2021

The impact of long-acting progestin contraception on the vaginal microbiome

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

THE IMPACT OF LONG-ACTING PROGESTIN CONTRACEPTION ON THE VAGINAL MICROBIOME

by

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B.S., Northeastern University, 2016

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2021

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ACKNOWLEDGMENTS

I would like to thank all of the participants who enrolled in this study, Dr. Doyle Ward for his expertise and assistance in analyzing the dataset, and my mentor and supervisor, Dr. Wendy Kuohung, for her constant guidance and patience throughout this project.

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ABSTRACT

Progestins are synthetic progestogens that prevent pregnancy by thickening the mucous of the cervix to prevent sperm entry and by disrupting implantation via alteration of the timing of endometrial changes occurring during a normal menstrual cycle. Various hormonal birth control methods utilize progestins, with some of the most effective types of birth control methods being long-acting reversible contraceptives. These include hormonal injections such as depot medroxyprogesterone acetate (DMPA), hormonal implants such as Nexplanon, and hormone-releasing intrauterine devices (IUDs) such as Mirena. Although there have been many studies on the safety and effectiveness of these methods, fewer studies have examined how these hormonal methods may impact the bacterial environment of the vagina, better known as the vaginal microbiome. The health of the vagina relies heavily on the bacteria composing the microbiome. Changes in species composition correlate with higher risk of sexually transmitted infections (STIs) and adverse pregnancy outcomes. When women select their preferred hormonal contraceptive method, they should know if it will impact their vaginal microbiome and increase susceptibility to disease. Twenty-one patients enrolled in this study, with one patient initiating DMPA, 14 initiating levonorgestrel (LNG) IUD, and 6 initiating the etonogestrel subdermal implant (ESI). At initiation, 3 months post initiation, and 6 months post initiation, no differences were seen in the vaginal microbiomes of each of

the women enrolled in the study. Some differences in the vaginal microbiota of postpartum women and those who were not postpartum were seen. More specifically, enrichment of three families, *Lachnospiraceae, Ruminococcaceae*, and *Erysipelotrichaceae*, was seen in women who were more than 12 weeks postpartum, but the effects of those differences remain unclear. Although our sample size was small, the lack of changes in the vaginal microbiome in women initiating long-acting progestin contraception is reassuring; further study in this area is needed.

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LIST OF ABBREVIATIONS

AIDS ..... Acquired Immunodeficiency Syndrome

ANOVA ..... Analysis of Variance

APC ..... Antigen Presenting Cell

ART ..... Antiretroviral Therapy

ATP ..... Adenosine Triphosphate

BMC ..... Boston Medical Center

BMI ..... Body Mass Index

BV ..... Bacterial Vaginosis

ºC ..... Degrees Celsius

CD ..... Cluster of Differentiation

CFAR ..... Center for AIDS Research

CSTs ..... Community State Types

DMPA ..... Depot Medroxyprogesterone Acetate

DNA ..... Deoxyribonucleic Acid

ESI ..... Etonogestrel Subdermal Implant

FABMs ..... Fertility Awareness-Based Methods

FDA ..... Food and Drug Administration

GBS ..... Group B Streptococcus

HIV ..... Human Immunodeficiency Virus

HPV ..... Human Papillomavirus

HSV ..... Herpes Simplex Virus

IL ..... Interleukin

IM ..... Intramuscularly

IUD ..... Intrauterine Device

IVF ..... In Vitro Fertilization

LARCs ..... Long-Acting Reversible Contraception

LDA ..... Linear Discriminant Analysis

LefSe ..... Linear Discriminant Analysis Effect Size

LNG ..... Levonorgestrel

LNG IUD ..... Levonorgestrel Intrauterine Device

MaAsLin ..... Multivariate Association with Linear Models

MANOVA ..... Multivariant Analysis of Variance

NET-EN ..... Norethisterone Enanthate

OTU ..... Operational Taxonomic Units

PAMPs ..... Pathogen-Associated Molecular Patterns

pH ..... Potential of Hydrogen

PID ..... Pelvic Inflammatory Disease

PrEP ..... Pre-Exposure Prophylaxis

QIIME ..... Qualitative Insights into Microbial Ecology

rDNA ..... Ribosomal Deoxyribonucleic Acids

RNA ..... Ribonucleic Acid

SQ ..... Subcutaneously

SNPs ..... Single Nucleotide Polymorphisms

STI.....Sexually Transmitted Infection

TLRs ..... Toll-Like Receptors

TNF-α.....Tumor Necrosis Factor Alpha

UTI..... Urinary Tract Infection

VNAB ..... Vaginosis-Associated Bacteria

VVA..... Vulvovaginal Atrophy

WIHRI.....Women and Infants Hospital of Rhode Island

INTRODUCTION

Overview of Modern Contraception

There are many options available today in both hormonal, barrier, and behavioral birth control methods. Some of these methods are reversible and some are not. Overall, hormonal birth control methods are the most popular and widely used methods in the United States (Blumenthal, 2008).

Hormonal contraception may vary widely in terms of its effectiveness and delivery method. All forms of hormonal contraception include a progestogen, and many forms also include some type of estrogen for improved period control. Over the years, the dosage of estrogen has been reduced to curb unwanted side effects such as nausea, headache, weight gain, and episodes of venous thromboembolism. Today's combination contraceptives contain one of two forms of estrogen, ethinyl estradiol or estradiol. By including synthetic estrogen in these combination options, the levels of estrogen within the body remain more consistent, preventing a rise in follicle-stimulating hormone, the recruitment of a dominant follicle, and hence ovulation. The primary reason for including estrogen in combined hormonal contraception is to help regulate bleeding so it occurs at more predictable intervals (Blumenthal, 2008).

There are many different synthetic progestogens or progestins currently in use in various types of hormonal contraceptives today. The contraceptive effect of progestin occurs due to three mechanisms of action. Progestins have an antigonadotrophic effect that inhibits ovulation specifically by blocking the LH surge, although this does not

happen consistently at lower doses. Progestin contraception also relies on effects on the endometrium and cervical mucus. Progestins thicken the cervical mucus, thereby preventing sperm entry. They also disrupt implantation by altering the timing of endometrial changes occurring during a normal menstrual cycle, leading to thinning and atrophy of the endometrium (Regidor, 2018).

In addition to their contraceptive effects, combination contraceptives also offer other advantages, specifically due to their progestin component. These contraceptives offer some protection against certain types of endometrial and ovarian cancers, decrease menstrual flow, help with dysmenorrhea, and may lessen the negative effects of endometriosis. Additionally, combined contraceptive methods can often improve acne and reduce unwanted hair growth due to increases in sex hormone binding globulin that binds androgen. Combined contraceptives may also reduce menstrual migraine frequency and painful cramps associated with the menstrual cycle (Regidor, 2018).

There are some progestin-only forms of contraception that are ideal for women that cannot take estrogen due to other health issues or who are especially sensitive to side effects of estrogen-containing contraception. Women who benefit from progestin-only pills include those who smoke, have high blood pressure, are overweight, are nursing, or have a history of blood clots. There are many contraindications to using combination hormonal pills including metabolic and cardiovascular side effects. High doses of combined hormonal contraception have been associated with an increased incidence of glucose intolerance and with an increased risk of venous thromboembolism (deep vein thrombosis, stroke, and myocardial infarction (Regidor, 2018). As the amount of

estrogen in these contraceptives decreases, so do the adverse effects. Typically, when estrogens are formulated with newer progestins, the risk of adverse side effects also increases (De Leo, 2016). Some of these risks increase with both age, smoking, and weight. The higher the dose of estrogen in the pills, the more likely minor side effects are to occur including headaches and nausea (De Leo, 2016).

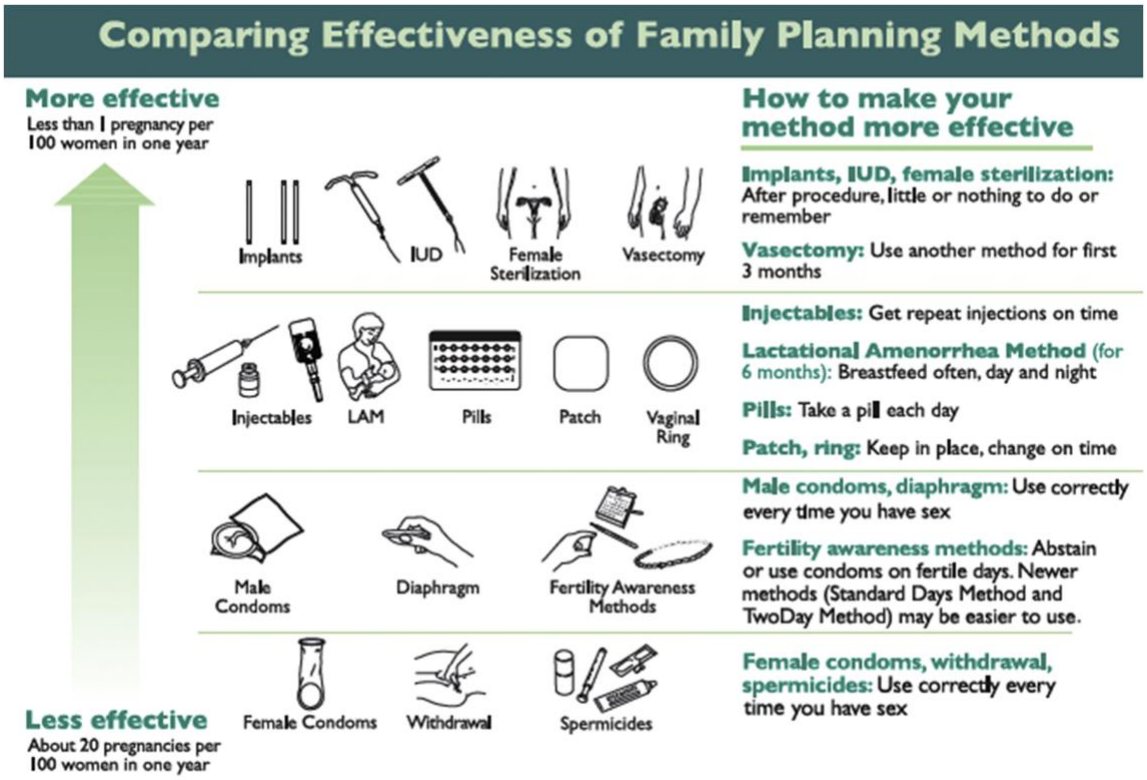

Figure 1: Overview of Modern Contraception. Effectiveness of various family planning methods from the Family Planning Global Handbook for Providers. (Festin, 2020)

Permanent birth control methods include sterilization of males or females. In women, sterilization may be achieved via tubal ligation, a surgical procedure that blocks

or cuts the fallopian tubes, preventing the sperm from encountering the egg. In men, a vasectomy is permanent birth control. In this procedure, shorter and less invasive than a tubal ligation, the vasa deferentia are blocked or cut, preventing sperm from entering the ejaculatory fluid. When looking at worldwide utilization of tubal ligations and vasectomies, nearly 20% of reproductive-aged women have undergone sterilization compared to only 3% of men (Festin, 2020).

After sterilization, the next most effective form of birth control is long-acting reversible contraceptive methods (LARCs). These include hormonal contraceptive implants and intrauterine devices (IUDs). IUDs consist of small plastic or metal devices in different shapes that may be inserted through the cervix and maintain their position in the triangular uterine cavity. The most commonly used IUDs in the United States are T-shaped devices These IUDs prevent pregnancy either by releasing progestin or by using copper. ParaGard® is a copper IUD that is nonhormonal and can provide contraception for up to 12 years. The copper is toxic to sperm, damaging sperm so they cannot swim to meet the egg. The copper IUD is advantageous for women trying to avoid hormonal methods of birth control. Additionally, the copper IUD can be used as emergency contraception if it is inserted within 120 hours after unprotected sex. Adverse effects of the copper IUD include painful periods and heavier menstrual bleeding. Just over 1 in 10 women who have a copper IUD placed will have it removed within the first year of use due to heavier bleeding and cramping (Hsia, 2016).

There are several hormonal IUDs available in the United States. They all contain progestin that prevents the sperm from meeting the egg by thickening the cervical mucus.

Each hormonal IUD differs slightly in duration of contraceptive protection, but they range from 3 to 7 years. The design is similar to a copper IUD except that the T-arms fold upwards instead of downwards during insertion. The most popular IUD in the United States is the Mirena® IUD. This IUD releases 20ug of levonorgestrel daily for the first few weeks. One year after insertion, the amount decreases to 18ug per day, and after 5 years, there is roughly 10ug per day released. Liletta® is a similar product to Mirena and is also commercially available in the United States. A smaller levonorgestrel-releasing IUD, Skyla®, is approved for 3 years of use and is available on the US market. IUDs are advantageous because once they are in place, there is nothing the user has to think about with regards to contraception other than protecting herself from sexually transmitted infections. Aside from their success as contraceptives, progestin IUDs can also help users with heavy menstrual bleeding and cramping and are considered a great nonsurgical and affordable option for users with anemia or irregular uterine bleeding (Hsia, 2016).

The most effective hormonal contraceptives are implants containing levonorgestrel or etonorgestrel. These devices, along with IUDs, are long-term contraceptive methods that slowly release hormones over a period of 3-5 years. Implants are subcutaneous devices that are typically implanted in the upper arm of the user. All implants prolong the action of a short-acting hormone, typically a progestin, by releasing a continuous amount of drug from a reservoir within the implant itself. Implants usually come as rods that have polymers mixed with steroid or as capsules filled with steroid crystals. One major obstacle in the way of more women utilizing implants as their choice

of contraception is the need for the provider to have specific training in order to implant and remove it safely (Benagiano, 2015).

Today, there are two main implants available worldwide. The Norplant-2® contains two rods filled with a polymer-levonorgestrel matrix. There is 140mg of active hormone contained in the rods, and it can continuously and effectively release hormone for up to five years. Implanon® is another implant option containing a single rod with 68mg of etonogestrel. This implant is effective for four years based on recent studies that show high serum etonogestrel levels through 48 months. Ovulation often resumes prior to year 3-4, but the thickened cervical mucus and endometrial dysregulation continue to prevent pregnancy. A newer version of Implanon is Nexplanon®. It is approved for 3 years and contains barium sulfate that helps locate it on X-ray. Nexplanon is more easily inserted and removed than Implanon. Within the last few years, removal was made easier by using ultrasound to mark the surface. With etonogestrel implants, 20% of users have it removed due to irregular bleeding before it is no longer effective (Benagiano, 2015).

Shorter acting hormonal contraceptives come in an oral (combination) pill form, injections, transdermal patches, and vaginal rings. Pills can be monophasic, biphasic, or triphasic. In monophasic pills, the active pills contain a constant dose of hormones, whereas in diphasic and triphasic formulations, the hormone dose increases once or twice, respectively, during the cycle. The diphasic and triphasic contraceptives are meant to mimic the normal physiology of the menstrual cycle, but there is no evidence that these formulations offer advantages to monophasic formulations (De Leo, 2016).

Combination pills can be taken different ways. Some pills are 21/7 or 24/4, where 21 days or 24 days are active hormone-containing pills and 7 or 4 pills are placebos, with the 24/4 having the advantage of a shorter placebo interval. There are also 21 or 28-day pill packs that contain only active pills and are meant to be taken back-to-back with no breaks in between. Women on these formulations will not have a period (De Leo, 2016). Lastly, there are 91-day pill packs designed for women who only want a period 4 times a year, so 84 days will be hormonal pills and the last 7 days will be placebo pills (Davis, 2010). There is also a progestogen-only pill (minipill) that is safer for women who are breastfeeding or who have elevated cardiovascular risks (Regidor, 2018). The risk of breakthrough bleeding with the progestin-only pill is higher as the stabilizing effect of estrogen on the endometrium is lacking.

Injectable contraceptives are usually combination or progestin-only formulations. The two main types of progestin-only contraceptives are depot medroxyprogesterone acetate (DMPA) or norethisterone enanthate (NET-EN). DMPA can be injected intra-muscularly (IM) in a 150-mg dose or subcutaneously (SC) in a 104-mg dose and is good for 3 months because it is slowly released into the bloodstream after injection. Subcutaneous injections of DMPA are more easily performed by the patient. DMPA, similar to typical hormonal methods containing progestin, has a three-pronged effect in preventing pregnancy: it inhibits ovulation, thickens the cervical mucus, and thins the lining of the endometrium. When utilizing DMPA, normal fertility takes longer to return, with an average of 5-8 menstrual cycles (Yland, 2020). DMPA is safe and effective

immediately after an abortion, immediately postpartum if the user is not breast feeding, or 6 weeks postpartum in users who are breast feeding (Blumenthal, 2008).

Those who are pregnant or have breast cancer should not use DMPA. The most common side effect with DMPA is irregular bleeding, which is also the main reason that women opt for alternative contraceptive options. DMPA also has some noncontraceptive benefits including a reduction in endometrial carcinoma. DMPA users often see a reduction in anemia and ovarian cysts as well. Other effects of DMPA include stabilizing the erythrocyte membrane and increasing the hematocrit (by decreasing blood loss) in users with sickle cell anemia, increasing the seizure threshold in users with epilepsy, and protecting users at higher risk factor for pelvic inflammatory disease (PID) by decreasing the risk of infections by thickening the cervical mucus. There are some risks associated with DMPA, including weight gain and reversible decreases in bone density, especially in younger women. Patients who are heavy smokers, elite athletes with amenorrhea, or steroid users, as well as patients with anorexia nervosa, should find alternative methods due to their increased risk of bone density loss (Blumenthal, 2008).

NET-EN, the other main type of progestin-only injectable, needs to be given every 2 months and is also injected either IM or SC. NET-EN prevents ovulation and upon stopping, fertility will return in 3 to 4 months on average. Although not currently available in the U.S., there are many combination injectables available. These formulations may include either medroxyprogesterone acetate with estradiol cypionate or NET-EN with estradiol valerate. Unlike progestin-only options, these need to be injected

monthly and it takes slightly longer, on average 5 months, for fertility to return after method discontinuation (Festin, 2020).

The contraceptive patch contains a combination of progestin and estradiol. The patch is slightly smaller than a 2x2-inch square that is placed on the upper outer arm, buttocks, abdomen, or upper torso. Hormones are released into the skin, where they are absorbed into the bloodstream. The patch is changed weekly for three weeks. The fourth patch-free week is when the user's period should begin. Each week, the patch may be rotated to a different location in order to avoid irritation. Like combination pills, the patch may increase the risk of blood clots, especially in users over 35 years of age who smoke. The patch may be less effective in users over 200 pounds (Benagiano, 2006).

Vaginal rings offer another type of combination hormonal contraceptive. These rings are inserted into the vagina, where they release a continuous level of hormones into the bloodstream via the vaginal epithelium. Currently, there are two rings available on the market, the NuvaRing® and Annovera®. NuvaRing has been available for over two decades; it releases 15 mg of ethinyl estradiol and 120 mg of etonogestrel and is changed monthly (Benagiano, 2006). The ring should be inserted for 21 days followed by 7 days where the ring is removed to induce a period (Lee, 2020). The Annovera contains 17.4 mg of ethinyl estradiol and 103 mg of segesterone acetate and contains enough hormone to last for one year.

The expulsion rate of Annovera is higher than the NuvaRing, with about 25% of women reporting a complete expulsion at least one time during their year of use. Adverse effects of both rings are similar to the effects seen with other combination

hormonal contraceptives including dysmenorrhea, vaginal discharge, irregular bleeding, headache, nausea, and breast tenderness. About 12-15% of users will discontinue use due to these adverse effects. The effectiveness of contraceptive rings is similar to other hormonal contraceptives, with about 1-3 pregnancies per 100 women each year (Lee, 2020).

There are also barrier methods used by the female that are slightly-less effective forms of birth control, including diaphragms, sponges, cervical caps, spermicides, and female condoms. The male and female condoms are the best protection against STIs. The major benefit of barrier methods is that they are safe for women who have a contraindication to hormonal methods. Diaphragms are circular and made of silicone. They are filled with spermicidal jelly and inserted into the vagina before intercourse, and they cover the cervix, preventing sperm from being able to enter the uterus to fertilize the egg. They should be left in the vagina for 6 hours after intercourse but no longer than 24 hours post intercourse. There is a "one-size-fits-all" diaphragm that does not require fitting by a physician, the Caya® diaphragm, now available on the market. Currently, the Today Sponge is the only sponge approved for use in the United States. The sponge is circular, made of polyurethane foam, and contains a spermicide. It is inserted before intercourse and removed 6 or more hours after intercourse. The Today Sponge may be washed in between uses for re-use. The efficacy rate for the Today Sponge, when used correctly, is between 84% and 87% (Tracy, 2017).

Cervical caps are similar to diaphragms. There is only one commercially available cervical cap on the market in the United States, the FemCap. It is made of

silicone and circular in shape. Similar to most diaphragms, a prescription is needed for a cervical cap, and it needs to be properly sized by a clinician. There are a few sizes available: a 22 mm, generally for women who have never been pregnant; 26 mm, for anyone who has been pregnant for any period of time, and 30 mm for women who have given birth via vaginal delivery. It is often recommended to use a spermicide with the cervical cap. Success rates of the cervical cap range from 71% to 86%. The FemCap should be left in the vagina for at least 6 hours after intercourse, but not longer than 2 days.

A commercially available spermicide in the United States is Noxonyl-9. It is available as a film, gel, foam, cream, or suppository. Its recommended use is with a diaphragm or cervical cap, but it can be used alone if it is placed in the vagina at least 10 minutes before intercourse. Spermicides work by chemically immobilizing sperm (Tracy, 2017).

Both female and male condoms are available without a prescription. Female condoms are 75- 92% effective at preventing pregnancy and also protect against STIs. Female condoms are larger than male condoms and contain two rings. Typically, they are made of nitrile or polyurethane. The ring at the closed end is inserted into the vagina to cover the cervix. The other ring on the open side of the condom sits on the outside of the vulva. Male condoms are a popular choice of contraception, especially among younger adults, that also offering protection against STIs. The male condom, like the female condom, is single use and should be used consistently when relying solely on it for contraceptive purposes. The male condom should be placed on the penis after it

becomes erect, avoiding trapping air in the tip. Water-based lubricants should always be used with latex condoms to avoid weakening them. Male condoms, when used correctly, are 82% effective at preventing pregnancy (Tracy, 2017).

Lastly, there are also behavioral contraceptive methods available for couples. Most of the behavioral contraceptive methods are referred to as fertility awareness-based methods (FABMs). FABMs rely on day-to-day female physiological data to determine fertile periods. Based on whether she is fertile or not, a woman utilizing FABMs may abstain from sex or use a barrier method such as a condom, diaphragm, sponge, spermicide or cervical cap. On days when she is not fertile, intercourse can proceed without any forms of contraception. Couples who are trying to get pregnant would utilize the fertile days to increase their chances of conceiving. Some couples not wanting to conceive are completely abstinent during fertile days because their beliefs do not support the use of contraception. This is referred to as the Natural Family Planning Method (Simmons, 2020). Another relatively popular behavioral method is coitus interruptus or the withdrawal method, where the male removes his penis from the vagina prior to ejaculation. Although the withdrawal method has advantages such as its lack of cost and its total access, it is one of the least effective methods of contraception, with 1 in 5 women getting pregnant using this method alone (Nguyen, 2020).

Common Sexually Transmitted Infections

Most methods of birth control can prevent pregnancy but cannot protect against sexually transmitted infections (STIs). The only birth control methods that can protect

against both unwanted pregnancies and transmission of STIs are (external) male condoms and (internal) female condoms. STIs can be transmitted by bacteria, viruses, or other types of pathogens. Common bacterial STIs include syphilis, gonorrhea, and chlamydia. Syphilis is caused by *Treponema (T.) pallidum.* Each year, there are approximately 6 million new syphilis infections around the world, with more infections occurring in men having sexual intercourse with men. Syphilis is transmitted orally, anally, or through genital contact. Normally, a sore, or chancre, will appear at the infection site three weeks after contact with an infected partner. At this point, antibodies to the bacteria can be detected. There are four stages of syphilis infection, including the latent asymptomatic third phase. All four stages can be treated with penicillin. Untreated syphilis may result in death, or it can resolve by itself after the second stage (Buder, 2019).

Gonorrhea is transmitted by *Neisseria gonorrhoeae* during sexual intercourse or childbirth. Each year there are just over 70 million cases of gonorrhea reported worldwide. Unfortunately, about half of those who are infected do not realize it because they are asymptomatic, and will transmit the infection even without symptoms. When gonorrhea does present with symptoms, typically they occur 1-14 days after transmission and affect the mucous membranes at and around the site of infection. In women, the cervical canal is most commonly infected, accompanied by symptoms such as vaginal discharge and painful urination. In men, there can be some discharge from the urethra 2-6 days after infection, accompanied by painful urination. The only effective antibiotic for treating gonorrhea infections is ceftriaxone, and the threat of an untreatable or multi-drug resistant strain of *N. gonorrhoeae* surfacing in the near future is real (Fuchs, 2014).

Chlamydia is caused by *Chlamydia trachomatis* which has two biovars, the trachoma biovar that causes urogenital infections and is one of the top three most common causes of STIs worldwide, and the lymphogranuloma venereum biovar that is much more invasive, causing systemic spread via the lymphatic system upon infection. Chlamydia usually presents as an infection of the respiratory tract or urogenital tract, but it can also infect the conjunctivae. Many infections in both men and women are largely asymptomatic, so many chlamydial infections go undiagnosed and may lead to infertility in both men and women. Undiagnosed infections can cause reactive arthritis 1-2 months after exposure. Some men experience inflammation of the urethra after exposure, leading to discharge and painful urination. Very few women experience symptoms that may include pelvic pain, discharge, and bleeding from cervical infection. Urogenital chlamydial infection is treated with doxycycline or azithromycin, depending upon patient circumstances/adherence (Buder, 2019).

Trichomonas infection is a parasitic infection caused by *Trichomonas vaginalis* that can also be treated with antibiotics. Often, those who are infected are asymptomatic as with many bacterial STIs. The few men who have symptoms will present with inflammation of the urethra, epididymis, or prostate. When women have symptoms, they present with vaginal discharge. Those infected with trichomonas, as is the case with many other STIs, are at an elevated risk of contracting other STIs, especially human immunodeficiency virus (HIV). When asymptomatic, trichomonas infection can last for a few months up to a few years. This STI is treated with either metronidazole or tinidazole (Wagenlehner, 2016).

There are several common viral STIs including human papillomavirus (HPV), human immunodeficiency virus (HIV), and herpes simplex virus (HSV). Human papillomavirus consists of over 100 strains and is spread by skin-to-skin contact. Certain strains are more high-risk for development of cancer than others. HPV types 6 and 11 are known to cause genital warts (condyloma acuminata). These are lower-risk strains that can be treated at the site of the wart with cryotherapy, trichloroacetic acid, or ablative techniques such as laser therapy. When local treatment does not work, topical antimitotic creams such as podophyllotoxin, low doses of chemotherapy agents such as 5-fluoruracil, or immunosuppressants such as cyclosporine can be used to treat the genital warts. High-risk HPV types include 16 and 18. These strains can cause malignant growths. Nearly 100% of cervical cancers are HPV positive, while 90% of anal cancers demonstrate HPV. HPV also causes 70% of carcinomas seen in the penis, vulva, and vagina, and 30% of carcinomas of the throat and tonsils. There is a multivalent HPV vaccine available that is recommended to boys and girls ages 11-12 who have not yet had any form of sexual contact (Wagenlehner, 2016).

Human immunodeficiency virus, like herpes simplex virus, has the two strains HIV-1 and HIV-2, with HIV-1 (HIV) more commonly leading to end-stage acquired immunodeficiency syndrome (AIDS). HIV is a retrovirus that infects cells by reverse transcribing its single-stranded ribonucleic acid (RNA) into deoxyribonucleic acid (DNA) before incorporating its viral genome into the host DNA, allowing the virus to continue replicating. HIV is difficult to treat and to cure because the virus is excellent at overcoming the immune system of the host and the effects of the drugs used to treat it.

The virus eludes many mechanisms used to fight it through its characteristic variability, highlighting three odd features of HIV. The three features that contribute to viral variation include the error prone enzymes that often introduce at least one genomic mutation each time the virus replicates, the rapid replication cycle that allows the virus to make many copies of itself each day, and the high likelihood of genetic recombination between two viral particles within the host. HIV is spread to open skin regions via infected secretions or blood. Without treatment, most people infected with HIV die from AIDS within 11 years of infection. Although there is no cure for HIV or AIDS, antiretroviral therapy can slow the progression of HIV and limit complications due to secondary infections (Fanales-Belasio, 2010).

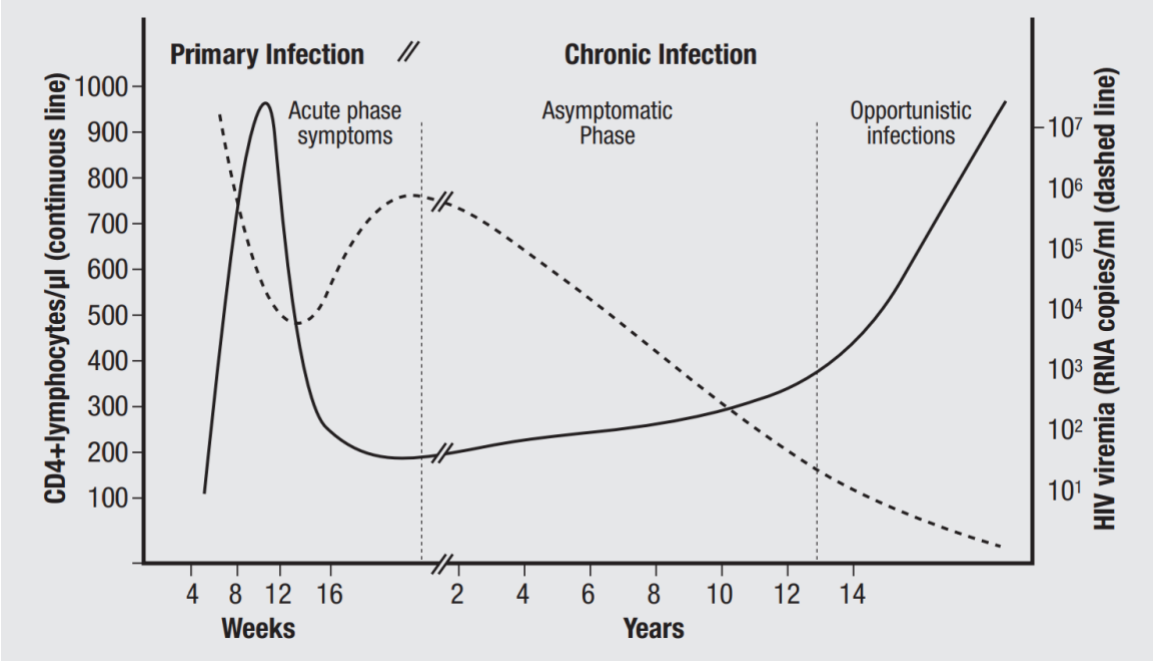

Figure 2: Course of Human Immunodeficiency Virus. During the asymptomatic phase, the virus is still replicating continuously while evading many immune mechanisms. As it is counteracting these mechanisms and replicating, the virus is creating a state of chronic inflammation that makes way for opportunistic infections, which are hallmarks of untreated HIV infections (Fanales-Belasio, 2010).

There are two types of herpes simplex virus, HSV-1 and HSV-2. Genital herpes infections are predominantly caused by HSV-2, which is transmitted via genital contact. HSV-1 is usually transmitted through oral secretions via oral-non-genital contact or oral-genital contact. Viral shedding and recurrence is much less common with genital HSV-1 infections. Infection with herpes virus can be silent, accounting for greater than 75% of viral transmission. Typically, HSV-1 and 2 present similarly, with painful ulcers on the anus or genitalia being the hallmark of initial infection. Often, those experiencing an initial infection also present with flu-like symptoms. Episodes occurring after the initial infection are usually less severe, but in some cases the virus can cause severe disease and complications involving pneumonitis, systemic infection, meningitis, encephalitis, and hepatitis. When HSV is symptomatic, patients should receive antiviral therapy immediately after their first episode. Antivirals used to treat genital HSV infections include acyclovir, famciclovir, and valacyclovir (Siracusano, 2014).

The Vaginal Microbiome

The human microbiota consists of various commensal and symbiotic microorganisms that live within and on the human body. Microorganisms within our body's microbiota include bacteria, archaea, protists, fungi, and viruses. Bacteria can be classified according to how they produce energy. Facultative anaerobic bacteria can generate energy in the form of adenosine triphosphate (ATP) either with or without oxygen, obligate anaerobic bacteria produce energy and survive in environments without

oxygen, and obligate aerobic bacteria produce energy and survive in environments with oxygen. The genomes of these microorganisms are what compose the metagenome of the human body, and they have nearly 150 times more genes than the genome of humans. The major microbiome within the human body is the gut microbiome, but in women, the cervical and vaginal microbiota are believed to be of great importance in diseases and infections central to those regions. Many diseases are believed to have some association with microbiotal disruption (Ata, 2019).

Within the vaginal microbiome, the interaction between the host and the organisms present is especially important. Typically, the microbes in the vagina exist in a symbiotic relationship with their host. The microorganisms found in the vaginal microbiome form the first line of defense against various pathogens that attempt to enter the body through the vagina. The ecosystem of the vagina consists of an outer layer of stratified squamous epithelium and an inner mucosal layer lubricated with cervicovaginal fluid containing mucins, antimicrobial products, and antibodies. The fluid of this mucus layer is largely responsible for maintaining the microbiota of the vagina (Torcia, 2019).

During a woman's lifetime, the vaginal microbiome may go through vast changes. Puberty, pregnancy, and menopause all cause major changes in the vaginal microbiome, while daily choices may cause minor changes. The vaginal microbiome is less diverse than the gut microbiome; typically, the more variation within the vagina, the worse the outcome in terms of disease and infection (Smith, 2017).

In women of reproductive age, it has been determined that there are five major community state types (CSTs), or vaginal microbial communities. Four of the CSTs are

comprised of various types of lactic-acid producing *Lactobacilli* including *L. crispatus* (CST-I), *L. gasseri* (CST-II), *L. iners* (CST-III), or *L. jensenii* (CST-V). Although these are the main *Lactobacilli* species seen, over 20 types of lactobacilli have been detected in the vagina. Typically, the fewer bacterial species composing the vaginal community, the healthier the vagina is likely to be (Huang, 2014). The 4th CST (CST-IV) lacks lactobacilli and is a mixture of anaerobes and strict anaerobes. CST-IV has some species of the genera *Gardnerella, Atopobium, Mobiluncus, Prevotella,* as well as taxa from the *Clostridiales* order (Smith, 2017).

The four CSTs that produce lactic acid are largely responsible for the acidic potential of hydrogen (pH) of the vagina, that is typically less than 4.5. The epithelial cells of the vagina produce glycogen that the *Lactobacilli* species feed on, fermenting it to produce D- and L-lactic acid. Some of these *Lactobacilli* species also increase the production of mucus, the first line of immune defense of the vagina. The acidic environment is thought to prevent colonization of the vagina by unwanted pathogens (Smith, 2017).

The *Lactobacilli*-dominant CSTs inhibit growth of non-native pathogens through the production of hydrogen peroxide and bacteriocins, which are peptides produced by bacterial strains that are toxic and lethal to bacterial strains that are closely related to the host bacteria (Smith, 2017). Some researchers believe that the growth of non-native pathogens is predominantly due to the bacteriocins and not hydrogen peroxide because the *Lactobacilli* species can't produce high enough levels of the hydrogen peroxide to actually inhibit growth, as seen in bacterial vaginosis (BV), a condition characterized by

an overgrowth of non-native bacteria in the vagina Lactic acid, however, can suppress the growth of bacteria causing BV (Huang, 2014). Some of the bacteria seen in CST-IV such as *Megasphera, Prevotella, Gardenella, and Sneathia* are often associated with the presence of BV (Gupta, 2019).

It is also believed that some species of *Lactobacilli* found in the vaginal microbiome keep non-native pathogens from colonizing because they outcompete these pathogens for nutrients and host cell surface receptors. Common non-native pathogens (some of which cause STIs and bacterial vaginosis) that are often out-competed by the native *Lactobacilli* include *Gardnerella vaginalis, Neisseria gonorrhoeae, Candida albicans, Staphylococcus aureus, group B Streptococcus (GBS), Pseudomonas aeruginosa, Streptococcus agalactiae, Escherichia coli, and Prevotella bivia. Lactobacilli* species have a higher affinity for vaginal host cell receptors and are able to displace these non-native pathogens before they can alter the vaginal microbiome. In some instances, *Lactobacilli* species co-aggregate with non-native pathogens, allowing for easier clearance of invading pathogens (Huang, 2014).

Although the presence and dominance of the *Lactobacilli* species are considered the hallmark of a healthy vaginal microbiome, there are women who are both asymptomatic and healthy overall who have a lower percentage of *Lactobacilli* species present within their vaginal microbiota. It is believed that along with the healthy bacteria present in the vagina, the cycling of hormones during the menstrual cycle causes glycogen release by the vaginal epithelial cells that are turned over after release. Bacteria attached to these epithelial cells are turned over as well, allowing for constant turnover of

cells and bacteria within the vagina, keeping some of the non-native bacteria from colonizing. So although *Lactobacilli* species are largely responsible for vaginal microbiome health, there are other mechanisms that help to keep the vagina healthy (Huang, 2014).

In women who lack the larger numbers of Lactobacilli species usually seen in healthy vaginal microbiomes, a larger and more diverse variety of other microbes are seen instead, including anaerobic or facultative bacteria from the genera *Atopobium, Corynebacterium, Anaerococcus, Peptoniphilus, Prevotella, Mobiluncus, Gardnerella*, and *Sneathia*. These bacteria are associated with elevated pH levels and often a diseased state, but in the presence of an otherwise healthy vaginal microbiome, it is believed that these bacteria can contribute to either health or disease depending on other conditions within the microbiome. Perhaps the joint presence of some of these species enables them to serve as commensal bacteria contributing to vaginal microbiome health (Huang, 2014).

The composition of vaginal microbiota differs between women of different ethnic groups. Specifically, the amount of *Lactobacilli* species present varies depending on ethnicity. Women who are Asian or Caucasian typically have a larger proportion of *Lactobacilli* species than Black or Hispanic women. Roughly 80-90% of the vaginal microbial communities in Asian and Caucasian women are dominated by *Lactobacilli* species. This proportion is roughly 60% in women who identify as Black or Hispanic (Huang, 2014). This could be due to genetic or environmental factors or a combination as the direct cause is unknown. Environmental factors include personal habits such as

vaginal douching that more Black women report doing (47% vs. 17% in Caucasian women and 12.5% in Mexican American women; Arbour, 2009), although these do not completely explain the the findings in Hispanic women (Gupta, 2019). In women who are Black or Hispanic, CST-IV is more common (~40%), and the pH of the vagina is higher than that observed in both Asian and Caucasian women (Ravel, 2011).

The Vaginal Microbiota Across the Reproductive Lifespan

The composition of the vaginal microbiome is largely dependent on age and life stage. Early in childhood, the vaginal microbiome is quite different in its composition compared to what is observed during reproductive age. Infants up to 6 weeks have vaginal microbiomes with an acidic pH that includes the common *Lactobacilli* species along with *Streptococci, Enterobacteriaceae, S. epidermidis, and Enterococci.* After 6 weeks of age until puberty, the vaginal microbiome has a neutral pH where *Lactobacilli* species are largely absent, and other species present during infancy are present along with *Ureaplasma, Gardnerella, and Mycoplasma.* After reaching puberty, the vagina microbiome acquires an acidic pH and includes all prepubertal microbiota in addition to *Lactobacilli* species (Gupta, 2019).

The microflora present upon entering puberty is especially important because the bacteria present in the vaginal microbiomes of women of reproductive age impact the health of the pregnancy. Research has shown that the composition of microbes throughout the reproductive tract is important in pregnancy. Women of reproductive age have higher estrogen levels than in other life stages. This elevation in estrogen increases

the amount of glycogen stored in the vaginal walls. Glycogen is the main food source for *Lactobacilli* species, which explains the abundance of these microbes present during puberty. Newborn infants have microbiomes largely resembling that of their mothers due to persistence of maternal hormones, specifically estrogen (Gupta, 2019). There have been some studies showing that infants born vaginally have early microbiota communities on the skin, in the gut, and in the oral and nasopharyngeal passages that have similar characteristics to their mother's vaginal microbiome. Babies delivered via cesarean section have microbiomes most closely resembling the microorganisms found on their mother's skin. It has also been observed that the mother's intestinal bacteria influences the fetus's intestinal microbiome communities *in vitro* as they often closely resemble each other (Huang, 2014).

Lactobacilli species are present in the highest numbers between puberty and menopause, when estrogen levels are relatively high (Gupta, 2019). Postmenopausal women have a vaginal microbiome showing a decline in *Lactobacilli* species. The change in CST is due to low estrogen levels in postmenopausal women that affects the epithelial proliferation and maturation within the vagina. With thinning of the vaginal epithelium in menopause, there is less accumulation of glycogen in the vagina and less nutrition available for *Lactobacilli* species (Smith, 2017).

Many women who are postmenopausal experience various unpleasant symptoms that are hallmarks of menopause including insomnia, vaginal dryness, and hot flushes. Between 25-50% of postmenopausal women will experience vulvovaginal atrophy (VVA) due to a decrease in estrogen levels. Symptoms of VVA include pain during

intercourse, bleeding post intercourse, burning sensation when urinating, vaginal itching and soreness, and foul-smelling discharge. With the decrease in estrogen and subsequently glycogen, the vaginal microbiota of postmenopausal women will attract harmful microbes as the intravaginal pH increases. Specifically, increases in *Escherichia coli*, *Candida*, *Enterobacter*, and *Gardnerella* are seen. Some postmenopausal women benefit from topical estrogen treatments that can help alleviate some of the symptoms of VAA; women with contraindications for estrogen therapy may use vaginal lubricants for relief. Both lubricants and estrogen therapy have side effects; new non-hormonal options are needed (Gupta, 2019).

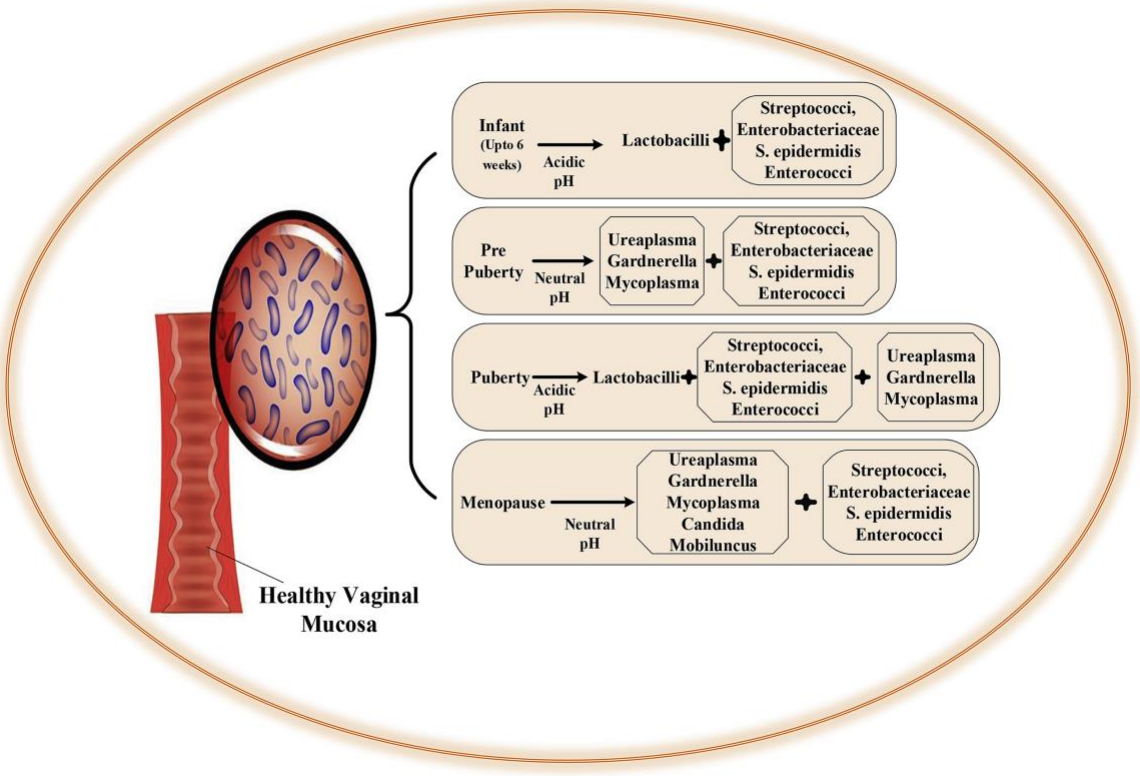

Figure 3: Microbial Composition of the Vaginal Microbiome During Different Stages of Life. Upon reaching puberty, reproductive aged women have the largest

presence of *Lactobacilli*, with a similar *Lactobacilli* composition seen in infants, while these species are largely absent from pre-puberty and menopausal women (Gupta, 2019).

Disruptions to the normal vaginal microbiome can happen daily. Behavioral habits such as dietary choices, exercise frequency, and prevalence of sexual activities can change the vaginal microbiome, along with the use of hormonal contraceptives and lubricants. Menstruation, hygiene practices, and the use of antibiotics may also affect the vaginal environment. Douching or intravaginal cleansing, especially after sex or menstruation, eliminates much of the healthy, protective bacteria from the vagina, making this habit a risk factor for increased frequency of vaginal infections. With antibiotics, prolonged use can lead to unwanted anaerobes becoming more numerous and active, leading to BV and increased susceptibility to STIs and urinary tract infections (UTIs) (Gupta, 2019). Exposure to spermicides has also been shown to decrease the prevalence of *Lactobacilli* species, increasing the susceptibility of the user to vaginal infections (Huang, 2014).

Other habits such as smoking and drinking can also affect the relationship between vaginal microbes and disease. Smoking is known to decrease two of the *Lactobacilli* species commonly found in the vagina, *L. crispatus and L. iners.* Smokers are more prone to developing BV as well as STIs. Stress, obesity, vitamin D deficiency, diabetes, and climate are additional factors that can harm the disease state of the vagina due to their negative effects on the beneficial bacteria within its microbiome (Huang, 2014).

The genetic factors that affect the vaginal microbiome are not completely understood, but some genetic variants have been linked to alteration of the vaginal

microbiome. Innate immunity is especially important in the urogenital tract for fighting off foreign invaders. Various innate immune responses are triggered when toll-like receptors (TLRs) interact with pathogen-associated molecular patterns (PAMPs). Many of the responses involve inflammatory cytokines including interleukins (IL) 4 and 10. Single nucleotide polymorphisms (SNPs) in IL-4 and IL-10 have been linked to abnormal responses to the bacteria that cause BV in relation to preterm births. Additionally, similar abnormal responses have been seen with polymorphisms in TLR4 and Tumor necrosis factor (TNF)-α, also part of the innate immune response within the vaginal microbiome (Huang, 2014).

The Vaginal Microbiome and STIs

Recent data suggests that the common STIs are more prevalent in certain women because of factors making the transmission of bacteria and viruses easier (Wessels, 2018). Certain behaviors as well as genetic and environmental factors may increase the likelihood of contracting an STI by decreasing healthy bacteria and increasing pathogenic microbes within the vagina. Healthy bacteria are the *Lactobacilli* species, and a decrease in their presence signals an increase in anaerobes that are correlated with a higher risk of bacterial vaginosis, preterm births, and STIs. In particular, vaginal dysbiosis has been linked to an increased risk of acquiring chlamydia, gonorrhea, Trichomonas, HSV, HPV, and HIV (Torcia, 2019). The microbiome profile lacking *Lactobacilli* is especially susceptible to contracting disease because of the increase in

inflammatory cytokines that can cause epithelial disruption and recruitment of cells to the areas targeted by the bacteria and viruses (Liu, 2019).

Bacterial vaginosis (BV) is the most common genital tract infection seen in women who are of reproductive age. It is not an STI but increases the likelihood that the woman will contract an STI. Typically, BV is characterized by a loss of *Lactobacilli*, causing the vaginal pH to increase to about 4.5. Symptoms include a foul-smelling discharge, irritation, and a fishy odor (Torcia, 2019). Microbes associated with BV vary, but infections usually result from the abundance of multiple anaerobic microorganisms. Along with increasing susceptibility to STIs, BV also increases the risk of preterm delivery in pregnant women. Treating BV in asymptomatic pregnant women, however, does not decrease the risk of preterm delivery, possibly due to effects of antibiotic treatment (Nygren, 2008). BV may be treated with the antibiotics metronidazole and single-dose secnidazole (Kaambo, 2018). The recurrence rates for BV are high. For BV infections that repeatedly recur, treating with first-line antibiotics, using estrogen-containing contraceptive methods, and ensuring male partners are circumcised can all help (Van de Wijgert, 2017).

Chlamydia trachomatis, the bacteria responsible for causing chlamydia infection, is able to more easily infect women with weakened reproductive tract barriers. Many of these protective barriers are made up of *Lactobacilli* species, so when other species of bacteria dominate the microbiome, *Chlamydia trachomatis* takes advantage. After invading the host, *Chlamydia trachomatis* triggers the production of IL-12, an inflammatory cytokine that stimulates the production of other cytokines as well as the

recruitment of inflammatory cells to the site of infection. *Chlamydia trachomatis* needs the amino acid tryptophan to survive, so within the immune response to the invading bacteria, enzymes are also produced that eliminate free and available tryptophan. Unfortunately, microbiome environments lacking *Lactobacilli* dominance often have plentiful amounts of *Prevotella*, a bacterium that makes tryptophan. *Prevotella* is also implicated in causing BV infections. Although the immune system can activate to combat a chlamydial infection, the dysregulated microbiota not only creates an easier entry for Chlamydia, but can also support its growth by supplying needed tryptophan (Molenaar, 2018).

Another bacterial STI, gonorrhea, also has an increased prevalence of infection among women with vaginal microbiomes including lower levels of *Lactobacilli* and with subsequently higher pH levels. *Neisseria gonorrhoeae* usually enters the body through the particularly vulnerable columnar cells of the endocervical glands. The main site of infection for this STI is the cervix. When trying to prevent *N. gonorrhoeae* from entering the body, the presence of L*actobacilli* species is the best defense. In particular, the metabolites of Lactobacilli, hydrogen peroxide and lactic acid, are crucial in preventing infection. The pH of the vagina is closely linked to gonorrhea risk (Zeng, 2019).

Trichomoniasis, an STI caused by *T. vaginalis*, is more prevalent with vaginal microbiomes that are CST-IV dominant. Whether the CST-IV was initially present and led to an increased rate of infection, or whether the *T. vaginalis* caused the switch to a CST-IV-dominant vaginal microbiome needs to be further studied. *T. vaginalis* is known

to use the lactic acid produced by *Lactobacilli* to help itself adhere to the vaginal epithelium and gain entrance to the host. After infection, the parasite modifies the vaginal microbiome via phagocytosis of the *Lactobacilli* species as well as by challenging the host mechanisms that protect the vaginal environment. A higher pH caters to growth and proliferation of *T. vaginalis* after it has entered the host (Kalia, 2020).

The acquisition of Human Papillomavirus (HPV) has also been linked to vaginal dysbiosis. It has been shown that women who are HPV-positive often have either CST-III dominant (*L. iners*-dominated*)* or a low presence of *Lactobacilli* species (CST-IV) in their vaginal microbiomes. Women who have CST-II dominant microbiomes have the ability to clear HPV with more success than women who have other CST profiles. Women who are HPV positive also have microbiomes containing lower levels of glutathione metabolites. The presence of lower concentrations of both reduced and oxidized metabolites of glutathione could indicate increased oxidative stress that lead to damage and death of various organelles within the cell (Borgogna, 2019).

Past studies have established an association between a decrease in *Lactobacilli* species and recurrent BV infections with herpes simplex virus type 2 infection. Simultaneous infection by BV and HSV causes increased shedding of herpes. After becoming infected with HSV, additional vaginal dysbiosis occurs due to several hallmarks of HSV infection. HSV has active and dormant periods. When HSV is reactivated, the immune system is activated within the vaginal environment, causing additional changes to the microbiome that favor an increase in bacteria and pathogens

commonly associated with higher STI prevalence. HSV reactivation also causes more iron to become available. The increase in iron availability supports increased growth of *Gardnerella vaginalis*, a bacterium often seen in higher abundance in BV (Torcia, 2019).

When the vagina is in a dysbiotic state, it can affect the T cells involved in the adaptive immune response. Much of this suppression is due to IL-33, secreted in response to the dysbiosis and epithelial damage, that inhibits T cells by interfering with cytokines that recruit them to the site of infection. With a suppressed T-cell response, it becomes harder for the host to clear HSV, making the likelihood of the host contracting other STIs such as HIV greater. When HSV is reactivated, it also increases the likelihood of acquiring HIV because the reactivated HSV disrupts the barrier of the vaginal epithelium and recruits cluster of differentiation (CD) cells to the site of disruption. HIV can target CD4+ lymphocytes at the site of the HSV lesion. Additionally, proteins involved in the regulation of HSV have been implicated in upregulating HIV (Torcia, 2019).

Women who are infected with HIV have been shown to have a higher proportion of CST-IV vaginal microbiomes. Compared to CST-I, women who have microbiomes dominated by CST-IV have a four-fold greater likelihood of acquiring HIV. On the molecular level, there are several mechanisms that could link vaginal dysbiosis and increased HIV incidence in women. Firstly, when *Lactobacilli* species decrease in abundance, specifically *L. crispatus*, there is an accompanying decrease in availability of their metabolic product, D-lactate, which helps trap viruses by facilitating the reaction between mucins and HIV viral surface proteins (Torcia, 2019). Additionally, an increase

in CST-IV increases the concentrations of enzymes available to degrade mucin, so there is less mucin to bind foreign particles. Lastly, antigen-presenting cells (APCs), likely upregulated by bacteria present in CST-IV, produce cytokines that recruit CD4+ lymphocytes to the vaginal epithelium (Torica, 2019).

Inflammatory responses are important and necessary in combating disease, but in the case of HIV, which targets the mucosal layer of the genital tract, women who are experiencing an inflammatory response are more likely to contract the virus (Noël-Romas, 2020). Pre-exposure prophylaxis (PrEP), antiviral medication taken by individuals at high risk of contracting HIV, is very effective in preventing HIV transmission via sex or injection drug use. However, it has been shown that the efficacy of PrEP in preventing sexual transmission of HIV is greatly reduced in women who have a lower presence of *Lactobacilli* in their vagina (Velloza, 2017).

Untreated HIV-positive women usually have an increased amount of anaerobes that cause vaginal inflammation, a larger HIV viral load in the genitals, and more shedding of the virus. These women, who have uncontrolled HIV infection, have a higher likelihood of transmitting HIV to sexual partners (Liu, 2019). For women who have undergone treatment for their HIV using antiretroviral therapy (ART), it has been shown that certain microbiome community types are correlated with lower concentrations of ART because the diversity of the vaginal microbiome, which affects the pH of the vagina, can determine how drugs treating the HIV can disperse into the vagina. Certain drugs chosen to treat HIV-positive women such as Tenofovir® and Atazanavir® show the highest drug concentration ratio, vagina:plasma, when the vaginal microbiome

exhibits intermediate diversity as compared to either high vaginal species diversity or low vaginal species diversity. The authors of this study, who found very different taxa dominating the low and high diversity microbiomes, hypothesized that the presence of different strains of bacteria could be changing the uptake or degradation of the type of ART being used (Donahue Carlson, 2017).

The Effect of LARCs on the Vaginal Microbiome

There is evidence both for and against the concept that certain contraceptive methods modify the vaginal microbiome. As previously discussed, the composition of the microbiome impacts the susceptibility to certain STIs, so if contraceptives alter the microbiome, it may mean that the contraceptive user is at higher risk for contracting certain STIs.

In one study looking at DMPA usage in Hispanic W hite and Black women, the vaginal microbiome at baseline was compared to samples taken at 1-month and 3-month timepoints. When examining the data as one large group, no significant changes in the vaginal microbiome were apparent three months after beginning DMPA contraceptive use. However, when analyzing the Hispanic White women and the Black women as separate groups, DMPA use in the Black women was associated with an increased diversity of vaginosis-associated bacteria (VNAB) and *Prevotella* species within their vaginal microbiomes. The Hispanic White participants did not see an enrichment of VNAB upon beginning DMPA contraception or at 3-months post DMPA start. Differences in the Black women's vaginal microbiome makeup, were noted when

comparing the baseline data to the 3-month post DMPA usage data. In summary, DMPA usage did appear to alter the vaginal microbiomes of Black women after 3 months or more of usage (Yang, 2019).

In previous studies, there was evidence that linked DMPA usage to an increased risk of HIV transmission. Women with a predominance of *Lactobacilli* species in their vaginal microbiomes who using DMPA were three times more likely to contract HIV than women who were *Lactobacilli* dominant but using NET-EN or COC as contraceptives. This difference in HIV acquisition was not seen in women who did not have *Lactobacilli*-dominant microbiomes. (Polis, 2016). A similar study examining only DMPA usage and its risk of STI acquisition among teenage women found that there was no significant correlation between 3 months of DMPA usage and the incidence of STIs such as gonorrhea, trichomoniasis, and chlamydia. A higher risk of STIs was only seen in participants who had a greater number of sexual partners during the study (Romer, 2013).

In a study using a mouse model for HSV-2 infection, DMPA and LNG usage were compared in relation to the susceptibility of treated mice to genital herpes infection. Both hormonal contraceptives reduced the expression of desmosomal cadherin desmoglein-1α (DSG1α). Additionally, both DMPA and LNG appeared to increase the number of inflammatory cells present in the genital tissue region by allowing these cells to more easily pass through the more permeable mucosal epithelium. When examining the effects of the contraceptives on untreated mice, it was found that DMPA increased the likelihood of vaginal infection by certain microorganisms by promoting increased

permeability and inflammation. When uninfected mice were treated with DMPA and an additional estrogen delivered intravaginally, the mice were protected from HSV-2 infections. Breakdown of the mucosal barrier, the first line of defense against pathogens in the vaginal microbiome, should be considered when starting a hormonal contraceptive regimen (Quispe Calla, 2016).

Another study compared the effects of three different contraceptive methods, combined hormonal pills, DMPA, and LNG-IUD on the vaginal microbiome. The study looked at 682 women who reported using either condoms, combined contraceptive pills, DMPA, or LNG-IUD. The women included in the study reported only using one type of birth control method at the time they were enrolled in the study, and all participants were on their choice of birth control for at least 1 month. The results indicated that women using combined hormonal pills had much higher levels of *Lactobacilli* species and less BV-associated bacterial species than women who used condoms. DMPA users were more likely to have vaginal microbiomes that were *Lactobacilli* dominant than condom users. DMPA users had fewer BV infections, but some of these users had higher levels of BV-associated species (*A. vaginae and P. bivia*) present in their vaginal microbiomes, while other users had lower levels of BV-associated species (*G. vaginalis*) present in their vaginal microbiomes. In the LNG-IUD group, the amount of BV-associated species in the vaginal environment were much higher than that of women who used combined hormonal pills (Brooks, 2017).

One study only examined the effect of LNG-IUD use on the vaginal microbiome. This study involved only Caucasian women and compared the vaginal microbiome 1

week before method initiation through 12 weeks of LNG-IUD use. None of the women had a recent history of pregnancy or hormone use. The sample size of 11 women was small, but over 400 samples were collected. Of the *Lactobacilli* species, *L. crispatus* was the most prevalent species in the vaginal microbiome, representing 48.9% of the total bacteria present. In longitudinal samples from the same participant, the vaginal microbiome did not change significantly over 12 weeks of LNG IUD use (Jacobson, 2014).

In a study focused on the effects of DMPA, copper IUD, and LNG implant usage on STI risk, 7,829 women from South Africa aged 16-35 years old were included. They examined prevalence of chlamydia and gonorrhea after use of certain contraceptives as risk factors for HIV acquisition. At the start, 18% of the women had chlamydia and 5% had gonorrhea. After 18 months, the prevalence of chlamydia did not differ between the women on DMPA or using the copper IUD, or between the women using the copper IUD or LNG implant. However, the risk of chlamydia was lower in those using DMPA than in those using the LNG implant (14% compared to 17%). When examining gonorrhea prevalence after 18 months, 4% of DMPA users, 6% of copper IUD users, and 5% of LNG implant users were infected. The prevalence of gonorrhea in the women using copper IUDs was significantly higher than that of women using DMPA (Deese, 2020).

Another study compared the usage of a copper IUD and a LNG-IUD. This study included 76 women, and data was collected at baseline (prior to contraceptive use) and at 6 months after the start of contraceptive use. A third sample was collected 12 months after initiation of contraception for 69 of the participants. Participants had one of three

characteristic microbiome types: a mixed *Lactobacilli* species dominant environment, an *L. iners* dominant environment, or an *L. crispatus* dominant environment. Although some changes were observed from the start of the study through 12 months after contraception initiation, the dominant vaginal species at a given time was not associated with either copper IUD or LNG-IUD usage (Bassis, 2017).

One study that examined various hormonal contraceptives and a copper IUD did not see any changes in the vaginal microbiomes of study participants at 1 month, 3 months, or 6 months post contraception initiation. The authors did find that usage of a copper IUD was correlated with an increase in BV diagnosis at 1, 3, and 6 months post contraception initiation. In participants using the copper IUD, the amount of *Lactobacilli* species did not decline, but the prevalence of bacterial species often implicated in BV increased, including *G. vaginalis* and *A vaginae*. Although there are multiple factors that can contribute to vaginal microbiome changes, the women in this study utilizing copper IUDs were having less sexual intercourse than their counterparts, so their increased incidence of BV was likely not linked to more sexual intercourse (Achilles, 2018).

In an additional study examining the effects of copper IUDs and LNG-IUDs on cervicovaginal cytokines, 20 women were enrolled who were STI-free. Data was collected before insertion of the IUD and 4 weeks post IUD insertion. Various cytokines were included in the analysis. Levels of IL-α, IL- β, IL-6, and TNFα were increased and the chemokine MCP-1 was also increased four weeks post IUD insertion. The cytokines that increased are pro-inflammatory cytokines that increase inflammation at the sites they act on, which may or may not increase the risk of STIs. Although an increase in pro-

inflammatory cytokines was seen with both IUDs, the copper IUD caused a more classical inflammatory response expected in a mucosal environment (Sharma, 2018).

SPECIFIC AIMS

Perturbations in the normal vaginal microbiota, or community of microorganisms inhabiting the vaginal body niche, are thus known to affect the risk of transmission of STIs. Vaginal microflora regulate the innate immunity of the epithelium that contributes to barrier function against infections. Studies have shown altered vaginal microbiota with DMPA injection, and conflicting studies support both altered and preserved vaginal microbiota with the LNG IUD. Few studies have compared several progestin LARC methods head-to-head or used culture-independent sequencing methodology.

We proposed a prospective pilot study to evaluate the impact of different long-acting progestin contraceptive formulations on the vaginal microbiome. Women who were planning to initiate DMPA, LNG IUD, and ESI contraception as well as controls seeking sterilization were recruited for the study from Boston Medical Center (BMC) and Women and Infants Hospital of Rhode Island (WIHRI), tertiary care centers with racially and socioeconomically diverse patient populations. Women had longitudinal follow-up with self-sampling of the vagina for sexually transmitted infection testing and metagenomics analysis at method initiation, 2-3 months, and 6 months.

We identified and compared metagenomics profiles associated with DMPA, LNG IUD, and ESI contraceptive use by community analysis of vaginal swab samples from women collected longitudinally after contraceptive method initiation. We hypothesized that DMPA would increase community diversity in the vaginal microbiota, whereas the LNG IUD and ESI would not affect the balance of microorganisms in the vagina.

This study was innovative because of its prospective and longitudinal profiling of vaginal microbiota in women initiating long-acting progestin hormonal contraception, direct comparison between three highly effective contraceptive methods, the leveraging of cutting-edge sequence technologies to characterize complex communities and computational tools to aid in handling "big data," and the racially and socioeconomically diverse patient cohort at BMC. Establishing the safest long-acting progestin contraceptive alternative will promote effective contraception use and lower rates of STI acquisition worldwide.

METHODS

Recruitment

Women between the ages of 18-40 initiating long-acting progestin contraception or who were seeking tubal sterilization were enrolled at a routine office visit at the BMC or WIHRI Ob/Gyn ambulatory practice/family planning clinics. Exclusion criteria included women who were non-English speaking, had used hormonal contraception within the past 3 months (barrier contraception use including condoms or diaphragm were acceptable and to be recorded), were currently menstruating, had had vaginal intercourse within 48 hours of the visit, had known or suspected pregnancy within the past 6 weeks, were using the LARC for a primary indication other than contraception (e.g. pelvic pain, menorrhagia), had a current STI or vaginitis (yeast or BV), use tampons, douche regularly, had recent antibiotic use within the last 4 weeks, or were HIV positive or on immunosuppressive therapy (organ transplant, chemotherapy).

Enrollment

Enrollment took place over a period of 6 months to allow for longitudinal follow-up and analyses. For this pilot study, we were seeking a total of 30 long-acting progestin contraceptive initiators with 10 subjects in each method group [Depo Provera (depot medroxyprogesterone acetate, DMPA), Mirena IUD (levonorgestrel intrauterine device, LNG IUD), or Nexplanon (etonogestrel subdermal implant, ESI)] and 6 age and race-

matched control patients seeking tubal sterilization were selected for longitudinal STI screening and vaginal sampling for microbiome analysis.

During study recruitment, staff were alerted to potentially eligible patient. After a subject gave consent to participate in the study, a brief interview was conducted to review recruitment criteria to confirm eligibility. Informed consent was then obtained from the subject by study staff.

A trained staff member administered a brief sociodemographic/habits questionnaire that included questions on age, race, body mass index, gravidity/parity, date of last menstrual period, smoking status, substance abuse (alcohol or drugs), history of STIs and most recent episode, history of vaginitis and most recent episode, weekly frequency of coitus, number of lifetime sexual partners, and prior contraceptive use. Some but not all of these questions would have been asked as part of standard clinical care for contraception initiation.

Vaginal swabs for STI screening and microbiome analysis were collected from subjects. Subjects were instructed to self-sample the vagina, or to have the provider sample the vagina if not comfortable with self-sampling. Swabs were collected at three time points including method initiation, 2-3 months later, and 6 months later. Each visit involved sampling with one APTIMA swab for STI testing and two Dacron swabs (stored dry). Swabs were then immediately stored at -20°C and then moved to -80°C for longer term storage. At visits after the initial visit, subjects were asked about interval STIs or vaginitis. At the second visit, a short questionnaire and the same swabs were collected; the participant was given a $25 gift card. At the third visit, a short questionnaire and the

same swabs were collected; the participant was given a $50 gift card. The first visit took place during the patient's initial visit to initiate long-acting contraception or to discuss surgical sterilization and thus lasted 30-40 minutes. The second and third visits were briefer and lasted 15-20 minutes.

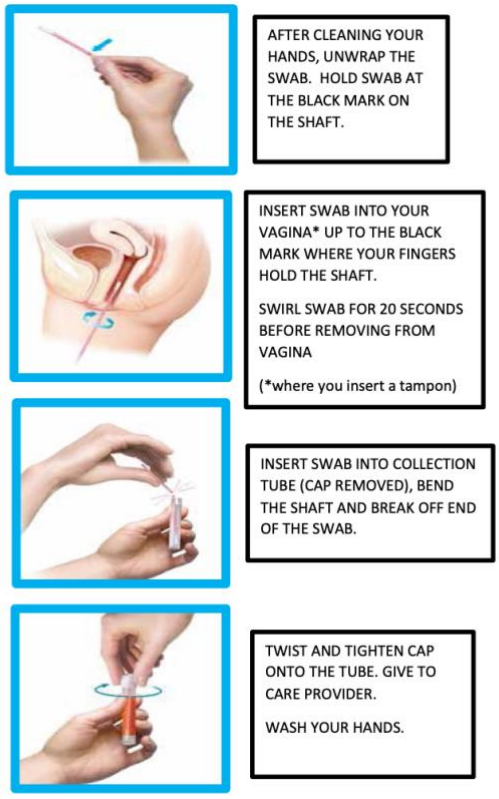

Figure 4: Picture Guide to Patient Vaginal Self-Sampling. This guide was given to patients who wished to self-sample the vagina for each visit.

Specific Experimental Interventions

APTIMA vaginal swab specimens were assayed for Trichomonas vaginalis, Chlamydia trachomatis, and Neisseria gonorrhoeae using DNA amplification in the

clinical lab. Nugent scores and pH correlate strongly with certain microbiome profiles and thus did not need to be performed separately.

Microbiome samples collected at each time point were analyzed and compared to the initiation sample to detect longitudinal differences. Microbial species composition and abundance sampled with self-collected vaginal swabs have been shown to be equivalent to those from swabs collected by clinicians (Forney, 2010). However, if the patient was uncomfortable with self-sampling, the clinician would administer the swabs. Swabs were inserted so that the entire swab head was completely within the orifice, and then the swab was rotated 6 times or for 20 sec while in contact with the vaginal wall. Both swabs could be inserted at the same time. Swabs were delivered to the respective laboratories at BMC or WIHRI shortly after sampling and stored immediately at -80ºC prior to DNA extraction.

In the laboratory, DNA was isolated from the vaginal swabs with QIAGEN DNeasy Blood &Tissue kits following the tissue spin-column protocol with minor modifications. Swabs were thawed on ice and vortexed vigorously for 5 min to resuspend cells before analysis. Ultra-high-throughput data generation for microbial community profiling employed well-established protocols using the 16S ribosomal DNA (rDNA), 515F and 806R universal primers, and sequencing on the Illumina MiSeq platform (Kozich, 2013). Overlapping, paired-end read pairs were 'stitched' to generate error-corrected sequences spanning the length of the amplicon.

DNA extraction and sequencing of samples was performed by Dr. Doyle Ward at University of Massachusetts Medical Center. De novo and reference-based clustering of

sequences into operational taxonomic units (OTUs) was applied using a combination of UPARSE (Edgar, 2013) and QIIME (Bolyen, 2019) packages to generate sample-based OTU tables and classifications and corresponding phylogenetic trees for the sample sequences. Dr. Ward is experienced with all data production and processing activities described above. REDCap was used for data management because it is a standardized software system that supports rapid setup of secure, web-based study-specific database systems and associated forms.

Data Analysis

Use of the following two approaches provided preliminary data for future larger studies from this limited pilot study. If our hypothesis of a vaginal microbial shift with Depo Provera (DMPA) use was confirmed (our PRIMARY analysis), these tools would enable visualization of the differences. The tables of relative abundance of taxonomic groups were used to identify differential bacterial biomarkers present among patient and sample classes. Two bioinformatics tools particularly useful for biomarker discovery are linear discriminant analysis effect size (LefSe) (Segata, 2011) and multivariate association with linear models (MaAsLin) (Mallick, 2021). LefSe used the non-parametric factorial Kruskal-Wallis test to detect features with significant differential abundance with respect to class comparison of interest (e.g. control vs. DMPA use).

Biological consistency was subsequently tested using pairwise tests among subclasses (e.g. smokers vs. nonsmokers) using the (unpaired) Wilcoxon rank-sum test. Linear Discriminant Analysis (LDA) with bootstrapping was used to rank differentially abundant features based on their effect sizes, which was an effective approach to detect

the microorganisms that most differentiate two microbial communities with respect to p values. Significance tests were performed at all clade levels for frequencies that differ significantly with respect to the different categories. A significance alpha of 0.05 and an effect size threshold of 2 was used for all biomarkers discussed in this study.

MaAsLin is a multivariate statistical framework that finds associations between clinical metadata and microbial community abundance or function. The clinical metadata can be of any type: continuous (age and body mass index), Boolean (sex), or discrete/factor (cohort groupings and phenotypes). MaAsLin performs boosted, additive general linear models between one group of data (metadata – the predictors) and another group (in our case microbial abundance – the response). The boosting was used to select metadata that showed some potential to be associated with abundances. Selected metadata was used in a general linear model using metadata as predictors and OTU arcsin-square root transformed abundance as the response (to account for sparse data characteristic of metagenomic studies).

Effects of different sources of variation (e.g. smoking status, presence of STI) on community structure was assessed in multivariant analysis of variance (MANOVA) models, and for OTUs of interest, analysis of variance (ANOVA). Analyses used either frequencies of OTUs or principal component analysis (PCA) values from major axes. Effects of various sources of variation on community diversity was analyzed by ANOVA on diversity indices, obtained from qualitative insights into microbial ecology (QIIME).

To classify community samples based on sources of variation, discriminant analysis was used, with the likelihood that a community was assigned to a given class

being estimated using individual frequencies of OTUs as predictor variables. This method identified specific community samples as ‘typical’ or ‘atypical’ members of a given class and identified OTUs significantly affecting classification of community samples, thus associated with use of a particular contraceptive. Directed identifications at strain level across samples and their combinations in context of health and disease status was evaluated by t-tests, ANOVA, or appropriate non-parametric statistics.

The Dirichlet-multinomial distribution allowed the analyst to perform tests of hypotheses (e.g., compare microbiomes across groups) and estimated parameters describing microbiome properties. The Dirichlet-multinomial distribution prevented Type I error inflation by accounting for the over-dispersion in count data in the microbiome. This model allowed comparison of microbiome populations between more than two groups of subjects (i.e. Mirena IUD users, DMPA users, Nexplanon implant users). This test was analogous to an analysis-of-variance test in classical statistics.

RESULTS

Subject Enrollment

583 potential subjects were screened for the study, and 21 patients were enrolled. Of the twenty-one women enrolled, 1 initiated DMPA, 14 initiated LNG IUD, 6 initiated ESI, and there were no controls.

Table 1: Demographics of the 21 subjects enrolled in the study.

Demographic	No (%)
Ethnicity	
Non-Hispanic	18 (85.7)
Hispanic	3 (14.3)
Race	
White	5 (23.8)
Black/African American	10 (47.6)
Asian	2 (9.5)
Native American, Native Hawiian/Pacific Islander	0
Other	4 (19)

Patient Histories

Prior histories of each subject were taken before enrollment in the study. History included information about types of contraception used prior to three months ago, any past vaginitis episodes (yeast, BV) and any previously contracted STIs (Table 2).

Table 2: Prior History of Contraceptive Use, Vaginitis, and STIs

Demographic	No (%)
Use of hormonal contraception PRIOR TO last 3 months	***13 (61.9)***
OCP	5 (26.3)
LNG IUD	5 (26.3)
DMPA	7 (36.8)
Condom	2 (10.5)
Patch	1 (5.3)
Other (withdrawal, pregnancy, postpartum, not sexually active since baby)	5 (26.3)
Prior history of vaginitis	***5 (23.8)***
→ Yeast vaginitis	3 (60)
→ Bacterial vaginosis	2 (40)
Prior history of STI	**4 (21.1)**
Gonorrhea	2 (11.8)
Trichomonas	1 (5.9)
Other	1 (5.6)

Population Characteristics

After histories were taken, participants were asked how sexually active they were and how many sexual partners they have had in their lifetime (Table 3). Data was collected on tobacco use, alcohol use, and substance use (Table 3).

Table 3: Participant Drug, Alcohol, and Tobacco Use and Prior Sexual History

Population Characteristics: Tobacco, Alcohol, and Drug Use	No (%)
Current smoker	0
Former smoker	3 (14.3)
Never smoker	18 (85.7)
Current alcohol use (median 1 drink per day)	3 (14.3)
Current drug use (marijuana)	1 (4.8)
Population Characteristics: Sexual History	Median
Number of vaginal intercourse episodes/week	2
Number of lifetime sexual partners	3

Data for Patient Follow-up Appointment #2

At the second appointment (3 months post initiation of contraception) patients were run through the initial exclusion criteria to better to determine which specific variables could be influencing the data collected at that time interval (Table 4). At the second appointment, there were 10 swabs collected. Of the 10 swabs collected, 7 of the women were on LNG IUD and 3 were on ESI. None of the participants from the second appointment were using DMPA for contraception. All STI testing was negative (Table 4).

Table 4: Demographics and STI Results from Visit 2. Visit 2 was 3 months post-contraception initiation. At this visit swabs were collected from 10 subjects including 7 in the LNG IUD group and 3 in the ESI group. No data was collected from the DMPA group.

Demographics	No (%)
Vaginal intercourse <48hrs	1 (10)
Pregnancy, Douching, Tampon, Condom, Diaphragm	0
Antibiotic use since last visit	1 (10)
Axillary abscess, I&D, unknown abx	14d
Vaginitis, STI since last visit	0
STI Results	No (%)
Chlamydia	0
Gonorrhea	0
Trichomonas	0

Data for Patient Follow-up Appointment #3

At the third appointment (6 months post initiation of contraception) patients were run through the initial exclusion criteria to better to determine which specific variables

could be influencing the data collected at that time interval (Table 5). At the third appointment, there were 8 swabs collected. Of the 8 swabs collected, 3 of the women were on LNG IUD and 5 were on ESI. None of the participants from the third appointment were using DMPA for contraception. All STI testing was negative (Table 5).

Table 5: Demographics and STI Results from Visit 3. Visit 3 was 6 months post-contraception initiation. At this visit swabs were collected from 8 subjects including 3 in the LNG IUD group and 5 in the ESI group. No data was collected from the DMPA group.

Demographics	No (%)
Vaginal intercourse <48hrs	0
Pregnancy, Douching, Tampon, Condom, Diaphragm	0
Antibiotic use since last visit	0
Axillary abscess, I&D, unknown abx	0
Vaginitis, STI since last visit	0
Results	No (%)
Chlamydia	0
Gonorrhea	0
Trichomonas	0

Vaginal Microbiome of Women Who Are Postpartum Differs From Those Who Are Not Postpartum

All baseline and subsequent STI testing was negative. Additionally, there were no significant differences both by LefSe and MaAsLin between any of the LNG contraceptives (Mirena IUD, DMPA injection, and Nexplanon implant) used. After analyzing the different contraceptive methods used, an analysis by race (Black vs. non-

Black) was also done. No differences in the vaginal microbiota profiles were seen based on race, likely due to the small sample size of the study.

The analysis then examined differences in vaginal microbiota between subjects who were either not postpartum (i.e. remote from pregnancy), 12 weeks or less postpartum, and more than 12 weeks postpartum. To identify significant taxa between these three groups, data was evaluated using LefSe and MaAsLin. Many of the analyses by MaAsLin did not find significant differences between the respective groups being analyzed while LefSe did. Because of this, LefSe figures only are included for simplicity. Significant MaAsLin data is summarized in Table 6. The most significant differences seen by MaAsLin were between the women who were not postpartum and those who were more than 12 weeks postpartum (Table 6).

The first analysis consisted of grouping all women who were postpartum together and examining differences between that group and all women who were not postpartum. For the postpartum group, the first sample was used from those 12 weeks or less postpartum and for the women who were not postpartum, and for the women who were 12 weeks or more postpartum, the last sample was used. This was done to maximize the number of subjects that could be included in the analysis. Analysis by LefSe indicated that there was enrichment with the family *Steptococcaeceae* for women who were postpartum (Figure 5). Analysis by MaAsLin indicated no significant differences between women who were postpartum and women who were not postpartum.

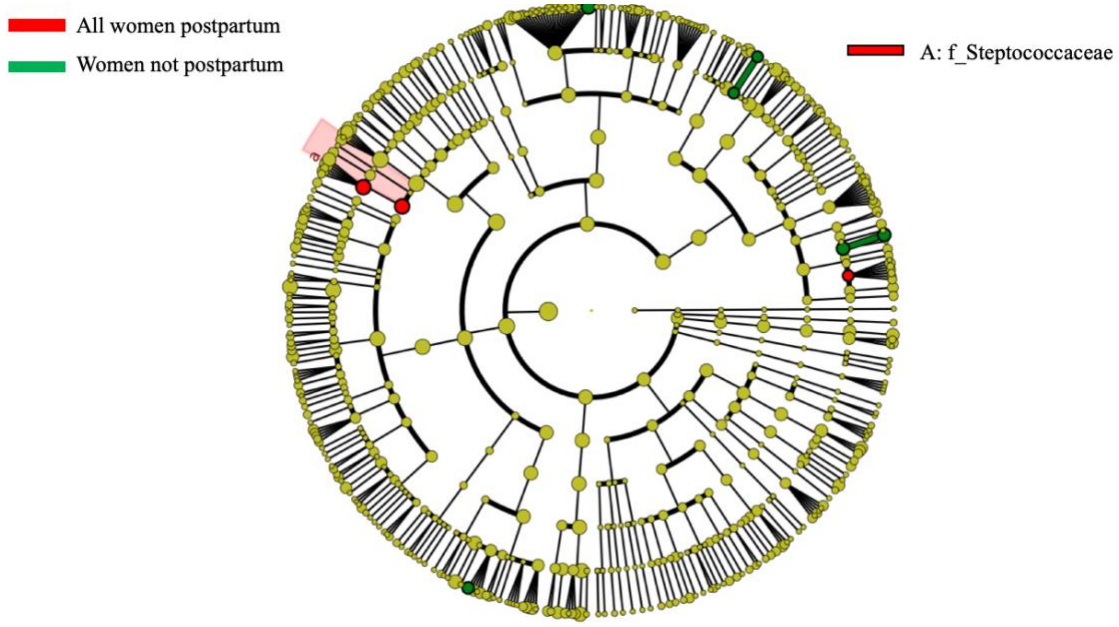

Figure 5: Diversity of the Vaginal Microbiome Between Women Who Are Postpartum and Those Who Are Not Postpartum. The diversity between women who are postpartum and those who are not is shown by LefSe. The cladogram indicates that there is enrichment of the family *Streptococcaceae* for women who are postpartum.

The second analysis examined differences between the two postpartum groups, women who were 12 weeks or less postpartum (early postpartum) and women who were more than 12 weeks postpartum (late postpartum). For the 12 weeks or less postpartum group, the first sample was used from those 12 weeks or less postpartum and for the women who were 12 weeks or more postpartum, the last sample was used. Analysis by both LefSe and MaAsLin indicated no significant differences in the vaginal microbiota of women who were early or late postpartum. The LefSe data is shown in Figure 6.

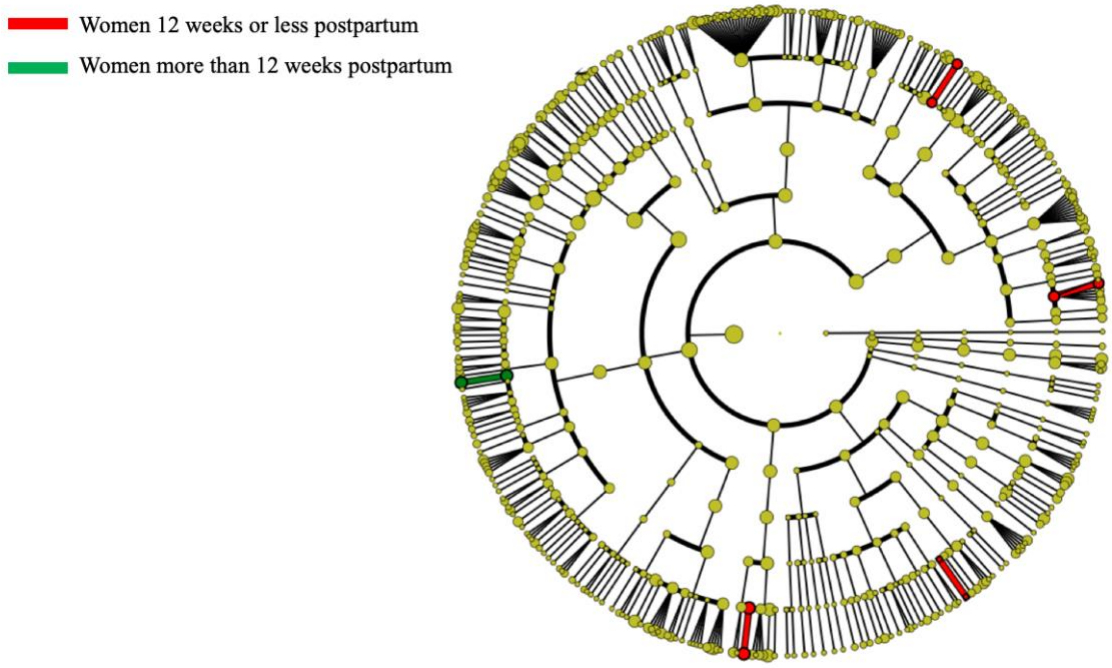

Figure 6: Diversity of the Vaginal Microbiome Between Women Who Are 12 Weeks or Less Postpartum and Women Who Are More Than 12 Weeks Postpartum. The diversity between women who are 12 weeks or less postpartum and those who are more than 12 weeks postpartum is shown by LefSe. The cladogram indicates that there is no significant differences in the diversity of the vaginal microbiota between the two postpartum groups.

Vaginal Microbiome of Women Who Are More Than 12 Weeks Postpartum Differs from Those Who Are Not Postpartum

The third analysis looked at women who were more than 12 weeks postpartum and women who were not postpartum. For both groups, the last sample collected was used. Analysis by both LefSe and MaAsLin found significant differences between the two groups. LefSe analysis indicated enrichment of 8 different taxa for the women who are more than 12 weeks postpartum, when compared to those women who are not postpartum (Figure 7). For women who were not postpartum, their vaginal microbiota

profiles were enriched for families *Actinomycetacea, Prevotellaceae, Lachnospiraceae, Desulfovibrionaceae,* and *Enterobacteriacea*; orders *Bacteroidales, Desulfovibrionaceae,* and *Enterobacteriales;* and classes *Bacteroidia* and *Deltaproteobacteria*. The LefSe analysis was enriched for the family *Enterbacteriacea* and the order *Enterobacteriales* when examining the vaginal microbiota of women who were more than 12 weeks postpartum.

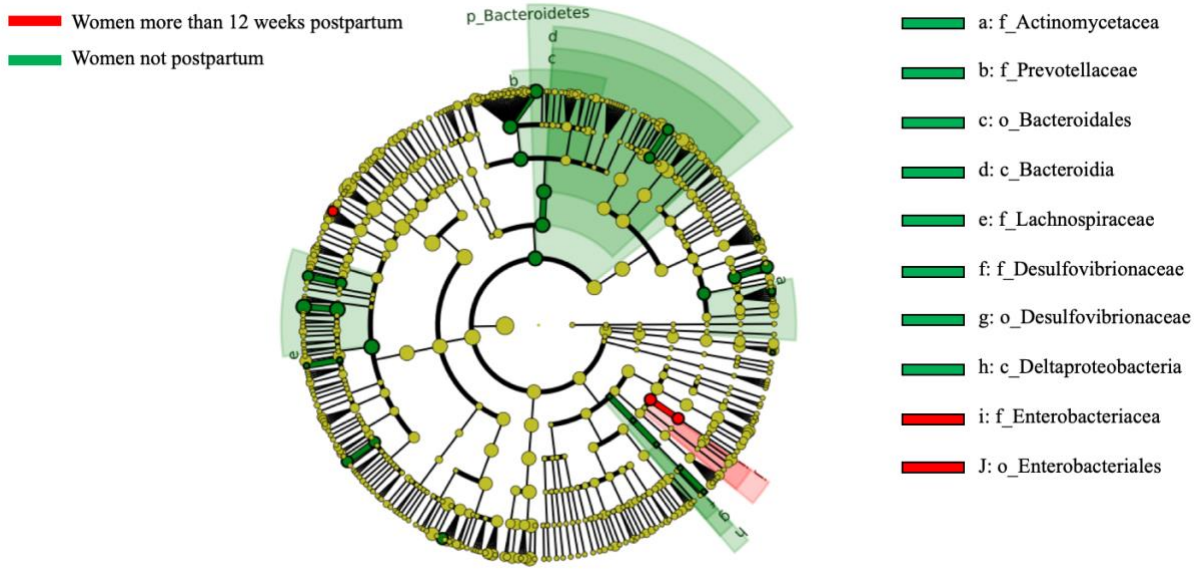

Figure 7: Diversity of the Vaginal Microbiome Between Women Who Are More Than 12 Weeks Postpartum and Women Who Are Not Postpartum. The diversity between women who are more than 12 weeks postpartum and women who are not postpartum is shown by LefSe. The cladogram indicates that there are significant differences in the diversity of the vaginal microbiota between the two postpartum groups. Compared to women who were more than 12 weeks postpartum, the vaginal microbiota profiles of non-postpartum women were enriched for families *Actinomycetacea, Prevotellaceae, Lachnospiraceae, Desulfovibrionaceae,* and *Enterobacteriacea*; orders *Bacteroidales, Desulfovibrionaceae,* and *Enterobacteriales;* and classes *Bacteroidia* and *Deltaproteobacteria*. The LefSe analysis indicated enrichment for the family *Enterobacteriacea* and the order *Enterobacteriales* for women who are more than 12 weeks postpartum.

The MaAsLin data indicated significance (q value < 0.05) for women not postpartum for 2 families, *Aerococcaceae* and *Streptococcaceae* (Table 6). The same analysis indicated significance for women who were more than 12 weeks postpartum for 3 families: *Lachnospiraceae, Ruminococcaceae,* and *Erysipelotrichaceae* (Table 6).

Table 6: Diversity of the Vaginal Microbiome Between Women Who Are More Than 12 Weeks Postpartum and Women Who Are Not Postpartum. The diversity between women who are more than 12 weeks postpartum and women who are not postpartum is summarized in a table after MaAsLin analysis. The table indicates that there are significant differences in the vaginal microbiota between the two postpartum groups. The MaAsLin data indicated significance (qval < 0.05) for women not postpartum for 2 families, *Aerococcaceae* and *Streptococcaceae* (indicated in green), and for women more than 12 weeks postpartum for 3 families: *Lachnospiraceae, Ruminococcaceae,* and *Erysipelotrichaceae* (indicated in red).

feature	coef	pval	qval
d_Bacteria/p_Firmicutes/c_Bacilli/o_Lactobacillales/f_Aerococcaceae	-0.10923822	0.0005803	0.04061969
d_Bacteria/p_Firmicutes/c_Bacilli/o_Lactobacillales/f_Streptococcaceae	-0.16020773	0.0072972	0.13617228
d_Bacteria/p_Firmicutes/c_Clostridia/o_Clostridiales/Lachnospiraceae	0.30010755	0.00910586	0.13617228
d_Bacteria/p_Firmicutes/ c_Clostridia/ o_Clostridiales/f_Ruminococcaceae	0.04537156	0.00972659	0.13617228
d_Bacteria/p_Firmicutes/c_Erysipelotrichia/o_Erysipelotrichales/f_Erysipelotrichaceae	0.00571035	0.00657374	0.13617228

Vaginal Microbiome of Women Who Are 12 Weeks or Less Postpartum Differs from Those Who Are Not Postpartum

The fourth analysis compared women who were 12 weeks or less postpartum and women who were not postpartum. For the 12 or less weeks postpartum group, the first sample was used, and for the women who were not postpartum the last sample was used.

Analysis by LefSe indicated that there was enrichment with the family *Steptococcaeceae* and within the class *Gammaproteobacteria* for women who were 12 weeks or less postpartum (Figure 8). Analysis by MaAsLin indicated no significant differences between women who were 12 weeks or less postpartum and women who were not postpartum.

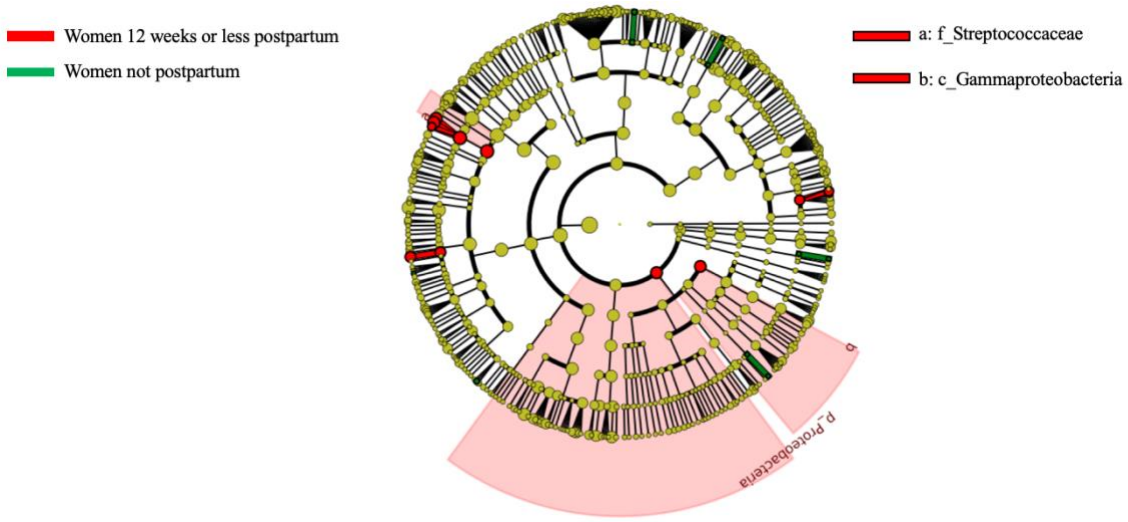

Figure 8: Diversity of the Vaginal Microbiome Between Women Who Are 12 Weeks or Less Postpartum and Those Who Are Not Postpartum. The diversity between women who are 12 weeks or less postpartum and those who are not postpartum is shown by LefSe. The cladogram indicates that there is enrichment of the family *Streptococcaceae* and the class *Gammaproteobacteria* for women who are 12 weeks or less postpartum.

DISCUSSION

In the vaginal microbiome, less diversity is more beneficial to health. Individuals with more diverse species within their vaginal microbiomes have higher risks of infection (Smith, 2017). Microbiome disruptions and changes may result from behavioral habits such as dietary choices, exercise frequencies, and hygiene practices (Gupta, 2019). Smoking, alcohol use, stress, obesity, vitamin D deficiency, diabetes, and climate are additional factors that may negatively affect the disease state of the vagina due to disruption of commensal bacteria within its microbiome (Huang, 2014).

Many previous studies, mostly in the context of increased risk of STIs, have examined the effects of different types of contraception on the vaginal microbiome. The relationship between different contraceptives and the increased risk of STI infection due to disruption of the vaginal microbiome is not completely clear. Head-to-head comparisons of the vaginal microbiome impact of long-acting progestin contraceptives, some of the most effective contraceptive options available, are lacking. We hypothesized that DMPA would increase community diversity in the vaginal microbiota, whereas the LNG IUD and ESI would not affect the balance of microorganisms in the vagina.

We compared the vaginal metagenomic profiles of women initiating either DMPA, LNG IUD, or ESI contraceptive use. Vaginal swabs were collected prior to beginning contraception, 3 months post contraception initiation, and 6 months post contraception initiation. DNA was extracted, and 16S rDNA gene sequencing was performed to characterize vaginal microbiome composition. LefSe and MaAsLin were used in our statistical data analyses. We found no differences over time in the vaginal

microbiota of the women who initiated each type of contraceptive. We also found no link between contraceptive use and STIs as baseline tests and subsequent analysis for chlamydia and gonorrhea were all negative. Because we found no link between vaginal microbiome profiles and type of progestin contraceptive being used, we also looked at other variables including race and postpartum status. When looking at race, we found no clear patterns between Black women and nonBlack women in terms of their microbiomes at each time point. This was likely due to low sample size in the study.

On examining the microbiomes of women who were recently postpartum (less than 12 weeks), late postpartum (over 12 weeks postpartum), or who were remote from pregnancy, there were some differences in the metagenomic profiles. When grouping all postpartum women together, regardless of how recently they had given birth, and comparing them to women who were remote from pregnancy, analysis by LefSe indicated that there was enrichment with the family *Steptococcaeceae* for women who were postpartum.

When comparing women who were more than 12 weeks postpartum to those who were not postpartum, LefSe analysis indicated enrichment of 8 different taxa for women who were not postpartum including the families *Actinomycetacea, Prevotellaceae, Lachnospiraceae, Desulfovibrionaceae,* and *Enterobacteriacea*; orders *Bacteroidales, Desulfovibrionaceae,* and *Enterobacteriales;* and classes *Bacteroidia* and *Deltaproteobacteria*. The LefSe analysis was enriched for the family *Enterobacteriacea* and the order *Enterobacteriales* when examining the vaginal microbiota of women who were more than 12 weeks postpartum. The MaAsLin data indicated significance (qval <

0.05) for women not postpartum for 2 families, *Aerococcaceae* and *Streptococcaceae*. The same analysis indicated significance for women who were more than 12 weeks postpartum for 3 families *Lachnospiraceae, Ruminococcaceae,* and *Erysipelotrichaceae*.

When comparing the women who were 12 weeks or less postpartum to women who were not postpartum, analysis by LefSe indicated that there was enrichment with the family *Steptococcaeceae* and within the class *Gammaproteobacteria* for women who were 12 weeks or less postpartum.

Pregnancy does appear to alter the vaginal microbiome. One study that examined the vaginal microbiome throughout pregnancy and postpartum found that the composition of the microbiome after pregnancy shifts from favoring *Lactobacilli* species to increased alpha diversity. This was the trend regardless of race or ethnicity. The study did note some differences with race in the vaginal microbiome profiles of pregnant women. They found that amongst the British population in their study, there was a predominance of *L. jensenii* amongst Caucasian and Asian women. In the samples from Black women, they observed the absence of *L. gasseri* in many of the samples (MacIntyre, 2015). This general trend of less *Lactobacilli* species present in the vaginal microbiomes of Black women compared to their Asian and Caucasian counterparts is something that has been observed in multiple studies (Huang, 2014).

Several other studies have confirmed that the pregnant vaginal microbiome is less diverse than that of women who are not pregnant. The less diverse pregnant vagina does appear to be more *Lactobacilli*-dominant when compared to women who are not pregnant. Studying the vaginal microbiome during pregnancy and just after giving birth

is important because it may shed light on the genesis of obstetrical complications. It is believed that the vaginal microbiome and the species that dominate it during pregnancy may contribute to preterm labor and adverse pregnancy outcomes (Gupta, 2020).

Other studies have found that the decrease in *Lactobacilli* species and the increase in diversity of various species in the postpartum vagina is likely due to falling estrogen levels post pregnancy. Researchers have found that the postpartum vagina begins to mimic the gut microbiome with its diversity, likely due to the possible translocation of stool microbiota to the vagina. This relationship needs to be explored further. Regardless, a very diverse and less stable vaginal microbiome postpartum is significant because this may impact STI and future obstetrical risks (Gupta, 2020).

In another study examining a subset of women in the US, the vagina was found to be more diverse postpartum, with lower amounts of *Lactobacilli* species and increased levels of *Streptococcus anginosus* and *Prevotella bivia*. Postpartum vaginal secretions also appeared to differ from pre-pregnancy and pregnancy secretions, with much decreased levels of lactic acid and increased amounts of HSP70 and hyaluronan (Nunn, 2021), indicating the presence of a higher vaginal pH and inflammation. A similar study examining the postpartum vaginal microbiomes of women in rural Malawi found a similar trend, with a decrease in *Lactobacilli* species and an increase in *G. vaginalis*. As the time from pregnancy increased, the authors found an increase in *Lactobacilli* species, but *G. vaginalis* remained dominant in many of the women in the study up to a year postpartum (Doyle, 2018).

Although more significance was seen when comparing the various postpartum groups to women remote from pregnancy with Lefse, the data that is most relevant are the differences seen with both Lefse and MaAsLin because q-values adjust for more confounding factors than p-values when examining and correcting for many taxonomic units. The MaAsLin data found enrichment of three families, *Lachnospiraceae, Ruminococcaceae,* and *Erysipelotrichaceae,* in women who were more than 12 weeks postpartum. *Lachnospiraceae* has been found to be strongly correlated with an increased susceptibility to BV infections (Hummelen, 2010), and *Ruminococcaceae* is not known to cause BV but has been seen in association with active BV infections (Hao, 2011). BV is more prevalent in women with more diverse vaginal microbiomes, and postpartum vaginal microbiomes are more diverse than pregnant vaginal microbiomes. This relationship between the dominant species present in the vaginas of postpartum women and their susceptibility to other infections should be further examined.

Study strengths included the successful collaboration between hospitals in two different states, the large number of subjects screened for the study in a short amount of time, and the use of a combined metagenomics approach to fully examine all variables and correlations in the data set. Weaknesses include the exclusion criteria that made enrollment especially challenging because it was very strict, low sample numbers, and study subject loss to follow-up after enrollment.

Although we did not see any differences in vaginal microbiota in women using different types of long-acting progestin contraception, this is a positive outcome because changes in vaginal microbiota in response to contraception use would be undesirable.

We were able to see some differences in the vaginal microbiota of postpartum women and those who were not postpartum, but the effects of those differences remain unclear. Future studies should further probe the relationship between postpartum vaginal microbiomes and disease. It would also be beneficial to follow women postpartum who are trying to conceive again to attempt to draw correlations between the recovering postpartum vaginal microbiome and adverse pregnancy outcomes.

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