A Description of the THRIVE (The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments) Bacterial Vaginosis Observational Study

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ABSTRACT

Objectives: Bacterial vaginosis (BV) contributes to poor reproductive health and is characterized by a displacement of *Lactobacillus* in the vaginal microbiome. However, treatment for BV is limited to antibiotics and half of the women treated experience recurrence within a year. THRIVE (The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments) is a prospective study in Winnipeg, Manitoba, Canada, which is designed to capture the daily variation of the microbiome and host mucosal immunity during treatment. The objective of this study is to identify host and bacterial factors that associate with vaginal microbiome stability to better inform therapeutic interventions.

Methods: Women treated for BV, and controls, are followed for 6 months collecting daily vaginal swabs and monthly questionnaires. Comprehensive mucosal sampling, including swabs, cytobrushes, biopsies, and blood are collected at baseline, months 1 and 6 post-enrolment.

Results: We performed analysis on the first 52 participants, (19 BV+, 33 BV−). Molecular profiling by 16s RNA sequencing showed 20 women with non-*Lactobacillus*-dominant microbiomes and 32 with *Lactobacillus*-dominant microbiomes, with increased microbial diversity in non-*Lactobacillus*-dominant microbiomes ($P = 3.1E-05$). A pilot analysis in 2 participants demonstrates that multi-omics profiling of self-collected daily swabs provides high-quality data identifying 73 bacterial species, 1773 mucosal proteins and 117 metabolites. Initial flow cytometry analysis showed an increased cluster of differentiation (CD)4+ T cells and neutrophil activation ($CD11b+CD62L^{neg/dim}$) in the positive participant at baseline, while after treatment these shifted and resembled the control participant.

Conclusions: This study provides a framework to comprehensively investigate the kinetics of vaginal mucosal microbiome alterations, providing further insight into host and molecular features predicting BV recurrence.

Keywords: bacterial vaginosis; cohort studies; microbiome; mucosal immunity; reproductive health

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RÉSUMÉ

Résumé : La vaginose bactérienne contribue à une mauvaise santé reproductive et se caractérise par un remplacement des lactobacilles dans le microbiome vaginal. Cependant, le traitement de la vaginose bactérienne se limite aux antibiotiques et la moitié des femmes traitées connaissent une récidive dans l'année qui suit.

Objectifs : THRIVE (The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments) est une étude prospective menée à Manitoba, Canada et conçue pour mesurer les variations quotidiennes du microbiome et de l'immunité

muqueuse de l'hôte pendant le traitement. L'objectif de cette étude est de déterminer les facteurs hôtes et bactériens qui sont associés à la stabilité du microbiome vaginal afin de mieux orienter les interventions thérapeutiques.

Méthodes : Les femmes traitées pour une vaginose bactérienne, ainsi que les témoins, sont suivies pendant 6 mois au moyen de prélèvements vaginaux quotidiens et de questionnaires mensuels. Une analyse détaillée des muqueuses a été effectuée au recrutement puis à 1 et 6 mois après le recrutement par divers prélèvements, à savoir par écouvillon, cytobrosse, biopsie et prise de sang.

Résultats : Nous avons effectué une analyse auprès des 52 premières participantes (19 VB+, 33 VB−). Le profilage moléculaire par séquençage de l'ARN 16S a montré que 20 femmes avaient un microbiome à dominance non-lactobacille et 32 avaient un microbiome à dominance lactobacille, la diversité microbienne étant accrue dans les microbiomes à dominance non-lactobacille ($P = 3{,}1E\text{-}05$). Une analyse pilote sur 2 participantes démontre que le profilage multiomique d'écouvillonnages quotidiens autoadministrés fournit des données de haute qualité permettant d'identifier 73 espèces bactériennes, 1773 protéines des muqueuses et 117 métabolites. L'analyse initiale par cytométrie en flux a montré une élévation des cellules T CD4+ et une activation des neutrophiles (CD11b+CD62L$^{\text{nég/faible}}$) chez la patiente positive au recrutement, alors qu'après le traitement, ces cellules ont changé pour finalement ressembler à celles de la participante témoin.

Conclusion : Cette étude donne un cadre pour étudier de manière exhaustive la cinétique des altérations du microbiome de la muqueuse vaginale, ce qui permet de mieux comprendre les caractéristiques de l'hôte et les caractéristiques moléculaires prédisant la récidive de la vaginose bactérienne.

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INTRODUCTION

Bacterial vaginosis (BV) is experienced in 23%−29% of women globally,[1] and causes detrimental effects on a female's quality of life physically, emotionally, sexually, and socially.[2] Clinical symptoms of BV include watery, white or grey vaginal discharge, vaginal discomfort/itchiness, and a strong fish-like odour.[3] BV is commonly diagnosed using a combination of clinical symptoms and laboratory tests such as Amsel's criteria[4] or molecular diagnostic tests.[5] A Nugent score capturing bacterial morphotypes using microscopy to determine a simplified ecology of the microbiome species present in a sample with a standard Gram stain is rarely used due to the time it takes to receive results.[6] However, Nugent-BV is used particularly in epidemiology research to define BV in large cohort studies and is associated with adverse reproductive health outcomes (such as pre-term birth, miscarriages, and premature rupture of membranes), as well as increased sexually transmitted infections (STIs) and cervical cancer.[7−10] Though clinically defined BV is not associated with overt inflammation causing redness or pain, subclinical inflammation with increased pro-inflammatory cytokines and chemokines is observed and associated with up to a 4-fold increase in risk in HIV acquisition.[7] BV treatment is often limited to the use of metronidazole or clindamycin, and clearance or reduction of BV is often transient with approximately 50% of women experiencing recurrence within 1 year post-treatment.[11] The development of better treatment options would significantly benefit female health, but the etiology and mechanisms underlying the development of BV remain poorly understood, limiting our ability to design interventions that extend the longevity of symptom resolution.

Molecular tools (16S ribosomal RNA sequencing, metagenomics, metaproteomics) provide classifications of microbial communities which are defined by the predominant bacterium and allow researchers to better define the communities based on the sequencing tools used.[12] Common patterns seen in vaginal microbial communities are dominance by individual species, or pairs of species of lactobacilli such as *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii* and others.[13] While heterogeneity exists, a *Lactobacillus*-dominant (LD) vaginal microbiome is considered optimal because of its ability to generate a pathogen-limiting environment through the generation of bacteriocins, hydrogen peroxide, and lactic acid which lowers the vaginal pH[14] and has antimicrobial and immunomodulatory effects.[14] The presence of "non-optimal" vaginal microbiota such as *Gardnerella*, *Fannyhessea*, *Prevotella* and *Mobiluncus* is classified as Molecular-BV.[12] Molecular-BV is strongly correlated with clinically defined BV through Amsel's criteria (Amsel's BV) or Nugent-BV, however, current research has shown that only a fraction of BV is symptomatic. There are instances of asymptomatic Amsel's BV captured in clinical trials which would not be presented in a normal clinical setting. These asymptomatic BV cases are also medically relevant as the subclinical inflammation associated with increased STI risks and poor reproductive health outcomes is also associated with a non-*Lactobacillus*-dominated (nLD) microbiome. To complicate matters certain *Lactobacillus* species, such as *L. iners*, do not have some of the protective properties as other lactobacilli species, indicating not all *Lactobacillus* are equal in their molecular functional profiles. Therefore, a better understanding of the molecular factors that contribute to poor health outcomes is

necessary to define diagnostic tools targeting individuals needing treatment irrespective of symptoms.

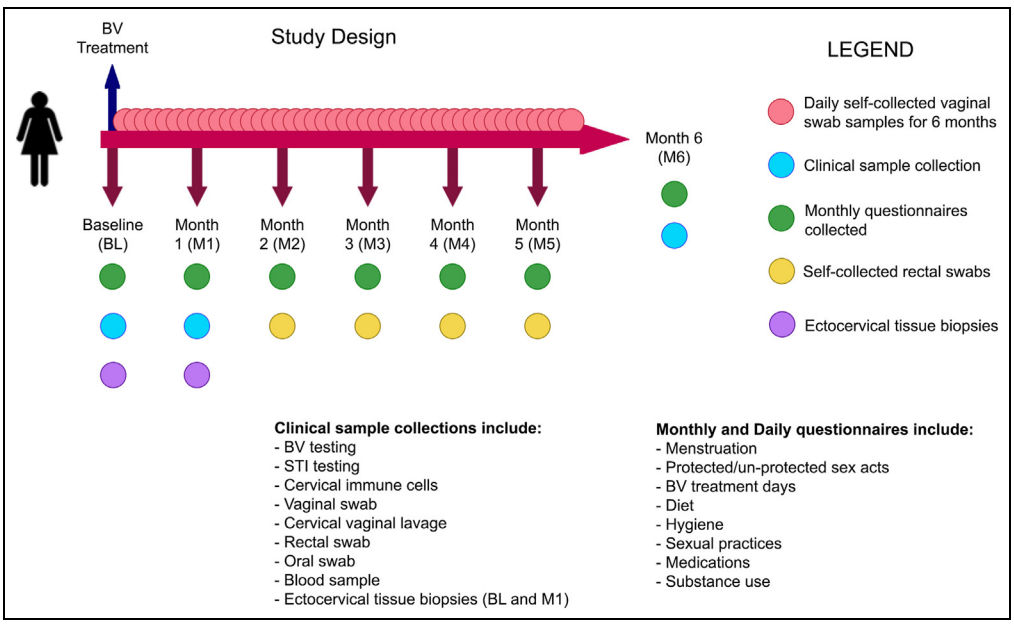
The THRIVE study ("The study of Host-bacterial Relationships and Immune function in different Vaginal Environments") is designed to characterize the dynamics and variation of the vaginal microbiome with high sampling frequency, alongside comprehensive mucosal immunity profiling, behavioural, medical, substance use, and dietary information. The objective of this study is to identify molecular drivers of microbiome stability which will provide mechanistic insight into factors that affect treatment of BV. THRIVE is designed to clarify bacterial functions and microenvironmental changes that underlie symptomatic and asymptomatic BV profiles. Understanding these mechanisms will allow us to investigate novel therapeutic targets aimed at shifting the vaginal microbiome to an optimal state. In this paper, we present a detailed description of the THRIVE study cohort with preliminary results and analysis strategies that will be utilized once we have reached our target enrolment.

METHODS

Study Design

The THRIVE sample collection study design is illustrated in Figure 1. Females (women assigned as female at birth) between the ages of 18–55 years with and without BV are recruited into the study and followed over 6 months. Exclusion criteria include menopausal women (those not menstruating for over a year), pregnancy, or women with a hysterectomy. Participants collect daily vaginal and monthly rectal swabs which are stored without any buffer (as per optimization protocols for omic samples described previously[15]) at -20°C before being brought to the lab at monthly intervals. Participants also complete detailed monthly questionnaires and daily diaries. Clinical visits occur at baseline (BL), 1 month (M1), and 6 months (M6) after enrolment. Samples collected during clinical visits include cytobrushes for flow cytometry, cervical biopsies, blood (hormone levels, peripheral blood mononuclear cells and serum), vaginal, rectal, and oral swabs. Participants are considered BV+ in the clinic using Amsel's criteria and treated. Treatments varied; however, most participants were prescribed oral metronidazole for 7–14 days, with some also using vaginal creams (dalacin, clindamycin) beyond this timeframe. As an observational study, treatments are provided based on a history of BV. However, we aim to recruit enough participants to have sufficient power to look at oral and vaginal antibiotics in a sub-analysis. Our major study outcome is to capture underlying molecular drivers of recurrent BV, where the microbiome switched back to a BV-like environment irrespective of the treatment provided. The M1 visit occurs after these treatment

Figure 1. THRIVE study design. Women with and without BV were followed for 6 months, with BV treatment provided at the enrolment visit if needed. Comprehensive sampling is performed during clinical visits at BL and months 1 and 6, including the collection of cervicovaginal rectal, oral and blood samples. Participants also collected daily vaginal swabs throughout the study and monthly rectal swabs. Cervical biopsies are collected pre- and post-treatment (BL and M1).



BL, baseline; BV, bacterial vaginosis; M1, month 1; THRIVE, The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments.

Table. Clinical, behavioural, and demographic characteristics of THRIVE study participants at the baseline enrolment visit

Cohort (N = 52)				
Variables		BV negative control group (n = 33)	BV-positive treated group (n = 19)	*P* value
Average age (range)		32.6 (20–49)	31.6 (22–48)	0.812
Ethnicity	White	25 (74%)	12 (63%)	0.696
	Aboriginal	1 (3%)	1 (5%)	
	Metis	4 (12%)	1 (5%)	
	Filipino	0 (0%)	1 (5%)	
	Latino	1 (3%)	1 (5%)	
	South Asian	1 (3%)	1 (5%)	
	Southeast Asian	0 (0%)	1 (5%)	
	Chinese	1 (3%)	0 (0%)	
	Black	1 (3%)	1 (5%)	
Gram stain results	Positive	0 (0%)	13 (68%)	**1.12E-10**
	Negative	31 (94%)	2 (11%)	
	Indeterminant	2 (6%)	4 (21%)	
Whiff test	Positive	0 (0%)	18 (95%)	**4.45E-13**
	Negative	33 (100%)	1 (5%)	
Nugent score		0.303 (0–4)	6.74 (0–10)	**5.22E-09**
Vaginal pH		4.52 (4.0–6.5)	5.54 (4.4–7.0)	**1.68E-09**
Microbiome dominance (16S sequencing)	*Lactobacillus*	32 (97%)	0 (0%)	**2.62E-13**
	non-*Lactobacillus*	1 (3%)	19 (100%)	
Microbiome dominance (MS metaproteomics)	*Lactobacillus*	30 (97%)	1 (6%)	**1.24E-10**
	non-*Lactobacillus*	1 (3%)	16 (94%)	
Previous BV	No	11 (33%)	2 (11%)	0.181
	Do not know	2 (6%)	2 (11%)	
	Yes	20 (61%)	15 (79%)	
Number of partners in last month		1 (0–3)	1.1 (0–3)	0.792

One BV+ participant was considered BV indeterminant via Nugent score at study enrolment but was treated for BV 2 weeks later. This individual was included in the BV+ group. Birth control and sexual behaviour data were collected based on frequency engaged within the past month prior to the questionnaire. Bold faced *P* values indicate significant differences between comparison groups.

BV: bacterial vaginosis; MS: mass spectrometry.

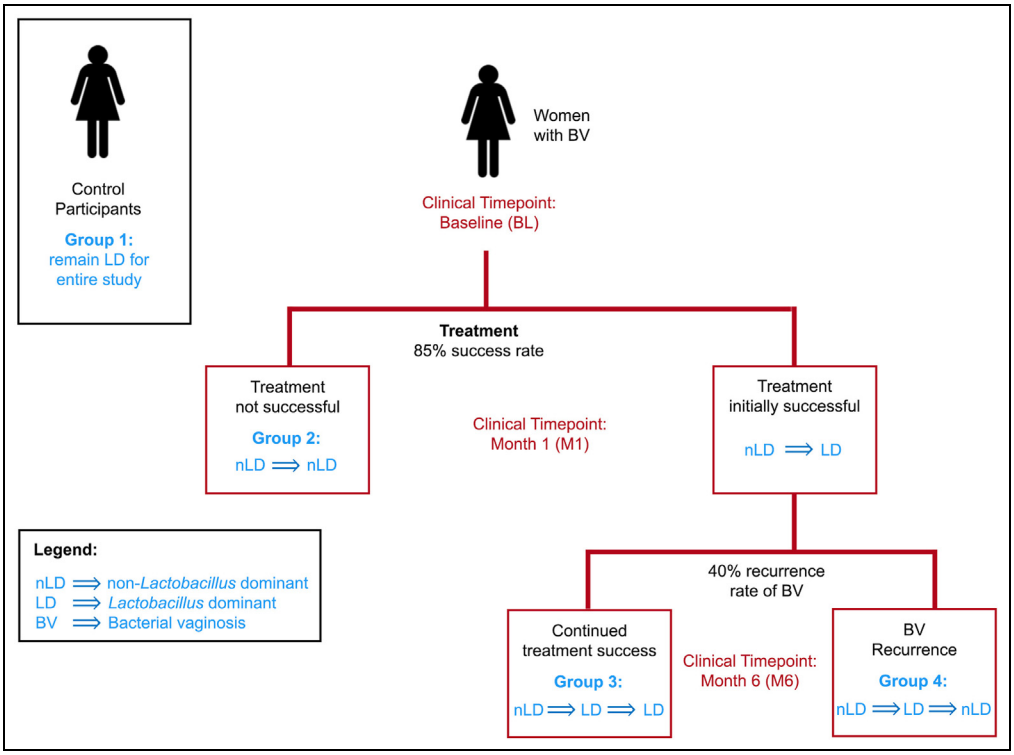
regimens have been initiated, ensuring the capture of vaginal microenvironmental changes during treatment. Participants are tested for STIs (chlamydia and gonorrhea) and BV Nugent scoring at each clinical visit. Information on previous STIs was collected in the questionnaire (Table). Currently, only 1 participant had a positive STI result (at BL; chlamydia) and was appropriately treated.

Primary Outcome and Covariate Assessment

We aim to characterize the stability and temporal dynamics of the vaginal microbiome by comparing microbiomes of women who had successful treatment of BV and did not experience recurrence, in comparison to those who experienced recurrence. According to previous literature,[11] initial treatments of clinically diagnosed BV are expected to be successful in approximately 85% of women, and 40% of those successfully treated will experience recurrence within 6 months. We used this information to generate our target study size of 115 women with BV which will provide at least 20 individuals for each comparison group (Figure 2). Currently, this estimation is proving accurate. For the first 12 BV+ women, 10 symptomatically responded to treatment (83%) and 4 of those had recurrent BV symptoms by M6 (40%).

For fold difference detection estimates and adjusted alpha level, we assumed a minimum sample size of 20 per group and estimated the coefficients of variation, number of measured taxa and bacterial proteins, immune cells, and metabolites from our previous multi-omics data analyses

Figure 2. Expected cohort grouping. According to previous literature, initial treatment of clinically diagnosed BV is expected to be successful in approximately 85% of women, and 40% of those successfully treated will experience recurrence within approximately 6 months. We used this information to generate our expected comparison groups, illustrated here. To enrol at least 20 participants per group at approximately 85% initial success rate of treatment for BV, and an expected 40% recurrence of BV after treatment, we will aim to recruit a total of 115 women with BV who are treated within the first month of the study.



BV, bacterial vaginosis.

and pilot data from the THRIVE cohort. All power calculations are performed using pwr (v1.3-0) package in R. Behavioural questionnaires completed at each study visit will be used to provide associations between topics such as vaginal hygiene practices or sexual practices, BV, and the microbiome using Fisher exact test, chi-squared test, Mann-Whitney-Wilcoxon test and Kruskal-Wallis tests as applicable. We will also use unsupervised hierarchical clustering for generating heatmaps of factors associated with the vaginal microbiome or clinical BV status.

A major outcome variable will be LD versus nLD microbiomes as measured using sequencing assays including both 16S sequencing (bacterial composition) and mass spectrometry (MS) (metabolically active bacteria species). Methods of microbiome sequencing, host proteomics and metabolomics can be found in the Supplementary Methods and are similar to our previous publications.[15–17] To determine microbiome groups, we use R programming software to calculate the distance matrix between samples using Euclidean distance, then perform complete-linkage hierarchal clustering on the distance matrix to assign microbiome groups from the resulting dendrogram. Euclidean distance is the distance between 2 points on a plane, and in our data, this indicates how similar in bacterial composition 2 samples are, using bacterial proportions in each sample. Those samples that are measured with the shortest Euclidean distance between the samples are considered the most similar. Using this grouping, we can determine the differences between host and microbiome factors and compare them with factors that are associated with clinically diagnosed symptomatic BV in our cohort. Other analyses can be performed once we have enough power, including comparing individuals who are successfully treated with those who do not respond to treatment.

RESULTS

Study Recruitment

Women 18+ years attending various clinics in Winnipeg are asked to participate in the study during scheduled appointments, or by calling into Women's Health Research Support Unit advertised with posters. Recruitment started

in December 2018, was put on hold in the spring of 2020 due to the COVID-19 global pandemic, and relaunched in June 2021. This manuscript focuses on describing the cohort and showing preliminary data collected from individuals recruited prior to the pandemic.

Cohort Characteristics

The clinical, behavioural, and demographic characteristics are summarized in Table, with an expanded table to include behavioural characteristics in Supplementary Table 1, including 19 women with and 33 women without BV. Most women (71.1%) self-identified as Caucasian. BV+ women had a similar age range, sexual behaviours, smoking habits, hormonal contraceptive use, vaginal hygiene practices, concurrent STIs, and other characteristics to those without BV. BV+ women were more likely to have a higher vaginal pH and an nLD microbiome compared to women without BV. A total of 54 women were enrolled into the study pre-pandemic, and of these 19 were lost to follow-up for reasons including painful routine vaginal exam and decided against enrolment (n = 2; no data collected), some who moved away (n = 1), moved into rehab (n = 1), scheduling conflicts (n = 9), or no longer responded to emails and phone calls from the study nurses (n = 6). We aim to address loss to follow-up in future recruitment by offering different regimes of sample collection, collecting less frequently (every other day, 3 times a week) if sample collections become too onerous. In addition, participants may opt out of certain sample collections, such as blood draws or biopsies, if these will impede follow-up visits. However, investigation of withdrawal rates up to date between controls and BV+ individuals using the Fisher exact test showed no significant differences. Overall, 11/33 (33%) BV− participants withdrew, while 6/19 (32%) BV+ participants withdrew.

Vaginal Microbiome Profiling of Study Participants

We performed MS analysis to assess vaginal microbial composition and function using cervicovaginal lavage specimens collected at enrolment. In total, 46 BL samples passed quality controls and several microbiome groups were identified based on the predominant bacterial taxa (Figure 3A, Supplementary Figure 1). The main microbiome groups defined by unsupervised hierarchical clustering included those dominated by *Gardnerella* (21.7%), *L. iners* (30.4%), and a group that was a non-species-specific *Lactobacillus*-dominant group (34.8%). In addition, there were 2 smaller groups, one which was a *Gardnerella/Lactobacillus* co-dominant profile (4.3%) and the other having a polymicrobial microbiome (8.7%). All but 1 participant with an nLD status (microbiome groups 1, 3, and 5) also tested positive for BV and was treated at BL.

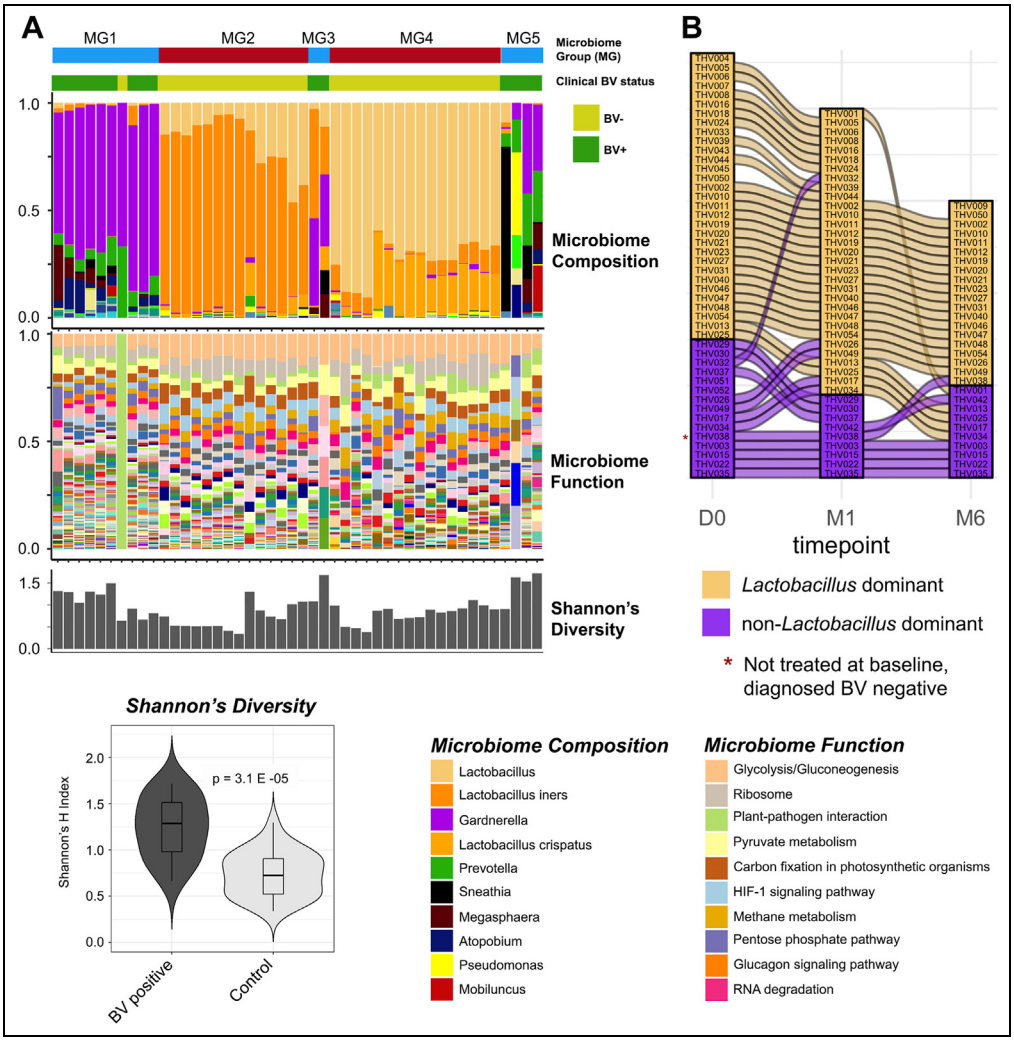
Shannon's diversity was calculated between the clinically determined BV+ (median = 1.29) and BV− groups (median = 0.73) and was found to be significantly more diverse in the BV+ group (P = 3.1E-5). Bacterial proteins were used to annotate bacterial functions based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Bacterial functions significantly increased in the BV+ group including biosynthesis of ansamycins, adipocytokine signalling pathway and vitamin B6 metabolism (adj. P = 2.2E-5). The top significant bacterial functions increased in the control group included methane metabolism (adj.P = 2.41E-5) and fructose and mannose metabolism (adj.P = 6.3E-5).

We then identified microbiome compositional changes using 16S sequencing at BL and follow-up visits (Figure 3B). One participant who had an nLD status was not diagnosed with BV at BL, however, she was diagnosed with BV at M1 and subsequently treated successfully (red asterisks in Figure 3B). Of those who received treatment at BL, we had follow-up visit information for 12 women, 42% (n = 5) who went from nLD to LD, while 33% (n = 4) women stayed nLD. Analysis of how this switchover correlates to the resolution of symptoms is ongoing.

Self-Collected Vaginal Swab Samples Capture Molecular Changes During BV Treatment

We acquired a total of 4817 self-collected swabs from 35 study participants over the 6-month enrolment period. We evaluated the quality of self-collected vaginal swabs in 2 study participants (THV001, THV002) collected over their first month post-enrolment (Figure 4A, Supplementary Figure 2). We measured over 1700 host proteins, 117 metabolites, and 213 bacterial genera with 73 species-level identifications, and 139 KO-level functions were annotated to bacterial proteins. THV001 was BV+ at enrolment and started metronidazole treatment during her first month in the study which was delayed due to personal reasons. However, her treatment corresponded with a switch in microbiome composition (nLD to LD) shortly after she started it (Figure 4A). The control participant THV002, in comparison, showed a consistent microbiome composition over time. Concurrent changes in the functional microbiome, host proteome and vaginal metabolites were also observed following treatment for THV001. Top bacterial strains, bacterial functions, host proteins and metabolites significantly different after treatment were assessed by comparing the average expression prior to treatment start (n = 14 Days) and the average expression after treatment

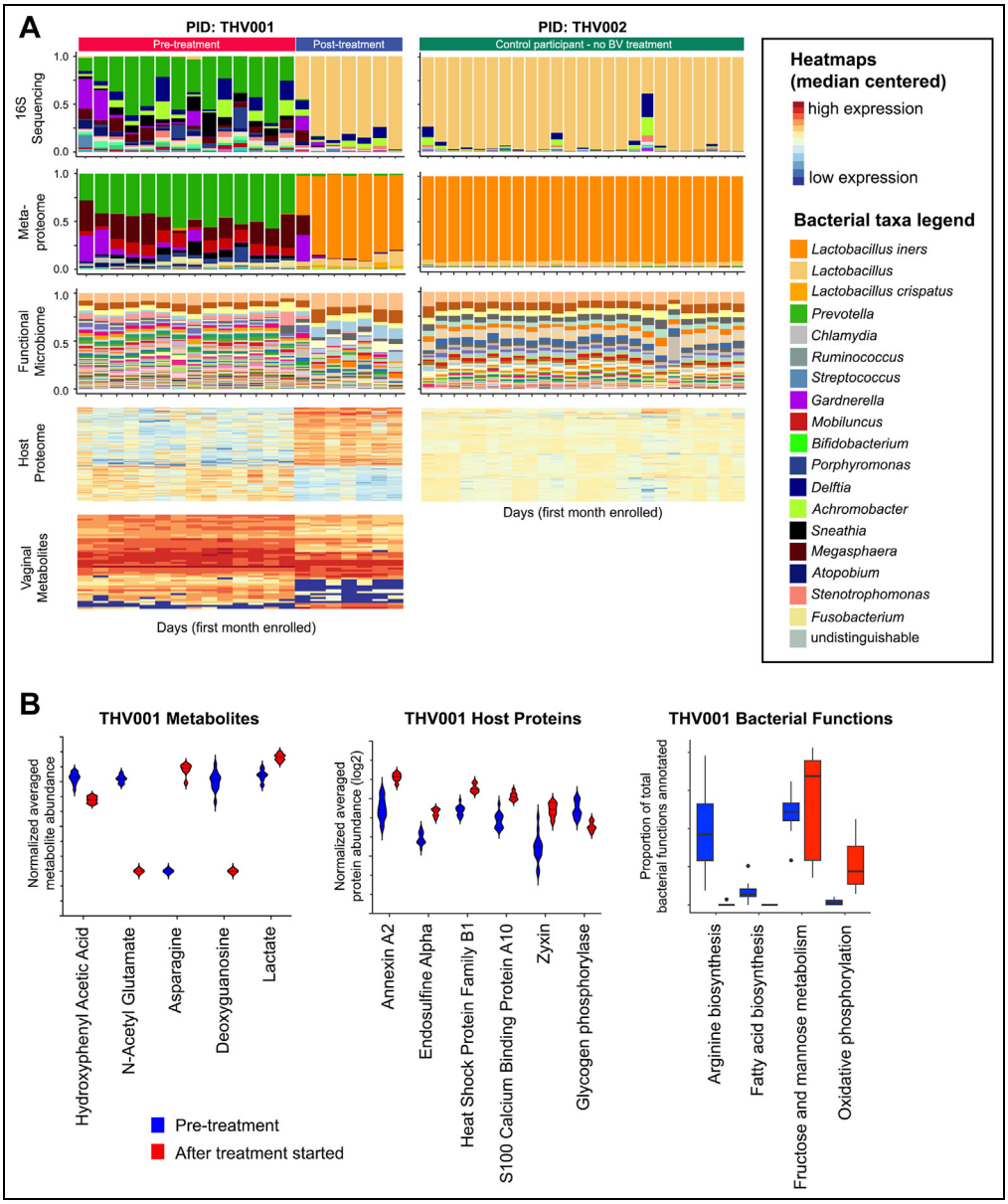
Figure 3. Vaginal microbiome analysis using mass spectrometry of THRIVE participants. (A) Baseline samples of 46 participants in the THRIVE study. Hierarchical clustering grouped participants into 5 main MG, with 3 groups having 5+ participants. The groups are MG1: *Gardnerella* dominant (n = 10), MG2: *L. iners* dominant (n = 14), MG3: *Gardnerella*/*Lactobacillus* co-dominant (n = 2), MG4: *Lactobacillus* dominant (n = 16), MG5: polymicrobial (n = 4). Shannon's diversity indicated significantly more diverse bacterial profiles for women with clinically positive BV diagnosis compared to the control group (*P* = 3.5E-5). Bacterial proteins were also used to annotate bacterial functions based on KO-level KEGG pathway analysis. (B) Microbiome composition using 16S sequencing was performed on the baseline and follow-up clinical visit samples (Baseline = D0, month 1 = M1, month 6 = M6). This identified women who experienced recurrence over the course of the study, or other microbiome switchover events. Data is shown as *Lactobacillus* dominant (yellow) or non-*Lactobacillus* dominant (purple) groups. The top 10 bacteria taxa and microbiome functions by abundance are shown in the legend. A full legend is shown in Supplementary Figure 1.



BV, bacterial vaginosis; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; M, month; MG, microbiome groups; THRIVE, The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments.

(n = 7 days), to account for sampling and biological variability. Bacterial functions that decreased after treatment started included arginine biosynthesis (−6.6 Log2 fold change [FC]) and fatty acid biosynthesis (−8.2 FC), while bacterial functions that increased included fructose and mannose metabolism (0.1 FC) as well as oxidative phosphorylate on (3.8 FC) (Figure 4B). Most significant host proteins changed in abundance included endosulfine alpha (0.4 FC), S100 calcium-binding protein A10 (0.3 FC), zyxin (0.7 FC), annexin A2 (0.4 FC), heat shock protein family B1 (0.3 FC) and glycogen phosphorylase (−0.3 FC) (Figure 4B). Most significant metabolites changed in abundance included hydroxyphenyl acetic acid (−0.3 FC), n-acetyl glutamate (−2.99 FC), asparagine (4.3 FC), deoxyguanosine (−2.88 FC), and lactate (0.21 FC) (Figure 4B).

Figure 4. Multi-omic analysis (16S sequencing, proteomics, metaproteomics, metabolomics) on daily vaginal swabs self-collected by THRIVE participants. (A) Daily swabs collected by participants 001 (BV+) and 002 (control, BV−) from their first month of enrolment were eluted and used for multiple assays to determine the viability of sample processing protocols and self-collections. (B) Metabolomics and host proteins pre- and post-treatment were analysed for significant changes in the mucosal fluid and the top 5 factors for each analysis are shown here. The top bacteria taxa by abundance for both participants are shown in the legend. A full legend is shown in the Supplementary Figure 2.

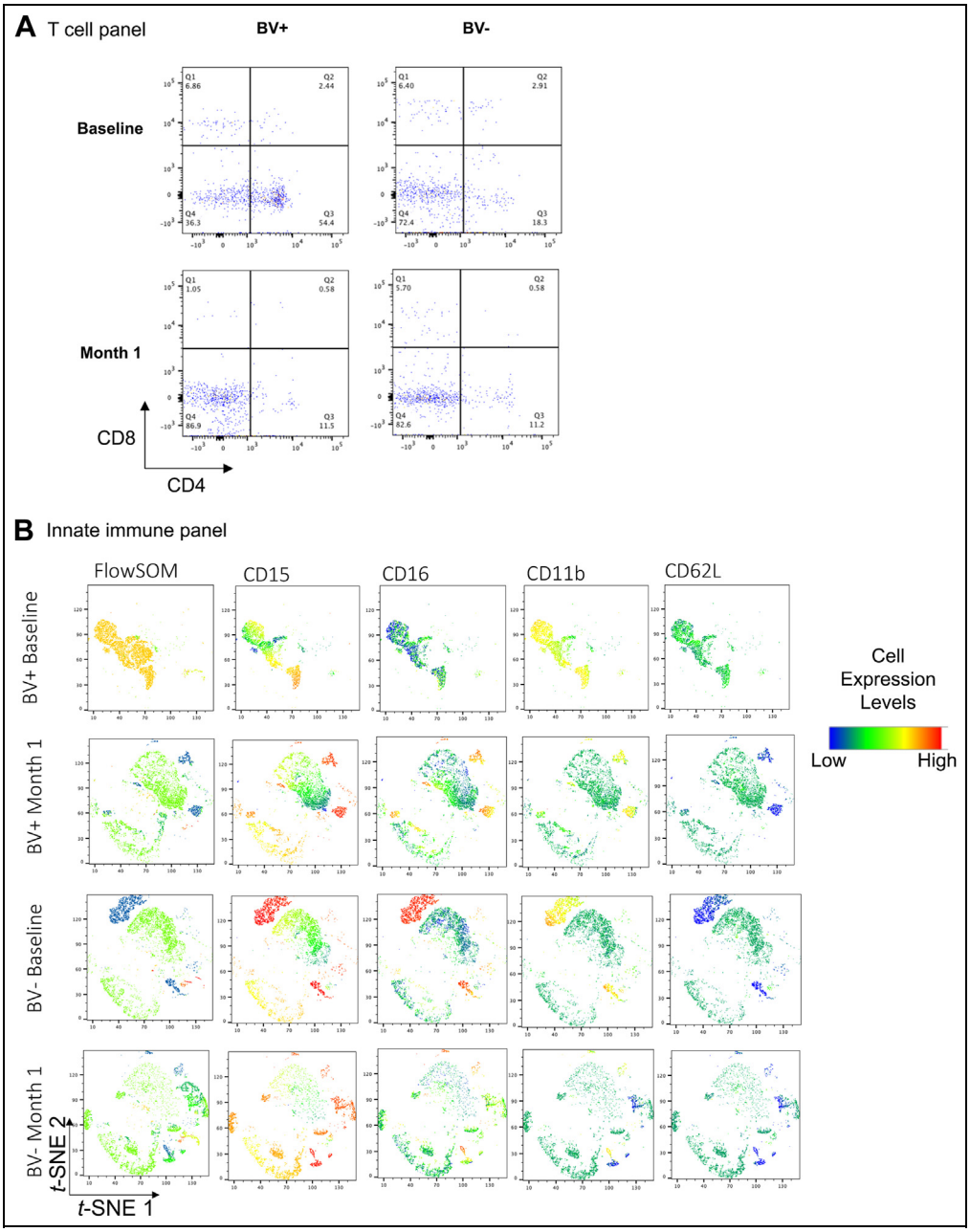


BV, bacterial vaginosis; THRIVE, The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments.

BV Treatment Induces an Immune Profile That Resembles That of an Untreated, Control Participant

We performed real-time flow cytometry on samples collected during the clinical visits and performed a preliminary analysis on 2 participants. Figure 5A shows the T cell panel comparing cluster of differentiation (CD)4+ (helper T cell) and CD8+ (cytotoxic T cell) marker expression. Supplementary Figure 3 shows the gating strategy for analyses and Supplementary Table 2 shows the antibodies used for both panels. At BL, the majority of T cells detected in the BV+ participants were CD4+ (54.4%). After BV treatment there was a shift in the T cell subsets with the majority double negative T cells (86.9%), resembling the control. Figure 5B shows the *t*-distributed stochastic neighbour

Figure 5. Example immune profiles of 1 BV+ participant and 1 control (BV−) individual at study enrolment and M1. (A) Live, CD3+ cells were gated and cell numbers were normalised between samples before examining the expression of CD4 (helper T cells) and CD8 (cytotoxic T cells). (B) Live cells were gated, and cell numbers were normalised between samples. Samples were concatenated then *t*-SNE and FlowSOM were run. CD3+CD19+CD56+ cells were gated out and FlowSOM populations and expression of neutrophil associated markers were examined. FlowSOM is an algorithm that organises high dimensional flow cytometry data using a Self-Organizing Map (SOM) based on the similarity of marker expression.[18] Eight populations were identified in the data and each colour represents one of these populations. CD15 (a marker of granulocytes), CD16 (a marker of neutrophils), CD11b and CD62L (markers of neutrophil activation), with expression levels across different *t*-SNE islands shown as a gradient from low (blue) to high (red). The cell composition of both the T cells and neutrophils was different at baseline between a BV+ and BV- participant. However, after BV treatment the cell composition of the BV+ participant resembled the BV- participant more than their initial visit.



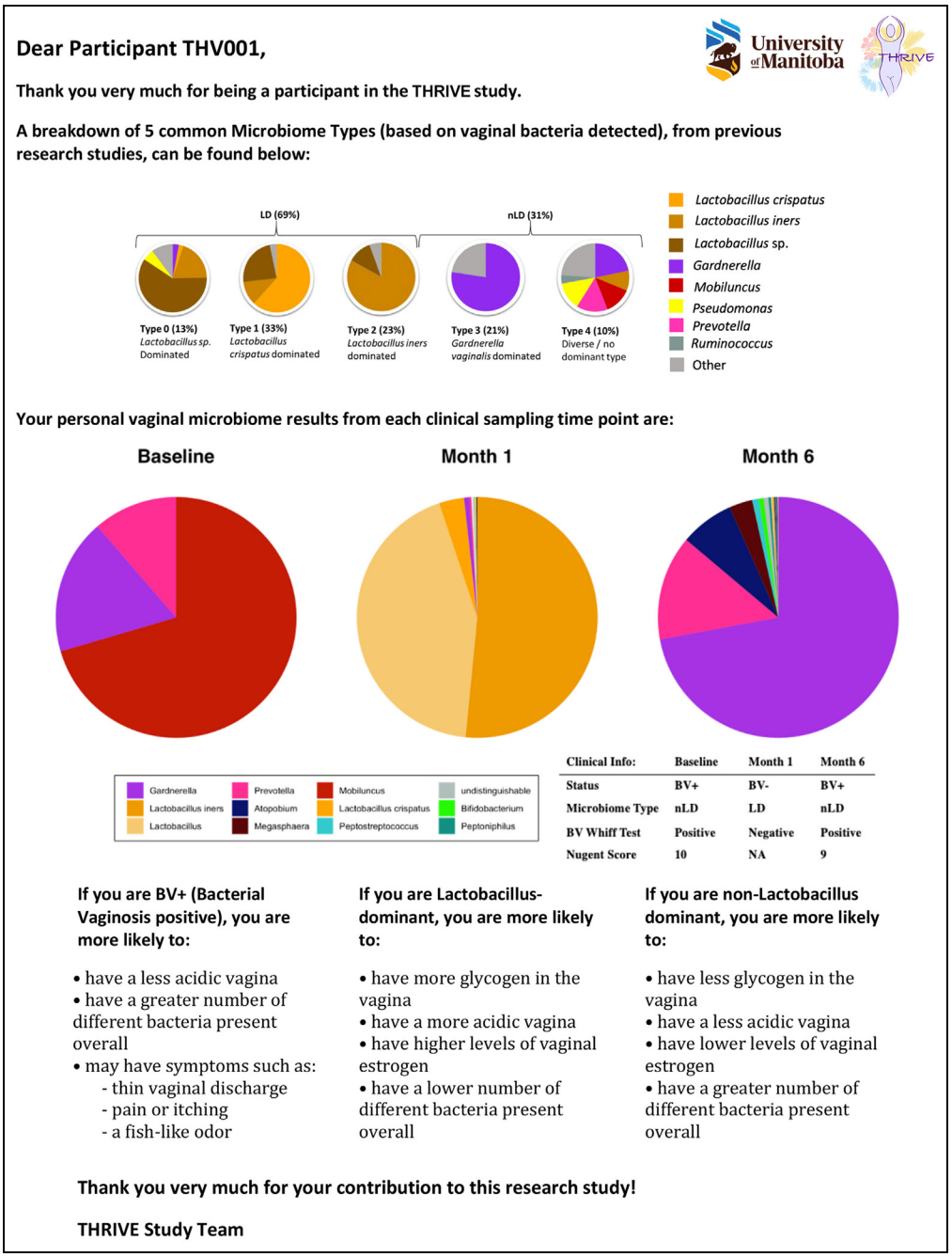
BV, bacterial vaginosis; CD, the cluster of differentiation; M, month; SOM, Self-Organizing Map; *t*-SNE, *t*-distributed stochastic neighbour embedding; THRIVE, The Study of Host-Bacterial Relationships and Immune Function in Different Vaginal Environments.

embedding (*t*-SNE) islands coloured by FlowSOM groupings as a colour gradient (low = blue; red = high), CD15, CD16, CD11b, and CD62L for the innate immune panel. At BL, the BV+ participant had high levels of granulocytes (CD66b+CD14-, 46.7%) that were activated (CD11b+), as well as Natural killer cells (38.7%). After treatment for BV, this participant had decreased levels of both these cell types which closely resembled the BV participant.

Participant Information Sharing

Participants who are interested are provided with their vaginal molecular profiles from their clinical time points (Figure 6) during a one-on-one scheduled meeting with the clinical lead to discuss the implications of the data. This information sheet provided participants with an overview of different types of vaginal microbiome communities commonly found in women which can be visually compared to their own results over time. Information on the clinical

Figure 6. Participant information sheet provided to participants upon completion of the study. A one-on-one meeting with their attending physician is scheduled to go through the information and answer any questions while informing them this is not a diagnostic tool.



implications of the different types of microbiome profiles was also provided and discussed during their in-person meeting. Feedback from participants was collected to help adjust recruitment strategies and overall protocols.

DISCUSSION

Principal Findings

Here we describe our established comprehensive cohort aimed to characterize BV recurrence with the resolution to capture bacterial, host and metabolic features that may play a role in vaginal microbiome stability. Our preliminary analyses provide validation that the proposed sampling protocol is sufficient to capture alterations in the vaginal microbiome and can even capture minor changes in the microbiome and host cellular functions in the control participants over time.

We addressed the question of sample quality by processing samples collected from our first 2 participants in the clinic as well as self-collected samples and showed our results in Figures 3–5. We had good coverage of the vaginal microbiome from both 16S and MS analyses, taken from both clinically collected samples (Figure 3) and self-collected samples (Figure 4), where we also looked at metabolomic and host proteomics. We determined that cervical mononuclear cells were viable and able to be processed using flow cytometry and presented example results from 2 participants (Figure 5).

To assess the feasibility of obtaining good adherence of participants to a daily swab routine and compliance with the lengthy questionnaires, an evaluation of participants who have completed the study was performed. On average, participants completed 96.5% of all the questions from the monthly questionnaires. Participants also collected 78.3% of their daily vaginal swabs during the entire 6-month study. This assessment shows that participants adhered well to daily swabbing, and we will have sufficient coverage for our analysis.

Strengths and Limitations

The limitation of this cohort is the lack of minority populations in our current recruitment profile. We are investigating avenues of increasing enrolment from different populations but may be limited to the local diversity. We acknowledge that our local cohort will be largely represented by women who are more associated with a *Lactobacillus*-dominant microbiome, however by targeting enrolment of women who have symptoms of BV, we will be able to capture non-*Lactobacillus*-dominant microbiomes. We also have data from over 2000 vaginal samples collected from women in other countries including Africa and the United States, in a range of demographic backgrounds and ethnicities. We expect that findings from our current study showing the kinetics of microbiome stability or changes over time can be used to look at this large dataset in comparison. Though these global samples aren't collected in the daily frequency as our THRIVE BV study, we can extrapolate findings to validate signatures of non-*Lactobacillus*-dominant profiles. If our initial study provides promising data, we can apply for additional funding to collect daily samples from different populations.

Interpretation

Our current enrolment validated the expected recurrence rate and treatment success, indicating our targeted population size is appropriate for sufficient power. Feedback from past participants regarding the study was captured for future improvements. Many women found the study gratifying to help women's health research and felt that our study team provided them with a personalized approach to their vaginal health. Though most women had no negative comments regarding the study, the most common was not being able to collect vaginal swabs daily. To alleviate this burden, we offer flexible sampling to continue follow-up visits. We recently updated our protocol to include follow-up visits at 1 and 2 years after enrolment to capture additional BV recurrences.

This study assessed the acceptability of women enrolling in a high-frequency sampling study. Though initially expecting difficulty enrolling healthy women to provide extensive and frequent mucosal sampling, we have been able to recruit more controls than BV+. Many participants expressed a desire to help ongoing research in women's health and understood the importance of our studies.

Generalisability

The vaginal microbiome composition has been previously shown to fluctuate rapidly over time with daily[19,20] and even hourly variations,[21] and may be influenced by the menstrual cycle,[22] hygiene practices[23] and sexual activity.[24,25] While this shows perturbations to the mucosal microenvironment can influence the vaginal microbiome, it remains unknown how microbial communities containing certain bacterial species are suddenly outcompeted by others. This is likely a complex process that is governed by both intrinsic and extrinsic factors, including species and strain diversity of the microbial community, nutrient availability, localized inflammation, dietary (i.e., micronutrient intake), probiotic/antibiotic use, and environmental factors.

Understanding functional and compositional features of the vaginal microbiome, the kinetics of variability, and

how this relates to host immunity could provide a blueprint for the rationale design of new BV interventions and restoration of vaginal mucosal homeostasis. Future research needs to consider interactions between both the pathogen and the host. Our comprehensive sampling strategy with daily vaginal swabs will provide a high-resolution dataset enabling us to better characterize drivers of microbiome stability and understand this dynamic interplay.

ETHICS

Ethics was required and obtained through the University of Manitoba Research Ethics and Compliance. Approval # HS21961 (B2018:071).

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AUTHOR CONTRIBUTIONS

Conceptualization, ADB, VP, ARB, CFZ; Methodology, ADB, VP, ARB, CFZ, PM, KDB; Software, LNR, SK, ARB KDB; Investigation, ARB, CFZ, KDB, MDL, KK, TT, SB; Validation, ARB, SK, LNR, Resources, VP, HP, SK, LNR; Data Curation, SK, ARB, LNR; Visualization, ARB, SK; Writing – Original Draft, ARB; Writing – Review and Editing, ADB, VP, KB, LNR, HP, CFZ, ARB; Supervision, VP, ADB; Funding Acquisition, ADB, VP.

SUPPLEMENTARY MATERIAL

Supplementary material related to this article can be found at https://doi.org/10.1016/j.jogc.2024.102667.

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