



BMJ Open Microbial interactions among *Gardnerella*, *Prevotella* and *Fannyhessea* prior to incident bacterial vaginosis: protocol for a prospective, observational study

Christina A Muzny ¹, Jacob H Elnaggar,² Lúcia G V Sousa,³ Ângela Lima,³ Kristal J Aaron,¹ Isaac C Eastlund,¹ Keonte J Graves,¹ Chaoling Dong,¹ Olivia T Van Gerwen,¹ Meng Luo,^{2,4} Ashutosh Tamhane,^{5,6} Dustin Long,⁷ Nuno Cerca,^{3,8} Christopher M Taylor ²

To cite: Muzny CA, Elnaggar JH, Sousa LGV, *et al.* Microbial interactions among *Gardnerella*, *Prevotella* and *Fannyhessea* prior to incident bacterial vaginosis: protocol for a prospective, observational study. *BMJ Open* 2024;**14**:e083516. doi:10.1136/bmjopen-2023-083516

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (<https://doi.org/10.1136/bmjopen-2023-083516>).

Received 22 December 2023
Accepted 16 January 2024



© Author(s) (or their employer(s)) 2024. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

For numbered affiliations see end of article.

Correspondence to
Dr Christina A Muzny;
cmuzny@uabmc.edu

ABSTRACT

Introduction The aetiology of bacterial vaginosis (BV), a biofilm-associated vaginal infection, remains unknown. Epidemiologic data suggest that it is sexually transmitted. BV is characterised by loss of lactic acid-producing lactobacilli and an increase in facultative and strict anaerobic bacteria. *Gardnerella* spp are present in 95%–100% of cases; *Gardnerella vaginalis* has been found to be more virulent than other BV-associated bacteria (BVAB) in vitro. However, *G. vaginalis* is found in women with normal vaginal microbiota and colonisation is not sufficient for BV development. We hypothesise that *Gardnerella* spp initiate BV biofilm formation, but incident BV (iBV) requires incorporation of other key BVAB (ie, *Prevotella bivia*, *Fannyhessea vaginae*) into the biofilm that alter the transcriptome of the polymicrobial consortium. This study will investigate the sequence of microbiologic events preceding iBV.

Methods and analysis This study will enrol 150 women aged 18–45 years with normal vaginal microbiota and no sexually transmitted infections at a sexual health research clinic in Birmingham, Alabama. Women will self-collect twice daily vaginal specimens up to 60 days. A combination of 16S rRNA gene sequencing, qPCR for *Gardnerella* spp, *P. bivia* and *F. vaginae*, and broad range 16S rRNA gene qPCR will be performed on twice daily vaginal specimens from women with iBV (Nugent score 7–10 on at least 2 consecutive days) and controls (with comparable age, race, contraceptive method and menstrual cycle days) maintaining normal vaginal microbiota to investigate changes in the vaginal microbiota over time for women with iBV. Participants will complete daily diaries on multiple factors including sexual activity.

Ethics and dissemination This protocol is approved by the University of Alabama at Birmingham Institutional Review Board (IRB-300004547) and written informed consent will be obtained from all participants. Findings will be presented at scientific conferences and published in peer-reviewed journals as well as disseminated to providers and patients in communities of interest.

STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ This study will investigate changes over time in the vaginal microbiota preceding incident bacterial vaginosis (iBV) among sexually active, reproductive-age cisgender women compared with controls maintaining normal vaginal microbiota; all women will be followed up to 60 days.
- ⇒ A combination of 16S rRNA gene sequencing, qPCR of several key BV-associated bacteria (*Gardnerella* spp, *Prevotella bivia* and *Fannyhessea vaginae*) and broad range 16S rRNA gene qPCR will be performed on vaginal specimens collected from women who develop iBV and controls with comparable age, race, contraceptive method and menstrual cycle days.
- ⇒ Daily diaries including data on sexual behaviours, menses, antibiotic use, sex toy use, sexual partner gender(s), douching and vaginal symptoms will be obtained from all women for the duration of the study.
- ⇒ One limitation is the feasibility of participants self-collecting twice daily vaginal specimens for 60 days; compliance with the study protocol will be monitored at weekly vaginal specimen drop-off visits.
- ⇒ This study primarily focuses on the role of *Gardnerella* spp, *P. bivia* and *F. vaginae* in the pathogenesis of iBV; however, future work should also include research on the role of other common BVAB in iBV pathogenesis.

INTRODUCTION

Bacterial vaginosis (BV), the most common cause of vaginal discharge, is associated with preterm delivery, pelvic inflammatory disease (PID) and increased risk of acquisition of HIV and other sexually transmitted infections (STIs).^{1–9} Epidemiological data suggest that BV is sexually transmitted.^{10–11} The rate of recurrence after therapy is >60%,¹²



yet BV aetiology remains unknown despite decades of research. BV is characterised by loss of protective lactic acid-producing vaginal lactobacilli (ie, *Lactobacillus crispatus*) and increases in facultative (ie, *Gardnerella* spp) and strict anaerobic bacteria.¹³ *Gardnerella* spp,¹⁴ previously described only as *Gardnerella vaginalis*, are present in 95%–100% of BV cases.^{15 16} The *Gardnerella* genus has recently been found to consist of at least 13 unique genomic species, 4 of which have been named: *G. vaginalis*, *G. piotii*, *G. swidsinskii* and *G. leopoldii*.¹⁴ Some *Gardnerella* spp such as *G. vaginalis* have been found to be more virulent than other BV-associated bacteria (BVAB) in vitro.^{17 18} *G. vaginalis* was originally thought to be the primary BV pathogen¹⁹; however, it has also been found in women with normal vaginal microbiota²⁰ and colonisation is not sufficient for BV development²¹; thus other *Gardnerella* spp may play important roles in incident BV (iBV) pathogenesis.

A notable feature of BV is the appearance of a multi-species biofilm on vaginal epithelial cells.^{22 23} While the biofilm likely contributes to high BV recurrence rates after therapy,^{12 24–26} it remains largely uncharacterised. It is known to contain abundant *Gardnerella* spp, fewer *Fannyhessea vaginalis* (formerly known as *Atopobium vaginalis*)²⁷ and other various undefined bacterial species.^{22 23} *Gardnerella* spp can displace *L. crispatus* from HeLa cells and adhere in high concentrations to initiate biofilm formation.²⁸ When certain BVAB are incorporated into the *Gardnerella* spp biofilm, *Gardnerella* spp virulence genes are upregulated.²⁹ Thus, our central hypothesis is that *Gardnerella* spp initiate BV biofilm formation, but iBV requires incorporation of other key BVAB into the biofilm that alter the transcriptome of the polymicrobial consortium.³⁰ This hypothesis is consistent with our finding that, among cisgender women who have sex with women (WSW), the mean relative abundance of *Prevotella bivia*, *Gardnerella* spp and *F. vaginalis* became sequentially higher prior to iBV.³¹ We propose that a similar distribution of these bacterial species will increase prior to iBV in cisgender women who have sex with men (WSM). *Gardnerella* spp and *P. bivia* exhibit a symbiotic relationship in vitro³² but whether *P. bivia* incorporates into the BV biofilm in vivo is unknown. *F. vaginalis* rarely appears in the absence of *Gardnerella* spp, suggesting synergism between these microorganisms and is specific for BV.³³ *F. vaginalis* stimulates an innate immune response from vaginal epithelial cells in greater magnitude than *Gardnerella* spp, leading to localised cytokine and β -defensin production,³⁴ suggesting that it is a potent component of the host response to BV. This may contribute to adverse outcomes associated with BV (ie, preterm birth and PID), as increased vaginal inflammatory cytokines and neutrophils are predictive of both.^{35 36}

To better determine the exact timing of the increase of these three key BVAB (among others) prior to iBV, more frequent vaginal sampling (two times per day) is necessary than has been previously done.³¹ In addition, a better understanding of how these key BVAB interact

in vivo and the molecular markers produced during BV biofilm formation is needed.

Here, we describe a prospective, observational iBV pathogenesis study with WSM. This study has intensive two times per day vaginal specimen collection to perform 16S rRNA gene sequencing and qPCR to characterise changes in the vaginal microbiota over time at high resolution, focusing on events preceding the development of iBV. The results will help determine if similar events in iBV pathogenesis occur in WSM (as seen in WSW), supportive of key roles of *Gardnerella* spp, *P. bivia* and *F. vaginalis* in iBV pathogenesis. We will also investigate potential behavioural (ie, sexual activity, douching, etc) factors, along with hormonal shifts and menstrual activity, which may be associated with iBV.³¹ Our long-term goal is to better understand the pathogenesis of BV to improve diagnosis, treatment and prevention.

Objectives

The primary aim of this study is to investigate changes in the vaginal microbiota over time preceding iBV in a longitudinal study of cisgender WSM. We hypothesise that, prior to iBV, the mean relative abundance and inferred absolute bacterial abundance (IAA) of *Gardnerella* spp, *P. bivia* and *F. vaginalis* will become sequentially higher compared with women maintaining normal vaginal microbiota for the majority ($\geq 85\%$ of days) of the study. Based on prior data,^{31 37} we anticipate that this will occur within 14 days prior to iBV.

Starting at study enrolment and continuing for 60 days thereafter, 150 women with normal vaginal microbiota (Nugent score 0–3) will self-collect 3 vaginal specimens two times per day and perform two times per day smears for vaginal Gram stain and Nugent score³⁸ determination. Participants will also complete daily diaries to document douching, menses, sexual behaviour data, vaginal symptoms and medication use (oral and/or intravaginal) while in the study to determine their influence on the vaginal microbiota. For women who develop iBV (cases), 16S rRNA sequencing, qPCR for *Gardnerella* spp, *P. bivia*, *F. vaginalis* and broad range 16S rRNA gene qPCR will be performed on all twice daily vaginal specimens for the 14 days prior to iBV as well as the day of iBV and 3 days afterwards. Cases with iBV will be comparable to controls (women maintaining normal microbiota) with regard to age, race, contraceptive method and menstrual cycle days; an equal number of controls to cases will be selected. In addition to standard 16S vaginal microbiome analysis, the qPCR for broad range 16S rRNA gene data will be used to calculate the inferred absolute bacterial abundance (IAA)³⁹ to account for variation in overall absolute bacterial abundance in the specimens. Changes in microbial community composition over time will be visualised via time course heatmaps along with characterisation of community state types (CSTs)⁴⁰ which will be determined by VALENCIA (VAGinal community state type Nearest Centroid Classifier).⁴¹

Figure 1 Study recruitment flyer.

METHODS AND ANALYSIS

Study recruitment

Between November 2020 and August 2024, potential participants will be recruited from the Birmingham, AL metropolitan area using study flyers (figure 1), word-of-mouth, advertising at local events, local newspaper and radio advertisements, and social media campaigns on Facebook, Instagram and Snapchat (figure 2). After contacting research staff about study participation, interested individuals will be scheduled to have a study screening visit at the University of Alabama at Birmingham (UAB) Sexual Health Research Clinic (SHRC), located on the UAB campus.

Study design

This is a prospective, longitudinal cohort study of WSM with normal baseline vaginal microbiota (no Amsel criteria⁴² and a normal Nugent score of 0–3³⁸ with no *Gardnerella* spp morphotypes). There are two phases: screening and enrolment.

For screening, women will be included based on being assigned female sex at birth, ages 18–45 years, a history of sex with men and a current male sexual partner (table 1). Women will be excluded from the study if they have a history of oral or intravaginal antibiotic usage within the last 14 days, self-reported HIV infection, are currently

Figure 2 Social media campaign advertisement. Interested individuals clicking on this advertisement are directed to a landing page providing basic information about the study as well as a section to include their information to be contacted about participation.

pregnant, are on their menses or have a history of sex with women. At the screening visit, women will be asked to self-collect one vaginal swab for determination of the Amsel criteria⁴² and Nugent score,³⁸ complete a brief screening visit questionnaire and provide urine for a pregnancy test. Non-pregnant women with no Amsel criteria,⁴² a Nugent score of 0–3³⁸ (determined by a research clinician in the clinic, confirmed by a second reader in the research laboratory of author CAM) and no *Gardnerella* spp morphotypes on vaginal Gram stain will be invited to enrol (table 1).

Enrolled participants will complete a study questionnaire on sociodemographics, alcohol/tobacco/drug use, recent antibiotic use, sexual history including STI history, douching history and contraception use. A pelvic exam will be performed with a vaginal swab obtained for *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium* nucleic acid amplification testing using the Roche cobas 6800 CT/GC⁴³ and TV/MG assays.⁴⁴ Women will complete a one-page daily diary (a yes/no checklist on their oral, vaginal, and anal sexual activities, antibiotic use, sex toy use, partner gender and type, douching, and vaginal symptoms) for 60 days. They will be taught to self-collect three vaginal specimens two times per day for 60 days, one of which will be smeared on a Gram stain slide for subsequent Nugent scoring by research laboratory staff. Written instructions for home collection of specimens will be provided (box 1). Participants will be given a 1-week supply of specimen collection materials in an insulated ‘research-only lunch cooler’ and hard copies of daily diaries. They will

Table 1 Screening and enrolment: inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Screening visit	
18–45 years of age	Use of oral or intra-vaginal antibiotics within the past 14 days
Female sex	HIV infection
History of sex with men	Pregnancy
Current male sexual partner	Current menses
	Sex with women
Enrolment visit	
No Amsel criteria	<i>Trichomonas vaginalis</i> on vaginal wet mount
Nugent score 0–3 with no <i>Gardnerella</i> spp morphotypes	Symptomatic vaginal yeast infection
	Positive for chlamydia, gonorrhoea, trichomonas or <i>Mycoplasma genitalium</i> at baseline by NAAT testing
	Pregnancy
	Self-reported HIV infection
NAAT, nucleic acid amplification test.	

also be given a specimen collection calendar and weekly drop-off appointment. Gram stain slides will be placed in a plastic slide holder and stored at room temperature in the participant's home. The three self-collected vaginal swabs will be placed in separate specimen collection tubes, stored in biohazard bags and kept in the 'research-only lunch cooler' in their home refrigerators (4°C) until weekly drop-off of materials at the study site. Each participant will also be asked to obtain a sampling control at the beginning of the study (ie, a blank swab opened and waved in the air for 20 seconds in the home sampling environment). This specimen will capture any background microbial contamination from the home that could confound vaginal microbiota analyses.⁴⁵

Participants will bring all daily diaries, vaginal Gram stains and vaginal specimen tubes on ice to the study site weekly for 60 days or until iBV occurs (Nugent score of 7–10 on ≥4 consecutive specimens). Women testing positive for *T. vaginalis*, *C. trachomatis*, *N. gonorrhoeae* or *M. genitalium* at baseline will be dropped from the study as these STIs (and their treatment) may alter the vaginal microbiota.^{46–50} Once the slides and specimens are dropped off and sent to the research laboratory of author CAM at UAB, they will be archived at –80°C until shipped to the Louisiana State University Health Sciences Center (LSUHSC) for 16S rRNA gene sequencing and qPCR (vaginal specimen 1). Vaginal specimens 2 and 3 will be used for additional BV pathogenesis research beyond the

Box 1 Home instructions for self-collection of vaginal specimens for glass slide preparation and tube insertion

1. Remove the first 'FLOQ' swab (flocked swab) from the packaging material by tearing off the top of the packaging. Be careful not to touch the tip of the swab.
2. Using your non-dominant hand (not your writing hand) open the labia (lips of the vaginal area) to allow entrance of the swab into the genital tract (vagina).
3. Insert the swab 2 inches into the genital tract being careful not to touch the tip of the swab anywhere else in the genital area.
4. Twist the swab several times while inside of the genital tract.
5. Remove the swab the same way that you did for insertion. Again, be careful not to touch the tip of the swab outside of the genitals.
6. Roll the swab across the length of the glass slide from left to right.
7. Place the glass slide in the slide carrier and close the top of the carrier.
8. Put the *slide* back into its bag.
9. Place this swab into the orange capped tube containing liquid.
10. Break off the handle of the swab at the score line (ie, the line pointed out to you during your visit) and discard the handle. Screw the top of the collection tube on *firmly*, but do not overtighten.
11. Put the *tube* back into its bag and keep the tube in an upright position.
12. Repeat steps 1–11 for the second and third genital swabs.
13. Store swabs in your study lunchbox in the refrigerator (at 4°C) until weekly drop-off at the University of Alabama at Birmingham Sexual Health Research Clinic.

scope of this manuscript (performed by authors LGVS, AL, CD and NC).

If women experience any vaginal symptoms (ie, discharge, itching, odour, etc) during the study, they will be encouraged to call the study site to schedule an interval, standard-of-care visit. If symptomatic BV is diagnosed at this visit (by Amsel criteria), they will be treated⁵¹ and dropped from the study, as the primary outcome of iBV will have been reached. If an incident STI is diagnosed during the study, participants will be treated⁵¹ and dropped.

All participants will be paid US\$30 at screening, US\$25 at enrolment, US\$30 at weekly drop-offs (weeks 1–8) and US\$50 at the final drop-off (week 9). All study-related data will be entered into a REDCap database.⁵²

Study setting

Participants will be enrolled at the UAB SHRC. As previously mentioned, vaginal specimens and Gram stain slides will be stored in the UAB research laboratory of CAM once returned to the study site. Select vaginal specimens will be shipped to LSUHSC for 16S rRNA gene sequencing and qPCR assays. The resulting sequence data will be analysed by JHE and CMT at LSUHSC for bioinformatics analysis and AT and DL at UAB will perform statistical analyses.

Data sources and variables

The online supplemental file includes the REDCap case report forms (CRFs) for the screening visit, enrolment visit and daily diaries. Additional CRFs include an

interval, standard-of-care visit for women who develop vaginal symptoms during the study and an end-of-study document. The REDCap database for this study is hosted by the UAB Department of Medicine Information Technology REDCap Team and managed by the UAB STD Research Programme Data Manager, author KJA.

Bias

While participants may experience social desirability bias, anonymity on the CRFs is expected to mitigate this bias.

DNA extraction, 16S and qPCR methods

DNA will be extracted from select vaginal specimens by author ML from women with iBV and women maintaining normal vaginal microbiota using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Germantown, Maryland, USA). Controls will be included at all steps to monitor for reagent contamination. DNA quality will be monitored by gel electrophoresis and fluorescent dsDNA assays. Extracted DNA will be divided into two aliquots for 16S rRNA gene sequencing and qPCR measurements.

To prepare the sequencing library, two amplification steps will be performed using the AccuPrime Taq high fidelity DNA polymerase system (Thermo Fisher/Invitrogen/Life Technologies, Carlsbad, California, USA). First, the 16S ribosomal DNA hypervariable region V4⁵³ will be amplified using 20 ng of genomic DNA and the gene-specific primers with Illumina adaptors: forward 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGCCAGCMGCCGCGGTAA 3'; reverse 5' GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAG GGACTACHVGGGTWTCTAAT 3'. Second, purified amplicon DNA from the last of 25 PCR cycles will be amplified for 8 cycles using the primers with different molecular barcodes: forward 5' AATGATACGGCGACCACCGAGATCTACAC [i5] TCGTCGGCAGCGTC 3'; reverse 5' CAAGCAGA AGACGGCATAACGAGAT [i7] GTCTCGTGGGCTCGG 3'. The normalised and pooled libraries will be run with paired-end sequencing on an Illumina MiSeq (Illumina, San Diego, California, USA) using the 500 base pairs (bp) V2 sequencing kit (2×250 bp paired end reads).

A qPCR assay will be used to measure the copy number of the 16S rRNA gene using the following primers that target the V6 conserved region: 16S926F 5'-AAACTCAAAGAATTGACGG-3' and 16S1062R 5'-CTCACRR-CACGAGCTGAC-3'.⁵⁴ These primers will be synthesised and high-pressure liquid chromatography (HPLC) purified by Integrated DNA Technologies (Coralville, Iowa, USA). Core reagents for the SsoAdvanced Universal SYBR Green Supermix will be obtained from Bio-Rad (Hercules, California, USA). In a 20 µl reaction system, the final concentrations for both the forward and reverse primers will be added at 0.4 µM and probe concentration will be 0.2 µM. The reagent master mix will be filtered to minimise contamination using a Microcon YM-100 centrifugal filter unit (Millipore) at 2000 rpm for 35 min, 5000 rpm for 5 min and 8000 rpm for 5 min. Reactions will start at 50°C for 2 min on the Bio-Rad CF96 real-time

cycler followed by initial denaturation at 98°C for 3 min. Reactions will then undergo 40 cycles of amplification at 98°C for 15 s and 60°C for 15 seconds. Mixed vaginal plasmid standards made up of *Gardnerella*, *Prevotella* and *Lactobacillus*⁵⁵ will be run for each reaction ranging from 10⁷ to 100 gene copies; values will be reported as 16S rRNA gene copies/specimen to estimate the total bacterial load. Measurements for total bacterial load will be used to adjust relative abundance measures acquired from vaginal microbiome sequencing data. Additionally, HPLC primers will be ordered for targeted qPCR of *Gardnerella* spp (Fw 5'-CACATTGGGACTGAGATACGG-3', Rv 5'-AGGTACACTCACCCGAAAGC-3'), *P. bivia* (Fw 5'-CGCACAGTAAACGATGGATG-3', Rv 5'-ATGCAGCACCTTCACAGATG-3') and *F. vaginae* (Fw 5'-TATATCGCATGATGTATATGGG-3', Rv 5'-CATTTCACCGCTACACTTGG-3').⁵⁶ Each qPCR assay will be performed in triplicate.

Bioinformatics analysis of 16S rRNA gene sequence data and qPCR data

Fastq files from the Illumina MiSeq run will be processed through the DADA2 V.1.26 software package in R⁵⁷ at LSUHSC to determine amplicon sequence variants. Parameters based on quality profiles of the sequencing run will be used to retain high-quality data and trim off amplicon primers. The DADA2 algorithm will be used to build an error profile for the vaginal specimens, isolate amplicon sequence variants and detect and remove chimaeras. The Silva database V.138⁵⁸ will be used for taxonomic classification. Sequence variants will be placed into a sequence table indicating frequency for each specimen and decontam V.1.18⁵⁹ will be used with sampling controls for each participant to remove sequence variants identified as contaminants. We will apply methods developed by Tettamanti Boshier *et al.*³⁹ to complement our vaginal microbiome sequencing data with bacterial load estimates in order to infer the absolute abundances of bacterial species present and adjust the measured relative abundance metrics to IAA measures. Phyloseq V.1.42⁶⁰ will be used to calculate and visualise the alpha and beta diversity and provide a taxonomic summary of the vaginal specimens for an overview of the data. CSTs will be constructed using the Bray-Curtis distance to calculate pairwise sample distances followed by denoising of this distance matrix selecting the most significant Principal Coordinates Analysis eigenvectors.⁶¹ Custom scripts will be written to investigate hypotheses and produce custom analyses and visualisations.

Statistical power

Table 2 illustrates standardised effect sizes that can be detected with ≥80% power using a two-tailed type I error rate of 0.05, assuming various autocorrelation (rho) and iBV event rates using a repeated measures study design with a sample size of 150 women and 28 vaginal specimens (2 specimens per day × 14 days prior to iBV). The standardised effect size is calculated by

**Table 2** Statistical power for various standardised effect sizes

iBV event rate	Sample size=150*		Rho	Statistical power		
	With iBV	Normal microbiota		80%	85%	90%
10%	15	75	0.2	0.38	0.41	0.44
10%	15	75	0.3	0.45	0.48	0.52
10%	15	75	0.4	0.51	0.55	0.60
14%	21	69	0.2	0.33	0.36	0.39
14%	21	69	0.3	0.40	0.43	0.46
14%	21	69	0.4	0.45	0.49	0.52
18%	27	63	0.2	0.31	0.33	0.36
18%	27	63	0.3	0.37	0.39	0.43
18%	27	63	0.4	0.42	0.45	0.48

*We anticipate that the remaining participants will have an intermediate Nugent score of 4-6.
iBV, incident bacterial vaginosis.

dividing the difference between the two group means ($\text{mean}_{\text{iBV}} - \text{mean}_{\text{normalvaginalmicrobiota}}$) by a common SD. The autocorrelation will account for the fact that repeated observations for the same woman are not independent observations; non-independence in repeated measurements increases variation. As the autocorrelation increases (table 2), larger effect sizes are required to achieve adequate statistical power. Higher iBV rates will permit detection of smaller differences. Table 2 shows that, across various autocorrelations and event rates, the study will have adequate power to detect small-medium standardised effect sizes. The circled table cell can be interpreted as follows: assuming an iBV rate of 18%⁶² (27 women with iBV and 63 women maintaining normal vaginal microbiota), an SD of 10%, and an autocorrelation of 0.20, the study will have 85% power to detect a mean difference of 3.3% ($0.33 \times 10\%$) in the mean relative and absolute abundance of a given bacterial species between the two groups. Similarly, with an SD of 20%, a mean difference of 6.6% ($0.33 \times 20\%$) will be detected. If the final sample size for analysis is 10% smaller than expected (ie, $n=135$, $\text{iBV}=24$, $\text{normal}=57$) due to STI infection at enrolment or loss-to follow-up, the effect size for the circled table cell will still be 0.35. Power calculations were estimated using Power and Sample Size software, V.14 (NCSS, Kaysville, Utah, USA).

We anticipate that approximately 400 WSM will need to be screened in order to enrol 150 women, based on the prevalence of normal vaginal microbiota in our prior study.⁶³

Statistical analysis

Women with iBV will be compared with those maintaining normal vaginal microbiota with regard to various characteristics including the mean relative abundance and IAA of *Gardnerella* spp, *P. bivia*, *F. vaginae* and other common BVAB.³¹⁻³⁹ Continuous variables such as age

will be compared using an unpaired t-test or Wilcoxon rank sum test, as appropriate. Sociodemographics, STI history, sexual behaviour history and contraception use at enrolment will be compared between groups using χ^2 or Fisher's exact tests. Longitudinal patterns in the sequencing data will then be examined using different approaches such as repeated measures analysis of variance (ANOVA) and linear mixed models which will account for missing data. We will compare the results using these approaches and will report the result for which the assumptions will be most reasonable. If the data demonstrate extreme skewness, we will use bootstrapping to conduct a non-parametric analysis; 10 000 bootstrapped samples will be drawn with replacement from women with iBV and women maintaining normal vaginal microbiota to create 95% CIs comparing the mean relative abundance difference between groups. Our bootstrapping approach will select the available set of longitudinal values for each participant to maintain and account for the correlation structure among the repeated observations. Mean relative abundance differences between women with iBV and women maintaining normal vaginal microbiota, after adjustment for antibiotic use and other characteristics, will be calculated within each bootstrap for each sample. Bootstrap CIs will be estimated using a Bonferroni correction (conservative approach) adjusting the 0.05 type I error rate for the available number of samples (ie, $0.05/28=0.0017$ when 28 samples are available). If the Bonferroni corrected bootstrap empirical CIs do not include zero, we will conclude that the groups differ significantly at that time point. Secondary analyses will examine the association of various sociodemographic characteristics, menses, douching and sexual risk behaviours (collected in the enrolment questionnaire and the daily diaries) with iBV. Statistical significance will be set at 0.05 (two tailed) except while examining individual samples when the Bonferroni correction will be applied, as stated above. IAA of vaginal bacteria of interest, including *Gardnerella* spp, *P. bivia* and *F. vaginae*, will be obtained by multiplying each bacteria's relative abundance (measured by 16S rRNA gene sequencing) by their total bacterial load (measured by broad range 16S rRNA gene qPCR).³⁹ The relationship between the IAA and targeted qPCR absolute abundance of *Gardnerella* spp, *P. bivia* and *F. vaginae* will be examined by scatter plots and Pearson/Spearman correlation coefficients. Furthermore, agreement between the two measures will be examined by the Bland-Altman method for repeated measures.⁶⁴⁻⁶⁶ If agreement is high (ie, 80% of the points within 2 SD), the IAA of other vaginal bacteria will be used in our analyses as targeted qPCR will only be performed for *Gardnerella* spp, *P. bivia* and *F. vaginae*. If agreement is not high, we will instead use absolute abundance data of *Gardnerella* spp, *P. bivia* and *F. vaginae* from targeted qPCR in our analyses. All analyses will be conducted using SAS V.9.4.

Data management and confidentiality

Participating in this study involves the disclosure of sexual behaviour to research personnel. Every effort will be made to create a secure and trustworthy environment in the privacy of an examination room at the clinic. In order to mitigate potential disclosure of sensitive health information (on the daily diaries) to persons close to or cohabitating with participants, all study materials will be labelled in a generic manner and not contain any indication that the study is related to sexual health subject matter. Hard copy signed screening and enrolment consent forms as well as CRF forms will be stored in study binders in a locked cabinet in a locked office at the study site. All hard copy CRF data will be entered by study personnel into the REDCap database. This database will be stored on a password-protected UAB server and managed by the UAB STI Research Programme Data Manager, author KJA. Participants will be assigned a unique study identification number and no identifiers will be collected on the CRFs. Electronic transfer of CRF data by author KJA to CAM, AT and DL will occur through the UAB intranet via Box. Deidentified sequence data generated by authors JHE and CMT at LSUHSC will be sent via Box to authors to CAM, AT and DL at UAB, for statistical analysis. These data will be kept on password-protected servers on UAB and LSUHSC-encrypted computers. As this study is not a clinical trial, a Data and Safety Monitoring Board is not required.

Patient and public involvement

None.

ETHICS AND DISSEMINATION

This protocol is approved through the single IRB mechanism by the UAB IRB (Protocol # IRB-300004547); written informed consent will be obtained from all participants. The LSUHSC New Orleans Human Research Protection Programme is an external relying site. This study is also approved by the Ethics Committee for Research in Life and Health Sciences (CEICVS) at the University of Minho in Braga, Portugal (CEICVS 147/2019). Findings will be presented at national and international scientific conferences and published in peer-reviewed journals as well as disseminated to providers and patients in communities of interest.

DISCUSSION

This prospective cohort study will investigate longitudinal changes in the vaginal microbiota of WSM via intensive vaginal specimen collection over a 60-day period. This study is novel and important for several reasons. First, prior longitudinal studies of the vaginal microbiota only collected vaginal specimens daily, weekly or quarterly, for short periods of time (ie, 7–14 days), or did not specifically focus on the days leading up to iBV.^{61 67–70} Additionally, there are little data on the temporal dynamics

of the vaginal microbiota before, during and after BV episodes.^{31 71} Our proposed study is well powered and includes twice daily vaginal specimen collection over 60 days. A combination of 16S sequencing and qPCR will be performed on these specimens for the 14 days leading up to iBV, with the data compared with women maintaining normal vaginal microbiota. The application of methods for IAA integrating qPCR data with 16S rRNA gene sequencing data³⁹ will allow for a more rigorous assessment of microbiome changes over time in this cohort. This design provides a unique opportunity to better define events in the vaginal microbiota occurring prior to iBV.

Despite the study strengths, there are limitations. One limitation is that participants may not collect three vaginal specimens two times per day for 60 days. We will assess their compliance with the study protocol at each weekly specimen drop-off visit and remind them of the importance to collect their specimens. Based on our power calculations, small-to-medium standardised effects can still be detected even if we only have one set of daily vaginal specimens for the 14 days leading up to iBV (not shown). Additionally, study questionnaires and daily diaries are subject to social-desirability bias given the sensitive nature of the questions. To minimise this, we will encourage participants to complete these documents on their own, without an interviewer. The enrolment questionnaire will also include a brief social desirability bias scale.⁷² Finally, our study primarily focuses on the role of *Gardnerella* spp, *P. bivia*, and *F. vaginae* in the pathogenesis of iBV; however, future work should also include research on the role of other common BVAB in iBV pathogenesis.

Author affiliations

¹Division of Infectious Diseases, University of Alabama at Birmingham, Birmingham, Alabama, USA

²Department of Microbiology, Immunology, and Parasitology, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA

³Centre of Biological Engineering, Laboratory of Research in Biofilms Rosário Oliveira (LIBRO), University of Minho—Gualtar Campus, Braga, Portugal

⁴Microbial Genomics Resource Group, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA

⁵Division of Nephrology, University of Alabama at Birmingham, Birmingham, Alabama, USA

⁶Center for Clinical and Translational Sciences, University of Alabama at Birmingham, Birmingham, Alabama, USA

⁷Department of Biostatistics, University of Alabama at Birmingham, School of Public Health, Birmingham, Alabama, USA

⁸LABELS—Associate Laboratory, Braga, Guimarães, Portugal

Twitter Christina A Muzny @ChristinaMuzny

Acknowledgements The authors would like to thank Jane Schwabke, MD and William Edward Swords, PhD, for helpful discussions regarding protocol development. The authors would also like to thank Joy Lewis, Zoe (Alex) Sherman, Brenda Ocegueda, Faith George, Jenna Bae, Saralyn Richter, RN and Angela Pontius, CRNP for their assistance in recruitment and enrolment in this study and Maya Caves for her assistance with participant kit preparation and processing of vaginal specimens. Additionally, the authors thank the research laboratory of Barbara Van Der Pol, PhD, MPH for performance of the Roche cobas CT/GC and TV/MG assays used in this study. The authors also thank Caleb Ardizzone, BS and the research laboratory of Allison Quayle, PhD, for their assistance with the qPCR methods used in this protocol. Finally, the authors thank the UAB Marketing and Communications

Department for their assistance in developing the advertisements for the social media campaign for this study.

Contributors CAM, JHE, LGVS, AL, KJA, ICE, KJG, CD, OTVG, ML, AT, DL, NC and CMT contributed to development of the study protocol. CAM, OTVG and KJA developed the study case report forms for the REDCap database. The manuscript was written and revised by CAM and CMT with input and review from JHE, LGVS, AL, KJA, ICE, KJG, CD, OTVG, ML, AT, DL and NC. All authors read and approved the final manuscript.

Funding This work is supported by the National Institute of Allergy and Infectious Diseases (grant R01AI146065-01 to CAM). LGVS and AL are supported by the Portuguese Foundation for Science and Technology (FCT), individual PhD fellowships 2020.04912.BD and 2022.13112.BD, respectively. CMT is partially supported for this work by grants P20GM103424 (National Institute of General Medical Sciences), UL1TR003096 (National Center for Advancing Translational Sciences), and 2018936 (National Science Foundation/LSUHSC Campus Cyberinfrastructure Compute).

Competing interests CAM has received research grant support to her institution from NIH/NIAID, Lupin Pharmaceuticals, Gilead Sciences, Visby Medical and Abbott Molecular. CAM also reports honorarium and/or consulting fees from Scynexis, Cepheid, BioNTech, BioMed Diagnostics, Visby Medical, Elsevier, UpToDate, Abbott Molecular and Roche. OTVG has received research grant support to her institution from NIH/NIAID, Lupin Pharmaceuticals, Gilead Sciences and Abbott Molecular and has served on a scientific advisory board for Scynexis. KJG has received research grant support to his institution from NIH/NIAID and Lupin Pharmaceuticals. The other authors have no competing interests to declare.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

ORCID iDs

Christina A Muzny <http://orcid.org/0000-0002-4005-3858>

Christopher M Taylor <http://orcid.org/0000-0002-7983-5275>

REFERENCES

- Leitch H, Bodner-Adler B, Brunbauer M, et al. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *Am J Obstet Gynecol* 2003;189:139–47.
- Martin HL, Richardson BA, Nyange PM, et al. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J Infect Dis* 1999;180:1863–8.
- Cherpes TL, Meyn LA, Krohn MA, et al. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clin Infect Dis* 2003;37:319–25.
- Brotman RM, Klebanoff MA, Nansel TR, et al. Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. *J Infect Dis* 2010;202:1907–15.
- Myer L, Denny L, Telerant R, et al. Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. *J Infect Dis* 2005;192:1372–80.
- Hillier SL, Nugent RP, Eschenbach DA, et al. Association between Bacterial Vaginosis and Preterm Delivery of a Low-Birth-Weight Infant. *N Engl J Med* 1995;333:1737–42.
- Wiesenfeld HC, Hillier SL, Krohn MA, et al. Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. *Clin Infect Dis* 2003;36:663–8.
- Ness RB, Kip KE, Hillier SL, et al. A Cluster Analysis of Bacterial Vaginosis-associated Microflora and Pelvic Inflammatory Disease. *Am J Epidemiol* 2005;162:585–90.
- Lokken EM, Balkus JE, Kiarie J, et al. Association of Recent Bacterial Vaginosis With Acquisition of Mycoplasma genitalium. *Am J Epidemiol* 2017;186:194–201.
- Muzny CA, Schwabke JR. Gardnerella vaginalis: Still a Prime Suspect in the Pathogenesis of Bacterial Vaginosis. *Curr Infect Dis Rep* 2013;15:130–5.
- Forcey DS, Vodstrcil LA, Hocking JS, et al. Factors Associated with Bacterial Vaginosis among Women Who Have Sex with Women: A Systematic Review. *PLoS One* 2015;10:e0141905.
- Bradshaw CS, Morton AN, Hocking J, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* 2006;193:1478–86.
- Hillier SL, Marrazzo J, Holmes KK. Bacterial Vaginosis. In: Holmes KK, Sparling PF, Mardh PA, eds. *Sexually transmitted diseases*. 4th edition. New York: McGraw-Hill, 2008: 737–68.
- Vanechoutte M, Guschin A, Van Smaey L, et al. Emended description of Gardnerella vaginalis and description of Gardnerella leopoldii sp. nov., Gardnerella pitotii sp. nov. and Gardnerella swidsinski sp. nov., with delineation of 13 genomic species within the genus Gardnerella. *Int J Syst Evol Microbiol* 2019;69:679–87.
- Hill GB. The microbiology of bacterial vaginosis. *Am J Obstet Gynecol* 1993;169:450–4.
- Catlin BW. Gardnerella vaginalis: characteristics, clinical considerations, and controversies. *Clin Microbiol Rev* 1992;5:213–37.
- Patterson JL, Stull-Lane A, Girerd PH, et al. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of Gardnerella vaginalis relative to other bacterial-vaginosis-associated anaerobes. *Microbiology (Reading)* 2010;156:392–9.
- Alves P, Castro J, Sousa C, et al. Gardnerella vaginalis outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using an in vitro biofilm formation model. *J Infect Dis* 2014;210:593–6.
- GARDNER HL, DUKES CD. Haemophilus vaginalis vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *Am J Obstet Gynecol* 1955;69:962–76.
- Aroucheva AA, Simoes JA, Behbakht K, et al. Gardnerella vaginalis isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin Infect Dis* 2001;33:1022–7.
- Hickey RJ, Forney LJ. Gardnerella vaginalis does not always cause bacterial vaginosis. *J Infect Dis* 2014;210:1682–3.
- Swidsinski A, Mendling W, Loening-Baucke V, et al. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 2005;106:1013–23.
- Machado A, Cerca N. Influence of Biofilm Formation by Gardnerella vaginalis and Other Anaerobes on Bacterial Vaginosis. *J Infect Dis* 2015;212:1856–61.
- Sobel JD, Ferris D, Schwabke J, et al. Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. *Am J Obstet Gynecol* 2006;194:1283–9.
- Muzny CA, Schwabke JR. Biofilms: An Underappreciated Mechanism of Treatment Failure and Recurrence in Vaginal Infections. *Clin Infect Dis* 2015;61:601–6.
- Swidsinski A, Mendling W, Loening-Baucke V, et al. An adherent Gardnerella vaginalis biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 2008;198:97.
- Nouioui I, Carro L, García-López M, et al. Genome-Based Taxonomic Classification of the Phylum Actinobacteria *Front Microbiol* 2018;9:2007.
- Castro J, Alves P, Sousa C, et al. Using an in-vitro biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis Gardnerella vaginalis isolates. *Sci Rep* 2015;5:11640.
- Castro J, Machado D, Cerca N. Unveiling the role of Gardnerella vaginalis in polymicrobial Bacterial Vaginosis biofilms: the impact of other vaginal pathogens living as neighbors. *ISME J* 2019;13:1306–17.
- Muzny CA, Taylor CM, Swords WE, et al. An Updated Conceptual Model on the Pathogenesis of Bacterial Vaginosis. *J Infect Dis* 2019;220:1399–405.
- Muzny CA, Blanchard E, Taylor CM, et al. Identification of Key Bacteria Involved in the Induction of Incident Bacterial Vaginosis: A Prospective Study. *J Infect Dis* 2018;218:966–78.
- Pybus V, Onderdonk AB. Evidence for a commensal, symbiotic relationship between Gardnerella vaginalis and Prevotella bivia

- involving ammonia: potential significance for bacterial vaginosis. *J Infect Dis* 1997;175:406–13.
- 33 Bradshaw CS, Tabrizi SN, Fairley CK, *et al.* The association of Atopobium vaginae and Gardnerella vaginalis with bacterial vaginosis and recurrence after oral metronidazole therapy. *J Infect Dis* 2006;194:828–36.
- 34 Libby EK, Pascal KE, Mordechai E, *et al.* Atopobium vaginae triggers an innate immune response in an in vitro model of bacterial vaginosis. *Microbes Infect* 2008;10:439–46.
- 35 Ramsey PS, Lyon MD, Goepfert AR, *et al.* Use of vaginal polymorphonuclear to epithelial cell ratios for the prediction of preterm birth. *Obstet Gynecol* 2005;105:139–44.
- 36 Yudin MH, Hillier SL, Wiesenfeld HC, *et al.* Vaginal polymorphonuclear leukocytes and bacterial vaginosis as markers for histologic endometritis among women without symptoms of pelvic inflammatory disease. *Am J Obstet Gynecol* 2003;188:318–23.
- 37 Muzny CA, Lensing SY, Aaron KJ, *et al.* Incubation period and risk factors support sexual transmission of bacterial vaginosis in women who have sex with women. *Sex Transm Infect* 2019;95:511–5.
- 38 Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 1991;29:297–301.
- 39 Tettamanti Boshier FA, Srinivasan S, Lopez A, *et al.* Complementing 16S rRNA Gene Amplicon Sequencing with Total Bacterial Load To Infer Absolute Species Concentrations in the Vaginal Microbiome. *mSystems* 2020;5:e00777–19.
- 40 Ravel J, Gajer P, Abdo Z, *et al.* Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 2011;108:4680–7.
- 41 France MT, Ma B, Gajer P, *et al.* VALENIA: a nearest centroid classification method for vaginal microbial communities based on composition. *Microbiome* 2020;8:166.
- 42 Amsel R, Totten PA, Spiegel CA, *et al.* Nonspecific vaginitis. *Am J Med* 1983;74:14–22.
- 43 Van Der Pol B. A profile of the cobas(R) CT/NG assay on the cobas(R) 6800/8800 system for detection of Chlamydia trachomatis and Neisseria gonorrhoeae. *Expert Rev Mol Diagn* 2020;20:375–80.
- 44 Van Der Pol B. A profile of the cobas(R) TV/ MG test for the detection of Trichomonas vaginalis and Mycoplasma genitalium. *Expert Rev Mol Diagn* 2020;20:381–6.
- 45 Eisenhofer R, Minich JJ, Marotz C, *et al.* Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. *Trends Microbiol* 2019;27:105–17.
- 46 van der Veer C, Bruisten SM, van der Helm JJ, *et al.* The Cervicovaginal Microbiota in Women Notified for Chlamydia trachomatis Infection: A Case-Control Study at the Sexually Transmitted Infection Outpatient Clinic in Amsterdam, The Netherlands. *Clin Infect Dis* 2017;64:24–31.
- 47 Martin DH, Zozaya M, Lillis RA, *et al.* Unique vaginal microbiota that includes an unknown Mycoplasma-like organism is associated with Trichomonas vaginalis infection. *J Infect Dis* 2013;207:1922–31.
- 48 Ketterer MR, Rice PA, Gulati S, *et al.* Desialylation of Neisseria gonorrhoeae Lipooligosaccharide by Cervicovaginal Microbiome Sialidases: The Potential for Enhancing Infectivity in Men. *J Infect Dis* 2016;214:1621–8.
- 49 Shafer WM. Does the Cervicovaginal Microbiome Facilitate Transmission of Neisseria gonorrhoeae From Women to Men? Implications for Understanding Transmission of Gonorrhea and Advancing Vaccine Development. *J Infect Dis* 2016;214:1615–7.
- 50 Molenaar MC, Singer M, Ouburg S. The two-sided role of the vaginal microbiome in Chlamydia trachomatis and Mycoplasma genitalium pathogenesis. *J Reprod Immunol* 2018;130:11–7.
- 51 Workowski KA, Bachmann LH, Chan PA, *et al.* Sexually Transmitted Infections Treatment Guidelines, 2021. *MMWR Recomm Rep* 2021;70:1–187.
- 52 Harris PA, Taylor R, Thielke R, *et al.* Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;42:377–81.
- 53 Van Der Pol WJ, Kumar R, Morrow CD, *et al.* In Silico and Experimental Evaluation of Primer Sets for Species-Level Resolution of the Vaginal Microbiota Using 16S Ribosomal RNA Gene Sequencing. *J Infect Dis* 2019;219:305–14.
- 54 Bacchetti De Gregoris T, Aldred N, Clare AS, *et al.* Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J Microbiol Methods* 2011;86:351–6.
- 55 Einaggar JH, Ardizzone CM, Cerca N, *et al.* A novel Gardnerella, Prevotella, and Lactobacillus standard that improves accuracy in quantifying bacterial burden in vaginal microbial communities. *Front Cell Infect Microbiol* 2023;13:1198113.
- 56 Machado A, Jefferson KK, Cerca N. Interactions between Lactobacillus crispatus and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 2013;14:12004–12.
- 57 Callahan BJ, McMurdie PJ, Rosen MJ, *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13:581–3.
- 58 Quast C, Pruesse E, Yilmaz P, *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41:D590–6.
- 59 Davis NM, Proctor DM, Holmes SP, *et al.* Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 2018;6:226.
- 60 McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;8:e61217.
- 61 DiGiulio DB, Callahan BJ, McMurdie PJ, *et al.* Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci U S A* 2015;112:11060–5.
- 62 Muzny C, Aaron K, Pontius A, *et al.* P375: Risk factors for incident bacterial vaginosis among heterosexual women. Abstracts for the STI & HIV World Congress (Joint Meeting of the 23rd ISSTD and 20th IUSTI), July 14–17, 2019; Vancouver, BC Canada: STI & HIV World Congress, 2019
- 63 Olson KM, Boohaker LJ, Schwebke JR, *et al.* Comparisons of vaginal flora patterns among sexual behaviour groups of women: implications for the pathogenesis of bacterial vaginosis. *Sex Health* 2018;15:61.
- 64 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–10.
- 65 Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Methods Med Res* 1999;8:135–60.
- 66 Bland JM, Altman DG. Agreement between methods of measurement with multiple observations per individual. *J Biopharm Stat* 2007;17:571–82.
- 67 Verstraelen H, Verhelst R, Claeys G, *et al.* Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol* 2009;9:116.
- 68 Gajer P, Brotman RM, Bai G, *et al.* Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;4:132ra52.
- 69 Srinivasan S, Liu C, Mitchell CM, *et al.* Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One* 2010;5:e10197.
- 70 Romero R, Hassan SS, Gajer P, *et al.* The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2014;2:4.
- 71 Ravel J, Brotman RM, Gajer P, *et al.* Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. *Microbiome* 2013;1:29.
- 72 Crowne DP, Marlowe D. A new scale of social desirability independent of psychopathology. *J Consult Psychol* 1960;24:349–54.