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Dynamics of the vaginal microbiome during the menstrual cycle of HIV positive and negative women in a sub-urban population of Kenya

Teresa N. Kiama[1]*, Mario Vaneechoutte[2], Paul M. Mbugua[1], Hans Verstraelen[3], Benson Estambale[4], Marleen Temmerman[3] and Rita Verhelst[2]

[1]Department of Medical Physiology, University of Nairobi, Kenya.
[2]Laboratory for Bacteriology Research, Faculty of Medicine and Health Sciences, University of Ghent, Belgium.
[3]Department of Obstetrics & Gynaecology, Ghent University hospital, Belgium.
[4]University of Nairobi Institute of Tropical and Infectious Diseases (UNITID), Kenya.

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Vaginal microbiome (VM) is dominated by Lactobacillus for maintenance of vaginal health. The objective of this study was to characterize changes in the VM during the menstrual cycle of HIV positive (HIV+) and HIV negative (HIV-) women in a sub-urban population of Kenya. In this longitudinal study of 38 women-20 HIV+,18 HIV-high vaginal swabs were for genomic DNA and Gram stain and quantitative PCR (qPCR). qPCR nested on Gram stain showed high concentration of *L. iners* in normal VM, increasing during bacterial vaginosis (BV) and high levels of *L. jensenii* in women with BV while *L. crispatus* was absent. *G. vaginalis* increased from normal to BV. *A. vaginae* was absent in normal but detectable in intermediate gram stain and increased during BV. Gram stain showed BV was absent in HIV-ve women using condoms. Both groups had high concentration *L. iners* and *G. vaginalis*, harboured *A. vaginae*. Frequency and concentration of *L. crispatus* were less in HIV+ women, *L. jensenii* undetectable but condom use significantly higher. Menstrual cycle showed high concentration of *L. iners* and *G. vaginalis*. *L. crispatus* increased while *A. vaginae* decreased. At the initial phase, *L. jensenii* was low and undetectable thereafter. In this Kenyan population L. iners predominates normal VM, increased during BV. Both groups had high concentration of *L. iners* and *G. vaginalis*. Concentration of *L. crispatus* increased while *A. vaginae* decreased. Condoms and *L. crispatus* show protection against BV while *L. jensenii* does not. Both the presence and quantities of *L. crispatus* determine healthy VM.

Key words: Vaginal microbiome, HIV, qPCR, Lactobacilli, menstrual cycle.

INTRODUCTION

The vaginal micro-environment is dynamic and undergoes changes during the menstrual cycle in women of reproductive age. These changes correlate with the accompanying hormonal changes (Owen, 1975; Hay,

*Corresponding author. E-mail: kiamatn777jes@hotmail.com. Tel: +254-717857852.

2005; Farage et al., 2009). Studies show that changes occur in the vaginal microbiome during the normal menstrual cycle. However, most studies have been carried out in populations outside Africa such as in the United Kingdom (Wilks and Tabaqchali, 1987; Keane et al., 1997), Japan (Fujisawa et al., 1992) and in the United States of America (Onderdonk et al., 1986; Schwebke et al., 1999; Eschenbach et al., 2000; Ness et al., 2006). These studies found the most profound changes in vaginal microbiome to occur during menses. Only one study of African women has related the changes occurring in the vaginal microbiome with cyclic menstrual changes (Morison et al., 2005).

The recurrent imbalance that occurs in the equilibrium of vaginal microbiome favors the overgrowth of bacterial vaginosis (BV)-associated bacteria (Nyirjesy, 2008). The relative depletion of the resident Lactobacilli has been shown to be most profound at the time of menses, indicating that the vaginal microbiome become less stable during this event (Bartlett et al., 1977; Sautter and Brown, 1980; Onderdonk et al., 1986; Keane et al., 1997; Hay et al., 1997; Schwebke et al., 1997, 1999; Eschenbach et al., 2000; Wilson et al., 2007). This phenomenon has generally been attributed to the premenstrual decline in circulating levels of estrogen (Bradshaw et al., 2006; Wilson et al., 2007), which in turn affects the colonisation strength of the vaginal Lactobacilli, for example by limiting their capacity for epithelial adherence. The vaginal *Lactobacillus* microbiome are probably further challenged by the menstrual flooding of the vagina (Wilson et al., 2007), which is accompanied by a sharply rising pH, further exacerbating the unfavorable conditions for epithelial adherence of the Lactobacilli. Under such conditions the colonization resistance offered to BV-associated anaerobes can easily be overcome (Hay, 2005). Menses have also been shown to induce a wash-out of Lactobacilli. These mechanisms might explain why BV is less prevalent during pregnancy (Hay et al., 1994; Riggs et al., 2007; Song et al., 2020) and in postmenopausal women (Hillier and Lau, 1997), despite the relative estrogen deficiency especially in the latter group. To further demonstrate the important role that hormones might play in maintaining the balance in the vaginal ecosystem, one longitudinal study found women using hormonal contraceptives to have less prevalence due to greater remission of BV (Riggs et al., 2007).

Menses therefore represent a presumably critical challenge to the maintenance of the normal, Lactobacilli-dominated microbiome during the index cycle. This was clearly demonstrated in a previous study (Keane et al., 1997) where daily Gram-stained vaginal smears were prepared over the whole duration of a menstrual cycle among 21 volunteers. Seven of them who initially presented with basically normal vaginal microbiome showed BV-like overgrowth on further study follow-up, which was in all the cases preceded by a decrease of Lactobacilli within the first few days of the menstrual cycle. It is conceivable that following the decline in estrogenization of the vaginal epithelium, and the subsequent vaginal flooding with menstrual blood, Lactobacilli must replenish the vagina in due time within the first few days of the menstrual cycle. This study postulates that it is this re-colonization effort that determines the balance for vaginal microbiome stability during the rest of the cycle. Following disruption of the pH, it takes a normal population of Lactobacilli several hours to re-establish the known acidity (Boskey et al., 1999). In a similar subsequent study of 74 women, the 50 who had BV showed the overall rate of recovery of heavy growth of *Lactobacillus* to increase over the menstrual cycle. However, their results showed that this was not the case for the hydrogen peroxide (H_2O_2)-producing Lactobacilli (Eschenbach et al., 2000), suggesting that the acquisition of these species during the early follicular phase may determine the vaginal microbiome status during the entire cycle. Failure to recover a stable *Lactobacillus* microbiome apparently sets the scene for overgrowth of BV-associated anaerobic species during the index cycle as suggested in the study by Keane et al. (1997). In their observations, the development of BV was consistently preceded by decreased numbers of Lactobacilli during menses. In addition to these inescapable physiologic events, behavioural factors during menses may further compromise the delicate balance between the H_2O_2-producing Lactobacilli and BV-associated microorganisms. Douching after menses for instance has been found to act as a strong predictor of subsequent development of BV (Schwebke et al., 2004). This may be explained by the detrimental effect that douching has on the H_2O_2-producing Lactobacilli (Ness et al., 2002; Beigi et al., 2005), in addition to the antagonizing effect of menses (Brotman et al., 2008). A similar observation has been made for sexual intercourse during menses (Ness et al., 2004).

Beyond the critical menstrual period, the vaginal microbiome may continue to display transient shifts away from a Lactobacilli dominance (Priestley et al., 1997; Schwebke et al., 1999). Such episodes could represent a window phase of heightened vulnerability to the development of a true episode of BV, such as is induced by douching or sexual contact. A recent study showed that during menses the diversity of vaginal microbiome increased while on the contrary the concentration of *Lactobacillus* species decreased. The use of hormonal contraceptives in turn increased the Lactobacilli (Song et al., 2020). Of note in this respect is the observation in the Keane et al. (1997) study that, two of seven women converted to intermediate microbiome or BV, and had decreased Lactobacilli on the day prior to a change in the status as shown by Gram stain (Keane et al., 1997). The implications of these observations are critical to the understanding of the pathogenesis of BV as it may well be that established risk factors do not necessarily disrupt

a healthy vaginal microbiome, but merely superimpose on an already imbalanced microbiome. In one outstanding, example from a large prospective cohort study on vaginal douching, Hutchinson et al. (2007) concluded that contrary to consensus, douching may in fact sufficiently disrupt an already imbalanced flora to create BV, but not induce *de novo* BV or the acquisition of BV vaginosis-associated micro-organism. Similarly, Vallor et al. (2001) found sexual intercourse to be a risk factor for BV, an indication that coitus only poses a threat to the *Lactobacillus* if they are not granted sufficient time to recover following transient instability. In the latter study, antibiotic use and frequency of intercourse were shown to be factors that make it more difficult for H_2O_2-producing lactobacilli to recolonize the vagina. Recolonization also depends on the *Lactobacillus* spp. present, *Lactobacillus crispatus* and *Lactobacillus jensenii* showing more colonization resistance than *Lactobacillus gasseri* (Vallor et al., 2001). The latter study demonstrated the transient nature of *Lactobacillus* colonization. Within the study duration of 8 months, two thirds of the 101 women studied either lost or acquired colonization by *Lactobacillus*. Leppäluoto (2011) observed that temporary imbalance of the vaginal microbiome towards a BV-like profile following intercourse could be attributed to the accompanying changes in pH.

It therefore appears that the delicate balance of whether *Lactobacillus* will recolonize the vagina after menses is determined by certain factors, the key one being ability of the *Lactobacillus* strain present to overcome the prevailing resistance. Resistance to recolonization may be due to prolonged times of elevated pH as may happen in frequent intercourse. Other factors may include the systemic effects of hormones that may play a role at the receptor level in the vaginal epithelium. Srinivasan et al. (2010) observed that during menses the population of *G. vaginalis* increased alongside that of *Lactobacillus iners* while quantities of *L. crispatus* and *L. jensenii* decreased simultaneously. This trend was reversed at the end of menses. Similar observations were made in an earlier study (Schwebke et al., 1997). The former authors also observed that *Gardnerella vaginalis* was present in women with and without BV, and that the increase during menses may be linked to availability of a substrate in the menses, namely iron. Indeed, *G. vaginalis* contains vaginolysin that can perforate erythrocytes to release iron and activate immune markers of the vaginal epithelium (Gelber et al., 2008), causing the inflammation observed in BV. Vaginolysin, found in another Gram-positive genus (Gelber et al., 2008), belongs to cholesterol-dependent cytolysins (CDCs) produced by organisms that colonize and cause disease at mucosal surfaces (Tweten, 2005). In most cases, toxin production has been shown to be essential for maintenance of colonization and pathogenesis of invasive disease. Further, *G. vaginalis* species have been shown to produce sialidase (Santiago et al., 2011) that could exacerbate the pathogenesis of BV. Systemic effects of hormones may then only contribute to ongoing inflammation at the vaginal epithelium.

The pathogenesis of BV may further be unravelled through the appraisal of our current knowledge on the vaginal microbiome in relation to the menstrual cycle, when instability is critical to whether or not BV develops. Transient changes in the vaginal microbiome occur predominantly in the first part of the menstrual cycle, where the quantities of lactobacilli are significantly reduced (Keane et al., 1997; Morison et al., 2005; Song et al., 2020). Vaginal colonization by Lactobacilli is believed to confer multiple benefits to women, among them being the inhibition of the development of BV, a condition associated with many undesirable effects such as preterm delivery (Hillier et al., 1995; Leitich et al., 2003; Wilks et al., 2004; Fettweis et al., 2019) and increased vulnerability to other STIs (Allsworth et al., 2008; Cherpes et al., 2003; Kaul et al., 2007; Doerflinger et al., 2014; Bayigga et al, 2019), including HIV infection (Cohen et al., 1995; Sewankambo et al., 1997; Taha et al., 1998; Martin et al., 1999). Studies show that vaginal microbiome dominated by non-*Lactobacillus* bacteria increases the risk of HIV infection (Wang et al., 2023). Further it has been shown that a combination of specific *Lactobacillus* predominance as well as its concentration may both be crucial for maintenance of the dynamic physiologic balance of healthy vaginal microbiome (De Seta et al., 2019). The purpose of the current study was to describe by Gram stain and quantitative PCR, the dynamics of the vaginal microbiome of HIV^+ and HIV^- premenopausal women during the menstrual cycle in a sub-urban population of Kenya.

METHODS

Study population

The characteristics of the study population are described in detail elsewhere (Kiama et al., 2014) and summarized here in Table 1. The study was approved by the ethical review board of Kenyatta National Hospital/University of Nairobi (Registration No. P122/8/2005).

Laboratory screening tests

The study participants were screened for HIV-1 infection, pregnancy, and syphilis. Two high vaginal swabs were taken for *Trichomonas vaginalis* culture and for the diagnosis of candidiasis as assessed by microscopic examination for the presence of budding yeasts or pseudohyphae in a drop of 10% KOH. One endocervical swab was used for the combined *Clamydia trachomatis* and *Neisseria gonorrhoeae* PCR. These screening tests were carried out as described previously (Kiama et al., 2014). The $CD4^+$ T cell count was performed by flow cytometry on Becton Dickinson FACS automatic Count System with fluorochrome-labeled antibodies and a fluorescent beads standard for enumeration. Stained samples were analysed on a Facs-Calibur

Table 1. Population characteristics of participants at enrolment into the study.

Parameter	HIV$^+$ (n=20)	HIV$^-$ (n=18)	P value
Age (years)			
21-28	4 (20.0)	4 (22.2)	0.78
29-36	10 (50.0)	7 (38.9)	
37-44	6 (30.0)	7 (38.9)	
CD4 count (cells/µL)			
250-500	4 (20.0)	1 (5.6)	0.001
501-750	11 (55.0)	1 (5.6)	
751-1000	3 (15.0%	8 (44.4)	
1001-1500	2 (10.0)	8 (44.4)	
Gram stain			
Grade 0	0 (0.0)	1 (5.6)	
Grade I	14 (70.0)	14 (77.8)	NA
Grade II	0 (0.0)	0 (0.0)	
Grade III	5 (25.0)	2 (11.1)	
No score	1 (5.0)	1 (5.6)	
Antibiotic prophylaxis			
Yes	13 (65.0)	0 (0.0)	<0.001
No	7 (35.0)	18 (100.0)	
Marital status			
Married	15 (75.0)	9 (50.0)	0.01
Single/separated	2 (10.0)	9 (50.0)	
Widow	3 (15.0)	0 (0.0)	
Condom use			
Yes	12 (60.0)	4 (22.2)	0.02
No	8 (40.0)	14 (77.8)	
Level of schooling			
Primary and below	9 (45.0)	4 (22.2)	0.33
Secondary	10 (50.0)	13 (72.2)	
Tertiary	1 (5.0)	1 (5.6)	

machine using CELLQuest Software (Becton Dickinson).

Sample collection and grading during follow-up

Specimens were collected three times per cycle for two consecutive menstrual cycles as follows: Follicular phase (Day 5-8); Ovulaton phase (Day 12-15); Luteal phase (Day 19-22) to allow for flexibility of scheduled visits.

For the 38 women (20 HIV$^+$ and 18 HIV$^-$) that were followed up, two high vaginal swabs were obtained at subsequent visits as follows: with a non-lubricated speculum in place, sterile cotton swabs were consecutively inserted into the vaginal vault. Each swab was rotated against the lateral vaginal wall at the mid-portion of the vault and carefully removed to prevent contamination with the vulva and introitus microbiome. The first swab was used to make a Gram stain. The second swab was transported to the laboratory in a dry sterile tube for DNA extraction. Gram stain specimens were analysed for the composition of the vaginal microbiome by microscopy according to the Nugent criteria (Nugent et al., 1991). An additional category of the Gram stains known as grade 0 was included to represent the smears lacking bacteria cells (Verhelst et al., 2005).

Statistical analysis

Prevalence rates were compared between groups through Chi-square test or Fischer's Exact Test. Statistical significance was accepted at the significance level α=0.05. All analyses were

Table 2. Distribution of Nugent scored vaginal microbiome grades among HIV+ve and HIV-ve women during different phases of two menstrual cycles. Percentages in parentheses.

Grade	HIV +ve (n = 41)			HIV -ve (n = 33)		
	Follicular	Ovulation	Luteal	Follicular	Ovulation	Luteal
0	2 (2.4)	4 (4.9)	0 (0.0)	1 (1.5)	1 (1.5)	0 (0.0)
I	42 (51.2)	49 (59.8)	53 (64.6)	41 (62.1)	45 (68.2)	44 (66.7)
II	18 (22.0)	12 (14.6)	15 (18.3)	11 (16.7)	8 (12.1)	9 (13.6)
III	17 (20.7)	16 (19.5)	14 (17.1)	11 (16.7)	12 (18.2)	11 (16.7)
Missing	3 (3.7)	1 (1.2)	0 (0.0)	2 (3.0)	0 (0.0)	2 (3.0)
Total	82	82	82	66	66	66

performed with statistical software package PASW v18.0 (Chicago, IL).

DNA extraction from dry vaginal swabs

The dry swab specimen from each patient was swirled for 15 s in 1200 µl of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton). Aliquots of 200 µl were prepared. To each aliquot was added 5 µl 10% SDS to final concentration of 0.25%. Fifty units of mutanolysin (25 U/µl) (Sigma, Bornem, Belgium) were added and the samples were incubated for 15 min at 37°C. After the addition of 20 µl Proteinase K (25 mg/ml) the samples were incubated for 15 min at 55°C and vortexed every 5 min. Afterwards, 1800 µl Nuclisens Easymag buffer (BioMérieux, La Balme-les-Grottes, France) was added and the samples were incubated for 10 min at room temperature. Subsequently, 2.0 ml of the processed sample was added to Easymag disposable caps. A 100 µl mixture of magnetic silica and Easymag extraction buffer 3 added. DNA extraction from the pretreated swab medium was then performed on the NucliSENS EasyMAG system according to the manufacturer's instructions. The DNA extracts were stored at -80°C for later use.

Real-time PCR (qPCR)

The qPCR Core Kit for SYBR Green I (Eurogentec, Luik, Belgium) was used and analysis performed on the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA).

Reactions were done in PCR mixtures containing 2.5 µl of DNA extract, 2.5 µl of 10x Reaction Buffer, 3.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.625 U HotGoldStar *Taq* polymerase, 0.75 µl SYBR® Green I, diluted 10-fold in DMSO and the appropriate primer concentration. Core Kit qPCR primers were used. The reaction mixture was adjusted to 25 µl with HPLC grade water. Each run included a standard series, and each sample was run in triplicate. In case the result was not in the range of the standard curve, the samples were diluted tenfold and re-analyzed in triplicate. The median $\log_{10}$ cells/ml was expressed as per 1 ml elution buffer.

Statistical analysis

Data were analyzed under the non-parametric assumption, taking into consideration the $\log_{10}$ [count] distributions of species under study did not approximate the normal distribution. For any given category (grades I-IV), the distribution of concentrations ($\log_{10}$ cells/ml) of each species was expressed as the median count and the accompanying interquartile range (IQR). Between-group comparisons of distributions were performed with the Mann-Whitney U-test for two groups and with the Kruskal Wallis test for multiple groups. Correlations between the different species were determined by the Spearman (rank) test and reported as Spearman's rho value (r). All analyses were performed using SPSS v15 software (Chicago, Illinois).

RESULTS

Cohort characteristics

The baseline demographic characteristics of the 20 HIV^+ and 18 HIV^- women are presented in Table 1. All the participants were sufficiently literate to follow the appointment schedule. For most parameters measured at enrolment, no significant differences existed between the two groups. The HIV^+ women had significantly lower CD4+ T cell counts (p=0.001). Another difference in the groups directly related to the HIV-status was antibiotic prophylaxis intake among the HIV^+ group (p<0.001). The Kenya national guidelines for treatment and care for HIV persons allow continuous antibiotic intake for prevention of malaria and recurrent bacterial infections. Furthermore, 75% of the HIV^+ participants were married compared to 50% HIV^- ones (p=0.01).

The Gram stain and culture results for this population were previously analyzed in Kiama et al. (2014).

Nugent score

Table 2 shows the number of grades I, II and III Gram stains was similar throughout the phases of the menstrual cycle in both study groups.

Fluctuation of the Gram stain score

Table 3 shows the fluctuation in Gram stain score over two menstrual cycles. While BV was virtually absent in HIV-ve women using condoms, half of the HIV+ve women using condoms had a disturbed microbiome on three or more visits. Of the women not using condoms, majority had one or more episodes of BV during the scheduled visits.

Table 3. Percentages of the fluctuation of the Gram stain scores of HIV -ve and HIV +ve women taken at six visits each, spanning two menstrual cycles.

Total Number	HIV -ve		HIV +ve	
	Condom Use			
	No (27)	Yes (6)	No (17)	Yes (24)
Invariably normal	33.3	83.3	47.1	20.8
Single intermediate or BV episode	11.1	16.7	11.8	29.2
Two intermediate or BV episodes	14.8	0	5.8	0
Three or more intermediate or BV episodes	29.6	0	17.6	37.5
Invariably intermediate or BV episode	11.1	0	17.6	12.5

Real-time PCR

Figure 1 shows the results of real-time PCR analysis of different bacterial species based on Gram stain category. There was a high load of *L. iners* in the normal vaginal microbiome, which increased during BV. There were high levels of *L. jensenii* in half of the women with BV, while *L. crispatus* was absent in BV cases. *G. vaginalis* concentration increased progressively from normal to BV microbiome while *Atopobium vaginae* was absent in normal microbiome, but was detectable in intermediate Gram stain and increased progressively during the BV phase.

Figure 2 shows the results of real-time PCR analysis based on HIV status. There were high concentrations of *L. iners* and *G. vaginalis* in both the HIV^+ and HIV^- groups. The frequency and concentration of the protective *L. crispatus* were less in the HIV^+ group of women. *A. vaginae* was present in both groups, but *L. jensenii* was not detectable in the HIV^+ women.

Figure 3 shows the real-time PCR analysis based on phase of menstrual cycle. There was high concentration of *L. iners* and *G. vaginalis* throughout the menstrual cycle. The concentration of *L. crispatus* increased, while the concentration of *A. vaginae* decreased across different phases of the menstrual cycle. *L. jensenii* was not detectable after the initial phase of the menstrual cycle, where its concentration was comparatively low.

DISCUSSION

This is the first study to use a combination of Gram stain and real-time PCR to investigate the vaginal microbiome of African HIV^+ and HIV^- women. Vaginal colonization by Lactobacilli is believed to confer multiple benefits to women, among them being the inhibition of the development of BV, a condition associated with many undesirable effects such as preterm delivery (Hillier et al., 1995; Leitich et al., 2003; Wilks et al., 2004; Fettweis et al., 2019) and increased vulnerability to other STIs (Allsworth et al., 2008; Cherpes et al., 2003; Kaul et al., 2007; Doerflinger et al., 2014; Bayigga et al, 2019), including HIV infection (Cohen et al., 1995; Sewankambo et al., 1997; Taha et al., 1998; Martin et al., 1999). Thus, molecular characterization of the Lactobacilli and other bacteria species that colonize the vaginas of HIV^+ and HIV^- women has a high potential to help in understanding the dynamism of microbiome in health and disease. Both the specific *Lactobacillus* predominance as well as its quantities are crucial for maintenance of the dynamic physiologic balance of healthy vaginal microbiome (De Seta et al., 2019). We found interesting patterns of dynamism of the vaginal microbiome to occur during the menstrual cycle by using real-time PCR to quantify *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae*.

Gram stain and condom use

BV was found to be absent in all HIV-ve women using condoms. This situation was replicated in about 50% of HIV+ve women who also used condoms. Not using condoms appears to predispose to the acquisition of BV. A previous study (Hutchinson et al., 2007) also found the use of condoms to be associated with decreased risk for acquisition of BV as well as the spread BV-associated microflora. It has previously been postulated that the presence of BV increases the risk for HIV acquisition (Wang et al., 2023). It may thus be that the HIV+ve women in this study started using condoms when they already had BV. The presence of BV at the initiation of the study has previously shown to have no impact on incidence (Yotebieng et al., 2009). Recurrence may however be prevented. Condoms can offer protection against BV as well as other sexually-transmitted infections (Fethers et al., 2008).

Real-time PCR analysis based on Gram stain category

Application of real-time PCR to quantify vaginal microbiome has previously been used by other groups. Molecular quantification eliminates culture bias and

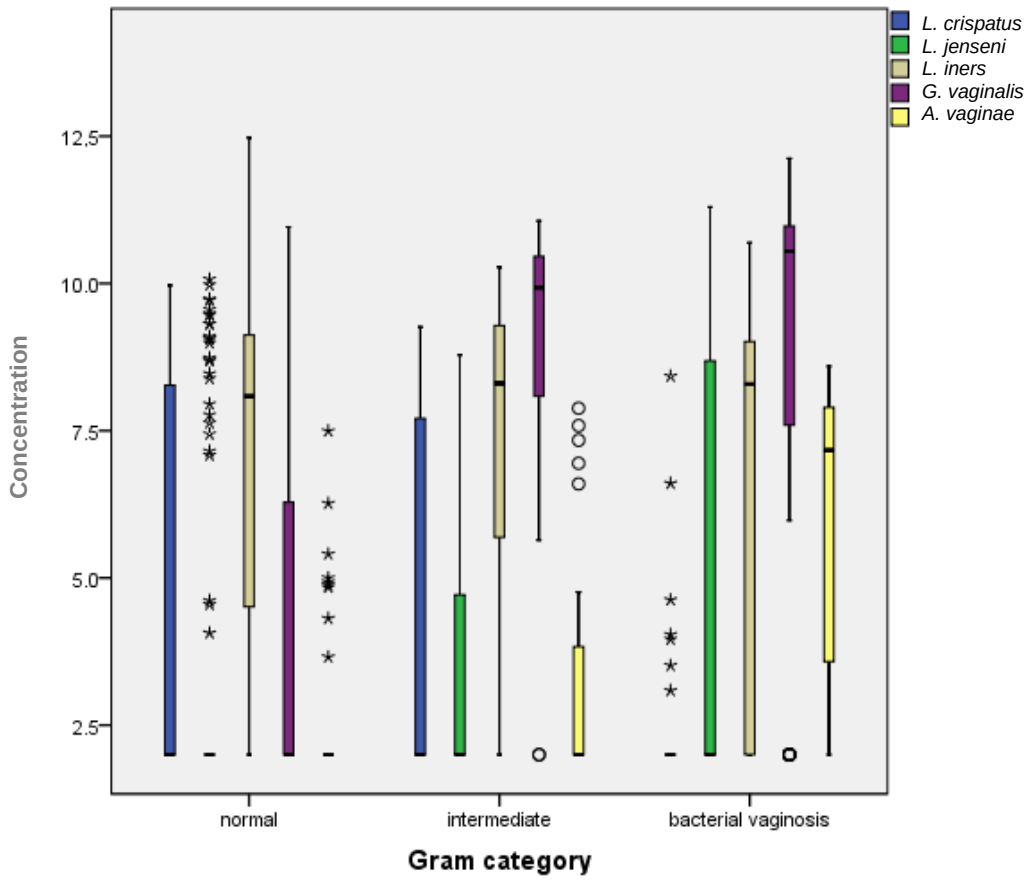

Figure 1. Real-time PCR analysis based on Gram stain category.

provides for objective interpretation of results and also gives more detailed information of resident species irrespective of density, and can lead to the recognition of novel species (Rodriguez-Jovita et al., 1999; Verhelst et al., 2004). It also allows detection of biologically inactive biofilm communities (Swidsinski et al., 2005) that are inevitably missed out by culture methods. In the vagina of healthy premenopausal women, Lactobacilli are the predominant bacteria (Swidsinski et al., 2005; Zhou et al., 2009). However, in this study, we found a high load of *L. iners* to be equally represented in normal, intermediate and BV microbiome. Further, De Backer et al. (2007) found *L. iners* to be increasingly associated with disturbed vaginal microbiome unlike previously held presumption that Lactobacilli are only associated with normal flora. *L. iners* was also present in normal and intermediate Gram stains, which is in agreement with Anukam et al. (2006) who found *L. iners* to be the predominant Lactobacilli in healthy premenopausal Nigerian women (64% of 241). Other studies have also reported *L. iners* to be one of the predominant lactobacilli in vaginal microbiome of BV-free Caucasian women in Sweden (Vasquez et al., 2002) and Canadian women (42% of 19) without symptoms or signs of urinary tract infection (Burton et al., 2003). Although previous studies omitted *L. iners* due to culture bias (De Backer et al., 2007), culture-independent studies have recently shown that *L. iners* is one of the predominant *Lactobacillus* in vaginal microbiome (Vasquez et al., 2002; Fredricks et al., 2005; Zhou et al., 2007). Here, we show that *L. iners* is the predominant *Lactobacillus* in these HIV^+ and HIV^- Kenyan women, irrespective of the Nugent grading of their vaginal microbiome. It was concluded that *L. iners* is part of biologically inactive biofilm community in the vaginal Microbiome.

Also in agreement with De Backer et al. (2007) was the finding that *L. jensenii* was present sporadically in all the Nugent grades. There were high levels of *L. jensenii* in half of the women with BV and very low levels in the women with normal vaginal microbiome. Our results are also in agreement with Anukam et al. (2006) who detected *L. jensenii* only in a few women with normal Nugent scores from their Nigerian study population, using PCR-denaturing gradient gel electrophoresis (DGGE).

In this study, we found *L. crispatus* to be either absent or in very low concentration in women with BV, which is consistent with the known protective role of this species in normal vaginal microbiome (Verstraelen et al., 2009).

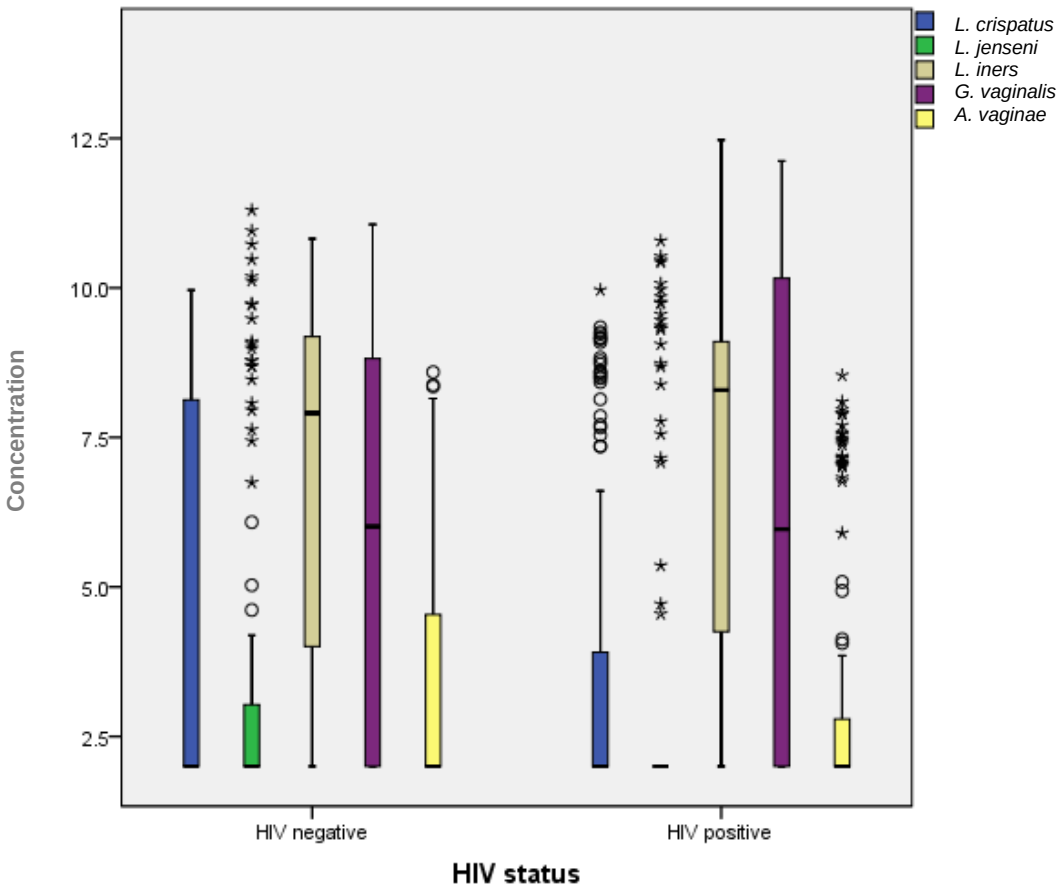

Figure 2. Real-time PCR analysis based on HIV status.

L. crispatus has also been found to offer vaginal colonization resistance against Group B Streptococci during pregnancy (Starc et al., 2022). Although De Backer et al. (2007) found *L. crispatus* to be present in varying concentrations across all Nugent grades, they used a cohort inclusive of pregnant Belgian women. However, the present study specimen collection was based on the menstrual cycle, and most BV cases coincided with the early phase, as was previously shown (Morison et al., 2005). It has also been shown that infrequent specimen collection can lead to underestimation of BV or to omission of the dynamism of vaginal microbiome (Brotman et al., 2010).

L. crispatus and *L. jensenii* showed an interesting inverse relationship where *L. crispatus* decreases from the normal microbiome to almost being undetectable in BV; *L. jensenii* is almost undetectable in normal microbiome but increases progressively to peak at BV microbiome. This study provides evidence to contradict the currently held hypothesis that both *L. crispatus* and *L. jensenii* must be present in normal vaginal microbiome. Vasquez et al. (2002) also found *L. crispatus* to be the single predominant *Lactobacillus* in the vagina. The findings of the present study correspond with those of Hawes et al. (1996) who found that lack of vaginal H_2O_2-producing lactobacilli or presence of only non-H_2O_2-producing lactobacilli were risk factors for acquisition of BV. It appears that *L. jensenii* alone, though known to be a H_2O_2-producer (Hawes et al., 1996; Antonio et al., 1999), is not protective since it was very low in normal microbiome and high in BV. *L. jensenii* has previously been shown to exhibit both poorer colonization strength and poorer colonization resistance, in comparison to *L. crispatus* (Verstraelen et al., 2009). We confirm that it is *L. crispatus* that apparently tips the balance in these women. We propose that in these women only *L. crispatus* is critical in vaginal health, and that possibly, the depletion of this *Lactobacillus* contributes to development of BV. Fettweis et al. (2019) found low concentrations of *L. crispatus* in the vagina to be implicated in preterm delivery. It has previously been suggested that existing differences in microbial communities of Caucasian and African women may well account for discrepancies in their susceptibility to BV and other vagina infections (Royce et al., 1999; Zhou et al., 2007). It is notable that all grades of vaginal microbiome

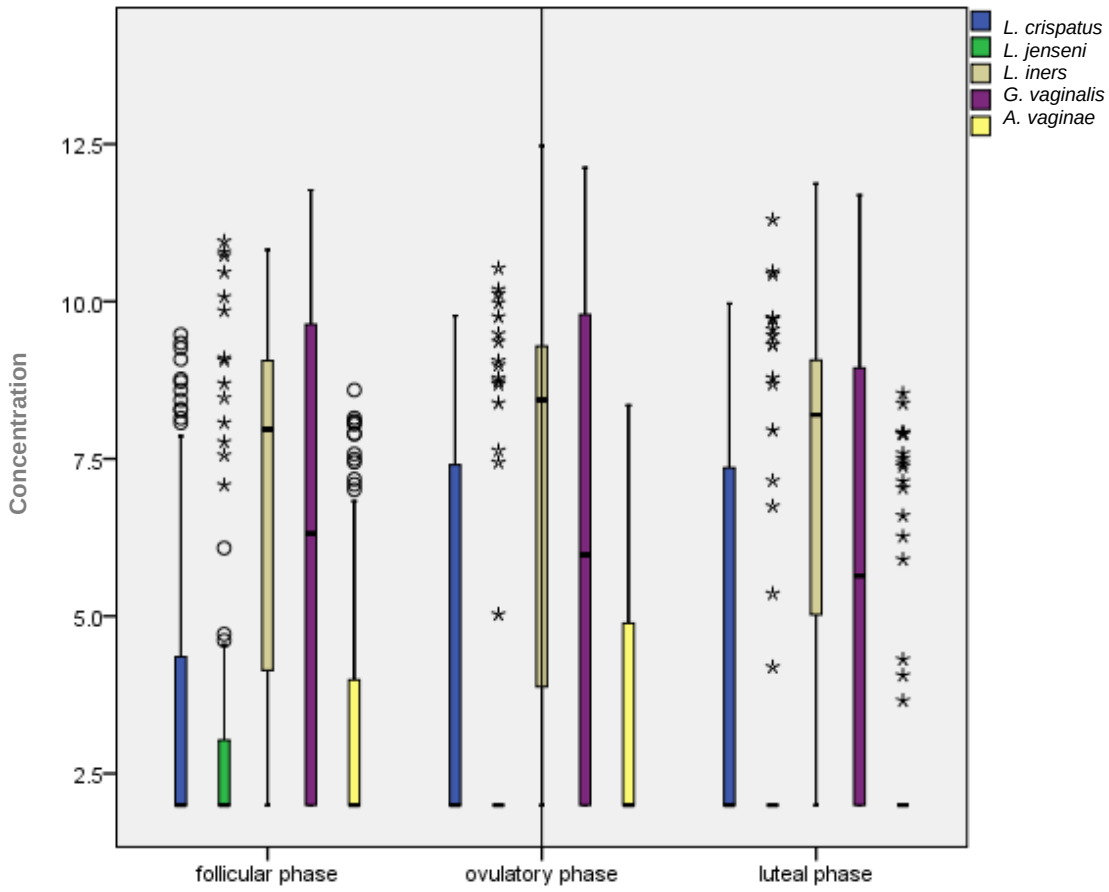

Figure 3. Real-time PCR analysis based on phase of menstrual cycle.

had at least two species of *Lactobacillus*, which is in conformity with the proposal that the function of lactic acid production in vaginal microbiome is highly conserved (Zhou et al., 2007; Ravel et al., 2010), which underscores its importance.

In this study, the concentration of *G. vaginalis* increased progressively to reach a peak in BV-associated microbiome. This finding is in agreement with that of De Backer et al. (2007) whom, also using real-time PCR, found *G. vaginalis* to be present in all grades of the vaginal microbiome. Burton et al. (2003) using DGGE and sequence analysis found that the presence of *G. vaginalis* did not necessarily exclude *Lactobacilli* from the vaginal ecosystem; this contradicts an earlier hypothesis that *G. vaginalis* only occurred where *Lactobacilli* were depleted (Martin et al., 1999; Baeten et al., 2009). We found a strong positive correlation of occurrence between *G. vaginalis* and *L. iners,* irrespective of the Nugent score. It was proposed that these two species possibly contribute to the comparatively higher pH found in women of African descent (Royce et al., 1999; Ravel et al., 2010), which may play a role in the high prevalence of BV in this population. Ravel et al. (2010) found that vaginal bacterial communities not dominated by *L. crispatus* tend to have slightly higher pH values. The vaginal communities in this study population were dominated by *L. iners* irrespective of Nugent grade, which may imply that these women naturally have higher vaginal pH values. This implies that they tend to have a reduced colonization resistance, a factor that could easily predispose to other less resilient species, leading to development of BV. The differences in the *Lactobacillus* communities present in African women as shown in this study and others (Anukam et al., 2006; Ravel et al., 2010) compared to Caucasian women (Eschenbach et al., 1989; Hawes et al., 1996), coupled with sexual habits and practices may together influence the high susceptibility of African women to BV (Bukusi et al., 2006; Allsworth and Peipert, 2007; Brotman et al., 2008). It has been reported that *L. crispatus*, even when accompanied by the other *Lactobacillus* species, offers significant stability to vaginal microbiome in contrast to *L. iners* dominated microbiome (Verstraelen et al., 2009).

The concentration of *A. vaginae* was found to be either very low or absent in normal microbiome, detectable in intermediate Gram stain and increasing progressively

during the BV phase. Other recent studies have documented the presence of *A. vaginae* in vaginal microbiome. Since its discovery (Jovita et al., 1999) and subsequent association with BV (Verhelst et al., 2004; Fredricks et al., 2005), some researchers have proposed that *A. vaginae* can reliably be used to indicate diagnosis for BV (Feris et al., 2004; Burton et al., 2005; De Backer et al., 2007). These suggestions were based on the observation that *A. vaginae* was present in a high percentage of BV patients. Burton et al. (2005) detected *A. vaginae* in 50% of Canadian BV patients and Ferris et al. (2004) found that *A. vaginae*-specific PCR assays were negative in all women with normal vaginal Gram stains (35 women in total). They suggested that *A. vaginae* is rarely if ever a component of normal vaginal flora. De Backer et al. (2007) found high concentrations of *A. vaginae* in grade III samples. In this study, we found in addition that *A. vaginae* had an inverse relationship with *L. crispatus*. In BV microbiome where *A. vaginae* was the highest, *L. crispatus* was absent or not detectable, confirming these earlier reports. It is notable that in our results, the PCR analysis of the normal microbiome resembles that of the luteal phase, confirming earlier reports that vaginal microbiome is most unstable in the early phase of the menstrual cycle (Keane et al., 1997; Eschenbach et al., 2000; Morison et al., 2005). The very low presence of *A. vaginae* in normal microbiome and the subsequent resurgence in BV microbiome may be explained by Walker's hypothesis (Walker, 1992) of microbial community structures referred to as "drivers and passengers" theory. It postulates that for an ecosystem to function there are "driver" species (in our case *L. crispatus*) that strongly influence the community structure where they occur. Other species in the community constitute "passenger" species that have no major influence. Further, Ravel et al. (2010) postulated that undetectable members of a community (e.g. *A. vaginae* in normal Gram stain) may serve as a "seed bank" of species whose numbers multiply when favourable conditions arise, as would happen for example in this case when *L. crispatus* becomes depleted for one reason or another (in the BV-associated microbiome).

Real-time PCR analysis based on HIV status

So far only a few studies have compared the vaginal microbiome of HIV^+ and HIV^- women (Spear et al., 2008; Spear et al., 2011; Apalata et al., 2021). This is the first study to compare the vaginal microbiome of African HIV^+ and HIV^- women, sampled severally during the menstrual cycle, using culture-independent methods. All the HIV^- women had CD_4 counts above 500, except one with 488. 56.1% of the HIV^+ women had CD_4 counts of 250≥ while the rest had a count above 500, and none were on antiretroviral therapy. Analysis of PCR results based on HIV status showed a high concentration of *L. iners* and *G. vaginalis* in both the HIV^+ and HIV^- groups. Although condom use was significantly higher in HIV^+ women, the number of women harbouring the protective *L. crispatus* as well as the concentration of *L. crispatus* were remarkably lower compared to HIV^- women. *A. vaginae* was present in both groups, but *L. jensenii* was not detectable in the HIV^+ women. Spear et al. (2011) also found *L. jensenii* to be absent or present at relatively low levels in all except one out of 46 women studied. Since *L. jensenii* is known to have poor colonization resistance (Verstraelen et al., 2009), it is possible that increased biodiversity in HIV^+ women (Spear et al., 2008; Kiama et al., 2014) leads to the displacement of this species. We did not find that HIV infection influences the incidence of BV, which is in agreement with earlier reports (Greenblatt et al., 1999; Watts et al., 2006). However, other researchers (Apalata et al., 2021) found a higher incidence of BV in HIV^+ women compared HIV^- ones based on Nugent scoring. Since it has been shown that vaginal microbiome dominated by non- *Lactobacillus* bacteria increases the risk of HIV infection (Wang et al., 2023), it can be concluded that it is the absence of Lactobacillus that causes the higher incidence of BV in HIV^+ women. The observation that both groups of women in the current study harbored *L. crispatus* and *A. vaginae* may explain why we did not find differences in the prevalence of BV between them, since *A. vaginae* was suggested to be a reliable indicator for BV. It appears that *L. iners* and *G. vaginalis* are a fixed structure of the vaginal communities of these women irrespective of the prevailing circumstances. It is suggested that the short-term temporal dynamics of vaginal communities are determined by the species proportions of *L. crispatus* and *L. jensenii*, which in turn determine the relative resistance and resilience to the inevitable challenges of systemic homeodynamism, local disturbances as well as pertinent individual behaviors, habits, and practices. Given such an ecosystem, opportunistic and pathogenic species will more likely gain a foothold in communities that exhibit low stability (Hobbs and Huenneke, 1992; Verstraelen *et al*., 2009). This is a likely situation where *L. iners* predominates, since it is known to offer less colonization resistance due to low H_2O_2 production (Eschenbach et al., 1989; Hillier et al., 1993; Verstraelen et al., 2009), coupled with the observation that such communities tend to have slightly higher pH values (Ravel et al., 2010) and are less stable than those where *L. crispatus* predominates (Verstraelen et al., 2009).

Data obtained from HIV^+ pregnant indicate that a disturbed vaginal microbiome raises the risk for horizontal transmission of the virus (Frank et al., 2012). The current study however used non-pregnant reproductive-age women. It has been found that the presence of BV increases the risk for acquisition of HIV as well as other sexually transmitted infections because of the compromised integrity of the vaginal mucosal barrier (Doerflinger et al., 2014; Bayigga et al., 2019).

Real-time PCR analysis based on phase of menstrual cycle

Analysis based on the phase of the menstrual cycle, showed a high concentration of *L. iners* and *G. vaginalis* throughout the menstrual cycle. In all the three angles of analysis done here, these two species appear present in high concentrations. We propose that they are a fixed structure of the vaginal communities of these women irrespective of the prevailing circumstances. The unaltered presence of *L. iners* in normal vaginal microbiome of African women was also document by other researchers (Jespers et al., 2017). The concentration of *L. crispatus* increased while concentration of *A. vaginae* decreased throughout the menstrual cycle. This trend is exactly a reversal of what we observed in PCR analysis based on Gram stain scores. It has been previously reported that vaginal microbiome tends to be less stable during the early phase of the menstrual cycle (Keane et al., 1997; Eschenbach et al., 2000; Morison et al., 2005). This instability appears to be coupled to the decrease and subsequent displacement of *L. jensenii* and *A. vaginae* from follicular through to the ovulation phase. *L. jensenii* was not detectable after the initial phase of the menstrual cycle where it was comparatively low. Dynamic systemic changes in hormonal levels occurring during the cycle are usually accompanied by dramatic changes in the vaginal epithelium that influence the type and volume of secretions, the pH as well as the adherence capabilities of resident communities in the face of menstrual flooding (Owen, 1975; Brotman et al., 2008; Farage et al., 2009). During the follicular phase all the three lactobacilli studied were present together with *G. vaginalis* and *A. vaginae*. This state of vaginal microbiome is mirrored by the intermediate Gram stain category. It appears to be a transient stage that the microbiome goes through before the full effects of the systemic and local changes take their full effect. Immediately after, in the ovulatory phase, instability is seen in the displacement of *L. jensenii* and the accompanying steady concentration of *A. vaginae*. The luteal phase is driven by increased secretion of two groups of steroids, namely progesterones and estrogens (Owen, 1975; Farage et al., 2009). The qPCR Figure 3 of the vaginal microbiome during this phase resembles closely the one obtained from the normal microbiome where both *L. jensenii* and *A. vaginae* were either absent or undetectable. We provide evidence here that the instability witnessed in the early stage of the menstrual cycle is due to decreasing levels of *L. jensenii* as well as increasing levels of *A. vaginae*. Other researchers found this trend to be associated with increased concentrations of proinflammatory cytokines (Jespers et al., 2017).

Srinivasan et al. (2010) observed that during menses the population of *G. vaginalis* increased alongside that of *L. iners* while quantities of *L. crispatus* and *L. jensenii* decreased simultaneously. Similar observations were made in an earlier study (Schwebke et al., 1997). The former authors also observed that *G. vaginalis* was present in women with and without BV. *G. vaginalis* contains vaginolysin that can perforate erythrocytes to release iron and activate immune markers of the vaginal epithelium (Gelber et al., 2008), causing the inflammation observed in BV. Vaginolysin belongs to cholesterol-dependent cytolysins produced by organisms that colonize and cause disease at mucosal surfaces (Tweten, 2005). The implication is that in these women inflammation of the vaginal epithelium constantly poses the danger of development of BV. Toxin production has been shown to be essential for maintenance of colonization and pathogenesis of invasive disease (Tweten, 2005). Systemic effects of hormones may then only contribute to ongoing inflammation at the vaginal epithelium.

Conclusions

This study shows that *L. iners* is the predominant *Lactobacillus* in these HIV^+ and HIV^- Kenyan women, irrespective of the Nugent grading of their vaginal microbiome. This study provides evidence to contradict the currently held hypothesis that both *L. crispatus* and *L. jensenii* must be present in normal vaginal microbiome. We propose that only *L. crispatus* is critical in vaginal health, and that possibly, the depletion of this *Lactobacillus* contributes to development of BV. We suggest that *L. iners* and *G. vaginalis* are a fixed structure of the vaginal communities of these women irrespective of the prevailing circumstances. Condom use was found to be protective against BV.

The results showed that *A. vaginae* had an inverse relationship with *L. crispatus*. In BV-associated microbiome where *A. vaginae* was the highest, *L. crispatus* was absent or undetectable. On the other hand, the concentration of *L. crispatus* increased while the concentration of *A. vaginae* decreased throughout the menstrual cycle. We confirm that the instability observed in the vaginal microbiome during the early phase of the menstrual cycle is coupled to the decrease and subsequent displacement of *L. jensenii* and *A. vaginae* from follicular through to the ovulation phase.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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