CSTs were predominantly mono-stable and rarely transitioned to completely different CSTs,
302 which could explain the persistence of these bacteria in the CVMB even after HIV acquisition
303 (37). It is also possible that the influence of maternal age, racioethnicity and geography (**Fig.**
304 **S2**) distorts the impact of HIV status on the CVMB in this meta-analysis as seen before
305 (47,48,74,75). Therefore, we tentatively conclude that HIV infection is associated with
306 changes in the CVMB of pregnant people as seen in a recent study with similar populations
307 (76). However, future research will have to explore the causative associations of HIV on the
308 pregnant CVMB and how this alters pregnancy outcomes.
309

310 **Predicted metabolic pathways were differentially abundant**

311 **among CSTs in the CQI-PMTCT cohort**

312 The CST framework classifies the CVMB based on taxonomic composition (15).
313 However, even when microbial communities differ in their taxonomic composition, they
314 might have functional redundancy (77) or conversely, diverse functional capabilities in the
315 same species (78). Hence, functional prediction of the CVMB may yield more specific
316 insights into potential phenotypic differences between CST, and thus host responses. Here,
317 we predicted the function of the CVMB, stratified by CST, using PICRUSt2 based on the
318 KEGG (Kyoto Encyclopedia of Genes and Genomes) database (79,80). We predicted 6466
319 KEGG Orthologs (KOs), which were assigned to 162 KEGG pathways. Of note, these are
320 predicted functions and not direct readouts by the bacterial species.

321 Using multiple differential abundance analyses (see **Methods**), we identified four (4)
322 KEGG pathways that were differentially abundant between CSTs (**Fig. 5**), namely G protein-
323 coupled receptors (GPCRs, KO04030), amino sugar and nucleotide sugar metabolism
324 (KO00520), fatty acid metabolism (KO01212) and polycyclic aromatic hydrocarbon
325 degradation (KO00624). Fatty acid metabolism and polycyclic aromatic hydrocarbon
326 degradation had higher relative abundance in CST IV compared to CST III, with the highest
327 abundance of both in CST II. G protein-coupled receptors and amino sugar and nucleotide
328 sugar metabolism were more abundant in CST III, followed by CST IV and lastly CST II (**Fig.**
329 **5**).

330

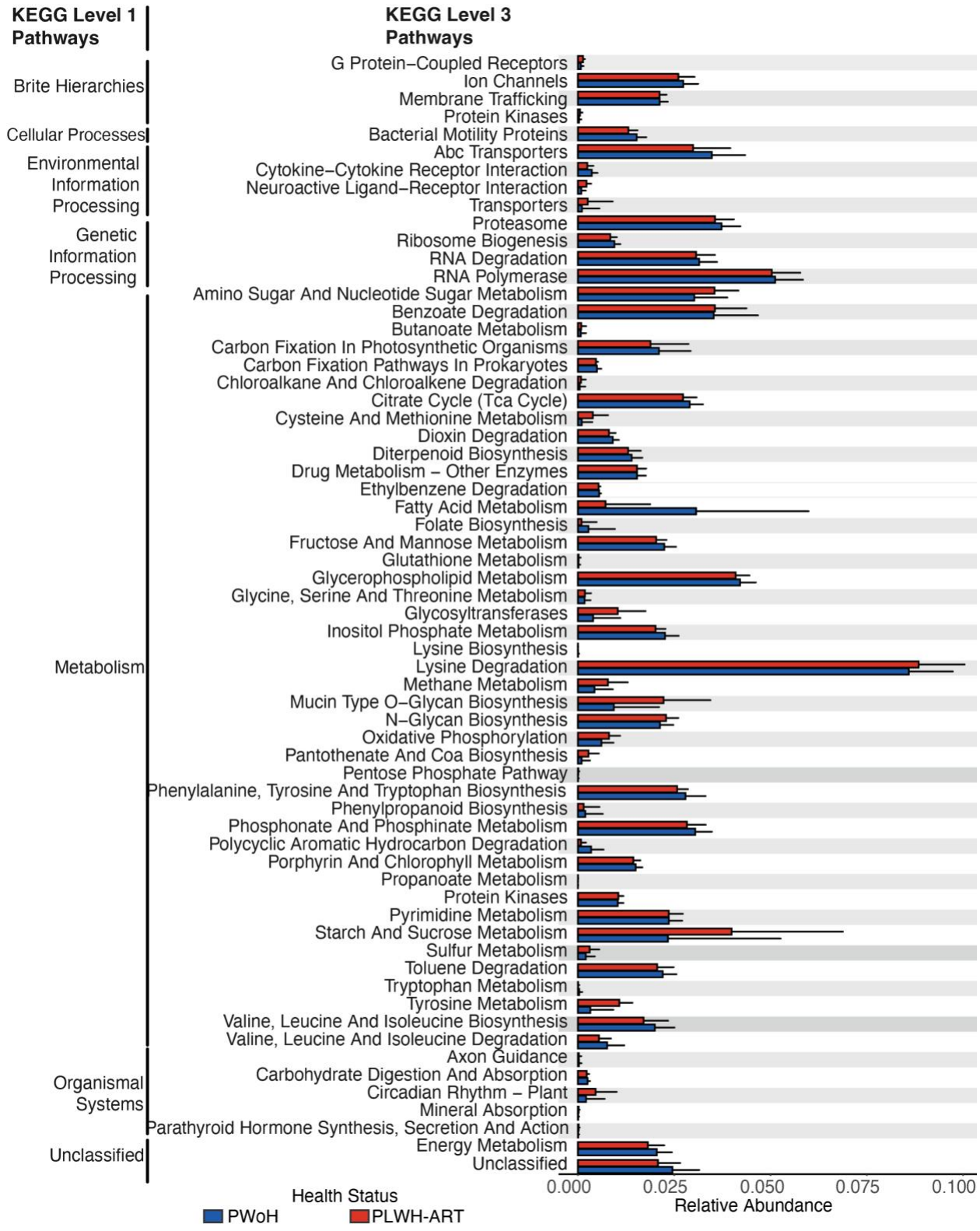


Fig 5. Predicted KEGG pathways differentially abundant between CSTs in the CQI-PMTCT cohort as determined using three differential abundance analysis methods (see **Methods)**.

334

335 Because there were only two samples that were assigned CST II, we will focus our
336 discussion on CST III and IV only. A combination of predicted CVMB genotypes suggests a
337 plausible mechanism for disruption of host homeostasis. Short-chain fatty acid (SFCA)
338 metabolism was more abundant in CST IV compared to CST III CVMBs, indicating greater
339 presence of bacteria capable of producing SFCAs. CST IV bacteria play a large role in SCFA
340 production, and the increased presence of SFCAs like propionate, acetate and butyrate
341 have been associated with cervicovaginal dysbiosis (81). Furthermore, the production of
342 SCFA leads to decreased *Lactobacillus* abundance and increased pH in the vagina (81–83).
343 Based on this, we can posit that there were high concentrations of cervicovaginal SFCAs in
344 this cohort. GPCRs were slightly, yet still significantly, more abundant in CST III compared
345 to CST IV CVMSs. GPCRs mediate the immunoregulatory effects of SFCAs (82) and
346 specifically, GPCRs 41, 43 and 109A, can inhibit histone deacetylases (HDACs) which
347 subsequently induce a cellular and humoral immune response (82,84). Simultaneously
348 increased SFCAs and decreased GPCRs may induce a local pro-inflammatory response in
349 this cohort. Given that inflammation in the cervicovaginal tract is associated with adverse
350 birth outcomes, like preterm birth in pregnant PLWH-ART (21), which may be linked to
351 increases in SFCA production and decreases in the immunoregulators in the CVMB.

352 Amino sugar and nucleotide sugar metabolism was another pathway that might be
353 implicated in birth outcomes in pregnant PLWH-ART. For bacteria, these are core
354 intermediates involved in peptidoglycan and lipopolysaccharide, among other
355 macromolecule, biosynthesis, energy metabolism, and other processes. In this cohort, this

356 pathway was more abundant in CST III compared to CST IV CVMBs. One study found that
357 amino sugar and nucleotide metabolism was negatively associated with mammalian target
358 of rapamycin (mTOR), a kinase that regulates cell growth and proliferation (85). In the same
359 study, mTOR was negatively correlated with *L. iners* and positively correlated with
360 polymicrobial, CST IV bacteria (85). While some of this association may be due to major
361 structural features of the CVMB, i.e., the presence of a lipopolysaccharide layer of some
362 members but not others, these metabolic products from the CVMB of pregnant PLWH-ART
363 may interact with the mTOR pathway, activating it (by CST III bacteria) or inhibiting it (by CST
364 IV bacteria). This interaction could maintain or disrupt cervicovaginal epithelial integrity,
365 potentially determining whether birth outcomes are adverse or normal through a
366 proinflammatory or anti-inflammatory response and either increasing or decreasing the risk
367 of STIs.

368 Lastly, polycyclic aromatic hydrocarbon (PAH) degradation was also differentially
369 more abundant in CST IV than in CST III CVMBs. PAHs, which are ubiquitous in the
370 environment can impact the CVMB (9) which can lead to adverse birth outcomes like
371 intrauterine growth restrictions and preterm birth (86,87). The polymicrobial state type, CST
372 IV, tends to have varying abundances of putative PAH-degrading bacterial genera including
373 *BVAB2* and *Megasphaera, Sphingomonas*, *Acinetobacter*, *Micrococcus*, *Pseudomonas*,
374 and *Ralstonia,* and two of the genera, *Megasphaera* and *Pseudomonas* are present in the
375 CQI-PMTCT cohort (88–90). Therefore, somewhat unexpectedly, a more diverse,
376 polymicrobial CST IV CVMB that may be better able to degrade PAHs might offer some
377 protection against their harmful effects. Perhaps, because the Sub-Saharan African region

378 is exposed to a substantial amount of PAHs (91,92), a CST IV CVMB might be advantageous
379 and help explain its prevalence in Sub Saharan African populations (17,48,93). However, a
380 multidisciplinary research study, across life and social sciences, would need to be
381 conducted to investigate this claim (94,95), which is an important next step. Overall, these
382 findings provide the direction for further studies to clarify these differences with more
383 granularity and the role of bacterial metabolic pathways on reproductive and birth outcomes
384 in pregnant PLWH-ART in vulnerable and underrepresented populations. Additionally, future
385 multi-omics studies should be conducted to progress the CST framework from taxonomy-
386 only based classification to microbial function and taxonomy-based classification.
387

388 Meta-analysis reveals predicted metabolic pathways were 389 differentially abundant between PLWH-ART and PWoH.

390 We used PICRUSt2 to predict 4,534 KOs which were assigned to 130 KEGG pathways.
391 Of those, 62 KEGG pathways were differentially abundant between pregnant PWoH and
392 PLWH-ART CVMBs (**Fig. 6**).
393

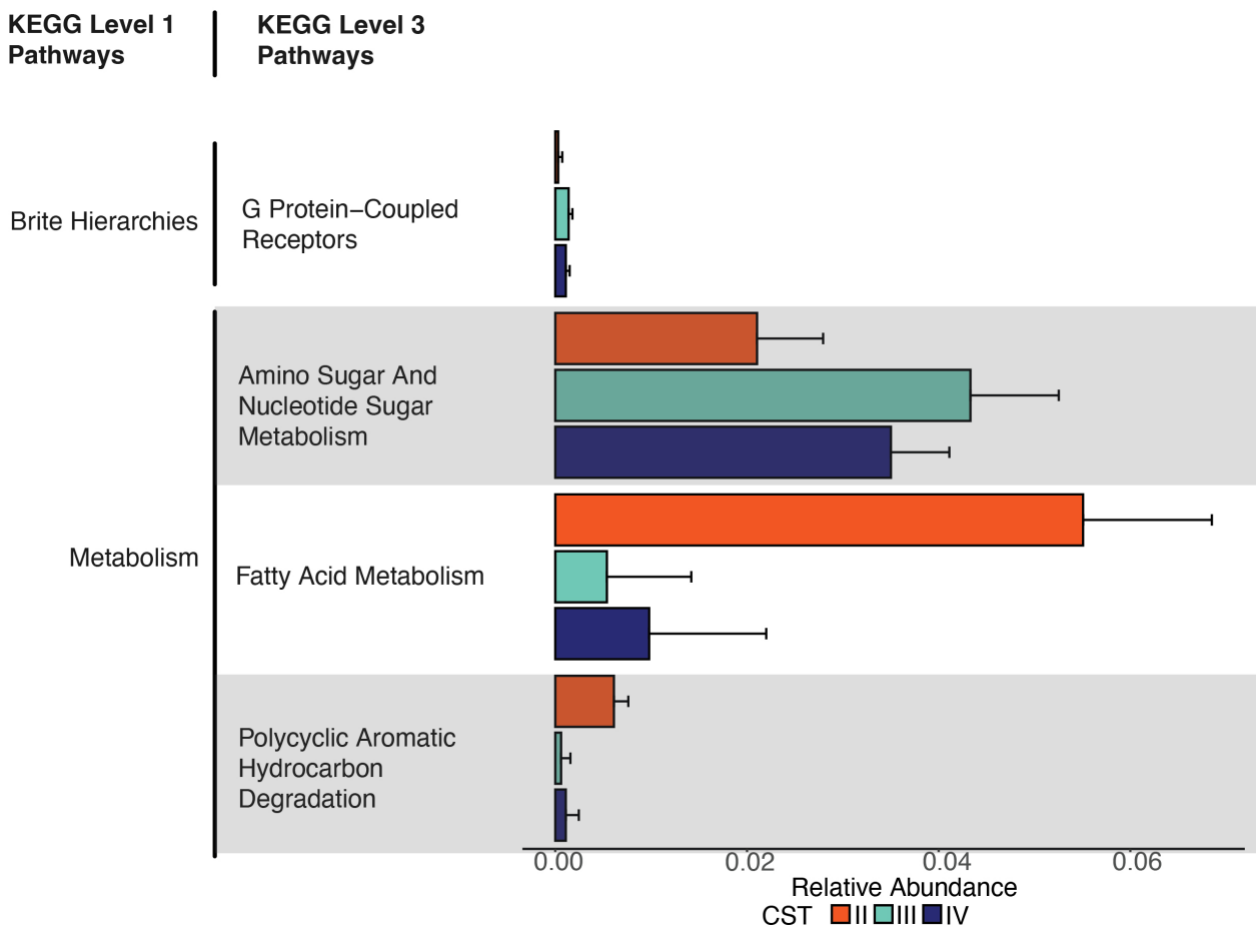


Fig 6. KEGG pathways differentially abundant between pregnant PLWH-ART and pregnant PWoH as determined using three differential abundance analysis methods (see **Methods)**.

Most of these pathways were primarily involved in metabolism. This could indicate that HIV-associated changes to the pregnant CVMB may alter microbial function, which could influence pregnancy and birth outcomes and susceptibility to other cervicovaginal infections in PLWH-ART. For this study, we will focus on only a few pathways that might have relevance with HIV, and pregnancy and birth outcomes. Lysine degradation (KO00310) was differentially more abundant in the PLWH-ART group compared to the PWoH group. Products of lysine degradation — cadaverine and pipecolate — were increased in

405 cervicovaginal fluids of women with BV (96). BV, indicative of CST IV, has been associated
406 with adverse birth outcomes in PLWH-ART (20,21,28) and increased risk of HIV acquisition
407 (17). Cysteine and methionine metabolism (KO00270) was differentially more abundant in
408 the PLWH-ART group compared to the PWoH group, and the opposite is true for folate
409 biosynthesis (KO00790). Levels of cysteine in maternal plasma or cervicovaginal fluid have
410 been associated with adverse pregnancy and birth outcomes (97–99). These findings
411 provide direction for further research to investigate how and if HIV-associated changes to
412 the CVMB function impact pregnancy and birth outcomes. One pathway, mucin O-glycan
413 synthesis (KO00512) was differentially more abundant in PLWH-ART and this has been
414 shown to increase the infectivity of sexually transmitted viral pathogens like HIV (69). Future
415 research should investigate how this pathway is involved in pregnancy and birth outcomes
416 in PLWH-ART.
417

418 CVMB characteristics are correlated with some plasma immune 419 factors in the CQI-PMTCT cohort

420 Next, we asked if there was an association between plasma immune factors and the
421 CVMB in the CQI-PMTCT cohort. The correlation and causative associations between CVMB
422 and cervicovaginal cytokines and chemokines have been well studied (17,24,67,100). Few
423 studies have identified the link between the systemic immune system and the CVMB in
424 PLWH-ART. To this end, we used linear models to identify the CVMB metrics (Shannon
425 diversity, CST and bacterial species abundance) that were associated with the systemic

426 immune system (see **Methods**). We hypothesized that CST IV bacteria or highly diverse
427 CVMBs would be associated with plasma immune factors that are linked to adverse birth
428 outcomes.

429 Shannon diversity was positively correlated with plasma MMP-2 (r = 0.214, p = 0.051)
430 and CXCL13 (r = 0.353, p = 0.005) (**Fig. 7A-B**). When looking at individual taxa, *L. iners* was
431 negatively correlated with sCD163 (r = -0.32, p =0.013 (**Fig. 7C**). In contrast, sTNFRSF1A
432 concentrations were significantly higher in individuals with the CST III CVMBs vs those with
433 CST IV CVMBs (**Fig. 7D**). However, in most cases, CST IV associated bacteria were positively
434 correlated with immune markers of inflammation (**Fig. S3**). *L. crispatus,* a bacterium
435 associated with low diversity in the CVMB, was negatively correlated with MMP-2 and
436 CXCL13 and other biomarkers of inflammation (**Fig. S3**). In contrast, *L. gasseri,* also in low
437 diverse CVMBs, was positively correlated with pro- and anti- inflammatory immune factors
438 (**Fig. S3)**.
439

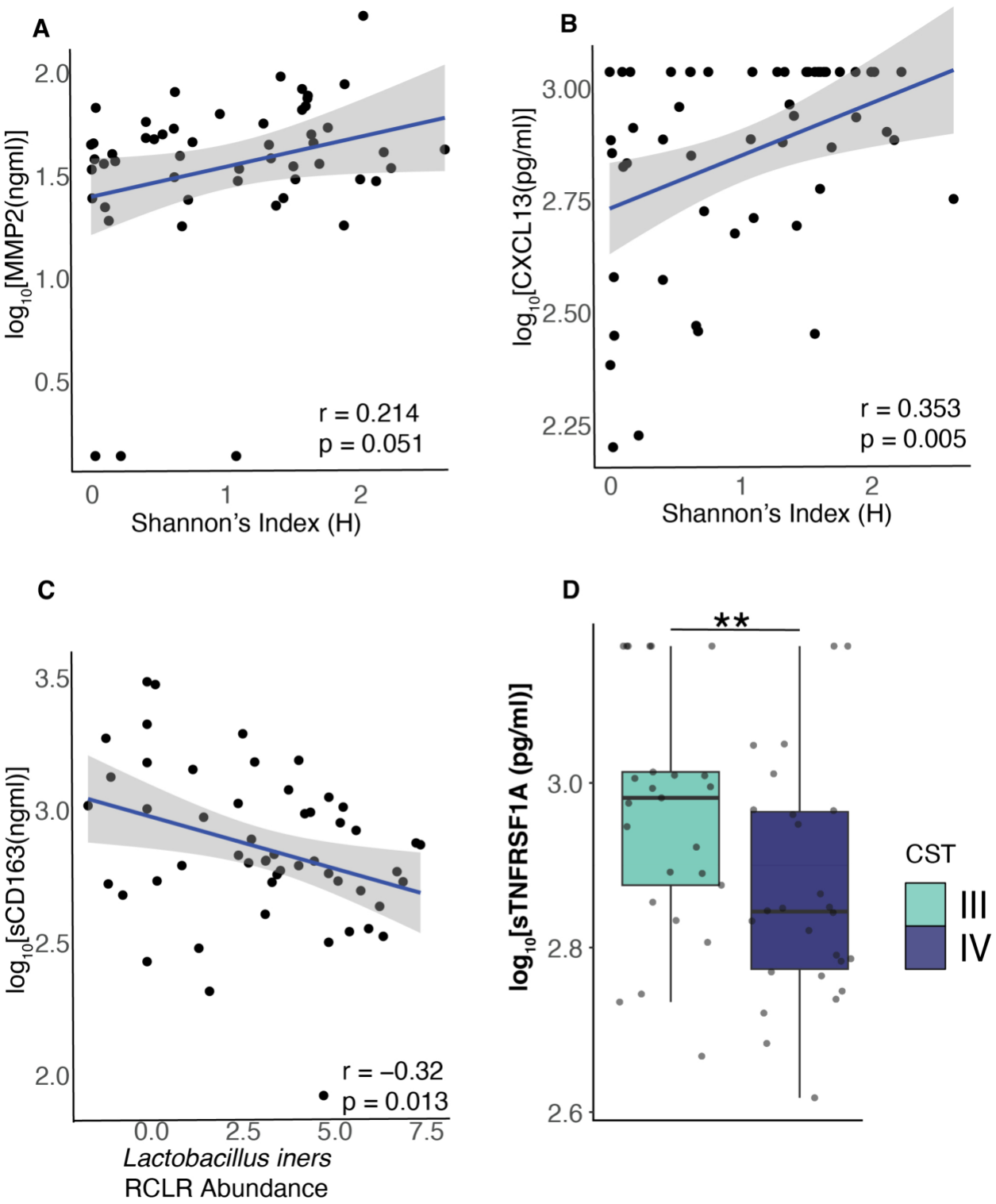


Fig 7. Correlations of CVMB metrics to plasma immune mediators A-B) Spearman correlation of MMP2 and CXCL13 to Shannon diversity **C)** sCD163 to *L. iners* abundance and

443 **D)** Levels of sTNFRSF1A between CST III and IV (Wilcoxon Test p = 0.0188). Only statistically
444 significant correlations (not corrected) are shown and only the top 3 abundant taxa are
445 shown. Linear regression lines of A-C are shown in blue, and the shaded regions represent
446 95% confidence intervals. Full abbreviations of immume factors are in **Table S3**
447

448 Genital inflammation, linked to cervicovaginal dysbiosis, has been associated with
449 adverse pregnancy and birth outcomes (21,101,102). Here, we investigate whether systemic
450 inflammation is also linked to the CVMB. MMP-2 and CXCL13 were positively correlated with
451 Shannon diversity. Abnormal concentrations and varying expression of MMP-2 during
452 pregnancy have been implicated in adverse pregnancy outcomes like preeclampsia
453 (103,104), recurrent spontaneous abortion (105), preterm birth (106) and gestational
454 trophoblastic disease (107) and pregnancy outcomes may lead to adverse infant outcomes
455 that impact infant mortality and development (108). The same is true for CXCL13 as it has
456 been associated with placental inflammation (109) and preterm birth (110). Similarly,
457 adverse pregnancy and birth outcomes, have been linked with highly diverse CVMBs,
458 particularly those with CST IV communities (8,14,21,28). Altogether, this suggests that there
459 is a complex interplay between the CVMB and systemic inflammation in PLWH-ART that
460 impacts pregnancy and birth outcomes.

461 *L. iners* was negatively correlated with sCD163, an immune factor that has also been
462 linked to preterm birth (111) and is a predictor of mortality in PLWH-ART (112,113) However,
463 one study showed that sCD163 concentrations varied during pregnancy in PLWH-ART,
464 declining in the first trimester and then remaining stable (114). All of this suggests that the

465 role cervicovaginal bacteria have in modulating the systemic immune system in pregnant
466 PLWH-ART — and therefore pregnancy and birth outcomes — is complex and may depend
467 on the host's immunological state. Indeed, the impact gut bacteria have on the systemic
468 immune system might greatly confound the effect of cervicovaginal bacteria on the systemic
469 immune system (115–117). Given that the local cervicovaginal immune profile is distinct
470 from the systemic immune profile, cervicovaginal bacteria may have very little effect on the
471 systemic immune profile (118). However, these associations between the plasma immune
472 factors and maternal CVMB may be important for understanding the biological mechanisms
473 underlying HIV-associated pregnancy and associated infant complications.
474

475 Limitations, future opportunities and conclusions

476 The power of our study is limited by the lack of a pregnant PWoH group for
477 comparison. This study was nested within a larger longitudinal study from the CQI-PMTCT
478 study that was evaluating the long-term effects of ART usage in pregnant and chestfeeding
479 people (119). As such, samples were only collected from pregnant PLWH-ART. Subsequent
480 studies will have samples from pregnant PLWH-ART and PWoH and will also include larger
481 sample sizes for better statistical inference. We did not present the impact of CVMB on birth
482 outcomes in the CQI-PMTCT cohort as there were not enough samples in each adverse birth
483 outcome to reach statistical significance.
484 Even acknowledging these limitations, our study revealed some important details about
485 the CVMB of PLWH-ART in DRC and globally. We showed that the CVMB of pregnant PLWH-
486 ART is largely CST III and IV both of which differ in their predicted functions. We found

487 associations between the systemic immune system and the CVMB, providing direction for
488 more studies to investigate this association as it might have implications on maternal and
489 infant health. In a meta-analysis, we found that the HIV-associated CVMB and predicted
490 function in pregnant PLWH-ART is distinct from PWoH, contrary to previous studies.
491 Subsequent studies with larger cohorts in the same population in Kinshasa, DRC, will utilize
492 shotgun metagenomic sequencing for genome-resolved analyses and better functional
493 inference of bacterial and viral populations and their association with adverse birth
494 outcomes in PLWH-ART. This will allow us to infer any strain level differences in the CVMB
495 that may cause different host phenotypes, therefore ecosystem roles that are not detected
496 with 16S rRNA gene sequencing. The CST framework would benefit to move on from
497 taxonomy only based classification to microbial function and taxonomy-based
498 classification that will give a better insight on the role of the CVMB on health and disease.

499 Materials and methods (1590words)

500 Ethics approval and consent to participate

501 The Ohio State Institutional Review Board (number 2015H0440), Albert Einstein
502 College of Medicine Institutional Review Board (number 2020-12018), and the University of
503 Kinshasa School of Public Health Ethical Committee, and the University of Kinshasa School
504 of Public Health Institutional Ethical Committee (ESP/CE/046/2018) granted approval for
505 conducting this study and for protocol related specimen testing. All research was
506 conducted in accordance with relevant guideline and regulations. Participants in the CQI-
507 PMTCT study provided signed consent before undergoing study procedures

508

509 ## Study cohort and sample collection

510 Participants in this analysis were enrolled in Kinshasa, Democratic Republic of
511 Congo. This was a pilot study nested within the larger in the Continuous Quality Intervention:
512 Preventing Mother to child transmission (CQI-PMTCT) study, a longitudinal study evaluation
513 the long term outcomes of ART on pregnant people (NCT03048669) (120). From October
514 2020 to May 2021, we collected cervicovaginal swabs and peripheral blood from 82
515 participants at enrolment in their second or third trimesters who could access medical
516 facilities during the SARS-CoV-2 pandemic. The 82 participants represent 40% of total
517 participants who consented to provide cervicovaginal swabs. Clinicians collected
518 cervicovaginal swabs and stored them in Puritan DNA/RNA shield (ZymoResearch, R1108)
519 and transported to the central laboratory at Protestant University of Congo to be stored at -
520 80C until shipped to Columbus, OH on dry ice for processing. Plasma collected from the
521 peripheral blood was processed immediately and stored at -80C until shipment to
522 Columbus, OH for processing (**Fig. 1A**).

523 Demographic data including participant age, gestational age, ART regimen, gravidity,
524 socioeconomic status, depression scores were collected at enrolment. The first principal
525 component analysis (PCA) of the factors indicative of the participants socioeconomic status
526 (SES) was used to categorize the SES status to quartiles with 0 as the lowest and 3 as the
527 highest (121). Adverse birth outcomes including preterm birth, stillbirth and low birth weight
528 were collected at delivery.

529

530 ## Study selection, search strategy and criteria for meta-analysis

531 16S rRNA gene CVMB studies were selected by conducting a thorough and manually
532 curated search of the Web of Science Core Collection of Thomson Reuters (WOS) and the
533 European Nucleotide Archive (ENA) for studies published until December 2022. The search
534 terms, hits and justification for exclusion or inclusion are shown in **Fig. S1** and information
535 about the selected studies are in **Table S1**. The inclusion criteria were as follows: (i)
536 pregnant healthy or living with HIV (iii) availability of raw data and (iii) Illumina paired-end
537 short read amplicon sequencing of the V3-V4 hypervariable region. Only studies with
538 sufficient information for comparison were included (i.e., metadata and minimally
539 reproducible 16S rRNA gene sequence processing pipeline). Longitudinal studies were
540 excluded from this analysis. Metadata from each study was obtained from NCBI, and/or via
541 correspondence with the authors of the selected studies. Among these studies, three were
542 from Europe –one from Italy (122) and two from Finland (123,124), one from the United
543 States (125), and one from Uganda (126). Only the Ugandan study included samples from
544 PLWH-ART (n = 5). In total, including this study, 1861 samples were analyzed. More details
545 on the studies can be found in **Table S1.**

546

547 ## DNA extraction, 16S rRNA gene sequencing and processing

548 Bacterial DNA from cervicovaginal swabs in the CQI-PMTCT cohort were extracted
549 using the Qiagen AllPrep DNA/RNA Mini kit (Qiagen, 80204) following the manufacturer's
550 manual. These samples were low microbial biomass, hence, to reduce contamination,

551 samples were extracted in a clean room and all materials were treated with 70% ethanol,
552 RNAse and UV light for ~15minutes before DNA extraction. Additionally, reagent-only
553 controls were extracted in parallel with each DNA extraction batch. The PCR products of the
554 reagent-only controls were examined on 1.2% gels, and no bands were detected.

555 Samples were sent to SeqCenter (https://www.seqcenter.com/) for amplification of
556 the V3-V4 hypervariable region of the 16S rRNA gene using the 341F (5'-
557 CCTACGGGDGGCWGCAG-3') and 806R (5'-GACTACNVGGGTMTCTAATCC-3') primers
558 using the Zymo Research's Quick-16S kit. The V3-V4 region was chosen as it has been shown
559 to identify the most taxa in cervicovaginal samples (127). The PCR products were sequenced
560 on an Illumina NextSeq2000 to generate 2x301bp paired-end reads.

561 16S rRNA gene reads were processed following the DADA2 SOP (128). Sequence
562 quality was assessed using FASTQC v0.11.5 and any adapters or primer sequences were
563 trimmed using Trimmomatic v0.36 (129,130). Generally, sequences were trimmed at the
564 first base with a quality score of Q<25. Forward and reverse reads were merged using the
565 mergePairs function with default parameters (minOverlap = 12, maxMismatch = 0), and
566 chimeras removed using removeBimeraDenovo with default parameters. Taxonomy was
567 assigned using the Greengenes2 database (131). Sequences were filtered to remove
568 sequences of mitochondria and chloroplast, samples with less that 100 reads and taxa with
569 less than 10 reads, less than 0.5% abundance across all samples and not present more than
570 2 times in 10% of samples were removed. The resultant ASV table was agglomerated to
571 species-level and robust centered log-ratio (RCLR) transformation was performed on the
572 table using the decostand function on the vegan package v2.6-6. This transformation was to

573 mitigate the inherent negative correlation bias in compositional data (132,133) The
574 transformed table was used in all subsequent analyses.

575 For the five (5) selected studies for the meta-analysis, sequences were processed on
576 a per-study basis as described above. Reads were filtered based on read quality for each
577 study. Per study processing parameters are shown in **Table S2.** Generated ASV tables per
578 study were combined and taxonomy was assigned on the combined table (34,871 ASVs and
579 1861 samples). The aggregated table was further filtered by removing ASVs not assigned to
580 any taxonomic level. To reduce data complexity (134), bacterial ASVs were filtered if
581 observed at frequencies of 0.0005% study-wide and observed in only 2 samples. Samples
582 with fewer than 2000 reads or with excess zeroes were removed using the PreFL function
583 from the PLSDAbatch package v1.0 (135). After filtering, ASVs were agglomerated at the
584 species level resulting in an abundance table with 74 species-level taxa and 664 samples. A
585 centered log-ratio (CLR) transformation was performed on the abundance table. Before the
586 CLR transformation, zeros were replaced with a constant value smaller than the detection
587 limit (e.g., 65% of the detection limit) (136,137).

588 PICRUSt2 version v2.4.2 (79) was used to predict functional composition from the
589 16S rRNA gene sequences. Data were normalized based on the abundance of 16S rRNA
590 gene copy numbers. Metagenome predictions were used to estimate KEGG pathways and
591 any unassigned KEGG IDs were mapped using KEGGREST v3.19 (138).

592

593 ## Quantification of plasma cytokine, chemokine and soluble
594 ## factors (immune factors)

595 Levels of cytokines, chemokines and soluble factors were measured from maternal
596 plasma were quantified using the LEGENDplex COVID-19 Cytokine Storm Panel 1 & 2
597 (BioLegend, 741095), LEGENDplex Human Proinflammatory Chemokine Panel 1
598 (BioLegend, 740984) and LEGENDplex Vascular Inflammation panel 1 (BioLegend, 740551).
599 Additional details related to measurement of immune factors are described in
600 Supplemental Methods.

601 ## Statistical analyses

602 For the CQI-PMTCT study, statistical analyses were as follows. Community state
603 types (CST) were assigned to each sample using VALENCIA, which assigns CSTs to each
604 samples based on their similarity to a reference centroid (139). Alpha diversity indices
605 (Chao1, Simpson, and Shannon) were estimated using an asymptotic statistical approach
606 (140,141). Differences in alpha diversity were tested using the Kruskal-Wallis test, followed
607 by Wilcoxon pairwise comparison tests if significant.

608 Beta diversity analyses were computed using principal component analysis (PCA) on
609 Robust Aitchison distances. Significance of PCAs used ANOSIM from the vegan package
610 v2.6-10. To identify associations between CVMB metrics (alpha diversity and bacterial
611 abundance) and the log transformed plasma cytokine, chemokine and soluble factors
612 concentrations, linear regression models were computed with the with Spearman's rank

correlation. Adjustments for multiple hypothesis testing were not performed due to this being an exploratory study unless as a sensitivity analysis for our main findings (142)

For the meta-analysis, data were analyzed as following. CSTs were assigned as described above using VALENCIA. Batch effects were accounted for using Partial Least Square Discriminant Analysis (PLSDA-batch) after evaluation of several batch removal methods (see Supplemental Methods for more details). Alpha diversity indices and beta diversity on Aitchison distances were computed as above.

To identify differentially abundant species and predicted functions, three statistical methods were used: LinDA from the MicrobiomeStat package v.1.1 (143,144) , ANCOM-BC v.2.2.1(145) and ALDEx2 v.1.32.0 (146) with the formula "abundance ~ health_status + study". A species was considered differentially abundant if it met the following criteria: p-adjusted value < 0.05 in LinDA; q-value < 0.05 in ANCOM-BC; or effect size > 0.5 in ALDEx2. Additional details on differential abundance analysis are described in Supplemental Methods. The STORMS checklist (147) (**Table S5**) is included for standardization and reproducibility of data.

Data availability

The datasets supporting the conclusions of this article are available in the Sequence Read Archive (SRA) under BioProject PRJNA1234224 and scripts and data used to analyses all the data are available on https://github.com/SulliVagOSU/16S_meta_analysis.

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644

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