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Exploring Associations between the Vaginal Microbiome and Health Behaviors, Sociodemographic Factors, and Vaginal Symptoms in Postmenopausal Women

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By

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Exploring Associations between the Vaginal Microbiome and Health Behaviors, Sociodemographic Factors, and Vaginal Symptoms in Postmenopausal Women submitted to the Faculty of the
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Abstract

Exploring Associations between the Vaginal Microbiome and Health Behaviors, Sociodemographic Factors, and Vaginal Symptoms in Postmenopausal Women

By Gaea Daniel

Background: Postmenopausal women are more likely to have greater bacterial diversity and less protective *Lactobacillus* (*L*. spp.) within their vaginal microbiome than premenopausal women due to decreased estrogen levels. It is unclear whether health behaviors and sociodemographic factors affect the vaginal microbiomes of postmenopausal women and if vaginal symptoms are related to unhealthy changes in the vaginal microbiome.

Purpose: To characterize the vaginal microbiomes of postmenopausal women, and to assess associations between the vaginal microbiome of these women and their health behaviors, sociodemographic factors, and vaginal symptoms.

Methods: A secondary analysis of an ongoing study of postmenopausal women was conducted. Twenty-one participants with physician-collected vaginal swabs and self-reported responses to questionnaires about their health behaviors, sociodemographic factors, and vaginal symptoms were available for analysis. Alpha and beta diversity were used to assess microbial diversity within and between samples. The Wilcoxon and Kruskal-Wallis rank sum tests, relative abundances, Bray-Curtis and Jaccard distances, PERMANOVA, and Linear Decomposition Model were used to evaluate associations between the vaginal microbiome and health behaviors, sociodemographic factors, and vaginal symptoms.

Results: Alpha diversity was significantly higher for participants who used antibiotics ($p<0.05$) and trended towards significance for participants who used oral probiotics ($p=0.16$). Four vaginal microbiome samples had a relative abundance of *L*. spp. ≥90%. Relative abundance of other genera was negligible in these samples.

Conclusion: Use of antibiotics is associated with increase diversity in the vaginal microbiome, and oral probiotic use may potentially be associated with decreased diversity. The association between *L*. spp.-dominance and health promoting and disruptive behaviors warrants further study. A larger sample size is needed to confirm associations. Both researchers and clinicians should thoroughly assess health promoting and health disruptive behaviors when evaluating the vaginal microbiome and gynecological health.

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Article One: A Framework for Studying the Vaginal Microbiome

Introduction

A person whose biological sex is female ages with time and naturally transitions into the postmenopausal life stage, indicating the end of reproductive years. Although a female is beyond childbearing years, their gynecological health still plays an important role in health outcomes. For example, the drying and thinning of the vaginal wall in postmenopausal women, combined with reduced condom utilization due to the inability to get pregnant, may increase the risk of contracting a sexually transmitted infection (STI) (Minkin, 2010). Furthermore, the risk of endometrial, ovarian, and vulvar cancers increases during the postmenopausal years of life (Furau et al., 2011). Gynecological health is influenced by multiple factors including health behaviors of the host and the sociodemographic factors that characterize the host. Recent research has identified another factor that may influence vaginal health, the vaginal microbiome (VM). To date, most research has focused on premenopausal women and the association among vaginal microbial communities and preterm births. However, more recent work has begun to assess how the vaginal microbiome may impact and promote gynecological health or disease, although most of this research has continued to ignore postmenopausal women. Based on the limited current literature, a framework (Figure 1) was developed to explore the interactions among the vaginal microbiome, sociodemographic factors (SDFs), health behaviors, and vaginal symptoms.

The vaginal microbiome (VM) is the symbiotic microbial community inhabiting the vagina and is composed of microorganisms, including bacteria, fungi, and archaea. The VM has long been associated with gynecological health in premenopausal women relative to the dominating presence of *Lactobacillus* species (*L.* spp.) in the vagina. Growth of other bacterial species is inhibited by *L*. spp.'s production of lactic acid, hydrogen peroxide, and proteins that are toxic to some pathogens (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, Vaginal Microbiome, et al., 2014). Therefore, *L*. spp. supports a protective acidic vaginal environment through bactericidal and bacteriostatic properties. These characteristics lead to decreased diversity (the number of different types of bacteria) in the VM, which is considered healthy. Dysbiosis of the VM occurs when *L*. spp. is lacking and other, often pathogenic bacteria dominate the VM. This dysbiotic state is associated with increased susceptibility to poor gynecological health outcomes such as increased susceptibility to STIs and uncomfortable vaginal symptoms like discharge, dryness, and itching (Low et al., 2011a; Muhleisen & Herbst-Kralovetz, 2016).

Sociodemographic Factors and the Vaginal Microbiome

Postmenopausal women are at greater risk than premenopausal women of experiencing a dysbiotic VM since estrogen, which supports the abundance of *L*. spp. in the VM, decreases as a woman transitions into menopause. When it occurs naturally, most women transition into the menopausal stage at ages between their late 40s to early 50s and will spend about 40% of their lives in the postmenopausal state (Takahashi & Johnson, 2015). Thus age both a SDF and biologic factor that affects the amount of *L*. spp. in the VM due to temporal changes in the sex hormone estrogen (Muhleisen & Herbst-Kralovetz, 2016). Education, unlike age, has not been shown to directly affect the amount of *L*. spp. in the VM. Although few have assessed the relationship between education and the vaginal microbiome, a recent study revealed that higher education level is associated with *L*. spp.-dominant VMs (Virtanen et al., 2019). Health insurance is a common proxy for socioeconomic status when household income is not available since household income is not usually collected in hospital data (Foraker et al., 2010). Inability to afford healthcare or lack of access to healthcare may indirectly affect the VM, as women may self-treat for vaginal symptoms. No significant relationships have been found between the VM and income, but a trend in association was found between income and dysbiosis (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, Vaginal Microbiome, et al., 2014).

Race as an SDF may increase susceptibility to infections and diseases due to shared social, cultural, and physical environments. Race is a social construct used to categorize individuals to align with historical US health data (DeSantis et al., 2016). It is within the racial context that distinct differences are seen in the VM that increase susceptibility to human papillomavirus (HPV) (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, The Vaginal Microbiome, et al., 2014; Mitra et al., 2016). The explicit physiological differences in VMs based on race and ethnicity may be linked to factors like biological processes (i.e. DNA methylation from stress) and socioeconomic status (SES), which can be tied to the effects of racism and promote race-based differences (Burger et al., 2016).

A longitudinal cohort study funded through the Vaginal Human Microbiome Project found that the mean localized alpha diversity (vaginal microbial diversity within one sample) of Black women's VMs is significantly greater than that of White women, and that Black women were 2.9 times more likely to be diagnosed with bacterial vaginosis (BV), a form of dysbiosis that may be asymptomatic (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, The Vaginal Microbiome, et al., 2014). The same group from the Vaginal Human Microbiome Project more recently conceded that the cause of the differences seen in the VM by race and ethnicity is not clear, and that "genetic and environmental influences are likely contributors" (Serrano et al., 2019). While acknowledging race-based differences in the VM, caution must be taken to avoid speculation that the differences are only genetically determined by the host and redirect the focus towards “human habits and practices” for explanation of gynecological disparities (Ravel et al., 2011). These “human habits and practices” are addressed in the framework (Figure 1) as health behaviors. Some behaviors, such as intravaginal practices and cigarette smoking, have been associated with an unhealthy increase in VM diversity.

Health Behaviors and the Vaginal Microbiome

Intravaginal practices (IVPs) refer to the types of practices women use to clean inside their vaginas and are generally considered unhealthy. A wide variety of products, including commercial douching products, antiseptics, detergents, soaps, and herbs, are placed or pushed inside the vagina with different types of tools, including fingers, bottles, wipes, and commercial tools, by women all over the world to make their vaginas feel clean, control odor, or to enhance sexual pleasure for themselves or their partners (Alcaide et al., 2015; Alcaide, Rodriguez, Fischl, Jones, & Weiss, 2017; Amaral et al., 2007; Brown et al., 2016; Chisembele, Rodriguez, Brown, Jones, & Alcaide, 2018; Crann, Cunningham, Albert, Money, & O'Doherty, 2018; Masese et al., 2013; McCarthy, Nii-Trebi, Musah, & Asmah, 2015; Ness et al., 2001; Sivapalasingam et al., 2014; van de Wijgert et al., 2000). Details of IVPs are not well-described, especially in the US, and no validated metrics exist to assess IVPs. Therefore, an in-depth description of this practice is provided here.

Researchers in countries other than the US have documented use of homemade, household, and natural products used for IVPs (Alcaide et al., 2015; Alcaide et al., 2017; Amaral et al., 2007; Brown et al., 2016; Chisembele et al., 2018; Crann et al., 2018; Masese et al., 2013; McCarthy et al., 2015; Ness et al., 2001; Sivapalasingam et al., 2014; van de Wijgert et al., 2000). A well-known form of IVPs in the US is douching where women irrigate their vaginas with a liquid solution. A variety of douching products are sold commercially and can be found on shelves of stores. Douching is a significant risk factor for dysbiosis associated with race since Black women are at least two times more likely than White women to douche (Fettweis et al., 2014, Mitra et al., 2016). Women may try to remedy malodor caused by BV or sexual intercourse with douching without the realization that douching can exacerbate the problem.

Intravaginal practices, including douching but also the use of other products, are often a result of misperceptions about appropriate vaginal hygiene and are associated with younger ages of sexual debut (Ness et al., 2003). Products used for IVPs can cause dysbiosis by depleting or killing *L.* spp. (Aslan & Bechelaghem, 2018). In one study conducted in Sub-Saharan African women, IVPs are associated with dysbiosis in the VM leading to BV and increase risk of STIs, including persistent, oncogenic strains of HPV and human immunodeficiency virus (HIV) (Low et al., 2011b).

Some studies in the US have yielded insignificant associations between IVPs and dysbiosis in the VM (Brotman, He, et al., 2014; Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, Vaginal Microbiome, et al., 2014). However, IVPs were not consistently and thoroughly assessed in these studies, and primarily focused on douching with commercial products, ignoring the myriad of additional IVPs women may use.

A few studies conducted in the US have conducted more in-depth investigations of IVP use and have found that women reported use of soap and water; use of a cloth, finger, sponge, rag, or wipe to clean inside their vaginas; or use of lubricants, oils, and petroleum jelly for lubrication during sexual activities (Brown et al., 2013). In addition, even on the rare occasions when IVP use is assessed, the purpose of use is rarely assessed; however, when asked, some women have indicated that their use of IVPs is related to hygiene and sex (Alcaide et al., 2017). The frequency of IVP use has not been regularly assessed. The reason why women use IVPs varies; women state that they want to feel and smell fresh and want to get rid of any odor especially concerning peri-sexual activities, thus they clean inside of their vaginas to prepare for sex and may clean themselves after sex (Amaral et al., 2007). Additionally, products are also inserted related to sex for lubrication during sexual activities to reduce vaginal dryness and discomfort, while some women may insert products to promote vaginal dryness, believing it increases pleasure for their male sex partners (Sivapalasingam et al., 2014).

At least one study has reported that some women use IVPs to satisfy their sexual partner’s request and misguided belief that IVPs maintain or improve vaginal health. They are often taught these practices by their mothers or grandmothers, some other relative, friends, or sexual partner(s) (Masese et al., 2013). Some women teach themselves IVP use, and other women are influenced by media advertisements (Martin Hilber et al., 2010).

There are aspects of sexual behaviors that may have unhealthy consequences for the VM. Having both male and female sex partners and multiple sex partners are all associated with dysbiosis (Koumans et al., 2007; Muzny, Harbison, Pembleton, & Austin, 2013). Use of condoms may decrease the unhealthy effects of sex on the VM (Jespers et al., 2014). Related to sexual activity, vaginal lubricants used to alleviate vaginal dryness may have a negative effect on the VM depending on the ingredients of the lubricants. Though no studies have been conducted to our knowledge to evaluate the use of lubricants on the VM, it has been hypothesized that the use of lubricants will disrupt the VM (Brotman, 2016).

Smoking cigarettes, a behavior that is linked to several poor health outcomes, is consistently associated with dysbiosis in the VM compared to women who do not smoke (Bagaitkar, Demuth, & Scott, 2008; Brotman, He, et al., 2014). One study also observed nicotine and its metabolites in the vagina from smoking cigarettes has be detected (Nelson et al., 2018).

While some behaviors are unhealthy and can be considered risky concerning the VM, others have been associated with a healthy decrease in VM diversity or an increase in *L*. spp. Hormonal replacement therapy (HRT), either oral or topical estrogen, have both been associated with increased vaginal *L*. spp. (Heinemann & Reid, 2005; Shen et al., 2016; Si, You, Yu, Sung, & Ko, 2017).

Ingested products have also been associated with *L*. spp.-dominant VMs. Oral probiotics are associated with increased *L*. spp. in the VM after two weeks of use; and vaginal symptoms from dysbiosis decreased with ingestion of yogurt after four weeks of use (Laue et al., 2018; Petricevic, Unger, Viernstein, & Kiss, 2008). Both of these oral products contained *L*. spp. It is unclear if the abundance of *L*. spp. is maintained after the consumption of probiotics or yogurt ceases. Alcohol consumption in moderation is likely to yield less pathogenic bacteria in the VM than women who do not consume alcohol; however, excessive alcohol consumption is considered an independent risk factor for dysbiosis (Baisley et al., 2009; Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, Vaginal Microbiome, et al., 2014). The amount where alcohol consumption crosses the threshold to become excessive and negatively affect the VM is unknown.

These behaviors, both healthy and unhealthy influence the diversity of the VM, and some specifically affect the abundance of *L*. spp. Decreased diversity tends to lead to optimal gynecological health outcomes, while increased diversity can lead to poor outcomes like vaginal symptoms. However, beyond diversity, the actual composition and abundance of healthy vaginal microbial communities has yet to be defined.

Vaginal symptoms, including dryness and discharge, are common in about half of postmenopausal women and are associated with dysbiosis of the VM (Muhleisen & Herbst-

Kralovetz, 2016). The pain and discomfort associated with these vaginal symptoms impact sexual health and overall quality of life (Freedman, 2002; Muhleisen & Herbst-Kralovetz, 2016). Understanding the association among vaginal microbial communities and vaginal symptoms is important for symptom management. An assessment of the literature characterizes the VM as a moderator for vaginal symptoms, thereby making it an important target for assessment and intervention since dysbiosis of the VM is associated with vaginal symptoms (Brotman, Shardell, et al., 2014; Muhleisen & Herbst-Kralovetz, 2016).

Discussion

Based on the literature, the model proposed herein is appropriate for studying the VM when aims include 1) characterizing the VM by participant characteristics, 2) identifying racial/ethnic disparities in the VM composition, 3) exploring associations between the VM and social, cultural, and environmental factors, and 4) examining outcomes as a result of vaginal microbial dysbiosis. Future research should delve further into identifying the non-biological contributing factors of healthy and dysbiotic VM in order to test appropriate and feasible interventions. Clinicians should assess IVP use and educate patients accordingly. Encouragement of smoking cessation and safe sex practices would also be beneficial.

In summary, the vaginal microbiome is dynamic and complex, and its composition is not based on one factor or biological process, but rather an interaction of sociodemographic factors and health behaviors. Researchers should further assess social, cultural, and environmental influences to better understand which factors determine healthy microbial composition in postmenopausal women.

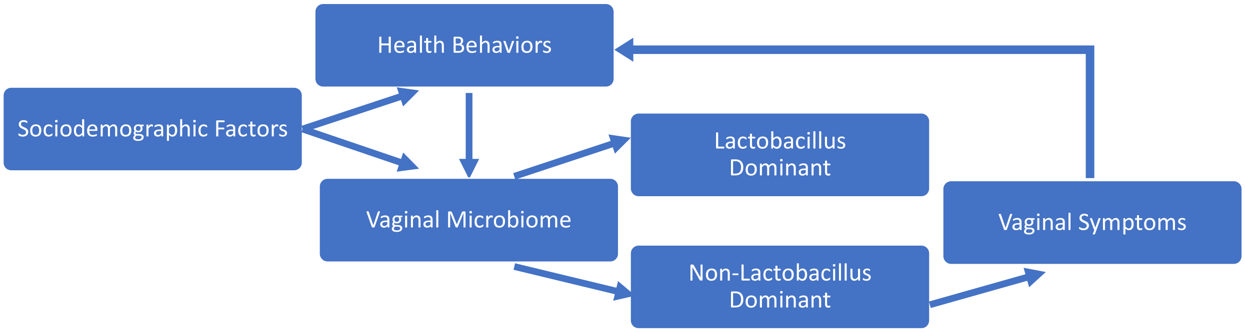

Figure 1. A model displaying the relationships among the vaginal microbiome, health behaviors, and sociodemographic factors as a framework for studying the vaginal microbiome.

References

Alcaide, M. L., Chisembele, M., Malupande, E., Arheart, K., Fischl, M., & Jones, D. L. (2015). A cross-sectional study of bacterial vaginosis, intravaginal practices and HIV genital shedding; implications for HIV transmission and women's health. *BMJ Open, 5*(11), e009036. doi:10.1136/bmjopen-2015-009036

Alcaide, M. L., Rodriguez, V. J., Fischl, M. A., Jones, D. L., & Weiss, S. M. (2017). Addressing intravaginal practices in women with HIV and at-risk for HIV infection, a mixed methods pilot study. *Int J Womens Health, 9*, 123-132. doi:10.2147/IJWH.S125883

Amaral, R., Giraldo, P. C., Gonçalves, A. K., Junior, J. E., Santos-Pereira, S., Linhares, I., & Passos, M. R. L. (2007). Evaluation of hygienic douching on the vaginal microflora of female sex workers. *International Journal of STD and AIDS, 18*(11), 770-773. doi:10.1258/095646207782212333

Aslan, E., & Bechelaghem, N. (2018). To 'douche' or not to 'douche': hygiene habits may have detrimental effects on vaginal microbiota. *Journal of Obstetrics and Gynaecology, 38*(5), 678-681. doi:10.1080/01443615.2017.1395398

Bagaitkar, J., Demuth, D. R., & Scott, D. A. (2008). Tobacco use increases susceptibility to bacterial infection. *Tobacco Induced Diseases, 4*, 12. doi:10.1186/1617-9625-4-12

Baisley, K., Changalucha, J., Weiss, H. A., Mugeye, K., Everett, D., Hambleton, I., . . . Watson-Jones, D. (2009). Bacterial vaginosis in female facility workers in north-western Tanzania: Prevalence and risk factors. *Sexually Transmitted Infections, 85*(5), 370-375. doi:10.1136/sti.2008.035543

Brotman, R. M., He, X., Gajer, P., Fadrosh, D., Sharma, E., Mongodin, E. F., . . . Rath, J. M. (2014). Association between cigarette smoking and the vaginal microbiota: a pilot study. *BMC Infectious Diseases, 14,* 471. doi:10.1186/1471-2334-14-471

Brotman, R. M., Shardell, M. D., Gajer, P., Fadrosh, D., Chang, K., Silver, M. I., . . . Gravitt, P. E. (2014). Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause, 21*(5), 450-458. doi:10.1097/GME.0b013e3182a4690b

Brown, J. M., Hess, K. L., Brown, S., Murphy, C., Waldman, A. L., & Hezareh, M. (2013). Intravaginal practices and risk of bacterial vaginosis and candidiasis infection among a cohort of women in the United States. *Obstetrics and Gynecology, 121*(4), 773-780. doi:10.1097/AOG.0b013e31828786f8

Brown, J. M., Poirot, E., Hess, K. L., Brown, S., Vertucci, M., & Hezareh, M. (2016). Motivations for Intravaginal Product Use among a Cohort of Women in Los Angeles. *PloS One, 11*(3), e0151378. doi:10.1371/journal.pone.0151378

Burger, E. A., Lee, K., Saraiya, M., Thompson, T. D., Chesson, H. W., Markowitz, L. E., & Kim, J. J. (2016). Racial and ethnic disparities in human papillomavirus-associated cancer burden with first-generation and second-generation human papillomavirus vaccines. *Cancer, 122*(13), 2057-2066. doi:10.1002/cncr.30007

Chisembele, M., Rodriguez, V. J., Brown, M. R., Jones, D. L., & Alcaide, M. L. (2018). Intravaginal practices among young HIV-infected women in Lusaka, Zambia. *International Journal of STD and AIDS, 29*(2), 164-171. doi:10.1177/0956462417721438

Crann, S. E., Cunningham, S., Albert, A., Money, D. M., & O'Doherty, K. C. (2018). Vaginal health and hygiene practices and product use in Canada: a national cross-sectional survey. *BMC Women's Health, 18*(1), 52. doi:10.1186/s12905-018-0543-y

DeSantis, C. E., Siegel, R. L., Sauer, A. G., Miller, K. D., Fedewa, S. A., Alcaraz, K. I., & Jemal, A. (2016). Cancer statistics for African Americans, 2016: Progress and opportunities in reducing racial disparities. *CA: A Cancer Journal for Clinicians, 66*(4), 290-308. doi:10.3322/caac.21340

Fettweis, J. M., Brooks, J. P., Serrano, M. G., Sheth, N. U., Girerd, P. H., Edwards, D. J., . . . Buck, G. A. (2014). Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology, 160*(Pt 10), 2272-2282. doi:10.1099/mic.0.081034-0

Fettweis, J. M., Brooks, J. P., Serrano, M. G., Sheth, N. U., Girerd, P. H., Edwards, D. J., . . . Buck, G. A. (2014). Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology, 160*(Pt 10), 2272-2282. doi:10.1099/mic.0.081034-0

Foraker, R. E., Rose, K. M., Whitsel, E. A., Suchindran, C. M., Wood, J. L., & Rosamond, W. D. (2010). Neighborhood socioeconomic status, Medicaid coverage and medical management of myocardial infarction: atherosclerosis risk in communities (ARIC) community surveillance. *BMC Public Health, 10,* 632. doi:10.1186/1471-2458-10-632

Freedman, M. A. (2002). Quality of life and menopause: the role of estrogen. *J Womens Health (Larchmt), 11*(8), 703-718. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12570037

Furau, G., Dascau, V., Furau, C., Paiusan, L., Radu, A., & Stanescu, C. (2011). Gynecological Cancer Age Groups at the "Dr. Salvator Vuia" Clinical Obstetrics and Gynecology Hospital during the 2000-2009 Period. *Maedica (Buchar), 6*(4), 268-271. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/22879839

Heinemann, C., & Reid, G. (2005). Vaginal microbial diversity among postmenopausal women with and without hormone replacement therapy. *Canadian Journal of Microbiology, 51*(9), 777-781. doi:10.1139/w05-070

Jespers, V., Crucitti, T., Menten, J., Verhelst, R., Mwaura, M., Mandaliya, K., . . . Vaginal Biomarkers Study, G. (2014). Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: a cross-sectional study. *PloS One, 9*(10), e109670. doi:10.1371/journal.pone.0109670

Koumans, E. H., Sternberg, M., Bruce, C., McQuillan, G., Kendrick, J., Sutton, M., & Markowitz, L. E. (2007). The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sexually Transmitted Diseases, 34*(11), 864-869. doi:10.1097/OLQ.0b013e318074e565

Laue, C., Papazova, E., Liesegang, A., Pannenbeckers, A., Arendarski, P., Linnerth, B., . . . Schrezenmeir, J. (2018). Effect of a yoghurt drink containing Lactobacillus strains on bacterial vaginosis in women - a double-blind, randomised, controlled clinical pilot trial. *Benef Microbes, 9*(1), 35-50. doi:10.3920/BM2017.0018

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H., . . . Hilber, A. M. (2011a). Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS Medicine, 8*(2), e1000416. doi:10.1371/journal.pmed.1000416

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H. H. M., . . . Hilber, A. (2011b). Intravaginal practices, bacterial vaginosis, and HIV infection in women: Individual participant data meta-analysis. *PLoS Medicine, 8*(2). doi:10.1371/journal.pmed.1000416

Martin Hilber, A., Hull, T. H., Preston-Whyte, E., Bagnol, B., Smit, J., Wacharasin, C., . . . Group, W. G. S. (2010). A cross cultural study of vaginal practices and sexuality: implications for sexual health. *Social Science and Medicine, 70*(3), 392-400. doi:10.1016/j.socscimed.2009.10.023

Masese, L., McClelland, R. S., Gitau, R., Wanje, G., Shafi, J., Kashonga, F., . . . Kurth, A. (2013). A pilot study of the feasibility of a vaginal washing cessation intervention among Kenyan female sex workers. *Sexually Transmitted Infections, 89*(3), 217-222. doi:10.1136/sextrans-2012-050564

McCarthy, F. N. A., Nii-Trebi, N. I., Musah, B. O., & Asmah, R. H. (2015). Intravaginal practices and lactobacilli colonization among women in Accra, Ghana. *BMC Women's Health*. doi:10.1186/s12905-015-0205-2

Minkin, M. J. (2010). Sexually transmitted infections and the aging female: placing risks in perspective. *Maturitas, 67*(2), 114-116. doi:10.1016/j.maturitas.2010.05.003

Mitra, A., MacIntyre, D. A., Marchesi, J. R., Lee, Y. S., Bennett, P. R., & Kyrgiou, M. (2016). The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? *Microbiome, 4*(1), 58. doi:10.1186/s40168-016-0203-0

Muhleisen, A. L., & Herbst-Kralovetz, M. M. (2016). Menopause and the vaginal microbiome. *Maturitas, 91*, 42-50. doi:10.1016/j.maturitas.2016.05.015

Muzny, C. A., Harbison, H. S., Pembleton, E. S., & Austin, E. L. (2013). Sexual behaviors, perception of sexually transmitted infection risk, and practice of safe sex among southern African American women who have sex with women. *Sexually Transmitted Diseases, 40*(5), 395-400. doi:10.1097/OLQ.0b013e31828caf34

Nelson, T. M., Borgogna, J. C., Michalek, R. D., Roberts, D. W., Rath, J. M., Glover, E. D., . . . Brotman, R. M. (2018). Cigarette smoking is associated with an altered vaginal tract metabolomic profile. *Scientific Reports, 8*(1), 852. doi:10.1038/s41598-017-14943-3

Ness, R. B., Hillier, S. L., Richter, H. E., Soper, D. E., Stamm, C., Bass, D. C., . . . Aral, S. (2003). Why women douche and why they may or may not stop. *Sexually Transmitted Diseases, 30*(1), 71-74. doi:10.1097/00007435-200301000-00014

Ness, R. B., Soper, D. E., Holley, R. L., Peipert, J., Randall, H., Sweet, R. L., . . . Bass, D. C. (2001). Douching and endometritis: Results from the PID evaluation and clinical health (PEACH) study. *Sexually Transmitted Diseases, 28*(4), 240-245. Retrieved from http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L32244957

Petricevic, L., Unger, F. M., Viernstein, H., & Kiss, H. (2008). Randomized, double-blind, placebo-controlled study of oral lactobacilli to improve the vaginal flora of postmenopausal women. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 141*(1), 54-57. doi:10.1016/j.ejogrb.2008.06.003

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., . . . Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America, 108 Suppl 1*, 4680-4687. doi:10.1073/pnas.1002611107

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., . . . Buck, G. A. (2019). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. *Nature Medicine, 25*(6), 1001-1011. doi:10.1038/s41591-019-0465-8

Shen, J., Song, N., Williams, C. J., Brown, C. J., Yan, Z., Xu, C., & Forney, L. J. (2016). Effects of low dose estrogen therapy on the vaginal microbiomes of women with atrophic vaginitis. *Scientific Reports, 6,* 24380. doi:10.1038/srep24380

Si, J., You, H. J., Yu, J., Sung, J., & Ko, G. (2017). Prevotella as a Hub for Vaginal Microbiota under the Influence of Host Genetics and Their Association with Obesity. *Cell Host Microbe, 21*(1), 97-105. doi:10.1016/j.chom.2016.11.010

Sivapalasingam, S., McClelland, R. S., Ravel, J., Ahmed, A., Cleland, C. M., Gajer, P., . . . Kurth, A. E. (2014). An effective intervention to reduce intravaginal practices among HIV-1 uninfected Kenyan women. *AIDS Research and Human Retroviruses, 30*(11), 1046-1054. doi:10.1089/aid.2013.0251

Takahashi, T. A., & Johnson, K. M. (2015). Menopause. *Medical Clinics of North America, 99*(3), 521-534. doi:10.1016/j.mcna.2015.01.006

van de Wijgert, J. H. H. M., Mason, P. R., Gwanzura, L., Mbizvo, M. T., Chirenje, Z. M., Iliff, V., . . . Padian, N. S. (2000). Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. *Journal of Infectious Diseases, 181*(2), 587-594. doi:10.1086/315227

Virtanen, S., Rantsi, T., Virtanen, A., Kervinen, K., Nieminen, P., Kalliala, I., & Salonen, A. (2019). Vaginal Microbiota Composition Correlates Between Pap Smear Microscopy and Next Generation Sequencing and Associates to Socioeconomic Status. *Scientific Reports, 9*(1), 7750. doi:10.1038/s41598-019-44157-8

Article Two: Exploring the Vaginal Microbiome and Intravaginal Practices in Postmenopausal Women

Introduction

The Vaginal Microbiome

The human microbiome is the microbial community composed of trillions of microorganisms that lives in and on the body in a symbiotic relationship with the host (Turnbaugh et al., 2007). Microorganisms that have been identified in the microbiome include archaea, bacteria, and fungi, but for the purpose of the study, the focus is exclusively on bacteria (Blum, 2017). The vaginal microbiome (VM) is specific to the vagina, as the name implies, and promotes homeostasis when dominated by the bacterial genus *Lactobacillus* (Huang, Fettweis, Brooks, Jefferson, & Buck, 2014). The abundance of *Lactobacillus* species (*L*. spp.) is an important component of the VM since *L*. spp. produce lactic acid, which makes the vaginal environment acidic and protects it from infectious, pathogenic microorganisms such as viruses and some other bacteria (Fettweis et al., 2014; Zhou et al., 2007). Additionally, some *L*. spp. are able to inhibit the growth of other bacterial species with their production of hydrogen peroxide and proteins that are toxic to pathogenic bacteria (Fettweis et al., 2014). These characteristics support studies suggesting high abundance of *L*. spp. is associated with reproductive and gynecologic health (Fettweis et al., 2014; Green, Zarek, & Catherino, 2015; Mitra et al., 2016). Compared to other organs in the human body such as the colon, the VM has relatively few bacterial species, and the bacterial species within the VM vary among women. Ravel et al. groups the bacterial communities of maternal-aged pre-menopausal women into categories called community state types (CSTs), which are characterized by the dominant bacterial species in the VM (Ravel et al., 2011).

Community state type I, CST II, CST III, and CST V are dominated by *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, respectively (Ravel et al., 2011). CST IV is a group with lower proportions of *L.* spp. and higher proportions of anaerobic bacteria (Ravel et al., 2011). CST IV was later divided into two groups, CST IV-A (characterized by species of the *Anaerococcus*, *Peptonophilus*, *Prevotella*, and *Streptococcus* genera) and CST IV-B (*Atopobinum* and *Megasphaera* genera), by Gajer et al (Gajer et al., 2012). Brotman et al. found that the association among 87 pre-, peri-, and postmenopausal healthy women (67% White, 27% Black, and 6% other) between CST and menopausal transition status is statistically significant and that postmenopausal women are classified mostly to CST IV-A (Brotman, Shardell, Gajer, Fadrosh, et al., 2014). A decrease in estrogen levels as a result of naturally occurring or surgically induced menopause is associated with unhealthy changes in the VM, making this population important to study (Muhleisen & Herbst-Kralovetz, 2016). In addition, there is emerging evidence that intravaginal practices that women use to cleanse their vagina, reduce odor, or enhance sexual pleasure may be associated with unhealthy changes in the VM, however, to date, this work has only been conducted in premenopausal women.

Intravaginal Practices

Intravaginal practices (IVPs) are defined in this study as the insertion of substances (soaps, deodorizers, powders, and others listed in Figure 1) via finger(s), cloth, or specialized tool(s) into the vagina to cleanse, reduce odor, or enhance sexual pleasure (Alcaide et al., 2015; Alcaide, Rodriguez, Fischl, Jones, & Weiss, 2017; Amaral et al., 2007; Brown et al., 2016; Chisembele, Rodriguez, Brown, Jones, & Alcaide, 2018; Crann, Cunningham, Albert, Money, & O'Doherty, 2018; Masese et al., 2013; McCarthy, Nii-Trebi, Musah, & Asmah, 2015; Ness et al., 2001; Sivapalasingam et al., 2014; van de Wijgert et al., 2000). IVPs are used by nearly 60% of Black women versus approximately 30% of Whites (Brown et al., 2013; Chandra, Martinez, Mosher, Abma, & Jones, 2005; Low et al., 2011b). Misperceptions about cleaning inside the vagina and cultural norms influence IVP use, which are linked to younger ages of sexual debut (Alcaide, Cook, Chisembele, Malupande, & Jones, 2016; Ness et al., 2003). The purpose of IVPs is to cleanse, dry, and/or tighten the vagina for hygienic or sex-related reasons (Esber et al., 2016). However, IVPs are associated with unhealthy changes in the VM (Low et al., 2011b). Vaginal douching with commercial products is a common type of IVP where women irrigate their vagina with products that have been shown to contain bactericidal chemicals that affect *L.* spp. (Aslan & Bechelaghem, 2018). Overall types and frequencies of IVP use vary widely, and given most studies investigating use have had small to moderate sample sizes, statistical power is limited from which to draw conclusions (Lewis et al., 2017). Additionally, no valid, reliable way to measure IVP use is available in the literature.

IVPs are associated with disruptions in the VM, including bacterial vaginosis (BV), and those disruptions increase the risk of sexually transmitted infections (STIs), including human papillomavirus (HPV) and human immunodeficiency virus (HIV) in pre- but not postmenopausal women (Cherpes, Meyn, Krohn, & Hillier, 2003; Gillet et al., 2012; Low et al., 2011b; Short et al., 2010; Tsai, Shepherd, & Vermund, 2009; Viens et al., 2016). While the adverse effects of IVPs are consistent worldwide, the association between the VM and IVPs beyond douching specifically in the US is not well understood, especially in postmenopausal women.

The purpose of this pilot study was 1) to characterize the VMs of postmenopausal women, 2) to identify IVP use, types, and evaluate the frequency of these practices in this cohort of postmenopausal women, and 3) to explore associations among VM communities and IVP use in postmenopausal women.

Methods

Setting and Study Participants

This pilot study is a secondary analysis assessing the control arm of an ongoing parent study (GEMSTONE; PI: Bruner) of the VM in postmenopausal women with gynecological cancer and healthy controls. Postmenopausal women in this study were recruited from hospitals and clinics in metro Atlanta, Georgia. Inclusion criteria for the control group included women who were postmenopausal, 18 years of age or older, willing and able to provide informed consent, and able to read, write, and speak English in order to complete patient reported questionnaires. Exclusion criteria for the control group included the following: history of cancer and co-morbidities that may cause severe vaginal toxicities (cystic fibrosis, poorly controlled diabetes, autoimmune disease, STIs, and current fungal infection). Participants enrolled in the healthy control cohort completed a Demographic Short Form (DSF) questionnaire for sociodemographic data and the Vaginal Cleansing Practices (VCP) questionnaire to assess IVPs. Participants completed the questionnaires in paper and pencil format and also provided physician-collected vaginal swab samples for VM assessment. The data were collected at the baseline study visit after obtaining informed consent.

Vaginal Cleansing Practices (VCP) Questionnaire

Intravaginal practices were obtained from the VCP questionnaire. Since a validated questionnaire was not available in the literature, the VCP questionnaire was developed specifically for this study. Items were derived from a literature review of how women in the United States have reported use of home remedies or products for vaginal hygiene. Items underwent expert review by a nurse scientist with expertise in gynecological cancer research. The VCP questionnaire has four items: one item addresses postmenopausal status (yes/no). Another item addresses whether vaginal cleansing practices changed after menopause (yes/no), since IVPs are associated with menstrual hygiene practices (Mitra et al., 2016). Two questions address the types of products used to clean inside and outside of the vagina (including douching, vinegar/water, soap/water, vaginal deodorants or suppositories, natural herbs, and other product not previously mentioned) and how often (select all that apply) they were used. Thus, IVP use was questioned in the same manner to assess practices before and after menopause. Since the VM changes significantly once women reach menopause, and VM samples were collected only for postmenopausal women, thus this pilot study analyzed practices used after menopause only. The type and frequency of products used were assessed by providing participants with a list of practices with frequency options for each practice. In addition, an “other” category was included so participants could add practices and frequencies not on the instrument list. The “other” response was created to provide options other than douching with commercial products to capture additional information previously not studied in US samples (Brotman, Shardell, Gajer, Tracy, et al., 2014; Fettweis et al., 2014; Zhou et al., 2007). Examples of questions and options regarding IVP use from the VCP questionnaire can be found in Table 1. All survey data were entered into and managed using REDCap database at Emory University (Harris et al., 2009).

Vaginal microbiome samples

Vaginal microbiome samples in the parent study were collected by a physician using a validated and standardized protocol from the Human Microbiome Project as described in Aagaard et al. (2013). The samples were collected during a routine visit to the gynecologist. Vaginal swabs were obtained from the mid vagina using Catch-All™ sample collection swabs (Epicentre Biotechnologies, Madison WI); they were immediately placed in 750 µL of MoBio buffer contained in sterile MoBio Power Bead tubes (Mo Bio Laboratories, Inc., Carlsbad, CA) and stored at −80°C until deoxyribonucleic acid (DNA) extraction. The VM data were obtained from files containing raw sequences of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene from collected vaginal swab samples.

Statistical Analyses

De-identified data for postmenopausal women in the parent study's healthy control group were extracted from REDCap. Women who self-identified as more than one race or as a race other than black or white were categorized as "other." Amazon Web Services was used to build a virtual machine to run Quantitative Insights Into Microbial Ecology (QIIME), through which the raw sequences of the VM samples were analyzed. Through the QIIME™2 (qiime2018.11) pipeline, the classifier was trained on the Greengenes reference taxonomy database with the sequences trimmed to include 250 base pairs. Forty-one genus-level taxonomy was chosen over species-level due to missing assignment of most operational taxonomic units (OTUs) to species. An OTU, or operational taxonomic unit, is a way to classify or categorize closely related units, or in the case of this study, bacteria. The genus-level taxonomy table and the corresponding metadata were imported into RStudio for the analyses (2016). The relative abundance was calculated for the genera.

To characterize the VMs of postmenopausal women, the mean relative abundance of each genus was calculated for each sample. Relative abundance is the count of a specific type of bacterium relative to other bacteria in a sample and is important in assessing the microbiome. Women with a VM having ≥50% *L*. spp. relative abundance were considered to have *L*. spp.-dominant VMs. If another bacterial genus represented ≥50% of the bacteria in the VM, it was recoded accordingly. The six types of IVPs used by this cohort were identified from the VCP questionnaire, from which the frequencies of the identified IVP types were evaluated. Women who reported using any type of IVP use at any frequency after menopause were categorized as current postmenopausal IVP users.

Several measures of alpha diversity (the diversity within a sample) and beta diversity (the diversity between samples) exist to assess the microbiome. Species richness (the count of different bacteria in a sample), Shannon’s diversity index (the count of different bacteria in a sample and how many of each type of different bacteria), Inverse Simpson index (similar to Shannon’s diversity index, but less affected by rare bacteria), and Chao1index (estimates bacterial diversity from abundance and is affected by rare bacteria) are measures of alpha diversity. Measures of beta diversity include Bray-Curtis distance, which is based on the number of each type of bacteria present, and Jaccard distance, which is based on the ratio of the size of the symmetric difference to the union of the groups. While there are multiple ways to measure diversity, each strategy should be employed since each one is measured differently and will most likely not account for every species in a sample. Associations between the VM and IVP use were explored by calculating alpha diversity using species richness, Shannon Index, Inverse Simpson Index, and Chao 1 Index; and beta diversity using Bray-Curtis, and Jaccard distances. For association testing, our primary variable was IVP use (yes or no), which classified the participants by whether they used IVPs. The Wilcoxon rank-sum test was used to compare each univariate measure of the VMs, such as an alpha diversity metric or relative abundance of a specific OTU, between the VMs of women who used IVPs and women who did not. The permutational multivariate analysis of variance (PERMANOVA) was used to associate each beta diversity metric to the IVP use status or the IVP use frequency. The linear decomposition model (LDM) package in RStudio was used to test the global association of the overall microbiome profile and the IVP use status, as well as to detect individual OTUs that contributed to the global association.

Results

Twenty-one women met inclusion criteria for this analysis which included VM data at baseline and completion of the VCP questionnaire. The sociodemographic, *L*. spp.-dominance of the VM samples, and IVP characteristics of the participants collected at one time point (baseline) are described in Table 2. The average age of the women was 64.2 years (range: 55-74 years), and 66.7% of the women self-reported as White, 23.8% as Black, and 9.5% as Other.

The most abundant known bacterial genus found overall in the VM samples was *Lactobacillus* (35.7%). Following in descending order of the most abundant known bacterial genera found were: *Prevotella* (21.4%), *Bifidobacterium* (8.8%), *Gardnerella* (4.7%), *Atopobium* (4.3%), and *Streptococcus* (4.0%) (Figure 2). However, only five VM samples (GEM-B, GEM-C, GEM-I, GEM-K, and GEM-R) possessed a relative abundance of *L*. spp. greater than 75%.

Ten (48%) women reported never using IVPs since menopause, while the remaining 11 (52%) reported using at least one type of IVP since menopause. The most common type of IVP was the use of soap and water to clean inside the vagina, with seven participants reporting it. One participant reported using two types of IVPs (douching and other), but none reported using vinegar and water mixtures or natural herbs. Table 3 describes the frequency of use for each IVP listed in the VCP questionnaire.

Due to a general lack of data beyond species level resulting from the low resolution of 16S rRNA marker gene, only four VM samples were able to be identified according to the Brotman classifications. Two participant VM samples could be classified as CST III (GEM-C and GEM-R) and two other participants could be classified as CST IV-A (GEM-E and GEM-I) (Brotman, Shardell, Gajer, Fadrosh, et al., 2014).

Although *L*. spp. was the most abundant genus among all samples combined, the abundance of *L*. spp. was concentrated within the five (23.8%) VM samples that exhibited *L*. spp.-dominance. The remaining VM samples possessed less than 25% relative abundance of *L*. spp., with 14 of 16 non-*L*. spp.-dominant containing less than 3% relative abundance of *L*. spp. The heatmap in Figure 3 illustrates the relative abundance of each of the top known bacterial genera by self-reported IVP use of each participant, which also emphasizes the large relative abundance of *L*. spp. in the five *L*. spp.-dominant VM samples.

Alpha diversities were not significantly different between IVP users and non-users for Shannon Index (p=0.8094), Inverse Simpson Index (p=0.6539), and Chao1 Index (p=0.8783) using the Wilcoxon rank-sum test as evidenced by the boxplots in Figure 4. Beta diversities were not significant for Bray-Curtis (p=0.8351) (Figure 5) or Jaccard (p=0.1292) (Figure 6) distances revealing that the frequency or presence-absence of which each bacteria genera occur does not account for the variance in the difference we see between those who do and do not use IVPs. Furthermore, the LDM test indicated a lack of global association of the overall microbiome profile and the IVP use status.

Discussion

In this study of postmenopausal women, *L*. spp. was the most dominant bacterial genus present in their VM. This is in contrast to a previous study of 87 women (of which 28 postmenopausal) which used 16S rRNA gene analysis indicating that postmenopausal women are most likely to have vaginal microbiomes Brotman grouped as CST IV-A, characterized by

Anaerococcus, *Peptonophilus*, *Prevotella*, and *Streptococcus* genera (Brotman, Shardell, Gajer, Fadrosh, et al., 2014).

Intravaginal practices were more common (52%) in this cohort than previously identified in studies by Fettweis et al. and Mitra et al. reporting only douching at rates of 11% and 22%, respectively; however, douching by itself in this study fell within the previously identified rates at 14% (Fettweis et al., 2014; Mitra et al., 2016). However, IVPs were less common in other studies reporting IVPs beyond douching (Alcaide et al., 2017; Brown et al., 2016). The use of soap and water for IVPs was the most common IVP in this cohort as seen in previous studies (Low et al., 2011a). Fifty-seven percent of non-White women and 50% of the White women had used IVPs since menopause. While this finding aligned with previous studies where women of color were found to use IVPs more than White women, the magnitude of IVP use was less in our study compared to a study by Chandra and colleagues which reported 59% of non-White women and 27% of White women reported IVP use (Chandra et al., 2005).

After the analyses of the VMs, four women from the study warranted further exploration. The VMs of these four women (GEM-B, GEM-C, GEM-K, and GEM-R) were of particular interest due to their VMs being dominated by *L.* spp. (at least 90% composition) with almost no relative abundance of any other bacteria from the known top twelve genera. The one Black and three White participants ranged in age from 58 to 73 years old. Two of them used IVPs and two of them did not. The two participants who used IVPs reported using soap and water to clean inside the vagina. While species-level data were not available for all the bacteria found in the VMs of the participants in this study, *L. iners* was one of the unique taxa able to be identified, and each bacterium identified as *L. iners* was found in both of the VMs of GEM-C and GEM-R but neither of the other two participants with VMs dominated by at least 90% *L.* spp. The presence of *L. iners* is important since it is considered a transitional species of *Lactobacillus* where some strains are thought to have pathogenic or less protective effects compared to other *L.* spp. (Ferris, Norori, Zozaya-Hinchliffe, & Martin, 2007). This observation suggests that *L. iners* has the ability to dominate the VM, but additional research is needed to identify the effects of *L. iners* in the VMs of postmenopausal women and the function of the different strains of *L. iners*.

Limitations to this pilot study should be noted. The sample size was small and underpowered to draw conclusions; however, the findings can be considered hypothesis generating. Another limitation was that the VCP questionnaire has not been validated and requires additional testing.

In summary, IVPs may be used by women regardless of race and menopausal status. Clinicians should thoroughly assess IVP use to help determine whether the women may be at risk for poor gynecological health outcomes including STIs. Future research should employ a mixed methods research design to understand what IVPs are common in the US, why participants do or do not use IVPs, and which IVPs are associated with dysbiosis in the VM.

Cleaning

- Antiseptics
- Commercial douching products
- Deodorants
- Detergents
- Disinfectants
- Lemons
- Salt
- Vinegar

Lubrication

- Cream
- Saliva
- Petroleum jelly
- Oils/Lotions

Drying

- Herbs
- Flowers
- Leaves
- Powder

Other

- Carbonated beverages
- Homemade formulas
- Sticks/Stones/Rocks

Figure 1. Reports of intervaginal practices from around the globe. (Alcaide et al., 2015; Alcaide et al., 2017; Amaral et al., 2007; Brown et al., 2016; Chisembele et al., 2018; Crann et al., 2018; Masese et al., 2013; McCarthy et al., 2015; Ness et al., 2001; Sivapalasingam et al., 2014; van de Wijgert et al., 2000)

Questions to access intravaginal practices	Have you experienced menopause (either naturally or as a result of surgery or other treatment)?
	Have your common vaginal cleansing practices changed since menopause?
	Since menopause, what has been your common vaginal cleansing practice(s)? Please check all that apply and indicate frequency of use.
Options for products used in vaginal cleansing	Douche
	Natural herbs
	Soap/water
	Vaginal deodorants
	Vinegar/water
	Other product

Table 1. Example questions and options from the Vaginal Cleansing Practices questionnaire

Characteristic	**Total**
Age, mean yrs (sd)	64.2 (5.8)
Race, n (%)	
White	14 (67)
Black	5 (24)
Other (includes more than one race and other	2 (9)
Lactobacillus-dominant, n (%)	
Yes	5 (24)
No	16 (76)
Intravaginal Practice Use, n (%)	
Yes	11 (52)
No	10 (48)

Table 2. Sociodemographic, vaginal microbiome, and intravaginal practice characteristics of postmenopausal participants.

	Frequency		
IVP Types	Never	Occasionally	No Answer
Douching	16	3	2
Vinegar/water	17	-	4
Soap/water	11	7	3
Vaginal deodorants or suppositories	17	1	3
Natural herbs	18	-	3
Other	16	1	4

Table 3. Participant results from the Vaginal Cleansing Practices questionnaire assessing the type and frequency of intravaginal practices (IVPs) use. One participant reported using two IVPs while the other reported only using one.

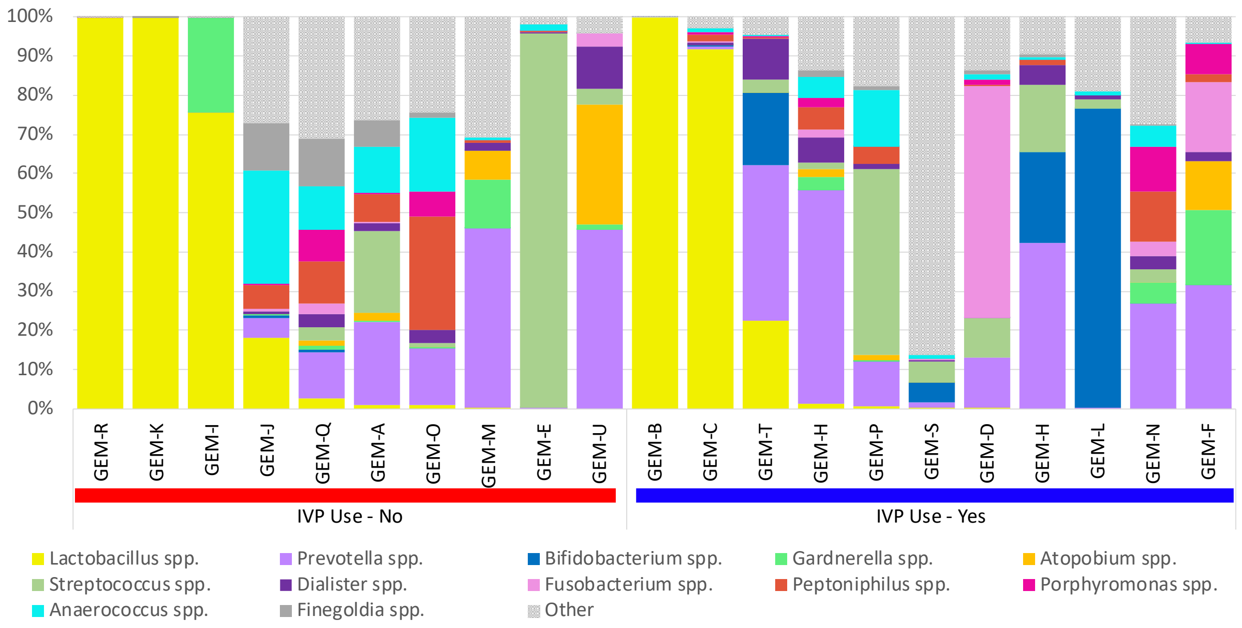

Figure 2. Taxonomy bar plot for relative frequency of vaginal bacteria present for each participant by the relative frequency of *Lactobacillus* species grouped by IVP use.

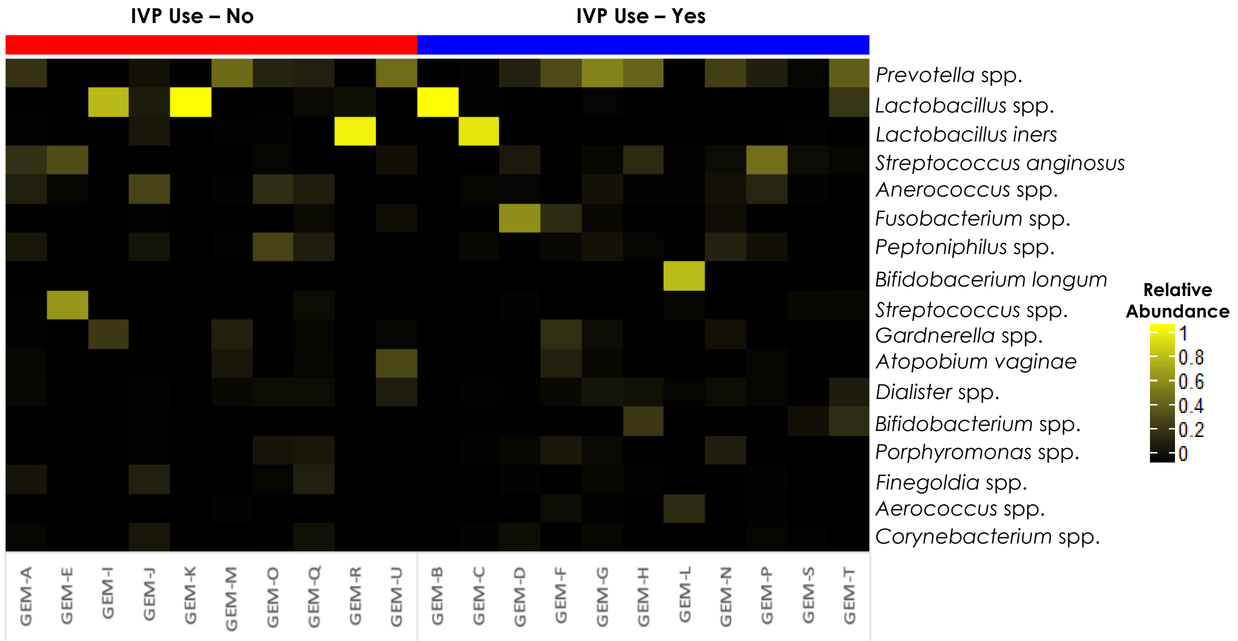

Figure 3. Heatmaps emphasizing the relative abundance of the top twelve known bacterial genera by IVP use.

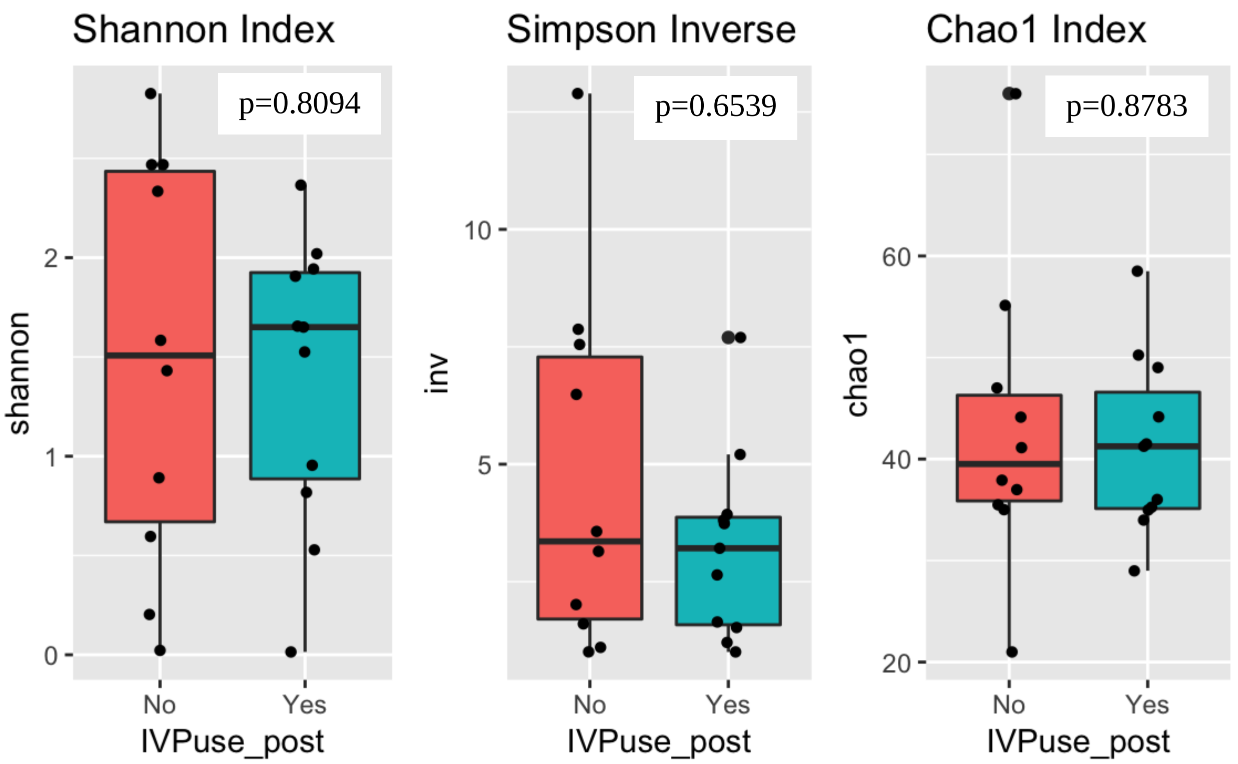

Figure 4. Alpha diversity boxplots for VM samples of women who reported no use IVPs during menopause versus those who did.

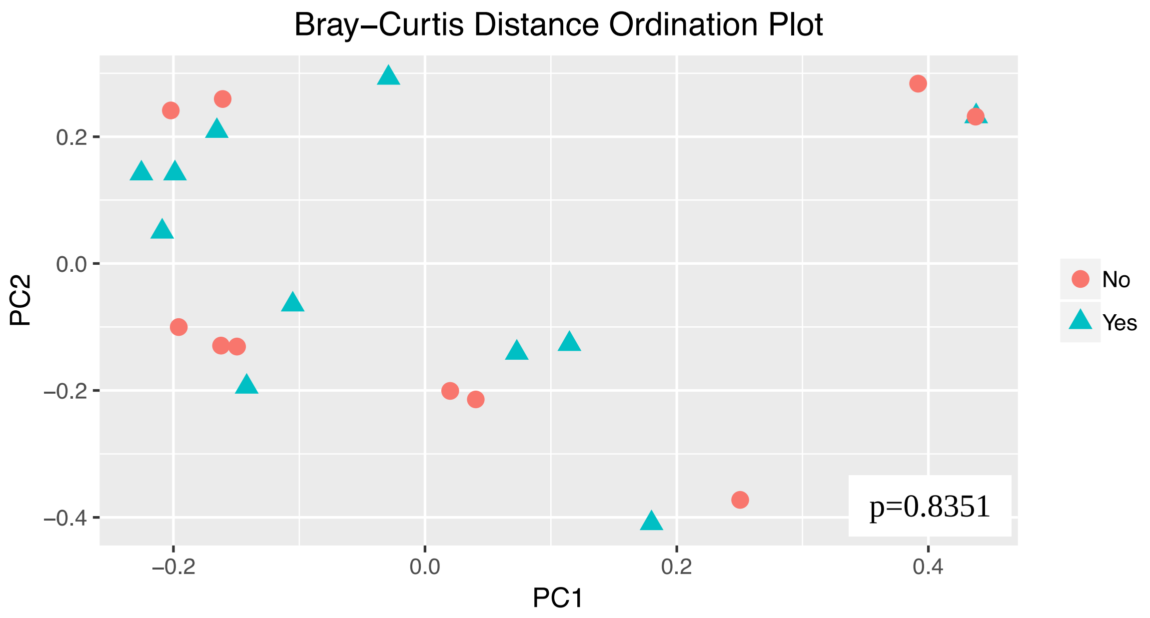

Figure 5. Bray-Curtis Distance Ordination Plot displaying no difference between groups based on intravaginal practices.

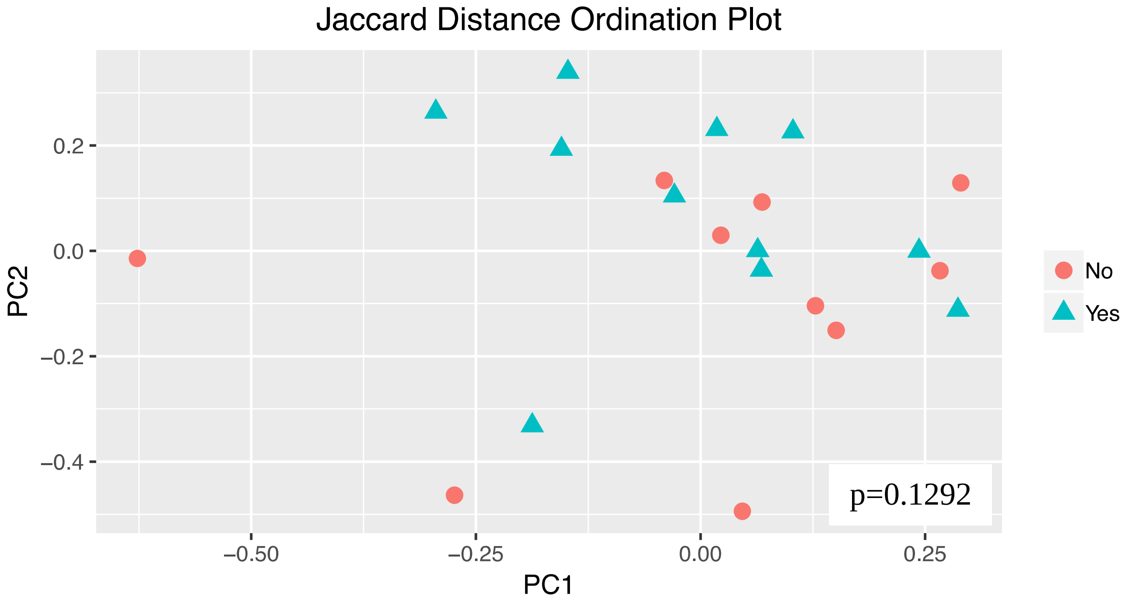

Figure 6. Jaccard Distance Ordination Plot displaying no difference between groups based on intravaginal practices.

References

Aagaard, K., Petrosino, J., Keitel, W., Watson, M., Katancik, J., Garcia, N., . . . Versalovic, J. (2013). The Human Microbiome Project strategy for comprehensive sampling of the human microbiome and why it matters. *FASEB Journal, 27*(3), 1012-1022. doi:10.1096/fj.12-220806

Alcaide, M. L., Chisembele, M., Malupande, E., Arheart, K., Fischl, M., & Jones, D. L. (2015). A cross-sectional study of bacterial vaginosis, intravaginal practices and HIV genital shedding; implications for HIV transmission and women's health. *BMJ Open, 5*(11), e009036. doi:10.1136/bmjopen-2015-009036

Alcaide, M. L., Cook, R., Chisembele, M., Malupande, E., & Jones, D. L. (2016). Determinants of intravaginal practices among HIV-infected women in Zambia using conjoint analysis. *International Journal of STD and AIDS, 27*(6), 453-461. doi:10.1177/0956462415585447

Alcaide, M. L., Rodriguez, V. J., Fischl, M. A., Jones, D. L., & Weiss, S. M. (2017). Addressing intravaginal practices in women with HIV and at-risk for HIV infection, a mixed methods pilot study. *Int J Womens Health, 9*, 123-132. doi:10.2147/IJWH.S125883

Amaral, R., Giraldo, P. C., Gonçalves, A. K., Junior, J. E., Santos-Pereira, S., Linhares, I., & Passos, M. R. L. (2007). Evaluation of hygienic douching on the vaginal microflora of female sex workers. *International Journal of STD and AIDS, 18*(11), 770-773. doi:10.1258/095646207782212333

Aslan, E., & Bechelaghem, N. (2018). To 'douche' or not to 'douche': hygiene habits may have detrimental effects on vaginal microbiota. *Journal of Obstetrics and Gynaecology, 38*(5), 678-681. doi:10.1080/01443615.2017.1395398

Blum, H. E. (2017). The human microbiome. *Advances in Medical Sciences, 62*(2), 414-420. doi:10.1016/j.advms.2017.04.005

Brotman, R. M., Shardell, M. D., Gajer, P., Fadrosh, D., Chang, K., Silver, M. I., . . . Gravitt, P. E. (2014). Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause, 21*(5), 450-458. doi:10.1097/GME.0b013e3182a4690b

Brotman, R. M., Shardell, M. D., Gajer, P., Tracy, J. K., Zenilman, J. M., Ravel, J., & Gravitt, P. E. (2014). Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection. *Journal of Infectious Diseases, 210*(11), 1723-1733. doi:10.1093/infdis/jiu330

Brown, J. M., Hess, K. L., Brown, S., Murphy, C., Waldman, A. L., & Hezareh, M. (2013). Intravaginal practices and risk of bacterial vaginosis and candidiasis infection among a cohort of women in the United States. *Obstetrics and Gynecology, 121*(4), 773-780. doi:10.1097/AOG.0b013e31828786f8

Brown, J. M., Poirot, E., Hess, K. L., Brown, S., Vertucci, M., & Hezareh, M. (2016). Motivations for Intravaginal Product Use among a Cohort of Women in Los Angeles. *PloS One, 11*(3), e0151378. doi:10.1371/journal.pone.0151378

Chandra, A., Martinez, G. M., Mosher, W. D., Abma, J. C., & Jones, J. (2005). Fertility, family planning, and reproductive health of U.S. women: data from the 2002 National Survey of Family Growth. *Vital Health Stat 23*(25), 1-160. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/16532609

Cherpes, T. L., Meyn, L. A., Krohn, M. A., & Hillier, S. L. (2003). Risk factors for infection with herpes simplex virus type 2: Role of smoking, douching, uncircumcised males, and vaginal flora. *Sexually Transmitted Diseases, 30*(5), 405-410. doi:10.1097/00007435-200305000-00006

Chisembele, M., Rodriguez, V. J., Brown, M. R., Jones, D. L., & Alcaide, M. L. (2018). Intravaginal practices among young HIV-infected women in Lusaka, Zambia. *International Journal of STD and AIDS, 29*(2), 164-171. doi:10.1177/0956462417721438

Crann, S. E., Cunningham, S., Albert, A., Money, D. M., & O'Doherty, K. C. (2018). Vaginal health and hygiene practices and product use in Canada: a national cross-sectional survey. *BMC Women's Health, 18*(1), 52. doi:10.1186/s12905-018-0543-y

Esber, A., Rao, N., Norris, A., Carr Reese, P., Kandodo, J., Nampandeni, P., . . . Norris Turner, A. (2016). Intravaginal Practices and Prevalence of Sexual and Reproductive Tract Infections Among Women in Rural Malawi. *Sexually Transmitted Diseases, 43*(12), 750-755. doi:10.1097/OLQ.0000000000000531

Ferris, M. J., Norori, J., Zozaya-Hinchliffe, M., & Martin, D. H. (2007). Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. *Journal of Clinical Microbiology, 45*(3), 1016-1018. doi:10.1128/JCM.02085-06

Fettweis, J. M., Brooks, J. P., Serrano, M. G., Sheth, N. U., Girerd, P. H., Edwards, D. J., . . . Buck, G. A. (2014). Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology, 160*(Pt 10), 2272-2282. doi:10.1099/mic.0.081034-0

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., . . . Ravel, J. (2012). Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine, 4*(132), 132ra152. doi:10.1126/scitranslmed.3003605

Gillet, E., Meys, J. F., Verstraelen, H., Verhelst, R., De Sutter, P., Temmerman, M., & Vanden Broeck, D. (2012). Association between bacterial vaginosis and cervical intraepithelial neoplasia: systematic review and meta-analysis. *PloS One, 7*(10), e45201. doi:10.1371/journal.pone.0045201

Green, K. A., Zarek, S. M., & Catherino, W. H. (2015). Gynecologic health and disease in relation to the microbiome of the female reproductive tract. *Fertility and Sterility, 104*(6), 1351-1357. doi:10.1016/j.fertnstert.2015.10.010

Harris, P. A., Taylor, R., Thielke, R., Payne, J., Gonzalez, N., & Conde, J. G. (2009). Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *Journal of biomedical informatics*, *42*(2), 377-381.

Huang, B., Fettweis, J. M., Brooks, J. P., Jefferson, K. K., & Buck, G. A. (2014). The changing landscape of the vaginal microbiome. *Clinics in Laboratory Medicine, 34*(4), 747-761. doi:10.1016/j.cll.2014.08.006

Lewis, B. A., Billing, L., Schuver, K., Gjerdingen, D., Avery, M., & Marcus, B. H. (2017). The relationship between employment status and depression symptomatology among women at risk for postpartum depression. *Womens Health (Lond), 13*(1), 3-9. doi:10.1177/1745505717708475

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H., . . . Hilber, A. M. (2011a). Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS Medicine, 8*(2), e1000416. doi:10.1371/journal.pmed.1000416

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H. H. M., . . . Hilber, A. (2011b). Intravaginal practices, bacterial vaginosis, and HIV infection in women: Individual participant data meta-analysis. *PLoS Medicine, 8*(2). doi:10.1371/journal.pmed.1000416

Masese, L., McClelland, R. S., Gitau, R., Wanje, G., Shafi, J., Kashonga, F., . . . Kurth, A. (2013). A pilot study of the feasibility of a vaginal washing cessation intervention among Kenyan female sex workers. *Sexually Transmitted Infections, 89*(3), 217-222. doi:10.1136/sextrans-2012-050564

McCarthy, F. N. A., Nii-Trebi, N. I., Musah, B. O., & Asmah, R. H. (2015). Intravaginal practices and lactobacilli colonization among women in Accra, Ghana. *BMC Women's Health*. doi:10.1186/s12905-015-0205-2

Mitra, A., MacIntyre, D. A., Marchesi, J. R., Lee, Y. S., Bennett, P. R., & Kyrgiou, M. (2016). The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? *Microbiome, 4*(1), 58. doi:10.1186/s40168-016-0203-0

Muhleisen, A. L., & Herbst-Kralovetz, M. M. (2016). Menopause and the vaginal microbiome. *Maturitas, 91*, 42-50. doi:10.1016/j.maturitas.2016.05.015

Ness, R. B., Hillier, S. L., Richter, H. E., Soper, D. E., Stamm, C., Bass, D. C., . . . Aral, S. (2003). Why women douche and why they may or may not stop. *Sexually Transmitted Diseases, 30*(1), 71-74. doi:10.1097/00007435-200301000-00014

Ness, R. B., Soper, D. E., Holley, R. L., Peipert, J., Randall, H., Sweet, R. L., . . . Bass, D. C. (2001). Douching and endometritis: Results from the PID evaluation and clinical health (PEACH) study. *Sexually Transmitted Diseases, 28*(4), 240-245. Retrieved from http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L32244957

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., . . . Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America, 108 Suppl 1*, 4680-4687. doi:10.1073/pnas.1002611107

Short, V. L., Totten, P. A., Ness, R. B., Astete, S. G., Kelsey, S. F., Murray, P., & Haggerty, C. L. (2010). The demographic, sexual health and behavioural correlates of Mycoplasma genitalium infection among women with clinically suspected pelvic inflammatory disease. *Sexually Transmitted Infections, 86*(1), 29-31. doi:10.1136/sti.2009.037721

Sivapalasingam, S., McClelland, R. S., Ravel, J., Ahmed, A., Cleland, C. M., Gajer, P., . . . Kurth, A. E. (2014). An effective intervention to reduce intravaginal practices among HIV-1 uninfected Kenyan women. *AIDS Research and Human Retroviruses, 30*(11), 1046-1054. doi:10.1089/aid.2013.0251

Tsai, C. S., Shepherd, B. E., & Vermund, S. H. (2009). Does douching increase risk for sexually transmitted infections? A prospective study in high-risk adolescents. *American Journal of Obstetrics and Gynecology, 200*(1), 38 e31-38. doi:10.1016/j.ajog.2008.06.026

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. *Nature, 449*(7164), 804-810. doi:10.1038/nature06244

van de Wijgert, J. H. H. M., Mason, P. R., Gwanzura, L., Mbizvo, M. T., Chirenje, Z. M., Iliff, V., . . . Padian, N. S. (2000). Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. *Journal of Infectious Diseases, 181*(2), 587-594. doi:10.1086/315227

Viens, L. J., Henley, S. J., Watson, M., Markowitz, L. E., Thomas, C. C., Thompson, T. D., . . . Saraiya, M. (2016). Human Papillomavirus-Associated Cancers - United States, 2008-2012. *MMWR: Morbidity and Mortality Weekly Report, 65*(26), 661-666. doi:10.15585/mmwr.mm6526a1

Zhou, X., Brown, C. J., Abdo, Z., Davis, C. C., Hansmann, M. A., Joyce, P., . . . Forney, L. J. (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J, 1*(2), 121-133. doi:10.1038/ismej.2007.12

Article Three: The Vaginal Microbiome in Postmenopausal Women: Associations with Health Behaviors, Sociodemographic Factors, and Vaginal Symptoms

Introduction

The Vaginal Microbiome

The healthy vaginal microbiome (VM) is a symbiotic community of microorganisms (bacteria, archaea, fungi, etc.) that is specific to the vagina. Gynecological health is directly associated with the types (composition) and amounts (abundance) of bacteria in the VM. Unlike the well-known gut microbiome, low bacterial diversity in the VM is conducive to a healthy environment. Particular *Lactobacillus* species (*L.* spp) are associated with low bacterial diversity due to their ability to produce lactic acid to reduce or eliminate the presence of pathogenic, infectious microorganisms (Mitra et al., 2016; Reid, 2016; Stapleton, 2016). Certain species of *Lactobacillus* are considered protective and, when dominant, are used in most of the literature as synonymous with a healthy VM. These *L.* spp. inhibit the growth of other bacterial species with their production of hydrogen peroxide and toxic proteins (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, The Vaginal Microbiome, et al., 2014).

Microbial imbalance, or dysbiosis, of the VM is characterized as decreased amounts of *L.* sp. and increased amounts of pathogenic bacteria like *Gardnerella* and *Prevotella* (Huang, Fettweis, Brooks, Jefferson, & Buck, 2014). Dysbiosis may lead to increased susceptibility to infections. For example, *Gardnerella* and *Prevotella* are associated with bacterial vaginosis (BV), which is a type of dysbiosis that is the most common vaginal infection (Muzny & Schwebke, 2016). Many factors can contribute to dysbiosis including hormonal status, sociodemographic factors and individual behaviors. Dysbiosis can also occur as a result of menopause (Lee et al., 2013). Postmenopausal women are more likely to have VMs that are classified into a group characterized by species of the *Anaerococcus*, *Peptonophilus*, *Prevotella*, and *Streptococcus* genera (Brotman, Shardell, et al., 2014). Few studies have explained the composition of the VM in postmenopausal women or explored the impact that health behaviors and sociodemographic factors have on the VM in this specific population. Furthermore, it is unclear whether the composition of the VM is associated with vaginal symptoms such as dryness, discharge, and itching. The bacterial composition of the VM is dynamic over a woman's lifespan with short-term fluctuations occurring in response to hormonal changes; however, various health behaviors related to socially-, culturally-, and environmentally-influenced decisions can also contribute to changes in the VM (Hickey, Zhou, Pierson, Ravel, & Forney, 2012).

Health Behaviors

Individuals choose to participate in activities that impact their health with positive and negative outcomes. Intravaginal practices (IVPs) are the methods women use to clean inside their vaginas for hygienic and sexual purposes although negative health outcomes may result from this behavior. (Esber et al., 2016; Hilber, Chersich, van de Wijgert, Rees, & Temmerman, 2007; Low et al., 2011b). Women insert many different types of homemade, household, natural, or commercial products into their vaginas with cloth, specialized tools, and their fingers to maintain cleanliness or to increase sexual desire and pleasure for their partners or themselves (Esber et al., 2016; Low et al., 2011b). Douching means to irrigate the vagina with a liquid, and some IVPs can be classified as such. However, women may not consider or admit their IVPs as douching with non-commercial products or products not specifically labeled for douching. Additionally, some women use non-liquid products or leave products inside the vagina for an extended period of time and are practices not defined as douching. IVP use not classified as douching, such as the use of leaves, stones, and homemade or “traditional” products, has been associated with dysbiosis in the VM of women in countries outside of the United States, but few studies have characterized the VM of postmenopausal women, and none have focused on postmenopausal women using IVPs (Low et al., 2011a).

Recreational health behaviors have been associated with changes in the VM. While not well understood, women who consume alcohol have been found to be *less* likely to have pathogenic bacteria in the VM than women who do not consume alcohol (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, The Vaginal Microbiome, et al., 2014). However, increased or perhaps excessive alcohol consumption has also been deemed an independent risk factor for BV (Baisley et al., 2009). Women who smoke tobacco are consistently more likely to have pathogenic bacteria linked to BV in their VM than women who do not smoke tobacco (Bagaitkar, Demuth, & Scott, 2008; Brotman, He, et al., 2014). One study concludes that increased pathogenic bacteria in the VM is associated with increased metabolites related to nicotine in smokers, as well as increased biogenic amines in smokers that are known to contribute to vaginal malodor and to impact the virulence capabilities of pathogens (Nelson et al., 2018). Currently it is unclear whether alcohol and tobacco use are associated with IVP use and exacerbate vaginal dysbiosis.

Sexual activity is an important behavior for evaluating potential causes of vaginal dysbiosis. Frequent sexual (vaginal) intercourse and multiple, new or an increased number of male partners are associated with vaginal dysbiosis (Cherpes, Hillier, Meyn, Busch, & Krohn, 2008; Fethers, Fairley, Hocking, Gurrin, & Bradshaw, 2008; Jespers et al., 2015). Also, the VMs of women who have sex (anal, vaginal, and/or oral) with women may be more likely to have pathogenic bacteria that is associated with dysbiosis, whereas the VMs of women who have sex with men have higher bacterial diversity (Muzny, Harbison, Pembleton, & Austin, 2013). Additionally, a study evaluating the effects of over-the-counter feminine hygiene products found that vaginal lubricant related to sexual activity can be bactericidal (Fashemi, Delaney, Onderdonk, & Fichorova, 2013).

Recreational behaviors are not the only types of behaviors that affect the VM. Different types of medications may be related to changes in the VM as well. For instance, alterations in the VM occur as a result of antibiotic use (Ferrer, Mendez-Garcia, Rojo, Barbas, & Moya, 2017). Antibiotics can clear bacteria in the VM, and pathogenic bacteria have been shown to quickly reemerge, potentially causing dysbiosis (Mayer et al., 2015). Conversely, postmenopausal women who used various forms of prescribed hormone replacement therapy (HRT) and topical estrogen possessed vaginal microbial communities dominated by *L*. spp. (Gliniewicz et al., 2019). While more research is needed, there may be over-the-counter products that benefit the VM. One randomized control trial found that postmenopausal women who received oral probiotic strains of *L*. spp. reported statistically significant improvements in vaginal symptoms (Petricevic, Unger, Viernstein, & Kiss, 2008). Vaginal dysbiosis and symptoms have been shown to decrease with oral consumption of yogurt containing *L*. spp. strains (Laue et al., 2018).

Health behaviors themselves may be influenced by sociodemographic factors which may in turn have a direct effect on the VM, as explored below.

Sociodemographic Factors

Sociodemographic factors (SDFs) are the social, economic, and physical conditions of a person's environment that have the potential to interact with and influence individual health behaviors and contribute to health outcomes. SDF variables that have been suggested to be related to the VM include age, education, health insurance, and race. These variables may impact how an individual’s social environment influences their behavior, the quality of healthcare they receive, and the type of care needed for optimal health.

Age is a biological SDF that is related to menopausal status, which affects the vaginal microbial community (Muhleisen & Herbst-Kralovetz, 2016; Office of Disease Prevention and Health Promotion, 2018). Age may be also associated with the use of IVPs. In a prospective observational study of 1,199 women in the US age range 13 to 36, 75% African American, women who used IVPs tended to be older with 59% of the ≥30 age range cohort using IVPs vs only 31% in the 13-18 younger age cohort (Ness et al., 2005). Level of education is another SDF that may play a role in IVP use and vaginal dysbiosis. Lower educational attainment has been associated with a higher prevalence of douching and attitudes more likely to perceive IVPs as positive or healthy behaviors (Chandra, Martinez, Mosher, Abma, & Jones, 2005; Ness et al., 2003); however, as of yet, no studies have documented associations between education and the VM. Health insurance, another SDF, serves as a proxy for access to care and household income. Access to care has yet to be studied in association with vaginal microbial communities or IVPs. With the exception of White women, lower household income is associated with greater IVP use, but little is known about its impact on the VM (Brotman et al., 2008; Chandra et al., 2005; Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, The Vaginal Microbiome, et al., 2014; Ness et al., 2005).

Race is the SDF that has received the most attention in studies of the VM. Race can serve as a weak proxy for social norms and attitudes experienced by people of color since multiple ethnicities and enormous cultural diversity exists within a racial category. Studies have consistently demonstrated that IVPs are more often used by Black women compared to White women (Chandra et al., 2005; Ness et al., 2005). Studies also reveal a difference between the vaginal microbial communities of mostly premenopausal Black and White women with Black women's vaginal communities having significantly fewer *L.* spp. and more pathogenic bacteria in their VM than White women (Fettweis et al., 2014; Ravel et al., 2011). Aligning with these differences, Black women in the United States also have higher rates of HPV, HIV, BV, and cervical cancer than White women (Center for Disease Control and Prevention, 2018; DeSantis et al., 2016; Fettweis et al., 2014; Viens et al., 2016). Since race is not biological, we posit that other factors like health-related behaviors are the contributors to the disparities as opposed to one's race.

Vaginal Symptoms

Hormonal changes from menopause are also associated with disruptions in the VM and symptoms (Lee et al., 2013; Muhleisen & Herbst-Kralovetz, 2016). The menopausal-related decrease in *L.* spp. and other healthy vaginal microbiota are associated with vulvovaginal atrophy (also known as atrophic vaginitis). Vulvovaginal atrophy is characterized by uncomfortable, sometimes painful symptoms including dryness, discharge and itching (Brotman, Shardell, et al., 2014; Shen et al., 2016). Vaginal symptoms, including discharge, dryness, and itching, related to menopause significantly affect a woman's quality of life (Hummelen et al., 2011). Additionally, 25-50% of postmenopausal women experience vaginal symptoms such as dryness, but the degree to which these symptoms are associated with specific bacteria in the VM is understudied (Sturdee, Panay, & International Menopause Society Writing, 2010). A study of menopausal women concluded that increased vaginal symptom severity was associated with increased microbial diversity in the VM, but many health behaviors with the potential to influence the VM were exclusionary criteria, so the effects of health behaviors on the VM were not able to be explored (Hummelen et al., 2011). Studies are needed to assess whether SDFs and current health behaviors contribute to dysbiosis and presence of vaginal symptoms of postmenopausal women.

Therefore, the purpose of this pilot study was to evaluate the relationships among the VM, health behaviors, SDFs, and vaginal symptoms in postmenopausal women by assessing 1) whether the vaginal microbial community is associated with health behaviors, 2) whether the vaginal microbial community is associated with SDFs, 3) whether the vaginal microbial community is associated with vaginal symptoms, and 4) whether health-promoting and health-disruptive behaviors are associated with *L*. spp. dominance.

Methods

Setting and Study Participants

This secondary analysis of an ongoing primary study assessing the VM in healthy vs women treated for gynecologic cancers, GEMSTONE, includes post-menopausal women from hospitals and clinics in metro Atlanta, Georgia. Twenty-one participants enrolled in the healthy control cohort of the primary study who were eligible for this study. Eligibility included completion of the Demographic Short Form questionnaire [DSF], Female Sexual Function Index questionnaire [FSFI], Pre-Exam questionnaire [PE-Q], and Vaginal Cleansing Practices questionnaire [VCP], and provision of a vaginal swab sample for vaginal microbiome assessment. The data were collected at the baseline study visit.

Questionnaires: All questionnaires from the primary study are given in pencil-and-paper format at the study visit to assess self-reported health behaviors, SDFs, and vaginal symptoms. The VCP assessed self-reported IVPs as described elsewhere (Daniel et al., 2019). Alcohol consumption and tobacco smoking information were obtained from the DSF. Alcohol was categorized by the following levels: <1 drink/week, 1-4 drinks/week, and 5-14 drinks/week. Tobacco smoking was defined as smoking at least 100 cigarettes, cigars, or e-cigarettes ever, and the options available from which the participant was able to choose were no; yes, but quit; and yes, currently smoke. Sexual intercourse defined as penile penetration (entry) of the vaginal was the health behavior obtained from the FSFI. The FSFI is reliable and psychometrically and clinically valid (Rosen et al., 2000). This questionnaire obtained participant engagement in sexual intercourse over the past four weeks. The FSFI asks questions in the context of sexual activities (Rosen et al., 2000). The participant is given an option to choose an answer from a Likert-type scale that best describes their situation or indicates that they have had no sexual activity over the last four weeks. The PE-Q is a questionnaire designed by the GEMSTONE study team to assess use of medical and dietary products that may affect the VM and symptoms experienced by the participant related to the VM. Additional health behaviors obtained from the PE-Q included the following: antibiotic(s) use during the last four weeks (yes/no), current or past HRT use (yes/no), probiotic(s) use over the last four weeks (yes/no), topical estrogen use over the last four weeks (yes/no), vaginal lubricant(s) use over the last four weeks (yes/no), and regular oral consumption of (non-frozen) yogurt (yes/no).

Sociodemographic characteristics such as age, education, healthcare insurance type, and race were obtained from the DSF. Vaginal symptoms were assessed with the PE-Q. Vaginal discharge, dryness, and itching were specific vaginal symptoms assessed. The participants who answered the questionnaires also provided biological samples from their VMs.

Vaginal microbiome samples

Vaginal microbiome samples were obtained from physician-collected swabs using Aagaard et al.’s validated and standardized protocol from the Human Microbiome Project (2013). Files containing raw sequences of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene from collected vaginal swab samples were used to assess the vaginal microbiome. The samples were collected during a routine visit to the gynecologist; collection procedures are described elsewhere (Article #2).

Statistical Analyses

De-identified data from the questionnaires for the healthy control group from the primary study of postmenopausal women were extracted from REDCap. Due to the small sample size and low number of responses across response options, several items were collapsed or dichotomized. As previously reported, data for *L*. spp. dominance was recoded “yes” if the relative abundance of *L*. spp. was ≥50% or “no” if it was <50%; IVPs were recoded as “yes” or “no” based on the participants’ report of any IVP use during menopause; and race was recoded as “other” for the participants who self-reported as Asian and two races (Black and Asian). (Daniel et al., 2019). Additionally, alcohol, tobacco, and sexual intercourse were recoded. Alcohol use for participants who consumed <1 drink/week were recoded as “no” for alcohol use, and alcohol use for participants who consumed ≥1 drink/week were recoded as “yes” for alcohol use. Tobacco smoking for participants who answered “no” or “yes, but quit” were recoded as “no” for tobacco smoking, and tobacco smoking for participants who answered “yes, currently smoke” were recoded as “yes” for tobacco smoking. Sexual intercourse for participants who answered “No sexual activity” was recoded as “no” for sexual intercourse, and the remaining answers were recoded as “yes” for sexual intercourse.

The QIIME™2 (qiime2018.11) pipeline was employed through Amazon Web Services to analyze the raw sequences of the VM samples. Missing assignment of most operational taxonomic units (OTUs), or bacterial classifications for identification, to species dictated the decision to choose genus-level taxonomy. That taxonomy table was imported into RStudio, along with the corresponding metadata, for analyses, and relative abundance was calculated for the genera.

Alpha diversity, diversity within a sample, of the VM samples was calculated using Shannon Index, Inverse Simpson Index, and Chao 1 Index to assess whether differences within the VM sample were related health behaviors, SDFs, and vaginal symptoms. Many metrics are used to assess diversity since each one measures alpha diversity differently. The Wilcoxon rank sum test was used to compare alpha diversity between each health behavior (coded as “yes” or “no”) used by postmenopausal women. Similarly, this test was used to assess SDFs and vaginal symptoms where appropriate. The Kruskal-Wallis rank sum test was used to compare alpha diversity among ages, races, insurances, and vaginal symptom types. The LDM package in RStudio was used to assess whether health behaviors, SDFs and vaginal symptoms were significantly associated with the VM.

Results

Twenty-one participants from the primary study met inclusion criteria. Participants racially self-identified as White (n = 14; 67%), Black (n = 5; 24%), and Asian (n = 2; 9%). Age ranged from 55 to 74 years, with a mean age of 64.2 years (SD = 5.8). More than half of the participants used IVPs and experienced vaginal symptoms. Table 1 displays the sample demographic characteristics, IVP use, and symptoms.

The vaginal microbiome genus, abundance, and diversity has been reported elsewhere (Daniel et al., 2019). Of all the study participants, only four (GEM-B, GEM-C, GEM-K, and GEM-R) displayed VMs that were *L.* spp.-dominant as displayed in the heatmap (Figure 1). The VM of those four participants also displayed no other genera in the top ten known bacteria. Three of the four self-identified as White (21% of all Whites in the study), and one as Black

(20% of all Blacks). This similarity in racial percentages further removes race as a factor for the differences in relative abundance of *L*. spp. in the VM for this study. Their ages ranged from 58 to 73 years old, with the oldest and youngest having the highest relative abundance of *L*. spp., so age also did not appear to be a contributing factor in the relative abundance of *L*. spp.

The p-values for all alpha diversities using the Wilcoxon or Kruskal Wallis rank sum tests are listed Table 1. Alpha diversity assessed using the Wilcoxon rank sum test was significant for only the Inverse Simpson Index of antibiotic use (p=0.046). The boxplot in Figure 2 depicts this significant difference in alpha diversity for antibiotic use. Although alpha diversity was significantly associated with antibiotic use, the LDM test indicated a lack of global association of the overall microbiome profile and antibiotic use (p=0.66).

Discussion

This study aimed to enhance the understanding of the relationship between the VM and health behaviors, SDFs, and vaginal symptoms in postmenopausal women. Age and level of education while not significantly associated with the VM in this study may be important for future exploration. As women reach menopause, estrogen levels decrease, which may decreased diversity in the VM, possibly leading to increased susceptibility to vaginal symptoms, bacterial vaginosis, and sexually transmitted infections. Level of education may be a confounder for increased diversity in the VM since education level is associated with the use of IVPs, and IVPs are associated with increased diversity in the VM. Additional research is needed to further explore the association between the VM and SDFs with a larger sample size since women who have no insurance or are underinsured may be more likely to self-treat vaginal symptoms due to lack of access to care.

While no association between the VM and race was found in this study, previous studies reveal a difference between the vaginal microbial communities of Black and White women with Black women's vaginal communities having significantly fewer *L.* spp. and more pathogenic bacteria in their VM than White women (Fettweis, Brooks, Serrano, Sheth, Girerd, Edwards, Strauss, The Vaginal Microbiome, et al., 2014; Ravel et al., 2011). The discordance between this sample and others may be due to small sample size, or as described by Ravel, "human habits and practices" that strongly influence the association between the VM and race, which were assessed differently from previous studies (Ravel et al., 2011).

A few noteworthy health behaviors that bear further study were observed. Three of the four women with the *L.* spp.-dominant VMs had used topical estrogen. This could have contributed to the high relative abundance of *L.* spp. as estrogen is related to the abundance of *L.* spp. in the VM (Muhleisen & Herbst-Kralovetz, 2016). Two other women without *L.* spp.-dominant VMs also used topical estrogen. However, one was underweight based on her BMI (17.7kg/m^2) and the other reported using corticosteroids, both of which have been found to be associated with decreased estrogen levels, which is associated with decreased *L.* spp. (Gong et al., 2008; Muhleisen & Herbst-Kralovetz, 2016; Ziomkiewicz, Ellison, Lipson, Thune, & Jasienska, 2008).

Another health behavior explored was the use of probiotics and the consumption of yogurt. Three of the four women with *L.* spp.-dominant VMs took probiotics, and a different group of three women with *L.* spp.-dominant VMs consumed yogurt on a regular basis. The content of these products and the specific regimen in which they were consumed is unknown but could be related to the relative abundance of *L.* spp. or could support the maintenance of a *L.* spp.-dominant VM as evidenced in prior studies (Barrons & Tassone, 2008; Laue et al., 2018).

This study suffered from a number of limitations. Only 21 participants were available for analysis in this pilot study and therefore the study was underpowered to draw definitive conclusions, hence the data is considered hypothesis generating. Future research with larger samples should further investigate the associations between the VM and SDFs and health behaviors described in this pilot study and how the influence vaginal microbial diversity and risk of disease and/or vaginal symptoms. Another limitation in this study was the inability to access frequency of sexual activity and number of sexual partners as health behaviors since these were not collected in the primary study. Sexual activity should be described and evaluated in future studies, as it is associated with increased diversity from semen and vaginal fluid exchange during sex, as well as multiple sex partners. Sexual activity is also important because many women use IVPs before sex to prepare for the activity and after sex for hygienic purposes (Alcaide, Rodriguez, Fischl, Jones, & Weiss, 2017).

In summary, health-related behaviors may influence a woman's ability to acquire and/or maintain a potentially healthy *L.* spp.-dominant VM. Sociodemographic factors should not be ignored, and understanding and focusing on behaviors that affect the VM will help in the development of interventions that modify behaviors to support a *L.* spp.-dominant VM.

	Characteristic	Frequency, n (%)	Shannon Index (p-value)	Inverse Simpson Index (p-value)	Chao1 Index (p-value)
Vaginal Microbiome	*Lactobacillus* species-dominant^		-	-	-
	Yes	5 (23.8)			
	No	16 (76.2)			
Health Behaviors	Intravaginal practice use (since menopause)^		0.8094	0.6539	0.8783
	Yes	10 (47.6)			
	No	11 (52.4)			
	Alcohol consumption (past one year)^		0.393	0.6842	0.8695
	Yes	10 (47.6)			
	No	10 (47.6)			
	Missing	1 (4.8)			
	Tobacco smoking (current)^		-	-	-
	Yes	0 (0)			

	No	21 (100)			
	Sexual intercourse^		0.7394	0.8534	0.2799
	Yes	10 (47.6)			
	No	10 (47.6)			
	Missing	1 (4.8)			
	Antibiotic use (last 4 weeks)^		0.1061	**0.0462***	0.7012
	Yes	6 (28.6)			
	No	13 (61.9)			
	Missing	2 (9.5)			
	Hormone replacement therapy use (last 4 weeks)^		0.3837	0.5208	0.2083
	Yes	8 (38.1)			
	No	12 (57.1)			
	Missing	1 (4.8)			
	Topical estrogen use (last 4 weeks)^		0.7791	0.9044	0.4940
	Yes	6 (28.6)			
	No	14 (66.7)			
	Missing	1 (4.8)			
	Oral probiotics use (current)^		0.3507	0.3507	0.1574
	Yes	7 (33.3)			
	No	13 (61.9)			
	Missing	1 (4.8)			
	Vaginal lubricant use (last 4 weeks)^		0.6158	0.7649	0.3579
	Yes	3 (14.3)			
	No	17 (80.9)			
	Missing	1 (4.8)			
	Yogurt consumption^		0.5531	0.6116	0.3949
	Yes	15 (71.4)			
	No	5 (23.8)			
	Missing	1 (4.8)			
Sociodemographic Factors	Age (years), mean (range)~	64.2 (55-74)			
	Education^		0.3023	0.3809	0.6643
	<Bachelor's degree	6 (28.6)			
	≥Bachelor's degree	15 (41.4)			
	Insurance~		0.4928	0.3710	0.9022
	Public	5 (23.8)			
	Private	9 (42.8)			
	Both	7 (33.3)			
	Race~		0.3710	0.4643	0.5566
	White	14 (66.7)			
	Black	5 (23.8)			
	Other	2 (9.5)			
Vaginal Symptoms	Vaginal symptoms (current)^		0.3054	0.2380	0.3837
	Yes	12 (57.1)			
	No	8 (38.1)			
	Missing	1 (4.8)			
	Vaginal symptom type~		0.2475	0.2121	0.3679
	Discharge	1 (4.8)			
	Dryness	7 (33.3)			
	Itching	4 (19.0)			
	Missing	1 (4.8)			

Table 1. Participant (n=21) characteristics and resulting p-values of the vaginal microbiome, health behaviors, sociodemographic factors, and vaginal symptoms.

^- Wilcoxon rank sum test

~-Kruskal-Wallis rank sum test

*-Significant

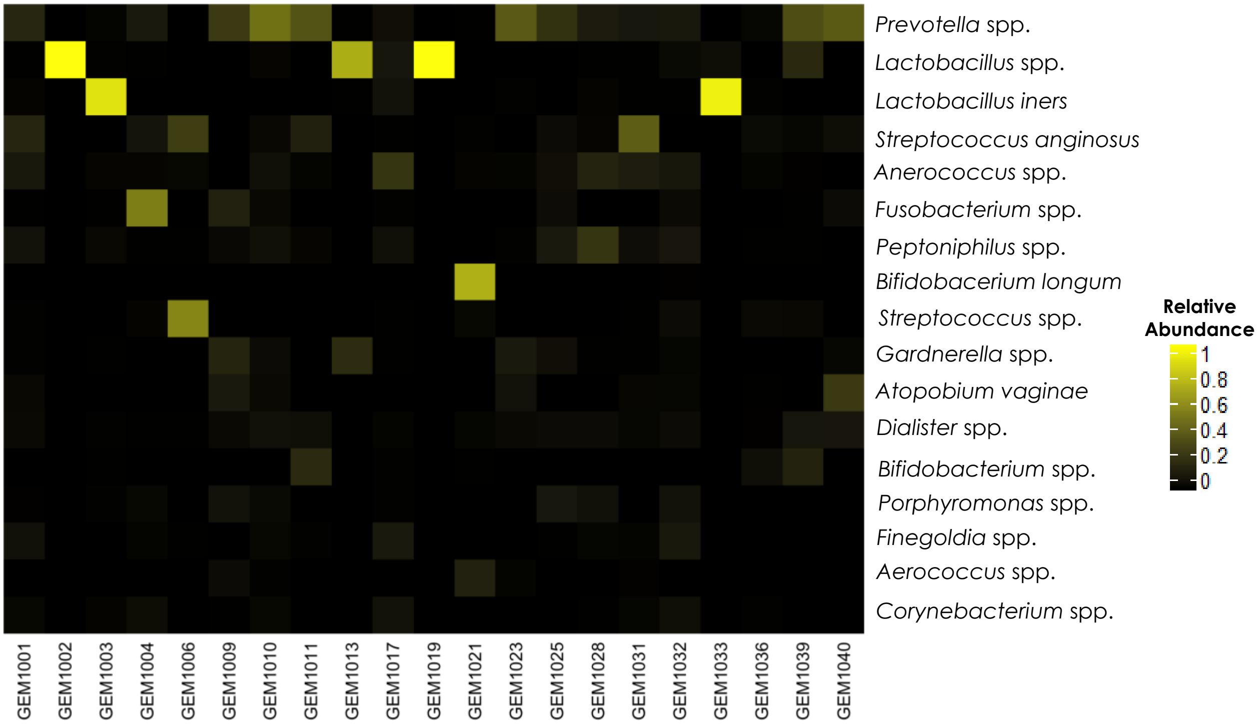

Figure 1. Heatmap emphasizing the relative abundance of the top known bacterial genera by subject identification. *L*. spp. being the most abundant overall.

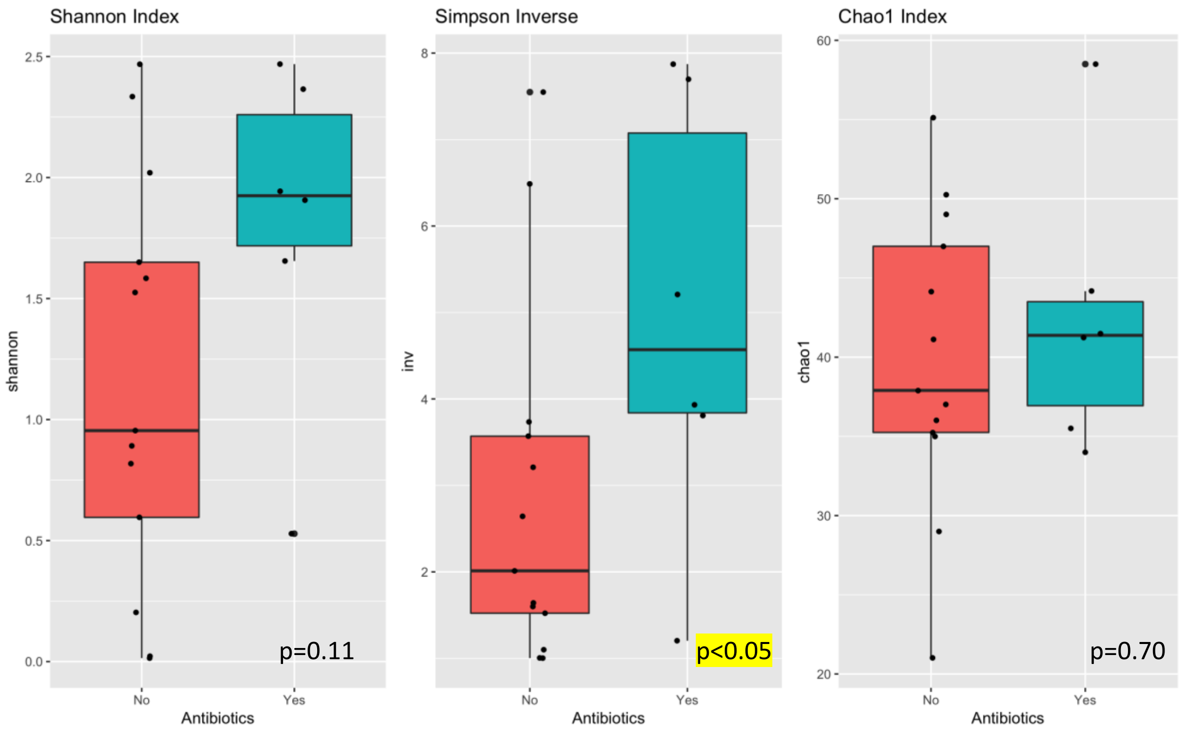

Figure 2. Alpha diversity (Shannon, Inverse Simpson, and Chao1 Indices, respectively) boxplots for antibiotic use.

References

Aagaard, K., Petrosino, J., Keitel, W., Watson, M., Katancik, J., Garcia, N., . . . Versalovic, J. (2013). The Human Microbiome Project strategy for comprehensive sampling of the human microbiome and why it matters. *FASEB Journal, 27*(3), 1012-1022. doi:10.1096/fj.12-220806

Alcaide, M. L., Rodriguez, V. J., Fischl, M. A., Jones, D. L., & Weiss, S. M. (2017). Addressing intravaginal practices in women with HIV and at-risk for HIV infection, a mixed methods pilot study. *Int J Womens Health, 9*, 123-132. doi:10.2147/IJWH.S125883

Bagaitkar, J., Demuth, D. R., & Scott, D. A. (2008). Tobacco use increases susceptibility to bacterial infection. *Tobacco Induced Diseases, 4*, 12. doi:10.1186/1617-9625-4-12

Baisley, K., Changalucha, J., Weiss, H. A., Mugeye, K., Everett, D., Hambleton, I., . . . Watson-Jones, D. (2009). Bacterial vaginosis in female facility workers in north-western Tanzania: Prevalence and risk factors. *Sexually Transmitted Infections, 85*(5), 370-375. doi:10.1136/sti.2008.035543

Barrons, R., & Tassone, D. (2008). Use of Lactobacillus probiotics for bacterial genitourinary infections in women: A review. *Clinical Therapeutics, 30*(3), 453-468. doi:https://doi.org/10.1016/j.clinthera.2008.03.013

Brotman, R. M., He, X., Gajer, P., Fadrosh, D., Sharma, E., Mongodin, E. F., . . . Rath, J. M. (2014). Association between cigarette smoking and the vaginal microbiota: a pilot study. *BMC Infectious Diseases, 14*, 471. doi:10.1186/1471-2334-14-471

Brotman, R. M., Klebanoff, M. A., Nansel, T. R., Andrews, W. W., Schwebke, J. R., Zhang, J., . . . Scharfstein, D. O. (2008). A longitudinal study of vaginal douching and bacterial vaginosis - A marginal structural modeling analysis. *American Journal of Epidemiology, 168*(2), 188-196. doi:10.1093/aje/kwn103

Brotman, R. M., Shardell, M. D., Gajer, P., Fadrosh, D., Chang, K., Silver, M. I., . . . Gravitt, P. E. (2014). Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause, 21*(5), 450-458. doi:10.1097/GME.0b013e3182a4690b

Chandra, A., Martinez, G. M., Mosher, W. D., Abma, J. C., & Jones, J. (2005). Fertility, family planning, and reproductive health of U.S. women: data from the 2002 National Survey of Family Growth. *Vital and Health Statistics. Series 23: Data from the National Survey of Family Growth*(25), 1-160. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/16532609

Cherpes, T. L., Hillier, S. L., Meyn, L. A., Busch, J. L., & Krohn, M. A. (2008). A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sexually Transmitted Diseases, 35*(1), 78-83. doi:10.1097/OLQ.0b013e318156a5d0

Daniel, G., Hu, Y., Jhaney, C., Tsementzi, D., Yeager, K., Hu Y. J., & Bruner, D. (2019). Exploring the Vaginal Microbiome and Intravaginal Practices in Postmenopausal Women. Manuscript submitted for publication.

DeSantis, C. E., Siegel, R. L., Sauer, A. G., Miller, K. D., Fedewa, S. A., Alcaraz, K. I., & Jemal, A. (2016). Cancer statistics for African Americans, 2016: Progress and opportunities in reducing racial disparities. *CA: A Cancer Journal for Clinicians, 66*(4), 290-308. doi:10.3322/caac.21340

Esber, A., Rao, N., Norris, A., Carr Reese, P., Kandodo, J., Nampandeni, P., . . . Norris Turner, A. (2016). Intravaginal Practices and Prevalence of Sexual and Reproductive Tract Infections Among Women in Rural Malawi. *Sexually Transmitted Diseases, 43*(12), 750-755. doi:10.1097/OLQ.0000000000000531

Fashemi, B., Delaney, M. L., Onderdonk, A. B., & Fichorova, R. N. (2013). Effects of feminine hygiene products on the vaginal mucosal biome. *Microbial Ecology in Health and Disease, 24*. doi:10.3402/mehd.v24i0.19703

Ferrer, M., Mendez-Garcia, C., Rojo, D., Barbas, C., & Moya, A. (2017). Antibiotic use and microbiome function. *Biochemical Pharmacology, 134*, 114-126. doi:10.1016/j.bcp.2016.09.007

Fethers, K. A., Fairley, C. K., Hocking, J. S., Gurrin, L. C., & Bradshaw, C. S. (2008). Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clinical Infectious Diseases, 47*(11), 1426-1435. doi:10.1086/592974

Fettweis, J. M., Brooks, J. P., Serrano, M. G., Sheth, N. U., Girerd, P. H., Edwards, D. J., . . . Buck, G. A. (2014). Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology, 160*(Pt 10), 2272-2282. doi:10.1099/mic.0.081034-0

Gliniewicz, K., Schneider, G. M., Ridenhour, B. J., Williams, C. J., Song, Y., Farage, M. A., . . . Forney, L. J. (2019). Comparison of the Vaginal Microbiomes of Premenopausal and Postmenopausal Women. *Frontiers in Microbiology, 10*, 193. doi:10.3389/fmicb.2019.00193

Gong, H., Jarzynka, M. J., Cole, T. J., Lee, J. H., Wada, T., Zhang, B., . . . Xie, W. (2008). Glucocorticoids antagonize estrogens by glucocorticoid receptor-mediated activation of estrogen sulfotransferase. *Cancer Research, 68*(18), 7386-7393. doi:10.1158/0008-5472.CAN-08-1545

Hickey, R. J., Zhou, X., Pierson, J. D., Ravel, J., & Forney, L. J. (2012). Understanding vaginal microbiome complexity from an ecological perspective. *Translational Research: The Journal of Laboratory and Clinical Medicine, 160*(4), 267-282. doi:10.1016/j.trsl.2012.02.008

Hilber, A. M., Chersich, M. F., van de Wijgert, J. H., Rees, H., & Temmerman, M. (2007). Vaginal practices, microbicides and HIV: what do we need to know? *Sexually Transmitted Infections, 83*(7), 505-508. doi:10.1136/sti.2007.028597

Huang, B., Fettweis, J. M., Brooks, J. P., Jefferson, K. K., & Buck, G. A. (2014). The Changing Landscape of the Vaginal Microbiome. *Clinics in Laboratory Medicine, 34*(4), 747-761. doi:https://doi.org/10.1016/j.cll.2014.08.006

Hummelen, R., Macklaim, J. M., Bisanz, J. E., Hammond, J. A., McMillan, A., Vongsa, R., . . . Reid, G. (2011). Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness. *PloS One, 6*(11), e26602. doi:10.1371/journal.pone.0026602

Jespers, V., van de Wijgert, J., Cools, P., Verhelst, R., Verstraelen, H., Delany-Moretlwe, S., . . . Vaginal Biomarkers Study, G. (2015). The significance of Lactobacillus crispatus and L. vaginalis for vaginal health and the negative effect of recent sex: a cross-sectional descriptive study across groups of African women. *BMC Infectious Diseases, 15*, 115. doi:10.1186/s12879-015-0825-z

Laue, C., Papazova, E., Liesegang, A., Pannenbeckers, A., Arendarski, P., Linnerth, B., . . . Schrezenmeir, J. (2018). Effect of a yoghurt drink containing Lactobacillus strains on bacterial vaginosis in women - a double-blind, randomised, controlled clinical pilot trial. *Benef Microbes*, *9*(1), 35-50. doi:10.3920/BM2017.0018

Lee, J. E., Lee, S., Lee, H., Song, Y. M., Lee, K., Han, M. J., . . . Ko, G. (2013). Association of the vaginal microbiota with human papillomavirus infection in a Korean twin cohort. *PloS One*, *8*(5), e63514. doi:10.1371/journal.pone.0063514

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H., . . . Hilber, A. M. (2011a). Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS Medicine, 8*(2), e1000416. doi:10.1371/journal.pmed.1000416

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H. H. M., . . . Hilber, A. (2011b). Intravaginal practices, bacterial vaginosis, and HIV infection in women: Individual participant data meta-analysis. *PLoS Medicine, 8*(2). doi:10.1371/journal.pmed.1000416

Mayer, B. T., Srinivasan, S., Fiedler, T. L., Marrazzo, J. M., Fredricks, D. N., & Schiffer, J. T. (2015). Rapid and Profound Shifts in the Vaginal Microbiota Following Antibiotic Treatment for Bacterial Vaginosis. *Journal of Infectious Diseases, 212*(5), 793-802. doi:10.1093/infdis/jiv079

Mitra, A., MacIntyre, D. A., Marchesi, J. R., Lee, Y. S., Bennett, P. R., & Kyrgiou, M. (2016). The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? *Microbiome, 4*(1), 58. doi:10.1186/s40168-016-0203-0

Muhleisen, A. L., & Herbst-Kralovetz, M. M. (2016). Menopause and the vaginal microbiome. *Maturitas, 91*, 42-50. doi:10.1016/j.maturitas.2016.05.015

Muzny, C. A., Harbison, H. S., Pembleton, E. S., & Austin, E. L. (2013). Sexual behaviors, perception of sexually transmitted infection risk, and practice of safe sex among southern African American women who have sex with women. *Sexually Transmitted Diseases, 40*(5), 395-400. doi:10.1097/OLQ.0b013e31828caf34

Muzny, C. A., & Schwebke, J. R. (2016). Pathogenesis of Bacterial Vaginosis: Discussion of Current Hypotheses. *Journal of Infectious Diseases, 214 Suppl 1,* S1-5. doi:10.1093/infdis/jiw121

Nelson, T. M., Borgogna, J. C., Michalek, R. D., Roberts, D. W., Rath, J. M., Glover, E. D., . . . Brotman, R. M. (2018). Cigarette smoking is associated with an altered vaginal tract metabolomic profile. *Scientific Reports, 8*(1), 852. doi:10.1038/s41598-017-14943-3

Ness, R. B., Hillier, S. L., Kip, K. E., Richter, H. E., Soper, D. E., Stamm, C. A., . . . Sweet, R. L. (2005). Douching, pelvic inflammatory disease, and incident gonococcal and chlamydial genital infection in a cohort of high-risk women. *American Journal of Epidemiology, 161*(2), 186-195. doi:10.1093/aje/kwi025

Ness, R. B., Hillier, S. L., Richter, H. E., Soper, D. E., Stamm, C., Bass, D. C., . . . Aral, S. (2003). Why women douche and why they may or may not stop. *Sexually Transmitted Diseases, 30*(1), 71-74. doi:10.1097/00007435-200301000-00014

Petricevic, L., Unger, F. M., Viernstein, H., & Kiss, H. (2008). Randomized, double-blind, placebo-controlled study of oral lactobacilli to improve the vaginal flora of postmenopausal women. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 141*(1), 54-57. doi:10.1016/j.ejogrb.2008.06.003

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., . . . Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National*

Academy of Sciences of the United States of America, 108 Suppl 1, 4680-4687. doi:10.1073/pnas.1002611107

Reid, G. (2016). Cervicovaginal Microbiomes–Threats and Possibilities. *Trends in Endocrinology and Metabolism, 27*(7), 446-454. doi:https://doi.org/10.1016/j.tem.2016.04.004

Rosen, R., Brown, C., Heiman, J., Leiblum, S., Meston, C., Shabsigh, R., . . . D'Agostino, R., Jr. (2000). The Female Sexual Function Index (FSFI): a multidimensional self-report instrument for the assessment of female sexual function. *Journal of Sex and Marital Therapy, 26*(2), 191-208. doi:10.1080/009262300278597

Shen, J., Song, N., Williams, C. J., Brown, C. J., Yan, Z., Xu, C., & Forney, L. J. (2016). Effects of low dose estrogen therapy on the vaginal microbiomes of women with atrophic vaginitis. *Scientific Reports, 6*, 24380. doi:10.1038/srep24380

Stapleton, A. E. (2016). The Vaginal Microbiota and Urinary Tract Infection. *Microbiol Spectr, 4*(6). doi:10.1128/microbiolspec.UTI-0025-2016

Sturdee, D. W., Panay, N., & International Menopause Society Writing, G. (2010). Recommendations for the management of postmenopausal vaginal atrophy. *Climacteric, 13*(6), 509-522. doi:10.3109/13697137.2010.522875

Viens, L. J., Henley, S. J., Watson, M., Markowitz, L. E., Thomas, C. C., Thompson, T. D., . . . Saraiya, M. (2016). Human Papillomavirus-Associated Cancers - United States, 2008-2012. *MMWR: Morbidity and Mortality Weekly Report, 65*(26), 661-666. doi:10.15585/mmwr.mm6526a1

Ziomkiewicz, A., Ellison, P. T., Lipson, S. F., Thune, I., & Jasienska, G. (2008). Body fat, energy balance and estradiol levels: a study based on hormonal profiles from complete menstrual cycles. *Human Reproduction*, *23*(11), 2555-2563. doi:10.1093/humrep/den213

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