Effect of periconceptual vaginal microbiota disruption on fecundability among Kenyan women planning pregnancies

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A dissertation

submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Washington

2019

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Program Authorized to Offer Degree:

Epidemiology

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Abstract

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INTRODUCTION: Vaginal microbiota disruption is a common condition that is associated with sexually transmitted infection and HIV acquisition, spontaneous abortion, preterm birth, and poor in-vitro fertilization outcomes. Whether vaginal microbiota disruption is associated with non-medically assisted reproduction has not been thoroughly explored. This dissertation addresses the hypothesis that disruption of the optimal, *Lactobacillus* dominated vaginal microbiota during the periconception period is associated with reduced fecundability among HIV-negative Kenyan women.

METHODS: These analyses leveraged data from a prospective case-cohort study assessing the impact of periconceptual vaginal microbiota on women's risk of preterm birth (Chapter 2). At monthly preconception visits, participants reported the first day of last menstrual periods and recent sexual behavior, underwent a urine pregnancy test, and provided vaginal swab specimens. Discrete time proportional probabilities models with robust standard errors were used to estimate

fecundability ratios (FR) and 95% confidence intervals (CI) among menstrual cycles with and without vaginal microbiota disruption assessed using non-molecular methods of detection. Three non-molecular methods of detection were assessed including bacterial vaginosis (BV) by Nugent score (≥7) (Chapter 3), elevated sialidase activity (≥0.25 μg) detected by a point of care test for BV (Chapter 3), and cultivation of *Lactobacillus* on Rogosa agar (Chapter 4).

RESULTS: Among 273 eligible women, 35.5% (n=97) had BV, 33.3% (n=91) had elevated sialidase, and 30.8% (n=84) had no cultivable *Lactobacillus* at enrollment. Participants contributed 768 menstrual cycles and 128 became pregnant. The six-menstrual cycle cumulative pregnancy rate was 69.3% (95%CI 61.5-82.7). Neither BV (adjusted FR [aFR] 0.95, 95%CI 0.66-1.35), elevated sialidase (aFR 1.12, 95%CI 0.79-1.59), nor cultivable *Lactobacillus* (aFR 1.14, 95%CI 0.77-1.68) at the visit prior to each pregnancy test were significantly associated with fecundability. However, when considering two consecutive measurements per discrete menstrual cycle, the per-cycle probability of pregnancy among menstrual cycles with persistent BV was associated with a 34% reduction in fecundability that trended toward significance compared to cycles that were BV negative at both visits (aFR 0.66, 95%CI 0.42-1.03, p=0.07). In addition, the per-cycle probabilities of pregnancy in menstrual cycles with cultivable *Lactobacillus* at one or both consecutive visits were a non-significant 1.1-1.6 fold higher compared to cycles negative for *Lactobacillus* at both visits. Lastly, menstrual cycles with *Lactobacillus* morphotypes detected on Gram stain at both consecutive measurements had a 52% increased per-cycle probability of pregnancy that trended toward significance (aFR 1.52, 95%CI 0.97-2.41).

CONCLUSIONS: There were no significant associations between BV by Nugent score, elevated sialidase, or cultivable *Lactobacillus* at the visit prior to pregnancy testing and fecundability. However, the secondary analyses assessing the stability of the vaginal microbiota across two time points for each menstrual cycle provide some evidence that persistent vaginal microbiota

disruption may contribute to reduced fecundability, and that *Lactobacillus* may be associated with modestly increased fecundability. If persistent vaginal microbiota disruption is associated with reduced fecundity, this could have important implications for a large number of women who wish to conceive due to the high global prevalence of BV and infertility. Importantly, these results are preliminary as the target sample size was not reached by the analysis date. Future studies should consider utilizing both culture-dependent and molecular methods of detection to explore the association between the presence, viability, and functionality of vaginal bacteria and fecundability.

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ACKNOWLEDGEMENTS

Completion of this dissertation would not have been possible without the encouragement and dedication of my dissertation committee, the Microbiota and Preterm Birth (MPTB) participants and study team, the UW Department of Epidemiology, my fellow Epidemiology PhD students, and my family.

Thank you to my dissertation committee - Dr. Scott McClelland, Dr. Lisa Manhart, Dr. Charles Muller, and Dr. James Hughes - for their guidance, for providing important content expertise, and for their enthusiastic support of my training and this project. I would like to express my sincerest gratitude to the Chair of my committee, Dr. Scott McClelland, for his outstanding mentorship throughout my doctoral training, for the opportunity to lead the launch of the MPTB study, and for his constant promotion of my training as a STI/reproductive health epidemiologist. I would also especially like to thank Dr. Lisa Manhart for her ongoing mentorship throughout my graduate training starting in 2013. I have learned so much from her.

This research would not have been possible without the generosity and commitment of the MPTB study participants. In addition, thank you to the study teams in Nairobi and Mombasa, Kenya for their collaboration, for their rigorous work ethic, and for their warm welcomes during my trips to the research sites.

Thank you to Dr. Sheila Lukehart, Dr. Anna Wald, and the Center for STD/AIDS Research Training Fellowship Program for supporting three years of my predoctoral work and investing valuable time and resources to my academic training.

I am also grateful to Dr. Anjuli Wagner and Dr. Jennifer Balkus, who kindly provided me with guidance, epidemiological insights, STATA code, and friendship along the way.

To my fellow Epidemiology PhD cohorts, especially Dr. Emily Deichsel, Dr. Jennifer Velloza, Dr. Darcy Rao, and Dr. Jessica Long – It has been an honor to train alongside y'all, both in the classroom and in the mountains!

To my family…thank you to my mom, Cynthia, for passing on the gift of writing and for introducing me to public health all those years ago. To my dad, Roald, for passing on his scientific curiosity and for reading my general exam and the manuscripts I sent home just to see what I was up to. To my brother and sister, Neil and Elaine, for the comic relief.

And finally, to Jeff, who has been the calm presence by my side throughout this whole experience. On to the next adventure!

CHAPTER ONE - Introduction

Considerable efforts have been made to address unmet family planning need and improve maternal and neonatal health in low and middle income countries (1–3), but efforts to address infertility in these settings have received comparatively little support. Globally, infertility is considered low-priority due to a high burden of other competing health priorities, an emphasis on family planning to reduce unwanted pregnancies, and a lack of awareness about the burden of infertility and its consequences (4,5). Yet, per the World Health Organization's (WHO) Director of Reproductive Health and Research in 2016, "While infertility is rarely considered a national health priority by governments, its impact on the lives of individuals, couples, and their families…are enormous" (6). Therefore, efforts to understand and address preventable causes of infertility, reduce barriers to care, and improve awareness and education hold potential for substantial global public health benefit. **To that end, this dissertation addresses the overall hypothesis that disruption of the optimal, *Lactobacillus* dominated vaginal microbiota during the periconception period is associated with reduced fecundability among Kenyan women planning to conceive.**

Infertility: Global Burden, Causes, Psychosocial Implications, and Barriers to Care

Global Burden: Global infertility prevalence and trends over time are challenging to estimate. Inconsistent definitions of infertility (i.e., length of time at risk for pregnancy), the ultimate purpose for the estimate (i.e., burden or individual risk), whether the outcome is failure to conceive or failure to have a live birth, and limited data on male factor infertility contribute to the estimation challenge (4,7,8). Clinicians and epidemiologists use a different definition of infertility than demographers. Clinically, infertility is the failure to become pregnant despite well-timed condomless sex for 12 months (9). Globally, the median prevalence of clinical infertility is

estimated to be 9% (range: 3.5% to 16.7%), equating to more than 72 million couples (10). Demographic definitions of infertility estimate the absence of a live birth in women at risk for pregnancy after a defined period of at-risk time, and typically estimate infertility prevalence using demographic and reproductive health surveys (8). Demographic estimates range from 73 million women globally (11) to as high as 186 million ever-married women in developing countries alone (12).

Infertility can be described as primary or secondary. Primary infertility is the inability to conceive or have a first live birth, while secondary infertility is the inability to conceive or have a live birth after a prior pregnancy, independent of the outcome of the first pregnancy (7,13). Globally, secondary infertility prevalence is higher than primary infertility prevalence and disproportionally affects couples in developing countries (11,12,14). Secondary infertility is driven by pregnancy and delivery complications, reproductive tract infections (i.e., post-abortion or postpartum infection, STIs), and increasing maternal age (11,15). The first WHO/World Bank Report on Disability estimates that infertility due to maternal sepsis and unsafe abortion is the fifth highest burden of disability in women of reproductive age in low and middle income countries, affecting 32.5 million in 2011 (16).

Causes of Reduced Fecundity and Infertility: Fecundity and infertility are related, but distinct, terms. **Fecundity** is the "biologic capacity for reproduction, irrespective of pregnancy intentions" (9) and is assessed through a variety of outcomes including puberty onset, ovulation characteristics, menopause, hormonal profiles, semen quality, and conception. Fecundity can be studied by estimating **fecundability**, the per-menstrual-cycle probability of pregnancy, utilizing time-to-pregnancy (TTP) studies. While fecundability varies by study population, approximately 58-80% of couples become pregnant within six months of beginning to try and 75-95% conceive by 12 months (17–20). Delayed TTP is a marker of impaired fecundity and may be an indication

of underlying infertility (9). By studying TTP and fecundability, it may be possible to identify preventable causes of infertility. As noted earlier, **infertility** is defined as not becoming pregnant (sometimes lack of a live birth) after a defined period of time at risk for pregnancy (9).

Infertility diagnostics in women may include assessment of medical, reproductive, and menstrual history, tubal patency, hormonal profiles to assess ovulatory reserve, ovulatory function, and endometrial receptivity, and transvaginal ultrasonography (21,22). Biological causes of female infertility are described in the following paragraph. Men typically undergo semen analysis and often endocrine evaluation (21,22). Briefly, male factor infertility is driven by abnormalities in sperm production, sperm function, or ductal obstruction of a genetic, immunologic, hormonal or anatomical nature (e.g., congenital anomalies). Female and male factor infertility contribute equally to underlying fertility, and in many couples both the female and male partner are sub-fecund (20,23). Overall, while causes contributing to an infertility diagnosis can be identified in the majority of cases, up to 15% have unknown etiologies (23).

Biological causes of female factor infertility are hormonal, ovulatory, tubal, uterine, and cervical in nature (21,22). A frequent cause of infertility is ovulatory dysfunction presenting as absent or irregular ovulation, or luteal phase deficiency. Anovulation is common in women with high or low body mass index (BMI) and in women with polycystic ovarian syndrome (21). Other women may have primary ovarian insufficiency prior to the typical age for menopause. Tubal factor infertility (TFI) is estimated to contribute to approximately 25% of infertility cases (24). The majority of female factor infertility diagnoses in sub-Saharan Africa relate to tubal factors (14). Tubal factor infertility can be caused by pelvic inflammatory disease (PID) attributable to *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and potentially some bacterial vaginosis (BV) associated bacteria (25–31). Inflammation caused by these infections can lead to Fallopian tube obstruction and pelvic-peritoneal adhesions (24,31). Cumulative risk of TFI after

one episode of PID increases with disease severity (26) and with delayed treatment (32). *Mycoplasma tuberculosis* and schistosomiasis infections involving the reproductive tract have been associated with infertility, though diagnosis and estimating prevalence is challenging (31,33,34). These pathogens may contribute to unexplained infertility in settings where these infections are endemic. Post-abortion, intrapartum, and postpartum infections are common causes of secondary female factor infertility, particularly in sub-Saharan Africa (11,15). Less frequent causes of female factor infertility include abnormal uterine morphology, endometriosis, fibroids, and cervical mucus-sperm interaction abnormalities (21).

Advanced age and extremes of BMI are consistent contributors to both female and male sub-fecundity and infertility. In women, fecundity begins to decline around age 32, and more rapidly after age 37, reflecting decreases in egg quality and hormonal changes (19,35). Older women are also more likely to experience miscarriage (35). The evidence for reduced fecundity in older men is less strong, but increasingly of concern given age-effects on sperm and testicular function (19,36,37). Body mass index, both underweight and obese status, is also an important contributor to reduced fecundity and infertility (28,36,38–40). In women, hormonal changes due to both underweight and obese status are implicated in ovulatory dysfunction.

There is also a robust literature linking numerous lifestyle and environmental factors to reduced fecundity. Maternal and paternal smoking are consistently associated with reduced fecundity through mechanisms such as negative effects on sperm quality and ovulatory dysfunction (36,41,42). Approximately 13% of female infertility may be attributable to smoking (42). Preconception caffeine intake, alcohol use, maternal stress, diet, and sleep patterns may be associated with reduced fecundability (22,36,43–47), though data are more limited, and these associations are not observed in all studies. Chemicals and metals from environmental or occupational exposures, including cadmium, lead, mercury, and some perfluorochemicals and

polychlorinated biphenyls, have been associated with reduced fecundity in both males and females (48,49).

Psychosocial & Relationship Implications: Individuals suffering from infertility may experience poor mental health, including stress, feelings of guilt or shame, anxiety/depression, and suicide (4,12,15,50–53). In societies with high infertility associated stigma, women may experience a loss of social status and isolation, intimate partner and familial violence, divorce, and economic instability (4,12,15,50–54). This is particularly true in areas of the world where the social cost of childlessness is high, such as sub-Saharan Africa (4,50–52).

Barriers to Care: Cultural and religious beliefs, internalized and external stigma, and high costs are major barriers to infertility care (4,5,15,51). In addition, fear of diagnosis, lack of information, and misinformation can delay or prevent effective sub-fertility care (5,51). For example, in sub-Saharan Africa, use of traditional treatments, such as herbs and supplements, is common (53). An additional barrier to infertility care is the perception that it is "the woman's problem", which persists despite equal proportions of male versus female factor infertility (5,51,52). This gender bias increases the stigma women face and reduces effectiveness of biomedical intervention if the male partner refuses to participate. In addition, in societies and relationships where a woman has low financial autonomy, her ability to seek effective care without the support of her male partner is reduced (51,53). Lastly, while couples struggling to conceive can utilize assisted reproductive technologies including in-vitro fertilization (IVF), these services are expensive, frequently require multiple attempts, and are often unsuccessful. Access to infertility treatment is particularly limited sub-Saharan Africa (5,6,55).

Bacterial vaginosis and infertility

The vaginal microbiome is a complex, polymicrobial ecosystem that exhibits temporal changes within and between women, and becomes more diverse around menses, with exposure to semen, and in women with new or multiple sex partners (56–58). Bacterial vaginosis is characterized by disruption of the optimal, *Lactobacillus* dominated vaginal microbiota through the loss of lactobacilli and an overgrowth of anaerobic bacteria and other microorganisms (59). Bacterial vaginosis can be assessed clinically using Amsel's criteria or microbiologically using the criteria of Nugent and Hillier (60,61). Over the last 15 years, molecular techniques (e.g., broad-range 16s rRNA gene PCR with next generation sequencing and taxon-directed quantitative PCR) have contributed to the discovery of novel BV-associated microorganisms and descriptions of distinct vaginal microbial communities (56–58). Bacterial vaginosis is associated with increased risk of acquiring STIs including *N. gonorrhoeae*, *C. trachomatis* (62–64)*, M. genitalium* (65), *Trichomonas vaginalis* (66)*,* and salpingitis, endometritis, and PID (67–70). In turn, these infections are associated with ectopic pregnancy and tubal factor infertility (25–27,31,71).

Research to date on the relationship between BV and infertility is predominately among populations of infertile women seeking assisted reproduction services in the United States, Europe, and the Middle East. Cross-sectional studies comparing the prevalence of BV between infertile women and fertile women suggest that infertile women have a higher BV prevalence (72–78). In a non-randomized study of infertile women with polycystic ovarian syndrome (PCOS) or unexplained infertility receiving ovulation stimulation (but not undergoing IVF), the proportion of women with BV at baseline who become pregnant in six months was higher in couples who received monthly BV treatment with secnidazole compared to untreated couples (unexplained infertility: 24.5% versus 14.3%, p=0.04; PCOS: 49.1% versus 23.5%, p=0.001) (74). In contrast, a meta-analysis of pregnancy outcomes associated with BV among women undergoing IVF found that while BV was not associated with biochemical pregnancy (β-hCG detection in serum), clinical

pregnancy, or live birth rate, BV was associated with early spontaneous abortion at <12 weeks (RR: 1.68, 95%CI: 1.24-2.27) (77). Overall, these data suggest that BV may influence fertility outcomes in women undergoing IVF, but data remain sparse. Furthermore, the effect of BV in women trying to conceive naturally has not been characterized.

Hypothesized mechanisms linking disrupted vaginal microbiota and reduced fecundity

Vaginal microbiota disruption present near the time of conception may contribute to reduced fecundity and infertility through a number of biological mechanisms that might affect fertilization and implantation (**Figure 1.1**).

<u>Bacterial vaginosis effect on cervical mucus and inflammation in the upper reproductive tract</u> - Cervical mucus is a physiochemical barrier that prevents bacterial ascension from the vagina the upper reproductive tract (79). Sialidase produced by bacteria associated with BV, such as *Bacteroides spp., Prevotella spp.,* and *Gardnerella vaginalis* (80–83), is a mucin degrading enzyme. A breakdown of the cervical mucus can promote ascension of pathogenic vaginal bacteria into the upper reproductive tract. Sub-clinical inflammation of the upper reproductive tract or clinical PID associated with a disrupted vaginal microbiota may have deleterious effects on Fallopian tubes. Bacterial vaginosis and BV-associated bacteria have been associated with both clinical and sub-clinical PID (29,30,68–70,84–86), but the relationship between BV-associated PID and infertility is less clear (30,86).

In addition to impacts mediated through disruption of cervical mucus, ascension of vaginal bacteria may also exert a deleterious effect on implantation, most likely by causing endometrial inflammation. The interaction between the vaginal and endometrial microbiota, the uterine immunological environment, and success of implantation and early fetal development is a growing area of interest in assisted reproduction (87–90). Studies using molecular methods to assess the

vaginal or endometrial microbiota in women undergoing IVF have generated mixed findings. A higher vaginal bacterial diversity measured by the Shannon Index on samples collected prior to IVF cycles or at embryo transfer has been associated with decreased rates of clinical pregnancy (90) and live birth (90,91), but not biochemical pregnancy (90). In one of these studies, women with high concentrations of *Gardnerella vaginalis* or *Atopobium vaginae* detected by quantitative PCR (qPCR) had a lower clinical pregnancy rate (90). In two small studies assessing endometrial microbiota, a *Lactobacillus*-dominated endometrial microbiota was associated with increased implantation, pregnancy, and live birth rates in one study (92), but was not associated with ongoing pregnancy in another study (93). These conflicting findings may be explained by small sample sizes, different sampling locations, timing, and methods, and different pregnancy outcomes.

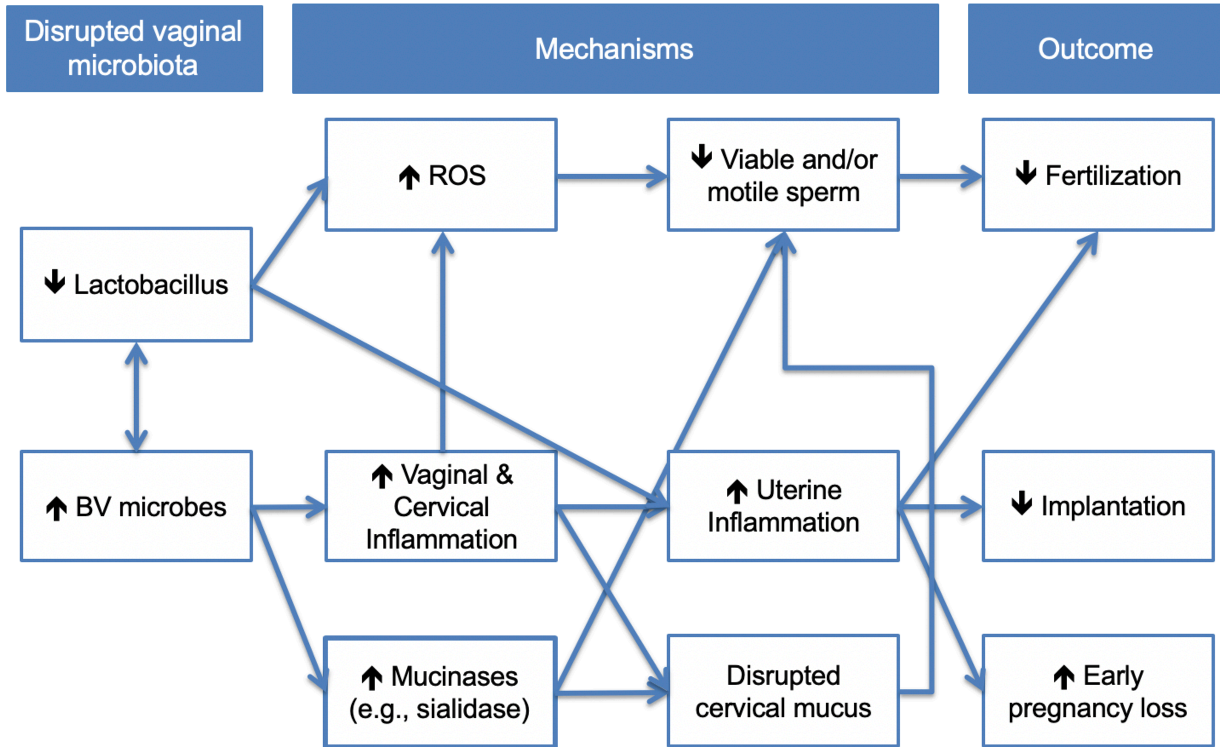
Bacterial vaginosis effect on sperm function – While sperm are protected by antioxidants and other proteins in the seminal fluid while in the vagina (94), microorganisms associated with a disrupted vaginal microbiota may exert deleterious effects on sperm at the cervix and in the upper reproductive tract where sperm are no longer protected by seminal fluid. In addition, the microarchitecture of healthy cervical mucus facilitates transportation of sperm from the vagina to the uterus and provides a barrier to sperm with poor motility or abnormal morphology (94,95). Cervical mucus may also play a role in sperm capacitation, an integral step in the sperm activation process (96). Damage to the physical and chemical properties of cervical mucus induced by vaginal microbiota disruption may, therefore, impede sperm transport from the vagina to the uterus. Moreover, while low levels of reactive oxygen species (ROS), such as nitric oxide and H_2O_2, are important for normal reproductive function (ex: sperm capacitation) (97,98), at increased levels such as during an inflammatory response, ROS can lead to oxidative stress, which in turn can cause sperm DNA fragmentation and reduce sperm motility (97). Lastly, sperm are coated in sialic acid, which mask cells from the immune system (99). High levels of sialidase

produced by BV-associated microorganisms may degrade sialic acids coating sperm, exposing these cells to the female immune response and leading to a decrease in fertilizing potential.

Dissertation: Hypothesis, Data, and Outline

The research to date and compelling biological mechanism hypotheses suggest that vaginal microbiota disruption may be associated with infertility and poor IVF outcomes. However, there is no data from a population of women without an infertility diagnosis who are planning to conceive through non-medically assisted reproduction. In addition, no studies have yet assessed the association between vaginal microbiota disruption and fecundability. Therefore, to fill this gap, this dissertation addresses the overall hypothesis that disruption of the optimal, *Lactobacillus* dominated vaginal microbiota during the periconception period is associated with reduced fecundability. This project utilized rigorously collected longitudinal data from a case-cohort study in Kenya assessing the impact of periconceptual vaginal microbiota on women's risk of spontaneous preterm birth (MPTB Study, R01 HD087346; PI McClelland) (100). **Chapter Two** describes the MPTB Study protocol. The PhD Candidate served as Study Coordinator and Data Manager for the MPTB Study throughout her doctoral work. **Chapter Three** examines the association between a disrupted vaginal microbiota, as measured by Gram stained vaginal specimens using the Nugent and Hillier criteria (≥7), and elevated sialidase as measured by positivity on a point of care test for BV, and fecundability. **Chapter Four** reports the results of an analysis assessing the association between cultivable *Lactobacillus* as a marker for optimal vaginal health and fecundability. **Chapter Five** provides an overall conclusion to this dissertation and outlines potential next steps. Overall, this dissertation aims to contribute to our understanding of infectious, and potentially preventable, causes of reduced fecundity and infertility.

Figure 1.1. Hypothesized mechanisms linking disrupted vaginal microbiota and decreased fecundity



CHAPTER TWO - *Impact of periconceptual vaginal microbiota and women's risk of spontaneous preterm birth: Protocol for a prospective case-cohort study*

NOTE: This chapter describes the MPTB Study protocol (R01 HD087346, PI McClelland). The PhD Candidate developed this protocol manuscript in consultation with Dr. Scott McClelland and in alignment with the original grant application and study specific procedures developed during the study (100). Throughout her doctoral work, the Candidate served as Study Coordinator and Data Manager for the MPTB Study. The fecundability analyses reported in Chapters 3 and 4 leverage preconception data from this parent study.

BACKGROUND

Globally, approximately 10% of births occur prior to 37 weeks of gestation and are considered preterm. Rates are as high as 18% in some low-resource countries (101). Preterm birth and its sequalae is the leading cause of death among children under five (102). Approximately 30% of preterm births are medically indicated. These deliveries are preceded by complications such as pre-eclampsia, fetal distress, and intra-uterine growth restriction, and require induction of labor or cesarean section. The majority of preterm deliveries are spontaneous preterm births (SPTB), and occur with or without preterm premature rupture of membranes (103). The cause of SPTB is often not known, but up to 40% may be associated with infection, such as urinary tract infections, sexually transmitted infections (STI) including HIV, and systemic infections (103,104). Other risk factors include socio-demographic characteristics (age, race, education) (103), extremes of body mass index (105), periodontal disease (106), pregnancy history (inter-pregnancy interval less than six months, prior SPTB), psychological characteristics (stress, depression), and substance use (alcohol, smoking) (103). Further elucidation of the causes of SPTB may provide insight into novel approaches for reducing the risk of early deliveries (100).

Bacterial vaginosis is a vaginal condition characterized by a shift from an optimal *Lactobacillus*-predominant microbiota to one characterized by high concentrations of diverse anaerobic species (59). Global prevalence of BV is as high as 30% in the general population (107). Numerous studies have shown that BV during pregnancy is associated with increased risk of SPTB (108). However, a meta-analysis of clinical trials of treatment of BV during pregnancy concluded that while antibiotic treatment prior to 20 weeks gestation was efficacious for eradicating BV, the risk of subsequent SPTB was not significantly reduced (109).

Molecular microbiology has transformed our understanding of vaginal microbiota composition from a binary differentiation between optimal microbiota and BV using Gram stain and Nugent score or clinical criteria (Amsel's criteria) (60,61), to a much broader spectrum of phenotypes. These phenotypes range from "low-diversity *Lactobacillus*-dominated bacterial communities to a heterogeneous group of high-diversity BV-associated communities (56)…Individual vaginal species have also emerged as strongly associated with BV (110), particular symptoms (57), and distinct metabolic profiles (111)" (100).

Molecular microbiological approaches are being used to characterize the relationship between the vaginal microbiota and SPTB. Small studies, primarily in the US and Europe, consistently identify a predominance of *Lactobacillus* species and decreased vaginal bacterial community diversity during pregnancy (112–116). Studies exploring the relationship between the vaginal microbiota and SPTB have yielded conflicting findings. Some found significant associations between increased species diversity and preterm delivery (117–121), while others have not (122–124). A number of studies have found that women with vaginal bacterial communities characterized by low relative abundance of *Lactobacillus* species and more diverse community state types with higher relative abundance of bacteria typically linked to BV, such as *Gardnerella vaginalis*, may be at higher risk of preterm birth (121,124–127). However, others found no

association between vaginal bacterial community type and preterm birth (117,119,122). In a recently published study of a large cohort of pregnant women, higher relative abundance of *Sneathia sanguinegens*, *Mobiluncus curtisii/mulieris*, *Megasphaera*, *Mageeibacillus indolicus*, *Porphyromonas asaccharolytica*, *Prevotella buccalis*, and *Atopobium spp* were associated with SPTB, particularly among African American women (124). Other studies have found that detection or higher concentrations of specific vaginal bacteria were associated with preterm delivery including *Leptotrichia/Sneathia* (121,128), BV-associated bacterium 1 (BVAB1) (121,128), *Megasphaera* (128), *G. vaginalis* (129,130), *Atopobium vaginae* (129), TM7-H1 (121), and some *Prevotella* species (121). Variability in results across studies assessing the vaginal microbiota and SPTB risk may be due to differences in race/ethnicity, underlying risk of SPTB, sampling timing and frequency, and gestational age of the preterm births under study (earlier versus later).

No previous study has addressed the hypothesis that the vaginal microbiota detected close to the time of conception may be mechanistically more tightly linked to SPTB than the vaginal microbiota during pregnancy (100). "Bacteria present around the time of conception could compromise the protective effects of cervical mucus (131), gaining access to the surface of the endometrium before fetal membrane development (132). These bacteria could, in turn, colonize and cause low-level inflammation in the decidua, placenta, fetal membranes, or amniotic cavity (133,134)" (100). To address this hypothesis, the Microbiota and Preterm Birth Study (MPTB) was established to enroll HIV-negative Kenyan women with immediate fertility intent into a prospective case-cohort study with frequent vaginal fluid sampling (100). The three aims of the MPTB study are to:

1) Compare the species diversity and richness of the vaginal microbiota sampled close to the time of conception in women with SPTB versus term delivery using broad-range 16S rRNA gene PCR and next generation sequencing.
2) Compare the presence and concentration of select bacterial genera/species based on published data (128) and results of Aim 1 using qPCR assays, comparing their presence

and concentrations in vaginal specimens sampled close to the time of conception in women with SPTB versus term births.

3) Perform species-specific qPCR assays on samples collected from fetal membranes and histological examination of membranes and umbilical cord to determine if vaginal bacteria ascend to the upper genital tract and cause inflammation in the fetal membranes and umbilical cord.

METHODS/DESIGN

Study design, setting, and timeline

The MPTB Study is a prospective case-cohort study. Eligible HIV-negative Kenyan women who are planning to become pregnant are enrolled into a prospective cohort and will be selected into the case-cohort sample, as detailed in the analysis section. Women are enrolled prior to conception and are followed through preconception, pregnancy, delivery, and until six-weeks postpartum. Study sites are located in Nairobi, at the Couples Counseling Center at Kenyatta National Hospital (KNH), and in Mombasa, at the Ganjoni Health Center with deliveries at Coast Provincial General Hospital (CPGH). Study enrollment began in Nairobi in April 2017 and in Mombasa in April 2018. Enrollment will continue through approximately December 2019.

Eligibility criteria and recruitment strategies (100)

The target population is HIV-negative women who are currently planning to become pregnant. Additional eligibility criteria include being ≤45 years old, having had a menstrual period in the prior three months or recently discontinued contraceptive methods that induce amenorrhea (implant, hormonal intrauterine device [IUD], depo medroxyprogesterone acetate injectable [DMPA]), willing to comply with study procedures, planning to remain in the study area, and able to provide informed consent. Minors aged 14-17 are only eligible if they are emancipated under Kenyan law. For women with known HIV-positive partners, their male partner must have a documented undetectable HIV viral load, or the participant must be taking pre-exposure prophylaxis (PrEP).

Additional exclusion criteria are current pregnancy, using contraception other than condoms for HIV/STI prevention, having a DMPA injection in the last three months, history of cervical or uterine surgery other than colposcopy, cryotherapy, loop electrosurgical excision procedure, or cesarean section, known autoimmune disease, antibiotic use in the prior four weeks, and history of seeking care for infertility.

Potential participants are recruited by study staff or referred by outside healthcare providers at multiple sites providing reproductive and maternal health care services, including family planning clinics, pediatric immunization clinics, safer conception clinics for HIV-serodiscordant couples, and HIV voluntary counseling and testing clinics. The study specifically targets recruitment of women attending family planning clinics to discontinue contraceptive implants or IUDs for the purpose of becoming pregnant. Health providers and study participants who refer potentially eligible women are reimbursed 500 Kenyan shillings (KSh; approximately $5.00) per referral to compensate their time and effort.

Study visits and procedures

Study participation takes place in six phases including screening/enrollment, periodontal examination, preconception, pregnancy, delivery, and postpartum (**Figure 2.1**) (100). Specimen collection, laboratory testing, and other clinical procedures are summarized in **Table 2.1**.

Screening and enrollment

Following written informed consent for screening, study staff perform rapid HIV testing using blood from a fingerstick according to Kenyan Guidelines (135). Potential participants also provide urine for a pregnancy test. A brief structured interview is conducted in English or Kiswahili to assess eligibility, as described previously. Eligible women can enroll immediately upon providing written informed consent. If enrollment does not occur on the day of screening, the participant provides

another urine sample for pregnancy testing to re-confirm eligibility prior to enrollment procedures. Enrollees complete a structured face-to-face interview regarding demographics, sexual behavior, substance use, depression symptoms (Patient Health Questionnaire-9), and reproductive, contraceptive, and medical history. A study clinician performs a general physical examination and speculum-assisted pelvic examination. If a woman is menstruating, the examination is deferred until completion of menses to avoid sampling when the vaginal microbiota undergoes rapid changes (136). Two vaginal fluid specimens are collected by rolling push-off Dacron swabs (FitzCo, Inc) three rotations against the lateral vaginal wall; these are stored for vaginal microbiota and vaginal inflammatory response evaluation. Additional genital specimens are collected for STI diagnosis [*N. gonorrhoeae, C. trachomatis, and T. vaginalis* by nucleic acid amplification testing (NAAT)], vaginal and cervical Gram stains, detection of prostate specific antigen (PSA) and elevated sialidase using a chromogenic point-of-care diagnostic test for BV. A vaginal specimen is also inoculated directly onto Rogosa agar for detection of cultivable *Lactobacillus*.

Syndromic management for STI is provided if indicated based on reported symptoms and identification of clinical signs, following WHO and Kenyan National Guidelines for Management of STIs (137). Additional therapy is provided at the first preconception visit based on NAAT results for STIs. Symptomatic BV is treated according to standard of care.

Women receive health counseling on a variety of preconception and pregnancy topics, including the importance of smoking cessation, refraining from vaginal washing, and maintaining a healthy diet. Study staff also evaluate participants' menstrual cycle history, discuss menstrual characteristics (i.e, regularity), and identify the probable fertile window using calendar-based methods. Ovulation is estimated to occur 14 days prior to the first day of the next predicted menses, with the most fertile days emphasized as the five days before and day of ovulation (138). Participants are also provided prenatal vitamins.

Periodontal Exam

Periodontal disease has been associated with SPTB (106) and oral microbiota have been detected in the placenta of women with SPTB (139). To account for this risk factor, each participant receives a periodontal examination. A periodontist at the University of Nairobi (UoN) Dental School conducts the examinations in Nairobi and trained a CPGH dentist to conduct the periodontal examinations for the Mombasa site. A bi-annual calibration process occurs, in which the UoN periodontist and a second UoN periodontist conduct periodontal exams on a series of patients to compare results. The primary UoN periodontist involved in the study then travels to Mombasa, where he serves as the gold standard comparator for the CPGH dentist. Intra-examiner assessment occurs at least once every fifty study examinations and involves repeating the examination on one participant to compare results between the first and second examination.

To avoid periodontal changes that occur with pregnancy, periodontal examinations are scheduled within four weeks of enrollment. Participants receive dental hygiene education and complete a modified version of the WHO's oral health questionnaire (140). Prior to the examination, subgingival plaque samples are collected with sterilized paper points at six sites (one paper point per site), representing molars, premolars, and anterior teeth. In a fully dentate patient, the Ramjord teeth are used. When partially dentate, a representative set of teeth at a minimum of four sites are used. Samples are stored at -80°C. Following sample collection, participants undergo oral examination, including the periodontal examination, Decay-Missing-Filled Index, and Gingival Index (140). Using a periodontal probe, the periodontist measures pocket depths and clinical attachment for diagnosis of periodontitis. The presence and severity of periodontitis are defined using the 2007 Centers for Disease Control and Prevention/American Academy of

Periodontology case definitions (141). Urgent dental care needs (i.e., fillings, extractions, root canals) identified on examination are addressed at no cost to participants.

Monthly Preconception Visits

Participants return at one-month intervals while trying to become pregnant. At each preconception visit, a structured interview is conducted to update sexual behavior, reproductive, and medical history. A urine pregnancy test is performed. Women self-collect vaginal swabs (with assistance if needed) for vaginal microbiota and inflammatory response analysis, vaginal Gram stain, PSA detection, *Lactobacillus* culture, and sialidase detection. Participants with genital symptoms are treated for STIs using syndromic management per Kenyan Guidelines (137). Counseling is provided to reinforce messages about healthy preconception behaviors. Participants are provided prenatal vitamins at each visit.

Women whose pregnancy test is positive are scheduled for a first trimester visit between nine and twelve weeks of gestation. Most women who remain non-pregnant after six months exit the study. Women who discontinued DMPA less than six months prior to enrollment are eligible for nine months of preconception trying time due to the delayed return to fertility after DMPA discontinuation (142,143).

First Trimester Visit (9-12 weeks of gestation)

At the first trimester visit, a structured interview is conducted to update information on sexual behavior and reproductive and medical history. Women self-collect vaginal swabs (with assistance if needed) for vaginal microbiota and inflammatory response analysis, vaginal Gram stain, PSA detection, and *Lactobacillus* culture. Women then undergo an obstetrical ultrasound examination to confirm gestational age. The ultrasound is conducted by sonographers at KNH in Nairobi and at a private radiology facility in Mombasa. Prior to conducting study ultrasounds, all

sonographers received a standardized refresher training on ultrasound determination of gestational age in the first trimester, early pregnancy complications, and MPTB study specific procedures. For each participant, an image of the crown rump length is saved to the MPTB Study Quality Assurance website. This website was adapted from the ultrasound quality assurance web platform developed for the Global Network for Women's and Children's Health Research *First Look* trial by RTI International with input from MPTB study staff (144). First trimester ultrasound image and crown rump length measurement quality are assessed by a Kenyan radiologist according to criteria developed for the International Fetal and Newborn Growth Consortium for the 21st Century (145). Sonographers are able to access the results of quality assurance assessments through the website and receive ongoing feedback from the Kenyan radiologist. The radiologist holds an annual meeting with sonographers in Nairobi to review quality metrics and provide refresher training on study procedures and quality assurance criteria and conducts ongoing one-on-one quality support to the Mombasa sonographers by phone.

Using the participant’s reported last menstrual period (LMP) and ultrasound derived gestational age, the participant’s estimated date of delivery is determined. Using the American College of Obstetrics & Gynecology’s 2014 guidelines, the ultrasound derived gestational age is utilized if it differs from that calculated using the LMP by more than 7 days before 16 weeks (146). If a later ultrasound is obtained, the sonographic dates are used if they differ from LMP dates by more than 10 days between 16 and 22 weeks, by more than 14 days between 22 and 28 weeks, and by more than 21 days after 28 weeks (100).

Pregnant participants are enrolled in a two-way short message service (SMS) system described in the ‘*Retention During Pregnancy’* section. They are also referred to routine antenatal care (ANC) at a clinic of the woman’s choosing. For participants who attend ANC at KNH, Ganjoni Health Center, or CPGH, data (i.e., HIV and syphilis results, haemoglobin, blood pressure) are

abstracted directly from their ANC records. If participants attend ANC elsewhere, their data are abstracted at the postpartum visit from their Mother & Child Health Booklet. This booklet is a resource provided to all pregnant women in Kenya that contains their ANC and delivery information.

If a participant suffers a miscarriage prior to or identified at the ultrasound visit, they are referred for obstetrical care. Management of the pregnancy loss is conducted by non-study clinicians according to the standard of care. Women who lose a pregnancy prior to 20 weeks of gestation are eligible to remain in the study if they would like to try for another pregnancy. Women who choose to re-enter for a second pregnancy attempt can return for up to six additional monthly preconception visits. Women who miscarry twice are not eligible to return for a third preconception attempt; they are referred for further obstetrical evaluation.

Retention During Pregnancy

To enhance retention, participants are offered enrollment into an adaptation of an automated two-way SMS platform that was initially designed to support HIV-positive Kenyan women during pregnancy and postpartum (147). Messages are sent at 16, 20, and 24 weeks gestation, bi-weekly beginning at 28 weeks, and weekly from 38 weeks through six weeks postpartum. In addition, study nurses call participants weekly starting at week 35 of gestation to confirm planned delivery location and ascertain whether delivery has occurred. Participants are instructed to call at the onset of labor so study staff can coordinate collection of delivery samples for deliveries occurring at KNH or CPGH, and to assist with identification of the delivery date for births occurring at other facilities (100).

Delivery Procedures

Obstetrical care during delivery follows standard obstetrical procedures and is not conducted by study staff. If a participant delivers at the Labor and Delivery (L&D) Ward at KNH or CPGH, delivery samples are collected. Delivery samples are not collected for participants who deliver at other facilities. Trained L&D nurses collect the samples (training details below). Upon delivery of the placenta by vaginal or caesarean delivery, it is placed in a sterile container. Once the participant and neonate are stable, samples are collected as soon as feasible and within two hours of delivery. Using sterile technique, a pair of trained nurses collects samples from between the amnion and chorion (148) (**Figure 2.2**). A sterile push-off swab (FitzCo, Inc) is collected from between the membranes, placed in a cryovial, and placed in a -4°C (Nairobi) or -20°C (Mombasa) freezer in the L&D ward for short-term storage. The placenta is placed in 10% neutral buffered formalin. The nurses notify study laboratory staff when samples are collected. Laboratory staff collect the samples and transport them to study laboratories at KNH and CPGH within 24 hours during the week. Samples obtained on weekends are transported to the laboratory on Monday morning. Fetal membrane swabs are transferred to a -80°C freezer. Laboratory staff also collect placental samples for histopathology. These include a four-centimeter fetal membrane roll from the ruptured edge of the membranes to the edge of the placental disc, a one-centimeter section of umbilical cord starting three centimeters from the placental disk, and a one-centimeter block of placenta with overlying membranes adjacent to the site of cord insertion (**Figure 2.2**). All pathological specimens are stored in 10% neutral buffered formalin.

Labor and delivery nurses were initially trained by Dr. Lannon, who developed the fetal membrane collection technique (148). Subsequent trainings are led by a Kenyan obstetrician-gynecologist based at KNH, who participated in the initial training by Dr. Lannon. The trainings take place in three steps. First, the trainer demonstrates sample collection between the fetal membranes for

vaginal and cesarean deliveries, and sample storage. Second, each nurse demonstrates collection of the fetal membrane swabs during a practical session. Third, each nurse completes a verbal quiz to confirm understanding of the steps. Refresher trainings are conducted as needed.

To capture additional details of pregnancy and delivery, study staff abstract data from L&D records including type of delivery (i.e., vaginal or caesarean; labored or did not labor; spontaneous or induced labor), pregnancy and delivery complications, live birth or stillbirth, baby's birth weight, and HIV test results, if conducted, during labor.

Postpartum Visit (Six Weeks)

Prior to their postpartum visits, participants are reminded to bring their delivery discharge report and their Mother & Child Health Booklet. At the study visit, study clinicians abstract any ANC and delivery details not previously captured through review of ANC and delivery records. Participants complete an interview about the infant's heath status, symptoms associated with illness, and immunization status. If participants are unable to come to the research clinic, the interview is conducted at a home visit or by phone.

Incentives

Participants receive 300 KSh (about $3.00) at enrollment, the periodontal exam, and each preconception visit. Women who become pregnant receive a free obstetrical ultrasound and 300KSh at the first trimester visit. Those who deliver at KNH or CPGH receive 1000KSh. Participants completing the postpartum interview receive 300KSh. These amounts are provided to compensate women for their transportation and communication costs, time, and effort. All women who deliver are offered a baby-item, such as a swaddling blanket, in recognition of their dedication to the study.

Laboratory methods (100)

Microscopy, Lactobacillus culture, STI testing, detection of sialidase, and detection of PSA in vaginal secretions

Vaginal Gram stained slides are evaluated for BV using the criteria of Nugent and Hillier (60). Saline and potassium hydroxide wet mounts are examined for the presence of motile trichomonads, clue cells, yeast, and sperm. Endocervical Gram stained slides are scanned at low power, and polymorphonuclear leukocytes in three nonadjacent oil immersion fields are counted and averaged to evaluate cervical inflammation. Clinicians inoculate vaginal specimens directly on Rogosa agar for detection of cultivable *Lactobacillus* species and store the plate in a candle jar until transportation to the laboratory (149). Hydrogen peroxide (H_2O_2) production is evaluated by subculture of *Lactobacillus* isolates on tetramethylbenzidine (TMB) agar containing horseradish peroxidase (150). A vaginal specimen is tested for *N. gonorrhoeae, C. trachomatis,* and *T. vaginalis* by NAAT (Aptima Combo-2 CT/NG Detection System and Aptima *Trichomonas vaginalis* assay, Hologic Corporation). One vaginal swab is used for detection of sialidase using a commercially available point of care diagnostic test for BV (Diagnosit BVBlue; Gryphus Diagnostics). The minimum detectable level of sialidase is 0.25 μg, and samples above this concentration are considered positive (151). Testing for PSA in vaginal samples is performed with a commercially available assay (ABAcard, Abacus Diagnostics), which can detect semen for 24-48 hours after condomless sex (152).

Molecular methods for identification of vaginal bacterial species (100)

Stored vaginal swabs for bacterial PCR will be transported on dry ice to the Fredricks Laboratory at the Fred Hutchinson Cancer Research Center (Fred Hutch) in Seattle, WA. “MoBio DNA extraction kits will be used to extract and purify DNA from vaginal swabs. This protocol uses bead beating and chaotropic lysis to break apart bacterial cells and recover DNA that is free of PCR inhibitors…Swabs that have not contacted a human surface will be processed in parallel to serve

as sham DNA extraction (negative) controls. Extracted DNA will be subjected to broad range 16S rRNA gene PCR using primers that anneal with highly conserved regions of the small subunit rDNA gene, amplifying a 470 base pair segment that contains a highly variable sequence useful for species identification. Bar coded primers will be used to multiplex samples (129). Libraries of 16S rDNA gene amplifications will be mixed for sequencing on the Illumina MiSeq platform using 300 bp paired-end reads. The assembled reads will be binned into individual study samples using the nucleic acid bar codes. Approximately 10,000-30,000 sequence reads will be generated per sample, providing robust detection of minority species in the vaginal bacterial community. Results will be analyzed using an internal analysis pipeline developed in the Fredricks laboratory specifically for this purpose. Chimeras will be detected and deleted. Sham extraction controls will also be processed in parallel to exclude bacterial contamination of reagents (100)". Bacterium-specific qPCR assays will also be performed. This study focuses on key species hypothesized to be associated with increased risk of SPTB, such as BVAB1, *Megasphaera,* and *Sneathi*a. The final set of species/genera tested will be fine-tuned following analysis of the deep sequencing data from Aim 1. "A standard exogenous jellyfish qPCR amplification control will be used to assess for PCR inhibitors (130)…A broad-range bacterial 16S rDNA gene qPCR assay will be used to measure total bacterial load in each sample (100)."

Histopathological examination of fetal membranes, placenta, and umbilical cord samples

Hematoxylin and eosin staining of membrane rolls, placental samples, and umbilical cord sections are examined according to published guidelines by an experienced Kenyan pathologist (Dr. Mandaliya) (131). "Acute and subacute inflammatory lesions will be graded and staged for both maternal and fetal components. Chronic inflammatory lesions will be characterized including any observation of chronic deciduitis or the presence of decidual plasma cells (100)."

Data collection and management

Clinical and laboratory data are collected on paper case report forms. Data are entered into a REDCap database on a daily basis through secure servers. The REDCap database is password protected and only accessible to authorized study staff. Weekly and monthly reports are generated by the data manager to support study retention efforts, monitor progress, and perform quality control checks on key exposure and outcome data.

Statistical analysis (100)

Sample size estimate and generating the case-cohort population

The largest sample size is required for Aim 2, so this was used to guide sample size estimation for the study. The prospective case-cohort "analysis set will include three women who delivered at term for each one with SPTB…A standard case-control method was employed to compute power (153)…[Assuming] three primary species/genera of interest (e.g. BVAB1, *Megasphaera,* and *Sneathia*), Simes' methodology was utilized to fix a type-1 error rate adjusted for three tests (154)…A sample of women with 80 SPTB and 240 term births would provide ≥80% power to detect a statistically significant 2.8-fold or greater difference in the odds of detecting a preconception vaginal bacterial species/genus in women with SPTB versus term delivery, assuming ≥10% prevalence of the organism at preconception visits in those delivering at term (100)".

To accrue the overall target of 80 SPTB cases, a total cohort of approximately 1100 women will be enrolled (100). Once 80 SPTB cases occur, the sample for the case-cohort will be defined. First, cases of spontaneous abortion (<20 weeks gestation) and medically indicated preterm births will be excluded. Next, "a random sample of the remaining women with delivery data will be selected such that, when added to the remaining SPTB cases, the full case-cohort population will have a term birth to SPTB ratio of 3:1…A sampling fraction f, with f solved using the formula: $nf +$

*(1-f)*80=320*, where *n* is the total number of women (cases and non-cases) with delivery data, will be used to select the random sample…[Lastly], all of the remaining SPTB cases will be added to the random sample creating the full case-cohort sample (100)".

Statistical analysis plan

The goal of Aim 1 is to characterize and compare preconception vaginal species diversity and richness between women with SPTB versus term birth. "All women with SPTB and a random sample of the same number of women with term birth from the case-cohort sample will be included…To describe the overall frequency and relative abundance of species, cumulative rank abundance plots will be generated for each group (155)…We will compare the cumulative distribution vaginal bacterial taxa between women with SPTB versus term birth using the Kolmogorov-Smirnov test. Rarefaction curves will be used to evaluate species richness (number of taxa at a 97% sequence similarity cutoff defining an operational taxonomic unit) in women with SPTB versus term birth…Finally, we will assess species diversity using the Shannon Diversity Index (156) and species richness using the Chao1 richness estimator (157) by comparing the mean values between women with SPTB versus term birth…To further explore the relationship between bacterial species relative abundance and SPTB, we will perform logistic regression with SPTB status as the outcome and species rank abundance percentage for each species separately. We will first determine the score statistics (with SPTB status as the outcome) for each variable, then rank the variables from largest to smallest score statistic. Next, we will perform the logistic modeling on each of these in rank order of score statistic until we reach a p-value of 0.2 in univariate logistic regressions. These data will be examined to refine targets for the primary hypothesis test in Aim 2 (100)."

For Aim 2, based on the literature and informed by Aim 1 results, "bacterial taxa will be selected for evaluation using qPCR assays, comparing their presence and concentrations in vaginal

specimens sampled periconceptually…These analyses will utilize data from the full case-cohort sample and will be weighted to account for the case-cohort sampling scheme. For each bacterial taxon, we will first perform unadjusted logistic regression to examine the association between the presence of that taxon and the risk of SPTB. Multivariable logistic regression analysis will be used to determine the independent contributions of bacterial species to the risk of SPTB. Species associated with SPTB in univariate analyses ($p<0.10$) will be included in the multivariable regression model after addressing collinearity (100)". A manual forward stepwise model building approach will be used to address confounding. "For the three species selected for the primary hypothesis test, $p<0.033$ will be considered statistically significant, using the Simes' correction for multiple comparisons (100)". Other bacterial taxa may be evaluated but will be considered exploratory. Additional exploratory analyses will be performed to investigate the relationship between periconceptual quantities of specific bacteria and risk of SPTB.

In Aim 3, the mechanism for the hypothesized association between vaginal bacterial species present at conception and SPTB will be explored by examining the association between specific vaginal bacteria detected both in preconception samples and in fetal membranes. This analysis will also evaluate the association between bacteria identified in the fetal membranes and histological evidence of deciduitis, chorioamnionitis, and funisitis. "The analysis will use data from the women in the case-cohort sample who contribute fetal membrane, placenta, and umbilical cord samples…[There are] three different binary exposures, defined as periconception detection of each of the three species tested for the primary hypothesis in Aim 2. The set of binary outcomes includes detection of the same bacterial species in fetal membranes, deciduitis, chorioamnionitis and funisitis (100)". Odds ratios will be estimated for each exposure using unadjusted logistic regression models, and multivariable logistic regression analyses will be utilized to adjust for potential confounding factors.

Ethics and dissemination plan

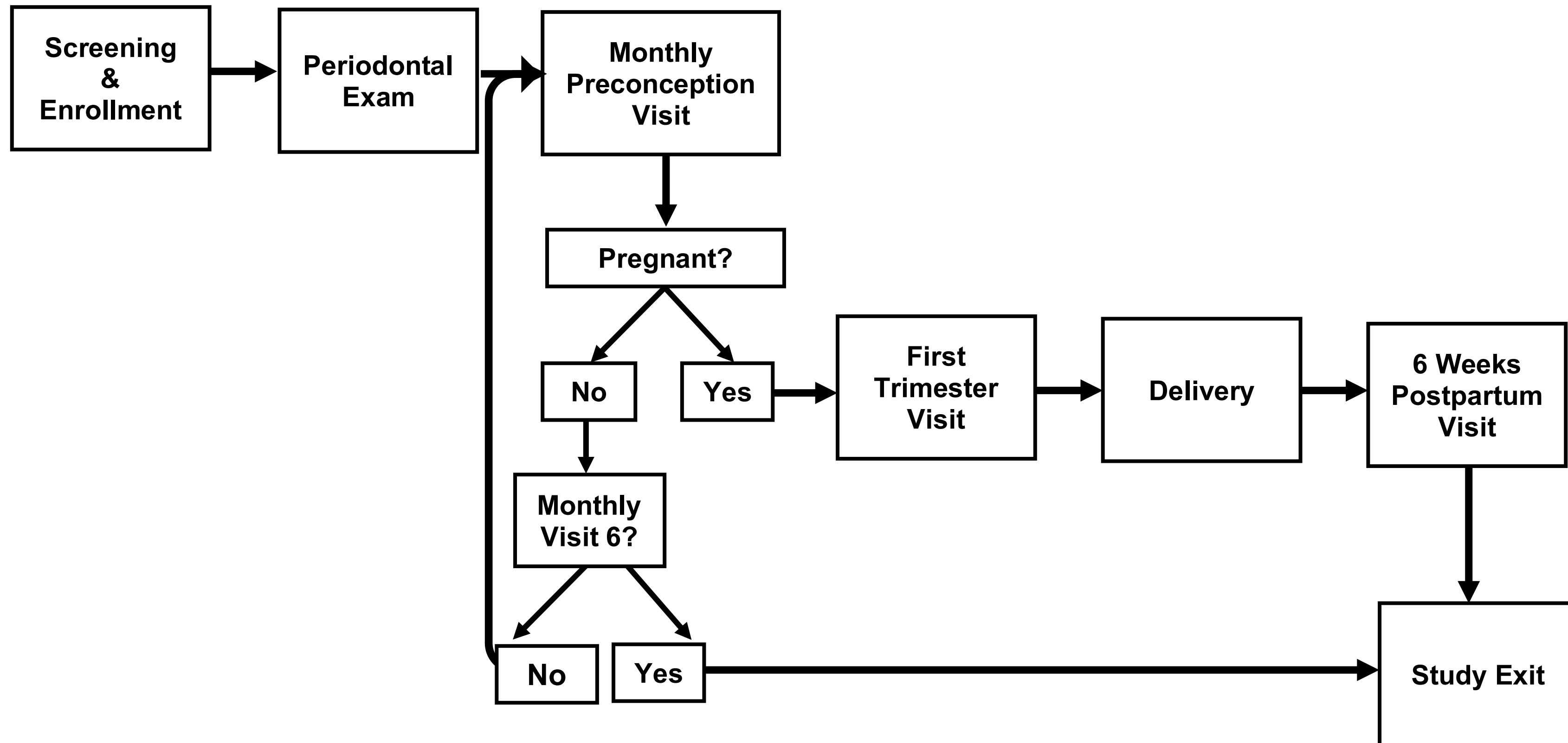
This study was approved by the ethics committees at Kenyatta National Hospital/University of Nairobi Ethics and University of Washington. There are inherent risks to both women and fetuses during pregnancy and delivery, but participation in this observational study does not increase these risks. Study staff do not conduct antenatal or delivery care. The risks of participating in this study are minimal. Risks include discomfort associated with the fingerstick for HIV testing at screening, sensitive questions about sexual behavior, the pelvic examination, and genital sample collection; stress associated with an HIV or STI diagnosis detected during the study; and breach of confidentiality. To minimize risks associated with participation, experienced study staff counsel participants on potential risks and explain that they are able to withdraw from the study at any time. The risk for breach of confidentiality is minimized by securing informed consents with patient names in locked cabinets in a restricted area, assigning participants a study identification number to de-identify their data, locking participant research files in a restricted area, and utilizing password-protected and encrypted computers to access and analyze the data (100). The results of this study will be published in peer-reviewed journals, disseminated to clinicians at study sites and partner institutions, and presented at local and international conferences.

DISCUSSION

While there are many known risk factors for SPTB, most cases occur without a known cause (103). Numerous studies have shown that BV during pregnancy is associated with increased risk of SPTB, but clinical trials of BV treatment in pregnancy have shown minimal or no reduction in subsequent risk of preterm birth (109). The MPTB Study addresses the hypothesis that the vaginal microbiota detected during the periconception period may be linked to SPTB (100). The findings could shift the paradigm for thinking about the mechanisms linking vaginal microbiota and prematurity, and could be used to guide the development and evaluation of interventions

aimed at lowering the risk of SPTB by identifying and eradicating high-risk vaginal bacteria prior to conception (100,158–160).

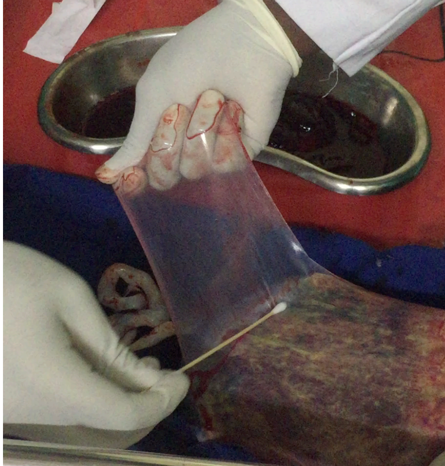
Figure 2.1. Microbiota and Preterm Birth Study Phases



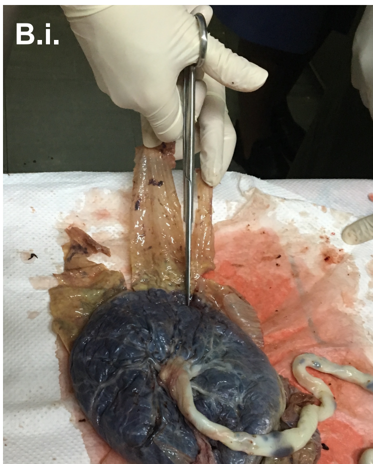
Additional participant pathways: 1) Participants who discontinued DMPA injectable contraception within 6 months of study enrollment are eligible for 9 months of preconception follow-up. 2) Participants who miscarry are eligible to re-enter preconception follow-up for a second pregnancy attempt. Participants who miscarry twice are not eligible to enter for a third preconception attempt. They are exited and referred for obstetrical consultation.

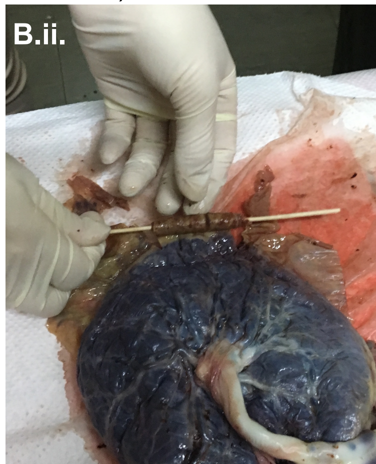
Figure 2.2. Fetal membrane swab and placenta sample collection

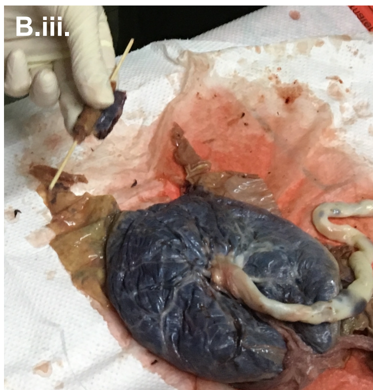
A. *Fetal membrane swab sampling (Rolling the swab between the amnion and chorion.)*

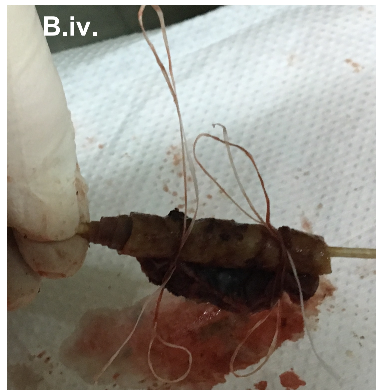


B. *Fetal membrane roll (i. Cutting strip of membranes from the ruptured edge toward the placental disc. ii. Rolling the membranes. iii. Cutting off the roll including a section of the placental disk. iv. Using dental floss, loosely tie around the membrane roll. Carefully push the roll off the wooden stick and store in formalin.)*

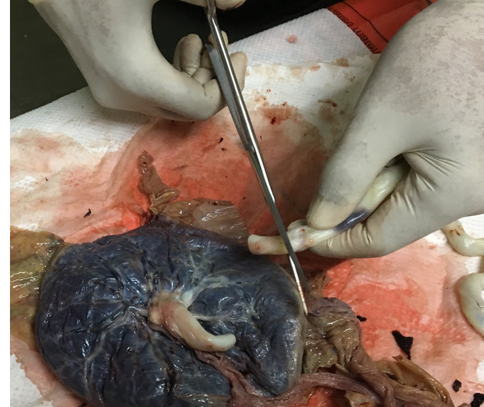




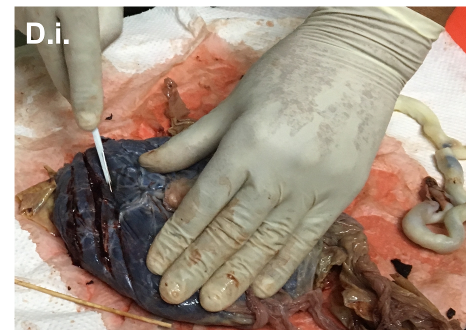




C. *Umbilical cord sampling (An alternative to the steps outlined in B is to roll the fetal membranes around the umbilical cord, securing with a pin.)*



D. *Placental punch (i-iii. Cutting a 1X1X1cm section of placenta with overlying membranes adjacent to the site of cord insertion.*



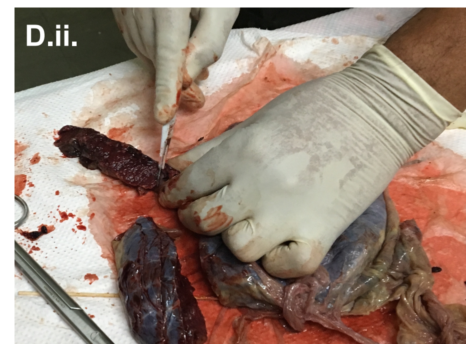


Table 2.1 Specimen collection, laboratory testing, and other procedures by study visit

Procedure Type	Screening	Enrollment	Periodontal Exam	Pre-conception	9-12 Week Gestation	Delivery[i]	Postpartum
Specimen Collection							
Urine pregnancy test	X	X		X			
Genital swabs							
APTIMA swab		X					
Push-off swabs[ii]		X		X	X		
Cotton & Dacron swabs		X		X	X		
Sialidase test swab		X		X			
Oral/gum paper-point[iii]			X				
Fetal membrane swab						X	
Placental punch						X	
Fetal membrane roll						X	
Umbilical cord						X	
Laboratory Testing							
HIV Rapid Test	X						
N. gonorrhoeae, C. trachomatis, & T. vaginalis		X					
Cervical Gram stain		X					
Vaginal pH		X					
Vaginal wet mount		X					
Vaginal Gram stain		X		X	X		
Lactobacillus culture[iv]		X		X	X		
Sialidase test		X		X			
PSA test		X		X	X		
Participant Questionnaires							
Eligibility screen	X						
Demographics		X					
Reproductive history		X					
Medical history & current symptoms		X		X	X		
Sexual behavior		X		X	X		
Substance use		X		X	X		
Dental health history			X				
Neonatal heath							X
Obstetric Ultrasound					X		

Abbreviations: PSA-prostate specific antigen

[i] Delivery follows standard obstetrical procedure and is not conducted by study staff
[ii] Storing for vaginal microbiota and vaginal inflammatory response testing
[iii] Storing for potential oral microbiome testing
[iv] Rogosa agar, followed by sub-culture for hydrogen peroxide production on tetramethylbenzidine agar containing horseradish peroxidase

CHAPTER THREE - Periconceptual bacterial vaginosis and elevated sialidase: The association with fecundability among Kenyan pregnancy planners

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INTRODUCTION

Bacterial vaginosis (BV) is a polymicrobial condition characterized by disruption of the optimal *Lactobacillus* dominated vaginal microbiota through the loss of lactobacilli and an overgrowth of anaerobic bacteria and other microorganisms (59). The distinctive clinical signs of BV are a thin, homogenous white/grey abnormal vaginal discharge and an amine odor. Though BV is not always symptomatic, it is the most common genital tract complaint in women of reproductive age (59). Bacterial vaginosis is associated with increased risk of sexually transmitted infections (STI) and conditions including *Neisseria gonorrhoeae*, *Chlamydia trachomatis* (62–64), *Mycoplasma genitalium* (65), *Trichomonas vaginalis* (66), cervicitis, salpingitis, endometritis, and pelvic inflammatory disease (PID) (67–70,161). These, in turn, are associated with ectopic pregnancy and tubal factor infertility (TFI) (25–27,31,71). In addition, BV during pregnancy is associated with spontaneous abortion and preterm birth (162,163). The effects of vaginal microbiota disruption and BV on infertility in the context of both medically assisted reproduction and in non-medically assisted reproduction (i.e., natural conception) are not completely understood.

In cross-sectional studies, infertile women have a higher BV prevalence compared to fertile women (72–78), and women with tubal factor infertility have a higher prevalence of BV than women with other infertility types (76,78,164–166). A meta-analysis of pregnancy outcomes associated with BV among women undergoing in-vitro fertilization (IVF) found that BV at pre-stimulation, oocyte collection, or embryo implantation was not associated with lower incidence of biochemical pregnancy, clinical pregnancy, or live birth. However, BV was associated with early spontaneous abortion (<12 weeks gestation; RR 1.68, 95% confidence interval [CI] 1.24-2.27) (77). Studies utilizing molecular methods to assess the vaginal or endometrial microbiota in women undergoing IVF have generated conflicting findings. A higher vaginal bacterial diversity

measured by the Shannon Index on samples collected prior to IVF cycles or at embryo transfer has been associated with decreased clinical pregnancy (90) and live birth (90,91), but not biochemical pregnancy (90). In one of these studies, there were no significant differences in biochemical or clinical pregnancy rate by community state type, but women with high concentrations of *Gardnerella vaginalis* or *Atopobium vaginae* detected by quantitative PCR had a lower clinical pregnancy rate (90). In two small studies assessing endometrial microbiota, one found that a *Lactobacillus*-dominated endometrial microbiota was associated with increased implantation, pregnancy, and live birth rates (92), while another identified no difference in the rate of ongoing pregnancy associated with endometrial *Lactobacillus* dominance (93). Differences in sampling location, timing, and method, small sample sizes, and varying pregnancy outcomes under study may explain these mixed results.

Vaginal microbiota disruption present near the time of conception may be a driver of infertility through ascension to and subsequent inflammation of the upper reproductive tract. Bacterial vaginosis and BV-associated bacteria have been associated with both clinical and sub-clinical PID (29,30,68–70,84–86), but the relationship between BV-associated PID and infertility has not been thoroughly explored (30,86). In a study of women with suspected PID, women without *N. gonorrhoeae* or *C. trachomatis* who tested positive for *Sneathia sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae*, and BVAB1 at the cervix and/or endometrium at baseline had a 3.4-fold increased risk of infertility compared to women who were negative for all four types of bacteria (30). In addition, pro-inflammatory responses to BV-associated bacteria may upset the carefully modulated fetal-maternal immune interaction, potentially affecting endometrial receptivity and contributing to implantation failure. However, the mechanisms and the frequency with which this may occur are unknown (167,168).

Bacterial vaginosis associated bacteria also exert deleterious effects on cervical mucus integrity, which may contribute to reduced fecundity. Cervical mucus contains inflammatory cytokines, immunoglobulins, and antimicrobial proteins that prevent vaginal bacteria from ascending and colonizing the upper reproductive tract (79). Healthy cervical mucus also facilitates transportation of sperm from the vagina to the uterus during specific phases of the menstrual cycle, provides a barrier to sperm with abnormal motility or morphology, and may contribute toward sperm capacitation, an integral step in the sperm activation process (94–96). Sialidase and other mucin degrading enzymes produced by bacteria associated with BV, such as *Bacteroides spp., Prevotella spp.,* and *G. vaginalis* (79–82), can degrade cervical mucus and disrupt its functions. In addition, sialidase activity may promote bacterial adhesion to epithelial cells (79). Moreover, as allogenic cells, sperm are targets of the immune response in the female reproductive tract. In addition to protection conferred by proteins and anti-oxidant properties of seminal fluid, sperm are coated in sialic acid, which mask cells from the immune system (94,99). High levels of sialidases produced by BV-associated microorganisms may disrupt the sialic acid coating protecting sperm, exposing these cells to the female immune response.

The limited research on vaginal and endometrial microbiota disruption and its association with fertility outcomes in women undergoing IVF is suggestive, but the results to date may not be generalizable to non-medically assisted conception attempts. Few prospective studies have been conducted to examine the association between BV and female infertility in the context of non-medically assisted conception (30,86). None have assessed fecundability, the per-menstrual cycle probability of pregnancy, and none have specifically studied a population of women who are all planning to conceive. To address these gaps, the objective of this study was to assess the associations between BV, elevated sialidase activity, and fecundability in a cohort of HIV-negative Kenyan women with immediate fertility intent.

METHODS

Study Design & Population

Participants in this prospective fecundability study were enrolled in the Microbiota and Preterm Birth (MPTB) Study, an ongoing cohort study in Nairobi and Mombasa, Kenya (Chapter 2). The MPTB Study follows participants with fertility intent through the preconception, pregnancy, and early postpartum periods to examine the relationship between periconceptual vaginal microbiota and the risk of spontaneous preterm birth. Eligibility criteria for the MPTB study included having immediate fertility intent, being HIV-negative, 14-45 years old (minors only eligible if emancipated under Kenyan Law), having a menstrual period in the prior three months or recently discontinued a contraceptive method that can cause amenorrhea [implant, hormonal intrauterine device (IUD), depo medroxyprogesterone acetate injectable (DMPA)], willing to adhere to study procedures, and able to provide informed consent. Women with HIV-positive male partners were eligible if their partner had a documented undetectable HIV viral load, or the woman was taking pre-exposure prophylaxis (PrEP) for HIV prevention. Additional exclusion criteria were current pregnancy, using contraception other than condoms for HIV/STI prevention, having a DMPA injection in the last three months, history of significant cervical or uterine surgery (excluding cesarean section), known autoimmune disease, antibiotic use in the prior four weeks, and history of seeking care for infertility. Participants in the MPTB Study were recruited at sites providing reproductive and maternal health services, which included family planning clinics (e.g., women discontinuing implants/IUDs to conceive), pediatric immunization clinics, and safer conception clinics for HIV-serodiscordant couples. The study was approved by the institutional review boards of Kenyatta National Hospital and the University of Washington. All participants provided written informed consent.

Participants in the MPTB Study who were enrolled and contributed at least one menstrual cycle between April 18, 2017 and April 30, 2019 were eligible for this fecundability study. Women

reporting a history of hospitalization for treatment of PID, ectopic pregnancy, polycystic ovarian syndrome, endometriosis, or more than three menstrual cycles of conception attempt time prior to enrollment were excluded. Women using condoms intermittently for HIV/STI prevention were eligible for this analysis. In Kenya, safer conception strategies are discussed and offered to HIV-serodiscordant couples, including but not limited to counseling to time condomless sex during the fertile window, treatment for the HIV-positive partner combined with viral load testing to identify when it is safe to try to conceive, and PrEP for the HIV-negative partner (169).

Clinical Procedures

The MPTB Study procedures, including enrollment, preconception, pregnancy, delivery and postpartum procedures have been previously presented (Chapter 2). Specific enrollment and preconception visit procedures relevant to this fecundability study are briefly described here. At enrollment, participants completed a structured face-to-face interview to collect data on demographics, sexual behavior, substance use, depressive symptoms, and reproductive and medical history. Study clinicians conducted a physical examination and speculum-assisted pelvic examination. Vaginal fluid specimens were collected for *N. gonorrhoeae, C. trachomatis, and T. vaginalis* diagnosis by nucleic acid amplification tests (NAAT), vaginal and cervical Gram stains, elevated sialidase detection, and detection of prostate specific antigen (PSA). The PSA test is a biological marker for recent condomless sex, and can detect PSA in vaginal secretions up to 24-48 hours after sex (152). If participants were menstruating, examination was deferred until completion of menses, as the vaginal microbiota may undergo rapid changes around menstruation (57,136).

Participants attended monthly visits during the preconception period. At each preconception visit, a brief interview was conducted to collect updated sexual behavior and reproductive history, and a urine pregnancy test was performed. Self-reported frequency of condomless sex and other

behaviors were reported by participants for the prior month and not for each discrete menstrual cycle. Women also self-collected vaginal swabs for Gram stain, elevated sialidase detection, and PSA detection. Women who remained non-pregnant after six months were exited. However, women who discontinued DMPA less than six months prior to enrollment were eligible for nine months of preconception trying time due to the delayed return to fertility after DMPA discontinuation (142,143).

At enrollment and preconception visits, women received counseling on healthy behaviors for preconception and pregnancy, including nutrition, smoking cessation, and recommendations to refrain from vaginal washing. Study clinicians also discussed each participant's menstrual cycle history and characteristics (i.e., regularity), and estimated the next fertile window using calendar methods (138). Participants with genital symptoms, including symptomatic BV, were treated using syndromic management per Kenyan Guidelines (137). Additional therapy was provided to the participants and offered to their partners at the first monthly preconception visit based on the results of STI testing performed at enrollment.

Laboratory Procedures

Microscopy, STI testing, and detection of sialidase and PSA in vaginal secretions were performed by laboratory staff at the study sites. Testing of enrollment genital specimens included saline and potassium hydroxide wet mounts examined for the presence of motile trichomonads, clue cells, yeast, and sperm. Endocervical Gram stained slides were scanned at low power, and polymorphonuclear leukocytes in three nonadjacent oil immersion fields were counted and averaged to evaluate cervical inflammation. Enrollment samples were also tested for *N. gonorrhoeae, C. trachomatis,* and *T. vaginalis* (Aptima Combo-2 CT/NG Detection System, Aptima *Trichomonas vaginalis* assay*;* Hologic Corporation). At enrollment and preconception visits, vaginal Gram stained slides were evaluated for BV using the criteria of Nugent and Hillier

(60). The Nugent score is a weighted score determined by microbiological evaluation for *Lactobacillus* morphotypes, *G. vaginalis* or Bacteroides (gram-variable and gram-negative rods), and curved gram-variable rods. A Nugent score of 0-3 is considered normal, 4-6 is intermediate microbiota, and 7-10 is BV. Detection of elevated vaginal sialidase concentrations indicative of BV (≥0.25 μg; Diagnosit BVBlue; Gryphus Diagnostics) and PSA (ABAcard, Abacus Diagnostics) were performed using commercially available assays according to the manufacturers' instructions.

Defining Time at Risk and Conception Outcome

At enrollment and at each preconception visit, women reported the first day of their last menstrual period (LMP). Women who missed preconception visits were asked to report any interim menstrual cycles. One hundred and thirty participants did not have the opportunity to report interim menstrual periods for missed visits occurring prior to July 2018 when this question was added to the interview. For these cases, cycles were imputed as needed based on the menstrual cycle duration reported at enrollment. For women with irregular cycles, a cycle length of 28 days was used to impute missed cycles. Five percent of the total cycles (n=41/768, 5.3%) were imputed and 3.9% (n=30/768) were reported by participants after missed visits

Discrete menstrual cycles were modeled utilizing the LMPs reported by participants and the imputed cycles (**Figure 3.1**). Only complete menstrual cycles with known outcomes were included in this analysis. Participants were considered pregnant in a cycle if the urine pregnancy test was positive. They were considered not pregnant in a cycle if their pregnancy test was negative and they reported a new LMP at their next visit, demonstrating that they did not become pregnant later in the cycle.

Statistical Analysis

The overall cumulative incidence of pregnancy was visualized using Kaplan Meier plots. Discrete time proportional probabilities models with robust standard errors were used to estimate fecundability ratios (FR) and 95% confidence intervals (95% CI) (170,171). The FR is interpreted as the per-menstrual cycle probability of pregnancy comparing exposed to unexposed participants; a FR of <1.0 represents reduced fecundability, while a FR >1.0 represents improved fecundability. To account for left truncation, participants reporting conception attempt time prior to enrollment were delayed entry into the analysis by the number of reported menstrual cycles of prior trying time (171). Censoring criteria during follow-up included initiation of biomedical infertility treatment, resumption of contraception excluding condoms for HIV/STI prevention, participant withdrawal (i.e., change in pregnancy intention, moved from the study area, discomfort with genital sample collection), loss to follow-up, or reaching the end of preconception follow-up without pregnancy.

Two primary time-varying measures of vaginal microbiota disruption were assessed in this study: BV by a Nugent score ≥7 (60) and elevated sialidase by the point-of-care test. Exposure data were lagged from the visit prior to each pregnancy test to align the vaginal microbiota disruption measurement as close to the preconception period for each menstrual cycle as possible. For cycles that were reported following a missed visit and for imputed cycles, the missing exposure and time-varying confounder data were imputed from the most recent preceding visit.

Age (<25, 25-29, 30-34, 35-39, 40-45) and frequency of condomless sex in the prior four weeks (time-varying; none, 1-4, 5-8, ≥9) were included *a priori* in the adjusted models due to known associations with both vaginal microbiota disruption and fecundity (19,20,59,172,173). Study site (Nairobi, Mombasa) was also included *a priori*. Additional potential confounding factors were selected based on known associations with both vaginal microbiota disruption and conception.

These included maternal body mass index (BMI; underweight ≤18.5, normal 18.5–24.9, overweight 25–29.9, and obese ≥30) (40,174,175), vaginal washing in the last month (time-varying, no/yes) (29,176,177), any condom use in the last month (time-varying, no/yes) (59,173), PSA in vaginal secretions (lagged, time-varying; no/yes) (152), maternal education (<8, 8-11, 12-15, ≥16 years), and household income (Kenyan Shillings per month, <2,500, 2,500-10,000, 10,000-30,000, 30,000-75,000,≥75,000). Maternal smoking, while associated with reduced fecundability and BV, was not considered as a confounder in the multivariate models due to the low prevalence of smoking in this cohort (reported at <1% of cycles). To assess potential confounders for inclusion in the adjusted models, bivariate analysis was performed with each potential confounding variable. These potential confounding variables were then added to a multivariate model that included the *a priori* variables using a manual forward stepwise model-building approach. None of the potential confounding factors changed the FR estimate by >10%, so none were included in the final adjusted models.

For both the BV and elevated sialidase analyses, a sensitivity analysis was conducted after excluding additional women based on strict criteria for identifying potential underlying infertility that is likely due to known causes other than disrupted vaginal microbiome. Strict exclusion criteria included *N. gonorrhoeae, C. trachomatis,* or *T. vaginalis* diagnosis at enrollment; provision of treatment for PID at enrollment; self-report of any PID diagnosis (regardless of hospitalization); self-report of a history of STI treatment for *N. gonorrhoeae, C. trachomatis, T. vaginalis*, or syphilis; self-report of fibroids or unknown uterine abnormality; and having a known HIV-seropositive partner (178). Sensitivity analyses were also conducted to examine the effects of imputing menstrual cycles and imputing missing data on the effect estimates by excluding these cycles. Finally, sensitivity analyses were conducted with inclusion of women who reported up to six months of trying time prior to enrollment, which is another common cutoff point for prior conception attempt time in in fecundability studies (46,179,180).

Predefined secondary analyses were conducted for both BV and elevated sialidase to further assess the association between vaginal microbiota and fecundability. The vaginal microbiota and Nugent score varies over time (57,136,181,182), but vaginal specimen sampling in this study only occurred once each month. To address this challenge of identifying the best measurement periconceptual vaginal microbiota disruption, and to assess stable versus dynamic vaginal microbiota over the periconceptual period, a variable was created combining the measurement from the visit prior to and the measurement at each cycle's ultimate pregnancy test. This method resulted in four categories: negative at both visits (optimal vaginal health); positive at visit prior but negative at current visit, negative at prior visit but positive at current visit, and positive at both visits (persistent disrupted). In addition, models were run utilizing enrollment measures only (time-independent). Lastly, a model was run to assess the association between an abnormal vaginal microbiota (Nugent score ≥4) at the visit prior and fecundability.

RESULTS

Baseline Characteristics

Of the 640 women screened for the MPTB Study, 514 were eligible and 458 (89.1% of eligible) enrolled. After applying the exclusion criteria for this fecundability analysis, 273 participants remained (**Figure 3.2**). Participants were a median of 30 years old (interquartile range [IQR] 26-35, **Table 3.1**). Most participants had 12 or more years of education (68.1%, n=186) and were gravid (n=250, 91.6%). The majority of participants reported a HIV-seronegative male partner (73.1%, n=198), while 6.3% (n=17) were in known HIV-serodiscordant relationships and 20.7% (n=56) were unaware of their partner's HIV status. Only one participant reported smoking in the month prior to enrollment (0.4%) and a minority of women report drinking alcohol (n=45, 16.5%). The most common methods of recent contraception were copper IUD (n=80, 29.3%), implant (n=76, 27.8%), and none (n=79, 28.9%). Participants reported a median of five (IQR 3-10) acts of condomless sex in the month prior to enrollment. *Chlamydia trachomatis* was diagnosed in 18

(6.6%) women, two women had *N. gonorrhoeae* (0.7%), and one had *T. vaginalis* (0.4%). Bacterial vaginosis (n=97, 35.5%) and elevated sialidase (n=91, 33.3%) were detected in one third of participants. There were some differences in baseline characteristics by study site (**Table 3.1**). Compared to eligible participants from the Mombasa site, eligible participants in Nairobi were older (≥30 years old: 55.5% versus 32.4%), more likely to have recently discontinued the IUD or implant (62.7% versus 21.6%), less likely to have reported 1-3 menstrual cycles of prior conception attempt time (17.4% versus 67.6%), and less likely to report vaginal washing behavior (32.6% versus 70.3%).

Of the 185 women excluded from this analysis, 53.5% (n=99) had not yet contributed follow-up time, 44.3% (n=82) reported more than three (or an unknown number of) menstrual cycles of prior conception attempt time at enrollment, and 2.2% (n=4) were excluded for solely clinical reasons (**Figure 3.2**). Women excluded from the analysis were mostly similar to those who were included, but there were some significant differences (**Table 3.2**). Women who were eligible for this analysis were more likely to be from the Nairobi site, have finished at least secondary school (≥12 years; 68.2% versus 55.7%), and have a monthly household income of ≥10,000 KSh (~$100; 71.1% versus 52.4%). The eligible participants were also more likely to have discontinued IUD/implant use (57.1% versus 22.7%), more likely to be gravid (91.6% versus 78.4%), report a lower frequency of condomless sex in the last four weeks (median: 5 versus 8 acts), and were more likely to have cultivable vaginal *Lactobacillus* (69.0% versus 50.8%).

Follow-up Characteristics & Cumulative Pregnancy Rates

Eligible participants attended 715 preconception study visits over 65.3 years of follow-up time. The median number of visits (including enrollment) was three (IQR 2-5) with a median of 28 days between visits (IQR 28-34). During this time, participants contributed 768 complete menstrual cycles. Since the Nairobi site began enrolling participants one year prior to the Mombasa site,

most menstrual cycles were contributed by participants at the Nairobi site (89.1%, 684/768). Seventeen percent of cycles (16.7%, 128/768) were positive for pregnancy. The median time-to-pregnancy was four menstrual cycles (IQR 2-7) and the cumulative six-cycle pregnancy rate was 69.3% (95%CI 61.5-82.7) (**Figure 3.3A**). In the sensitivity analysis including women with up to six cycles of prior trying time, 38 additional women were eligible for analysis bringing the total to 311 women, 876 menstrual cycles, and 138 pregnancies (**Figure 3.3B**).

At the time of analysis, 46.9% (128/273) of participants were pregnant, 17.2% (47/273) were still in the preconception follow-up, 13.9% (38/273) were not pregnant by the end of preconception follow-up and were exited from the study, 3.7% (10/273) had withdrawn from the study, and 18.3% (50/273) were lost to follow-up after contributing at least one complete menstrual cycle. Compared to women who withdrew or were lost to follow-up, women who completed the study were more likely have a monthly household income of ≥10,000 KSh (Completed study: 75.9% versus Withdrawn/Loss to follow-up: 56.7%) and recently discontinued IUD or implant for contraception (Completed study: 66.8% versus Withdrawn/Loss to follow-up: 31.7%). The retained women also reported a higher median frequency of condomless sex in the month prior to enrollment than women who withdrew or were lost to follow-up (6 versus 4) (**Table 3.3**).

Bacterial Vaginosis & Fecundability

Thirty percent (301/987) of study visits were BV positive (Nugent ≥7), though participants reported abnormal vaginal discharge at only 9.3% (28/300) of BV positive visits. Only 3.0% (9/301) of BV positive visits were accompanied by a metronidazole prescription. In the primary analysis, 34.8% (267/768) of menstrual cycles were considered to be exposed to BV at the most recent visit prior to pregnancy testing. The probability of pregnancy was similar in cycles with and without preconception BV (**Table 3.4**). In unadjusted analysis, BV at the visit prior to each pregnancy test was not associated with fecundability (FR 0.94, 95%CI 0.67-1.32) (**Table 3.5**). Adjusting for age,

frequency of condomless sex in the last four weeks, and study site did not change the estimate of the association (adjusted FR [aFR] 0.95, 95%CI 0.66-1.35). These results were robust to sensitivity analysis excluding 45 additional participants who did not meet the stricter set of additional inclusion criteria (**Tables 3.5 & 3.7**) and in analyses excluding the imputed menstrual cycles and menstrual cycles reported after a missed visit (**Table 3.5)**. The null association between BV and fecundability was also demonstrated in secondary analyses assessing abnormal vaginal microbiota at the visit prior (Nugent ≥4 time-varying, aFR 1.03, 95%CI 0.73-1.44), BV at enrollment only (aFR 1.00, 95%CI 0.71-1.42), and when including women with up to six cycles of prior conception attempt time (Nugent ≥7 time-varying, aFR 0.89, 95%CI 0.63-1.27).

When BV status was re-evaluated utilizing measurements at two consecutive visits, 60.8% of menstrual cycles were BV negative at both visits (n=467), 25.9% (n=199) had persistent BV (positive at both visits), and the remaining 13.2% (n=102) were BV positive at one but not the other visit (**Table 3.6**). Compared to menstrual cycles that were BV negative at both visits, BV at the visit prior to and no BV at the visit with pregnancy testing was associated with a significant 1.7-fold increased per-cycle probability of pregnancy (aFR 1.76, 95%CI 1.13-2.72), while persistent BV was associated with a 34% reduction in per-cycle probability of pregnancy (aFR 0.66, 95%CI 0.42-1.03; p=0.07); this association was not statistically significant at α=0.05 (**Table 3.6**). In the sensitivity analysis including women with up to six cycles of prior trying time, the association between persistent BV and fecundability was similar, but statistically significant (aFR 0.60, 95%CI 0.39-0.93).

Elevated Sialidase & Fecundability

The sensitivity and specificity of the test for elevated sialidase versus BV by Nugent score ≥7 as the gold standard was 76.3% and 90.3% at the enrollment visit. Thirty-two percent (32.4%, 249/768) of menstrual cycles were considered exposed based on detection of elevated sialidase

at the visit prior to pregnancy testing (**Table 3.4**). Similar to the BV analysis, there was no association between preconception elevated sialidase and fecundability when modeled as a time-varying exposure (aFR 1.12, 95%CI 0.79-1.59), or when utilizing the enrollment measurement (aFR 0.96, 0.67-1.38) (**Table 3.5**). In addition, when compared to menstrual cycles that were sialidase test negative at the visit prior and current visit, cycles that were sialidase test positive at the visit prior and negative at the current visit had a 1.7-times increased per-cycle probability of pregnancy (aFR 1.75, 95%CI 1.16-2.65), similar to the BV finding (**Table 3.6**). Persistent sialidase test positivity across two visits was associated with a non-significant reduction in fecundability (aFR 0.81, 95%CI 0.54-1.24).

DISCUSSION

Despite some suggestive data from studies assessing associations between vaginal and endometrial microbiota disruption and reduced IVF success (77,92), BV and elevated sialidase measured at the visit prior to each pregnancy test was not associated with reduced fecundability in this prospective cohort of HIV-negative Kenyan women trying to conceive naturally. However, when considering two consecutive measurements as a marker of persistent vaginal microbiota disruption, persistent BV was associated with a 34% lower fecundability that trended toward significance.

The results presented here, and future studies assessing this question, will need to be interpreted carefully as condomless sex is a strong confounder of the association between vaginal microbiota disruption and becoming pregnant. Specifically, condomless sex is associated with increased vaginal microbiota disruption and with increased pregnancy rates. Therefore, in the presence of residual confounding by condomless sex, results will invariably be biased toward an association between BV and increased fecundability, which is the opposite of the hypothesis under study. This strong biasing effect may, in part, explain the 75% increase in probability of pregnancy in

cycles that were BV positive at the visit prior but BV negative at the current pregnant test compared to cycles that were BV negative at both time points. In addition, this finding could be explained by the shift of the vaginal microbiota during pregnancy to one dominated by *Lactobacillus spp*. with decreased bacterial community diversity (112–116). This is thought to occur early in pregnancy (118,183) and may be more pronounced in women of African descent (183). Therefore, the shift from BV positive to BV negative across two consecutive study visits observed among some of the women who became pregnant may represent an early transition to a more optimal microbiota associated with pregnancy. Lastly, vaginal microbiota disruption is associated with menses (57,136), so pregnancy induced amenorrhea may also provide some explanation for this finding.

When considering two consecutive measurements of vaginal microbiota disruption, there was also a 34% reduction in the per-cycle probability of pregnancy in menstrual cycles with BV positivity at both time points. This association became statistically significant when including women with up to six cycles of conception attempt time prior to enrollment. These findings require confirmation but indicate that women with persistent BV may represent a group at increased risk for sub-fecundity. While BV is not associated with vaginal neutrophils and does not often present with clinically apparent inflammation, BV has been associated with elevated levels of the pro-inflammatory cytokine IL-1β and less consistently IL-8 and IL-6 (184). Bacterial vaginosis has also been associated with reduced levels of secretory leukocyte protease inhibitor (SLPI) which has anti-inflammatory activity (184). Persistent exposure to sub-clinical inflammation during the periconception period, paired with changes to the cervical mucus due to mucin degrading enzymes and ascension of bacteria into the upper reproductive tract, could contribute to BV-associated sub-fecundity.

The tools utilized in these analyses to measure vaginal microbiota disruption, including BV by Nugent score and elevated sialidase by a point of care test for sialidase activity indicative of BV, are non-specific methods of detection. In addition, endometrial microbiota disruption may be more strongly associated with fecundability than vaginal microbiota disruption. Studies utilizing molecular methods of detection have shown that the endometrium is frequently colonized by vaginal bacteria (92,185–187). However, overall bacterial load and concentrations of specific bacteria are lower in the endometrium than in the vagina and bacteria are not always detected in both locations at the same time (92,186,187). While periconceptual BV was not associated with fecundability in primary analysis, it is possible that distinct communities of vaginal or endometrial microbiota or quantities of specific high-risk bacteria may be associated with reduced fecundability in women attempting non-medically assisted conception.

Of the few fecundability studies conducted among the general population sub-Saharan Africa, none have utilized a prospective design, which is considered the gold standard for studying fecundability (20). To our knowledge, this fecundability analysis leveraging the MPTB Study's preconception data is the first prospective time-to-pregnancy study in sub-Saharan Africa. The six-cycle pregnancy rate was 69% and the median time-to-pregnancy was four months. The six-cycle pregnancy rate falls within the range of 58-81% reported for other prospective fecundability cohorts (17–19,188,189). Importantly, time-to-pregnancy estimates are population specific, varying regionally due to differences in the underlying fecundity of the population under study and by sexual behavior (20,190). Retrospective studies in South Africa (191) and Ethiopia (192) have reported slightly lower six-month pregnancy rates of 50% and 66%, respectively. In another study utilizing Nigerian Demographic and Health Survey data and the current duration approach to estimate fecundability, the median time to pregnancy was estimated to be 5.1 months (193). Additional information on time-to-pregnancy in African cohorts is available in the context of safer conception interventions for HIV-affected couples. Two studies report lower cumulative

pregnancy rates than the estimates for general population women described above. In a South African study, conception by six-months was 35% and risk of subfertility was higher in HIV positive women (178). In a Kenyan comprehensive safer conception intervention, the six and 12-month cumulative pregnancy rates were estimated to be 45% and 62%, respectively (194). In this current analysis of HIV-negative Kenyan women, only 6% of participants had a known HIV positive male partner and cumulative pregnancy rates did not differ by partner's HIV status.

This prospective fecundability analysis has several strengths. This was a prospective fecundability analysis, which is the gold standard study design for fecundability studies (20). The prospective design allowed for monthly vaginal specimen sampling and assessment of the association using a time-varying approach, which is important given the variation in Nugent score that can occur over time (181,182,195). Prospective fecundability studies, however, can be biased when a large proportion of the population under study reports trying time for pregnancy prior to enrollment (196). This can contribute to a loss of precision and to selection bias due to missing time-at-risk. Some specific concerns include increased enrollment of women with lower fecundity than the population at-large, including women who experienced unknown biochemical pregnancies or missed miscarriages whose reported trying time is therefore longer than experienced for the current pregnancy attempt, a higher likelihood of recall bias when reporting the number of cycles of prior trying time, and behavior change that may occur as a result of sub-fecundability. In this study, nearly 60% of participants were enrolled shortly after discontinuing their IUD and implants for conception so the first cycle at risk for pregnancy was observed for a majority of participants. Lastly, participants reported very low rates of smoking and alcohol use, so these results are unlikely to be confounded by these behaviors, which are associated with both BV and fecundability.

There were also a number of limitations. First, this fecundability analysis leveraged data from a parent study that was not originally designed for assessing predictors of fecundability. Women were unable to report interim menstrual cycles if they had missed a study visit for the first year of the study, so menstrual cycles were imputed for missed visits during this period. Importantly, results were similar in sensitivity analyses excluding these visits. Second, these analyses were subject to residual confounding related to condomless sex. Self-reported frequency of condomless sex was reported for the prior month (e.g., since the last study visit) and not for each discrete menstrual cycle. In addition, the parent study did not provide ovulation predictor kits, so measurement of sexual frequency in the fertile window was imprecise and anovulatory cycles were not identified. Due to this misclassification of an important confounder, the adjusted effect estimates may be artificially close to the unadjusted estimates (197). Third, one of the MPTB Study inclusion criteria was that women who were not recently discontinuing contraception known to disrupt ovulation had to have experienced a menstrual period in the prior three months. Excluding women without a menstrual cycle in the prior three months may have induced selection bias by excluding women with long or irregular menstrual cycles (i.e., less fecund women). This could have resulted in recruiting a cohort of women with a higher rate of fecundability than the general population. However, <2% (10/640) women screened for the MPTB Study were excluded for this reason. Fourth, 22.0% of participants were lost to follow-up or withdrew from the study early. This could bias study results if vaginal microbiota disruption or fecundability were associated with completing versus not completing preconception follow-up. Notably, the prevalence of BV as well as most other enrollment characteristics were similar in women who were lost or withdrew compared to women completing the study and to women still in preconception follow-up (BV: 33% versus 36% versus 36%). Lastly, the target sample size of 450 participants has not yet been reached for this analysis, so the present results are preliminary. Results will be finalized when the target sample size is accrued or in September 2020, whichever comes first.

In conclusion, in this cohort of Kenyan women attempting to conceive, vaginal microbiota disruption, defined as BV or elevated sialidase at the visit prior to pregnancy testing, was not associated with reduced fecundability. However, women with persistent BV during the periconception period may be at risk of reduced fecundability and represent a population of women for further study. Given the high global prevalence of BV and infertility, if persistent BV is associated with reduced fecundability in the context of non-medically assisted reproduction, this could have important implications for a large number of women who wish to conceive.

Figure 3.1. Defining time at risk and conception outcome A) MPTB Study visit schedule for a participant attending 3 preconception visits, including vaginal specimen sampling and pregnancy testing frequency, and defining discrete menstrual cycles. **B)** A four menstrual cycle to pregnancy scenario. C) Censoring a last reported cycle for a participant who did not get pregnant during follow-up (censored at the cycle prior to their last negative pregnancy test). **D)** Demonstrating a scenario where a (pregnant) participant reports the same LMP across multiple preconception visits.

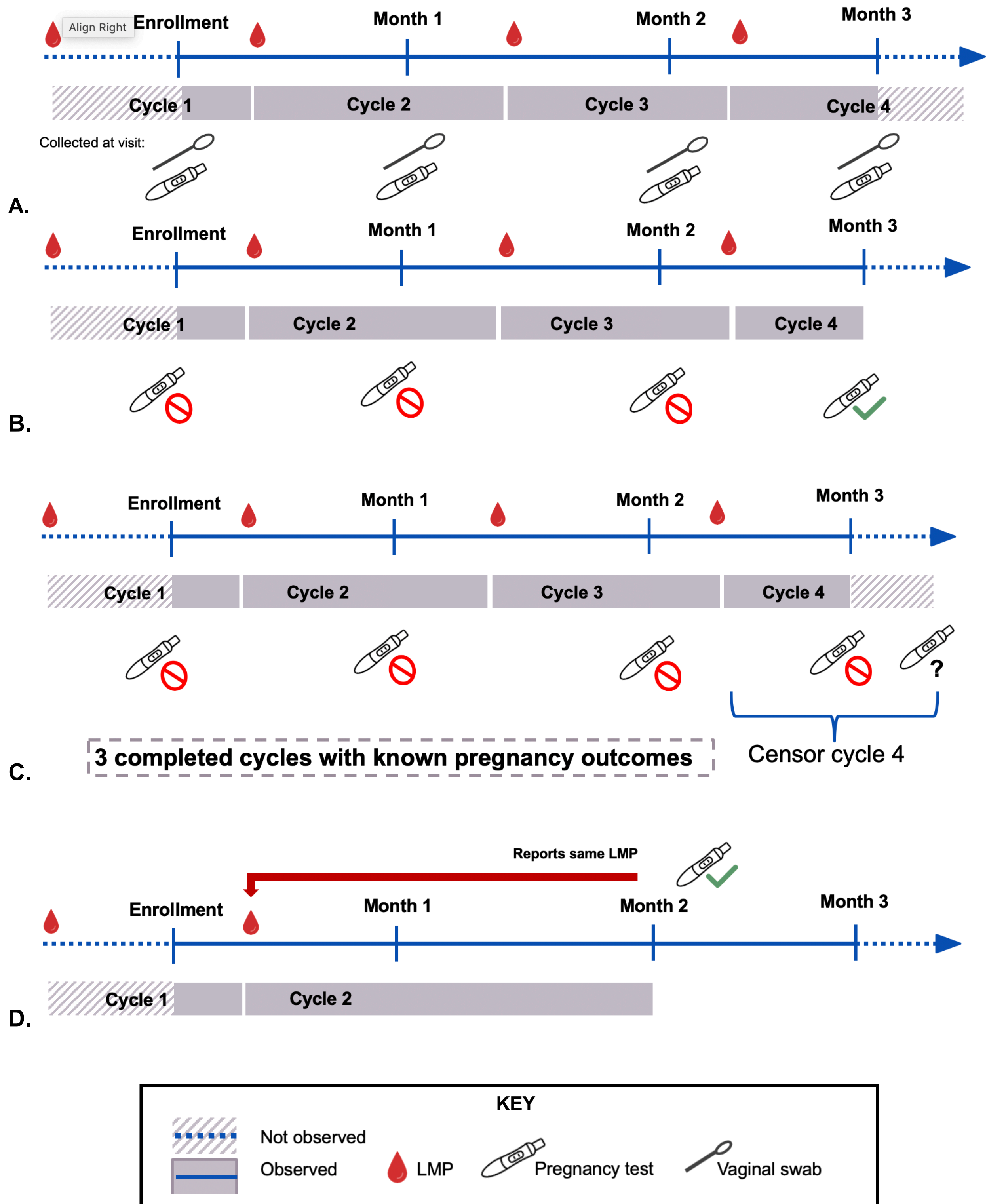
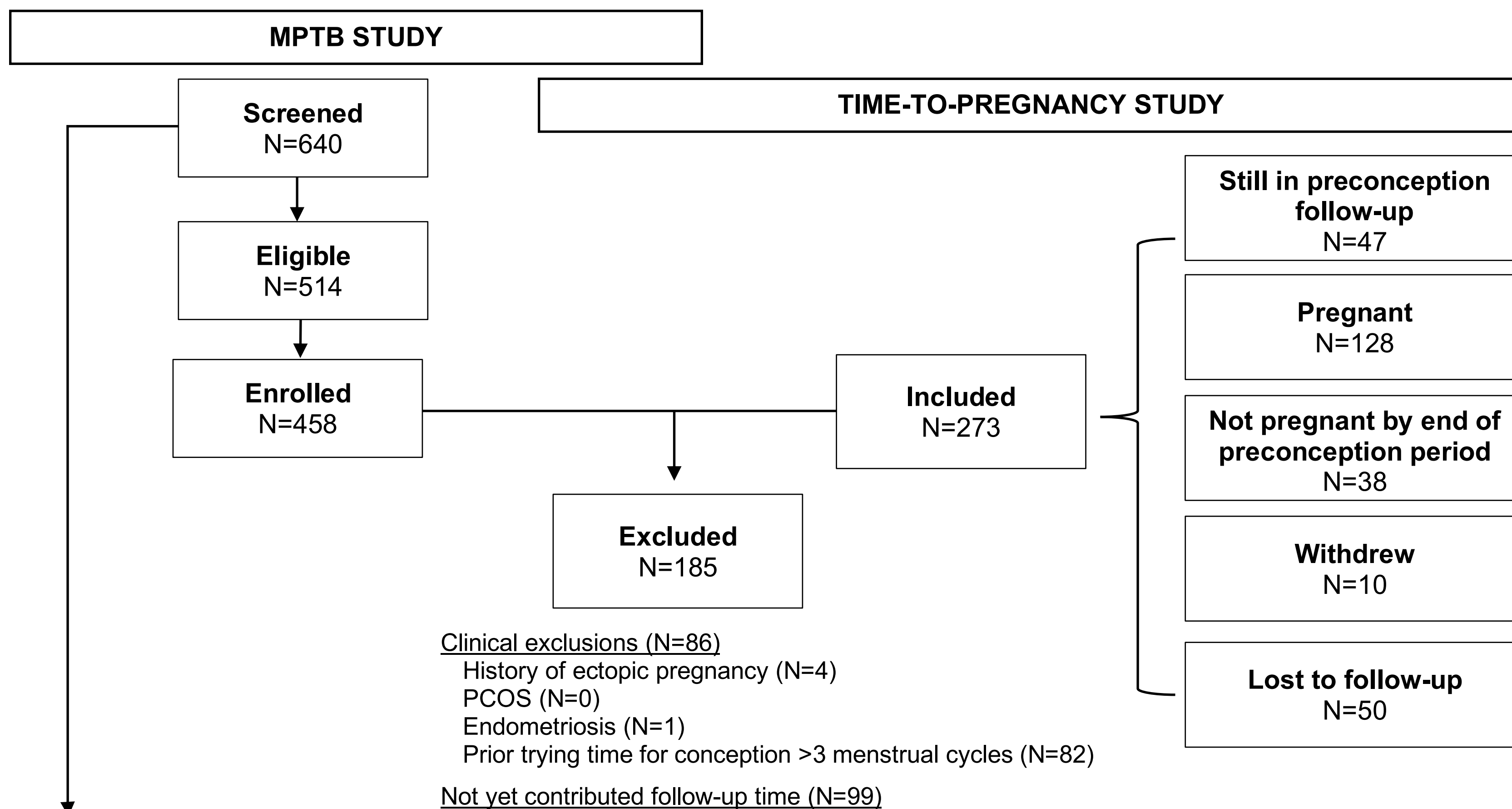


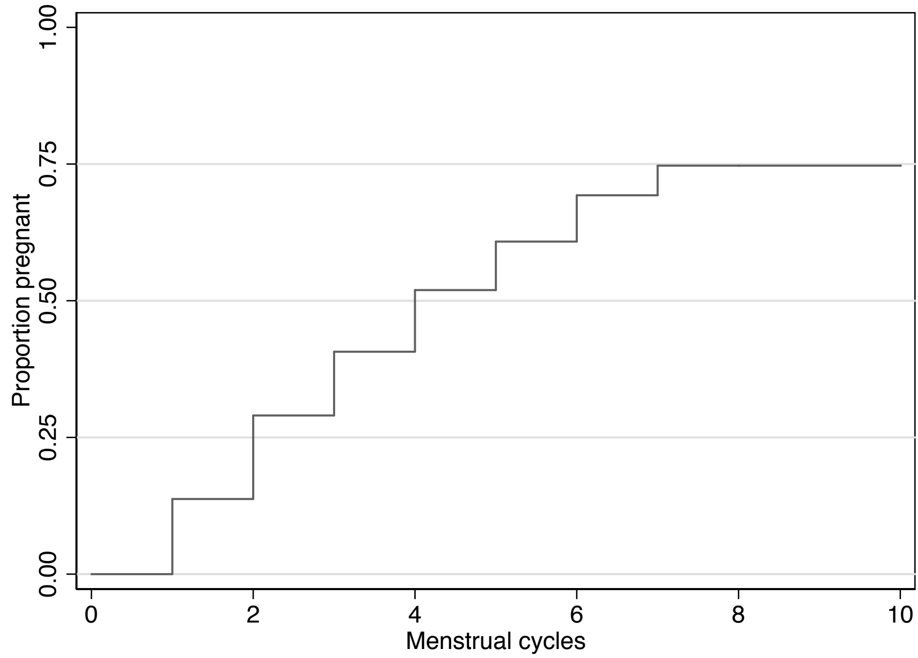
Figure 3.2. Participant eligibility and participation flow-chart



Exclusions (N=126)
No menstrual cycle in last 3 months and not recently discontinuing hormonal IUD, implant, DMPA (N=10)
Not planning a pregnancy in next 6 months (N=3)
Not willing to comply with study visits/procedures (N=6)
HIV positive (N=6)
Positive pregnancy test (N=12)
Currently using contraception, including vasectomy and DMPA injection <3 months ago (N=15)
History of cervical/uterine surgery (N=1)
Autoimmune disease (N=1)
Antibiotics in last 4 weeks (N=23)
History of seeking care for infertility (N=62)

Figure 3.3. Kaplan-Meier plot illustrating cumulative pregnancy rate among Kenyan pregnancy planners - ***A.*** *Primary analysis including 273 participants;* ***B.*** *Sensitivity analysis including participants with up to six cycles of prior conception time for a total of 311 participants)*

A.



B.

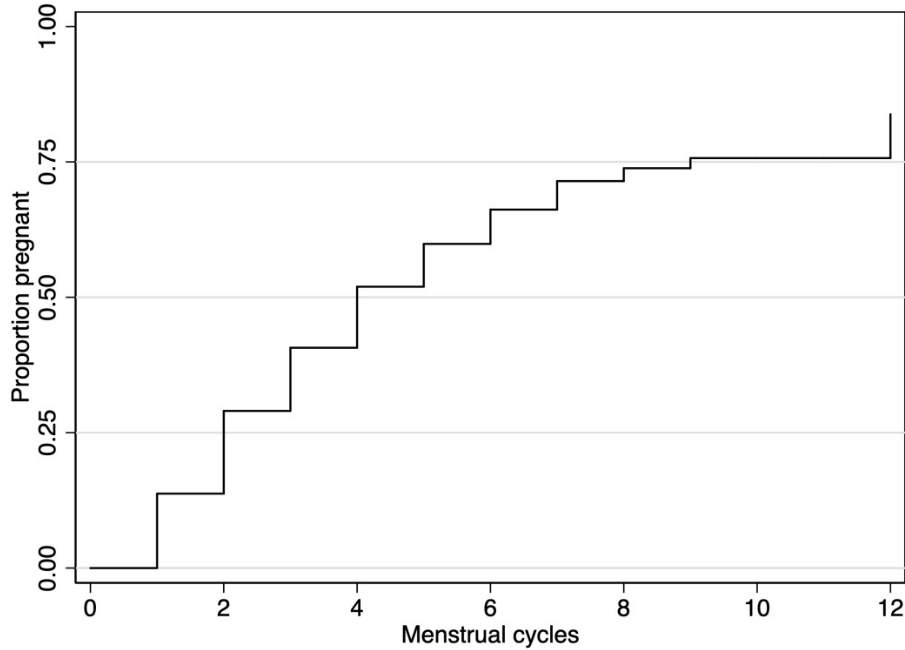


Table 3.1. Enrollment characteristics for 273 Kenyan women with fertility intent

Characteristic	N	All Eligible Total (N=273)		By Site Nairobi (N=236)		Mombasa (N=37)		p-value
Demographic & Partnership Characteristics								
Age	273							0.03
<25		47	(17.2)	37	(15.7)	10	(27.0)	
25-29		83	(30.4)	68	(28.8)	15	(40.5)	
30-34		73	(26.7)	63	(26.7)	10	(27.0)	
35-39		56	(20.5)	55	(23.3)	1	(2.7)	
40-45		14	(5.1)	13	(5.5)	1	(2.7)	
Education Level	273							0.30
<8 years		13	(4.8)	10	(4.2)	3	(8.1)	
8-11 years		74	(27.1)	62	(26.3)	12	(32.4)	
12-15 years		128	(46.9)	110	(46.6)	18	(48.7)	
≥16 years		58	(21.3)	54	(22.9)	4	(10.8)	
Monthly Household Income (KSh)	271							0.27
<2,500		9	(3.3)	8	(3.4)	1	(2.7)	
25,000-10,000		68	(25.1)	56	(23.9)	12	(32.4)	
10,000-30,000		112	(41.3)	94	(40.2)	18	(48.7)	
30,000-75,000		49	(18.1)	44	(18.8)	5	(13.5)	
>75,000		33	(12.2)	32	(13.7)	1	(2.7)	
Partner's age	273							0.37
<25		8	(2.9)	7	(3.0)	1	(2.7)	
25-29		46	(16.9)	37	(15.7))	9	(24.3)	
30-34		79	(28.9)	65	(27.4)	14	(37.8)	
35-39		70	(25.6)	62	(26.3)	8	(21.6)	
40-44		52	(19.1)	48	(20.3)	4	(10.8)	
≥45		18	(6.6)	17	(7.2)	1	(2.7)	
Partner's HIV-serostatus	271							0.006
HIV-seronegative		198	(73.1)	163	(69.7)	35	(94.6)	
HIV-seropositive		17	(6.3)	17	(7.3)	0	(0.0)	
Unknown		56	(20.7)	54	(23.1)	2	(5.4)	
Substances								
Smoke cigarettes	273	1	(0.4)	1	(0.4)	0	(0.0)	0.69
Frequency of alcohol use	273							0.44

None		228	(83.5)	195	(82.6)	33	(89.2)	
Monthly or less		37	(13.6)	33	(14.0)	4	(10.8)	
2-4 times per month		8	(2.9)	8	(3.4)	0	(0.0)	
≥4 times per month		0	(0.0)	0	(0.0)	0	(0.0)	
Depressive symptom screen (PHQ-9)	273							0.44
None/Minimal (0-4)		263	(96.3)	226	(95.8)	37	(100.0)	
Mild (5-9)		9	(3.3)	9	(3.8)	0	(0.0)	
Moderate/severe (≥10)		1	(0.4)	1	(0.4)	0	(0.0)	
Contraception								
Most recent contraceptive method[i]	273							<0.001
None		79	(28.9)	57	(24.1)	22	(59.5)	
Condoms		20	(7.3)	17	(7.2)	3	(8.1)	
OCP		6	(2.2)	6	(2.5)	0	(0.0)	
Injectable (DMPA)		10	(3.7)	6	(2.5)	4	(10.8)	
Copper IUD		80	(29.3)	79	(33.5)	1	(2.7)	
Implant		76	(27.8)	69	(29.2)	7	(18.9)	
Other		2	(0.7)	2	(0.9)	0	(0.0)	
Any DMPA injection in last 6 months	273	13	(4.8)	9	(3.8)	4	(10.8)	0.06
Reproductive History								
Gravid	273	250	(91.6)	227	(96.2)	23	(62.2)	<0.001
Parous	273	240	(87.9)	218	(92.4)	22	(59.5)	<0.001
Trying for pregnancy prior to study enrollment[ii]	273	66	(24.2)	41	(17.4)	25	(67.6)	<0.001
# of cycles trying to get pregnant (median/IQR)	66	1	(1, 2)	1	(1,2)	1	(1,2)	0.23
Regular menses	273	166	(60.8)	150	(63.6)	16	(43.2)	0.02
History of PID (not treated in a hospital)	273	1	(0.4)	1	(0.4)	0	(0.0)	0.69
Abnormal uterus[iii]								
Fibroids	272	2	(0.7)	2	(0.9)	0	(0.0)	0.58
Other/unknown	272	3	(1.1)	3	(1.3)	0	(0.0)	0.50
Sexual risk behavior in last 4 weeks								
Any vaginal washing	273	103	37.7	77	(32.6)	26	(70.3)	<0.001
Frequency of condomless sex	272							0.14
No condomless sex		30	(11.0)	27	(11.4)	3	(8.3)	
1-4		92	(33.8)	81	(34.3)	11	(30.6)	
5-8		72	(26.5)	66	(28.0)	6	(16.7)	
≥9		78	(28.7)	62	(26.3)	16	(44.4)	
Any condom use	271	17	(6.3)	16	(6.8)	1	(2.9)	0.37

Clinical					
BMI	273				0.19
Underweight		5 (1.8)	3 (1.3)	2 (5.4)	
Normal		103 (37.7)	86 (36.4)	17 (46.0)	
Overweight		103 (37.7)	92 (39.0)	11 (29.7)	
Obese		62 (22.7)	55 (23.3)	7 (18.9)	
History of STI[iv]	273	4 (1.5)	2 (0.9)	2 (5.4)	0.03
BV (Nugent ≥7)	273	97 (35.5)	83 (35.2)	14 (37.8)	0.75
Elevated sialidase	273	91 (33.3)	77 (32.6)	14 (37.8)	0.53
Any Cultivable *Lactobacillus*	273	189 (69.2)	168 (71.8)	19 (51.4)	0.01
H_20_2 Producing *Lactobacillus*	272				0.01
None		84 (30.9)	66 (28.2)	18 (50.0)	
Type of *Lactobacillus* growth unknown[v]		14 (5.2)	13 (5.6)	0 (0.0)	
Non-H_20_2 Producing *Lactobacillus*		45 (16.5)	37 (15.8)	8 (22.2)	
H_20_2 Producing *Lactobacillus*		129 (47.4)	118 (50.4)	10 (27.8)	
Lactobacillus morphotypes on Gram Stain	273	174 (63.7)	150 (63.6)	24 (64.9)	0.88
PSA	272	95 (34.9)	81 (34.5)	14 (37.8)	0.69
N. gonorrhoeae	272	2 (0.7)	2 (0.9)	0 (0.0)	0.57
C. trachomatis	272	18 (6.6)	17 (7.2)	1 (2.7)	0.30
T. vaginalis	272	1 (0.4)	1 (0.4)	0 (0.0)	0.69
Metronidazole prescription	273	1 (0.4)	1 (0.4)	0 (0.0)	0.69
Vaginal discharge (self-report)	273	28 (10.3)	28 (11.9)	0 (0.0)	0.03
Moderate/profuse vaginal discharge (clinician assessed)[vi]	270	104 (38.5)	100 (42.9)	4 (10.8)	<0.001

Abbreviations: KSh–Kenyan shillings, OCP–oral contraceptive pills, DMPA–depo medroxyprogesterone acetate, IUD–intrauterine device, PID–pelvic inflammatory disease, STI–sexually transmitted infection, BMI-body mass index, BV–bacterial vaginosis, PSA–prostate specific antigen

[i] Reported dates of IUD and implant removal and last date of DMPA were reviewed. Women reporting device removal >2 months prior to enrollment were re-classified as non-contraceptors (none) for the purpose of this analysis. Women reporting a last DMPA injection >6 months prior were reclassified as non-contraceptors.
[ii] Participants reporting >3 cycles were excluded from this fecundability analysis.
[iii] Self-reported
[iv] Self-report of syphilis, chlamydia, gonorrhea, and/or trichomoniasis
[v] Samples did not grow on sub-culture.
[vi] Excludes 3 participants with blood/menses observed on exam

Table 3.2. Enrollment characteristics by eligibility status for the fecundability analyses

Characteristic	Eligible (N=273)			Not Eligible (N=185)			P-value
	N	n	%	N	n	%	
Demographic & Partnership Characteristics							
Study Site	273			185			<0.001
Nairobi		236	(86.5)		76	(41.1)	
Mombasa		37	(13.6)		109	(58.9)	
Age	273			185			0.45
<25		47	(17.2)		42	(22.7)	
25-29		83	(30.4)		57	(30.8)	
30-34		73	(26.7)		45	(24.3)	
35-39		56	(20.5)		29	(15.7)	
40-45		14	(5.1)		12	(6.5)	
Education Level	273			185			0.01
<8 years		13	(4.8)		18	(9.7)	
8-11 years		74	(27.1)		64	(34.6)	
12-15 years		128	(46.9)		79	(42.7)	
≥16 years		58	(21.3)		24	(13.0)	
Monthly Household Income (Kenyan Shillings)	271			183			<0.001
<2,500		9	(3.3)		5	(2.7)	
25,000-10,000		68	(25.1)		81	(44.3)	
10,000-30,000		112	(41.3)		65	(35.5)	
30,000-75,000		49	(18.1)		26	(14.2)	
>75,000		33	(12.2)		6	(3.3)	
Partner's age	273			184			0.95
<25		8	(2.9)		5	(2.7)	
25-29		46	(16.9)		37	(20.1)	
30-34		79	(28.9)		48	(26.1)	
35-39		70	(25.6)		49	(26.6)	
40-44		52	(19.1)		33	(17.9)	
≥45		18	(6.6)		12	(6.5)	
Partner's HIV-serostatus	271			185			0.04
HIV-seronegative		198	(73.1)		153	(82.7)	
HIV-seropositive		17	(6.3)		10	(5.4)	

Unknown		56	(20.7)		22	(11.9)	
Substances							
Smoke cigarettes	273	1	(0.4)	185	1	(0.5)	0.78
Frequency of alcohol use	273			185			0.12
None		228	(83.5)		163	(88.1)	
Monthly or less		37	(13.6)		16	(8.7)	
2-4 times per month		8	(2.9)		4	(2.2)	
≥4 times per month		0	(0.0)		2	(1.1)	
Depressive symptom screen (PHQ-9)	273			167			0.03
None/Minimal (0-4)		263	(96.3)		167	(90.3)	
Mild (5-9)		9	(3.3)		17	(9.2)	
Moderate/severe (≥10)		1	(0.4)		1	(0.5)	
Contraception							
Most recent contraceptive method[i]	273			185			<0.001
None		79	(28.9)		117	(63.2)	
Condoms		20	(7.3)		20	(10.8)	
OCP		6	(2.2)		2	(1.1)	
Injectable		10	(3.7)		2	(1.1)	
Copper IUD		80	(29.3)		13	(7.0)	
Implant		76	(27.8)		29	(15.7)	
Other		2	(0.7)		2	(1.1)	
Any DMPA injection in last 6 months	273	13	(4.8)	185	2	(1.1)	0.03
Reproductive History							
Gravid	273	250	(91.6)	185	145	(78.4)	<0.001
Parous	273	240	(87.9)	185	130	(70.3)	<0.001
Trying for pregnancy prior to study enrollment	273	66	(24.2)	185	131	(70.8)	<0.001
# of cycles trying to get pregnant (median/IQR)	66	1	(1, 2)	115	6	(4, 10)	<.0001
Regular menses	273	166	(60.8)	185	114	(61.6)	0.86
History of ectopic pregnancy	273	0	(0.0)	175	4	(2.2)	0.02
History of pelvic inflammatory disease	273	1	(0.4)	185	3	(1.6)	0.16
Treated in hospital	4	0	(0.0)	0	0	(0.0)	-
PCOS (self-report)	273	0	(0.0)	185	0	(0.0)	-
Endometriosis (self-report)	273	0	(0.0)	185	2	(1.1)	0.09
Abnormal uterus (self-report)							
Fibroids	272	2	(0.7)	185	0	(0.0)	0.24
Other/unknown	272	3	(1.1)	185	2	(1.1)	0.98

Sexual risk behavior in last 4 weeks							
Any vaginal washing	273	103	37.7	185	40.5	40.5	0.55
Frequency of condomless sex (median, IQR)	272	5	(3, 10)	178	8	(3, 12)	0.03
Frequency of condomless sex	272			178			0.02
No condomless sex		30	(11.0)		17	(9.6)	
1-4		92	(33.8)		52	(29.2)	
5-8		72	(26.5)		33	(18.5)	
≥9		78	(28.7)		76	(42.7)	
Any condom use	271	17	(6.3)	178	12	(6.7)	0.84
Clinical							
BMI	273			183			0.59
Underweight		5	(1.8)		3	(1.6)	
Normal		103	(37.7)		74	(40.4)	
Overweight		103	(37.7)		58	(31.7)	
Obese		62	(22.7)		48	(26.2)	
History of STI (self-report)[ii]	273	4	(1.5)	185	0	(0.0)	0.10
BV (Nugent ≥7)	273	97	(35.5)	185	62	(33.5)	0.66
Elevated sialidase	273	91	(33.3)	185	61	(33.0)	0.94
Any Cultivable *Lactobacillus*	273	189	(69.2)	185	94	(50.8)	<0.001
H_20_2 Producing *Lactobacillus*	272						<0.001
None		84	(30.9)		91	(50.6)	
Type of *Lactobacillus* growth unknown[iii]		14	(5.2)		3	(1.7)	
Non-H_20_2 Producing *Lactobacillus*		45	(16.5)		25	(13.9)	
H_20_2 Producing *Lactobacillus*		129	(47.4)		61	(33.9)	
Lactobacillus morphotypes on Gram Stain	273	174	(63.7)	185	128	(69.2)	0.23
PSA	272	95	(34.9)	185	62	(33.5)	0.76
N. gonorrhoeae	272	2	(0.7)	176	2	(1.1)	0.69
C. trachomatis	272	18	(6.6)	184	14	(7.6)	0.68
T. vaginalis	272	1	(0.4)	171	3	(1.8)	0.13
Metronidazole prescription	273	1	(0.4)	185	2	(1.1)	0.35
Vaginal discharge (self-report)	273	28	(10.3)	185	19	(10.3)	1.0
Moderate/profuse vaginal discharge (clinician assessed)[iv]	270	104	(38.5)	183	51	(27.9)	0.02

Abbreviations: KSh – Kenyan shillings, OCP – oral contraceptive pills, DMPA – depo medroxyprogesterone acetate, IUD – intrauterine device, PID – pelvic inflammatory disease, PCOS – polycystic ovarian syndrome, STI – sexually transmitted infection, BMI – body mass index, BV – bacterial vaginosis, PSA – prostate specific antigen

[i] Reported dates of IUD and implant removal and last date of DMPA were reviewed. Women reporting device removal >2 m
enrollment were re-classified as non-contraceptors (none) for the purpose of this analysis. Women reporting a last DMPA i
were reclassified as non-contraceptors.
[ii] Self-report of syphilis, chlamydia, gonorrhea, and/or trichomoniasis
[iii] Samples did not grow on sub-culture.
[iv] Excludes 5 participants with blood/menses observed on exam

Table 3.3. Enrollment characteristics for 273 Kenyan women with fertility intent by follow-up status

Characteristic	N	All Eligible: Total (N=273)		By Follow-Up Status: Completed Follow-Up[i] (N=166)		Still in Preconception Follow-up (N=47)		Withdrawn or lost to follow-up[ii] (N=60)		p-value
Demographic & Partnership Characteristics										
Study Site	273									<0.001
Nairobi		236	(86.4)	151	(91.0)	33	(70.2)	52	(86.7)	
Mombasa		37	(13.6)	15	(9.0)	14	(29.8)	8	(13.3)	
Age	273									0.40
<25		47	(17.2)	22	(13.3)	12	(25.5)	13	(21.7)	
25-29		83	(30.4)	51	(30.7)	12	(25.5)	20	(33.3)	
30-34		73	(26.7)	51	(30.7)	12	(25.5)	10	(16.7)	
35-39		56	(20.5)	33	(19.9)	9	(19.2)	14	(23.3)	
40-45		14	(5.1)	9	(5.4)	2	(4.6)	3	(5.0)	
Education Level	273									0.27
<8 years		13	(4.8)	6	(3.6)	1	(2.1)	6	(10.0)	
8-11 years		74	(27.1)	42	(25.3)	13	(27.7)	19	(31.7)	
12-15 years		128	(46.9)	78	(47.0)	25	(53.2)	25	(41.7)	
≥16 years		58	(21.3)	40	(24.1)	8	(17.0)	10	(16.7)	
Monthly Household Income (KSh)	271									0.02
<2,500		9	(3.3)	6	(3.7)	1	(2.1)	2	(3.3)	
25,000-10,000		68	(25.1)	32	(19.5)	12	(25.5)	24	(40.0)	
10,000-30,000		112	(41.3)	64	(39.0)	25	(53.2)	23	(38.3)	
30,000-75,000		49	(18.1)	38	(23.2)	4	(8.5)	7	(11.7)	
>75,000		33	(12.2)	24	(14.6)	5	(10.6)	4	(6.7)	
Partner's age	273									0.43
<25		8	(2.9)	5	(3.0)	0	(0.0)	3	(5.0)	
25-29		46	(16.9)	24	(14.5)	8	(17.0)	14	(23.3)	
30-34		79	(28.9)	49	(29.5)	17	(36.2)	13	(21.7)	
35-39		70	(25.6)	42	(25.3)	13	(27.7)	15	(25.0)	
40-44		52	(19.1)	37	(22.3)	5	(10.6)	10	(16.7)	

≥45		18 (6.6)	9 (5.4)	4 (8.5)	5 (8.3)	
Partner's HIV-serostatus	271					0.08
HIV-seronegative		198 (73.1)	121 (73.3)	30 (63.8)	47 (79.7)	
HIV-seropositive		17 (6.3)	7 (4.2)	4 (8.5)	6 (10.2)	
Unknown		56 (20.7)	37 (22.4)	13 (27.7)	6 (10.2)	
Substances						
Smoke cigarettes	273	1 (0.4)	1 (0.6)	0 (0.0)	0 (0.0)	0.72
Frequency of alcohol use	273					0.24
None		228 (83.5)	134 (80.7)	43 (91.5)	51 (85.0)	
Monthly or less		37 (13.6)	25 (15.1)	3 (6.4)	9 (15.0)	
2-4 times per month		8 (2.9)	7 (4.2)	1 (2.1)	0 (0.0)	
≥4 times per month		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Depressive symptom screen (PHQ-9)	273					0.14
None/Minimal (0-4)		263 (96.3)	160 (96.4)	46 (97.9)	57 (95.0)	
Mild (5-9)		9 (3.3)	6 (3.6)	0 (0.0)	3 (5.0)	
Moderate/severe (≥10)		1 (0.4)	0 (0.0)	1 (2.1)	0 (0.0)	
Contraception						
Most recent contraceptive method[iii]	273					0.001
None		79 (28.9)	39 (23.5)	13 (27.7)	27 (45.0)	
Condoms		20 (7.3)	7 (4.2)	4 (8.5)	9 (15.60)	
OCP		6 (2.2)	3 (1.8)	0 (0.0)	3 (5.0)	
Injectable (DMPA)		10 (3.7)	5 (3.0)	3 (6.4)	2 (3.3)	
Copper IUD		80 (29.3)	61 (36.8)	9 (19.2)	10 (16.7)	
Implant		76 (27.8)	50 (30.1)	17 (36.2)	9 (15.0)	
Other		2 (0.7)	1 (0.6)	1 (2.1)	0 (0.0)	
Any DMPA injection in last 6 months	273	13 (4.8)	5 (3.0)	4 (8.5)	4 (6.7)	0.22
Reproductive History						
Gravid	273	250 (91.6)	158 (95.2)	39 (83.0)	53 (88.3)	0.02
Parous	273	240 (87.9)	154 (92.8)	38 (80.9)	48 (80.0)	0.009
Trying for pregnancy prior to study enrollment[iv]	273	66 (24.2)	36 (21.7)	17 (36.2)	13 (21.7)	0.11
# of cycles trying to get pregnant (median/IQR)	66	1 (1, 2)	1 (1, 2)	1 (1, 2)	1 (1, 2)	0.92
Regular menses	273	166 (60.8)	103 (62.1)	26 (55.3)	37 (61.7)	0.70
History of PID (not treated in a hospital)	273	1 (0.4)	1 (0.60)	0 (0.0)	0 (0.0)	0.72
Abnormal uterus[v]						

Fibroids	272	2	(0.7)	2	(1.2)	0	(0.0)	0	(0.0)	0.52
Other/unknown	272	3	(1.1)	1	(0.6)	0	(0.0)	2	(3.3)	0.16
Sexual risk behavior in last 4 weeks										
Any vaginal washing	273	103	37.7	55	(33.1)	20	(42.6)	28	(46.7)	0.14
Frequency of condomless sex (median, IQR)	272	5	(3, 10)	6	(3,10)	7	(4, 12)	4	(1,6.5)	<0.002
Frequency of condomless sex	272									0.02
No condomless sex		30	(11.0)	12	(7.3)	7	(14.9)	11	(18.3)	
1-4		92	(33.8)	57	(34.6)	9	(19.2)	26	(43.3)	
5-8		72	(26.5)	46	(27.9)	13	(27.7)	13	(21.7)	
≥9		78	(28.7)	50	(30.3)	18	(38.3)	10	(16.7)	
Any condom use	271	17	(6.3)	8	(4.9)	3	(6.4)	6	(10.0)	0.38
Clinical										
BMI	273									0.27
Underweight		5	(1.8)	2	(1.2)	2	(4.3)	1	(1.7)	
Normal		103	(37.7)	56	(33.7)	17	(36.2)	30	(50.0)	
Overweight		103	(37.7)	66	(39.8)	17	(36.2)	20	(33.3)	
Obese		62	(22.7)	42	(25.3)	11	(23.4)	9	(15.0)	
History of STI[vi]	273	4	(1.5)	2	(1.2)	1	(2.1)	1	(1.7)	0.89
BV (Nugent ≥7)	273	97	(35.5)	60	(36.1)	17	(36.2)	20	(33.3)	0.92
Elevated sialidase	273	91	(33.3)	54	(32.5)	16	(34.0)	21	(35.0)	0.94
Any Cultivable *Lactobacillus*	273	189	(69.2)	117	(70.5)	30	(63.8)	42	(70.0)	0.68
H_20_2 Producing *Lactobacillus*	272									0.87
None		84	(30.9)	49	(29.7)	17	(36.2)	18	(30.0)	
Type of *Lactobacillus* growth unknown[vii]		14	(5.2)	8	(4.9)	2	(4.4)	4	(6.7)	
Non-H_20_2 Producing *Lactobacillus*		45	(16.5)	30	(8.2)	8	(17.0)	7	(11.7)	
H_20_2 Producing *Lactobacillus*		129	(47.4)	78	(47.3)	20	(42.6)	31	(51.7)	
Lactobacillus morphotypes on Gram Stain	273	174	(63.7)	108	(65.1)	26	(55.3)	40	(66.7)	0.41
PSA	272	95	(34.9)	62	(37.6)	15	(31.9)	18	(30.0)	0.51
N. gonorrhoeae	272	2	(0.7)	0	(0.0)	2	(4.4)	0	(0.0)	<0.01
C. trachomatis	272	18	(6.6)	7	(4.2)	5	(10.9)	6	(10.0)	0.14
T. vaginalis	272	1	(0.4)	1	(0.6)	0	(0.0)	0	(0.0)	0.73
Metronidazole prescription	273	1	(0.4)	1	(0.6)	0	(0.0)	0	(0.0)	0.72
Vaginal discharge (self-report)	273	28	(10.3)	13	(7.8)	6	(12.8)	9	(15.0)	0.24

Moderate/profuse vaginal discharge (clinician assessed)[viii]	273	104	(38.5)	60	(36.8)	22	(44.7)	23	(38.3)	0.62

Abbreviations: KSh – Kenyan shillings, OCP – oral contraceptive pills, DMPA – depo medroxyprogesterone acetate, IUD – intrauterine device, PID – pelvic inflammatory disease, PCOS – polycystic ovarian syndrome, STI – sexually transmitted infection, BMI – body mass index, BV – bacterial vaginosis, PSA – prostate specific antigen

[i] Completed preconception follow-up = 1) Became pregnant, or 2) did not get pregnant during preconception trying time period
[ii] Withdraw reasons include no longer trying to get pregnant, participant request due to or not due to study procedures, moved out of area. Lost to follow-up is defined as a participant who did not attend their last possible preconception visit.
[iii] Reported dates of IUD and implant removal and last date of DMPA were reviewed. Women reporting device removal >2 months prior to enrollment were re-classified as non-contraceptors (none) for the purpose of this analysis. Women reporting a last DMPA injection <6 months prior were reclassified as non-contraceptors.
[iv] Participants reporting >3 cycles were excluded from this study.
[v] Self-reported
[vi] Self-report of syphilis, chlamydia, gonorrhea, and/or trichomoniasis
[vii] Samples did not grow on sub-culture.
[viii] Excludes 3 participants with blood/menses observed on exam.

Table 3.4. Cumulative probability of pregnancy by periconceptual BV and elevated sialidase status

	By Periconceptual BV status[i]					
	BV Negative			BV Positive		
Cycle	At risk	Pregnant	Cumulative % Pregnant (95%CI)	At risk	Pregnant	Cumulative % Pregnant (95%CI)
1	138	16	11.6 (7.3, 18.2)	73	13	17.8 (10.8, 28.7)
2	123	24	28.8 (22.1, 37.2)	69	10	29.7 (20.9, 41.2)
3	87	19	44.4 (36.3, 53.3)	53	4	35.0 (25.6, 46.7)
4	61	11	54.4 (45.9, 63.4)	39	8	48.4 (37.4, 60.7)
5	44	6	60.6 (51.9, 69.5)	21	6	63.1 (50.3, 75.9)
6	29	7	70.1 (60.9, 78.9)	8	1	67.7 (53.5, 81.2)
7	13	2	74.7 (64.7, 83.8)	4	1	75.8 (57.5, 90.5)
8	4	0	74.7 (64.7, 83.8)	-	-	- -
9	4	0	74.7 (64.7, 83.8)	-	-	- -
10	1	0	74.7 (64.7, 83.8)	-	-	- -

	By Periconceptual elevated sialidase status[i]					
	Elevated Sialidase Negative			Elevated Sialidase Positive		
Cycle	At risk	Pregnant	Cumulative % Pregnant (95%CI)	At risk	Pregnant	Cumulative % Pregnant (95%CI)
1	139	15	10.8 (6.7, 17.3)	72	14	19.4 (12.0, 30.6)
2	132	25	27.7 (21.2, 35.7)	60	9	31.5 (22.2, 43.5)
3	94	18	41.5 (33.8, 50.2)	46	5	39.0 (28.8, 51.3)
4	67	11	51.1 (42.9, 60.0)	33	8	53.8 (42.0, 66.4)
5	41	6	58.3 (49.4, 67.4)	24	6	65.3 (53.2, 77.2)
6	25	5	66.6 (56.9, 76.1)	12	3	74.0 (61.0, 85.4)
7	15	3	73.3 (62.7, 83.0)	2	0	74.0 (61.0, 85.4)
8	4	0	73.3 (62.7, 83.0)	-	-	- -
9	4	0	73.3 (62.7, 83.0)	-	-	- -
10	1	0	73.3 (62.7, 83.0)	-	-	- -

[i] Status at the visit prior to current pregnancy test, time-varying

Table 3.5. BV and elevated sialidase at the prior visit and fecundability

Exposure	Unadjusted FR (95% CI)		Adjusted FR (95% CI) [i]	
BV (Nugent ≥7)				
BV (lagged, time-varying)	0.94	(0.67, 1.32)	0.95	(0.66, 1.35)
BV at enrollment	1.07	(0.77, 1.48)	1.00	(0.71, 1.42)
Abnormal microbiota (Nugent ≥4, lagged, time-varying)	1.02	(0.74, 1.42)	1.03	(0.73, 1.44)
Sensitivity Analyses (Nugent ≥7, lagged, time-varying)				
Applying strict exclusion criteria	0.91	(0.62, 1.34)	0.95	(0.63, 1.44)
Including women with ≤ 6 cycles of prior trying time	0.88	(0.63, 1.23)	0.89	(0.63, 1.27)
Excluding participants with imputed cycles	0.95	(0.67, 1.35)	0.93	(0.64, 1.36)
Excluding imputed and reported missed cycles	0.95	(0.67, 1.34)	0.95	(0.66, 1.36)
Elevated Sialidase				
Sialidase test positive (lagged, time-varying)	1.12	(0.80, 1.57)	1.12	(0.79, 1.59)
Sialidase test positive at enrollment	1.01	(0.72, 1.42)	0.96	(0.67, 1.38)
Sensitivity Analyses (lagged, time-varying)				
Applying strict exclusion criteria	1.16	(0.80, 1.69)	1.18	(0.80, 1.74)
Including women with ≤ 6 cycles of prior trying time	1.06	(0.76, 1.48)	1.08	(0.78, 1.52)
Excluding participants with imputed cycles	1.11	(0.79, 1.57)	1.10	(0.77, 1.58)
Excluding imputed and reported missed cycles	1.12	(0.80, 1.56)	1.12	(0.79, 1.60)

[i] Adjusted for age, study site, and frequency of condomless sex in last four weeks

Table 3.6. Associations between BV and elevated sialidase status at the prior and current visit and fecundability

Exposure		Primary Analysis								Sensitivity analysis – Including women with up to 6 cycles of prior trying time							
Periconceptual BV Status		Cycles (N=768)		Pregnancy (N=128)		Unadjusted FR (95% CI)		Adjusted FR (95% CI)[i]		Cycles (N=876)		Pregnancy (N=138)		Unadjusted FR (95% CI)		Adjusted FR (95% CI)[i]	
Prior Visit	Current Visit	n	%	n	%					n	%	n	%				
No	No	467	(60.8)	78	(60.9)	Ref		Ref		533	(60.8)	86	(62.3)	Ref		Ref	
Yes	No	68	(8.9)	21	(16.4)	1.78	(1.15, 2.75)	1.76	(1.13, 2.72)	77	(8.8)	22	(15.9)	1.75	(1.14, 2.67)	1.73	(1.12, 2.65)
No	Yes	34	(4.3)	7	(5.5)	1.16	(0.57, 2.36)	1.22	(0.61, 2.46)	38	(4.3)	8	(5.8)	1.27	(0.66, 2.46)	1.33	(0.69, 2.57)
Yes	Yes	199	(25.9)	22	(17.2)	0.66	(0.42, 1.03)	0.66	(0.42, 1.03)	228	(26.0)	22	(15.9)	0.60	(0.39, 0.93)	0.60	(0.39, 0.93)
Periconceptual Elevated Sialidase		(N=765)		(N=125)						Cycles (N=872)		Pregnancy (N=134)					
No	No	462	(60.4)	73	(58.4)	Ref	-	Ref	-	524	(60.1)	80	(59.7)	Ref		Ref	
Yes	No	74	(9.7)	22	(17.6)	1.75	(1.14, 2.69)	1.73	(1.13, 2.65)	85	(9.8)	23	(17.2)	1.76	(1.15, 2.68)	1.75	(1.16, 2.65)
No	Yes	55	(7.2)	8	(6.4)	0.85	(0.42, 1.71)	0.81	(0.42, 1.59)	59	(6.8)	8	(6.0)	0.84	(0.42, 1.69)	0.80	(0.41, 1.57)
Yes	Yes	174	(22.8)	22	(17.6)	0.81	(0.52, 1.25)	0.81	(0.53, 1.24)	204	(23.4)	23	(17.2)	0.75	(0.49, 1.15)	0.77	(0.50, 1.16)

[i] Adjusted for age, study site, and frequency of condomless sex in last four weeks

Table 3.7. Enrollment characteristics - Sensitivity analysis excluding additional participants with potential underlying infertility

Characteristic[i]	N	Strict inclusion criteria: Eligible (N=228)		Not Eligible (N=45)		p-value
Demographic & Partnership Characteristics						
Cohort	273					0.14
Nairobi		194	(85.1)	42	(93.3)	
Mombasa		34	(14.9)	3	(6.7)	
Age	273					0.90
<25		38	(16.7)	9	(20.0)	
25-29		68	(29.8)	15	(33.3)	
30-34		61	(26.8)	12	(26.7)	
35-39		49	(21.5)	7	(15.6)	
40-45		12	(5.3)	2	(4.4)	
Education Level	273					0.30
<8 years		13	(5.7)	0	(0.0)	
8-11 years		59	(25.9)	15	(33.3)	
12-15 years		106	(46.5)	22	(48.9)	
≥16 years		50	(21.9)	8	(17.8)	
Monthly Household Income (KSh)	271					0.50
<2,500		8	(3.5)	1	(2.3)	
25,000-10,000		61	(26.9)	7	(15.9)	
10,000-30,000		90	(39.7)	22	(50.0)	
30,000-75,000		42	(18.5)	7	(15.9)	
>75,000		26	(11.5)	7	(15.9)	
Partner's age	273					0.83
<25		7	(3.1)	1	(2.2)	
25-29		39	(17.1)	7	(15.6)	
30-34		66	(29.0)	13	(28.9)	
35-39		56	(24.6)	14	(31.1)	
40-44		46	(20.2)	6	(13.3)	
≥45		14	(6.1)	4	(8.9)	
Partner's HIV-serostatus	271					<0.001
HIV-seronegative		174	(77.0)	24	(53.3)	
HIV-seropositive		*0*	*(0.0)*	*17*	*(37.8)*	
Unknown		52	(23.0)	4	(8.9)	

Substances						
Smoke cigarettes	273	1	(0.4)	0	(0.0)	0.66
Frequency of alcohol use	273					0.03
None		192	(84.2)	36	(80.0)	
Monthly or less		32	(14.0)	5	(11.1)	
2-4 times per month		4	(1.8)	4	(8.9)	
≥4 times per month		0	(0.0)	0	(0.0)	
Depressive symptom screen (PHQ-9)	273					0.36
None/Minimal (0-4)		218	(95.6)	45	(100.0)	
Mild (5-9)		9	(4.0)	0	(0.0)	
Moderate/severe (≥10)		1	(0.4)	0	(0.0)	
Contraception						
Most recent contraceptive method[ii]	273					<0.01
None		68	(29.8)	11	(24.4)	
Condoms		8	(3.5)	12	(26.7)	
OCP		5	(2.2)	1	(2.2)	
Injectable		10	(4.4)	0	(0.0)	
Copper IUD		70	(30.7)	10	(22.2)	
Implant		65	(8.5)	11	(24.4)	
Other		2	(0.9)	0	(0.0)	
Any DMPA injection in last 6 months	273	12	(5.3)	1	(2.2)	0.38
Reproductive History						
Gravid	273	207	(90.8)	43	(95.6)	0.29
Number of pregnancies (median, IQR)[iii]	273	2	(1,2)	2	(1,2)	0.82
Parous	273	200	(87.7)	40	(89.0)	0.83
Trying for pregnancy prior to study enrollment[iv]	273	55	(24.1)	11	(24.4)	0.96
# of cycles trying to get pregnant (median/IQR)	66	1	(1, 2)	1	(1, 2)	0.42
Regular menses	273	137	(60.1)	29	(64.4)	0.58
History of pelvic inflammatory	*273*	*0*	*(0.0)*	*1*	*(2.2)*	*0.02*
Abnormal uterus[v]						
Fibroids	*272*	*0*	*(0.0)*	*2*	*(4.4)*	*<0.001*
Other/unknown	*272*	*0*	*(0.0)*	*3*	*(1.1)*	*<0.001*
Sexual risk behavior in last 4 weeks						
Any vaginal washing	273	85	(37.3)	18	(40.0)	0.73
Frequency of condomless sex (median, IQR)	272	5	(3, 10)	5	(2, 8)	0.26
Frequency of condomless sex	272					0.21
No condomless sex		22	(9.7)	8	(18.2)	
1-4		79	(34.7)	13	(29.6)	

5-8		58	(25.4)	14	(31.8)	
≥9		69	(30.3)	9	(20.5)	
Any condom use	271	11	(4.9)	6	(13.6)	0.03
Clinical						
BMI	273					0.79
Underweight		5	(2.2)	0	(0.0)	
Normal		85	(37.3)	18	(40.0)	
Overweight		86	(37.7)	17	(37.8)	
Obese		52	(22.8)	10	(22.2)	
History of STI (self-report)[vi]	*273*	*0*	*(0.0)*	*4*	*(8.9)*	*<0.001*
BV (Nugent ≥7)	273	71	(31.1)	26	(57.8)	0.001
Elevated sialidase	273	67	(29.4)	24	(53.3)	0.002
Any cultivable *Lactobacillus*	273	162	(71.1)	27	(60.0)	0.14
H_2O_2 Producing *Lactobacillus*	272					0.33
None		66	(29.1)	18	(40.0)	
Type of *Lactobacillus* growth unknown[vii]		12	(5.3)	2	(4.4)	
Non-H_2O_2 Producing *Lactobacillus*		41	(18.1)	4	(8.9)	
H_2O_2 Producing *Lactobacillus*		108	(47.6)	21	(46.7)	
Lactobacillus morphotypes on Gram Stain	273	155	(68.0)	19	(42.2)	<0.001
PSA	272	78	(34.4)	17	(37.8)	0.66
N. gonorrhoeae	*272*	*0*	*(0.0)*	*2*	*(4.4)*	*<0.001*
C. trachomatis	*272*	*0*	*(0.0)*	*18*	*(40.0)*	*<0.001*
T. vaginalis	*272*	*0*	*(0.0)*	*1*	*(2.2)*	*0.02*
Metronidazole prescription	273	1	(0.4)	0	(0.0)	0.66
Vaginal discharge (self-report)	273	24	(10.5)	4	(8.9)	0.74
Moderate/profuse vaginal discharge (clinician assessed)[viii]	273	84	(37.2)	20	(45.5)	0.30

Abbreviations: KSh–Kenyan shillings, OCP–oral contraceptive pills, DMPA–depo medroxyprogesterone acetate, IUD–intrauterine device, PID–pelvic inflammatory disease, STI–sexually transmitted infection, BMI–body mass index, BV–bacterial vaginosis, PSA–prostate specific antigen

[i] The additional exclusion criteria for sensitivity analysis are italicized in the table.
[ii] Reported dates of IUD and implant removal and last date of DMPA were reviewed. Women reporting device removal >2 months prior to enrollment were re-classified as non-contraceptors (none) for the purpose of this analysis. Women reporting a last DMPA injection <6 months prior were reclassified as non-contraceptors.
[iii] Including pregnancies that ended in medical abortion, miscarriage, ectopic pregnancies, stillbirth and live birth
[iv] Self-reported by participants when asked "Have you been actively trying to get pregnant before entering this study (i.e., having regular condomless sex without using another form of contraception?". Participants reporting >3 cycles were excluded from this study.
[v] Self-reported

[vi] Including syphilis, chlamydia, gonorrhea, trichomoniasis
[vii] Samples did not grow on sub-culture.
[viii] Excludes 3 participants with blood/menses observed on exam

CHAPTER FOUR - Effect of cultivable Lactobacillus during the periconception period on fecundability among Kenyan pregnancy planners

Absence and low levels of vaginal *Lactobacillus* species have been associated with adverse reproductive health outcomes, including increased risk of bacterial vaginosis (BV), sexually transmitted infections (STI), HIV, preterm birth, and pregnancy loss (58,121,130,149). These associations have been identified using both culture-dependent and molecular methods of detection. *Lactobacillus crispatus* and *L. jensenii,* in particular, are associated with having an optimal vaginal bacterial community (58). The role of *L. iners*, which is detected in the majority of vaginal specimens, is unclear. Vaginal microbiota dominated by *L. iners* may represent an intermediate, or transitional, state between optimal and non-optimal vaginal ecosystems (56,198–200).

Lactobacillus species contribute to the optimal vaginal environment by producing lactic acid that maintains an acidic vaginal pH and prevents colonization by acid-intolerant bacteria (201). In addition, epidemiological studies have consistently demonstrated an association between hydrogen peroxide (H_20_2) producing *Lactobacillus* and a lower risk of adverse reproductive health outcomes (68,149,202–204). This has suggested a role for H_2O_2 in maintaining women's reproductive health, but the mechanism is debated (205). Hydrogen peroxide levels representing the *in-vivo* physiological capacity of H_2O_2-producing *Lactobacillus* are unable to inactivate 17 BV-associated bacteria *in vitro*, including *G. vaginalis*, *Prevotella* species, and *A. vaginae* (206). Moreover, the anti-oxidant properties of cervicovaginal fluid and semen neutralize H_2O_2 produced by *Lactobacillus* (207). An alternative explanation for the beneficial effects of H_2O_2-producing *Lactobacillus* is that the species that are most likely to be strong producers of H_2O_2 (e.g., *L. crispatus* and *L. jensenii*) produce their beneficial effects through a non-H_2O_2 mechanism (205,208).

Lactobacillus species may also contribute to improved outcomes for women undergoing in-vitro fertilization (IVF). In the 1990's and early 2000's several studies utilized culture-dependent techniques to isolate cervical and uterine bacteria at embryo transfer. These studies found reduced implantation, clinical pregnancy, and live birth rates in women with positive cultures for Gram-negative bacteria (including *Escherichia coli*), *Streptococcus spp*., and *Staphylococcus spp*. (209–214). In contrast, detection of cultivable H_20_2-producing *Lactobacillus* was associated with a higher live birth rate (212). The leading hypothesis for a vaginal or endometrial microbiota effect on assisted reproduction outcomes is that embryo implantation may be disrupted by the inflammatory response to BV-associated bacteria (215). With the advent of molecular methods for microbial detection including cultivation-resistant species, the question of endometrial bacteria and impact on IVF outcomes is being revisited. Studies of the vaginal and endometrial microbiota of women undergoing IVF have detected a high relative abundance of *Lactobacillus*, but have also identified BV-associated bacteria in the uterus (90–93,216–218). The evidence is mixed, however, whether *Lactobacillus* detected using molecular techniques is associated with IVF outcomes (92,93,218).

To date, however, no research has been conducted that examines the association between *Lactobacillus* detection and reproductive success in women without an infertility diagnosis attempting non-medically assisted reproduction. Therefore, the objective of this analysis was to examine the association between vaginal *Lactobacillus* and fecundability in a cohort of HIV-seronegative women in Kenya with immediate fertility intent.

METHODS

Study design, population, and procedures

The overall study design, population, and procedures for this prospective fecundability analysis have been presented in detail in **Chapter 3: Methods**. In addition to the vaginal specimen

samples previously described, one vaginal sample was inoculated directly onto Rogosa agar, which is selective for *Lactobacillus* growth. The agar plate was placed immediately in a candle jar and transferred within four hours to anaerobic jars for culture at 37°C for 72 hours. Plates were then assessed for *Lactobacillus* growth. *C*olonies identified on Rogosa agar were confirmed as *Lactobacillus* through the appearance of bacteria on a Gram stain. A sterilized wire loop was utilized to collect *Lactobacillus* colonies, which were then inoculated into serum vials containing skim milk and stored at -20°C. Every two months, samples were sub-cultured on Rogosa agar in anaerobic jars at 37°C for 72 hours and assessed for *Lactobacillus* growth. Negative samples were sub-cultured again; repeat negatives were recorded as negative for sub-culture. Positive samples were sub-cultured on tetramethylbenzidine (TMB) agar containing horseradish peroxidase in anaerobic jars at 37°C for 72 hours. On exposure to air, $H_2 0_2$ production by *Lactobacillus* was assessed by the intensity of the blue color produced (150).

Statistical Analysis

Discrete menstrual cycles, conception outcomes, and the discrete time proportional probabilities model for this analysis were the same as those defined in **Chapter 3: Methods** and **Figure 3.1**. The primary exposure for this analysis was presence of cultivable *Lactobacillus* (no/yes) at the visit prior to each pregnancy test to best align the with the preconception period of each menstrual cycle.

Age (<25, 25-29, 30-34, 35-39, 40-45) and frequency of condomless sex in the prior four weeks (time-varying; none, 1-4, 5-8, ≥9) were included as *a priori* confounders due to known associations with both optimal vaginal microbiota and becoming pregnant (19,20,177). Study site was also included *a priori*. Additional factors that were assessed as potential confounders included maternal body mass index (BMI; underweight ≤18.5, normal 18.5–24.9, overweight 25–29.9, and

obese ≥30) (20,40,174,175), vaginal washing in the last month (time-varying, no/yes) (65,176,177), any condom use in the last month (time-varying, no/yes) (59,173), maternal education (<8, 8-11, 12-15, ≥16 years), and household income (Kenyan Shillings, <2,500, 2,500-10,000, 10,000-30,000, 30,000-75,000,≥75,000). Potential confounders were selected using a manual forward stepwise model-building approach described in **Chapter 3**. None of the confounding factors assessed for inclusion changed the FR estimate by >10%. Therefore, none were retained in the final adjusted models.

Sensitivity analyses were conducted to assess the impact of analytic assumptions on the effect estimates. These included applying a set of strict exclusion criteria (**Chapter 3: Methods**), excluding imputed menstrual cycles, conducting a complete-case analysis to assess the effect of imputed missing data, and including women with up to six months of conception attempt time prior to enrollment to assess potential bias associated with exclusion of women with longer pre-enrollment conception attempts. Four secondary analyses were also conducted. First, an analysis using only the enrollment cultivable *Lactobacillus* status was conducted. Second, a model was run to assess the association between H_20_2-producing *Lactobacillus* at the visit prior and fecundability using a four-category variable: no cultivable *Lactobacillus*, *Lactobacillus* phenotype unknown (i.e., isolate did not grow upon sub-culture), non-H_20_2-producing *Lactobacillus*, H_20_2-producing *Lactobacillus.* Third, a variable combining the cultivable *Lactobacillus* result from the visit prior to and the measure at each cycle’s ultimate pregnancy test was generated to assess stable versus dynamic vaginal microbiota during periconception period. This method resulted in four categories: negative at both visits; positive at visit prior but negative at current visit, negative at visit prior but positive at current visit, and positive at both visits. Lastly, models assessing the association between the presence of *Lactobacillus* morphotypes detected on Gram stain at the visit prior and across two consecutive visits and fecundability were conducted. A *Lactobacillus* morphotype score of 0 was considered negative and a score of 1-4 was considered positive.

RESULTS

Baseline & Follow-up Characteristics

For these fecundability analyses, 273 participants contributed 768 menstrual cycles and 128 pregnancies (**Figure 3.2)**. Overall baseline (**Tables 3.1-3.3**) and follow-up characteristics were described in **Chapter 3: Results**. At enrollment, 69.2% (189/273) of participants had cultivable *Lactobacillus*, 47.4% (129/272) had H_20_2-producing *Lactobacillus*, and 63.7% (174/273) had *Lactobacillus* morphotypes detected on Gram stain (**Table 3.1**). Nearly three-quarters (194/273) of participants had concordant cultivable *Lactobacillus* and *Lactobacillus* by Gram stain results. Participants at the Nairobi site were more likely to have cultivable *Lactobacillus* (72.0% versus 51.4%, p=0.01) and less likely to report engaging in vaginal washing (32.6% versus 70.3%, p<0.001) compared to participants at the Mombasa site. Participants in the parent MPTB Study who were excluded from these fecundability analyses were less likely to have any cultivable *Lactobacillus* than those included in both the primary analysis (50.8% versus 69.2%, p<0.001, **Table 3.2**) and in the sensitivity analysis including women with up to six cycles of prior trying time (54.4.% versus 65.3%, p=0.03). At enrollment, Women with zero to three prior cycles of trying time were more likely to have any cultivable *Lactobacillus* than women with four to six cycles of prior trying time (69.2% versus 36.8%, p<0.001).

Lactobacillus **& Fecundability**

During pre-conception follow-up, participants had cultivable *Lactobacillus* detected at 65.9% (650/987) and H_20_2-producing *Lactobacillus* detected at 48.3% (470/974) of study visits. This resulted in 66.5% (511/768) of menstrual cycles positive for cultivable *Lactobacillus* at the visit prior and 47.5% (364/766) with H_20_2-producing *Lactobacillus* at the visit prior. Results from *Lactobacillus* culture were missing for 9.8% (75/768) of menstrual cycles; 41 were imputed cycles, 31 were missed cycles reported following a missed visit, and 4 were missing *Lactobacillus* results because participants declined examination.

The probability of pregnancy was similar for cycles with and without cultivable *Lactobacillus* species (**Table 4.1**). In unadjusted analysis, cultivable *Lactobacillus* at the visit prior was not significantly associated with fecundability (FR 1.15, 95%CI 0.80-1.66) (**Table 4.2**). These results were similar after adjusting for age, frequency of condomless sex in the last four weeks, and study site (adjusted FR [aFR] 1.14, 95%CI 0.77-1.68). The sensitivity analyses employing a strict set of additional exclusion criteria, assessing the effect of menstrual cycle imputation, and including women with up to six cycles of prior trying time did not markedly change the effect estimates (**Table 4.2**). Similarly, there was no association between enrollment measures of cultivable *Lactobacillus* (aFR 0.95, 95%CI 0.67-1.34), H_20_2-producing *Lactobacillus* at the visit prior, (aFR 1.12, 95%CI 0.74-1.70), or *Lactobacillus* morphotypes detected on Gram stain at the visit prior (aFR 0.98, 95%CI 0.69, 1.38) and fecundability.

When cultivable *Lactobacillus* status was re-evaluated based on the combined measurements at the visit prior to and at each pregnancy test, 25.4% (n=195) of menstrual cycles were negative for cultivable *Lactobacillus* at both visits, 8.6% (n=66) had cultivable *Lactobacillus* at the visit prior but were negative the visit with pregnancy testing, 8.1% (n=62) were negative for *Lactobacillus* at the visit prior to and positive for cultivable *Lactobacillus* at the current visit, and 57.9% (n=445) were *Lactobacillus* positive at both visits (i.e., optimal vaginal health) (**Table 3.2**). Compared to menstrual cycles considered *Lactobacillus* negative at both visits, cycles with at least one periconceptual measurement positive for cultivable *Lactobacillus* had a 1.14-1.63 fold higher probability of pregnancy, but none of the categories were statistically significant. These FRs increased in the sensitivity analysis including women with up to six cycles of prior trying time. There was a significant two times increased fecundability ratio (aFR 2.07, 95%CI 1.20-3.57) in menstrual cycles with cultivable *Lactobacillus* at the visit prior, but not at the current pregnancy testing visit, compared to cycles without *Lactobacillus* at either measurement. Similarly, when assessing presence of H_20_2-producing *Lactobacillus* across the two time points, menstrual cycles

with H_20_2-producing *Lactobacillus* at the visit before but not current visit, had a significant 1.7-fold increased per cycle probability of pregnancy (aFR 1.74, 95% CI 1.06-2.86); this increased to an adjusted FR of 2.14 (95%CI 1.34-3.42) when including women with up to six menstrual cycles of prior trying time. Lastly, compared to menstrual cycles with no detection *Lactobacillus* morphotypes at either visit, menstrual cycles with *Lactobacillus* morphotypes detected at one or both visits were associated with a 1.5-2.9 fold increased probability of pregnancy, which increased to significant aFRs of 1.7-3.1 when including women with up to six prior cycles of trying time at enrollment. Specifically, menstrual cycles with *Lactobacillus* morphotypes at both visits (i.e., optimal vaginal health) had 1.5-1.7 fold increased per-cycle probability of pregnancy (≤3 months of prior trying time: aFR 1.52, 95%CI 0.97-2.41; ≤6 months of prior trying time: aFR 1.65, 95%CI 1.05-2.59), and menstrual cycles without *Lactobacillus* morphotypes detected at the visit prior but with *Lactobacillus* morphotypes at the second visit had a statistically significant 2.9-3.1 fold increased FR (≤3 months of prior trying time: aFR 2.92, 95%CI 1.76-4.85; ≤6 months of prior trying time: aFR 3.14, 95%CI 1.90-5.19).

DISCUSSION

This prospective study of Kenyan women with immediate fertility intent is the first analysis to assess the association between cultivable vaginal *Lactobacillus* and fecundability in a population of women attempting non-medically assisted conception. In the primary analysis, there were small, non-significant, trends toward increased per-cycle probabilities of pregnancy in menstrual cycles with cultivable *Lactobacillus*. When menstrual cycles without cultivable *Lactobacillus* across two time points during the periconception period were compared to cycles with *Lactobacillus* detected by culture at one or both visits per conception attempt, the association between *Lactobacillus* detection and increased fecundability became stronger, though were still not statistically significant. In comparison, when considering detection of *Lactobacillus* morphotypes on Gram stain across two visits, menstrual cycles with *Lactobacillus* morphotypes

detected both visits were associated with a 52% increased fecundability that trended toward significance. Overall, these findings are consistent with the dogma that *Lactobacillus* species are associated with optimal reproductive health, but also points to the complex relationship between presence, viability, and functionality of specific vaginal *Lactobacillus* species and fecundability.

The adjusted FRs associated with *Lactobacillus* detected at one or both consecutive visits compared to neither were larger when *Lactobacillus* were detected on Gram stain versus culture on Rogosa agar. These differences in results reflect the strengths and limitations of the culture-dependent and microscopy-based methods of detection. Bacteria detected on Gram stain may not be viable, but a larger range of *Lactobacillus* species can be detected than on culture using Rogosa agar. Rogosa agar, while selective for *Lactobacillus*, does not support the growth of *L. iners* (219). *Lactobacillus iners* is common in women of African descent (56,220–222). If *L. iners* significantly contributes to fecundability in this study population, the culture-dependent analysis would not have been able to detect the association. However, its absence from the culture results increased the ability of these analyses to detect an association with cultivable *Lactobacillus* species that are more often considered to represent optimal vaginal microbiota (i.e., *L. crispatus*). In addition, *Lactobacillus* species likely have a differential ability to grow on culture, so some viable *Lactobacillus* may not have been detected due to growth restrictions or species competition. In this study, isolates grown on the Rogosa culture also were not speciated so the cultivated *Lactobacillus* species are unknown. For this reason, the analysis assessing *Lactobacillus* morphotype detection on Gram stain may have detected a wider range of *Lactobacillus* species such as *L. iners*, *L. gasseri*, and *L. vaginalis*, including some viable bacteria that were unable to grow on culture. Overall, these results suggest that underlying associations between specific *Lactobacillus* species, such as *L. crispatus* and *L. jensenii*, and their metabolomic profiles may contribute to improved fecundity and should be assessed in future studies.

The strongest associations between periconceptual *Lactobacillus* measured at two time points and improved fecundability compared to cycles negative for *Lactobacillus* at both time points were among menstrual cycles with *Lactobacillus* at one, but not both, of the study visits. This finding is perplexing since we hypothesized that women with *Lactobacillus* at both time points would have the highest relative fecundability ratio due to the well-documented associations between *Lactobacillus* colonization and improved reproductive health outcomes. This result could reflect a result of chance (Type 1 error), particularly in the context of multiple secondary analyses and smaller than planned sample size or could be a true association with yet known mechanisms. In addition, as discussed in the **Chapter 3: Discussion**, there is strong residual confounding by condomless sex that affect analyses assessing the association between vaginal microbiota disruption and fecundability. In addition, the vaginal microbiota becomes more *Lactobacillus* dominated in early pregnancy (118,183). This may also explain the increased fecundability identified in menstrual cycles without *Lactobacillus* at the visit prior but with *Lactobacillus* at the subsequent visit.

There were also small, but consistent, increases in the magnitude of associations for cultivable *Lactobacillus* at the visit prior and in the secondary analyses of cultivable *Lactobacillus*, H_20_2-producing *Lactobacillus*, and *Lactobacillus* morphotypes on Gram stain across two consecutive visits when including women with up to six menstrual cycles of prior conception attempt time at enrollment. Women with four to six menstrual cycles of unobserved conception attempt time were less likely to have any cultivable *Lactobacillus* at enrollment than those with zero to three cycles of prior trying time (50.8% versus 69.2%). While the differential cultivable *Lactobacillus* prevalence by prior trying time may be due to random variation, it could also reflect a *Lactobacillus* effect on entry time (i.e., differential left truncation by the exposure under study) and fecundability, which would result in a biased FR estimate (196). However, this does not likely explain the change in the effect estimates seen as a similar phenomenon occurred when assessing detection of

Lactobacillus morphotypes on Gram stain, which did not differ at enrollment between women with four to six versus zero to three prior cycles of trying time (69.2% versus 63.7%). Rather, this finding may more likely reflect the inclusion of 108 additional menstrual cycles and 10 additional pregnancies, providing more data and precision to these preliminary analyses.

Women undergoing medically assisted reproduction are not comparable to those who are attempting non-medically assisted reproduction, since they or their partners have already been diagnosed with infertility. In addition, the reproductive tract microbiota may be affected by infertility protocols, which may include high-levels of exogenous hormones, multiple intra-vaginal procedures, and often antibiotic therapy. Moreover, studies assessing vaginal or endometrial microbiota and IVF outcomes have primarily included only Caucasian women; results may not be generalizable to African women due to racial, ethnic, and regional differences in women's vaginal microbiota (56,220–223). Nonetheless, studies assessing the vaginal or endometrial microbiota in women undergoing IVF provide the only context that is currently available for comparison to the results of the present study. In a study of 91 women undergoing IVF, H_20_2-producing *Lactobacillus* by culture detected in vaginal fluids or on the catheter tip at embryo transfer had a higher live birth rate (vaginal fluid: 50% vs 21%, p=0.01; transfer tip: 70% vs 25%, p=0.01) (212). More recently, there have been a few studies utilizing molecular methods to assess vaginal or endometrial microbiota and IVF outcomes, but the results are conflicting. In a study of 33 women, *Lactobacillus* was the most abundant species identified in the endometrial microbiota detected on the catheter tip after embryo transfer among women with and without ongoing clinical pregnancies at 8 weeks post-embryo transfer (93). In another study, among 32 women contributing endometrial fluid samples collected prior to the embryo transfer cycle, those with a relative abundance of *Lactobacillus* >90% had significantly higher rates of implantation (60.7% versus 23.1%, p=0.02), pregnancy (70.6% versus 33.3%, 0.03), ongoing pregnancy (58.8% versus 13.3%, p=0.02), and live birth (58.8% versus 6.0%, p<0.01) compared to women with <90%

Lactobacillus. Similarly, when considering 150 vaginal samples collected just prior to embryo transfer, women with vaginal microbiota dominated by *L. crispatus* were more likely to have a biochemical pregnancy (p=0.039), clinical pregnancy (p=0.015), and live birth (p=0.021) than women not dominated by *L. crispatus* (218). Lastly, in a study of 75 women who underwent embryo transfer within two months of vaginal sampling, the researchers reported no significant differences in biochemical or clinical pregnancy rates among women with three community state types defined by high relative abundance of *L. crispatus*, high abundance of *L. iners*, and a diverse community of bacteria (biochemical pregnancies: 44% versus 59% versus 30%; clinical pregnancies: 33% versus 53% versus 10%; no p-values) (90). While the authors report these as non-significant differences, the biochemical and clinical pregnancy rates were highest in the women whose vaginal bacterial communities were dominated by *L. iners*. In this study, women with high concentrations of *G. vaginalis* or *A. vaginae* detected by qPCR had a lower clinical pregnancy rate (90). Comparing results across these studies is challenging, however, since they varied in sampling location (vaginal versus endometrial), sampling timing (in the month(s) prior to embryo transfer versus immediately prior to transfer versus after transfer), in methods (community state type versus relative abundance versus qPCR), and in outcomes under study (biochemical pregnancies versus ongoing clinical pregnancies versus live births), and had small sample sizes. Overall, while the evidence is mixed, these studies of a range of IVF outcomes and these present fecundability analyses provide evidence that *Lactobacillus* could be associated with fecundity and motivate future work in this area.

In assisted reproduction, the hypothesized mechanism to explain associations between vaginal and endometrial microbiota and reduced reproductive success is disruption of embryo implantation and early embryo development due to the immune response to pathogenic microbiota (215). In the context of non-medically assisted reproduction, this is also a plausible mechanism. In addition, vaginal and endometrial microbiota disruption may also contribute to

reduced fecundity by interrupting the physiochemical properties of cervical mucus and by exerting deleterious effects on sperm function. Cervical mucus prevents vaginal bacteria from ascending and colonizing the upper reproductive tract, facilitates sperm transport from the vagina to the uterus, provides a barrier to sperm with poor motility or morphology, and may contribute toward sperm capacitation (94–96). A reduction in the abundance of *Lactobacillus* species in the vagina and increase in BV-associated bacteria contributes to a degradation of the cervical mucus and ascension of microorganisms into the upper reproductive tract (79). Not only might this disrupt sperm transport through the cervix, but the inflammatory response may increase reactive oxygen species (ROS) production. These ROS can, in turn, lead to oxidative stress, damaging cells, causing sperm DNA fragmentation, and reducing sperm motility in the upper reproductive tract where sperm are no longer protected by the anti-inflammatory and anti-oxidant properties of seminal fluid (94,97,224).

Research to support, or refute, whether *Lactobacillus* species themselves exert a protective effect on sperm is sparse. One *in vitro* study found that a *Lactobacillus* probiotic containing *L. brevis*, *L. salivarius*, and *L. plantarum* protected against ROS induced lipid peroxidation of sperm and was associated with better sperm motility (225). These lactobacilli, however, are not those typically found in the vagina. In addition, several molecular studies assessing the seminal microbiota have identified *Lactobacillus* spp. as one of the most abundant species in semen (226–229). In two of these studies, men with normal semen parameters were more likely to have a higher relative abundance of *Lactobacillus* suggesting a beneficial effect on sperm health (227,229). How the seminal, vaginal, and endometrial microbiota collectively contribute to fecundity has not been explored.

Major strengths of this prospective fecundability analysis were the monthly vaginal specimen sampling and detection of *Lactobacillus* using three different methods, including culture on

Rogosa agar, sub-culture on TMB agar for H_2O_2 production, and Gram stain for *Lactobacillus* morphotype detection. Together, this allowed for assessment of the presence, viability, and functionality of *Lactobacillus* at multiple time points during the periconception period. Moreover, nearly 60% of participants discontinued oral contraceptive pills, implant, or IUD just prior to study enrollment. Therefore, the first completed menstrual cycle at risk for pregnancy was included for the majority of participants, which reduced selection bias associated with missing early cycles at risk.

In addition to not speciating the detected *Lactobacillus*, this fecundability analysis was also subject to a number of limitations related to the MPTB Study that that served as the parent study for this analysis. First, discrete menstrual cycles were created from reported LMP reported by participants at monthly preconception visits, but participants who missed preconception visits were not asked to report interim LMP during the first year of the study. Missed cycles were imputed using participants' menstrual cycle characteristics reported at baseline and their missing exposure data were imputed from the most recent measurement. However, sensitivity analysis excluding these cycles generated similar findings. Second, residual confounding by frequency of condomless sex is likely since the data were collected for the prior four weeks and not per discrete menstrual cycle. In addition, no measure of condomless sex within the fertile window was available. Third, prospective fecundability cohorts can suffer from high levels of participant withdrawal or loss to follow-up as pregnancy intention changes or due to intensive study procedures (230). In this study 22.0% of participants were lost to follow-up or withdrew from the study early. However, the proportion of participants with cultivable *Lactobacillus* at enrollment was similar among those who were retained (69%), withdrew (63.0%), and were lost to follow-up (70%), which minimizes risk of bias.

Given the predominance of *Lactobacillus* in the optimal vaginal environment and its association with protection against numerous adverse reproductive and pregnancy outcomes, it is plausible that *Lactobacillus* could also promote fecundity and fertility. In this cohort of HIV-negative Kenyan women trying to conceive, cultivable *Lactobacillus* in the vagina at the visit prior to each pregnancy test was associated with slightly higher fecundability, but the difference was not statistically significant. The magnitude of associations increased when *Lactobacillus* was detected by culture or on Gram stain at one or both consecutive measurements. There was an approximately 50% increase in fecundability in cycles with *Lactobacillus* morphotypes detected at two consecutive visits that trended toward significance. These exploratory results offer interesting findings that will need to be confirmed in future research. Future studies of the impact of *Lactobacillus* on fecundability in the context of natural conception would benefit from improved measurement of sex during the fertile window and from employing both culture-dependent and molecular methods of detection to explore the presence, functionality, and species of vaginal *Lactobacillus* during the periconception period.

Table 4.1. Overall cumulative probability of pregnancy and by periconceptual *Lactobacillus* status

Cycle	Overall				By Periconceptual Cultivable *Lactobacillus*[i]							
					No Cultivable *Lactobacillus*				Cultivable *Lactobacillus*			
	At risk	Pregnant	Cumulative % Pregnant (95%CI)		At risk	Pregnant	Cumulative % Pregnant (95%CI)		At risk	Pregnant	Cumulative % Pregnant (95%CI)	
1	211	29	13.7	(9.8, 19.2)	58	11	19.0	(11.0, 31.6)	153	18	11.8	(7.6, 18.0)
2	192	34	29.0	(23.5, 35.5)	60	9	31.1	(21.4, 43.9)	132	25	28.5	(22.0, 36.4)
3	140	23	40.7	(34.4, 47.7)	45	4	37.2	(26.8, 50.2)	95	19	42.8	(35.1, 51.3)
4	100	19	52.0	(45.1, 59.2)	40	4	43.5	(32.5, 56.4)	60	15	57.1	(48.6, 65.9)
5	65	12	60.8	(53.6, 68.2)	30	6	54.8	(42.8, 67.7)	35	6	64.4	(55.5, 73.3)
6	37	8	69.3	(61.5, 82.7)	15	3	63.9	(50.5, 77.1)	22	5	72.5	(63.0, 81.4)
7	17	3	74.7	(66.0, 82.7)	8	3	77.4	(61.6, 90.1)	9	0	72.5	(63.0, 81.4)
8	4	0	74.7	(66.0, 82.7)	1	0	77.4	(61.6, 90.1)	3	0	72.5	(63.0, 81.4)
9	4	0	74.7	(66.0, 82.7)	-	-	-	-	3	0	72.5	(63.0, 81.4)
10	1	0	74.7	(66.0, 82.7)	-	-	-	-	1	0	72.5	(63.0, 81.4)

[i] Status at the visit prior to current pregnancy test, time-varying

Table 4.2. Cultivable *Lactobacillus* and fecundability

Exposure	Unadjusted FR (95% CI)		Adjusted FR (95% CI) [i]	
Primary Analysis				
Cultivable *Lactobacillus* (lagged, time-varying)	1.15	(0.80, 1.66)	1.14	(0.77, 1.68)
Sensitivity Analyses (lagged, time-varying)				
Applying strict exclusion criteria	1.20	(0.80, 1.79)	1.17	(0.77, 1.78)
Including women with ≤ 6 cycles of prior trying time	1.27	(0.89, 1.83)	1.24	(0.84, 1.81)
Excluding participants with imputed cycles	1.24	(0.86, 1.79)	1.18	(0.81, 1.73)
Excluding imputed and reported missed cycles	1.20	(0.83, 1.73)	1.17	(0.79, 1.72)
Secondary Analysis				
Cultivable *Lactobacillus* at enrollment	1.01	(0.72, 1.41)	0.95	(0.67, 1.34)
H_20_2 Producing *Lactobacillus* (lagged, time-varying)				
No cultivable *Lactobacillus*	REF		REF	
Type of *Lactobacillus* growth unknown	0.90	(0.32, 2.48)	1.00	(0.34, 2.92)
Non- H_20_2 producing *Lactobacillus*	1.05	(0.62, 1.79)	1.05	(0.61, 1.81)
H_20_2-producing *Lactobacillus*	1.18	(0.80, 1.75)	1.12	(0.74, 1.70)
Lactobacillus morphotypes on Gram Stain (lagged, time-varying)	1.00	(0.71, 1.41)	0.98	(0.69, 1.38)

[i] Adjusted for age, study site, and frequency of condomless sex in last four weeks

Table 4.3. Associations between *Lactobacillus* status at the prior and current visit and fecundability

Exposure		Primary Analysis								Sensitivity analysis – Including women with up to 6 cycles of prior trying time							
Cultivable *Lactobacillus*		**Cycles (N=768)**		**Pregnancy (N=128)**		**Unadjusted FR (95% CI)**		**Adjusted FR (95% CI)[i]**		**Cycles (N=876)**		**Pregnancy (N=138)**		**Unadjusted FR (95% CI)**		**Adjusted FR (95% CI)[i]**	
Prior Visit	**Current Visit**	**n**	**%**	**n**	**%**					**n**	**%**	**n**	**%**				
No	No	195	(25.4)	27	(21.1)	Ref	-	Ref	-	233	(26.6)	27	(19.6)	Ref		Ref	
Yes	No	66	(8.6)	16	(12.5)	1.72	(0.98, 3.01)	1.63	(0.92, 2.89)	75	(8.6)	19	(13.8)	2.16	(1.26, 3.67)	2.07	(1.20, 3.57)
No	Yes	62	(8.1)	13	(10.2)	1.48	(0.80, 2.75)	1.35	(0.74, 2.45)	78	(8.9)	15	(10.9)	1.64	(0.92, 2.93)	1.50	(0.85, 2.62)
Yes	Yes	445	(57.9)	72	(56.3)	1.21	(0.79, 1.84)	1.14	(0.75, 1.72)	490	(55.9)	77	(55.8)	1.36	(0.90, 2.06)	1.28	(0.84, 1.94)
H_20_2-producing *Lactobacillus*		**(N=766)**		**(N=126)**						**(N=871)**		**(N=135)**					
No	No	325	(42.4)	43	(34.1)	Ref	-	Ref	-	378	(43.4)	44	(32.6)	Ref	-	Ref	-
Yes	No	74	(9.7)	19	(15.1)	1.89	(1.15, 3.11)	1.74	(1.06, 2.86)	87	(10.0)	23	(17.0)	2.27	(1.42, 3.62)	2.14	(1.34, 3.42)
No	Yes	77	(10.1)	19	(15.1)	1.78	(1.08, 2.93)	1.46	(0.89, 2.42)	91	(10.5)	21	(15.6)	1.96	(1.22, 3.16)	1.65	(1.02, 2.65)
Yes	Yes	290	(37.9)	45	(35.7)	1.19	(0.80, 1.79)	1.05	(0.70, 1.59)	315	(36.2)	47	(34.8)	1.29	(0.87, 1.91)	1.14	(0.76, 1.70)
***Lactobacillus* morphotypes on Gram stain**		**(N=768)**		**(N=128)**						**(N=876)**		**(N=138)**					
No	No	190	(24.7)	20	(15.6)	Ref	-	Ref	-	215	(24.5)	20	(14.5)	Ref	-	Ref	-
Yes	No	37	(4.8)	7	(5.5)	1.68	(0.75, 3.74)	1.80	(0.83, 3.92)	41	(4.7)	8	(5.8)	2.07	(0.97, 4.42)	2.27	(1.08, 4.80)
No	Yes	74	(9.6)	24	(18.9)	2.99	(1.77, 5.08)	2.92	(1.76, 4.85)	84	(9.6)	25	(18.1)	3.22	(1.91, 5.43)	3.14	(1.90, 5.19)
Yes	Yes	467	(60.8)	77	(60.2)	1.56	(0.99, 2.46)	1.52	(0.97, 2.41)	536	(61.2)	85	(61.6)	1.69	(1.08, 2.66)	1.65	(1.05, 2.59)

[i] Adjusted for age, study site, and frequency of condomless sex in last four weeks

CHAPTER FIVE - Conclusions

Both vaginal microbiota disruption and infertility are common reproductive conditions (10–12,107) that are associated with adverse sequelae including HIV/STI acquisition, spontaneous abortion, preterm birth (128,162,163,231), anxiety, depression, stigma, and sometimes relationship violence and dissolution (4,15). Vaginal and endometrial microbiota disruption may also be associated with poor IVF outcomes (77,92), but whether vaginal microbiota disruption affects non-medically assisted fecundability has not previously been explored. To address this gap, this dissertation utilized preconception data from a prospective case-cohort study of Kenyan pregnancy planners (**Chapter 2**) to assess whether vaginal microbiota disruption measured using non-molecular methods of detection was associated with fecundability.

The primary analyses found no significant associations between BV (**Chapter 3**), elevated sialidase (**Chapter 3**), or cultivable *Lactobacillus* (**Chapter 4**) at the visit prior to pregnancy testing and fecundability. However, in secondary analyses assessing these measures of vaginal microbiota across two time points to estimate microbial stability during discrete menstrual cycles, persistent BV was associated with a borderline significant 34% reduction in per cycle probability of pregnancy compared to women who were negative for BV at both time points. In addition, women with cultivable *Lactobacillus* at one or both cycle measurements had a non-significant increased per-cycle probability of pregnancy ranging from 1.1-1.6 compared to cycles without *Lactobacillus* and menstrual cycles with *Lactobacillus* morphotypes detected on Gram stain at both visits had a 50% increased per-cycle probability of pregnancy that trended toward significance. These findings should be interpreted as preliminary since the target sample sizes for the analyses have not yet been accrued.

While these dissertation analyses utilized non-molecular methods of detection, they provide important insights into the direction of this research, which may include transitioning to employing sophisticated molecular methods to explore the role of the vaginal microbiota on fecundability. Bacterial vaginosis assessed by Gram stain and Nugent score (Chapter 3), elevated sialidase by the BVBlue test (Chapter 3), and *Lactobacillus* by culture (Chapter 4) reflect related, but distinct, approaches to measuring the vaginal microbiota in optimal versus sub-optimal states. First, BV assessed by Gram stain provided an overall assessment of the vaginal microbiota on a scale of optimal to non-optimal based on the visualized quantities of *Lactobacillus* morphotypes, Gram variable rods, and curved rods, with the latter two morphotypes reflecting bacteria that are typically considered to be sub-optimal in the vaginal microbiota (60). Second, studying elevated sialidase activity provided a specific assessment of the function of sialidase producing BV-associated bacteria. Only 75-84% of women with BV have elevated sialidase activity, reflecting functional differences in this polymicrobial condition (80,232). Third, cultivation of *Lactobacillus* and assessment for H_20_2 production detected viable *Lactobacillus* species most consistently associated with optimal vaginal health (e.g., *L. crispatus* and *L. jensenii*). While reduced concentrations or absence of *Lactobacillus* is common among women with BV (59), the measures do not always overlap. Collectively, each of these measures had the potential to contribute unique insights to our understanding of the impact of a disrupted vaginal microbiota on fecundability.

No studies have compared the prevalence and concordance of these three non-molecular measures of vaginal microbiota disruption. At enrollment into the MPTB Study, 56.6% (259/458) of participants were positive for any of the three markers of vaginal microbiota disruption under study. Among these participants, two-thirds (67.6%, 175/259) were negative for cultivable *Lactobacillus*, 61.4% (158/259) had BV, and 58.7% (152/259) had elevated sialidase activity (**Figure 5.1**). The concordance was highest between BV by Nugent score and the test for elevated sialidase with 49.4% positive for both (128/259) (**Figure 5.1**). Importantly, nearly a third (29.0%,

75/259) of participants positive for at least one marker of vaginal microbiota disruption had BV, elevated sialidase, and were negative for cultivable *Lactobacillus*. These participants may represent a group with the most severe disruptions from optimal vaginal microbiota and could be explored as a unique sub-group in future analyses.

In settings without the supplies or microscopy expertise to assess BV by Amsel's criteria or by Gram stain and Nugent scoring, alternative modalities for testing may be a useful addition to clinical care. The point-of-care test was developed to fill this niche. However, there is little data on the operating characteristics of the test for elevated sialidase indicative of BV in sub-Saharan Africa. In non-pregnant women in the US, Canada, Australia, and Malaysia, the test's sensitivity (range: 73%-100%, median" 90%) and specificity (range: 95-99%, median: 97%) tend to be high compared to BV by Nugent score as the gold standard (233–237). However, the test performed poorly in one study at 10 sub-Saharan African sites and in an Indian study, where sensitivity estimates were 52% and 38% respectively in symptomatic women (238,239). Among 456 MPTB Study participants, sensitivity and specificity of the test compared to BV by Nugent score was 80.5% (95%CI 73.4-86.4) and 92.0% (95%CI 88.3-94.8), respectively. This is the largest sample size to date. One prior study found that the test performed less well among women with asymptomatic BV (233), which contributed to a hypothesis that sialidase activity was highest among women exhibiting abnormal discharge due to its mucin degrading properties. This hypothesis, however, is not consistent with the results from the MPTB study to date. Among the 47 women reporting abnormal discharge, the sensitivity and specificity were 64.3% (95%CI 35.1-87.2) and 90.9% (75.7, 98.1). Sensitivity in symptomatic women was lower than reported in the US, Australia, and Canada, but higher than the 52% reported for symptomatic women in the only other study in sub-Saharan Africa (238). Sensitivity and specificity were higher in the 411 MPTB participants who did not report abnormal discharge (82.1%, 95%CI 74.8-87.9; 92.1%, 95%CI 88.2-95.0). Overall, the lower performance of the test in sub-Saharan Africa may be due to racial,

ethnic, and geographic variations in the vaginal microbiota and in women's intravaginal and menstrual hygiene practices, which may contribute to differences in the operating characteristics of the test for elevated sialidase.

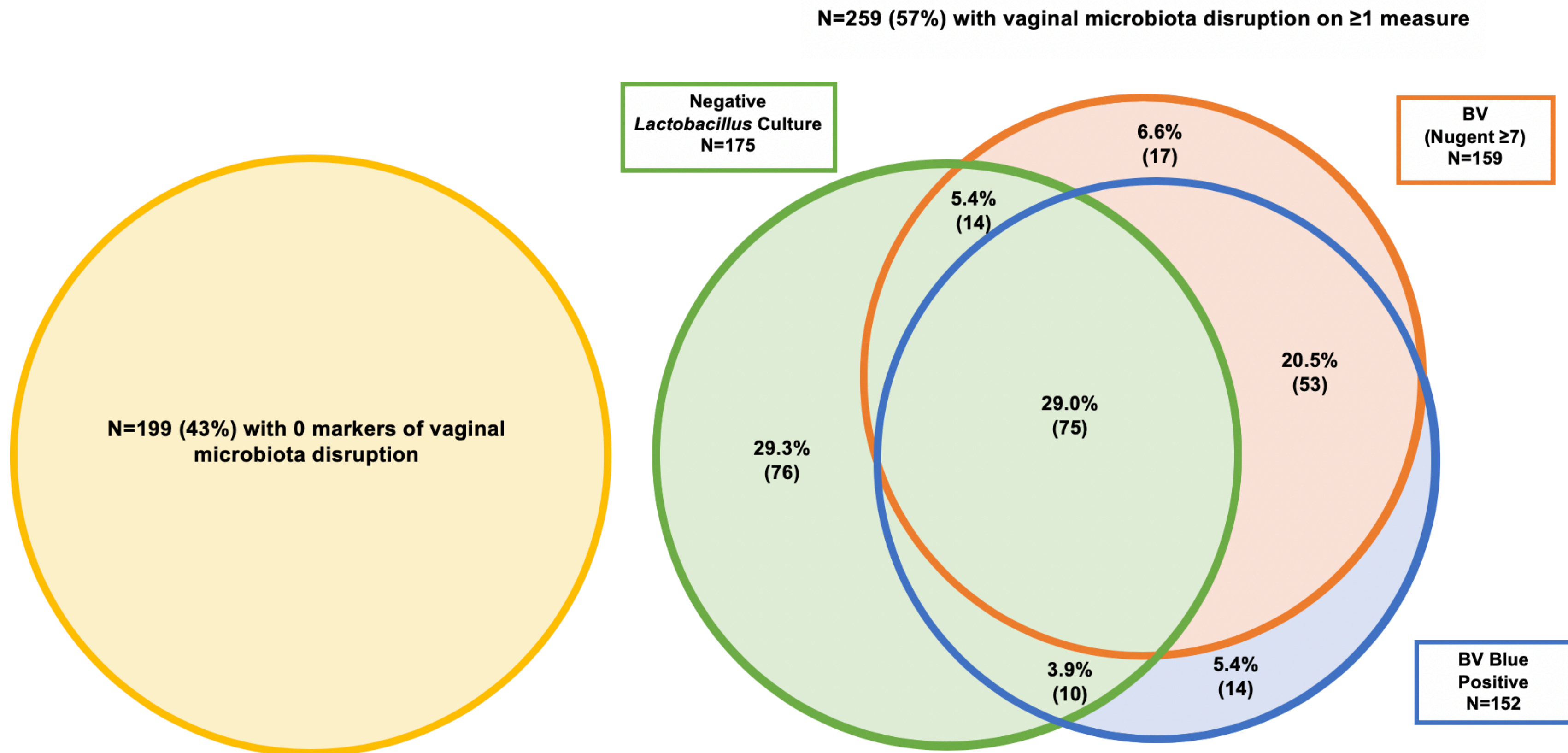
Future studies of vaginal health and fecundability should also assess the contribution of vaginal washing. Vaginal washing is associated with BV and with reduced detection of *Lactobacillus* (29,176,177,240). In addition, a 1996 study of married women in King County, WA found a 30% reduction in fecundability in women reporting vaginal douching compared to those who did not (176). Given this, vaginal washing was assessed as a potential confounder in these dissertation analyses, but it did not meet the pre-specified threshold (>10% change in FR) for inclusion in the adjusted models. However, when assessing the univariate association of vaginal washing in the last four weeks (time-varying) and fecundability, vaginal washing was significantly associated with 40% reduction in per-menstrual cycle probability of pregnancy (FR 0.59, 95%CI 0.38, 0.91). Given this intriguing univariate finding and the high prevalence of vaginal washing in MPTB Study participants, a future analysis utilizing MPTB Study preconception data is planned to assess the direct association between vaginal washing and fecundability. Ideally, this study would incorporate a mediation analysis to assess how fecundability may be impacted by the direct effects of vaginal washing and through indirect effects of vaginal washing on the vaginal microbiota.

Overall, these preliminary dissertation analyses point to the complex nature of studying the vaginal microbiota in the context of fecundability. Confirmatory studies should specifically collect sexual behavior data for discrete menstrual cycles and should include up to 12 months of follow-up to enable study of the infertility outcome as well. Future studies may also benefit from pairing traditional measures of assessment with molecular methods of detection to assess species-specific effects. In addition, given the temporal variability of the vaginal microbiota within women

and the null-findings when vaginal microbiota disruption status at the enrollment visit only was considered, future studies of vaginal microbiota disruption and fertility must also consider status over time. Increased vaginal sampling, such as weekly, and sampling close to the time of ovulation should also be considered. Moreover, as thoroughly discussed in Chapter 3, frequency of condomless sex is a strong confounder in any analysis of vaginal microbiota disruption and fecundability. To better assess this confounder, frequency of sex should be assessed more accurately and precisely through mechanisms such as daily or weekly diaries or calendars (paper or electronic), or a two-way SMS program collecting sex frequency data. Multiple biomarkers could also be employed, including PSA and Y-chromosome DNA detection in vaginal secretions (194,230). Participants should also be provided urine luteinizing hormone test kits for fertility monitoring to assess frequency of sex in the fertile window and to determine whether cycles were ovulatory (138). Lastly, future studies should include more data collection on the male partner since fecundability is a couple-based outcome. This could include medical history, semen parameter analysis, and even seminal microbiota assessment.

In summary, despite compelling biological mechanism hypotheses and data from the IVF literature, the effect of vaginal microbiota disruption in the context of non-medically assisted conception on fertility is not yet clear. While there were no associations between BV by Nugent score ≥7, elevated sialidase, or cultivable *Lactobacillus* in primary analysis, the secondary analyses assessing the stability of the vaginal microbiota across two time points provide some evidence that persistent vaginal microbiota disruption may contribute to reduced fecundability and that *Lactobacillus* may be associated with higher fecundability. Overall, given the high global prevalence of both vaginal microbiota disruption and infertility, if persistent vaginal microbiota disruption is associated with reduced fecundability, this could have important implications for large numbers of women wishing to conceive.

Figure 5.1 Prevalence of BV, negative cultivable *Lactobacillus,* and BVBlue positivity at MPTB Study enrollment for 458 Kenyan women trying to conceive – The proportions presented in the overlapping Venn diagram reflect the 259 participants with vaginal microbiota disruption on one of more of the measures. The area-proportional Venn diagram shapes were generated using www.biovenn.nl (241).



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