Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXXIV

Settore Concorsuale: 07/H1

Settore Scientifico Disciplinare: VET02

VAGINAL IMMUNOLOGY AND MICROBIOME COMPOSITION: BACTERIAL VAGINOSIS IN A MACAQUE MODEL

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Esame finale anno 2023

TABLE OF CONTENTS

ABSTRACT

Nonhuman primates (NHPs) are important animal models for the study of human health and disease. In particular, the use of NHPs to study the vaginal microbiome and susceptibility to infections (such as HIV and herpesvirus) is exceptionally valuable due to the similarity in anatomy and physiology. An important aspect to this is maintaining a healthy vaginal microbiome which then minimizes colonization by pathogens and resulting inflammation along the mucosa. In women, conditions such as bacterial vaginosis (BV) are frequently treated with antibiotics such as metronidazole or clindamycin. Due to the excessive use of antimicrobials in medicine and agriculture, alternative compounds and therapies are highly desired to treat infections. Approaches that have been developed and used for vaginal infections includes the use of natural antimicrobials such as essential oils, probiotics, and live cultures, which mimic and function like antibiotics but lack development of resistance like classic antibiotics. However, these approaches have been minimally studied in humans and animals. Effectiveness of essential oils are anecdotal at best. Microbiome manipulation on the other hand has been investigated more thoroughly. Novel products are being distributed for medical use and are monotherapies containing *Lactobacillus* which colonize the vaginal mucosa (Ali et al., 2020; Brichacek et al., 2013; Lagenaur, Sanders-Beer, et al., 2011). Unfortunately, these therapies have limitations due to durability and individual response in women. By evaluating the extent by which the NHP vaginal mucosa can be colonized with exogenously delivered bacteria, this animal model will highlight the NHP for use in translational studies which use essential oils and beneficial microbiome bacteria for vaginal delivery. These studies include the use of engineered vaginal tissue platforms and live NHPs to evaluate these manipulations and their responses, including presence of WBCs, cytokine release, histopathology, bacterial diversity, microbiome colonization, to name a few. Studying the

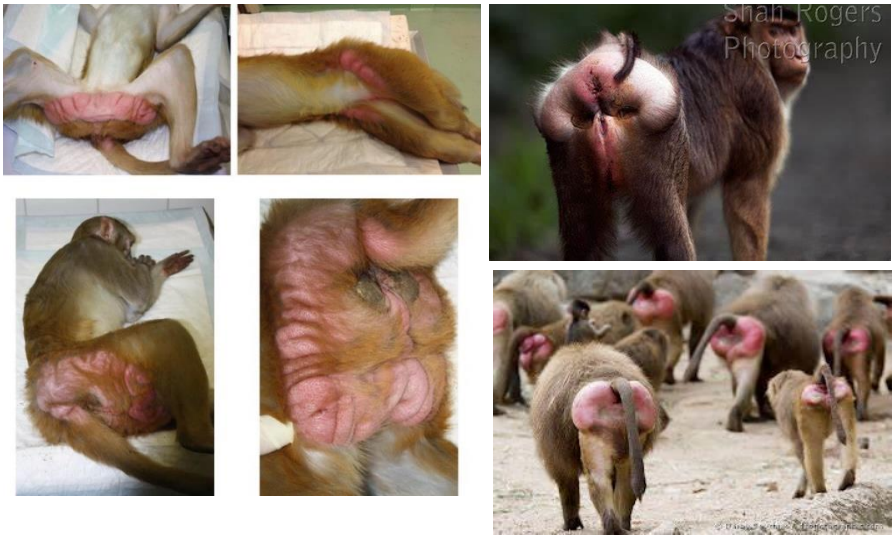
mechanisms underlying these associations is extremely difficult in humans, given the many confounding environmental factors such as diet, concurrent disease, menstrual cycle, sexual activity, and frequent sampling limitations. These shortcomings are less pronounced when studies are conducted in NHPs under controlled conditions. The translational impact of these studies is significant for women's health but can also provide similar therapies for the veterinary field.

1. INTRODUCTION

1.1 Female reproductive characteristics

Female nonhuman primates (NHP) are excellent models for studying women's reproductive health and disease. The anatomy and physiology are identical regarding the uterus, ovaries, cervix, and vagina. Macaques and baboons are most used for these studies due to their size and availability. Though similar, outward signs of menses in these species are quite different from each other and from women. Sex skin, outwardly visible in these images, is a secondary sex characteristic demonstrating estrogenic activity and receptivity (Schlabritz-Loutsevitch et al., 2016).

Figure 1. Baboon (left) pigtail macaque (right) and rhesus macaque (bottom) sex skin location. and appearance.



This redness and swelling can be found not only along the perineum (**Figure 1**), but can be found along the limbs, base of tail, and in rhesus on the face above the brow line (Casteleyn, Bakker. 2021). Much like human females, menses occurs monthly and lasts 28-30 days, with some variation depending on species. Female monkeys begin puberty around 3-4 years of age, again dependent on species. Most are seasonal breeders beginning in fall/autumn, with a gestation period

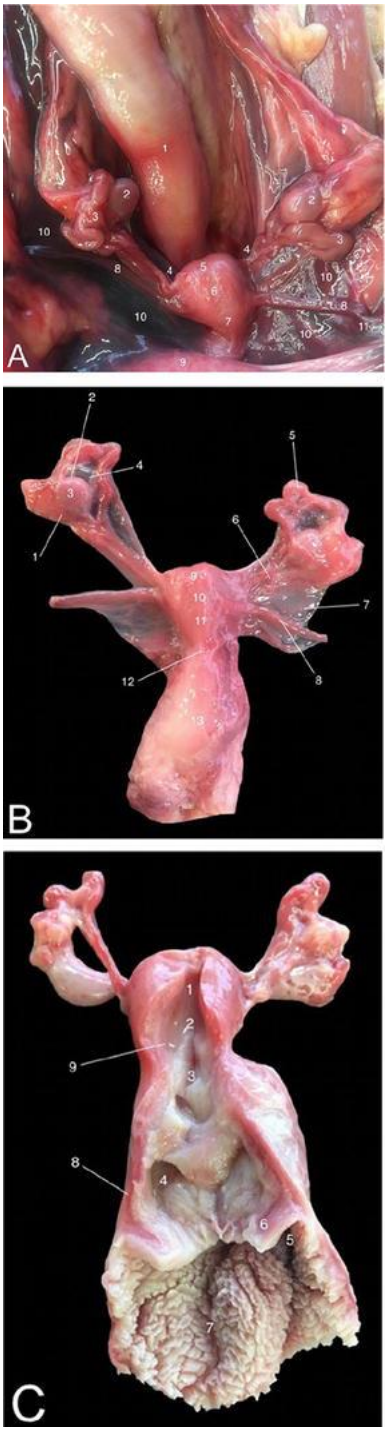


Figure 2. Gross anatomy. A. in situ ovaries and uterus; B. ex vivo uterus, ovaries, and fallopian tubes; C. transected reproductive tract to demonstrate uterine body, cervix, and vagina.

on average of 165 days (Casteleyn, Bakker. 2021). This then translates into most births beginning in February on the following year.

Reproductive anatomy in macaques and baboons is strikingly similar if not almost exact to that of women. **Figure 2** illustrates the various components of the anatomy including the in situ reproductive tract (A); ex vivo bilateral ovaries and fallopian tubes (B); and the simplex uterus, cervix, and vagina (C). The uterus being like that of a woman, single infants are most common, twinning occurring much less frequently. The vagina has three layers: the internal mucosal layer, the intermediate muscularis layer and the external adventitial layer (**Figure 3;** Casteleyn, Bakker. 2021). The mucosal layer continues from the uterine endometrium and is divided into two sublayers; an epithelial layer which rests on an underlying second layer of lamina propria. The vaginal epithelial layer consists of multiple layers of stratified squamous tissue which undergoes differentiation into layers known as strata. The vaginal epithelium outermost layer is the stratum corneum and is closest to the vaginal lumen. It

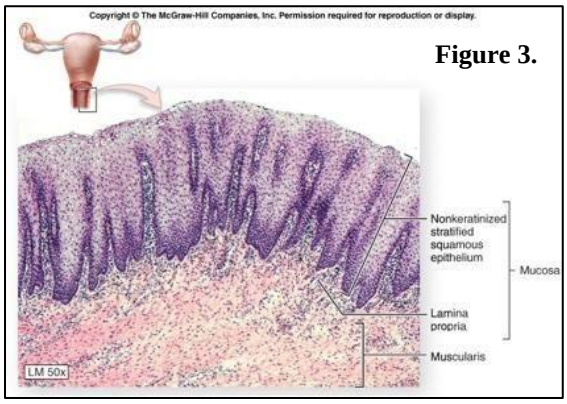


Figure 3.

undergoes shedding and regeneration throughout the menstrual cycle (Casteleyn, Bakker. 2021). These epithelial cells can be easily visualized when cytology of the mucosa is performed. This can also be a biological indicator of cycle phase and is commonly used in other species to aid in determination of ovulation.

Cells of various epithelial surfaces are responsible for initiating the innate immune response to counteract any foreign substance including pathogens, by triggering cellular mediators, like neutrophils, and expression of antimicrobial peptides. The adaptive immune response then comes into play in later stages of infection when development of immunological memory becomes dominant (Adapen et al., 2022; Al-Nasiry et al., 2020).

One important property of the vaginal mucosa is the microbiome, a complex environment of organisms carefully balanced by vaginal pH, mucosal immunity, and menstrual cycle. In women, the microbiome is mostly dominated by the organism genus *Lactobacillus*, L. *crispatus* and *L. iners* (Deka et al., 2021; Joseph et al., 2021). These organisms produce lactic acid which brings the vaginal pH between 3.5 and 4.5. This creates a milieu that allows for a favorable microbiome that promotes optimal vaginal health.

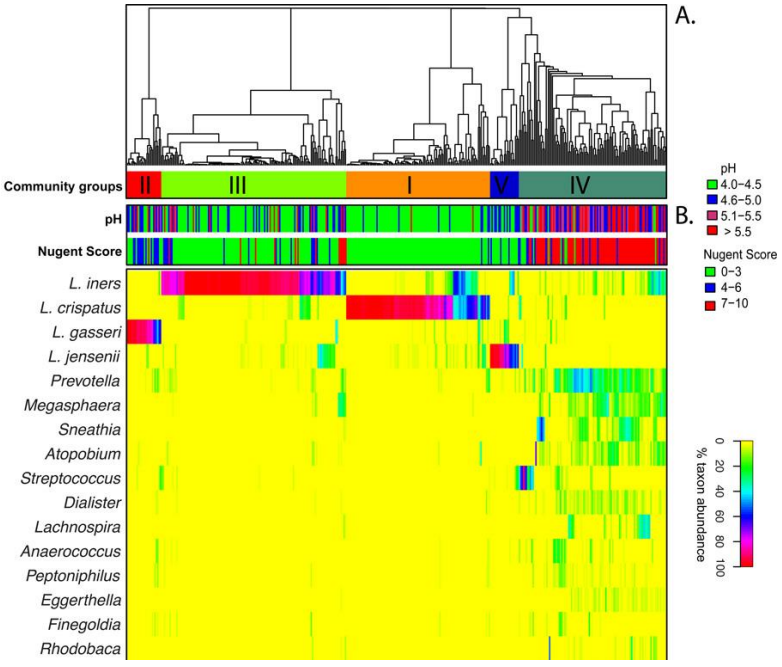


Figure 4. Heatmap of percentage abundance of microbes in vaginal microbial communities of 394 reproductive-age women. (A) Complete linkage clustering of samples based on species composition and abundance in communities defining five community state types (CST I–V). (B) Nugent scores and pH measurements for each of the 394 samples.

Figure 4 (Ma B, Ravel et al. 2012) demonstrates an important aspect of microbiome diversity, and the

specific populations that dictate optimal vaginal health compared to a compromised vaginal microbiome which can lead to conditions such as bacterial vaginosis (BV). In the figure, BV is associated with higher Nugent scores, higher pH, and a completely different distribution of microbes (Abou Chacra et al., 2021; Bradshaw & Sobel, 2016; van den Munckhof et al., 2019). Nugent scores are utilized in gynecology to rate the severity of bacterial vaginosis when associated with epithelial changes and the high prevalence of organisms such as *Prevotella*, *Sneathia*, *Gardnerella*, and *Megasphaera*. What is also most notable is the absence of *Lactobacillus* bacteria which are especially important in maintaining the lower pH.

Several therapies have been developed including the use of *Lactobacillus* given orally or as a suppository (de Vrese et al., 2019; Russo et al., 2018), and the use of agents such as boric acid, both important for lowering the vaginal pH. The ongoing research will aid in finding better therapies that lower pH but also add other beneficial organisms to help balance microbial diversity. Minimizing BV like organisms is essential when considering that this altered state allows a greater chance for transmission of pathogenic and dangerous organisms such as HIV, *Candida*, *Trichomonas*, and other pathogens (Fichorova et al., 2021; Foessleitner et al., 2021).

1.2 Examination of the cervix and vagina

Many NHPs are used for reproductive studies involving the cervicovaginal mucosa. However, the most extensively used species are *Macaca mulatta* (rhesus macaque) and *M. nemestrina* (pig-tailed macaque), although *M. fascicularis* (cynomolgus macaque) are also used. *M. fascicularis* are not as popular for cervicovaginal evaluation because of their small size, limited cervicovaginal visibility, and small vaginal canal space for sample collection and manipulation (Scorpio et al., 2008). Selection of a rhesus macaque rather than a pig-tailed or cynomolgus macaque will depend

on the human disease to model, variation in cervicovaginal anatomy, and similarity to humans regarding vaginal flora, cervicovaginal cellularity, and pH (Scorpio et al., 2008). Cervicovaginal evaluation for therapeutic and safety studies requires proper technique, equipment, supplies, and sequence of sample collection (Scorpio et al., 2008). Iatrogenic injury to the cervicovaginal mucosa is detrimental to interpretation of effects. In addition, having the proper equipment and supplies facilitates sample collection and visualization of the mucosa (**Figure 5**).

Colposcopy is performed first before instruments or swabs contact the cervicovaginal mucous membranes. The goal of colposcopy is to detect epithelial changes that may increase likelihood of microorganism transmission. Cervicovaginal microflora evaluation is the second procedure performed to prevent iatrogenic bacterial contamination of the mucous membranes. Microflora determination includes both aerobic and anaerobic bacterial cultures critical for assessing the normal microflora. pH determination along the mucosa is the next step before cervicovaginal lavage disturbs mucosal pH, which is important to monitor as imbalances in acidity or alkalinity could contribute to altered vaginal ecosystems and mucosal irritation. Cervicovaginal lavage is performed next before cervicovaginal biopsies as significant bleeding and inflammation can occur, which could affect interpretation of lavage sample cellularity, cytokines, and immunoglobulins (Ig). Baseline

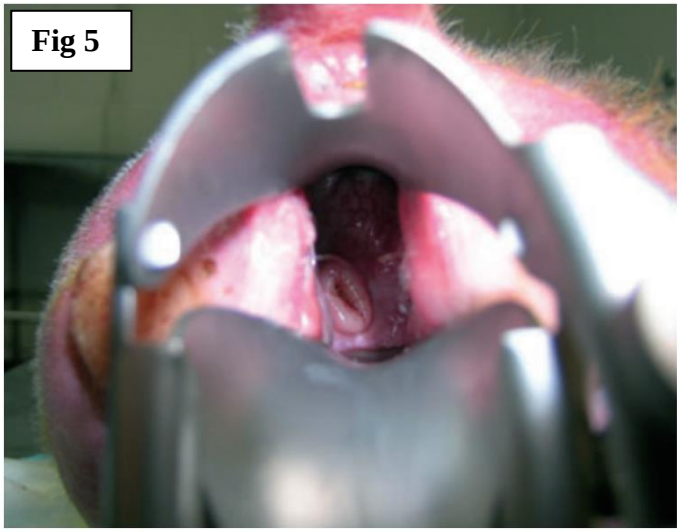


Fig 5 Pederson pediatric speculum, 70 x 15-mm size. (b). View of the vaginal vault and cervix through the inserted speculum. This is a post-partum rhesus macaque with optimal cervicovaginal visibility compared with the nulliparous macaques used in this study. The animal was positioned in ventral recumbency and the table positioned in Trendelenburg.

characterization of mucosal cellularity is important to adequately distinguish what is normal or abnormal for a specific macaque, such as altered cell distributions or cellular morphology. Lavage fluid collection is also beneficial in determining cytokine abundance and Ig profiles. Lastly, biopsy is performed, and this is because the biopsy itself causes bleeding and some tissue trauma, and avoids the contamination and variable introduced when assessing mucosal health and inflammation.

1.3 Essential oils for potential vaginal use.

Antimicrobial resistance has become a significant problem within medical communities due to overuse in both therapeutics and in the agricultural industries. As a result, new non-antibiotic compounds which can be utilized as microbicides have become especially attractive, such as essential oils. Essential oils are extracted from processes such as mechanical pressing or distillation. They are concentrated plant extracts that have a unique array of chemicals, and this variation affects absorption and effectiveness with use (Alvarez-Martinez et al. 2021). The chemical composition of an essential oil varies within the same plant species, and sometimes even plant to plant.

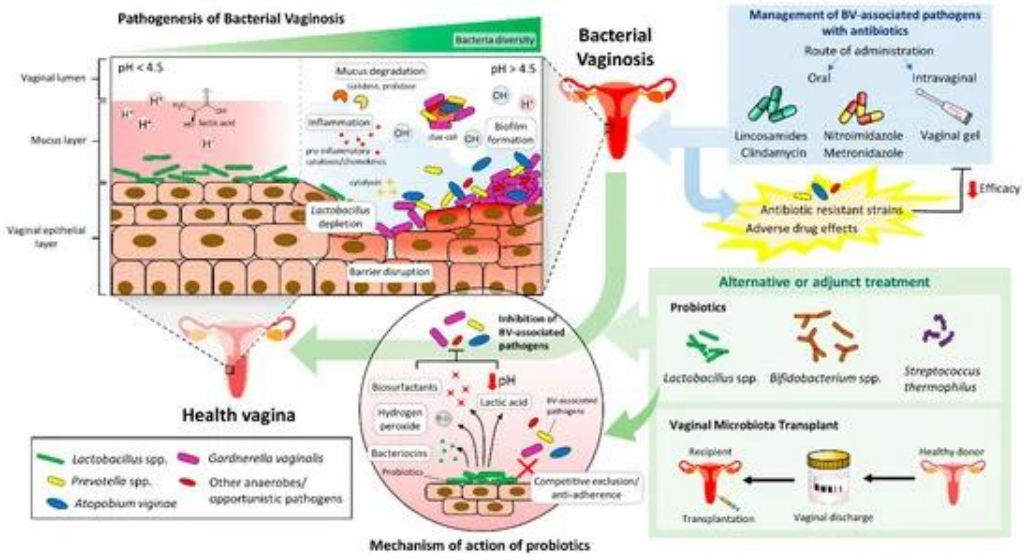
There is a paucity of studies in the published literature which have looked at safety and effectiveness of numerous possible candidates, including tea tree, rosemary, oregano, and other easily accessible compounds. Most studies have evaluated treatments for organisms such as *Trichomonas vaginalis*, *Gardnerella*, and *Candida*, using oregano, white thyme, garlic wood, clove, *Moringa*, *Eugenia pohliana*, and *Dracocephalum kotschyi* (Hashemi et al. 2021; Esmaeilifallah et al. 2022; Silva et al. 2021). These studies were conducted *in vitro* and have evaluated effects of the oils directly on specific pathogens. Few studies have evaluated the

effectiveness of essential oils in reproductive physiology and have focused more on spermatozoa and use of essential oils as antimicrobial agents for reproductive biotechnologies (Elmi et al. 2019). Most studies in essential oil therapeutics have focused on oral delivery for the gastrointestinal tract as a target. As a result, safety and efficacy in essential oil application to the reproductive mucosa is novel and innovative.

1.4 Microbiome model in macaques.

Studies conducted in women's health using female NHP's are numerous and include microbiome evaluation, sexually transmitted infections, microbicide testing, fetal-maternal interactions, and contraceptive safety, just to name a few (Spear et al., 2012). The most significant model utilizing the female macaque is that of SIV infection (model for HIV/AIDS), vaginal transmission, therapeutics, and vaccine development (Cheu et al., 2020; Eastment & McClelland, 2018; Schooley, 2018). Worldwide, HIV and AIDS threaten the health of millions of people. Sexual transmission from men to women is the most frequent mode of HIV infection. Susceptibility to infection increases when the female reproductive tract (FRT) is inflamed, as signified by elevated levels of chemokines and cytokines (Dabee et al., 2021; Jespers

Figure 6. Pathogenesis of bacterial vaginosis (BV) and the mechanism of action of in battling against BV (Joseph et al 2021).



et al., 2017; Yarbrough et al., 2015). However, the factors and pathways causing inflammation in the FRT are not well understood. Changes in amount and diversity of vaginal microbial communities are consistently associated with increased HIV risk, exemplified by a striking increase in HIV susceptibility with conditions such as bacterial vaginosis (BV), a significant shift in the microbiota caused by several factors including diet, sexual intercourse, hygiene, and many other causes **(Figure 6**). Inflammation due to microbial alterations may underlie this but has not been proven. HIV susceptibility varies throughout the menstrual cycle (Kersh et al., 2014; Vishwanathan et al., 2011; Wira & Fahey, 2008), which may be related to changes in the vaginal microbiome as well. Indeed, in humans, there is high fluctuation of bacterial populations throughout the menstrual cycle (Morison et al., 2005; Srinivasan et al., 2010). This is no different in NHPs, particularly macaques.

BV in women occurs when the normal protective microbiota in the vagina, dominated by lactic acid-producing *Lactobacillus* species, are replaced by strains of bacteria such as *Gardnerella vaginalis, Atopobium vaginae, Prevotella* spp., and other BV-associated bacteria (BVAB) (Schellenberg et al., 2012; Srinivasan et al., 2012). BV can be alleviated via antibiotic treatment (such as metronidazole), which rapidly eliminates BV-associated anaerobic species, but these species often reemerge over time (Mayer et al., 2015). BV is the most common cause of vaginitis worldwide, with high prevalence in developing countries, and is associated with a 60% increase in risk of HIV acquisition in women (Atashili et al., 2008; Mirmonsef, Krass, et al., 2012; Schellenberg et al., 2012). In addition, BVAB, such as

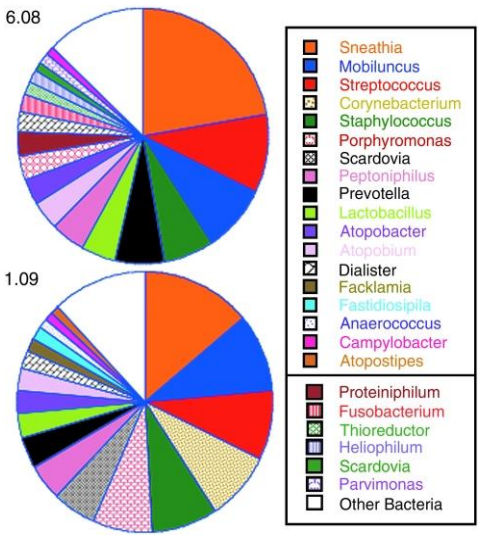


Figure 7. Predominant bacterial sequences in rhesus macaques (represents 2 animals).

BVAB-3, are associated with cervicitis in humans (Sycuro, 2012), thus specific BV-associated bacteria may induce inflammation in the cervix leading to recruitment of T cells and increased risk of SIV/HIV infection. Conversely, a healthy *Lactobacillus* dominated FRT is associated with improved protection from HIV transmission (Borgdorff et al., 2014). Studying the mechanisms underlying these epidemiological associations is extremely difficult in humans, given the many confounding factors such as sexual activity, vaginal douching and co-occurring STIs, as well as sampling limitations. Utilization of non-human primate (NHP) models to isolate the effects of the microbiome on inflammation can overcome these confounding variables. Non-human primates are the leading model for pre-clinical HIV infection and transmission studies because simian immunodeficiency virus (SIV) infection in macaques to a considerable extent recapitulates HIV infection in humans. Pigtail macaques (PTM) are highly desirable for HIV/SIV studies of the FRT, given that they have lunar menstrual cycles like women. However, few studies have been performed regarding the PTM vaginal microbiome or how it may affect transmission of SIV via vaginal inoculation. Studies that have been previously performed (Klatt, unpublished) suggest that PTM and rhesus macaques have a plethora of

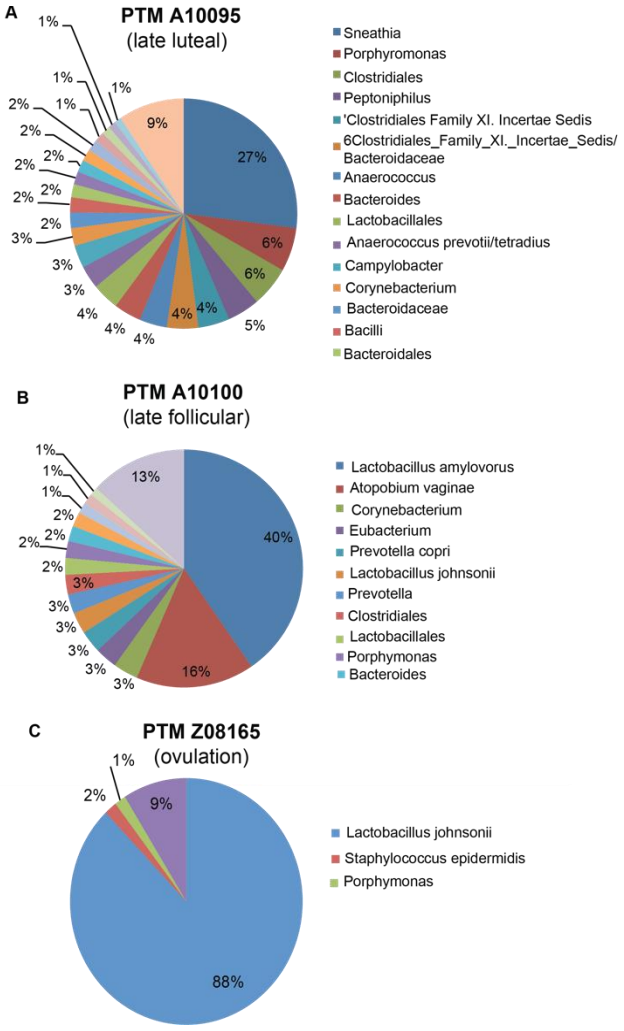


Figure 8. 16s microbiome sequencing in PTM (N. Klatt) showing A (late luteal); B (late follicular); and C (ovulation).

vaginal microbiota, not always similar to humans, with diversity that resembles BV and to a much lesser extent *Lactobacillus* dominant communities (Mirmonsef, Gilbert, et al., 2012; G. T. Spear et al., 2012) (**Figures 7 and 8**). In addition, studies in rhesus macaques have demonstrated that while there is rarely *Lactobacillus* found naturally (Mirmonsef, Gilbert, et al., 2012; G. Spear et al., 2012; Spear et al., 2010) it is also possible to colonize the RM vagina with *Lactobacillus* (Lagenaur, Lee, et al., 2011; Lagenaur, Sanders-Beer, et al., 2011; Lagenaur et al., 2015). Efforts to colonize the vaginal mucosa with *Lactobacillus* in macaques revealed that this bacteria could be sustained through one menstrual cycle, but was not sustained much beyond this time period.

These series of studies address key gaps in our knowledge regarding factors that may impact vaginal pathogen transmission and will allow testing modalities to assess whether a specific microbiome can drive inflammation and therefore HIV susceptibility, and whether the modification of the microbiome in a macaque model might mitigate this inflammation. The results of these studies inform the execution of pre-clinical non-human primate studies of HIV and other STD transmission and provides essential preliminary data for larger studies to better understand HIV/SIV susceptibility relative to the microbiome and inflammation in the FRT, and whether the macaque microbiome can be altered to improve concurrence between macaque and human studies. Improving the predictability of clinical outcomes through utilization of accurate, human-like, NHP models would provide immense benefit to the HIV prevention field.

The studies conducted here evaluate the use of healthy microbe inoculations (from human subjects) and essential oil application as safe and effective microbicides for use along the vaginal mucosa. These applications are hypothesized to promote a healthy microbiome without having any detrimental side effects to cervicovaginal tissues or the animal. This work also evaluates the vaginal microbiome throughout the menstrual cycle at baseline, after antibiotic treatment, and after

inoculation with vaginal fluid from women with or without BV (*Lactobacillus* dominant), which has never been reported before in primates (BV inoculation). Knowledge and development of the model drives a better understanding of vaginal microbial communities in the pigtail macaque model and alleviates concerns over pigtail macaques having microbial communities that do not represent most women. These studies also allow a highly innovative assessment of vaginal inflammation, including novel assessments of innate immunity. Better understanding of how the vaginal microbiome influences susceptibility throughout the cycle or after common BV antibiotic treatment, and development of a human representative FRT microbiome model in NHPs, will provide a more accurate method to assess how vaginal inflammation is associated with infection transmission in NHP, with the goal to develop novel therapeutic interventions aimed at decreasing vaginal transmission by reducing inflammation associated with BV.

2. EXPERIMENTAL METHODS

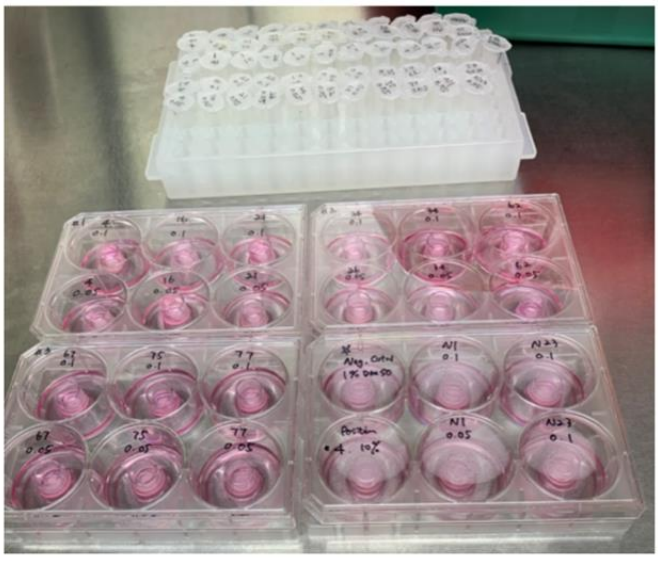
2.1 Essential oils study in vitro.

The aim of the present research was to develop and validate an in vitro model of cervicovaginal mucosal safety using essential oils, which is then aligned to our published work demonstrating essential oil use in an ex vivo model of porcine uterine mucosa evaluating potential effects of *M. alternifolia* and *R. officinalis* oils on endometrial tissue (Publication 1). An advantage to testing essential oils on in vitro artificial tissues or ex vivo is to evaluate potential effects before testing in animals. EpiVaginal (Mattek) has a a human vaginal and ectocervical tissue matrix for *in vitro* use. The bioengineered tissue is structurally similar to vaginal epithelium and compounds can be tested for effects on the tissue histopathologically, and supernatants can be tested for inflammatory markers. See Supplementary Materials (Labeled 1) which describes and illustrates the capabilities of this product. In wanting to explore a variety of essential oils for changes in tissue architecture

Figure 9 A

Sample #	Oil #	Oil Concentration (~% in 1% DMSO)	Cultured @ 37°C for 24 hours
1	#4	0.1	
2	#4	0.05	
3	#16	0.1	
4	#16	0.05	
5	#21	0.1	
6	#21	0.05	
7	#26	0.1	
8	#26	0.05	
9	#34	0.1	Collect Supernatant Store @-80°C
10	#34	0.05	
11	#62	0.1	
12	#62	0.05	"Luminex Performance Human Fixed Cytokine Discovery 14-plex" R&D Systems Cat# LKTM011
13	#67	0.1	
14	#67	0.05	
15	#75	0.1	https://www.rndsystems.com/products/luminex-performance-human-fixed-cytokine-discovery-14-plex_lktm011
16	#75	0.05	
17	#77	0.1	
18	#77	0.05	
19	#N1	0.1	
20	#N1	0.05	
21	#N23	0.1	
22	#N23	0.05	
23	Positive Control	Oil #4: 10%	
24	Negative Control	1% DMSO	

B



and cytokine responses, this system was applied and eleven different essential oils were used at 2 concentrations (0.05 and 0.1% in 1% DMSO) (**Figure 9A).** The oil types included tea tree, cinnamon, geranium, lemongrass, oregano, thyme, coriander, dillweed, clove, garlic, and grapefruit. A positive and negative control were used, with the positive control being 10% tea tree oil in 1% DMSO, and the negative control using only 1% DMSO, which was used to dilute the essential oils for use with this EpiVaginal system (**Figure 9 B).** Once oils were dispensed onto the tissues, the plate was incubated for 24 hours at 37C, and then supernatants were collected for cytokine analysis using the Luminex Performance Human Fixed Cytokine Disocvery 14-Plex (R&D Systems). Tissues were collected for histopathology, fixed in formalin, then cut into 5 micron sections for microscopy analysis, see Supplementary Material (Labeled 2). EpiVaginal tissues were then assessed to see if detrimental changes were noted. PDF results are shown for all oils (histopathology and cytokine analysis).

Regarding cytokine analysis, the attached PDF in Supplementary Materials (Labeled 3) displays results of 11 oils, at 2 concentrations, and 14 different cytokines, which are defined for their roles in inflammation as below:

IFNa: produced by nucleic acid immune complexes

IFNg: immunostimulatory innate immunity

IL-1a: constitutively produced by epithelial cells

IL-1b: involved in cell proliferation, differentiation, and apoptosis

IL- 1Ra: inhibits activity of IL-1a and IL-1b

IL-2: transient positive & negative feedback loops in mounting and dampening immune responses

IL-3: stimulates proliferation of all cells in the myeloid lineage

IL-4: in extravascular tissues promotes activation of macrophages

IL-6: stimulates acute phase protein synthesis

IL-7: produced locally by epithelial cells

IL-10: attenuation of inflammatory cytokines

IL-15: produced as a mature protein mainly by dendritic cells, monocytes and macrophages

IL-33: activating intracellular molecules that drive production of type 2 cytokines

VEGF: signal protein produced by cells that stimulates formation of blood vessels

2.2 Vaginal microbiome evaluation.

The main study included a cohort of ten female pigtail macaques (*Macaca nemestrina,* PTM) and 2 control cynomolgus macaques (*Macaca fascicularis*) to study how the vaginal microbiome relates to factors influencing inflammation and susceptibility to vaginal infection and mucosal transmission. We longitudinally assessed the microbiome and feasibility to measure white blood cells (WBC via CD45+ measurement via flow cytometry) throughout 2 menstrual cycles (**Phase I**). Animals were then treated with vaginal metronidazole (antibiotic commonly used to treat BV) and we again assessed the microbiome and WBC presence across 2 additional menstrual cycles, treating with metronidazole for the first week of each cycle (**Phase II**). We then split animals into two groups of 5 PTM and 1 control to assess how altering the microbiota by adding human vaginal bacteria affects the vaginal microenvironment. We treated with metronidazole one week prior to either (**Phase IIIA**) human BV fluid inoculation (isolated from women clinically diagnosed with BV) or (**Phase IIIB**) *Lactobacillus* dominant non-BV vaginal fluid containing *Lactobacillus spp,* and then animals were monitored and sampled through 3 cycles to determine the dynamics of colonziation following inoculation with the human bacteria. The microbiome was assessed bi-

weekly by vaginal swab, and cells collected for white blood cell screening from cervicovaginal lavage (CVL) and cervical cytobrush (CB) (**Figure 10 in Study Design**).

2.2.1 Introduction

Methods and experiments below include the use of nonhuman primates. All protocols were followed and approved by the Institutional Animal Care and Use Committee at the National Institutes of Health, Bethesda, Maryland. The work was financially supported by a loan repayment grant granted to the student, Diana Gerardi Scorpio.

2.2.2 Materials and Methods

Techniques developed for the proper evaluation of the external reproductive anatomy are necessary to create a standardized method for experimental studies (Scorpio et al., 2008).

Vaginal swab for microbiome analysis:

Wicking type vaginal swabs were used, and mucosal fluid was collected by placing a moistened swab (sterile saline) into the vaginal vault and applying light pressure along the swab tip to allow fluids to wick rather than swiping it across the mucosa, which leads to mucosal surface exfoliation and damage. Two swabs were taken from each animal.

Cervicovaginal cytobrush for flow cytometry and microbiome analysis:

The cytobrush allows for better isolation of cells by creating a sweeping action with soft bristles across the vaginal mucosa, which enhances and optimizes collection of cells. The brush was then placed into RPMI media and placed at room temperature.

Cervicovaginal lavage for cytokine analysis:

Using 3-5 ml of saline, a syringe with a catheter was inserted into the vaginal vault and fluid was expelled and recovered 3 times, and remaining fluid that was aspirated was placed into saline and kept at room temperature.

Metronidazole (test animals) and Nonoxynol 9 intravaginal cream (for positive control animals):

Both cream applications were dispensed using a plastic plunger which was placed into the vaginal vault.

Vaginal biopsy for determining inflammation:

A small alligator forceps was inserted into the vaginal vault. Three mucosal pinches were taken from each animal and then placed into 10% formalin for processing into slides. Five-micron sections were made, and histopathology reviewed via microscopy.

Human vaginal fluid inoculation for modulating the microbiome:

Bacterial lavage fluids were administered intravaginally using a small catheter. The animals were kept still for 5+ minutes to allow full absorption.

Microbiome metagenomic shotgun sequencing, 12M reads:

Microbiome sequencing was performed by a commercial vendor (COSMOS ID). DNA was extracted, followed by standardized library preparation and next generation sequencing. For each animal, 4 timepoints were analyzed to start. Following the cessation of metronidazole or N-9, the first bacterial inoculation was performed. After 4 weeks, a baseline sample was taken and then the second bacterial inoculation was performed. Samples were then analyzed at weeks 2, 4 and 6 after second inoculation, or weeks 4, 6, 8, and 10 after first inoculation. Data was then plotted using

stacked bar graphs and pie charts, which are included in the appendix. Shannon diversity was also determined and plotted as bar graphs, with higher numbers representing greater diversity.

WBC isolation from endocervical and vaginal brush and lavage samples for CD45+ analysis:

See Supplementary Materials (Labeled 4). For each animal, the frequency of CD45+ white blood cells were graphed over time. Cells yields evaluated both lavage and cytobrush samples to assess which sampling type was better for future analysis. A select sequence of 5 weeks (5 samples) was used to compare sample types.

Flow cytometry for CD45+ analysis:

Cells were prepared as published (Supplementary Materials Labeled 5), and a CD45+ fluorescent antibody used. FlowJo software was used to analyze data.

2.2.2.1 Study Design

Animals from Aims I-II (baseline microbiota assessed; effect of metronidazole assessed) were split into two groups (N=6). We assessed the changes in bacterial colonization in the vagina, with bi-weekly vaginal swabs and weekly vaginal biopsies and lavages (**Figure 10**). Bacterial colonization was measured by both broad range 16S rRNA gene PCR for entire microbial community analysis, and in real time by species-specific PCRs.

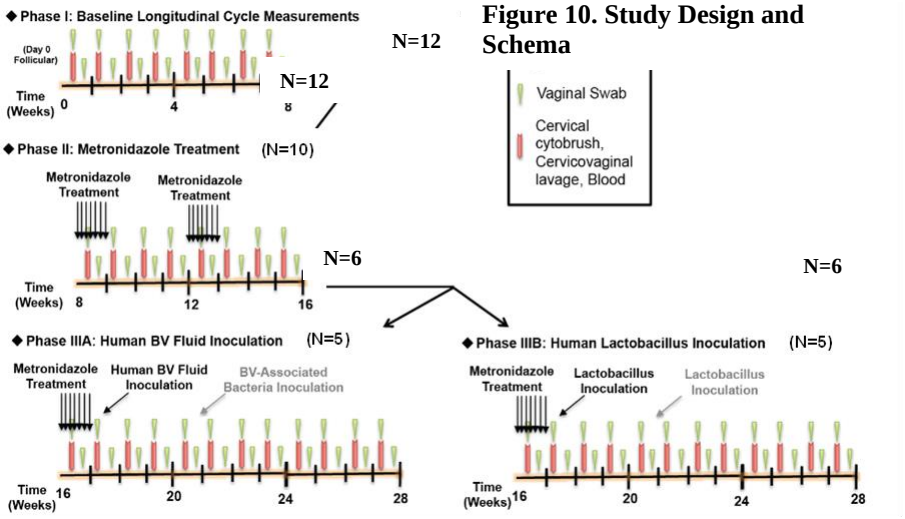


Figure 10. Study Design and Schema

Phase IIIA BV: Animals (n=6) were inoculated intravaginally with whole vaginal fluid acquired from women with clinically diagnosed BV or normal non-BV (WASH project, University of Miami), non-identifiable and only described by their clinical presentation, with Nugent scores of 7-10 and Amsel's BV positive criteria (REF), indicating BV at time of specimen collection. *Gardnerella vaginalis* and *Atopobium vaginae* have been implicated in biofilm formation in BV, and quantitation of these species predicts BV (Menard et al., 2008).

Phase IIIB *Lactobacillus*: Animals (n=6) were inoculated with human vaginal fluid predominant in *Lactobacillus* species to compare a healthy control and determine whether introduction of normal flora human bacteria versus BV-associated human bacteria alters inflammation. Previously collected whole vaginal fluid was used from women with Nugent scores of 0-3 and Amsel's BV negative criteria, and with a positive PCR for *Lactobacillus*.

2.2.2.2 Experiment 1 (Phase I). Determine the nature of fluctuations in microbial populations relative to inflammation and innate immunity longitudinally throughout the menstrual cycle. It was hypothesized that the risk of vaginal dysbiosis is greatest during the luteal phase of the menstrual cycle, when infection susceptibility is highest likely due to thinning of the mucosal

epithelium (Kersh et al., 2014; Vishwanathan et al., 2011). The nature of microbial changes over the course of the pigtail macaque menstrual cycle was determined. Increased bacterial diversity in the vaginal microbiome during the menstrual cycle is associated with increased white blood cell presence, which was assessed by flow cytometry analysis using CD45 + antibodies, CD3+ antibodies and neutrophil markers. Whether changes in bacterial species or communities could be associated with inflammation and innate immunity was determined.

The microbiome from bi-weekly vaginal swab collections over the course of two full menstrual cycles in PTM by broad-range 16S rRNA gene PCR and sequencing were sampled. Bacteria were identified to the species or genus level using a custom designed reference set of sequences performed by a commercial vendor (COSMOS ID). This method across selected time points during the study was used, demonstrating both similar and discordant bacterial communities in PTM and humans (**Fig. 8**). In preliminary studies conducted by N Klatt et al (unpublished), 18 PTM were identified with a BV-like environment (highly diverse bacteria species, with little or no *Lactobacillus;* **Fig. 8A**), while 3 PTM had a *Lactobacillus* dominated FRT, in addition to BV-associated bacteria (**Fig. 8B**), and 1 animal with *Lactobacillus* dominated without BV (**Fig. 8C**).

This preliminary data has also demonstrated an association with decreased innate inflammation in *Lactobacillus* dominated vs. BV-like vaginal microbiota in PTM using 2 cytokines as measures of inflammation, IL-1Ra and IL-8 (**Fig. 11**). However, these

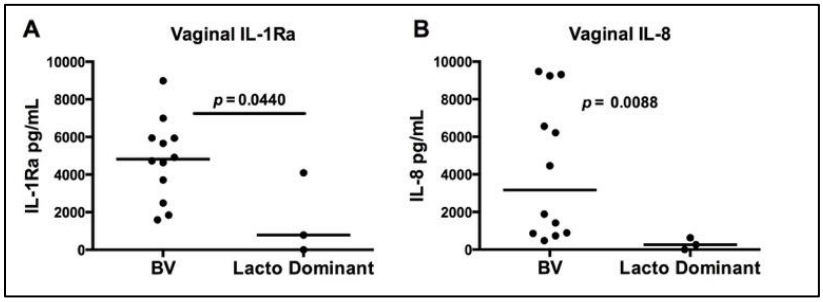


Figure 11. *Lactobacillus* dominant vaginal microbiota is associated with decreased innate inflammation, including IL-1Ra (A) and IL-8 (B).

data are from cross-sectional sampling at a single time point, and therefore longitudinal sampling is essential. This is the work we pursued for the current study.

2.2.2.3 Experiment 2 (Phase II): Determine how the macaque vaginal microenvironment is altered with antibiotic treatment.

The hypothesis developed was that the vaginal metronidazole treatment will result in microbiome alterations, with decreased concentrations of BV-associated anaerobes and increased abundance of *Lactobacillus* spp. PTM were treated with metronidazole vaginal gel for 7 days twice during the menstrual cycle, and two positive control macaques received nonoxynol-9 (which triggers inflammation and will disrupt a normal microbiome). Microbiome analysis was performed as well as basic measured determinants of WBC presence after antibiotic treatment (CD45+ by flow cytometry). After treatment, bi-weekly vaginal swabs were collected, as well as weekly CB and CVL sampling.

2.2.2.4 Experiment 3 (Phase III). Modulate macaque vaginal microbiome to reflect human microbiota.

While preliminary data demonstrates that PTM have a "BV-like" environment with diverse bacteria and little or no *Lactobacillus*, many bacteria present in the PTM FRT are not found in humans, and it is unclear if microbiota in macaques have similar potential effects on immunity or HIV susceptibility as in humans. Thus, the pigtail macaque vaginal microbiome was manipulated by introducing human microbiota to the macaque FRT. Successful modulation of the macaque microbiota to better represent the human microbiome would result in a more accurate model for pre-clinical HIV studies.

2.2.2.5 Statistical Analysis

Statistical analysis is performed only for microbiome analyses. Other data in results are descriptive in nature. Bioinformatics assessment includes the relationship with microbiota and the presence/absence of taxa, relative abundance of taxa, sequence read counts, and community profiles of bacteria derived from cluster analysis. The analyses include processing and taxonomic assignment of 16S rRNA sequence. A result of this analysis is a matrix, where entries represent counts of 16S sequences for each sample placed in the corresponding taxonomic group; richer phylogenetic representations are compared against less diverse taxa (Shannan diversity). Several hundred taxa are represented in vaginal microbial samples, only a small fraction of which are abundant and shown in the data.

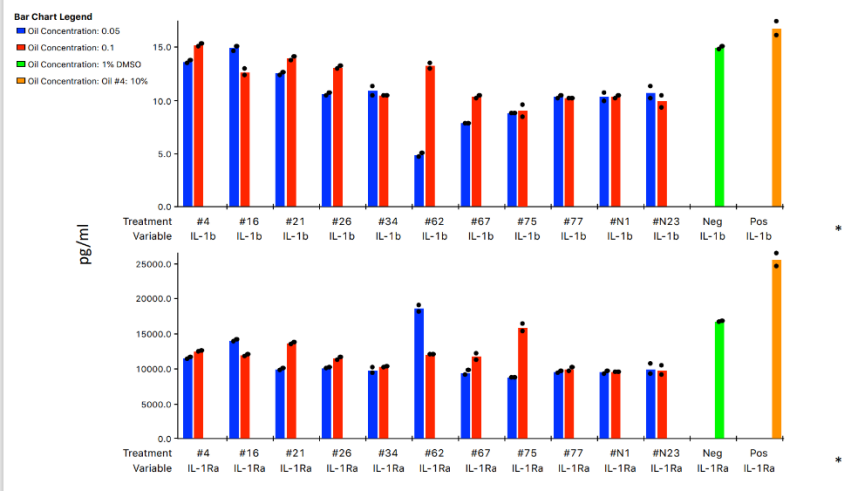
2.2.3 Results

2.2.3.1 Essential oils study in vitro.

For histopathology results, only the positive control (10% tea tree oil) shows evidence of mucosal disruption and cellular change, which is to be expected at this concentration. After consultation with the company (Mattek), they verified following review of the images that all tissues appeared to be within normal limits and none exhibited significant changes suggestive of toxicity. See Supplementary Materials (Labeled 2).

Results from the supernatant cytokine analysis demonstrates responses to all essential oils, as well as the controls (however controls were not consistent in response across the experiment). Oils which demonstrated a strong response compared to controls were considered as possible candidates for further analysis. These possible candidates included the following: EO #62 (0.05%) for IFNg; #62 and #67 (0.05%) for IL-1b; #62 (0.05%) for IL-1Ra; #62 (0.05%) for IL-6; #62 and #67 (0.05%) for IL-1a. Statistical analysis was not performed since only one experiment was conducted. However this platform has much promise as a screening tool to make decisions on candidates when hundreds of essential oils are tested at differing concentrations. See Supplementary Materials (Labeled 3), with cytokines IL-1b and IL-1Ra (**Figure 12**) .

Figure 12. Results from 2 cytokines demonstrating levels found in supernatants from Mattek platform. Oil #62 is an oil of interest for future investigation.



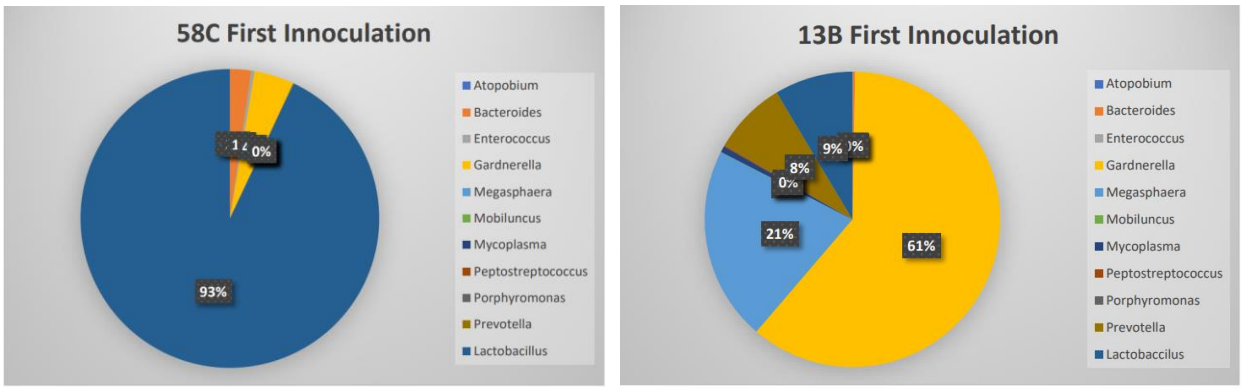
Oil #62 is garlic and appears to be a promising candidate for repeated experiments. Garlic oil has been shown in the peer reviewed literature to be effective for bacterial vaginosis, and has been used alongside other alternative therapies such as probiotics, boric acid, and yogurt (Asedi, Arezoo et al. 2022; Kalkan, Niyazi et al. 2021). Oil #67 is thyme which has been shown to have therapeutic promise as well. Thyme has been shown *in vitro* to have antimicrobial effects that require *in vivo* studies before efficacy can be proven. The experiment needs to be repeated several times to assure the controls respond reliably. However,

the consistent cytokine responses from the tissues across each oil demonstrates the usefulness of the platform for future experiments.

2.2.3.2 Vaginal microbiome evaluation (Phases I-III).

Microbiome sequencing data was compiled for each animal at 4 different timepoints described earlier. These timepoints spanned 3 phases and included baseline, antibiotic treatment, first inoculation right after antibiotic treatment, and second inoculation. Animals 65V and H38D have a missing baseline data set and therefore only have 3 sequences analyzed. Compiled data per animal is available in supplementray materials, example data shown in **Figure 13**. Of the animals in the *Lactobacillus* group, 5/6 animals showed either new or consistent colonization with a

Figure 13. Microbiome diversity shown for 58C (Lacto dominant) and 13B (BV dominant).



microbiome dominated by *Lactobacillus*. This finding does not confirm durability of the inoculation but does suggest the possibility that a degree of colonization is possible. In the BV group, the same occurred in that animals exhibited diversity and a microbiome pattern distribution similar to BV, in particular expression of bacteria such as *Gardnerella* and *Prevotella*. Of course, due to the fact that the microbiome can be affected by menstrual cycle, this variable would have

to be taken into consideration. Overall, it is evident that there is a variation in microbiome signatures dependent on the individual and sampling schema.

Another measurement which can be used to understand whether inoculation was effective is looking at microbiome diversity (Shannon diveristy index) across the two groups. BV has a hallmark of high bacterial diversity compared to the *Lactobacillus* group. As seen below in **Table 1**, the *Lactobacillus* dominant group shows a much greater number of animals with reduced diversity compared to the BV group. Although not every animal had a *Lactobacillus* dominant phenotype, the microbiome did move toward a more less diverse phenotype, suggesting some level of success minimizing a highly diverse population of "BV" organisms to expand significantly. It is of interest that the vaginal microbiomes of macaques do not commonly reveal a *Lactobacillus* dominant signature like women. Probiotic inoculations with *Lactobacillus* in macaques does show colonization, but durability lacks in most cases and returns to a more highly diverse "BV like" population. Our data recapitulated this finding in that more animals had *Lactobacillus* signatures in the LB group compared to animals in the BV group. See Supplementary Materials (Labeled 6) for individual animal microbiome results.

Shannon diversity.

Animal ID	Treatment	Human first treatment Nugent score*	Human second treatment Nugent score*	Baseline microbiome diversity	End of study diversity**
H31V control	Nonoxynol 9	0	0	1.92	2.07 (=)
58C	Lacto dominant	1	2	0.45	0.16 (D)
4D	Lacto dominant	0	2	2.46	1.94 (D)
95C	Lacto dominant	2	0	2.17	1.53 (D)
96A	Lacto dominant	3	3	2.40	0.89 (D)
24A	Lacto dominant	2	3	1.76	0.33 (D)
H38D control	Nonoxynol 9	10	7	N/A	1.69 (U)
126A	BV microbiome	8	7	0.12	2.06 (I)
17B	BV microbiome	9	10	1.45	2.68 (I)
13B	BV microbiome	10	9	2.18	2.25 (=)
69B	BV microbiome	8	8	2.00	0.57 (D)
65V	BV microbiome	9	7	N/A	1.26 (U)

Table 1. * Nugent Score: lower number suggests more *Lactobacillus* and higher number is consistent with BV.

****End of study diversity**: (D) – decreased; (I) – increased; (=) stayed the same; (U) - unknown

2.2.3.3 Vaginal biopsy and histopathology.

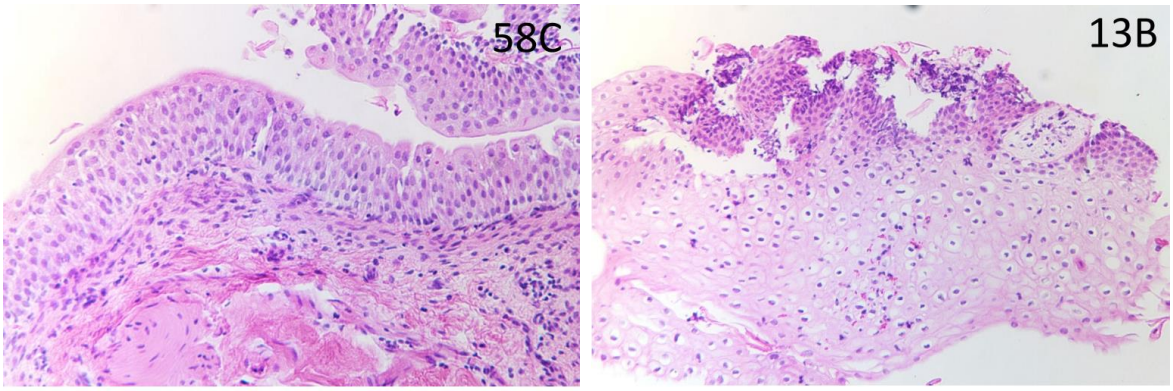
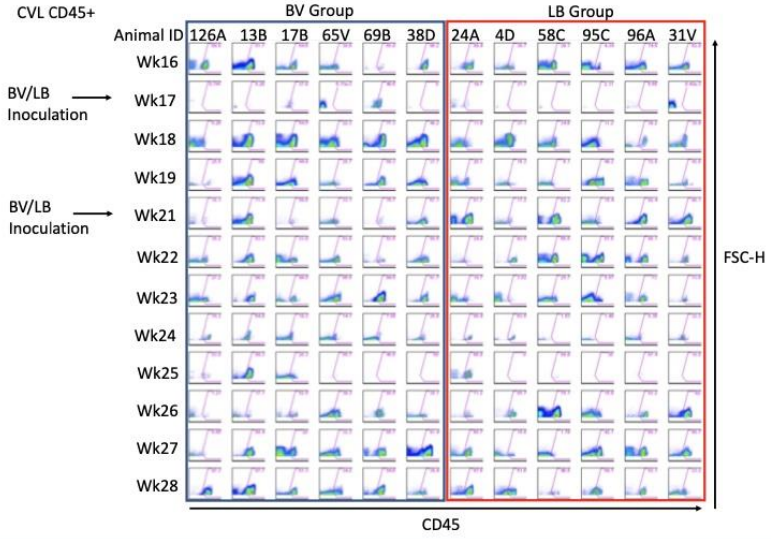


Figure 14. Biopsies from same animals in Figure 12, 20 X magnification, H&E stain at 16 wks (after antibiotic treatment at first inoculation)

Biopsies of the vaginal mucosa were performed monthly for each animal across the entire study and Phases (I-III). Images of each animal at each week were evaluated (**Figure 14**). Absence of images signifies a missing sampling point. Histopathology was relatively unremarkable across the study, given several study variables such as treatment with metronidazole or N9, menstrual cycle, and biospy sampling technique. Most images demonstrate a full thickness biopsy. In some tissues there was some evidence of mild, limited multifocal inflammation. However, this was not consistent for any animal nor conclusive for either group (Supplementary Materials Labeled 7).

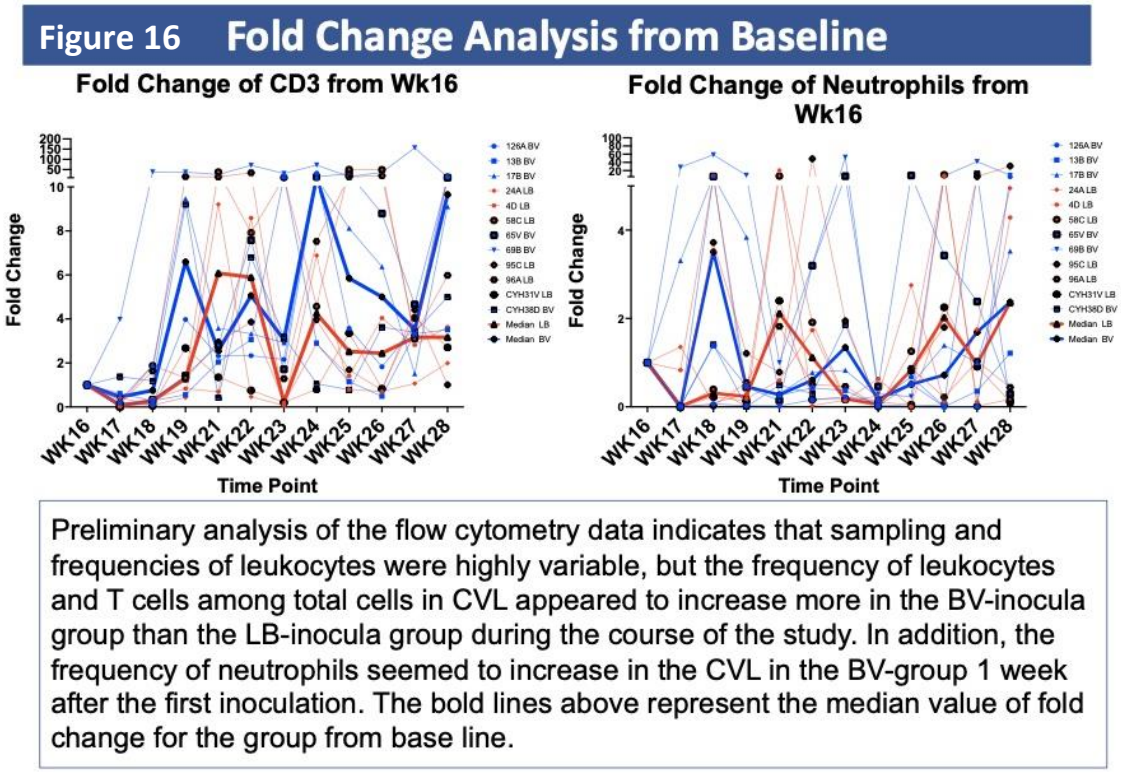
Figure 15 Flow Plots



Above are dot plots of CVL samples gated for CD45+ cells across the time course of the study. Over the course of the study the variable nature of the samples became clear, exemplified by these plots. The variability makes drawing definitive conclusions difficult often due to very low cell count or other factors such as the presence of RBC contamination from menstruation or the act of sampling itself. However CVL samples provided enough data that BV-inoculated animals appeared to have increased leukocyte and T cell populations compared to LB-inoculated animals.

2.2.3.4 CD45+ and WBC analysis via flow cytometry. Flow cytometry was used to assess how the different inocula effected the cell populations present over the course of the study. Thus far CD45+ cells, CD3+ T cells, and neutrophils have been analyzed**.** Preliminary analysis of the flow cytometry data indicates that sampling and frequencies of leukocytes were highly variable, but the frequency of leukocytes and T cells among total cells in CVL appeared to increase more in the BV-inocula group than the LB-inocula group during the course of the study (**Figure 15**). In addition, the frequency of neutrophils seemed to increase in the CVL in the BV-group 1 week after the first inoculation. The bold lines above represent median value of fold change for the group from baseline (**Figure 16**). Figures 15 and 16 are inlcuded in Poster 1, appended to the thesis.

Figure 16. CD3+ (leukocytes) and neutrophils over a 12 week period. Weeks 17 and 21 represent time of bacterial inoculations.



3. FINAL DISCUSSION AND CONCLUSIONS

Nonhuman primates, though a controversial biomedical model, provide an immense amount of information and insight into both human and animal health and disease. Regarding the female vaginal anatomy and physiology, only monkeys and apes recapitulate humans exactly, the one difference being the secondary sex characteristics such as sex skin. The reproductive physiology of the macaque does present complexities such as reproductive seasonality or lack thereof if animals are housed indoors, which is the case with the animals studied here. These complexities therefore need to be carefully considered when comparing indoor to outdoor housed animals. Otherwise, microscopic anatomy and localized physiology such as the vaginal mucosa provides a controlled *in vivo* platform for studying locally delivered therapeutics for treating infections, microbiome disturbances, and malignancies.

The essential oil experiments demonstrate that the EpiVaginal platform by Mattek has an unlimited ability to screen hundreds of compounds at different concentrations, with the capacity to study not only cytokine aberrations but also tissue histopathology and toxicity. We found that we could identify come promising oils such as garlic which is known to have many beneficial properties across many conditions, but there is an extraordinary paucity of information in the literature regarding its efficacy in vaginal health, outside of reports in vitro against *Candida* and *Trichomonas*. These studies in vitro establish a starting point for evaluating many essential oils and their important chemical components. Once safety and efficacy are established then animal studies can be initiated to evaluate further safety but most importantly efficacy against microbial challenge.

The vaginal microbiome of macaques is also complex, much more than that of humans, and represents a vast array of microbiome signatures, including to a lesser degree *Lactobacillus*

dominant and to a vast degree BV dominant (G. Spear et al., 2012; Zhu et al., 2015). There is some evidence in these studies that bacterial inoculation can have some influence on how a vaginal microbiome can be transformed, but long-term durability is questionable (Lagner, Brenchley et al. 2021). It is quite clear that animals cannot be compared to each other but must be studied longitudinally to come up with statistical conclusions. The microbial diversity across and within animals is substantial. This diversity does not recapitulate that of humans who tend to have a stable *Lactobacillus* dominant vaginal microbiota. However, the value in the NHP model is in the ability to create healthy versus diseased conditions to then evaluate inflammation and risk for viral transmission. This is the condition we wish to recapitulate from the female condition.

The study did show promise that a *Lactobacillus* dominant microbiome could be created in a macaque model, which naturally resembles a BV-like microbial diversity. *Lactobacillus* signatures are very uncommon in macaques (Lagner, Brenchley et al. 2021), yet we did show that out of 6 animals we could still demonstrate a high number of individuals with *Lactobacillus* abundant signatures.

Lastly, we were also able to demonstrate that leukocytes were isolated in adequate numbers to establish curves of quantities across the menstrual cycle and treatment regimen. More in-depth flow cytometric analyses could provide more correlative evidence associating microbial signatures and inflammation with risk assessment for increased pathogen transmission across the vaginal mucosa.

The ability to develop remedies that obviate the use of anti-microbials in reproductive health is a critically important initiative and these studies establish the early evidence that alternatives are available and cautiously beneficial. The nonhuman primate model will continue to show promise as a translational model for female reproductive health and disease.

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5. SUPPLEMENTARY MATERIALS (1-7)

1. Mattek EpiVaginal data sheet
2. Essential Oils Mattek vaginal histopathology for each oil
3. Essential Oils cytokine data from Mattek supernatant for each oil
4. Mononuclear leukocyte isolation from endocervical cytobrushes protocol
5. %CD45 results by study week and menstrual cycle
6. Individual microbiome signatures by group for each macaque
7. Vaginal histopathology results and images by study week and treatment group

6. PUBLISHED MANUSCRIPT

6.1 Preliminary Assessment of the Mucosal Toxicity of Tea Tree (Melaleuca alternifolia) and Rosemary (Rosmarinus officinalis) Essential Oils on Novel Porcine Uterus Models

POSTER

6.2 Keystone Global Priorities in Vaginal Health, Senior Author, 2021

7. ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals for their assistance, dedication, and support during the writing of this thesis**: Drs. Maria Laura Bacci (my mentor), Domenico Ventrella, Alberto Elmi, Francesca Barone, and the rest of the department of Veterinary Physiology.**

I would also like to thank my collaborators at the Vaccine Research Center and Texas Biomedical Research Institute for their assistance in my work along the years: **Kathryn Foulds, Mario Roederer, Elizabeth McCarthy, Dillon Flebbe, Saran Bao, Martha Lykes, the Division of Veterinary Resources at the NIH, and all other colleagues that have contributed their time and effort during this journey.**

Lastly, I would like to thank my parents, sister, and partner for always supporting me and understanding the time away from the family. Your love and commitment to this journey will never be forgotten.