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## Genital microbiota of women using a 90 day tenofovir or tenofovir and levonorgestrel intravaginal ring in a placebo controlled randomized safety trial in Kenya

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In a phase-IIa trial, we investigated the influence of 90 days continuous-delivery tenofovir (TFV) intravaginal rings (IVRs) with/without levonorgestrel (LNG) on the genital microbiota of Kenyan women. Eligible women (n = 27; 18–34 years; negative for HIV, sexually transmitted infections, and Amsel-bacterial vaginosis) were randomized 2:2:1 to use of IVRs containing TFV, TFV/LNG, or placebo. Using vaginal wall and IVR swabs at IVR insertion and removal, the genital microbial composition was determined using 16S rRNA gene sequencing. The presence of *Candida* spp. was determined using qPCR. The vaginal total bacterial burden appeared to decrease with TFV and TFV/LNG IVR use ( $\log_{10}0.57$  and  $\log_{10}0.27$  decrease respectively;  $p > 0.05$ ). The TFV/LNG IVR was more 'stabilizing': 50% of the participants' microbiota community state types remained unchanged and 50% shifted towards higher *Lactobacillus* abundance. Specifically, TFV/LNG IVR use was accompanied by increased abundances of *Lactobacillus gasseri/hominis/johnsonii/taiwanensis* (16.3-fold) and *L. fermentum/reuteri/vaginalis* (7.0-fold; all  $p < 0.01$ ). A significant shift in the overall microbial  $\alpha$ -diversity or  $\beta$ -diversity was not observed for either IVR, and IVR use did not influence *Candida* spp. prevalence. TFV/LNG and TFV IVRs did not adversely affect the genital microbiota and are safe to use. Our findings support further studies assessing their efficacy in preventing HIV/HSV-2 and unintended pregnancies.

With global estimates of 1.7 million incident HIV infections<sup>1</sup> and 121 million unintended pregnancies annually<sup>2</sup>, development of a multipurpose, user-controlled product that prevents heterosexual transmission of HIV and provides contraception for women is invaluable. Daily oral emtricitabine (FTC)/tenofovir disoproxil fumarate (TDF) has revolutionized HIV prevention as the first pre-exposure prophylaxis (PrEP) option. However, a high level of adherence with the dosing regimen is essential for protection and is often difficult to achieve<sup>3,4</sup>. Long-acting contraception options not requiring daily adherence have been pivotal in the prevention of unintended pregnancies. Innovative multipurpose technologies (MPT) could be developed by leveraging current long-acting contraceptive strategies with HIV PrEP co-delivery or co-formulation to improve protective coverage.

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	TFV + LNG n = 11 Median (IQR) or N (%)	TFV n = 11 Median (IQR) or N (%)	Placebo n = 5 Median (IQR) or N (%)
Age	21 [20–24]	25 [22–32]	23 [21–23]
BMI	20.2 [18.4–24.9]	22.9 [21.1–26.2]	23.7 [21.2–24.3]
Menstrual cycle length	31 [26.5–35.0]	28 [26.0–34.0]	31 [30.0–34.5]
<b>Contraceptive method at enrollment</b>			
None	2 (18.2%)	2 (18.2%)	1 (20.0%)
Condoms	8 (72.7%)	8 (72.7%)	4 (80%)
Cu-IUD	1 (9.1%)	1 (9.1%)	0 (0.0%)
<b>Nugent score at IVR insertion</b>			
0–3 (Negative)	7 (63.6%)	9 (81.2%)	3 (60.0%)
4–6 (Intermediate)	3 (27.3%)	1 (9.1%)	0 (0.0%)
7–10 (Positive)	1 (9.1%)	1 (9.1%)	2 (40.0%)
<b>Nugent score at IVR removal</b>			
0–3 (Negative)	6 (54.5%)	3 (27.3%)	2 (40.0%)
4–6 (Intermediate)	1 (9.1%)	3 (27.3%)	1 (20.0%)
7–10 (Positive)	4 (36.4%)	5 (45.4%)	2 (40.0%)
Vaginal pH at baseline	5.2 [4.6–5.5]	4.9 [4.6–5.2]	4.9 [4.9–4.9]
Vaginal pH at ring removal	5.2 [4.9–5.5]	5.2 [4.2–5.5]	4.6 [4.4–5.5]
Time on IVR, days	46 [21–89]	90 [40–91]	68 [67–91]

**Table 1.** Characteristics of women randomized to use 90 days intravaginal rings (IVR) with continuous delivery of tenofovir and levonorgestrel (TFV/LNG), tenofovir (TFV), or placebo, Kisumu Kenya, 2019. *IQR* interquartile range, *BMI* Body mass index, *Cu-IUD* Copper intrauterine device.

Intravaginal rings (IVRs) are a safe, discreet and effective option for simultaneous delivery of contraception and antiretrovirals<sup>5</sup>. An IVR with dapivirine, which was found to reduce HIV incidence by 27% in two efficacy trials<sup>6,7</sup>, with up to 75–91% reduction among women with higher adherence to the IVR<sup>8</sup>, received a positive opinion from the European Medicines Agency and the World Health Organization<sup>9,10</sup>. IVRs also allow for consistent contraceptive hormone release, eliminating the high peak experienced with injectables<sup>11</sup> or the daily fluctuations from oral contraceptives<sup>12</sup>.

Given their localized effects, the influence of IVRs on the genital microbiota is an important consideration. Microbiota shifts could influence genital health and ability of the female genital tract (FGT) to protect against infections including sexually transmitted infections (STIs). In one study of coitally-dosed 1% tenofovir (TFV) vaginal gel (a product that has not moved forward in development)<sup>13</sup>, efficacy was higher in the presence of a *Lactobacillus*-dominant microbiota but decreased in the presence of higher mucosal inflammation<sup>14,15</sup>.

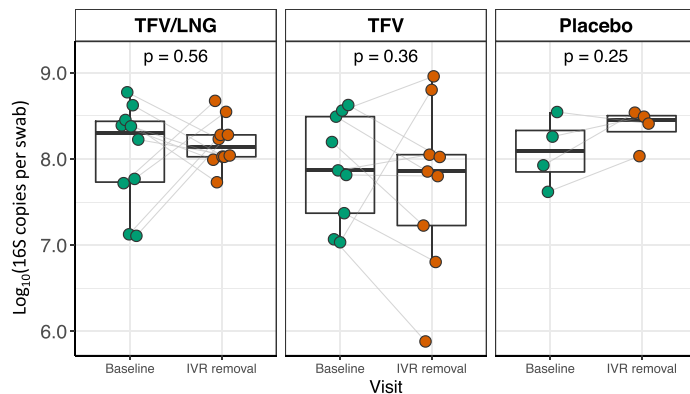
The CONRAD co-formulated TFV/levonorgestrel (LNG) and single agent TFV IVRs have been tested for safety in a phase-I trial among women from the United States (US) and the Dominican Republic<sup>16</sup>. However, the effect of these IVRs on the genital microbiota of sub-Saharan African Black women has not been evaluated. Women in this region face a disproportionate burden of HIV and their genital microbiota has been shown to be markedly different to women with different lived experiences and ethnicities. As part of the primary goals for this phase-IIa safety trial of 90 days use TFV and TFV/LNG IVRs, we determined the effect of each IVR relative to the placebo IVR on the genital microbiota of African women. In addition, we assessed the interplay between IVR use and genital microbiota on local TFV levels and the incidence of candidiasis.

## Results

**Participant characteristics.** Twenty-six of the twenty-seven randomized women had bacterial absolute abundance data available: TFV/LNG IVR (n = 11), TFV IVR (n = 10), and placebo IVR (n = 5). The median age was 22 years [interquartile range (IQR) 21–26], median body mass index was 22.4 [IQR 19.8–24.9], and demographic characteristics were similar across study arms (Table 1). Most women used male condoms during the study (74.1%), 7.4% previously used a copper-intrauterine device ( $\geq 6$  months before enrolling in the study), and 18.5% used no contraceptive method. Despite being asymptomatic and BV-negative by Amsel criteria at screening, 4/27 (14.8%) women were Nugent-BV-positive at the time of IVR insertion and 11/27 (41.0%) at the IVR removal visit. With the exception of an estimated increase in the prevalence of BV-positive Nugent score, differences in clinical characteristics at IVR removal were small.

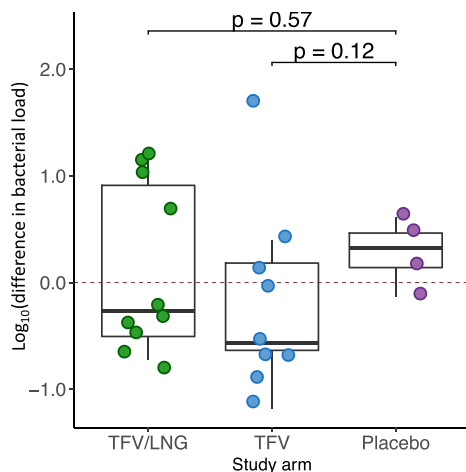
**Total bacterial load did not change with IVR use.** The total FGT 16S bacterial load did not change between the baseline and IVR removal visits for women in any arm (Fig. 1A; TFV/LNG:  $\log_{10}8.29$  vs  $\log_{10}8.14$ ,  $p = 0.56$ ; TFV:  $\log_{10}7.84$  vs  $\log_{10}7.94$ ,  $p = 0.36$ ; placebo:  $\log_{10}8.14$  vs  $\log_{10}8.45$ ,  $p = 0.25$ ) and the difference in the degree of change across arms relative to the placebo arm was small (Fig. 1B). At IVR removal, no participant-level differences were observed in any arms between the lateral vaginal wall bacterial load and that of the IVR surface (Fig. S1; TFV/LNG:  $\log_{10}8.14$  vs  $\log_{10}8.56$ ,  $p = 0.11$ ; TFV:  $\log_{10}7.94$  vs  $\log_{10}8.22$ ,  $p = 0.43$ ; placebo:

### A. Bacterial load in vaginal wall at baseline and IVR removal visits

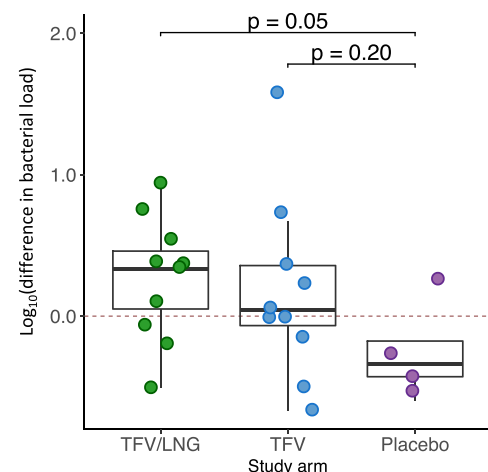


IVR	IVR insertion Median [IQR]	IVR removal Median [IQR]	p value
TFV/LNG	8.29 [7.72-8.45]	8.14 [8.03-8.28]	0.56
TFV	7.84 [7.07-8.49]	7.94 [7.23-8.59]	0.36
Placebo	8.14 [7.93-8.26]	8.45 [8.22-8.51]	0.25

### B. Difference in bacterial load in vaginal wall at baseline vs IVR removal



### C. Difference in bacterial load in vaginal wall vs IVR surface at IVR removal

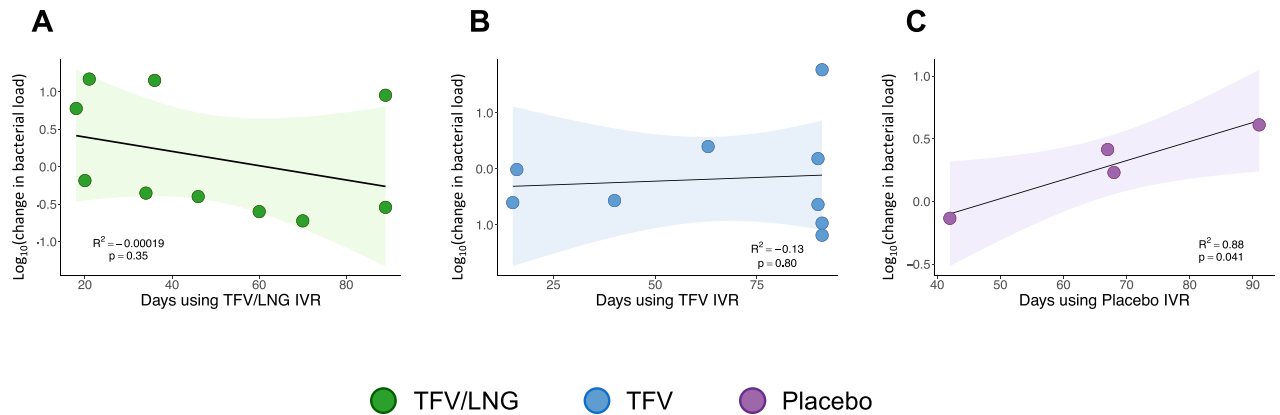


● TFV/LNG ● TFV ● Placebo

**Figure 1.** Change in bacterial load between intravaginal ring (IVR) insertion and removal among women randomized to use tenofovir/levonorgestrel (TFV/LNG), tenofovir (TFV), and placebo IVRs, Kisumu, Kenya, 2019. Comparing (A) vaginal  $\log_{10}$  total 16S copies per swab between the two visits, (B) the degree of change in vaginal wall  $\log_{10}$  total bacterial 16S copies from baseline to the IVR removal visit among women and (C) difference in total  $\log_{10}$  16S copies between the vaginal wall and IVR surface ( $\log_{10}$  bacterial load of vaginal wall  $-\log_{10}$  bacterial load of IVR) at the time of IVR removal. Values above and below the dotted red line indicate an increase and decrease in bacterial loads respectively. p values were determined using the Wilcoxon Signed Rank test (A) and Mann–Whitney U test (B,C).

$\log_{10}8.45$  vs  $\log_{10}8.18$ ,  $p=0.25$ ). Compared to the placebo arm, a higher estimated bacterial load was found on the TFV/LNG IVR surface (median difference of  $\log_{10}0.33$ ,  $p=0.05$ ) than on the TFV IVR (median difference of  $\log_{10}0.04$ ,  $p=0.20$  vs placebo median difference of  $\log_{10}-0.34$ , Fig. 1C) relative to the vaginal wall bacterial load. Overall, genital bacterial load was positively correlated with the total abundance of the *Lactobacillus* genus, with a  $\log_{10}0.95$  increase in total bacterial load with every  $\log_{10}$  increase in *Lactobacillus* abundance (Linear regression  $p=0.03$  after adjusting for multiple comparisons).

**Change in bacterial load was not dependent on length of IVR use.** There was no association between the vaginal bacterial load and the number of days a woman used the TFV/LNG IVR (Slope =  $-0.009$ ; 95% CI  $[-0.032, 0.012]$ ;  $p=0.34$ ; adjusted coefficient of determination ( $R^2$ ) =  $-0.00019$ ) or the TFV IVR (Slope =  $0.003$ ; 95% CI  $[-0.031, 0.012]$ ;  $p=0.80$ ; adjusted  $R^2$  =  $-0.13$ ). Women using the placebo IVR had a greater estimated increase in bacterial load with longer IVR use (Fig. 2;  $R^2=0.88$ ) although this estimate is highly



**Figure 2.** Impact of length of tenofovir/levonorgestrel (TFV/LNG), tenofovir (TFV), or placebo intravaginal ring (IVR) use on total bacterial load, Kisumu, Kenya, 2019. Linear regressions showing the association between number of days of IVR use and change in bacterial load between visits. The coloured shading represents the 95% confidence interval around the slope.

imprecise since only 4 women were in this arm. Similarly, the correlation between IVR surface bacterial load and length of time of IVR use was low for either arm (Fig. S2).

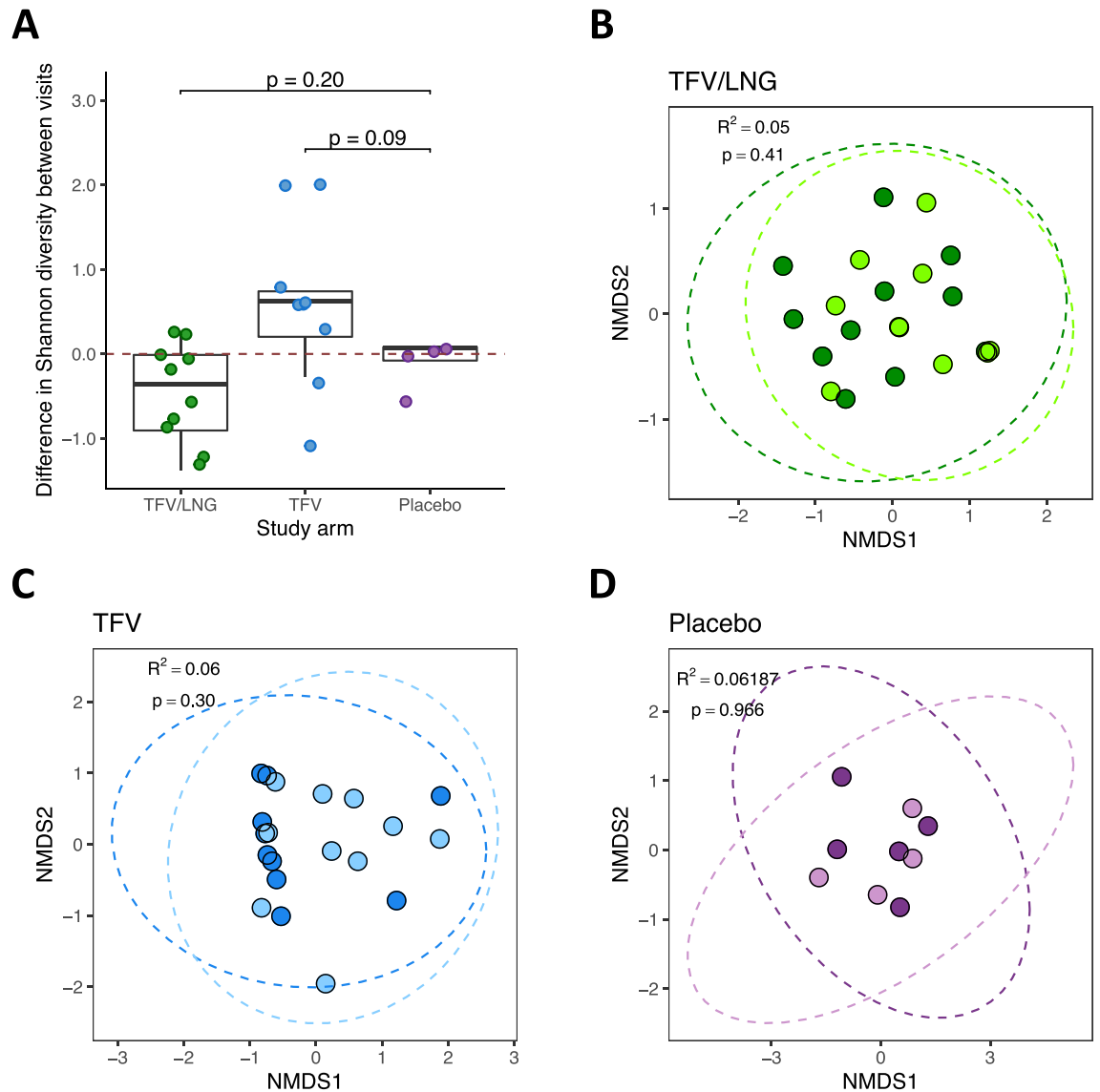
**The overall microbiota diversity did not change with IVR use.** To measure the effect of IVR use on the genital microbial composition of women, bacterial diversity was assessed using the Shannon diversity index and Bray–Curtis distances. We did not find a large change in overall microbiota diversity between baseline and IVR removal with use of any IVR (Fig. 3A–D). Microbial diversity decreased in women using the TFV/LNG IVR and increased with the TFV IVR (Shannon index difference of  $-0.36$  and  $0.62$  respectively; Fig. 3A); however both changes were not statistically significant. We also found no difference in the microbial diversity of the IVR microbiota compared to the vaginal microbiota at the IVR removal visit (Fig. S3; PERMANOVA TFV/LNG  $R^2 = 0.058$ ,  $p = 0.32$ ; TFV  $R^2 = 0.011$ ,  $p > 0.99$ ; placebo  $R^2 = 0.11$ ,  $p = 0.57$ ).

**TFV/LNG IVR use was associated with a shift towards a less diverse CST.** Based on hierarchical clustering of their dominant vaginal microbiota taxa, women were categorized as having community state type (CST) I if they had a genital microbiota dominated by *L. crispatus*, or CST III if their microbiota was dominated by *L. iners*. CST IVA had a higher abundance of the BV-associated bacterium *G. vaginalis* and CST IVB was composed of a broader range of BV-associated bacteria (Fig. S4). We found some discordance between Nugent-BV status and CST IV categorization. At baseline, of the 11/26 (42.3%) of women with a microbiota typically associated with microbial dysbiosis (CST IV A/B), 3/11 were Nugent-BV-positive, 3/11 had an intermediate Nugent score and 5/11 were Nugent-BV-negative. Further one participant with Nugent-BV was categorized as having a CST III microbiota.

During follow up, 50.0% (5/10) of women using the TFV/LNG IVR transitioned to a CST less associated with BV (2/5 transitioned from CST IVB to CST IVA) or a higher abundance of *Lactobacillus* species (1/5 transitioned from CST IVB to CST III, 1/5 from CST IVA to CST I, and 1/5 from CST III to CST I) while there were no CST shifts in the other 50.0% of women (Fig. 4A, Table S1). Among women using the TFV IVR, 22.2% (2/9) of women did not experience CST shifts, while the majority transitioned to a more diverse (44.4%; 4/9) or a less diverse CST (33.0%; 3/9). With the placebo IVR, 75.0% of women (3/4) did not transition to a different CST and one participant transitioned to a more diverse CST (CST IVA to CST IVB).

Overall, 41.6% (10/24) women had differences in assigned CST between the vaginal wall and the IVR surface at IVR removal (Fig. 4B), with 6/10 having a more diverse and 4/10 having a less diverse IVR CST, although none of these broad transitions were statistically significant (TFV/LNG  $p = 0.58$ ; TFV  $p = 0.57$ ; placebo  $p = 0.85$ ).

**Neither TFV/LNG nor TFV IVR use was associated with major increases in pathogenic bacterial taxa.** Longitudinal fold-changes in relative abundances of each individual bacterial taxa measured was determined for each study arm, accounting for individual variations in time between IVR insertion and removal. Using a threshold cutoff of  $\log_2 0.05$ , the placebo IVR was associated with the most fluctuations, with a significant fold-change in 23 bacterial taxa (Fig. 5, Table S2): including decreases in *Finnegoldia* spp. and *Dialister* spp. ( $\sim \log_2$  twofold), and increases in numerous taxa such as a  $> \log_2$  twofold increase in *Proteobacteria* phylum, *Atopobium vaginae*, *Corynebacterium coyleae/mucifaciens*, *Corynebacterium genitalium*, *Prevotella buccalis*, and *Porphyromonadaceae* as well as a  $\log_2 9.17$ -fold (standard error (SE) = 2.5) increase in *L. crispatus/acidophilus*. Women randomized to the TFV/LNG and TFV IVRs appeared to experience a more ‘stabilizing’ effect, with less change in vaginal bacterial taxa. Women using the TFV IVR were observed to have an average 5.42-fold (SE = 1.63) increase in *Dialister micraerophilus* abundance, a BV-associated bacterium. Women using the TFV/LNG IVR were observed to have a 3.79-fold (SE = 1.55) decrease in the pathogenic bacterium *Streptococcus*



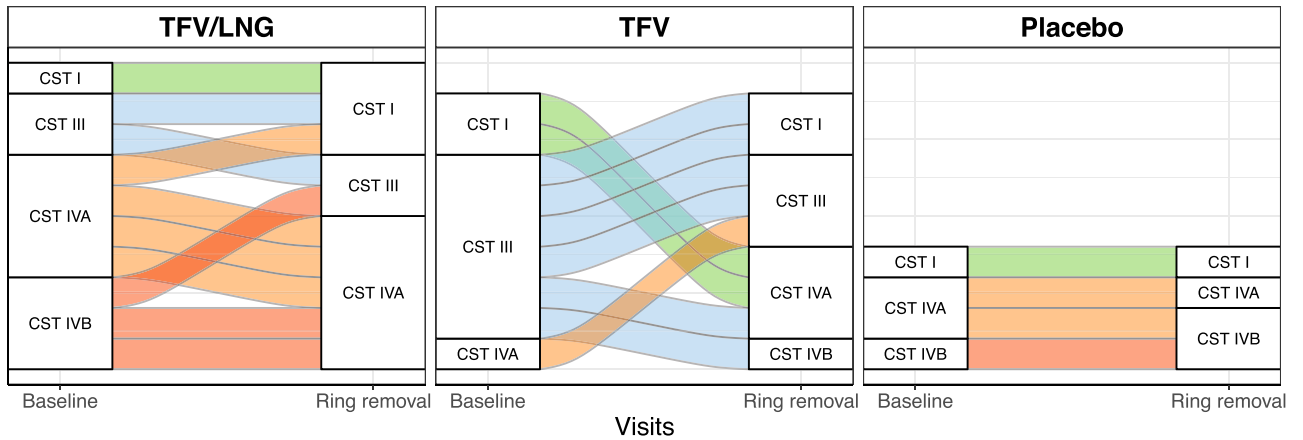
**Figure 3.** No change in overall microbiota diversity with use of tenofovir/levonorgestrel (TFV/LNG), tenofovir (TFV), or placebo intravaginal ring (IVR), Kisumu, Kenya, 2019. **(A)** Comparing the change in Shannon diversity (within-participant diversity) with each IVR. The dotted red line at zero indicates no change over time. **(B–D)** Principal component analysis plots showing the overlap between samples at baseline and the IVR removal visit for the TFV/LNG, TFV and Placebo IVRs, based on Bray–Curtis distances (between-participant diversity).  $p$  values were determined using the **(A)** Mann–Whitney U test or the **(B–D)** Adonis/PERMANOVA test based on permutations of distance matrices. *NMDS* non-metric multidimensional scaling.

*anginosus/milleri/sanguinis* and a 16.3-fold (SE = 2.09) and 7.0-fold (SE = 2.13) increases in *L. fermentum/reuteri/vaginalis* and *L. gasseri* respectively (all  $p \leq 0.01$ ; Fig. 5).

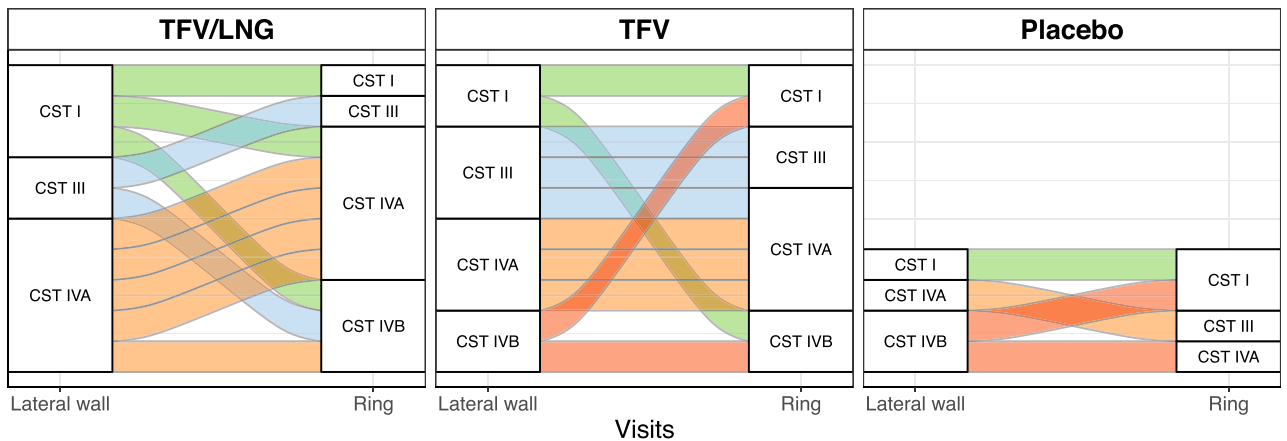
**Candidiasis was similar across IVR types.** The prevalence of *Candida* spp. were also measured at both visits. No *C. parapsilosis* was detected in any sample. There was an overall 30.8% prevalence of *Candida* at baseline and 44% at the IVR removal visit ( $p = 0.54$ ; Table 2). *C. krusei* (23%) and *C. albicans* (17%) were more common (Table 2, Fig. 6). The distribution of each *Candida* spp. did not differ across study arms at baseline (*C. albicans*  $p > 0.99$ ; *C. glabrata*  $p = 0.57$ ; *C. krusei*  $p > 0.99$ ) or at IVR removal (*C. albicans*  $p > 0.99$ ; *C. krusei*  $p = 0.64$ ). *C. glabrata* was not detected at the IVR removal visit. There were no differences in the overall microbial diversity (PERMANOVA  $p = 0.07$ ) or differences in abundances of individual bacterial taxa between women who remained *Candida*-negative and those who acquired a *Candida* infection by the IVR removal visit.

**Higher microbial diversity was associated with lower TFV levels.** At the IVR removal visit, we found a higher estimated concentration (84.6-fold) of vaginal TFV in women with lower microbial diversity (median 53550 ng/swab with CST I vs 632.5 ng/swab with CST IVB;  $p = 0.16$ ; Fig. 7), although sample sizes were

### A. Baseline vs IVR removal visit



### B. Vaginal wall vs IVR surface at IVR removal visit



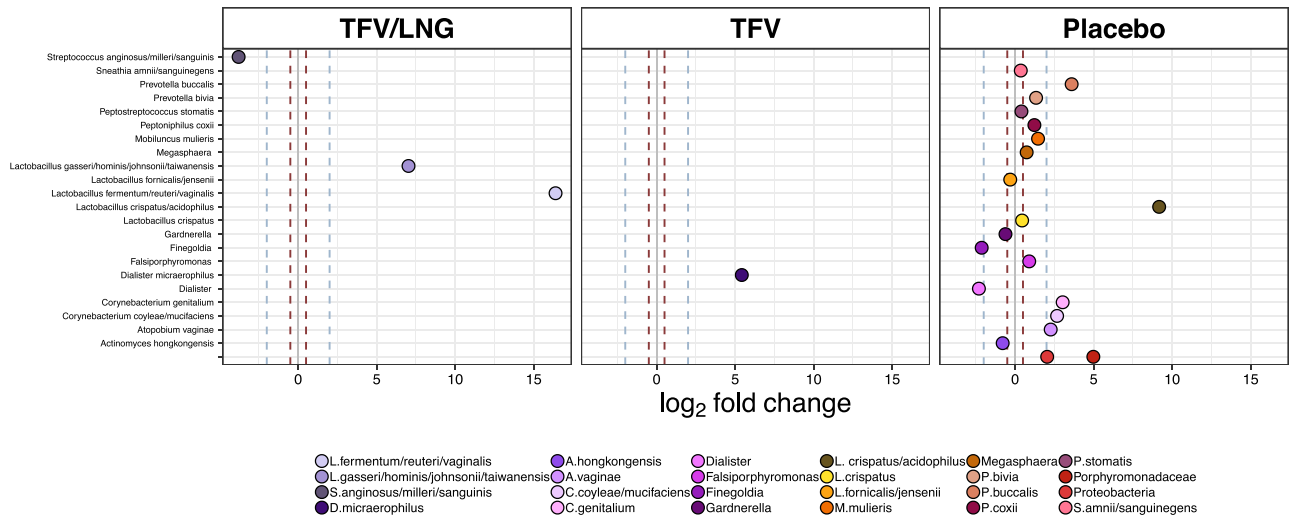
**Figure 4.** Transitions in bacterial communities within each study arm, Kisumu, Kenya, 2019. **(A)** Shifts in community state types (CSTs) in the genital tract from baseline to the intravaginal ring (IVR) removal visit. **(B)** Differences in CST between the genital tract and the IVR surface at the IVR removal visit. Each line represents one participant's transition within the tenofovir/levonorgestrel (TFV/LNG), tenofovir (TFV), or placebo study arms.

small. In addition, after adjusting for the number of days of IVR use, a  $\log_{10}$  increase in the absolute abundance of *Lactobacillus* genus was associated with a  $\log_{10}$  0.41 ng/swab increase in TFV concentrations ( $p > 0.99$ ).

### Discussion

Overall, we found no evidence that either the TFV/LNG or TFV IVR significantly affected a woman's genital bacterial or candidal composition. Our results are consistent with previous findings, including a recent safety study of the same IVR among women in the US and Dominican Republic<sup>16</sup>.

We mostly found small longitudinal changes in total bacterial load or in the overall microbial diversities in all study arms, suggesting that these IVRs do not induce major shifts in the genital microbiota. At the IVR removal visit, CSTs between the lateral vaginal wall and the IVR did not differ, similar to previous findings<sup>16</sup>. Despite no CST changes in most women using the placebo IVR, its use led to fluctuations in multiple bacterial taxa, with statistically significant fold changes in both *Lactobacillus* spp. and BV-associated bacteria. The TFV IVR was only associated with an increase in *D. micraerophilus* abundance, a BV-associated bacterium strongly associated with microbial diversity and high-inflammation states<sup>17–19</sup>. However, we did not see a shift to a more BV-associated microbial composition, likely because the increase in *D. micraerophilus* was insufficient to drive this change. Interestingly, there was a statistically significant decrease in *S. anginosus/milleri/sanguinis* abundance with the TFV/LNG IVR and a markedly larger fold-increase in *Lactobacillus* abundance, suggesting a positive effect of the TFV/LNG combination on microbial composition. Previous studies of vaginal rings found no significant destabilization of the genital microbiota, and an increase in *Lactobacillus* spp. in some cases<sup>16,20,21</sup>, which was attributed to ethinyl estradiol<sup>22–24</sup>. Here we found a similar stabilizing effect, with a decrease in microbial diversity with the TFV/LNG IVR despite containing LNG, a progestin, indicating that the decrease in microbial diversity



**Figure 5.** Fold changes in abundance of specific bacteria with the tenofovir/levonorgestrel (TFV/LNG), tenofovir (TFV), and placebo intravaginal rings (IVRs) respectively, Kisumu, Kenya, 2019. Dots on the right hand side of the gray solid line show a fold change increase in bacterial abundance and dots on the left hand side show a fold change decrease. The red and blue dotted lines represent 0.5-fold and twofold changes in bacterial abundance. Differential abundances with an adjusted p value of  $\leq 0.01$  are shown.

	TFV/LNG		TFV		Placebo	
	Baseline n/N (%)	IVR removal n/N (%)	Baseline n/N (%)	IVR removal n/N (%)	Baseline n/N (%)	IVR removal n/N (%)
All <i>Candida</i> species	4/10 (40.0%)	4/10 (40.0%)	3/9 (33.3%)	4/9 (44.4%)	0/4 (0.0%)	2/4 (50.0%)
<b><i>C. albicans</i></b>						
Prevalence	1/10 (10.0%)	2/10 (20.0%)	1/9 (11.1%)	2/9 (22.2%)	0/4 (0.0%)	1/4 (25.0%)
Mean concentration (log <sub>10</sub> copies/swab)	5.051	4.098	2.345	4.307	–	2.478
<b><i>C. krusei</i></b>						
Prevalence	3/10 (30.0%)	2/10 (20.0%)	2/9 (22.2%)	4/9 (44.4%)	0/4 (0.0%)	2/4 (50.0%)
Mean concentration (log <sub>10</sub> copies/swab)	5.392	2.081	3.371	6.561	–	2.224
<b><i>C. glabrata</i></b>						
Prevalence	0/10 (0.0%)	0/10 (0.0%)	1/9 (11.1%)	0/9 (0.0%)	0/4 (0.0%)	0/4 (0.0%)
Mean concentration (log <sub>10</sub> copies/swab)	–	–	–	–	–	–

**Table 2.** Prevalence of *Candida* species at baseline and removal of tenofovir/levonorgestrel (TFV/LNG), TFV or placebo intravaginal ring (IVR), Kisumu, Kenya, 2019. Only samples for which matched longitudinal data was available were included.

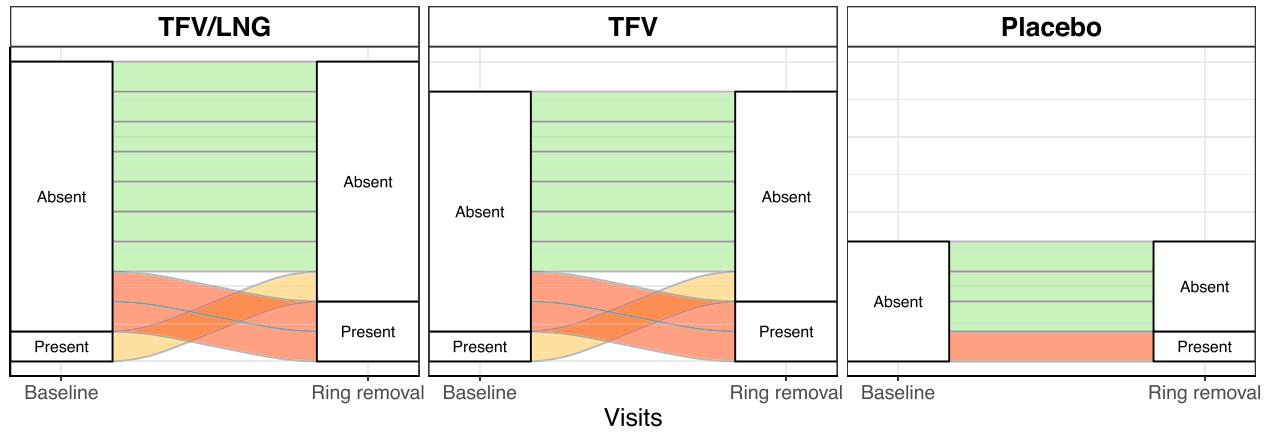
and increase in *Lactobacillus* spp. could be associated with progestin use, particularly LNG, in line with a previous study reporting an increase in genital *Lactobacillus* spp. concentration with LNG implant use<sup>25</sup>.

Although not statistically significantly so, we found that genital TFV concentrations at the IVR removal visit were positively associated with *Lactobacillus* spp. abundances. This is similar to other studies showing that TFV-based HIV PrEP products were less effective in *Lactobacillus*-deficient states<sup>14</sup>. There was a decreasing trend in microbial diversity with increased TFV concentration, with a 84.6-fold higher TFV concentration found with an *L. crispatus*-dominant microbiota (CST I) compared to a more diverse BV-associated microbiota (CST IVB). Based on our differential abundance analyses, this was not driven by *G. vaginalis* or *Prevotella* spp as previously described<sup>14,26</sup>. More work is needed to determine if this decrease is clinically relevant to impact the IVR capacity to prevent HIV or HSV-2. In particular, it would be important to assess the relationship between CSTs and concentrations of the TFV active metabolite, TFV-diphosphate, in mucosal tissues.

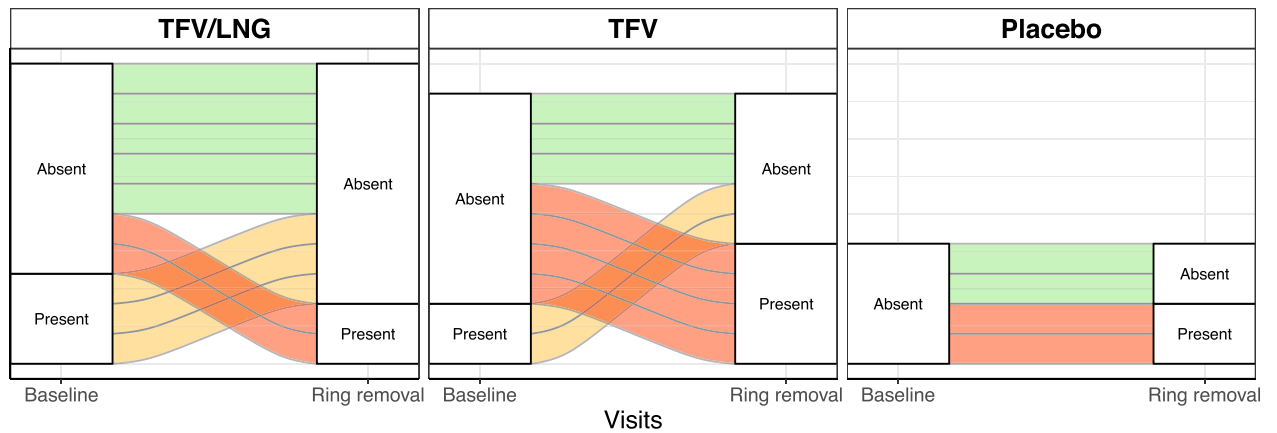
A primary limitation was the small sample size, due to the study being a phase-IIa safety trial. Thus, we presented substantial descriptive data that examine actual numbers and trends, rather than just statistical comparisons. More in-depth comparisons, in larger cohorts, that also include women with BV at IVR insertion, might better help identify potential genital microbial changes induced by these IVRs.

In this study measuring the safety of TFV/LNG and TFV polyurethane IVRs on the genital microbiota among women living in Kenya, we found no evidence of adverse changes to genital microbial health with up to 90 days of use. We found a trend towards the establishment of high *Lactobacillus* states with the TFV/LNG IVR, which could positively impact genital health in a population with a high BV prevalence<sup>27–29</sup>. These results indicate that

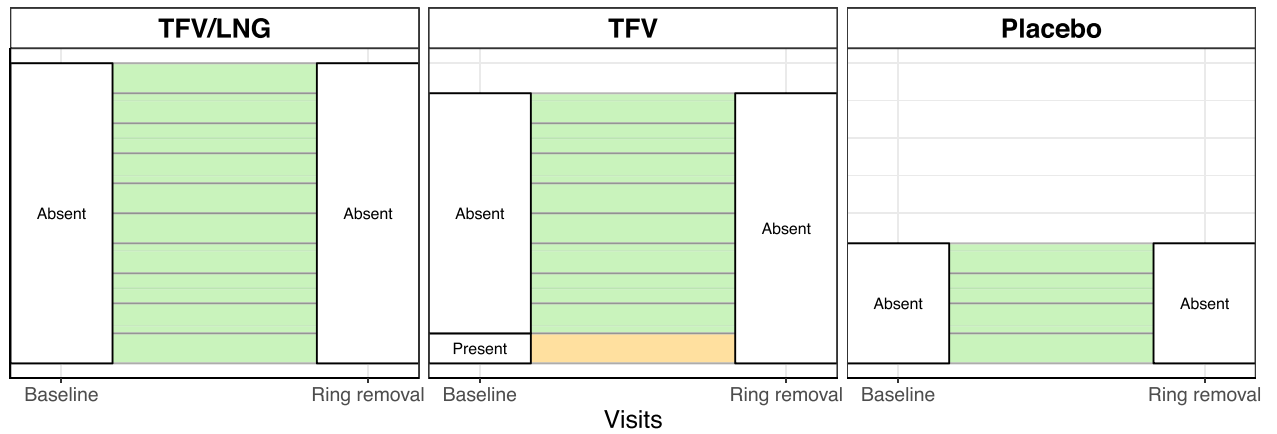
**A. *C. albicans***



**B. *C. krusei***



**C. *C. glabrata***

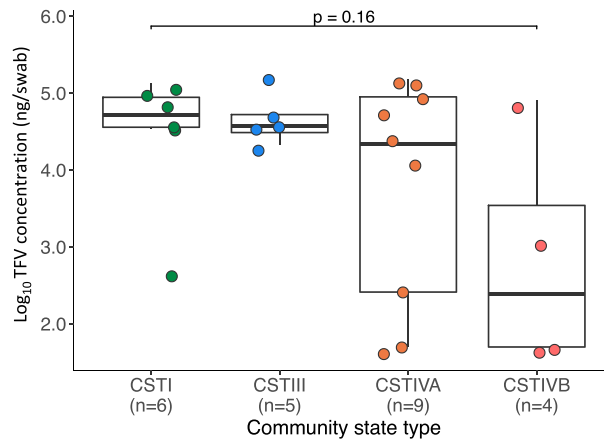


● Negative ● Cleared ● New infection

**Figure 6.** Longitudinal *Candida* status within each study arm for (A) *C. albicans*, (B) *C. krusei*, and (C) *C. glabrata*, Kisumu, Kenya, 2019. Women who remained *Candida*-negative are shown in green. Cleared infections are shown in yellow and new infections are shown in red.

TFV/LNG and TFV IVRs are likely safe to use and support further studies assessing their efficacy in preventing HIV, HSV-2, and unintended pregnancy.





**Figure 7.** Vaginal tenofovir (TFV) concentrations across community state types (CSTs), Kisumu, Kenya, 2019. Comparing the difference in log<sub>10</sub> vaginal TFV concentrations at the IVR removal visit among women using the TFV and tenofovir/levonorgestrel arms categorized by their CST. The p value was determined using the Mann–Whitney U test.

## Methods

**Participant recruitment.** CONRAD B17-144 was a phase-IIa randomised, placebo-controlled, investigational new-drug-enabling trial assessing the safety, pharmacokinetics, pharmacodynamics, tolerability, acceptability of, and adherence to two 90 days IVRs. Women were randomised in a 2:2:1 ratio to receive a TFV-only IVR<sup>30</sup> to prevent HIV/HSV, a combination TFV/LNG IVR that additionally may prevent pregnancy<sup>30–32</sup> and a placebo IVR<sup>31</sup>. All rings were composed of polyurethane tubing<sup>31</sup>.

This study recruited women at the Jaramogi Oginga Odinga Referral Hospital, Kisumu, Kenya, who were generally healthy, non-pregnant, at lower risk for HIV (based on a validated risk score<sup>33</sup>), seronegative for HIV and hepatitis B surface antigen, and bacterial vaginosis (BV)-negative by Amsel criteria. Women diagnosed with BV by Amsel criteria during the screening procedures were treated and permitted to be re-assessed for eligibility > 2 months after treatment, and enrolled if BV was not detected at that time. Eligible women were not using hormonal contraception at enrolment and had not been diagnosed or treated for STIs in the last three months (Supplementary Methods). The study was approved by the Scientific and Ethics Review Unit at the Kenya Medical Research Institute and the Human Subjects Division at University of Washington, and was registered with ClinicalTrials.gov (Identifier: NCT03762382; registered 03/12/2018). All participants provided written informed consent and all methods were carried out in accordance with relevant guidelines and regulations.

**Study procedures.** Results from the parent study will be published in a separate manuscript (Mugo et al.<sup>32</sup>, in preparation). Paired data from the baseline/IVR insertion and IVR removal visits were included for this analysis, focused on microbiota changes. IVR removal was scheduled for 90 days after IVR insertion or prior to the IVR expiry date, whichever date came first (At 90 days: 12/27; before expiry date: 9/27; other reasons including pregnancy or STIs: 6/27). In cases where matched clinical data from the IVR removal visit were not available, samples collected during the next visit, which was scheduled to occur 24 h later, were used for analyses.

**Sample collection at IVR insertion and removal.** After randomization, the IVR was inserted deeply into the vagina by a study clinician at the baseline visit and removed at the IVR removal visit. At both baseline and the IVR removal visits, vaginal swabs were collected for (1) TFV PK measurements, (2) Nugent score and vaginal pH and (3) microbiota 16S rRNA sequencing. A swab of the IVR surface was also collected at the IVR removal visit. Nugent-BV was defined as having a Nugent score ≥ 7; women with a Nugent score 4–6 were categorized as having an intermediate vaginal microbiota (Supplementary Methods).

**Genital TFV concentrations.** TFV concentrations from cervicovaginal fluid from swabs were determined using liquid chromatography tandem mass spectrometry as described previously<sup>34</sup> (Supplementary Methods).

**Microbial DNA extraction.** DNA extraction from lateral vaginal wall swabs was carried out (Qiagen All-Prep PowerViral<sup>+</sup> DNA/RNA kit; Supplementary Methods) and was stored at –20 °C until used for 16S rRNA sequencing, total 16S bacterial load using real-time polymerase chain reaction (qPCR), and *Candida* spp. quantitation (qPCR).

**16S rRNA gene sequencing and analysis.** The V3–V4 hypervariable region of the bacterial 16S rRNA was amplified using modified universal primers<sup>35</sup>. Samples were sequenced using the Illumina MiSeq platform and (300 bp paired-end). DADA2 v1.12.1<sup>36</sup> was used to process, merge and filter raw reads and samples with < 2000 reads were excluded from further analyses. Taxonomic annotation was carried out using the RDP database (v11.5) and a BLAST search was carried out for further taxonomic classification of unannotated

amplicon sequence variants (ASV). Taxonomic annotation was available for 993 ASVs, with 277 (27.9%) having species-level annotation. The ASVs were merged at the lowest available taxonomic level to generate relative abundances for a total of 105 bacterial taxa<sup>17</sup> (Supplementary Methods).

The total bacterial load per swab was determined using a qPCR of the 16S rRNA gene<sup>37</sup>. The BactQuant assay targets the V3–V4 region, and gives an estimate of the total 16S rRNA copies per swab. Based on these values and the relative abundances of taxa generated by 16S rRNA sequencing, absolute bacterial abundances (copies/swab) were calculated for each taxon. One sample (out of five), in the placebo arm, without sufficient 16S DNA to pass quality control checks was excluded from analysis (final  $n = 4$ ). All further analyses were carried out using absolute abundances for bacterial taxa.

**Candida spp. quantification.** Concentrations of *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis*, were measured using species-specific qPCRs at the baseline and IVR removal visits (Supplementary Methods). The final concentrations were reported as number of  $\log_{10}$  copies per swab.

**Statistical analysis.** All microbiota data analysis was carried out in R (v3.6.0). Paired comparisons between the ring insertion and ring removal visits were considered primary endpoints of this analysis. Participant microbiota were categorised into four CSTs using Ward clustering of weighted Unifrac dissimilarity matrices derived from bacterial absolute abundances. TFV and TFV/LNG study arms were compared to the placebo arm using Mann–Whitney U-tests for independent samples and permutational multivariate analysis of variance (PERMANOVA) for microbial diversity. Paired analyses comparing microbiota at IVR insertion and removal visits were carried out using Wilcoxon Signed Rank tests and generalized estimating equation models. Correlations between bacterial absolute abundance and variables such as time of IVR use and genital TFV concentrations were estimated using linear regression models. The number of days of IVR use was categorized as  $\leq 47$  days ( $< 2$  menstrual cycles), 48–71 days ( $\sim 2$  menstrual cycles) and 72–95 days ( $\sim 3$  menstrual cycles), and was adjusted for when measuring fold changes in individual bacterial taxa within study arms, using generalized linear models as part of the DeSeq2 package.  $\log_2$  fold-changes were determined only for bacterial taxa found in at least 15% of samples to minimize the influence of minority taxa. Due to the small sample size, the study was not powered to estimate differences between the study arms with a high degree of precision and analyses were descriptive in nature. As a result, p-values and confidence intervals (CI) were not adjusted for multiple comparisons unless explicitly stated, in which case a false discovery rate step down procedure was used<sup>38</sup> (Supplementary Methods).

## Data availability

The dataset generated and analyzed in this study has been deposited at NCBI SRA under Accession ID PRJNA834833 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA834833>). Linked data are available from the corresponding author on reasonable request.

Received: 10 December 2021; Accepted: 25 May 2022

Published online: 14 July 2022

## References

- UNAIDS. *Global HIV & AIDS Statistics—2020 Fact Sheet*. <https://www.unaids.org/en/resources/fact-sheet> (2020). Accessed Nov 2020.
- Bearak, J. *et al.* Unintended pregnancy and abortion by income, region, and the legal status of abortion: Estimates from a comprehensive model for 1990–2019. *Lancet Glob. Health* **8**, e1152–e1161 (2020).
- Van Damme, L. *et al.* Preexposure prophylaxis for HIV infection among African women. *N. Engl. J. Med.* **367**, 411–422 (2012).
- Marrazzo, J. M. *et al.* Tenofovir-based preexposure prophylaxis for HIV infection among African women. *N. Engl. J. Med.* **372**, 509–518 (2015).
- Griffin, J. B. *et al.* Vaginal ring acceptability and related preferences among women in low- and middle-income countries: A systematic review and narrative synthesis. *PLoS ONE* **14**, e0224898 (2019).
- van der Straten, A. *et al.* High acceptability of a vaginal ring intended as a microbicide delivery method for HIV prevention in African women. *AIDS Behav.* **16**, 1775–1786 (2012).
- Baeten, J. M. *et al.* Use of a vaginal ring containing dapivirine for HIV-1 prevention in women. *N. Engl. J. Med.* **375**, 2121–2132 (2016).
- Brown, E. R. *et al.* Greater dapivirine release from the dapivirine vaginal ring is correlated with lower risk of HIV-1 acquisition: A secondary analysis from a randomized, placebo-controlled trial. *J. Int. AIDS Soc.* **23**, e25634 (2020).
- European Medicines Agency. *Dapivirine Vaginal Ring 25 mg (Dapivirine)* (European Medicines Agency, 2020).
- WHO. *WHO Recommends the Dapivirine Vaginal Ring as a New Choice for HIV Prevention for Women at Substantial Risk of HIV Infection*. <https://www.who.int/news/item/26-01-2021-who-recommends-the-dapivirine-vaginal-ring-as-a-new-choice-for-hiv-prevention-for-women-at-substantial-risk-of-hiv-infection> (2021). Accessed Mar 2021.
- Thurman, A., Kimble, T., Hall, P., Schwartz, J. L. & Archer, D. F. Medroxyprogesterone acetate and estradiol cypionate injectable suspension (Cyclofem) monthly contraceptive injection: Steady-state pharmacokinetics. *Contraception* **87**, 738–743 (2013).
- van den Heuvel, M. W., van Bragt, A. J. M., Alhabawy, A. K. M. & Kaptein, M. C. J. Comparison of ethinylestradiol pharmacokinetics in three hormonal contraceptive formulations: The vaginal ring, the transdermal patch and an oral contraceptive. *Contraception* **72**, 168–174 (2005).
- Abdool Karim, Q. *et al.* Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* **329**, 1168–1174 (2010).
- Klatt, N. R. *et al.* Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* **356**, 938–945 (2017).
- McKinnon, L. R. *et al.* Genital inflammation undermines the effectiveness of tenofovir gel in preventing HIV acquisition in women. *Nat. Med.* <https://doi.org/10.1038/nm.4506> (2018).
- Thurman, A. R. *et al.* Vaginal microbiota and mucosal pharmacokinetics of tenofovir in healthy women using tenofovir and tenofovir/levonorgestrel vaginal rings. *PLoS ONE* **14**, e0217229 (2019).
- Lennard, K. *et al.* Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in South African adolescent females. *Infect. Immun.* <https://doi.org/10.1128/IAI.00410-17> (2018).

18. Sabo, M. C. *et al.* Associations between vaginal bacteria implicated in HIV acquisition risk and proinflammatory cytokines and chemokines. *Sex. Transm. Infect.* <https://doi.org/10.1136/sextrans-2018-053949> (2019).
19. De Seta, F., Campisciano, G., Zanotta, N., Ricci, G. & Comar, M. The vaginal community state types microbiome-immune network as key factor for bacterial vaginosis and aerobic vaginitis. *Front. Microbiol.* <https://doi.org/10.3389/fmicb.2019.02451> (2019).
20. Hardy, L. *et al.* Association of vaginal dysbiosis and biofilm with contraceptive vaginal ring biomass in African women. *PLoS ONE* **12**, e0178324 (2017).
21. Crucitti, T. *et al.* Contraceptive rings promote vaginal lactobacilli in a high bacterial vaginosis prevalence population: A randomised, open-label longitudinal study in Rwandan women. *PLoS ONE* **13**, e0201003 (2018).
22. Veres, S., Miller, L. & Burington, B. A comparison between the vaginal ring and oral contraceptives. *Obstet. Gynecol.* **104**, 555–563 (2004).
23. Huang, Y. *et al.* Effects of a one year reusable contraceptive vaginal ring on vaginal microflora and the risk of vaginal infection: An open-label prospective evaluation. *PLoS ONE* **10**, e0134460 (2015).
24. De Seta, F. *et al.* Effects of hormonal contraception on vaginal flora. *Contraception* **86**, 526–529 (2012).
25. Achilles, S. L. *et al.* Impact of contraceptive initiation on vaginal microbiota. *Am. J. Obstet. Gynecol.* **218**(622), e1–e10 (2018).
26. Hillier, S. L. *et al.* Impact of vaginal microbiota on genital tissue and plasma concentrations of tenofovir (2017).
27. Torrone, E. A. *et al.* Prevalence of sexually transmitted infections and bacterial vaginosis among women in sub-Saharan Africa: An individual participant data meta-analysis of 18 HIV prevention studies. *PLoS Med.* **15**, e1002511 (2018).
28. Jespers, V. *et al.* Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: A cross-sectional study. *PLoS ONE* **9**, e109670 (2014).
29. Masha, S. C. *et al.* High prevalence of curable sexually transmitted infections among pregnant women in a rural county hospital in Kilifi, Kenya. *PLoS ONE* **12**, e0175166 (2017).
30. Johnson, T. J. *et al.* A 90-day tenofovir reservoir intravaginal ring for mucosal HIV prophylaxis. *Antimicrob. Agents Chemother.* **56**, 6272–6283 (2012).
31. Clark, J. T. *et al.* Engineering a segmented dual-reservoir polyurethane intravaginal ring for simultaneous prevention of HIV transmission and unwanted pregnancy. *PLoS ONE* **9**, e88509 (2014).
32. Mugo, N. R. *et al.* Randomized, placebo-controlled trial of safety, pharmacokinetics, and pharmacodynamics of 90-day intravaginal rings (IVRs) releasing tenofovir (TFV) with and without levonorgestrel (LNG) among women in Western Kenya. In *HIV R4P* (2021).
33. Balkus, J. E. *et al.* An empiric HIV risk scoring tool to predict HIV-1 acquisition in African women. *J. Acquir. Immune Defic. Syndr.* **72**, 333–343 (2016).
34. Hendrix, C. W. *et al.* MTN-001: Randomized pharmacokinetic cross-over study comparing tenofovir vaginal gel and oral tablets in vaginal tissue and other compartments. *PLoS ONE* **8**, e55013 (2013).
35. Gohl, D. M. *et al.* Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat. Biotechnol.* **34**, 942–949 (2016).
36. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
37. Liu, C. M. *et al.* BactQuant: An enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiol.* **12**, 56 (2012).
38. Columb, M. O. & Sagadai, S. Multiple comparisons. *Curr. Anaesth. Crit. Care* **17**, 233–236 (2006).

## Acknowledgements

We are grateful to all the women who participated in this study, as well as the study teams at Kenya Medical Research Institute and the University of Washington. We would like to thank the Richard E. Haaland lab for providing the genital TFV measurements for all study participants. The authors would also like to acknowledge the efforts of the Kisumu Combined Ring Study Team: *Management Team* Nelly R Mugo (Center for Clinical Research (CCR), Kenya Medical Research Institute-KEMRI, Nairobi, Kenya & International Clinical Research Center (ICRC), Department of Global Health University of Washington (UW), USA), Victor Mudhune (Center for Global Health Research (CGHR)-KEMRI, Kisumu, Kenya), Renee Heffron (UW, Seattle, WA, USA), Centers for Disease Control and Prevention (CDC): Eleanor McLellan-Lemal, Siobhan O'Connor, Gustavo F Doncel (CONRAD, Eastern Virginia Medical School (EVMS)), Andrea R Thurman (CONRAD, EVMS), Betty Njoroge (CCR, KEMRI). *Implementing Study Site Team (KEMRI)* CGHR—KEMRI, Kisumu: Beatrice Nyagol, Eunice Ouma, Richard Ndivo, Maxcine Oguta, Haynet Opon, Dorine Awili, Anne Mithika, Epines Chavangi, Jecinter Oruko, John Okanda, Arthur Ogendo, Elizabeth Ayuo, Evans Odipo, Vitalis Sewe, Boaz Kerubo, Calvin Mbeda, Eucabeth Awuonda, Isdora Odero, Emily Anyango, Erica Mimba, Fredrick Oloo, Richard Odipo, Valarie Opollo, Emily Kerubo, Fredrick Omondi, Angelica Gende, Kelvin Wandera, Carolyne Juma, Alice Were, Phoebe Ogutu, Susan Aremo, Philister Madiega, Daniel Ogando, Judith Arego, Margaret Otieno, Rosemary Akello, Ken Ondeng'e, Teresa Omoro, Caren Amondi. CDC-HIV Branch, Kisumu: Kimberly McCarthy, Renee Ridzon. *Other Members of the KCRS Study Team* ICRC, Department of Global Health, UW, USA: Susan Morrison, Meighan Krows, Connie Celum; CONRAD -EVMS: Abenan L. Ouattara, Nazita Yousefieh, Jill Schwartz, Allison Matthews. United States Agency for International Development (USAID): Shannon Allen, Elizabeth Russell, Karen Near. Centers for Disease Control and Prevention, USA: Jeffrey Wiener, Taraz Samandari, Stacie R Deaton, Lee Claypool, Josh E. Betts. *Laboratory* CDC, USA: Richard E. Haaland, Amy Martin (Tenofovir (TFV) pharmacokinetics (PK) assessments), Jeffrey Fountain (cytokine analysis). CONRAD EVMS: Terry A. Jacot (CBQCA and glycerin assessments on Placebo rings, Anti-HSV on genital swabs and Anti-HIV genital on swabs). Endocrine Technologies Core, Oregon National Primate Research Center: David Erikson, Steven W. Blue (Serum Levonorgestrel (LNG) and Sex Hormone-Binding Globulin (SHBG) assessments. Seattle Children's Research Institute: Heather Jaspan, Smritee Dabee, Colin Feng, Melanie Gasper (Microbiota analysis on returned Intra Vaginal rings). Particle Sciences: Barrett Remington, Bruce L. Frank (residual drug assessment on returned Intra Vaginal rings). Nina Isoherranen (UW, TFV PK Assessment), Harald Haugen (ICRC, UW, USA). *Safety Monitoring Committee (SMC)* Jared Baeten, Katherine Thomas, Susan Morrison (UW, Global Health, ICRC), Doncel F Gustavo (CONRAD, EVMS), Victor Mudhune (CGHR-KEMRI, Kisumu, Kenya), Eleanor McLellan-Lemal (CDC), Siobhan O'Connor (CDC), Athena Kourtis (CDC), Naomi Tepper (CDC), Shannon Allen (USAID), Nelly Mugo (CCR-KEMRI, ICRC-UW). *Data management* was done by DF/Net, Inc. (Seattle, USA): Lisa Ondrejcek (President), Angela Williams (Clinical Data Manager), Matt Johnson, (Biostatistician), Joe Jiang (Data Science Lead). *Statistical analysis* Katherine Thomas, Sue Peacock (ICRC-UW, USA), Deborah Donnell (Fred Hutchinson Cancer Research Center, Seattle, WA, USA).

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## Funding

This work was supported by the United States Centers for Disease Control and Prevention and the United States Agency for International Development (USAID) under the U.S. President's Emergency Plan for AIDS Relief (PEPFAR) through cooperative agreement U01PS005183, 2017–2021 (registered at ClinicalTrials.gov under the identifier NCT03762382), and was made possible by the support of the American people through these governmental agencies and programs. Funding from USAID's Cooperative Agreements with CONRAD/Eastern Virginia Medical School (AID-OAA-A-14-00010 and AID-OAA-A-14-00011) was used to support the manufacturing and clinical qualification of the rings and CONRAD's personnel time and effort. The contents in this article are the sole responsibility of the authors, and do not necessarily reflect the official position of the institutions, PEPFAR, USAID, Centers for Disease Control and Prevention and/or the United States Government.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-022-13475-9>.

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