The Vaginal Microbiota Impact of Antibiotic and Probiotic Compounds and Point-of-Care Testing for Vaginal Dysbiosis and Sexually Transmitted Infections

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Marijn Verwijs

February 2020

ABSTRACT

THE VAGINAL MICROBIOTA IMPACT OF ANTIBIOTIC AND PROBIOTIC COMPOUNDS AND POINT-OF-CARE TESTING FOR VAGINAL DYSBIOSIS AND SEXUALLY TRANSMITTED INFECTIONS by Marijn Verwijs

Studies employing modern molecular techniques have shown that the vaginal microbiota (VMB) of most women is dominated by beneficial *Lactobacillus* species. However, about one-third of women worldwide at any given time point have a non-optimal VMB, referred to as vaginal dysbiosis. The most common bacterial vaginal dysbiosis is bacterial vaginosis (BV), which is characterised by a decrease in lactobacilli and an increase in BV-associated anaerobic bacteria. The most common type of fungal vaginal dysbiosis is vulvovaginal candidiasis (VVC), which is characterised by the presence of *Candida* yeasts, usually in the presence of lactobacilli. BV and VVC have been associated with HIV acquisition, and BV also with adverse pregnancy outcomes. BV can be treated with the antibiotics metronidazole or clindamycin, but recurrence is common. Better therapies to treat BV and prevent its recurrence are needed.

The prevalences of BV and sexually transmitted infections (STIs) are high worldwide but are highest in sub-Saharan Africa. Many sub-Saharan African countries rely on the World Health Organisation (WHO) syndromic management algorithms to treat symptomatic women given their limited clinic and laboratory infrastructure. These algorithms provide guidance on which urogenital infections to treat when certain urogenital symptoms (referred to as 'syndromes') are reported by the patient, without the need for diagnostic testing (and with pelvic examinations being optional). A syndrome is treated for all infections that might cause that syndrome. The performances of the WHO algorithms for vaginal discharge syndrome and lower abdominal pain syndrome in women are known to be poor, and asymptomatic infections are missed by definition. Support for the introduction of point-of-care testing (POCT) is mounting but has been hampered by the lack of ASSURED ('Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end users') POCTs for some urogenital infections.

This thesis describes the results of two studies that we conducted at a research clinic in Kigali, Rwanda, and the results of a systematic review. The first study (referred to as the Rwanda VMB study) investigated the impact of oral metronidazole and two different vaginal probiotics on the VMB and on BV recurrence. Sixty-eight HIV-negative and non-pregnant women with laboratory-confirmed BV and/or *Trichomonas vaginalis* (TV) received a seven-day oral metronidazole treatment course. We assessed their VMB before and after treatment by Gram stain Nugent scoring and 16S rRNA gene HiSeq sequencing (relative abundances) combined with 16S rRNA gene qPCR (to estimate bacterial concentrations). As expected, metronidazole decreased the overall bacterial load, and the relative abundances and concentrations of BV-anaerobes (all BV-anaerobes combined, as well as those of key BV-anaerobe taxa). However, metronidazole also increased the relative abundances and concentrations of lactobacilli (all lactobacilli combined, as well as increases in all key *Lactobacillus* taxa). Pre-treatment pathobionts concentration (defined as Proteobacteria, streptococci, staphylococci, enterococci, and a few others), and having a pre-treatment vaginal microbiota type containing more than 50% *Gardnerella vaginalis* (compared to BV-like VMB types with less than 50% *G. vaginalis*), were associated with increased likelihood of treatment failure, although the latter did not reach statistical significance (p=0.044 and p=0.084, respectively).

All 68 women were subsequently randomised to four groups (n=17 each): behavioural counselling only (negative control group), intermittent use of oral metronidazole, intermittent use of Ecologic Femi+ vaginal probiotic capsule (containing multiple *Lactobacillus* and one *Bifidobacterium* species; Winclove Probiotics, Amsterdam, Netherlands), or intermittent use of Gynophilus LP vaginal probiotic tablet (containing *L. rhamnosus* 35; Biose, Arpajon-sur-Cère, France). Participants used the interventions for two months and VMB assessments were done at baseline, Day 7, Month 1, Month 2, and Month 6. Adherence was assessed by triangulating multiple adherence assessments and was taken into account in our analyses. All three interventions were safe and the preliminary efficacies were

promising. BV (Nugent 7-10) incidence was 10.18 per person-year at risk in the control group, and lower in the metronidazole (1.41/person-year; p=0.004), Ecologic Femi+ (3.58/person-year; p=0.043), and Gynophilus LP groups (5.36/person-year; p=0.220). In mixed effects models adjusted for hormonal contraception/pregnancy, sexual risk-taking, and age, metronidazole and Ecologic Femi+ users, each compared to controls, had higher *Lactobacillus* and lower BV-anaerobes concentrations and/or relative abundances, and were less likely to have a dysbiotic VMB type by sequencing. However, inter-individual variability was high and effects disappeared soon after intervention cessation. We also assessed the acceptability of the interventions using structured questionnaires and semi-structured focus group discussions and in-depth interviews and found that acceptability was high. Finally, we identified a need for information, education and counselling campaigns to inform at-risk women about steps they can take to prevent BV, VVC, and STIs.

In a systematic review, we evaluated the impact of lactobacilli-containing vaginal probiotics on BV and VVC cure and/or recurrence, as well as VMB composition and vaginal detection of probiotic strains, to compare the results of our own study with the published literature. We identified 34 studies evaluating 22 different vaginal probiotics. Unfortunately, most identified studies were under-powered and of poor methodological quality (for example, only two studies – including our own – differentiated between probiotic and autologous lactobacilli), and methodological heterogeneity between studies was high. Our conclusions were that vaginal lactobacilli-containing probiotics are safe and hold promise for BV cure and prevention, but are much less promising for VVC cure and prevention. Vaginal detection of probiotic strains never lasted long beyond the dosing period, suggesting that the probiotic lactobacilli used to date do not colonise the vagina.

The aim of the second study (referred to as the WISH study) was to compare the performances of vaginal discharge/lower abdominal pain algorithms incorporating POCTs (the WISH algorithms), with traditional WHO syndromic management algorithms, and with nucleic acid amplification test (NAAT; gold standard) results. The POCTs we evaluated included the EcoCare vaginal pH swab (Merete Medical, Luckenwalde, Germany; pH≥5.0 was considered BV) and the TV OSOM assay (Sekisui Diagnostics, Lexington, USA) for BV and TV, both regardless of symptom-reporting. Women with a positive risk score were POC-tested for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (CT/NG; by GeneXpert, Cepheid, Sunnyvale, USA). VVC was treated presumptively based on reported symptoms. We enrolled 705 Rwandan women at risk of STIs, regardless of HIV and pregnancy status, and regardless of the presence or absence of urogenital symptoms. The study showed that POCT integration was feasible in a Rwandan setting, and acceptable to participants and staff. NAAT-based urogenital infection prevalences were: CT 8.5%, NG 7.1%, TV 16.1%, BV 18.1%, and VVC 8.6%. Infection-specific sensitivities of the WHO syndromic management algorithms compared to the gold standards ranged from 58.3-74.6%, and specificities from 44.7-50.6%. WISH POCT-based algorithms had good sensitivity (68.5-76.0%) and specificity (97.4-100%) for CT, NG, and TV but low specificity for BV (41.2%; sensitivity 95.2%), and modest sensitivity (64.4%) and specificity (69.4%) for VVC. Sensitivity (73.6%) and specificity (100%) for BV can be improved by screening all women for vaginal pH and conduct confirmatory testing (such as Nugent scoring or a molecular test) in women with a vaginal pH≥5.5 (n=275 in the WISH study). Based on these data, we concluded that existing POCTs should be introduced when feasible, and that POCT development should continue, including the development of ASSURED POCTs with multiple testing targets.

Taken together, the data in this thesis suggest that urogenital infection epidemics in sub-Saharan Africa could potentially be reduced by the introduction of existing POCTs, the development of additional ASSURED POCTs, the expansion of the evidence-base for lactobacilli-containing vaginal probiotics using improved trial designs that incorporate molecular techniques, and the development of improved vaginal probiotics and other therapies to cure and/or prevent BV. Such new tools may have the potential to ultimately lower incidence of HIV and adverse pregnancy outcomes in at-risk women.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Professor J. van de Wijgert and Professor A. Darby. Beste Janneke, ik denk dat toen we naast elkaar zaten in de KD groep in 2010, niet hadden verwacht dat dit een klein decennium later tot een PhD thesis zou leiden. Wat vliegt de tijd! Ik weet nog dat het e.e.a. begon met een discussie die we hadden in het WKZ over een artikel dat ik had gelezen in de NY Times over DMPA gebruik en HIV-acquisitie. Het is grappig dat dit thema nog altijd verweven zit in deze thesis. Bedankt voor al je niet-aflatende hulp: zonder jouw stroom aan commentaar, pogingen om figuren voor de tiende keer te verbeteren, en verzoeken tot heranalyses zou het niet zo'n mooi proefschrift zijn geworden. Bedankt voor je enthousiasme, jouw vermogen om altijd de big picture te zien ('the perfect is often the enemy of the good' – al weet ik dat je bij mij juist de perfectie vaak wat meer moest benadrukken), en jouw "can do"-attitude. Bedankt ook voor de vele koffiemomenten, en gezelligheid tijdens trips naar veel verschillende plekken. Ik hoop dat we in de toekomst nog samen kunnen werken! Dear Alistair, thank you so much for your always excellent mood, and your ability to change plans when things do not quite go as planned (e.g. buying me more expensive beads when it was clear that I had no talent at making them myself – do not worry, manufacturing beads is not a part of my future career plan). I enjoyed our discussions and your insights on microbiota and other topics.

I would like to thank the whole team at Rinda Ubuzima: murakoze cyane! Stephen, thank you for your enthusiasm, humour, and for working together so well on the VMB and WISH studies. Lambert & Viateur, thank you for your detail-oriented work in the lab. Marie Michele, thank you for the help with recruitment and your love for our cause, making sure RU continues its excellent work. Menne, bedankt voor je hulp bij de timings en observaties, en de gezelligheid tijdens de anderhalve-euro-lunch.

Prof. Ravel and Mike Humphrys, thank you for performing the BactQuant qPCR - this added a lot to the VMB study chapters and I am very grateful for this. Elke, Jessica, and Adrien, thank you for your insightful comments on chapters 3 and 4. Tania Crucitti, Vicky Jespers, en Vicky Cuylaerts: bedankt voor commentaar op het WISH artikel en het verrichten van de PCRs. Zonder jullie geweldige input zou de WISH paper veel minder toegevoegd hebben.

I would like to thank several people at IGH: Dan for help with R figures, and Christina Bronowski and Christina Gill for help with DNA extraction, PCR steps, and bioinformatics steps. Laura, thank you for your input and the discussions we had. I enjoyed working together on the PTB study! Connie en Ianthe, bedankt voor jullie hulp bij de reviews. Thank you to Prof. Gordon and Prof. Davies for being in my IPAP, and to Prof. Bogaert, Dr. Hay, and Prof. Geretti for being in my PhD examination committee. I am also very grateful to IGH for helping me with financial support for two years to write this thesis.

Hanneke, bedankt voor je hulp bij het zetten van mijn eerste stapjes in de wetenschap en voor al je tips; vooral aan je raad om niet iedere mail in vijf seconden te willen beantwoorden heb ik veel gehad! Ik wil van het Tergooi ziekenhuis Drs. Roos, Drs. Visser, Dr. Braams, Dr. Bon, Berrit, Lyke en Eline bedanken. Het SFG in Rotterdam: ik was er maar kort maar ik denk niet dat er een ziekenhuis bestaat op aarde met zo'n gezellige overdracht. Met name bedankt aan Dr. Van der Weiden en Dr. Hofhuis. Verder heel erg bedankt aan het hele team in het CWZ: ik vond het erg fijn om in zo'n warm en leuk team terecht te komen!

A big thank you to friends and family for love and support. Will, thank you for your comments on the introduction, I appreciate them a lot. Thanks to P&J and the link group for your friendship, especially to Beej. Ik wil BNL bedanken: Jordi, Niko, Daniël, Paul en Jouke. Niko, je bent het vast met me eens dat het een goede beslissing was om geen klassieke talen te studeren maar iets exacters: γνῶθι σεαυτόν, zou ik zeggen. Dank ook aan het Triage (Frank en Mantre): onze vriendschap mag wat mij betreft vanaf nu iets meer Lijn 4-gebaseerd zijn. We moeten ook nog eens op dezelfde plek met elkaar werken, of het nou een ziekenhuis of een koffietent is. Jelle, bedankt voor je gastvrijheid in Rotterdam: het bureau met het beste uitzicht ooit! Jouke & Stephanie, Roos & Erik, Joop & Justine, bedankt dat ik bij jullie bezoek mocht en/of logeren als ik in Nederland was – het was altijd erg gezellig! Teun en Joop, ik houd van jullie, bedankt voor alles, en ik hoop dat we jullie dit jaar vaker zullen zien. Papa, bedankt voor je enthousiasme over mijn onderzoek, je vele vragen en input, en je goede tips over van alles. Mama, dank voor je medeleven en ontelbare whatsappjes waarvan ik maar een fractie beantwoord heb, je humor, en dat je zo wijs bent. Sarah, thank you for being my best friend, and for taking the big leap to move to Liverpool - my life would have been quite different if you hadn't been here. Even though you may have wondered sometimes what the point was of me being awake at night thinking about possible improvements to an article figure, your support means a lot to me. I am looking forward to our next adventure!

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PUBLICATIONS

The following manuscripts that included data presented in this thesis were published, or submitted for publication:

Verwijs MC, Agaba SK, Sumanyi JC, Umulisa MM, Mwambarangwe L, Musengamana V, Uwineza M, Cuylaerts V, Crucitti T, Jespers V, van de Wijgert JHHM. Targeted point-of-care testing compared with syndromic management of urogenital infections in women (WISH): a cross-sectional screening and diagnostic accuracy study. *Lancet Inf Dis* 2019; 19(6):658-69.

van de Wijgert JHHM, Verwijs MC, Agaba SK, Bronowski C, Mwambarangwe L, Musengamana V, Uwineza M, Lievens E, Nivoliez A, Ravel J, Darby A. Intermittent lactobacilli-containing vaginal probiotic or metronidazole use to prevent bacterial vaginosis recurrence: safety and preliminary efficacy by microscopy and sequencing. *Scientific Reports* 2019; doi:10.1101/19001156 (*in press*).

van de Wijgert JHHM, Verwijs MC. Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs. *BJOG* 2019; doi:10.1111/1471-0528.15870.

Verwijs MC, Agaba SK, Darby AC, van de Wijgert JHHM. Impact of oral metronidazole treatment on the vaginal microbiota and correlates of treatment failure. *AJOG* 2019; doi:10.1016/j.ajog.2019.08.008.

Verwijs MC, Agaba SK, Umulisa MM, Uwineza M, Nivoliez A, Lievens E, van de Wijgert JHHM. Vaginal probiotic adherence and acceptability in high-risk Rwandan women participating in a pilot randomised controlled trial: a mixed-methods approach. *BMJ Open* 2019; (*accepted*).

ABBREVIATIONS

Abbreviations are also explained at their first occurrence in each chapter.

ASSURED Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end users
ASV Amplicon sequence variant
BV Bacterial vaginosis
BVAB Bacterial vaginosis-associated bacterium
CI Confidence interval
CT *Chlamydia trachomatis*
FGD focus-group discussion
GV *Gardnerella vaginalis*
HIV Human immunodeficiency virus
GUD Genital ulcer disease
IDI In-depth interview
LAP Lower abdominal pain
NG *Neisseria gonorrhoeae*
NPV Negative predictive value
OR Odds ratio
OTU Operational taxonomic unit
POCT Point-of-care test
PPV Positive predictive value
(q)PCR (quantitative) Polymerase chain reaction
STI Sexually transmitted infection
TV *Trichomonas vaginalis*
UTI Urinary tract infection
VDS Vaginal discharge syndrome
VMB Vaginal microbiota
VMB Study 'Preparing for a clinical trial of interventions to maintain normal vaginal microbiota for preventing adverse reproductive health outcomes in Africa' study
VVC Vulvovaginal candidiasis
WHO World Health Organisation
WISH Study 'Women's Improvement of Sexual and reproductive Health' study

Chapter 1 - General Introduction

1.1 Vaginal Microbiota Compositions

The term microbiota refers to 'the assemblage of microorganisms present in a defined environment', be it commensal, symbiotic, or pathogenic.[1,2] We have known since the end of the 19th century that the vagina of most women contains lactobacilli.[3,4] Modern molecular methods such as 16S rRNA gene sequencing and quantitative polymerase chain reaction (qPCR) have greatly increased vaginal microbiota (VMB) composition knowledge.[4] These studies have shown that most women have a low-diversity, optimal VMB that is dominated by beneficial lactobacilli. This optimal VMB state is also referred to as eubiosis.[5]

The VMB of most women is dominated by either *L. iners* or *L. crispatus*.[4] *L. iners* is more common in African and African-American women, while *L. crispatus* is more common in Caucasian and Asian women. Lactobacilli produce lactic acid which acidifies the vagina, thereby causing the vagina to have a low pH (<4.5) in normal conditions.[6] Lactic acid has important antimicrobial, antiviral, and immunomodulatory properties.[6,7] The role of *L. iners* is subject to debate, as it seems to protect women less well from other organisms than other *Lactobacillus* species: *L. iners* has been associated with more frequent transitions to non-optimal microbiota states than *L. crispatus* in longitudinal studies.[4,8–11] *L. iners* does produce lactic acid, but in lower quantities than *L. crispatus*. In some women the VMB is dominated by *L. gasseri, L. jensenii,* or *L. vaginalis* instead, but these species are less common and less well studied. The reasons for the ethnic differences in *Lactobacillus* domination are unclear as of yet but may depend on genetic, behavioural, and dietary factors.[4,8]

Some women have a non-optimal VMB in which there is no domination by lactobacilli. This is referred to as vaginal dysbiosis.[4,5,9] In this thesis, we use the term vaginal dysbiosis to refer to all VMB states in which there is a microbiological deviation from the optimal *Lactobacillus*-dominated low-diversity state, or states in which lactobacilli still form a majority but with important contributions of organisms with a relatively high pathogenicity index (which we refer to as pathobionts as explained later on in this chapter).[9] All forms of vaginal dysbiosis are associated with significant but differing degrees of vaginal inflammation, regardless of whether the affected woman experiences symptoms or not.[12] This thesis will therefore use the term vaginal dysbiosis regardless of patient symptomatology. The most common forms of vaginal dysbiosis are bacterial vaginosis (BV), low-diversity anaerobic dysbiosis (most commonly domination by *Gardnerella vaginalis*), bacterial dysbiosis characterised by high relative abundance of pathobionts, and the fungal dysbiosis vulvovaginal candidiasis (VVC).

BV is the most common vaginal dysbiosis. It is a state in which multiple fastidious anaerobic bacterial species dominate the VMB.[4] This process is associated with a loss of lactobacilli. The decrease in

Lactobacillus concentration results in a lower production of lactic acid and therefore an increase in the vaginal pH.[7] Examples of BV-associated anaerobic species that are often described are *Gardnerella vaginalis*, *Atopobium vaginae*, *Dialister* spp., BV-associated bacterium type 1 and 2 (BVAB-1 and BVAB-2), *Prevotella* spp., *Parvimonas* spp., *Mobiluncus* spp., *Megasphaera* spp., and *Mageeibacillus indolicus* (formerly BVAB-3).[4,13] These species often co-occur in BV in a highly diverse bacterial community, which is why the bacterial richness (total number of species observed in a given sample) and the alpha diversity index (a measure to quantify the diversity of the vaginal community in a given sample) of the VMB in these women is higher than in *Lactobacillus*-dominated women.[14,15] BV is associated with inflammation, but is not as inflammatory as the bacterial sexually transmitted infections (STIs) or VVC,[16] which is why the term 'vaginosis' was used instead of 'vaginitis'.[5] This may also explain why BV is often asymptomatic. BV is associated with important sequelae, such as increased rates of HIV transmission (both male-to-female and female-to-male), increased rates of the acquisition of other STIs, pelvic inflammatory disease, and adverse pregnancy outcomes such as stillbirth, invasive neonatal infections, and preterm birth.[9,17–20] These associations have also been found in asymptomatic women. Most medical guidelines worldwide recommend that BV is only treated when the woman has urogenital symptoms.[21–24] In some European countries, asymptomatic BV in pregnant women with a history of pre-term birth does get treated.[23] BV is generally not considered an STI but both its prevalence and recurrence are associated with sexual behaviours, and couples studies have shown that BV-associated anaerobic species are sexually transmitted.[4,25] BV is also positively associated with vaginal hygiene practices and menses, and negatively associated with hormonal contraception.[25–27]

Another type of anaerobic vaginal dysbiosis is when one single (facultative) anaerobic species dominates the VMB, such as *G. vaginalis*, *A. vaginae*, or *Prevotella* species. We will refer to this as low-diversity (anaerobic) dysbiosis.[5] Compared to classical BV, this form of vaginal dysbiosis has a low alpha diversity.[4,5,9] At the moment, classical high diversity BV and low diversity anaerobic dysbiosis are not considered separate entities from a clinical perspective,[5] and the treatment is the same.

A third type of bacterial vaginal dysbiosis is a VMB characterised by a high abundance or concentration of pathobionts.[9] Pathobionts have higher intrinsic pathogenicity than BV-associated anaerobes. They are often present in the VMB without causing problems, but they can cause serious illness in certain circumstances, for example when transmitted from a mother to a neonate.[5,28] Important examples in the vaginal niche are *Streptococcus agalactiae* (also known as Group B Streptococcus), *Staphylococcus aureus*, and several Enterobacteriaceae such as *Escherichia coli* (a complete list of organisms that we considered to be pathobionts is given in Appendix A). They generally do not dominate the VMB in the way BV-anaerobes or lactobacilli do, but are present at low or modest concentrations. At the moment, this type of vaginal dysbiosis is not recognised as a clinical entity and we will refer to it as 'pathobiont-dysbiosis' in the remainder of this thesis, regardless of symptomatology.

VVC is a type of vaginal dysbiosis caused by vaginal yeasts. The most common causative organism is *Candida albicans* which accounts for 70-95% of the cases.[29,30] *C. albicans* is highly inflammatory and it has been hypothesised that asymptomatic carriage only occurs at low concentrations.[30–32] Asymptomatic carriage is common, but when VVC is symptomatic, many clinicians believe that it is associated with a triad of typical symptoms (curd-like vaginal discharge, genital itching/ burning, and soreness) and typical signs (vulval erythema, excoriations, and oedema).[21] VVC often co-exists in a *Lactobacillus*-dominated VMB at a normal vaginal pH, and has been negatively associated with BV in many epidemiological studies.[4,9,30] VVC is also associated with an increased likelihood of HIV acquisition when exposed to HIV.[18]

Trichomoniasis is caused by a single-celled protozoa called *T. vaginalis* (TV). TV is sometimes described as a cause of vaginal dysbiosis as it often co-occurs with BV-anaerobes such as *Mycoplasma*, *Parvimonas* and *Sneathia*, and with BV in general.[33,34] However, TV is an STI, and is mostly asymptomatic in both men and women.[34] TV is also associated with other STIs, pelvic inflammatory disease, adverse pregnancy outcomes such as preterm delivery and premature rupture of membranes, as well as with increased HIV acquisition.[34,35]

1.2 Urogenital Infections Prevalences Worldwide and in Sub-Saharan Africa

BV is the most common curable vaginal infection worldwide, but other urogenital infections are also prevalent (note that BV is a polymicrobial condition but we will occasionally refer to it as a urogenital infection to improve readability). According to 2019 estimates, the prevalence of BV (defined as a Nugent score of 7-10 regardless of symptoms) ranges between 23-29% worldwide, and was estimated at 25% in sub-Saharan Africa.[36] However, the 2018 estimates from an individual participant meta-analysis of 18 HIV prevention studies in sub-Saharan Africa were much higher at 42.1% among 15-24-year-old women and 41.2% among women aged 25-49 years.[37] This meta-analysis included studies that preferentially enrolled participants with higher sexual risk behaviours than the general population.

2012 World Health Organisation estimates of the worldwide prevalence of the four main curable STIs among women were: *Chlamydia trachomatis* (CT) 4.2%, *Neisseria gonorrhoeae* (NG) 0.8%, TV 5.0% and syphilis 0.5%.[38] The combined incidence of these four curable STIs among men and women was estimated at one million cases per day. The aforementioned 2018 individual patient meta-analysis estimated the prevalence of these four STIs in HIV-negative sub-Saharan African women at 15.1% for CT, 4.6% for NG, 0.4% for active syphilis, and 7.9% for TV among 15-24-year-olds, and 7.0% for CT, 2.5% for NG, 1.0% for high-titer syphilis, and 8.6% TV among 25-49-year-olds.[37] Most studies included in the meta-analysis showed that TV is the most common of these four curable STIs.[34] Two common viral STIs other than HIV are herpes simplex virus type 2 and human papillomavirus. Both

viruses are incurable, and are more highly prevalent than the four curable STIs mentioned previously. The WHO estimated in 2012 that 416 million people were living with herpes simplex virus type 2, of which 267 million were women, corresponding to a global prevalence of 14.8% among women.[39] The 2018 meta-analysis among HIV-negative sub-Saharan African women estimated the prevalence of herpes simplex virus type 2 among women aged 15-24 years to be 39.3% and in women aged 25-49 years 77.8%.[37] The annual incidence of herpes simplex virus type 2 among 15-49-year-old women was estimated to be 0.6% worldwide and 3.4% in Africa.[39] The worldwide prevalence of human papillomavirus in women (with normal cytology) has been estimated at 10.4%, corresponding to 291 million women, and was highest in sub-Saharan Africa at 22.1%.[40,41]

The prevalences of VVC and pathobionts (except for group B *Streptococcus* by rectovaginal culture) are currently not precisely known. Current VVC estimates are often based on self-reporting by women rather than on laboratory-confirmed VVC.[42] In a 2018 systematic review, it was estimated that 138 million women are affected by recurrent VVC annually; the prevalence of recurrent VVC in women aged 25-34 years was estimated at 9%.[43] A 2013 study found that up to 50% of women had self-reported, health care provider-diagnosed vaginal yeast infections in their lifetime; around 23% of them suffer from recurrent VVC.[44] The presence of *C. albicans* (including low-concentration) carriage in the VMB of asymptomatic women is around 20%.[30] The epidemiology of pathobiont-dysbiosis is also largely unknown because it has not yet been recognised as a specific clinical entity. A 2016 meta-analysis estimated the worldwide prevalence of maternal *S. agalactiae* carriage by rectovaginal culture at 17.9%, and at 22.4% in sub-Saharan Africa.[45]

1.3 Impact of Antibiotics on the Vaginal Microbiota

Metronidazole and clindamycin are the most commonly prescribed antibiotics for vaginal dysbiosis.[46,47] These antibiotics are also prescribed for the syndromic management of vaginal discharge syndrome and pelvic inflammatory disease, intending to cover both BV and TV, or after laboratory-confirmed diagnosis of (symptomatic) BV and/or TV. Multi-day treatment of BV with metronidazole or clindamycin is often effective, with cure rates directly after treatment being between 65-90%.[22,46–49] Metronidazole and clindamycin can both be given in oral and vaginal formulations, and bio-availability in the vagina is excellent after oral administration.[50] The cure rates of oral and vaginal formulations of metronidazole and clindamycin do not differ significantly.[48,49] The recurrence of (symptomatic) BV after metronidazole treatment is high, at rates of over 50% within a year.[47,51,52] BV recurrence is thought not to be driven by antibiotic resistance, because resistance to metronidazole (contrary to resistance to clindamycin) has not commonly been described in culture-based BV treatment studies.[46,52,53] However, some studies contradict this and do report metronidazole resistance in women with recurrent BV, particularly in isolates with high *G. vaginalis* concentrations.[54–56] The efficacy of a seven-day oral

metronidazole treatment course for TV is high; a single-dose treatment is often given to maximise adherence but is not as effective.[57] Metronidazole is a nitroimidazole-class drug which is metabolised into nitroso radicals by anaerobic species and TV, which break microbial DNA and cause cell lysis.[58,59] Although it is sometimes assumed that metronidazole suppresses lactobacilli, culture-based studies show that lactobacilli are not sensitive to metronidazole.[60–63] The impact of metronidazole on the VMB using modern sequencing methods had not been extensively studied at the time of our study.

Clindamycin is also commonly prescribed for BV.[46] Culture-based studies show that *L. crispatus* and *jensenii* are (partially) sensitive to clindamycin, whereas other lactobacilli such as *L. iners* are not.[61,62] Treatment with clindamycin could therefore theoretically decrease both BV-anaerobes and *L. crispatus*, causing the non-optimal *L. iners* species to proliferate instead and increasing the risk of BV recurrence (compared to a *L. crispatus*-dominated VMB). However, this has not been studied in molecular studies. Clindamycin has also been described as being effective treatment for some pathobiont species such as *S. agalactiae*,[28,64] but this has not been studied in-depth in randomised controlled trials. No studies have comprehensively assessed the impact on the VMB of antibiotics given for non-gynaecological infections, but VVC has commonly been described as a side-effect of (systemic) antibiotic use.[65–67]

1.4 Impact of Vaginal Lactobacilli-Containing Probiotics on the Vaginal Microbiota

An alternative to antibiotics for the treatment or prevention of vaginal dysbiosis is the vaginal delivery of exogenous, beneficial live bacteria, most often lactobacilli.[68–70] These products are commonly called probiotics or, more recently, live biotherapeutic products (LBPs).[71] Probiotic therapy is designed to increase the number of beneficial lactobacilli and restore the dominance of lactobacilli in the vagina.[46,47,68] Probiotics do not cause antibiotic resistance by definition, and may prevent BV and VVC recurrence.[69] They can be given as main therapy for vaginal dysbiosis, or as adjuvant treatment together with antibiotics or antifungals. They can also be given after (successful) antibiotic/antifungal treatment to maintain an optimal VMB. It is unclear whether probiotics can only be successful if they colonise the vagina and therefore should be given at high concentrations to ultimately dominate the VMB, or whether probiotics can optimise the VMB without domination but by increasing lactic acid secretion, by modulation of local cervicovaginal mucosal immune responses, by the action of metabolites contained in the products, and/or by inhibition of biofilm formation.[6,12,72–74] Furthermore, it is unclear whether probiotics can decrease BV-anaerobes.

Some believe that probiotics to prevent BV or VVC could also be given orally. The rationale behind this is that there is a high degree of microbiological cross-talk between the gut/anal microbiota and the VMB: orally administered lactobacilli have been recovered from the vagina in some studies.[69,75] This recoverability may depend on multiple factors: the capacity of probiotic strains to survive the passage

through the stomach, the delay in vaginal colonisation after administration, and the *Lactobacillus* concentration that will finally be delivered in the vagina.[69] It is unclear whether oral probiotics are equally effective as vaginal probiotics: the effect of oral probiotics may be more diluted than that of vaginal probiotics as the former are not delivered directly at the intended target site. We have therefore focussed on vaginal probiotics in this thesis. In the past, probiotics could be marketed without the need of robust evidence for their effectiveness. The regulatory landscape for vaginal probiotics has, however, changed considerably in recent years. When health claims are made, the US Food and Drug Administration has required human drug approval for probiotics since 2016, and the European Medicines Authority will follow suit per 1 May 2020. As far as we know, only one of the products included in this review is being developed as a drug for human use: Lactin-V. It contains a *L. crispatus* strain (CTV-05) that was isolated from a healthy woman.[76]

1.5 Tests and Algorithms for Diagnosing Urogenital Infections

Gold standard laboratory tests of urogenital infections

BV is commonly treated by syndromic management or presumptively (see section 'Syndromic and presumptive management'). However, in some specialised urogenital disease clinics and in research settings, the Amsel criteria are used.[77,78] A woman is considered to have BV by Amsel criteria if three or more of the following criteria are present: 1) clue cells observed by microscopy on a wet mount preparation (often, a cut-off of ≥20% clue cells is used) 2) a positive whiff test, with a 'fishy odour' detected when adding 10% KOH to vaginal secretions 3) a vaginal pH of ≥4.5, and/or 4) the presence of a thin, homogenous vaginal discharge.[77] In some research settings (including in this thesis), a modified version of the Amsel criteria is used in which the fourth criterion is disregarded because of its subjective nature: the modified Amsel criteria are positive if two out of the three remaining objective criteria are present. Some argue that vaginal pH alone, for example in settings where microscopy is not available, might be sufficient to diagnose BV.[79] The Nugent score is generally used in research settings only, and is a standardised scoring system of a Gram stain preparation, in which three bacterial morphotypes are visualised: Gram-positive rods thought to be lactobacilli, small Gram-negative or Gram-variable rods thought to be *Gardnerella/Bacteroides*, and curved Gram-variable rods thought to be *Mobiluncus*. Vaginal smears receive a score of 0-10, with 0-3 indicating normal microbiota, 4-6 intermediate microbiota, and 7-10 BV.[78] While currently still considered the gold standard diagnostic test for BV, Gram stain Nugent scoring is not fully quantitative, cannot differentiate between different types of lactobacilli, does not comprehensively assess all BV-associated anaerobes, and sometimes misclassifies key species.[80–82] New qPCR-based multiplex tests (generally targeting *Lactobacillus* concentration and/or *Gardnerella* concentration) and scoring systems have been developed to improve BV diagnosis, but their performance has typically been compared to Nugent scoring rather than used to establish novel molecular-based definitions of BV and other types of vaginal dysbiosis.[83–86] One major obstacle is that

we currently do not know what the lactobacilli concentration threshold is below which we should diagnose and treat vaginal dysbiosis.

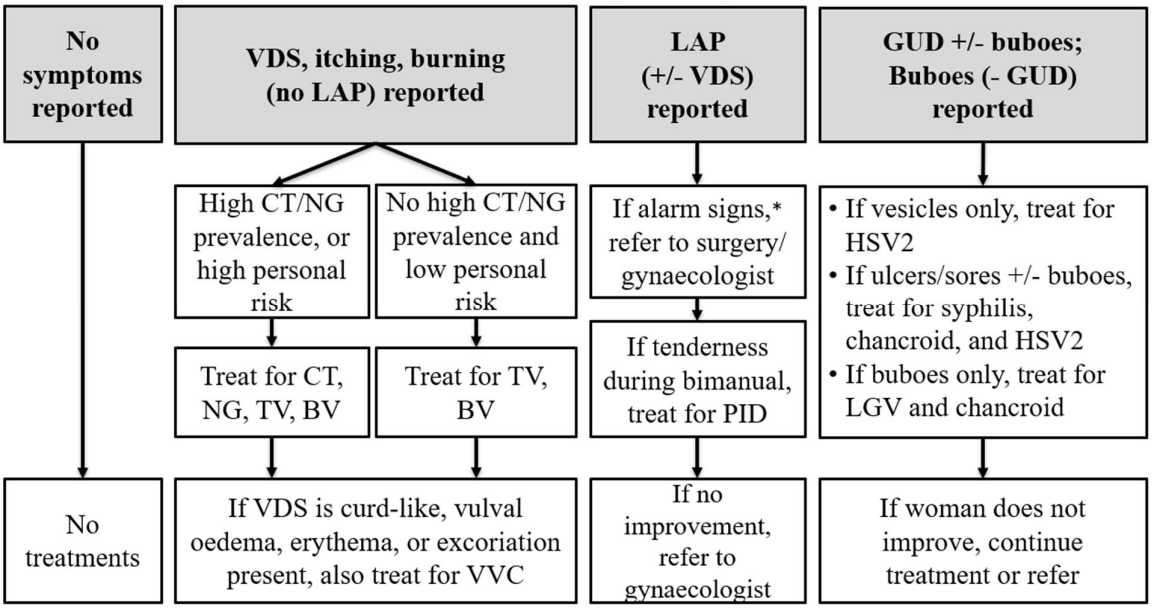
No gold standard test for VVC exists.[42,65,87] Some articles state that mycological culture is the gold standard (in symptomatic patients), because (q)PCR detection of *Candida* species would result in overtreatment of women with low *Candida* concentrations.[65,88] As with BV, a major obstacle is that we currently do not know what the *Candida* concentration threshold is above which we should diagnose and treat VVC.[30] However, *Candida* is highly inflammatory and asymptomatic carriage may be less harmless than often presumed.[65,89]

The gold standard test for TV is by nucleic acid amplification test (NAAT).[34] In many settings, microscopy (by observation of trichomonads on a wet mount) or TV culture are used, but these result in low sensitivity.[34,90] The gold standard laboratory tests for both CT and NG are also by NAAT.[91] Usually, laboratory tests for CT and NG are multiplexed in a single device or test.[92]

Syndromic and presumptive management

Urogenital infections, including BV and VVC, are most commonly diagnosed and treated by syndromic management in resource-poor settings, and presumptively in resource-rich settings. The WHO-recommended guidelines for the management of urogenital infections are based on reported symptoms alone, without the need for laboratory testing, to accommodate resource-poor countries and settings in which laboratory infrastructure is lacking and/or too expensive.[21] Each patient-reported symptom, potentially augmented by clinician-observed signs during a physical examination, is treated for all organisms that might cause that symptom. The four main syndromes in women are (figure 1.1): vaginal discharge (VDS) without lower abdominal pain (LAP); LAP regardless of the presence of VDS; genital ulcers regardless of the presence of inguinal buboes; and inguinal buboes without genital ulcers. The WHO recommends that VDS without LAP, the most common of the four,[93,94] is treated for BV and TV in all cases. VDS without LAP is also treated for CT and NG in the case of high risk due to a high CT/NG prevalence in the target population or high personal sexual risk (as determined by locally designed risk assessments). If the VDS is curd-like and vulval oedema, erythema, and/or excoriations are observed, the WHO recommends treatment for VVC as well.[21] Oedema, erythema, and excoriations can only be diagnosed by a clinician during a physical (preferably pelvic) examination, which are often not available. Local guidelines (including those in Rwanda) therefore often replace them with patient-reported VVC symptoms (e.g. curd-like discharge, genital burning and/or itching, soreness).[24] Syndromic management algorithms for urogenital symptoms in men are also available,[21,24] but are beyond the scope of this thesis.

Figure 1.1: Summary of the WHO syndromic management algorithms



BV bacterial vaginosis, *CT* Chlamydia trachomatis, *GUD* genital ulcer disease, *HSV2* Herpes simplex virus type 2, *LAP* lower abdominal pain, *LGV* lymphogranuloma venereum, *NG Neisseria gonorrhoeae*, *PID* pelvic inflammatory disease, *TV Trichomonas vaginalis*, *Vag* vaginal, *VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis.
The top rows are symptoms reported by participants. For details about the syndromic management algorithms, see WHO guidelines.[21]
*LAP requires examination. Alarm signs: missed/overdue period, recent delivery/abortion/miscarriage, abdominal guarding and/or rebound tenderness, abnormal vaginal bleeding, and abdominal mass. Treated for PID if no alarm signs but cervical motion, uterine, and/or adnexal tenderness are present, or when lower abdominal tenderness and VDS are present. PID treatment covers CT/NG/TV/BV. The VDS algorithm is followed if there are no alarm signs or if no PID is present.

In other settings, such as primary care clinics in Europe, urogenital symptoms are often treated presumptively.[95,96] This approach is less well standardised than syndromic management and relies heavily on individual clinical judgments. A physician will treat patient-reported symptoms for what s/he considers the most likely diagnosis, and will treat for the next most likely diagnosis if the symptoms persist after treatment. Diagnostic testing is typically only done when earlier treatment efforts fail.

The main problem of the syndromic and presumptive approaches is that all women with asymptomatic infections are missed by definition, as well as women with symptoms who do not seek care for those symptoms (which may or may not be a problem for BV and VVC as discussed in this thesis: asymptomatic BV and VVC have been associated with the same complications as symptomatic BV and VVC).[9,87] Syndromic management can lead to undertreatment when asymptomatic infections are untreated, and overtreatment when (multiple) urogenital infections are treated for without being present. The performance of four syndromic management algorithms in women are all suboptimal, with low sensitivity and specificity, even in studies that displayed a high degree of selection bias by only enrolling symptomatic women.[94,97–101] Furthermore, algorithms are often not applied well by clinicians, even further reducing their performances.[102,103]

Point-of-care testing

Syndromic management performs poorly and gold standard laboratory testing is often not feasible or cost-effective, not even in resource-rich settings. The introduction of point-of-care testing (POCTs) might offer a reasonable alternative, preferably enabling clinicians to provide treatment for positive results at the same visit without needing a patient to return and thereby minimising loss to follow-up.[104,105] POC testing could also be used to screen asymptomatic women at risk of STIs or pregnant women (for whom the risk of sequelae is higher than in the general population).[106] Additional advantages of POC testing include increased willingness to co-operate in partner notification and treatment due to the availability of a specific, laboratory-confirmed diagnosis, etc.[107] Many POCTs have been developed over the past decades, but their characteristics vary greatly.[106] For this reason, the WHO devised criteria that POCTs ideally should adhere to, the so-called ASSURED criteria: they should be 'Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end users'.[104,106,108]

ASSURED POCTs are available and widely used for HIV, syphilis, and pregnancy, including in resource-poor settings.[109,110] However, no ASSURED POCTs are available for CT, NG, TV, and BV. In addition, gold standard diagnosis for BV and VVC (as well as other types of vaginal dysbiosis) are currently suboptimal (see "Gold standard laboratory tests of urogenital infections"), which complicates POCT performance assessments.[105,106,108] Other challenges that may hinder POCT integration include: cost-effectiveness considerations,[79,111,112] increased waiting times in clinics when implementing POC or laboratory testing,[113] a lack of willingness to be tested in certain settings if a someone is asymptomatic,[114] and barriers at health-system level, whether local, regional, or national.[79] A short overview of the most promising POCTs available on the market for each urogenital disease, including the ones we chose to investigate in this thesis, is presented in Appendix E.

1.6 Aims of this Thesis and Overview of Chapters

The main aim of this thesis is to assess interventions that can potentially be used to improve sexual and reproductive health in sub-Saharan African women. The thesis consists of seven chapters: **Chapter 1** is a general introduction to this thesis. Chapters 2-4 describe the results of the Rwanda VMB study, conducted in Kigali, Rwanda. In **Chapter 2**, the impact of a seven-day oral metronidazole treatment course on the VMB of 68 women diagnosed with BV and/or TV is discussed. Their VMB characteristics before and after treatment are compared, as well as sociodemographic and microbiologic factors associated with treatment failure. In **Chapter 3**, the safety and preliminary efficacy results of a pilot clinical trial in which these same 68 women were enrolled are presented. In this trial, women were randomised to intermittent use of oral metronidazole or two different vaginal probiotics, each compared to a control group of women receiving behavioural counselling only. **Chapter 4** provides more detail on

the adherence to the interventions in the trial, as well as acceptability of the interventions as assessed by structured face-to-face interviews and semi-structured focus-group discussions and in-depth interviews. The chapter also includes the results of a structured face-to-face survey held among 131 Rwandan women (some of whom participated in the trial and some did not) about their knowledge of and attitudes towards urogenital infections. **Chapter 5** describes a systematic review of published lactobacilli-containing vaginal probiotic studies with BV and/or VVC cure or prevention outcomes. The chapter also includes the results of our own pilot trial as well as recommendations for future vaginal probiotic clinical trial designs. In **Chapter 6**, we report the results of the WISH study, which was also conducted in Kigali, Rwanda. The study evaluated the feasibility, acceptability and performance of POCT integration on urogenital disease case-finding and management in 705 women. Potential challenges related to POCT integration in STI or primary care clinics are discussed. **Chapter 7** is the final chapter, which synthesises and discusses the key findings of this thesis.

Chapter 2 - Impact of Oral Metronidazole Treatment on the Vaginal Microbiota and Correlates of Treatment Failure.

This chapter has been submitted to the international peer-reviewed journal *American Journal of Obstetrics and Gynecology*, and has been accepted for publication: Verwijs MC, Agaba SK, Darby AC, van de Wijgert JHHM. Impact of oral metronidazole treatment on the vaginal microbiota and correlates of treatment failure. *AJOG* 2019; doi:10.1016/j.ajog.2019.08.008. [Epub ahead of print]. The version presented here is the author-approved AJOG-accepted version with only minor modifications (numbering of figures, tables, and references). The methods used in Chapters 2 and 3 overlap considerably, and I have therefore combined the supplementary methods into one Appendix that applies to both chapters (Appendix A).

I reviewed the clinical data that had been collected by the Rinda Ubuzima team, and conducted the site close-out visit, in Kigali, Rwanda, under the supervision of Professor Janneke van de Wijgert (my primary supervisor). I subsequently coordinated the shipments of the stored vaginal samples from Kigali to Liverpool, and prepared them for sequencing at the University of Liverpool Centre for Genomic Research under the supervision of Professor Alistair Darby (my secondary supervisor). I processed the raw sequencing data, derived various VMB variables, and added them to the clinical database with the help of both my supervisors as well as fellow PhD student Christina Gill. I developed the analytical approach and performed the statistical analyses together with my primary supervisor. I wrote the first draft of the manuscript. All co-authors commented on and approved the final manuscript.

Abstract

Introduction: Metronidazole is the first-line treatment for bacterial vaginosis (BV), but cure rates are suboptimal and recurrence rates high.

Materials and methods: The objective of this study was to evaluate the impact of a standard course of oral metronidazole treatment (500 mg twice per day for seven days) on the vaginal microbiota of Rwandan bacterial vaginosis patients using microscopy and 16S rRNA gene sequencing, and to evaluate correlates of treatment failure. HIV-negative, non-pregnant women aged 18-45 with BV and/or *Trichomonas vaginalis* (TV; total N=68) were interviewed and sampled before and after metronidazole treatment. They were also screened, and treated if applicable, for other urogenital infections. The vaginal microbiota was assessed by Gram stain Nugent scoring, Illumina 16S rRNA HiSeq sequencing (relative abundances), and BactQuant 16S gene qPCR (estimated concentrations). Only women with a pre-treatment Nugent score of 7-10 and a valid post-treatment Nugent score (N=55) were included in metronidazole treatment failure analyses, with treatment failure defined as a post-treatment Nugent score of 4-10.

Results: The BV cure rate by Nugent scoring was 54.5%. The mean total vaginal bacterial concentration declined from 6.59 to 5.85 $\log_{10}$ cells/µl ($p<0.001$), which was mostly due to a reduction in mean BV-anaerobes concentration (all BV-associated anaerobe taxa combined) from 6.23 to 4.55 $\log_{10}$ cells/µl ($p<0.001$). However, only 16.4% of women had a BV-anaerobes concentration reduction of more than 50%, and only three women had complete eradication. The mean concentration of lactobacilli (all species combined) increased from 4.98 to 5.56 $\log_{10}$ cells/µl ($p=0.017$), with *Lactobacillus iners* being the most common species pre- and post-treatment. The mean concentration of pathobionts (defined as Proteobacteria, streptococci, staphylococci, enterococci, and a few others) did not change significantly: from 1.92 $\log_{10}$ cells/µL pre-treatment to 2.01 $\log_{10}$ cells/µL post-treatment ($p=0.939$). Pre-treatment pathobionts concentration, and having a pre-treatment vaginal microbiota type containing more than 50% *Gardnerella vaginalis* (compared to less than 50%), were associated with increased likelihood of treatment failure, but the latter did not reach statistical significance ($p=0.044$ and $p=0.084$, respectively).

Discussion: Metronidazole alone may not cure women with high *G. vaginalis* relative abundance, potentially due to biofilm presence, and women with high pathobionts concentration. These women may benefit from additional biofilm-disrupting and/or pathobiont-targeting treatments.

2.1 Introduction

Most women have an optimal vaginal microbiota (VMB) dominated by lactobacilli, but vaginal dysbiosis is highly prevalent.[4,9] The most common type of vaginal dysbiosis is bacterial vaginosis (BV), which is characterised by a reduction of lactobacilli and an increase of other anaerobes, usually leading to increased species diversity.[4,9] Other women carry microorganisms that do not necessarily dominate the VMB but have a higher pathogenic potential than BV-anaerobes, such as bacterial pathobionts (including most Proteobacteria, streptococci, staphylococci, and enterococci), *Candida albicans* (which is the main cause of vulvovaginal candidiasis) or *Trichomonas vaginalis*.[4,9]

Vaginal dysbiosis can cause symptoms but is often asymptomatic.[4,9] Both symptomatic and asymptomatic vaginal dysbiosis have been associated with pelvic inflammatory disease, HIV acquisition, and adverse pregnancy outcomes, with substantial attributable risk due to its prevalence.[9,17–19] Symptomatic dysbiosis is most commonly diagnosed empirically or syndromically without laboratory testing.[21] However, in research settings, BV is diagnosed by Nugent scoring of vaginal Gram stains or by the Amsel criteria.[77,78] BV is treated with antibiotics, of which oral and vaginal formulations of metronidazole or clindamycin are most commonly used.[47] While short-term BV cure rates of multi-day oral and vaginal metronidazole regimens are 65-90%,[48,49] recurrence rates are high.[51,52]

Metronidazole is a nitroimidazole-class drug. Under anaerobic conditions, anaerobic bacteria and some parasites (including *T. vaginalis*) metabolise metronidazole into nitroso radicals, which break microbial DNA and cause cell lysis.[58,115] Culture studies have shown that lactobacilli are not sensitive to metronidazole.[63] Metronidazole has thus far been associated with low levels of antimicrobial resistance.[50] The high recurrence rate is therefore often hypothesised to be due to vaginal mucosal biofilm formation by *G. vaginalis* and other BV-associated anaerobes, but has not been confirmed.[116]

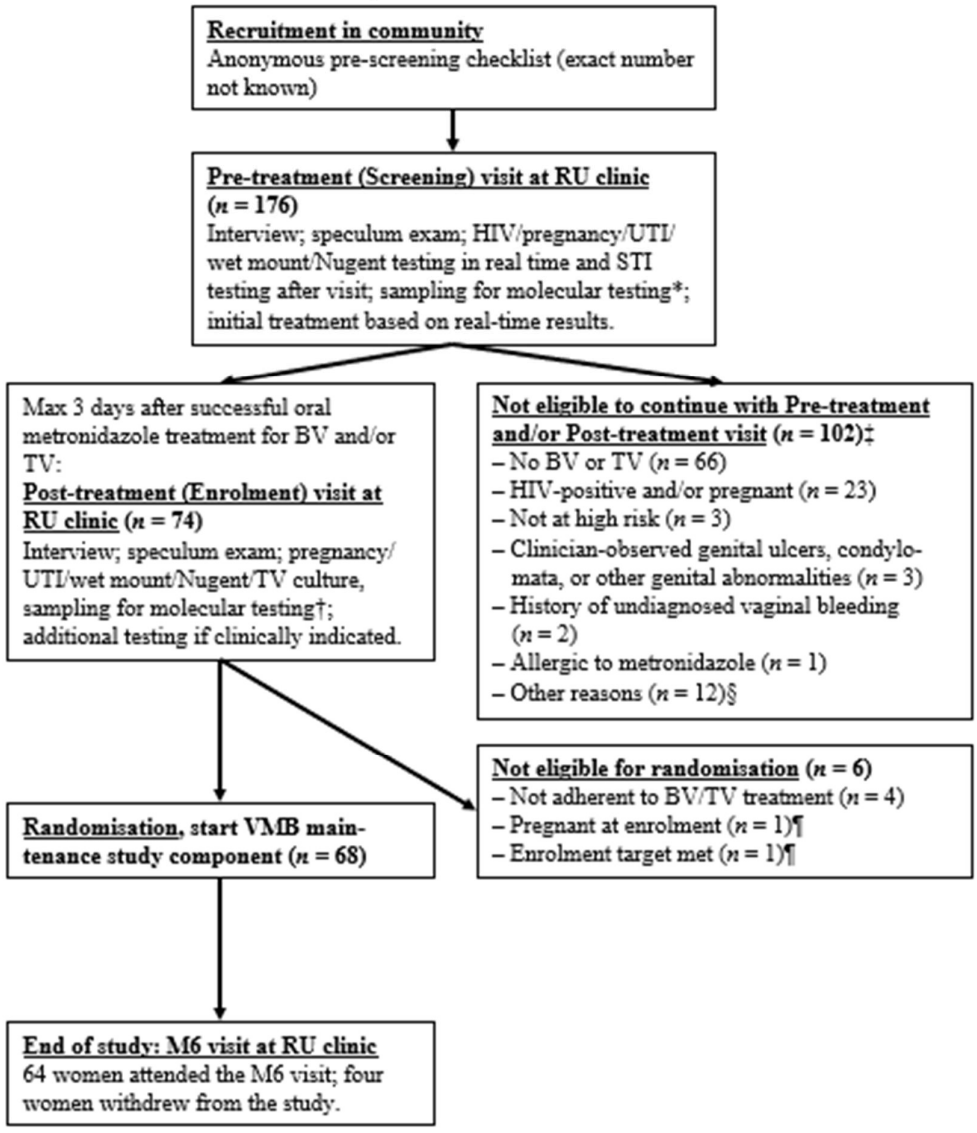
We investigated the impact of the most commonly used oral metronidazole treatment regimen, 500 mg twice per day for seven days, on the VMB of Rwandan women with BV and/or *T. vaginalis*. We compared their VMB compositions as assessed by microscopy and sequencing pre- and post-treatment, and determined sociodemographic and biological correlates of treatment failure.

2.2 Materials and Methods

Data were collected at the Rinda Ubuzima research clinic in Kigali, Rwanda, in 2015 (figure 2.1). HIV-negative, non-pregnant women, aged 18-45, and in good overall physical and mental health, were screened at a pre-treatment visit. Recruitment targeted women at high risk of BV/*T. vaginalis*, defined as having had more than one sex partner, or having been treated for BV and/or a sexually transmitted infection, in the last 12 months. Women who were BV-positive (by Nugent 7-10 and/or modified Amsel criteria as defined below), and/or *T. vaginalis*-positive (by wet mount and/or by culture), regardless of

symptomatology, were treated with seven days of 500 mg generic oral metronidazole (Tricozole; Laboratory & Allied ltd, Nairobi, Kenya) twice daily. Women were also tested, and treated or referred, for pregnancy, HIV, syphilis, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, vulvovaginal candidiasis, and urinary tract infections, using local guidelines.[24] Women returned for a post-treatment visit within three days after metronidazole treatment completion, and were re-tested for BV, *T. vaginalis*, and vulvovaginal candidiasis. Dacron vaginal swabs for molecular VMB testing were collected during speculum examinations at both visits, and stored dry at -80 °C.

Figure 2.1: Flowchart of the VMB study



BV bacterial vaginosis, *M6* Month 6 visit, *RU* Rinda Ubuzima, *STI* sexually transmitted infection, *TV Trichomonas vaginalis*, *UTI* urinary tract infection.

Flowchart of the VMB study, including the randomised vaginal microbiota maintenance component (Chapter 3).

*Valid Nugent data available for 67 women; valid rarefied sequencing data available for 67 women; valid qPCR-based estimated concentrations available for 66 women. †Valid Nugent data available for 66 women; valid rarefied sequencing data available for 67 women; valid qPCR-based estimated concentrations available for 63 women. Valid concentration data for both pre- and post-treatment were available for 61 women. ‡Totals to 110 reasons among 102 women because multiple reasons could have been reported by one woman. §Reasons: outside of metronidazole treatment window (n=5), enrolment target already met (n=4), has a mental disorder (n=1), did not complete screening

procedures and was subsequently lost to follow=up (n=1), withdrew consent during the pre-treatment (Screening) visit because she thought the reimbursement was too low (n=1) ¶No data are available for these two women; no vaginal swabs were taken as these women did not pass subsequent enrolment procedures. ||Successful treatment was defined as having a Nugent score of 7-10 before treatment and 0-3 after treatment (N=30), while treatment failure was defined as having a Nugent score of 7-10 before treatment and 4-10 after treatment (N=25). Thirteen women were excluded from these analyses because they did not have Nugent 7-10 at the pre-treatment visit (N=12) or did not have a valid Nugent result at the post-treatment visit (N=1).

Diagnostic procedures

BV was diagnosed by Gram stain Nugent scoring (a score of 0-3 was considered optimal, 4-6 intermediate microbiota, and 7-10 BV),[78] and by modified Amsel criteria (defined as the presence of at least two of the following criteria: vaginal pH>4.5, positive whiff test, and/or ≥20% clue cells).[77] Vaginal pH was measured by pressing a pH paper strip (pH range 3.6-6.1 with 0.3 increments; Machery-Nagel, Düren, Germany) against the vaginal wall. *T. vaginalis* was diagnosed when the *T. vaginalis* InPouch culture (Biomed Diagnostics, White City, OR, USA) was positive and/or if motile trichomonads were observed on wet mount. Vulvovaginal candidiasis was diagnosed when budding yeasts and (pseudo)hyphae were seen on wet mount. Other diagnostic testing is described in Appendix A.

Molecular VMB testing

DNA was extracted from one swab per woman per visit (N=136), using lysozyme lysis and bead-beating procedures combined with the Qiagen DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) (Appendix A).[117] The V3-V4 region of 16S rRNA genes were amplified and sequenced on an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) run in rapid mode, 2x300bp using a 250PE and 50PE kit. The panbacterial 16S rRNA gene copy concentration per sample was determined using the BactQuant quantitative PCR assay.[118]

Molecular data processing

Molecular data processing steps are described in Appendix A. Briefly, DADA2[119] in R 3.2.3 (R foundation for Statistical Computing 2016, Vienna, Austria) was used to assign reads to amplicon sequence variants (ASVs) using Silva v128 as the reference database,[120] with additional taxonomic assignments made using other databases (Appendix A). Relative abundances were rarefied at 1,111 reads using the *GUniFrac* 1.0 package in R.[121] The rarefied ASV relative abundance table consisted of 204 ASVs in 134 samples, mapping to species (133; 65.2%), genus (55; 27.0%), or higher taxonomic levels (16; 7.8%). Bacterial cell concentrations in cells/µl per ASV per sample were estimated by multiplying the ASV-specific copy-normalised relative abundance by the sample-specific 16S rRNA gene copies concentration (Appendix A). This yielded estimated concentrations of 204 ASVs in 129 samples, which were $\log_{10}$-transformed. Of the 204 ASVs, 108 ASVs were present at a relative abundance of at least 1% in at least one sample; the other 96 ASVs were minority species.

Data reduction was required for some biostatistical analyses, and was done in three different ways.

First, the inverse Simpson diversity index was calculated for each sample. Second, each ASV was assigned to one of four 'bacterial groups' based on the published literature (Appendix A; overview in table A.1): 1) lactobacilli; 2) BV-anaerobes (this group ended up containing all Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Tenericutes except those included in the other three groups); 3) pathobionts (most Proteobacteria, and streptococci, staphylococci, enterococci, *Spirochaetaceae*, *Listeria*, *C. trachomatis*, and *N. gonorrhoeae*); and 4) 'other bacteria' (a rest group, containing Actinobacteria that are known to be (facultative) aerobic skin bacteria, *Bifidobacterium* species, and seven difficult to classify minority species). Within each sample, read counts of ASVs belonging to the same bacterial group were summed (Appendix A). Third, we used hierarchical clustering based on Euclidean distance to pool samples into seven VMB types: 1) *L. iners*-dominated (Li; >75% lactobacilli of which *L. iners* was the most common; n=45 samples); 2) other lactobacilli-dominated (Lo; also >75% lactobacilli of which *L. jensenii* and *L. gasseri* were the most common; n=2); 3) lactobacilli and anaerobes (LA; 25-75% lactobacilli; n=30); 4) polybacterial *G. vaginalis*-containing (BV_GV; <25% lactobacilli and 10-50% *G. vaginalis*; n=30), 5) other polybacterial low-*G. vaginalis* (BV_noGV; <25% lactobacilli and <10% *G. vaginalis*; n=8), 6) *G. vaginalis*-dominated (GV; <25% lactobacilli and >50% *G. vaginalis*; n=12); and 7) pathobionts-containing (PB; >20% pathobionts; n=7). The samples in VMB types 1-6 had a maximum of 0.1-15.9% pathobionts per VMB type.

Statistical analyses

Statistical analyses were performed using Stata version 13 (StataCorp, College Station, TX, USA) and R. VMB characteristics pre- and post-treatment were compared using the Stuart-Maxwell test for matched categorical data, and Wilcoxon signed-rank test for matched continuous data, for all women, and women stratified by treatment success/failure (women who had Nugent 7-10 pre-treatment and a valid Nugent score at post-treatment; N=55), pre-treatment *C. trachomatis*/*N. gonorrhoeae* status (results became available after metronidazole treatment completion; N=67 due to one missing result), having received another antibiotic in addition to metronidazole at the pre-treatment visit or not (N=68), and having reported unusual vaginal discharge pre-treatment or not (N=68). Successful treatment was defined as Nugent 7-10 pre-treatment and Nugent 0-3 post-treatment, and treatment failure as Nugent 7-10 pre-treatment and Nugent 4-10 post-treatment. Kruskal-Wallis test for continuous variables, and Fisher's exact test for binary variables, were used for cross-sectional comparisons. Bivariable logistic regression was used to investigate associations between individual baseline sociodemographic and biological characteristics and treatment failure.

Ethical statement

All participants provided written informed consent. The study was conducted in accordance with the Helsinki Declaration, and approved by the National Ethics Committee of Rwanda and the University of Liverpool Research Ethics Subcommittee for Physical Interventions.

2.3 Results

We screened 176 women, and 68 women completed metronidazole treatment (ineligibility reasons in figure 2.1): 82.4% had BV alone, 2.9% had *T. vaginalis* alone, and 14.7% had both BV and *T. vaginalis*. Four of the 176 women were not eligible because they did not complete the treatment. The median age was 31 years (range 19-42) and most were sex workers (table 2.1).

Table 2.1: Baseline characteristics of participants

Sociodemographics and sexual behavior	**Pre-treatment (N=68)**	**Post-treatment (N=68)**
Age (median, IQR)	31 (27 – 35)	NA
Marital status (n %) - Never married - Married - Divorced - Widowed	 50 (73.5) 5 (7.4) 12 (17.6) 1 (1.5)	NA
Education level (n %) - No schooling - Primary school not completed - Primary school completed - Secondary school not completed - Secondary school completed	 14 (20.6) 31 (45.6) 17 (25.0) 6 (8.8) 0	NA
Number of sex partners in lifetime (median, IQR)	30 (7 – 463)	NA
Number of sex partners in last 12 months (pre-treatment) or month (post-treatment) (median, IQR)	11 (4 – 152)	5 (3 – 15.5)*
Exchanged sex for money/goods in past month (n %)	63 (92.6)	60 (92.3)*
Vaginal sex frequency last two weeks (median, IQR)	12 (8 – 18)	11 (8 – 19)*
Any condom use in past two weeks (n %) - Always - Sometimes but not always - Never - No sex in the past two weeks	 14 (20.6) 51 (75.0) 3 (4.4) 0	 23 (33.8) 36 (52.9) 6 (8.8) 3 (4.4)
Condom use during last sex act (n %)	36 (52.9)	44 (64.7)*
Currently using hormonal contraception (n %)	42 (61.8)	42 (62.7)*
Currently breastfeeding (n %)	14 (21.2)*	NA
Inserted anything inside the vagina in the last 12 months (n %)	NA	26 (38.2)
Had menses in the seven days prior to the visit (n %)	NA	11 (16.2)
Any current urogenital symptoms (at pre-treatment visit, including last two weeks), patient-reported (n %)	49 (72.1)	0
Current unusual vaginal discharge (at pre-treatment visit, including last two weeks), patient-reported (n %)	13 (26.5)	0
Received antibiotic in addition to metronidazole at pre-treatment visit (n %)†	18 (26.5)	NA
Received antifungal treatment at pre-treatment visit n (%)‡	6 (8.8)	NA
Laboratory results	**(N=68)**	**(N=68)**
HIV by serology (n %)§	0	NA
Positive urine pregnancy test (n %)§	0	0*
BV by Nugent 7-10 (n %)*	56 (83.6)	17 (25.8)
BV by modified Amsel criteria¶ (n %)	49 (72.1)	0
Trichomonas vaginalis on wet mount (n %)*	6 (8.8)	0
T. vaginalis by InPouch culture (n %)	11 (16.4)	0
Yeasts on wet mount (n %)	6 (8.9)	4 (5.9)
Positive urinalysis test (n %)	17 (25.0)	0*
Syphilis by serology (n %)	4 (5.9)	NA
Positive herpes simplex virus type 2 serology (n %)	44 (64.7)	NA
Chlamydia trachomatis by PCR (n %)	20 (29.4)	NA
Neisseria gonorrhoeae by PCR (n %)	13 (19.1)	NA

BV bacterial vaginosis, *IQR* inter-quartile range, *NA* not assessed, *PCR* polymerase chain reaction.
*1-3 missing values. †Includes ciprofloxacin for urinary tract infection and penicillin for syphilis. ‡Three women received both an antifungal and another antibiotic in addition to metronidazole. §All enrolled participants were HIV-negative and non-pregnant by design. ¶Two or more positive of: vaginal pH>4.5, positive whiff test, and/or ≥20% clue cells observed on wet mount.

Thirteen women (26.5%) reported unusual vaginal discharge at the pre-treatment visit. Some women (26.5%) received another antibiotic in addition to metronidazole for another condition, or had an ongoing *C. trachomatis* and/or *N. gonorrhoeae* infection during metronidazole treatment (38.2%; table 2.1). At the post-treatment visit, all women were BV-negative by modified Amsel criteria and *T. vaginalis*-negative by culture and wet mount, and no women reported urogenital symptoms (including unusual vaginal discharge), adverse events (including vomiting), or social harms. Of the 56 women with Nugent 7-10 pre-treatment, 30 (54.5%) had Nugent 0-3, 11 (20.0%) Nugent 4-6, and 14 Nugent 7-10 post-treatment (table 2.2).

Table 2.2: VMB characteristics before and after metronidazole treatment, including stratification by treatment success/failure

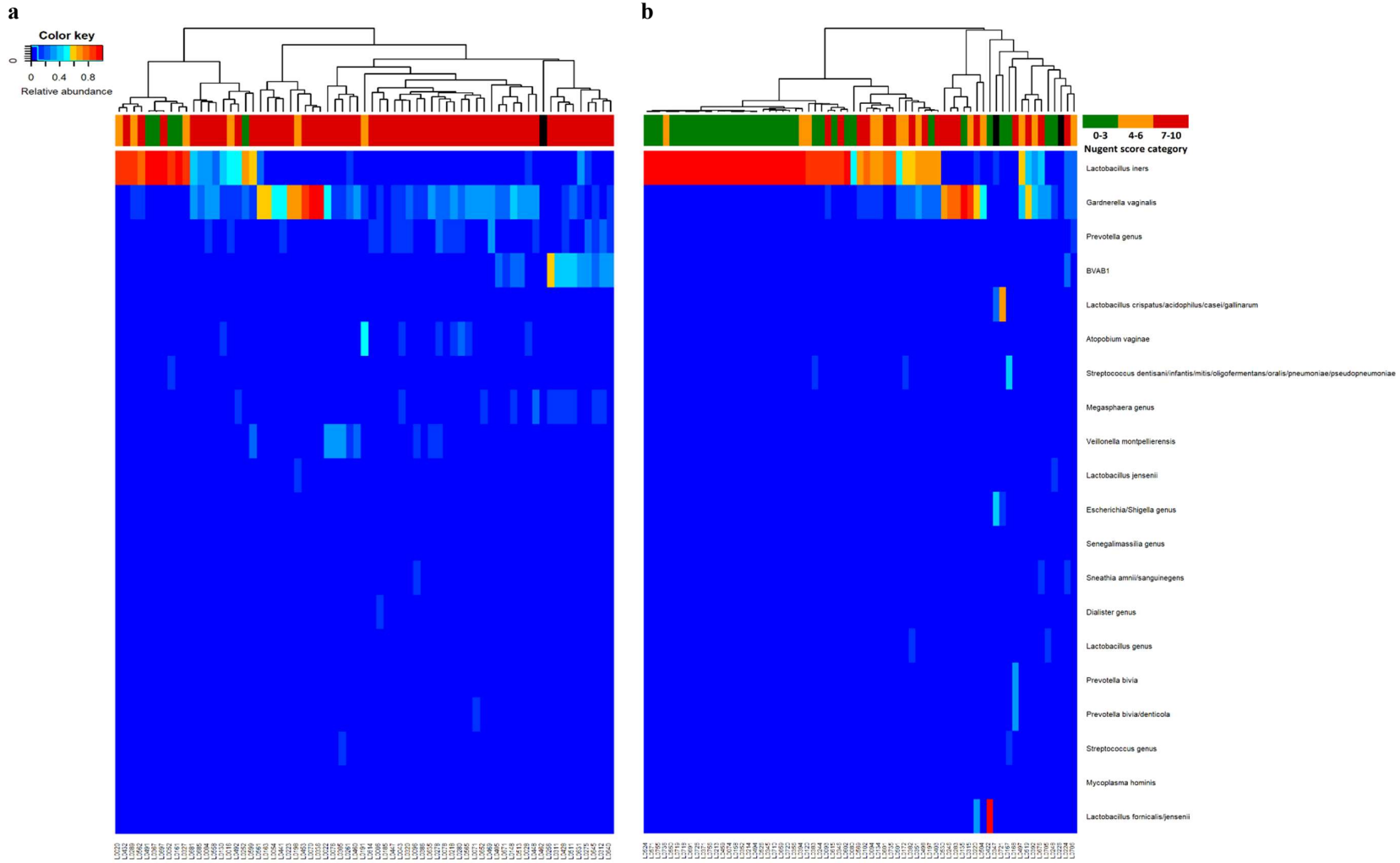
VMB Outcomes	All participants			Successful treatment†			Treatment failure†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 30)	Post-treatment (n = 30)	p*	Pre-treatment (n = 25)	Post-treatment (n = 25)	p*
Nugent categories (n %)‡ – 0-3 – 4-6 – 7-10	 5 (7.5) 6 (9.0) 56 (83.6)	 36 (54.6) 13 (19.7) 17 (25.8)	<0.001	 0 0 30 (100)	 30 (100) 0 0	NA§	 0 0 25 (100)	 0 11 (44.0) 14 (56.0)	NA§
Mean inverse Simpson diversity index (95% CI)¶	0.67 (0.60 – 0.73)	0.31 (0.25 – 0.38)	<0.001	0.70 (0.61 – 0.80)	0.13 (0.06 – 0.21)	<0.001	0.77 (0.70 – 0.85)	0.47 (0.39 – 0.56)	0.001
VMB type (n %)¶: – Li – Lo – LA – BV_GV – BV_noGV – GV – PB	 10 (14.9) 0 12 (17.9) 28 (41.8) 8 (11.9) 8 (11.9) 1 (1.5)	 35 (52.2) 2 (3.0) 18 (26.9) 2 (3.0) 0 4 (6.0) 6 (9.0)	<0.001	 2 (6.9) 0 7 (24.1) 13 (44.8) 4 (13.8) 3 (10.3) 0	 23 (79.3) 1 (3.5) 2 (6.9) 0 0 1 (3.5) 2 (6.9)	<0.001	 1 (4.0) 0 2 (8.0) 14 (56.0) 3 (12.0) 5 (20.0) 0	 6 (24.0) 0 14 (56.0) 2 (8.0) 0 2 (8.0) 1 (4.0)	0.002
Vaginal pH, median (IQR)	5.3 (5.0 – 5.6)	4.4 (3.6 – 4.6)	<0.001	5.3 (5.0 – 5.6)	4.1 (3.6 – 4.4)	<0.001	5.6 (5.0 – 5.6)	4.4 (4.4 – 4.7)	<0.001
Vulvovaginal candidiasis (n %)	6 (8.8)	4 (5.9)	0.527	1 (3.3)	3 (10.0)	0.317	1 (4.0)	1 (4.0)	1.00
Bacterial group relative abundances: mean (95% CI)¶									
Total lactobacilli	0.24 (0.15 – 0.32)	0.72 (0.64 – 0.80)	<0.001	0.18 (0.08 – 0.27)	0.88 (0.78 – 0.98)	<0.001	0.10 (0.02 – 0.18)	0.56 (0.45 – 0.68)	<0.001
Total BV-anaerobes	0.75 (0.67 – 0.83)	0.23 (0.16 – 0.30)	<0.001	0.81 (0.71 – 0.91)	0.07 (0 – 0.15)	<0.001	0.89 (0.81 – 0.97)	0.40 (0.29 – 0.52)	<0.001
Total pathobionts	0.02 (0.01 – 0.03)	0.05 (0.02 – 0.09)	0.050	0.01 (0 – 0.02)	0.05 (-0.02 – 0.11)	0.118	0.01 (0 – 0.03)	0.03 (0.01 – 0.05)	0.173
Total other bacteria	0 (0 – 0)	0 (0 – 0)	0.674	0 (0 – 0)	0 (0 – 0)	0.173	0 (0 – 0)	0 (0 – 0)	0.764
Bacterial group concentrations in log_{10} cells/µL: mean (95% CI)‖									
Total bacteria	6.59 (6.39 – 6.78)	5.85 (5.66 – 6.04)	<0.001	6.59 (6.31 – 6.86)	5.65 (5.38 – 5.91)	<0.001	6.68 (6.36 – 7.01)	6.23 (5.93 – 6.54)	0.028
Total lactobacilli	4.98 (4.61 – 5.35)	5.56 (5.34 – 5.78)	0.017	4.92 (4.36 – 5.49)	5.47 (5.16 – 5.77)	0.124	4.62 (3.92 – 5.31)	5.80 (5.38 – 6.21)	0.001
Total BV-anaerobes	6.23 (5.88 – 6.57)	4.55 (4.14 – 4.95)	<0.001	6.46 (6.14 – 6.78)	3.81 (3.23 – 4.38)	<0.001	6.62 (6.26 – 6.97)	5.79 (5.45 – 6.13)	0.003
Total pathobionts	1.92 (1.36 – 2.48)	2.01 (1.48 – 2.54)	0.939	1.09 (0.32 – 1.87)	1.48 (0.74 – 2.21)	0.649	2.30 (1.40 – 3.19)	2.66 (1.65 – 3.66)	0.637

VMB Outcomes	All participants			Successful treatment†			Treatment failure†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 30)	Post-treatment (n = 30)	p*	Pre-treatment (n = 25)	Post-treatment (n = 25)	p*
Total other bacteria	1.85 (1.36 – 2.35)	1.46 (1.01 – 1.92)	0.176	1.71 (0.96 – 2.45)	0.91 (0.36 – 1.47)	0.043	2.44 (1.56 – 3.31)	2.34 (1.41 – 3.27)	0.525
Individual bacteria concentrations in log_{10} cells/µL: mean (95% CI)‖									
L. iners	4.81 (4.38 – 5.24)	5.28 (4.94 – 5.62)	0.072	4.91 (4.34 – 5.48)	5.10 (4.54 – 5.65)	0.501	4.27 (3.89 – 5.14)	5.63 (5.10 – 6.17)	<0.001
*L. crispatus***	0.15 (-0.02 – 0.33)	0.51 (0.16 – 0.85)	0.089	0 (0 – 0)	0.47 (-0.08 – 1.01)	0.083	0.25 (-0.12 – 0.62)	0.55 (-0.08 – 1.19)	0.330
Other lactobacilli††	1.46 (0.97 – 1.94)	3.03 (2.57 – 3.48)	<0.001	0.67 (0.15 – 1.19)	2.62 (1.93 – 3.31)	<0.001	1.18 (0.45 – 1.91)	3.31 (2.45 – 4.17)	0.001
Gardnerella vaginalis	5.62 (5.20 – 6.03)	4.12 (3.63 – 4.61)	<0.001	6.00 (5.69 – 6.31)	3.29 (2.62 – 3.96)	<0.001	6.11 (5.74 – 6.47)	5.66 (5.30 – 6.01)	0.115
Atopobium vaginae	4.58 (4.00 – 5.16)	1.54 (1.06 – 2.02)	<0.001	4.91 (4.14 – 5.67)	1.44 (0.76 – 2.11)	<0.001	5.43 (4.83 – 6.03)	1.76 (0.78 – 2.73)	<0.001
Prevotella species	4.67 (4.18 – 5.16)	1.35 (0.90 – 1.79)	<0.001	5.07 (4.59 – 5.55)	1.31 (0.71 – 1.90)	<0.001	5.24 (4.47 – 6.00)	1.62 (0.69 – 2.54)	<0.001
Sneathia species	4.18 (3.63 – 4.73)	1.08 (0.63 – 1.54)	<0.001	4.38 (3.64 – 5.14)	1.10 (0.45 – 1.76)	<0.001	4.90 (4.16 – 5.63)	1.43 (0.51 – 2.36)	<0.001
Megasphaera species	3.17 (2.56 – 3.79)	0.22 (-0.01 – 0.44)	<0.001	3.96 (3.10 – 4.81)	0 (0 – 0)	<0.001	3.34 (2.32 – 4.35)	0.55 (-0.09 – 1.18)	0.001
Veillonella species	2.37 (1.75 – 3.00)	0.28 (0.01 – 0.56)	<0.001	1.85 (0.89 – 2.81)	0.27 (-0.12 – 0.66)	0.005	2.83 (1.79 – 3.87)	0.22 (-0.24 – 0.68)	0.002
BVAB1	1.76 (1.11 – 2.42)	0.46 (0.15 – 0.77)	<0.001	2.08 (0.97 – 3.19)	0.34 (0.01 – 0.67)	0.002	2.00 (0.84 – 3.15)	0.75 (-0.02 – 1.53)	0.067
Fusobacterium species	0.53 (0.17 – 0.89)	0 (0 – 0)	0.008	0.44 (-0.06 – 0.95)	0 (0 – 0)	0.008	0.68 (0.02 – 1.34)	0 (0 – 0)	0.046
Streptococcus species	1.47 (0.92 – 2.02)	1.34 (0.84 – 1.85)	0.453	0.83 (0.10 – 1.55)	0.85 (0.18 – 1.52)	0.286	1.76 (0.83 – 2.68)	2.05 (1.06 – 3.05)	0.767
Staphylococcus species	0.26 (0.05 – 0.47)	0.60 (0.27 – 0.93)	0.655	0.09 (-0.10 – 0.29)	0.34 (-0.01 – 0.70)	ND	0.34 (-0.05 – 0.72)	0.87 (0.12 – 1.63)	0.317
Escherichia/*Shigella* species	0.10 (-0.04 – 0.25)	0.86 (0.45 – 1.27)	0.317	0.24 (-0.10 – 0.59)	0.41 (0.00 – 0.83)	0.317	0 (0 – 0)	1.41 (0.52 – 2.29)	ND

BV bacterial vaginosis, *BVAB1* BV-associated bacterium type 1, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *CI* confidence interval, *GV G. vaginalis*-dominated, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *NA* not applicable, *ND* not determinable, *PB* pathobionts-containing, *VMB* vaginal microbiota.

*Stuart-Maxwell test for matched categorical data and Wilcoxon signed-rank test for matched continuous data. †Successful treatment was defined as having a Nugent score of 7-10 before treatment and 0-3 after treatment (N=30), while treatment failure was defined as having a Nugent score of 7-10 before treatment and 4-10 after treatment (N=25). Thirteen women were excluded from these analyses because they did not have Nugent 7-10 at the pre-treatment visit (N=12) or did not have a valid Nugent result at the post-treatment visit (N=1). ‡Valid Nugent data available for 67 participants at the pre-treatment visit and 66 participants at the post-treatment visit. §The definition of treatment success/failure was based on Nugent scores and these p-values are therefore meaningless. ¶Relative abundance, Simpson diversity indices, and VMB type data available for 67 participants at each visit. ‖Concentration data may contain at most five missing values (see Appendix A). **Includes all amplicon sequence variants attributed to *L. crispatus*, also those with multiple species assignments. ††Includes amplicon sequence variants attributed to *L. jensenii*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, and *Lactobacillus* genus, as well as 11 other minority amplicon sequence variants.

Figure 2.2: Heatmaps of 16S rRNA gene sequencing data pre- and post-treatment

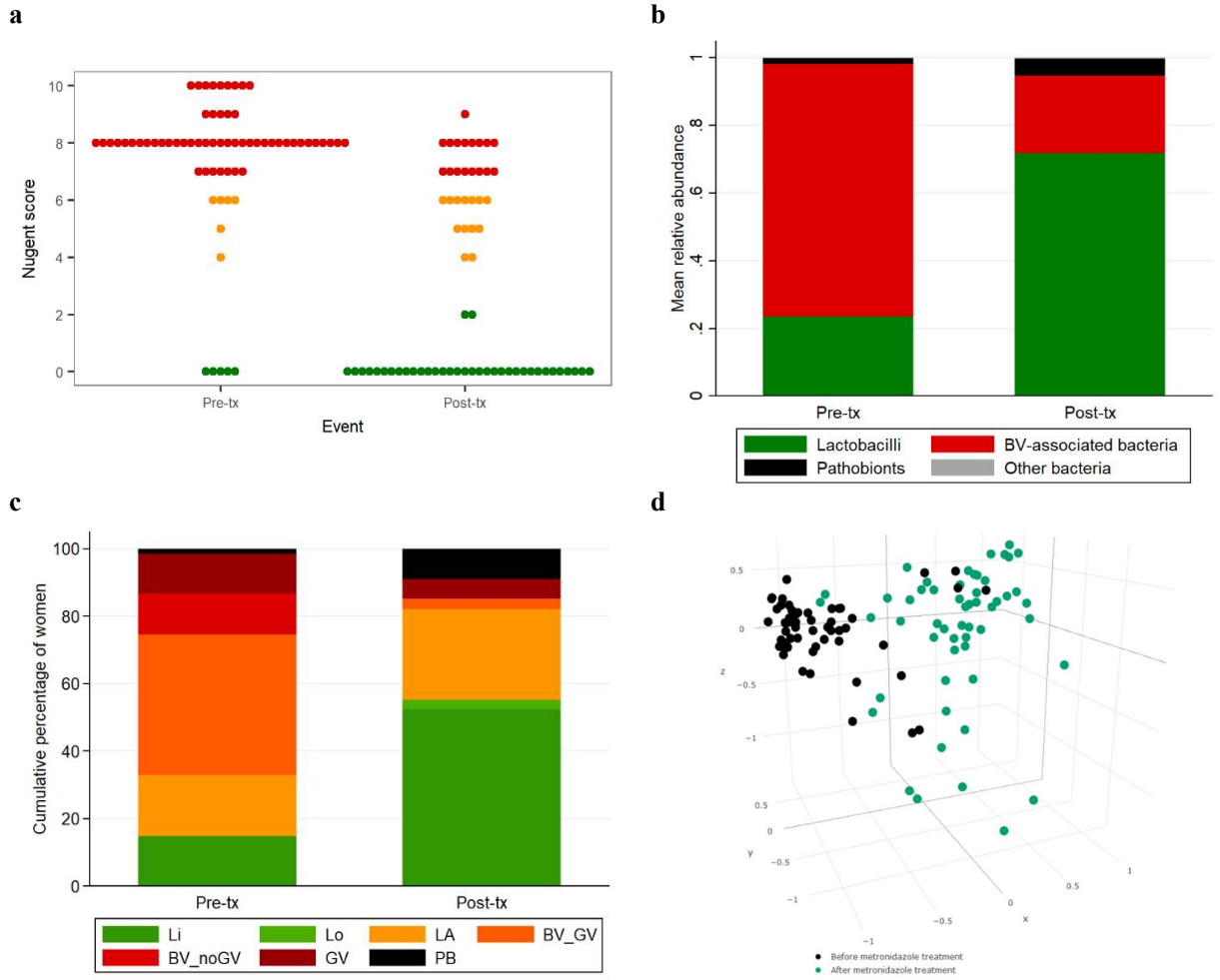


BVAB1 bacterial vaginosis-associated bacterium type 1.

a-b Heatmaps at the pre-treatment (**a**) and the post-treatment visit (**b**) depicting the 20 amplicon sequence variants with the highest mean relative abundance on the y-axis and samples (n=67 at each visit) on the x-axis. The dendrogram above the heatmap depicts vaginal microbiota clusters based on Euclidean distance. The bar below the dendrogram depicts Nugent score categories (see legend; black means no score available).

Pre- and post-treatment 16S microbiome data shows a shift towards increased relative abundance of lactobacilli (mainly *L. iners*) and decreased relative abundances of several BV-anaerobes (figure 2.2). The mean bacterial group relative abundance data confirmed this (table 2.2, figure 2.3b) and additionally showed that the mean relative abundance of pathobionts increased post-treatment (Wilcoxon signed rank p=0.050).

Figure 2.3: Nugent scores, mean bacterial group relative abundances, and vaginal microbiota type pre- and post-treatment



BV bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *GV G. vaginalis*-dominated, *IQR* inter-quartile range, *LA* lactobacilli and anaerobes, *Li Lactobacillus iners*-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts-containing, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit.
a-c Changes in vaginal microbiota characteristics before and after metronidazole treatment: Nugent scores (**a**), mean bacterial group relative abundances (**b**), vaginal microbiota types (**c**). **d** Three-dimensional non-metric multidimensional scaling plot based on rarefied relative abundances of samples before and after metronidazole treatment. The figure shows that samples cluster together by visit (and hence, treatment status).

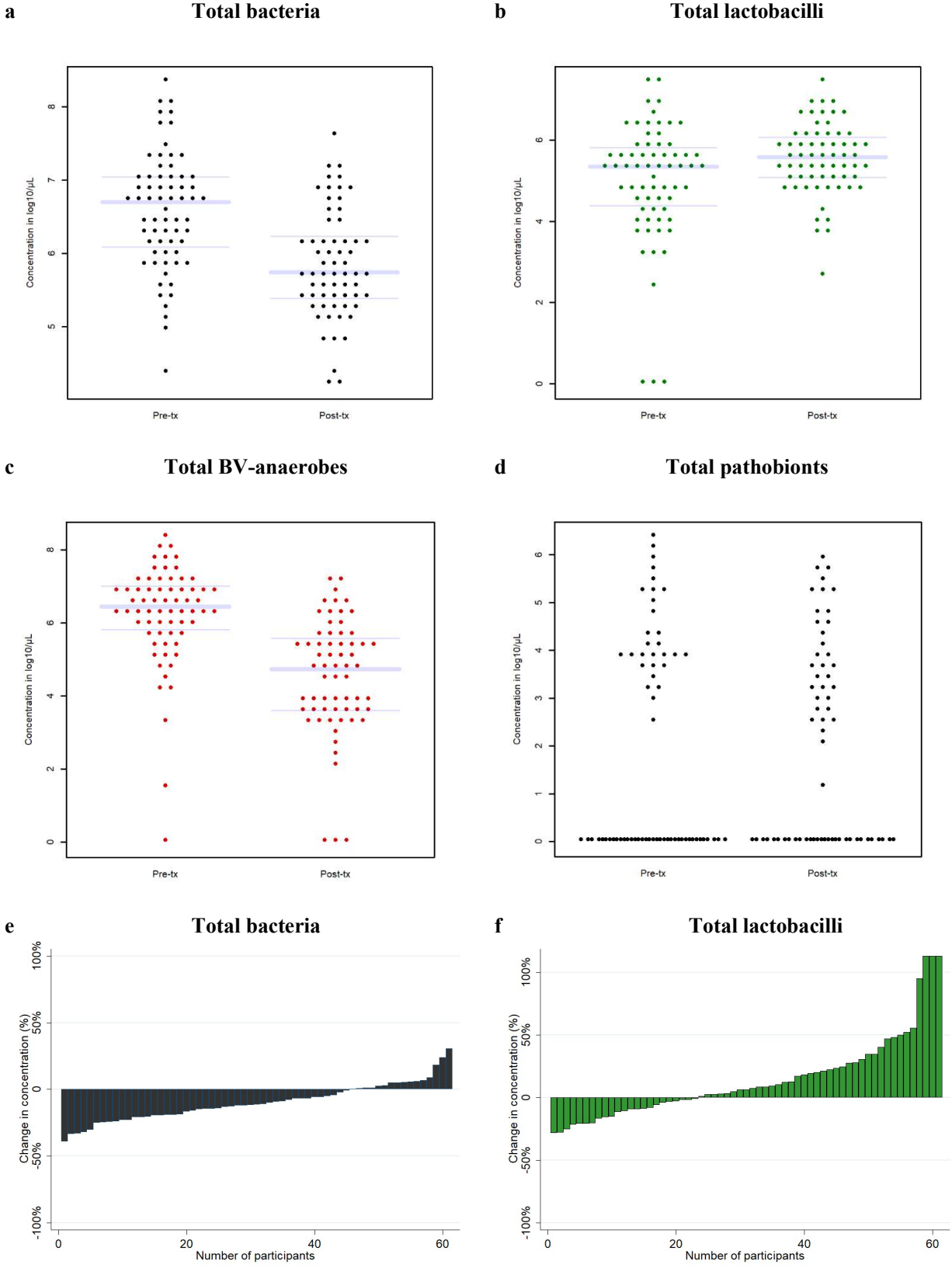
Metronidazole treatment was associated with a significant decrease in the mean concentration of total bacteria from 6.59 $\log_{10}$ cells/µL pre-treatment to 5.85 $\log_{10}$ cells/µL post-treatment (p<0.001; table 2.2). The mean BV-anaerobes concentration decreased from 6.23 $\log_{10}$ cells/µL to 4.55 $\log_{10}$ cells/µL (p<0.001), the mean *Lactobacillus* concentration increased from 4.98 $\log_{10}$ cells/µL to 5.56 $\log_{10}$

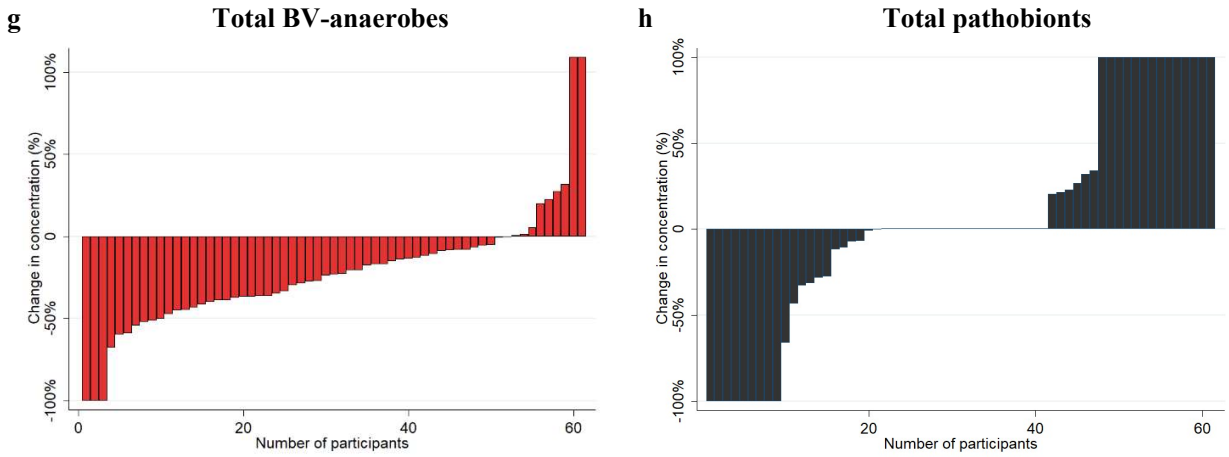
cells/μL (p=0.017), and the mean concentrations of pathobionts (1.92 $\log_{10}$ cells/μL pre-treatment and 2.01 $\log_{10}$ cells/μL post-treatment; p=0.939) and 'other bacteria' (1.85 $\log_{10}$ cells/μL pre-treatment and 1.46 $\log_{10}$ cells/μL post-treatment; p=0.176) did not change significantly. Among lactobacilli, the concentrations of *L. iners, L. crispatus,* and 'other lactobacilli' (mostly *L. jensenii* and *L. gasseri*) all increased, with *L. iners* having the highest concentrations before and after treatment, but 'other lactobacilli' achieving the greatest concentration increase (table 2.2). The median vaginal pH decreased from 5.3 to 4.4 (p<0.001). Among BV-anaerobes, the concentrations of the eight most common BV-anaerobes in our dataset (*Gardnerella*, *Atopobium*, *Prevotella*, *Sneathia*, *Megasphaera*, *Veillonella,* and *Fusobacterium* species, and BV-associated bacterium type 1) decreased (table 2.2). The concentrations of the three most common pathobionts in our dataset (*Streptococcus*, *Staphylococcus*, *Escherichia/Shigella* species) did not change significantly (table 2.2).

While the mean trends were clear, the inter-individual variability was high (figures 2.4a-h). Of the 61 participants for whom pre- and post-treatment concentration data were available, most had decreases in total bacterial concentration (n=45) and BV-anaerobes (n=52), but not everyone (figures 2.4e, 2.4g). BV-anaerobes were completely eradicated in only three women (4.9%), and reduced by more than 50% in an additional seven women (11.5%). Pathobiont concentrations showed the most inter-individual variability (figure 2.4h).

The mean inverse Simpson diversity index was 0.67 pre-treatment and 0.31 post-treatment (p<0.001; table 2.2). Metronidazole treatment changed the proportions of women with certain VMB types based on hierarchical clustering results: the proportions of women with lactobacilli-dominated VMB types (Li and Lo) and the mixed LA VMB type increased, whereas the proportions of women with the three BV-associated VMB types (BV_GV, BV_noGV, and to a lesser extent GV) decreased (table 2.2, figure 2.3c). The number of women with a PB VMB type increased from one (1.5%) pre-treatment to six (9.0%) post-treatment. Alluvial diagrams show that the majority of women transitioned from the three BV-associated VMB types into VMB types containing lactobacilli (Li, Lo, and LA; figures 2.5a-b). However, women with a BV_GV or BV_noGV VMB type transitioned more often into a lactobacilli-dominated VMB type (16/27 (59.3%), and 4/8 (50.0%), respectively) than women with a GV VMB type (1/8 (12.5%); Fisher's exact p=0.084 comparing the three groups; figure 2.5a).

Figure 2.4: Individual bacterial group concentrations pre- and post-treatment

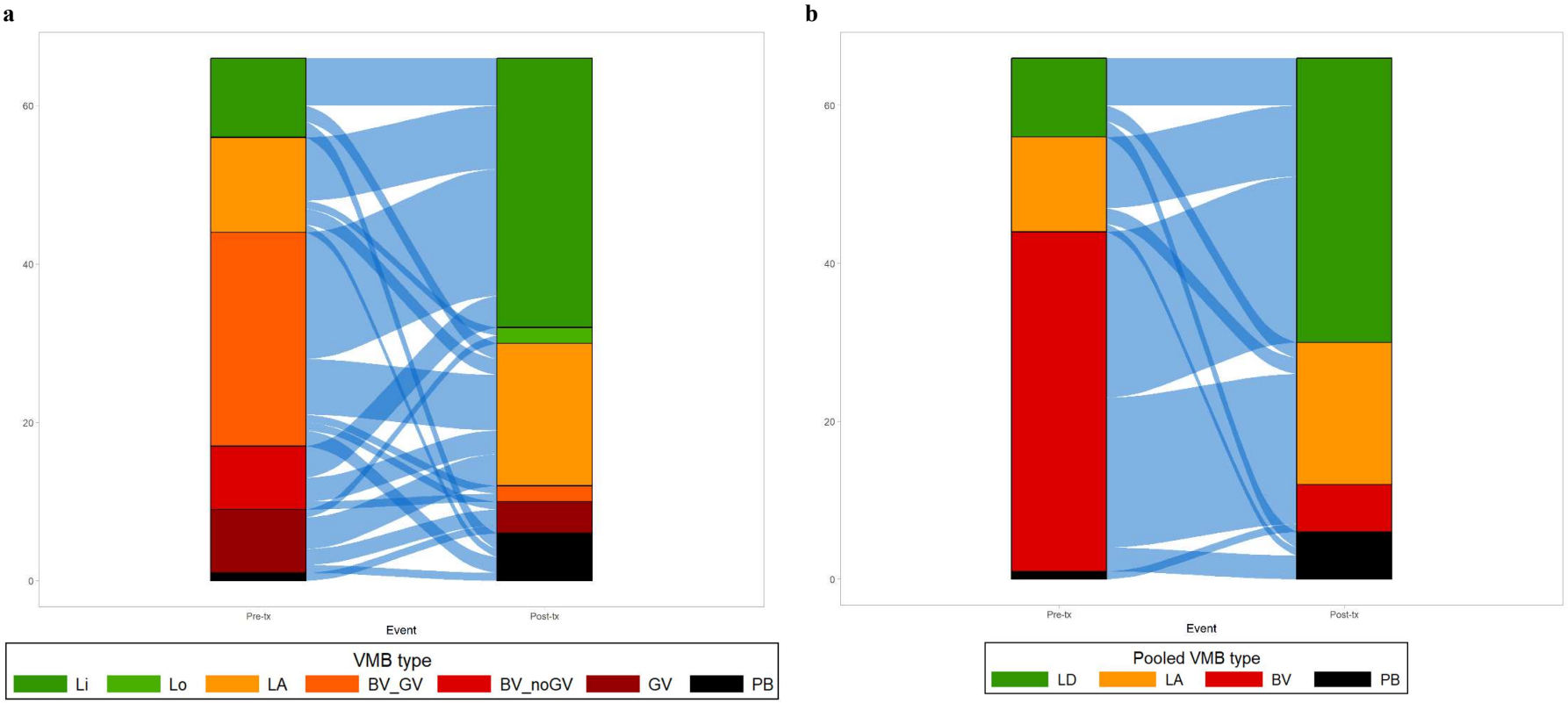




BV bacterial vaginosis, *conc* concentration, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit.
a-d Changes in total bacterial concentrations and bacterial group concentrations before (n=66) and after metronidazole treatment (n=63): total bacterial concentration (**a**), total lactobacilli (**b**), total BV-anaerobes (**c**) and total pathobionts (**d**; boxplot not shown because of high proportion of zero values). See table 2.2 for mean concentrations and 95% confidence intervals, and statistical significance. **e-h** Change in concentrations between pre- and post-treatment, expressed as a percentage for every individual participant with valid qPCR results at both visits (n=61), for total bacterial concentration (**e**), total *Lactobacillus* (**f**), total BV-anaerobes (**g**), and total pathobionts (**h**). In some women, concentration went from zero to non-zero; these increases were set at 100% or the greatest increase observed among the other participants, whichever was greatest.

Participants with treatment failure as defined by Nugent scoring had a lower mean lactobacilli relative abundance, smaller decreases in mean total bacteria and BV-anaerobes concentrations, and less often a lactobacilli-dominated VMB type, post-treatment (Appendix: figures B.1a-d). They also had a higher mean pathobionts concentration pre-treatment (table 2.3). Successfully treated participants had significant decreases in the mean concentrations of all eight most common BV-anaerobes in our dataset, but unsuccessfully treated participants did not have decreases in *G. vaginalis* and BVAB1 (table 2.2). Simpson diversity index did not differ by treatment success/failure (Appendix: figure B.1e). In logistic regression models, we did not identify any statistically significant sociodemographic or biological correlates of treatment failure, except for pathobionts concentration pre-treatment (p=0.044; table 2.4). Pre-treatment *C. trachomatis*/*N. gonorrhoeae* status, having received another antibiotic in addition to metronidazole, and reporting unusual vaginal discharge at the pre-treatment visit, did not modify the effects of metronidazole treatment on the VMB (Appendix: tables B.1, B.2, B.3, figures B.2a-f, B.3a-f, B.4a-f).

Figure 2.5: Alluvial diagrams of vaginal microbiota types pre- and post-treatment



BV bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *GV G. vaginalis*-dominated, *LA* lactobacilli and anaerobes, *LD Lactobacillus*-dominated, *Li L.* iners-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit, *VMB* vaginal microbiota.
a Changes in VMB types (n=66). Two participants with missing VMB types at either the pre-treatment (n=1) or post-treatment (n=1) visit are not shown. **b** Changes in pooled VMB types (n=66). VMB types were pooled into *Lactobacillus*-dominated (LD; combining VMB types Li and Lo), lactobacilli and anaerobes (LA), BV-like (combining VMB types BV_noGV, BV_GV, and GV) and pathobionts (PB).

Table 2.3: Bacterial group concentrations and relative abundances by treatment success

VMB outcomes at pre-treatment visit	All participants (n = 66)	Successful tx* (n = 29)	Unsuccessful tx† (n = 25)	p‡
Mean total bacterial concentration in $\log_{10}$ cells/µL (95% CI)§	6.59 (6.39 – 6.78)	6.59 (6.31 – 6.86)	6.68 (6.36 – 7.01)	0.656
Mean total *Lactobacillus* concentration in $\log_{10}$ cells/µL (95% CI)§	4.98 (4.61 – 5.35)	4.92 (4.36 – 5.49)	4.62 (3.92 – 5.31)	0.605
Mean total BV-anaerobes concentration in $\log_{10}$ cells/µL (95% CI)§	6.23 (5.88 – 6.57)	6.46 (6.14 – 6.78)	6.62 (6.26 – 6.97)	0.557
Mean total pathobionts concentration in $\log_{10}$ cells/µL (95% CI)§	1.85 (1.29 – 2.41)	1.09 (0.32 – 1.87)	2.30 (1.40 – 3.19)	0.037
Mean total other bacteria concentration in $\log_{10}$ cells/µL (95% CI)§	3.33 (2.84 – 3.83)	1.71 (0.96 – 2.45)	2.44 (1.56 – 3.31)	0.132
Mean RA total *Lactobacillus* (95% CI)	0.24 (0.15 – 0.32)	0.18 (0.08 – 0.27)	0.10 (0.02 – 0.18)	0.410
Mean RA total BV-anaerobes (95% CI)	0.74 (0.66 – 0.82)	0.81 (0.71 – 0.91)	0.89 (0.81 – 0.97)	0.263
Mean RA total pathobionts (95% CI)	0.02 (0.01 – 0.03)	0.01 (0.00 – 0.02)	0.01 (0.00 – 0.03)	0.079
Mean RA total other bacteria (95% CI)	0.01 (0 – 0.01)	0 (0 – 0)	0 (0 – 0)	0.099
Changes in VMB outcomes (comparing differences between visits)	**All participants (n = 66)**	**Successful tx* (n = 28)**	**Unsuccessful tx† (n = 25)**	**p‡**
Difference in mean total bacterial concentration in $\log_{10}$ cells/µL (95% CI)§	-0.71 (-0.95 – -0.48)	-0.96 (-1.29 – -0.64)	-0.44 (-0.82 – -0.06)	0.026
Difference in mean total *Lactobacillus* concentration in $\log_{10}$ cells/µL (95% CI)§	0.47 (0.11 – 0.83)	0.45 (-0.01 – 0.92)	1.06 (0.37 – 1.75)	0.095
Difference in mean total BV-anaerobes concentration in $\log_{10}$ cells/µL (95% CI)§	-1.61 (-2.07 – -1.15)	-2.68 (-3.28 – -2.09)	-0.81 (-1.32 – -0.31)	<0.001
Difference in mean total pathobionts concentration in $\log_{10}$ cells/µL (95% CI)§	0.09 (-0.56 – 0.74)	0.24 (-0.51 – 0.98)	0.29 (-1.06 – 1.64)	0.790
Difference in mean total other bacteria concentration in $\log_{10}$ cells/µL (95% CI)§	-1.63 (-2.24 – -1.03)	-0.79 (-1.62– 0.04)	-0.25 (-1.36– -0.86)	0.403
Difference in mean RA total *Lactobacillus* (95% CI)	0.47 (0.37 – 0.57)	0.69 (0.55 – 0.83)	0.47 (0.34 – 0.59)	0.002
Difference in mean RA total BV-anaerobes bacteria (95% CI)	-0.51 (-0.60 – -0.41)	-0.73 (-0.85 – -0.61)	-0.48 (-0.61 – -0.35)	0.001
Difference in mean RA total pathobionts (95% CI)	0.04 (0.00 – 0.07)	0.04 (-0.02 – 0.10)	0.02 (-0.01 – 0.05)	0.689
Difference in mean RA total other bacteria (95% CI)	0 (0 – 0)	0 (-0.01 – 0)	0 (-0.01 – 0)	0.457

BV bacterial vaginosis, *CI* confidence interval, *RA* relative abundance, *tx* treatment, *VMB* vaginal microbiota.
*Defined as having a Nugent score of 7-10 prior to treatment and 0-3 after treatment. †Defined as having a Nugent score of 7-10 prior to treatment and 4-10 after treatment. ‡By Mann-Whitney U test, comparing those with successful treatment to those with unsuccessful treatment. §Concentration data may contain at most five missing values due to invalid results.

Table 2.4: Sociodemographic and biological correlates of metronidazole treatment success

Sociodemographic correlates of treatment success*	**OR (95% CI)†**	**p†**
Age (continuous variable)	1.06 (0.97 – 1.17)	0.186
Education level:		
- primary school not completed‡	1.23 (0.26 – 5.90)	0.795
- primary school completed‡	1.80 (0.31 – 10.5)	0.514
- secondary school not completed‡	0.33 (0.02 – 4.74)	0.417
Exchanged sex for money/goods in month prior to post-treatment visit	5.40 (0.56 – 52.1)	0.145
Used a condom at the last vaginal sex act prior to post-treatment visit	0.99 (0.97 – 1.02)	0.678
Condom use in the two weeks prior to post-treatment visit		
- Always versus never	0.36 (0.03 – 3.92)	0.399
- Sometimes (but not always) versus never	0.22 (0.02 – 2.19)	0.196
Currently using hormonal contraception at the post-treatment visit	0.69 (0.23 – 2.05)	0.499
Currently breastfeeding at the pre-treatment visit	1.00 (0.97 – 1.03)	0.874
Inserted anything in vagina in the 12 months prior to post-treatment visit	1.03 (0.34 – 3.10)	0.959
Had menses in seven days prior to post-treatment visit	1.83 (0.41 – 8.23)	0.429
Reported any urogenital symptoms at the pre-treatment visit	0.67 (0.21 – 2.11)	0.496
Reported any urogenital symptoms at the post-treatment visit	NA§	NA§
Biological correlates of treatment success* [at pre-treatment visit]	**OR (95% CI)†**	**p†**
Total lactobacilli concentration	1.14 (0.80 – 1.62)	0.476
Total BV-anaerobes concentration	0.79 (0.41 – 1.53)	0.489
Total pathobionts concentration	0.76 (0.58 – 0.99)	0.044
Total other bacteria concentration	0.83 (0.63 – 1.10)	0.191
Gardnerella vaginalis concentration¶	0.86 (0.44 – 1.65)	0.640
Atopobium vaginae concentration‖	0.83 (0.59 – 1.17)	0.285
Prevotella concentration	0.93 (0.65 – 1.33)	0.691
Sneathia concentration	0.86 (0.63 – 1.16)	0.318
Megasphaera concentration	1.12 (0.89 – 1.42)	0.333
Veillonella concentration	0.85 (0.68 – 1.06)	0.157
BVAB1 concentration	1.01 (0.83 – 1.23)	0.912
Fusobacterium concentration	0.89 (0.61 – 1.30)	0.549
Vaginal microbiota type:		
- LA versus Li	1.75 (0.10 – 30.84)	0.702
- BV_GV versus Li	0.46 (0.04 – 5.75)	0.550
- BV_noGV versus Li	0.67 (0.04 – 11.29)	0.779
- GV versus Li	0.30 (0.02 – 4.91)	0.398
- BV_GV versus LA	0.27 (0.05 – 1.52)	0.136
- BV_noGV versus LA	0.38 (0.04 – 3.34)	0.383
- GV versus LA	0.17 (0.02 – 1.44)	0.104
Pooled vaginal microbiota type:		
- LA versus *Lactobacillus*-dominated [Li or Lo]	1.75 (0.10 – 30.84)	0.702
- [BV_GV or BV_noGV or GV] versus *Lactobacillus*-dominated [Li or Lo]	0.45 (0.04 – 5.40)	0.532
Yeasts on wet mount	0.83 (0.05 – 5.40)	0.896
Trichomonas vaginalis on wet mount	1.71 (0.15 – 20.1)	0.661
Positive urinalysis test	1.22 (0.33 – 4.44)	0.765
Any bacterial sexually transmitted infection (CT/NG/syphilis)	0.65 (0.22 – 1.91)	0.435
Positive herpes simplex virus type 2 serology	2.16 (0.70 – 6.69)	0.178

BV bacterial vaginosis, *BVAB1* BV-associated bacterium type 1, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis, CI* confidence interval, *CT Chlamydia trachomatis*, *GV G. vaginalis*-dominated, *LA* lactobacilli and anaerobes, Li *L. iners*-dominated, *Lo* other lactobacilli-dominated, *NG Neisseria gonorrhoeae*, *OR* odds ratio, *VMB* vaginal microbiota.

*Successful treatment defined as Nugent 7-10 pre- and 0-3 post-treatment (n=30 women) and treatment failure as Nugent 7-10 pre and 4-10 post-treatment (n=25 women). †Bivariable logistic regression models. ‡Compared to no schooling. §No women reported urogenital symptoms at the post-treatment visit. ¶Includes reads assigned to *Gardnerella* genus. ‖Includes reads assigned to *Atopobium* genus.

2.4 Discussion

In this study among high-risk women in Rwanda diagnosed with BV and/or *T. vaginalis*, the cure rate of seven-day oral metronidazole treatment by Nugent scoring was only 54.5%. The sequencing data showed a decrease in BV-anaerobes (but a reduction of more than 50% in only 16.4% of women), an increase in lactobacilli, and no change in pathobionts. Treatment failure was associated with higher levels of pre-treatment *Gardnerella vaginalis* or pathobionts levels, but not with sociodemographic factors.

Metronidazole treatment resulted in a mean BV-anaerobes concentration reduction (as well as *T. vaginalis* eradication), which is in agreement with a priori knowledge about the mechanism of action of metronidazole.[58,115] However, the extent of BV-anaerobes reduction was more modest than expected, with only 16.4% of women having a reduction of more than 50%. The mean lactobacilli concentration increased, and mean concentrations of pathobionts and 'other bacteria' were low pre- and post-treatment, resulting in an overall 5.5-fold reduction of total bacterial concentration (from 6.59 to 5.85 $\log_{10}$ cells/μL). The observed increase in lactobacilli is consistent with culture studies showing that lactobacilli are not sensitive to metronidazole,[63] but is inconsistent with claims made by some clinical researchers that the high BV recurrence rate may be due to detrimental effects of metronidazole on lactobacilli.[52] The reduction in BV-anaerobes concentration clearly allows lactobacilli, which are not affected by metronidazole, to expand.[60–62] In our study population of high-risk Rwandan women, *L. iners* was by far the most common *Lactobacillus* species pre- and post-treatment (4.81 and 5.28 $\log_{10}$ cells/μL, respectively), 'other lactobacilli' (which includes *L. jensenii*) increased the most during treatment (from 1.46 to 3.03 $\log_{10}$ cells/μL), and *L. crispatus* was uncommon and increased only slightly (from 0.15 to 0.51 $\log_{10}$ cells/μL). A metronidazole study in American women also showed that *L. iners* and *L. jensenii* concentrations increased more than the *L. crispatus* concentration.[122]

Women with a pre-treatment VMB type containing a relative abundance of >50% *G. vaginalis*, compared to ≤50%, were more likely to continue to have a dysbiotic VMB type post-treatment, but pre-treatment *G. vaginalis* concentration (as a continuous variable) was not associated with achieving Nugent 0-3 post-treatment. Both findings are consistent with earlier studies,[51,122] and with the *G. vaginalis*-containing biofilm hypothesis of treatment failure.[47] Metronidazole may eliminate dispersed *G. vaginalis* at low to modest concentrations, but may no longer be able to do so when a biofilm (containing a high concentration of *G. vaginalis*) has been established. However, other hypotheses have also been posited. A recent metatranscriptomics study showed that the VMB of BV patients with treatment failure contained *G. vaginalis* with upregulated clustered regularly interspaced short palindromic repeat-associated (CRISPR)-genes, which may protect the bacteria against metronidazole.[123] In our study, the concentrations of all other key BV-associated bacteria, including *A.*

vaginae, were effectively reduced by metronidazole and were not associated with treatment failure. This is in accordance to one study,[122] but in contrast to others that have suggested that pre-treatment presence or concentration of *A. vaginae* was associated with treatment failure.[124–126]

The concentrations of the pathobionts bacterial group were low pre- and post-treatment (1.92 and 2.01 $\log_{10}$ cells/μL, respectively). However, pathobionts have higher pathogenicity potential than BV-anaerobes,[9] and may therefore be clinically relevant even at low concentrations. Unfortunately, it is currently unknown at which concentrations or relative abundances pathobionts in the vagina should be treated to prevent complications, such as transmission to neonates. In our study, metronidazole treatment did not change the pathobionts concentration, but did increase the relative abundance due to the reduction in total bacterial concentration. Furthermore, a higher pre-treatment pathobionts concentration was associated with increased likelihood of treatment failure. None of the sociodemographic factors that have been associated with VMB composition in other studies, including menses in the seven days prior to the post-treatment visit,[127] were associated with treatment failure in our study.

Our study has several implications. Women with persistent or recurrent BV might benefit from vaginal biofilm-disrupting treatment, adjuvant therapy with lactobacilli-based live biotherapeutics, or treatment with drugs that specifically target all *G. vaginalis* clades. The former two are actively researched,[46,47,128] but the latter are not yet available. Whether these strategies are efficacious in real life would have to be evaluated in clinical trials. Furthermore, diagnostic tests to determine the presence of a biofilm or concentrations of *G. vaginalis* are not yet available to clinicians. Women at risk of complications caused by vaginal pathobionts (not just Group B streptococci), such as pregnant women, might benefit from targeted screening and treatment. We encourage the incorporation of quantitative molecular characterisation of both key individual bacteria with pathogenic potential, as well as bacterial communities and biofilms, in future intervention studies.

Limitations of our study include potentially limited generalisability of the results to lower risk and non-African populations, and the lack of vaginal biofilm detection and characterisation pre- and post-treatment. Recent studies have shown good correlations between the method that we used to quantify relative abundance data and species-specific qPCR results of non-minority species,[129,130] but additional validation studies are desirable. While Nugent-based studies have shown that oral and vaginal metronidazole of similar dose and duration of use have similar efficacy for BV,[48,49] molecular studies comparing the in-depth microbiological effects of different metronidazole formulations are desirable. A major strength of our study is that we used multiple laboratory and analytic methods to characterise the VMB, including methods that incorporated a priori knowledge about the pathogenic potential of specific micro-organisms and the types of communities in which they typically live.

Conclusions

Oral metronidazole treatment alone may not be sufficient for women with recurrent bacterial vaginosis, or for women at risk of complications caused by pathobionts (such as pregnant women). Additional treatments are urgently needed, including biofilm-disrupting treatments and drugs that specifically target all *G. vaginalis* clades or pathobionts.

Chapter 3 - Intermittent Lactobacilli-containing Vaginal Probiotic or Metronidazole Use to Prevent Bacterial Vaginosis Recurrence: Safety and Preliminary Efficacy by Microscopy and Sequencing

This chapter has been submitted to an international peer-reviewed journal and has been deposited on the pre-print server *MedRxiv*: van de Wijgert JHHM, Verwijs MC, Agaba SK, Bronowski C, Mwambarangwe L, Musengamana V, Uwineza M, Lievens E, Nivoliez A, Ravel J, Darby A. Intermittent lactobacilli-containing vaginal probiotic or metronidazole use to prevent bacterial vaginosis recurrence: safety and preliminary efficacy by microscopy and sequencing. *MedRxiv* 2019; doi:10.1101/19001156. The version presented here is the *MedRxiv* version with only minor modifications (numbering of figures, tables, and references). The methods used in Chapters 2 and 3 overlap considerably, and I have therefore combined the supplementary methods into one Appendix that applies to both chapters (Appendix A).

I reviewed the clinical data that had been collected by the Rinda Ubuzima team, and conducted the site close-out visit, in Kigali, Rwanda, under the supervision of Professor Janneke van de Wijgert (my primary supervisor). I subsequently coordinated the shipments of the stored vaginal samples from Kigali to Liverpool, and prepared them for sequencing at the University of Liverpool Centre for Genomic Research under the supervision of Professor Alistair Darby (my secondary supervisor). I processed the raw sequencing data, derived various VMB variables, and added them to the clinical database with the help of both my supervisors as well as fellow PhD student Christina Gill. I developed the analytical approach and performed the statistical analyses together with my primary supervisor. My primary supervisor and I wrote the first version of the manuscript together. All co-authors commented on and approved the final manuscript.

Abstract

Introduction: Bacterial vaginosis (BV) is associated with HIV acquisition, pelvic inflammatory disease, and adverse pregnancy outcomes. Recurrence rates after metronidazole or clindamycin treatment are high.

Materials and Methods: HIV-negative, non-pregnant Rwandan BV patients were randomised to four groups (n=17/group) after seven-day oral metronidazole treatment: behavioural counselling only (control), or counselling plus intermittent use of oral metronidazole, Ecologic Femi+ vaginal probiotic capsule (containing multiple lyophilised *Lactobacillus* and one *Bifidobacterium* species), or Gynophilus LP vaginal probiotic tablet (*L. rhamnosus* Lcr35) for two months. Vaginal microbiota assessments at all visits included Gram stain Nugent scoring and 16S rRNA gene qPCR and HiSeq sequencing.

Results: All interventions were safe. The BV (Nugent 7-10) incidence rate was 10.18 per person-year at risk in the control group, and lower in the metronidazole (1.41/person-year; p=0.004), Ecologic Femi+ (3.58/person-year; p=0.043), and Gynophilus LP groups (5.36/person-year; p=0.220). In mixed effects models adjusted for hormonal contraception/pregnancy, sexual risk-taking, and age, metronidazole and Ecologic Femi+ users, each compared to controls, had higher *Lactobacillus* and lower BV-anaerobes concentrations and/or relative abundances, and were less likely to have a dysbiotic vaginal microbiota type consisting of BV-anaerobes by sequencing; non-significant trends for Gynophilus LP users were in the same directions. Inter-individual variability was high and the beneficial effects disappeared soon after cessation of use.

Discussion: We conclude that lactobacilli-based vaginal probiotics warrant further evaluation because, in contrast to antibiotics, they are not expected to negatively affect microbiota or cause antimicrobial resistance. Next generation sequencing methods allow for much more rigorous efficacy evaluations than were possible in the past.

3.1 Introduction

Most women have a vaginal microbiota (VMB) that consists predominantly of lactobacilli.[4] The most common type of vaginal dysbiosis is bacterial vaginosis (BV), characterised by a decrease in lactobacilli and increase in fastidious anaerobes, which affects 30-40% of women worldwide.[87] Other types of bacterial dysbiosis, vulvovaginal candidiasis, and *Trichomonas vaginalis* (TV) are also common. These conditions are associated with vaginal inflammation, thereby increasing the risk of HIV acquisition.[12] They are also associated with pelvic inflammatory disease, infertility, and adverse pregnancy outcomes.[87]

The majority of women seeking care for vaginal symptoms receive antibiotic or antifungal treatment empirically or syndromically without any diagnostic testing.[21] In some specialised clinics, women might be offered limited diagnostic testing, such as vaginal pH determination and/or wet mount microscopy. In research settings, BV is typically diagnosed by the Amsel criteria or Gram stain Nugent scoring,[77,78] with the latter currently being considered the gold standard: Gram-stained vaginal smears are scored based on microscopic visualisation of three bacterial morphotypes with a score of 0-3 considered normal, 4-6 intermediate, and 7-10 BV regardless of symptoms. In the last 15 years, molecular methods have become more widely available, and have been applied to the VMB, although mostly in descriptive studies to date.[4]

Evidence is mounting that 'microbiological-BV' (by Nugent scoring or molecular methods) can cause long-term adverse outcomes in the presence or absence of vaginal symptoms.[87] BV is notoriously difficult to treat.[46,52,131] About 60-80% of patients are cured after a course of oral or vaginal metronidazole or clindamycin, but recurrence rates are high.[52] Therapies to restore and maintain a healthy VMB after antibiotic treatment are not standard practice, but some clinicians in Europe and North America recommend twice weekly 0.75% metronidazole vaginal gel for 4-6 months to lower the risk of BV recurrence.[52] This recommendation was tested in a randomised controlled trial in the USA, which showed a statistically significant reduction in BV recurrence (57% by Amsel criteria and 34% by Nugent scoring).[132] In addition, two African trials evaluating oral (2g monthly) and vaginal metronidazole (five nights every three months) for BV prevention showed significant beneficial effects, but the effects were modest, most likely due to infrequent dosing.[133,134]

Probiotic lactobacilli may be able to restore and maintain lactobacilli-dominated VMB, may be better able to prevent or disrupt BV-associated biofilms than antibiotics, and can likely be used safely for long periods without the risk of causing antimicrobial resistance.[72] Lactobacilli-containing vaginal probiotic clinical trials to date have shown mixed results, but eight of the 12 trials showed sufficiently promising efficacy for BV prevention to warrant further investigation.[72,135–145] Some trials used commercially

available probiotic strains (mostly derived from the gut or fermented foods) and others vaginal strains isolated from healthy women,[71] but currently available efficacy signals are similar for the two probiotic strain categories.[72,135–145] None of the trials reported major safety concerns or colonisation beyond the dosing period, but safety during pregnancy or in immunocompromised individuals has not been studied well. Because 'natural' strains do not seem to outperform commercially available strains, and for pragmatic reasons, we chose to evaluate two vaginal probiotics that are currently on the market. Our aim was to assess their impact on the VMB in much more detail than previous trials had done, and develop data analytic methods to enable use of high-dimensional 16S rRNA gene sequencing data for this purpose. The two probiotics that we evaluated were Ecologic Femi+ vaginal capsule (EF+; Winclove Probiotics, Amsterdam, The Netherlands) and Gynophilus LP vaginal tablet (GynLP; Biose, Aurillac, France). EF+ contains multiple lyophilised bacteria (*Bifidobacterium bifidum* W28, *Lactobacillus acidophilus* W70, *L. helveticus* W74, *L. brevis* W63, *L. plantarum* W21, and *L. salivarius* W24) in a total dose of $1.5x10^9$ colony forming units (CFU). GynLP contains $1.6x10^9$ CFU of Lcr Regenerans, a culture of the *L. rhamnosus* 35 (Lcr35) strain. The tablet disintegrates in the vagina after forming a gel to release Lcr35. Gynophilus (the same active ingredient as GynLP but a different formulation) had shown promise in preventing BV recurrence in a previous trial, but EF+ had not previously been studied for this indication.[136]

3.2 Materials and Methods

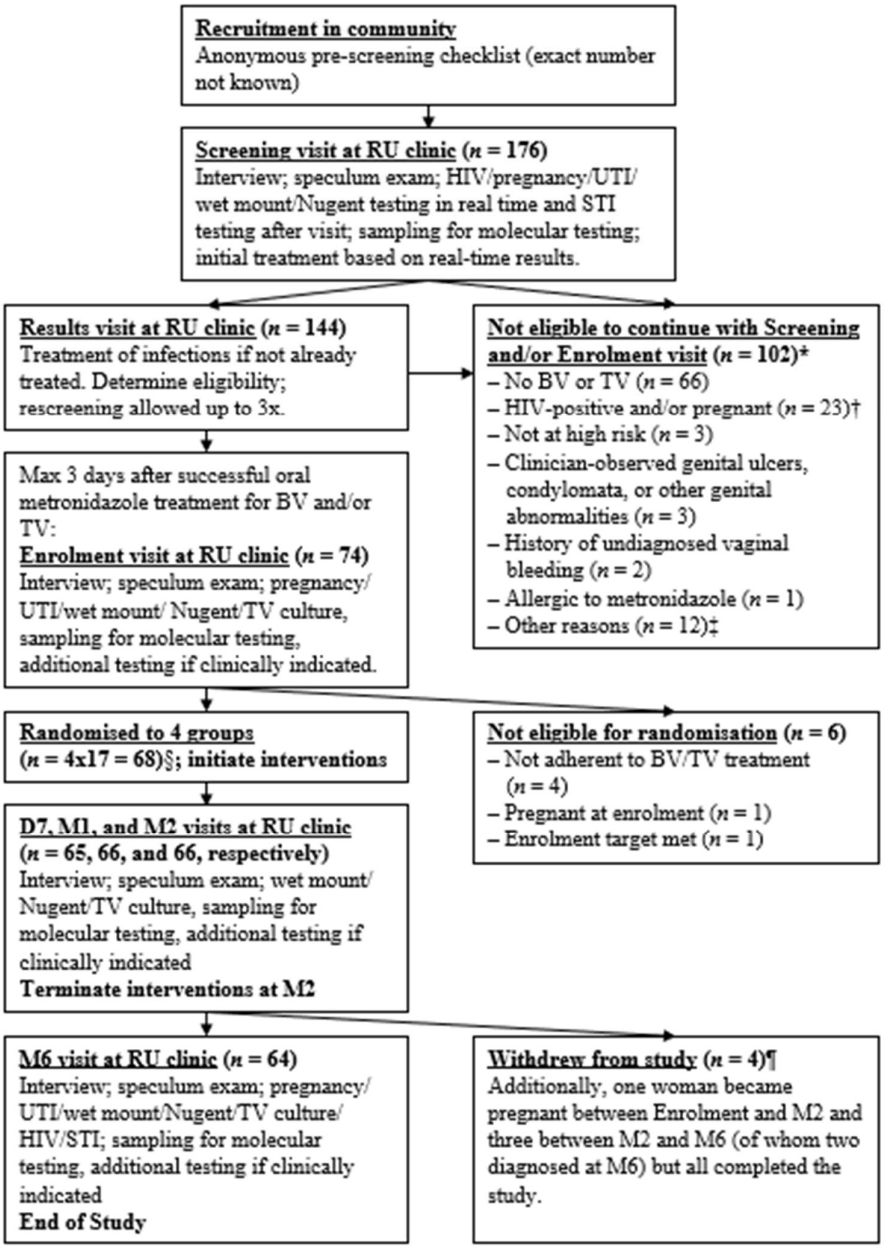
From June 2015 until February 2016, we conducted a randomised clinical trial in Kigali, Rwanda, to evaluate intermittent use of the above-mentioned vaginal probiotics as well as oral metronidazole (Tricozole, Laboratory & Allied ltd, Nairobi, Kenya) to prevent the recurrence of microbiological-BV after metronidazole treatment. The trial was a pilot trial with a modest sample size (N=68) at the request of the funder.

Eligibility

Women were eligible for screening if they were aged 18-45, in good overall physical and mental health, and at high urogenital infection risk defined as having had more than one sex partner in the last 12 months or having been treated for a sexually transmitted infection and/or BV in the last 12 months. They were eligible for enrolment if they were confirmed HIV-negative and non-pregnant, were diagnosed with BV (by Nugent score and/or Amsel criteria) and/or TV (by wet mount and/or culture), and were cured after seven days of oral metronidazole treatment (500 mg twice per day).[78] Cure was defined as no BV by Amsel criteria and no TV by wet mount.[77] We did not use Nugent scores and TV culture as tests-of-cure to allow for same day enrolment but results became available after enrolment. At enrolment, 51/68 women were BV-negative by Amsel and Nugent criteria, 17/68 women were BV-negative by Amsel but BV-positive by Nugent criteria, and all women were TV-negative by both wet

mount and culture and free of symptomatic vulvovaginal candidiasis, symptomatic urinary tract infection, syphilis, and clinician-observed genital abnormalities or vaginal discharge. We did not exclude women with chlamydia and/or gonorrhoea because the local testing turn-around time was slow. Positive herpes simplex type 2 serology was not a reason for exclusion. Additional exclusion criteria applied but these were rare (figure 3.1, Appendix A).

Figure 3.1: Flow diagram of numbers of women seen and study procedures at each visit



BV bacterial vaginosis, *D7* day 7 visit, *M1/2/6* Month 1/2/6 visit, *RU* Rinda Ubuzima, *STI* sexually transmitted infection, *TV Trichomonas vaginalis*, *UTI* urinary tract infection.
*Totals to 110 reasons among 102 women because there could be more than one reason per woman. †No speculum exam performed; molecular testing of self-sampled vaginal swabs. ‡Reasons: outside of metronidazole treatment window (n=5), enrolment target already met (n=4), has a mental disorder (n=1), did not complete screening procedures and was subsequently lost to follow=up (n=1), withdrew consent during the Screening visit because she thought the reimbursement was too low (n=1). §Three women in each randomisation

group were selected for self-sampling every other day during the first month of follow-up. ¶Reasons: moved away from Kigali (n=2), lost interest because symptoms resolved (n=1), and was verbally harassed by partner and sister about study participation (n=1).

Randomisation groups and visit schedule

Women were randomised to four groups (17 women per group) within three days of completing oral metronidazole treatment: 1) behavioural counseling only (control group); 2) counseling plus 500 mg oral metronidazole twice weekly for two months; 3) counseling plus EF+ vaginal capsule once per day for the first five days followed by thrice weekly for a total of two months; and 4) counseling plus GynLP once every four days for two months. Participants applied the first dose of their intervention under direct observation at the enrolment visit, and returned to the clinic after seven days (D7), one month (M1), two months (M2; cessation of product use), and six months (M6). They were allowed to cease vaginal product use temporarily during menstruation. Product adherence was assessed by review of diary cards, used and unused products, and by a self-rating adherence scale. Symptomatic BV, vulvovaginal candidiasis, and urinary tract infections, and laboratory-confirmed sexually transmitted infections, diagnosed during follow-up were treated using standard oral therapies, and women were urged to continue their interventions during treatment. Visit procedures are summarised in figure 3.1 and described in Appendix A.

Diagnostic testing

Women were tested for BV, TV, and vulvovaginal candidiasis at each visit. BV was diagnosed by Gram stain Nugent scoring[78] and Amsel criteria[77]; the vaginal pH was measured by pressing a pH paper strip (pH range 3.6 – 6.1 with 0.3 increments; Machery-Nagel, Düren, Germany) against the vaginal wall. TV was diagnosed when motile trichomonads were observed on wet mount and by InPouch culture (Biomed Diagnostics, White City, OR, USA). Vulvovaginal candidiasis was diagnosed when budding yeasts and/or (pseudo)hyphae were observed on wet mount. All other diagnostic tests (Appendix A) were only done at screening, M6, and when judged clinically necessary by the physician, with the exception of pregnancy and urinalysis tests, which were repeated at enrolment prior to randomisation.

16S rRNA gene sequencing and qPCR

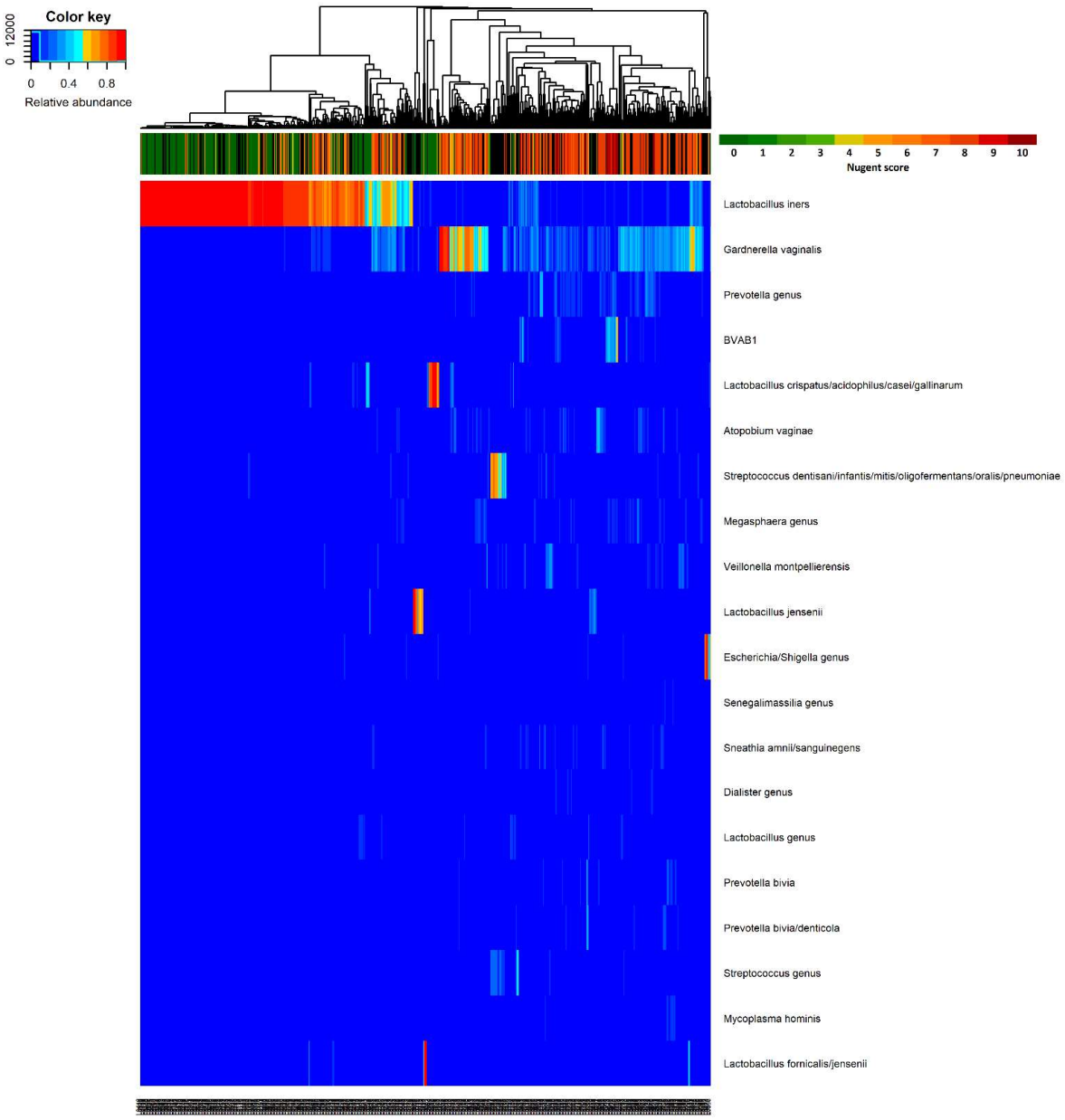
We collected vaginal swabs at 639 time points, including study visits of all 68 women and self-sampled swabs from 12 women (three per group) who had been asked to self-sample at home every Monday, Wednesday, and Friday during the first month. Self-sampled swabs were processed but were not included in analyses unless stated. DNA was extracted from one swab per time point per woman. Briefly, DNA was extracted using a combination of lysozyme lysis, Qiagen DNeasy Blood and Tissue kit (Qiagen, Manchester, UK), and bead-beating procedures. Purified DNA underwent two PCR rounds: amplification of the 16S rRNA gene V3-V4 region and dual-index barcoding allowing multiplexing of up to 384 samples. Samples were sequenced on an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA), run in rapid mode, 2x300bp using a 250PE and 50PE kit. All samples collected from

the same participant were included in the same run. Negative and positive controls (ZymoBiomics Microbial Community DNA standard; Zymo Research Corp, Irvine, USA) were incorporated throughout. The panbacterial 16S rRNA gene copy concentrations of physician-collected samples of enrolled women with valid sequencing results (N=393) were determined by BactQuant qPCR assay as previously described.[118]

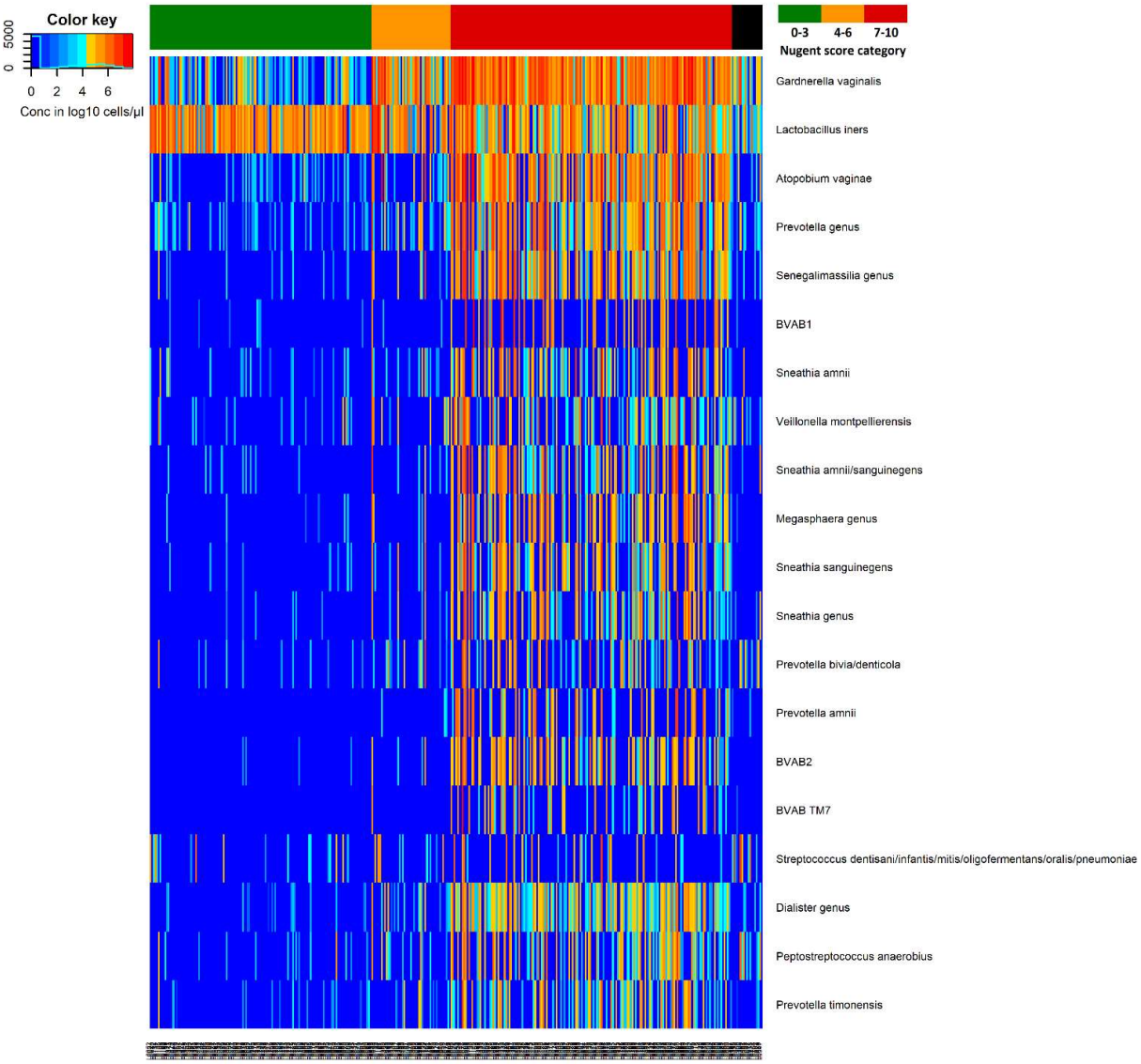
Molecular data processing

Reads were demultiplexed, and primer sequences removed using Cutadapt v1.2.1.[146] Error correction, dereplication, denoising, merging, removal of chimeras, and taxonomic assignment were performed in DADA2 v1.4.0 using Silva v128 as the reference database (Appendix A).[119,120] Further data processing included removal of rare, non-bacterial, and contaminant amplicon sequence variants (ASVs), and identification of vaginal and probiotic sequences that are not included in the Silva database using the NCBI *Needleman-Wunsch Global Align Nucleotide Sequences* function[136] (Appendix A). Silva-based taxonomic assignments of non-minority ASVs were double-checked using the NCBI *Microbial Nucleotide BLAST* function, using the non-redundant V3-V4 version of the Vaginal 16S rRNA Reference Database as a tiebreaker.[147,148] We rarefied at 1,111 reads using the *GUniFrac 1.0* package in R.[121] This yielded 401 unique ASVs in 629 samples, mapping to species (n=255; 63.6%), genus (n=116; 28.9%), or higher taxonomic level (n=30; 7.5%). Concentrations in cells/µl per ASV per sample were estimated by multiplying the ASV-specific copy-normalised relative abundance by the sample-specific 16S rRNA gene copies concentration.[129,130] Heatmaps of the 20 most common ASVs by sample are shown in figure 3.2a for relative abundances and figure 3.2b for concentrations. VMB data reduction was required for molecular efficacy analyses, and was done in three different ways. First, the Simpson diversity index (1-D) was calculated for each sample. Second, each ASV was assigned to one of four 'bacterial groups' based on the published literature (Appendix A, table A.1): 1) lactobacilli (all species combined, but with subcategorisation into EF+ strains, the GynLP strain, and 'natural lactobacilli' in some analyses); 2) BV-anaerobes (anaerobic bacteria that have consistently been associated with BV); 3) pathobionts (bacteria that are considered to have higher intrinsic pathogenicity than BV-anaerobes and have not consistently been associated with BV[9]); and 4) 'other bacteria' (a rest group, consisting mostly of skin bacteria and *Bifidobacterium* species). Within each sample, read counts of ASVs belonging to the same bacterial group were summed. This resulted in four relative abundances (one for each bacterial group) per sample, which sum to 1.0 for each sample. Third, we used hierarchical clustering based on Euclidean distance to pool samples into eight mutually exclusive VMB types (each sample was assigned to only one VMB type): 1) *L. iners*-dominated (Li, n=247 samples); 2) *L. crispatus*-dominated (Lcr, n=17); 3) other lactobacilli-dominated (Lo, n=28); 4) lactobacilli and anaerobes (LA, n=86); 5) polybacterial *Gardnerella vaginalis*-containing (BV_GV, n=138), 6) polybacterial but low-*G. vaginalis* (BV_noGV, n=23), 7) *G. vaginalis*-dominated (GV, n=41); and 8) pathobionts-containing (PB, n=49) (Appendix A).

Figure 3.2: Heatmaps of all study samples with relative abundance and concentration data a



b



BVAB1 BV-associated bacterium type 1, *BVAB2* BV-associated bacterium type 2, *BVAB TM7* BV-associated bacterium (phylum TM7), *conc* concentration.
a Heatmap depicting all samples (n = 629) on the x-axis and the 20 amplicon sequence variants with highest mean relative abundance on the y-axis. The dendrogram above the heatmap depicts VMB clusters based on Euclidean distance. The bar below the dendrogram depicts Nugent scores (black means Nugent score unavailable). **b** Heatmap depicting all samples (n = 379) with valid quantitative qPCR data on the x-axis and the 20 amplicon sequence variants with highest mean 16S rRNA gene copy-normalised concentration on the y-axis. The bar depicts Nugent score categories (black means Nugent score unavailable).

Downstream statistical analysis

Adverse events were coded using the Medical Dictionary for Regulatory Activities 19.1 (McLean, VA, USA). Data were analysed using Stata 13 (StataCorp, College Station, TX, USA). Most statistical comparisons were between randomisation groups (each intervention group compared to the control group) at screening, enrolment, and longitudinally over time. For cross-sectional analyses, we used Fisher's exact test for binary and categorical variables, and Kruskal-Wallis and Mann Whitney U tests

for continuous variables. For longitudinal analyses, we used incidence rates, incidence rate ratios, and mixed effects models. We conducted ITT analyses, and modified ITT analyses limited to women who were BV-negative by both Amsel and Nugent criteria at the time of randomisation (n=51 of 68). All mixed models included one VMB endpoint at a time as the outcome, participant identification number as the random effect, and randomisation group (an indicator variable with the control group as the reference group) as the main fixed effect. Models included samples collected during the intervention period only (including self-sampled time points), and adjusted for covariates that were associated with VMB composition in mixed effects models at $p<0.05$.

Ethical statement

All participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki, sponsored by the University of Liverpool, approved by the Rwanda National Ethics Committee and the University of Liverpool Research Ethics Subcommittee for Physical Interventions, and registered on ClinicalTrials.gov (NCT02459665).

3.3 Results

Participant disposition

Of the 68 randomised women, only four did not complete the trial (figure 3.1), resulting in 29.93 person-years of data. The median age was 31 (interquartile range (IQR) 27-35) (table 3.1, Appendix table C.1). The majority of women (92.6%) had exchanged sex for money or goods, and had had a median of five (IQR 2-18) sex partners, in the past month. All but three women used condoms, but mostly inconsistently. Two-thirds of the women (61.8%) were using hormonal contraception, and four women became pregnant during the trial. Short course metronidazole/tinidazole use for other indications during the intervention period was evenly distributed among randomisation groups (Appendix table C.2: n=1-3 per group; p=0.688), as was short course use of other oral antibiotics (Appendix table C.2: n=2-4 per group; p=0.781). Furthermore, these other antibiotics did not impact lactobacilli and BV-anaerobes concentrations significantly (Appendix, table C.2, figure C.1). No antifungals were used. Product adherence was assessed by review of diary cards, used and unused products, and by a self-rating adherence scale. Most women were adherent with their study product >90% of the time, but this percentage was non-significantly lower in the GynLP group (68.8%) than in the metronidazole (82.4%; Fisher's exact p=0.438) and EF+ groups (88.2%, Fisher's exact p=0.225; Appendix table C.3).

Table 3.1: Baseline characteristics

Sociodemographic characteristics at Scr/Enr	**Screened (*n* = 175)**	**Enrolled (*n* = 68)**	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 17)**	**p***
Age in years, median (IQR)	30 (27 – 34)	31 (27 – 35)	29 (24 – 36)	30 (27 – 34)	33 (28 – 35)	30 (27 – 35)	0.563
Sex partners last mo, median (IQR)	5 (2 – 16)	5 (2 – 18)	5 (3 – 20)	5 (2 – 10)	3 (2 – 15)	3 (2 – 20)	0.624
Condom during last sex act (n %)†	95 (54.6)	36 (52.9)	11 (64.7)	8 (47.1)	6 (35.3)	11 (64.7)	0.279
Currently using contraception (n %):							
- None	69 (40.2)	24 (35.3)	6 (35.3)	6 (35.3)	4 (23.5)	8 (47.1)	0.750
- Hormonal contraception	99 (57.6)	42‡ (61.8)	10 (58.8)	11 (64.7)	12 (70.6)	9 (52.9)	
- Copper IUD	4 (2.3)	2 (2.9)	1 (5.9)	0	1 (5.9)	0	
Laboratory results at Scr	**Screened (*n* = 173)**	**Enrolled (*n* = 68)**	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 17)**	**p***
Nugent score, mean (95% CI)†	4.7 (4.1 – 5.3)	7.4 (6.8 – 8.0)	7.6 (6.5 – 8.6)	6.8 (5.3 – 8.3)	7.1 (5.5 – 8.6)	8.2 (7.4 – 9.0)	0.333
Nugent categories (n %)†							
- 0-3	55 (38.2)§	5 (7.5)	1 (5.9)	2 (11.8)	2 (12.5)	0	0.075
- 4-6	20 (13.9)	6 (9.0)	0	4 (23.5)	0	2 (11.8)	
- 7-10	69 (47.9)	56 (83.5)	16 (94.1)	11 (64.7)	14 (87.5)	15 (88.2)	
BV modified Amsel¶ (n %)	63 (43.2)‖	49 (72.1)	11 (64.7)	12 (70.6)	13 (76.5)	13 (76.5)	0.933
Candida wet mount (n %)	14 (9.6)‖	6 (8.8)	1 (5.9)	2 (11.8)	3 (17.7)	0	0.493
TV by wet mount positive (n %)	9 (6.2)‖	6 (8.8)	3 (17.7)	1 (5.9)	1 (5.9)	1 (5.9)	0.707
TV InPouch positive (n %)	17 (11.8)§	11 (16.4)	3 (18.8)	1 (5.9)	5 (29.4)	2 (11.8)	0.282
UTI dipstick positive (n %)	33 (19.1)	17 (25.0)	4 (23.5)	6 (35.3)	4 (23.5)	3 (17.7)	0.760
CT PCR positive (n %)	30 (20.8)§	20 (29.4)	5 (29.4)	7 (41.2)	3 (17.7)	5 (29.4)	0.560
NG PCR positive (n %)	18 (12.5)§	13 (19.1)	5 (29.4)	4 (23.5)	2 (11.8)	2 (11.8)	0.555
HIV serology positive (n %)	17 (9.8)	0	0	0	0	0	NA
HSV2 serology positive (n %)	117 (67.6)	44 (64.7)	9 (52.9)	12 (70.6)	11 (64.7)	12 (70.6)	0.780
Syphilis serology positive (n %)	13 (7.5)	4 (5.9)	0	1 (5.9)	0	3 (17.7)	0.182
Pregnancy positive (n %)	7 (4.1)	0	0	0	0	0	NA
Laboratory results at Enr**	**Screened (*n* = 176)**	**Enrolled (*n* = 68)**	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 17)**	**p***
Nugent score, mean (95% CI)†	NA	3.1 (2.2 – 3.9)	3 (1.2 – 4.8)	3.3 (1.4 – 5.2)	1.7 (0.1 – 3.3)	4.3 (2.6 – 6.0)	0.187
Nugent categories (n %)†							
- 0-3	NA	36 (54.6)	8 (53.3)	9 (52.9)	13 (76.5)	6 (35.3)	0.149
- 4-6		13 (19.7)	5 (33.3)	2 (11.8)	1 (5.9)	5 (29.4)	
- 7-10		17 (25.8)	2 (13.3)	6 (35.3)	3 (17.7)	6 (35.3)	
BV by modified Amsel¶ (n %)	NA	0	0	0	0	0	NA
Candida on wet mount (n %)	NA	4 (5.9)	2 (11.8)	1 (5.9)	0	1 (5.9)	0.897
TV by wet mount/culture (n %)	NA	0	0	0	0	0	NA
VMB outcomes at Enr	**Screened (*n* = 176)**	**Enrolled (*n* = 67)**	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 16)**	**p***
Richness, mean (95% CI)	NA	12.8 (10.7 – 14.8)	17.8 (12.1 – 23.5)	12.1 (8.7 – 15.5)	7.5‡‡ (4.9 – 10.2)	13.7 (10.3 – 17.1)	0.003
Simpson diversity index, mean (95% CI)	NA	0.31 (0.25 – 0.38)	0.38 (0.21 – 0.54)	0.35 (0.20 – 0.50)	0.15§§ (0.05 – 0.25)	0.38 (0.25 – 0.50)	0.022
Total bacteria and bacterial group concentrations in log_{10} cells/µL, mean (95% CI)††							
Total bacteria	NA	5.85 (5.66 – 6.04)	5.75 (5.30 – 6.20)	5.79 (5.39 – 6.19)	5.54 (5.28 – 5.80)	6.34‡‡ (5.95 – 6.73)	0.019
Total *Lactobacillus*	NA	5.56 (5.34 – 5.78)	5.22 (4.63 – 5.82)	5.59 (5.20 – 5.97)	5.46 (5.19 – 5.73)	5.97 (5.44 – 6.49)	0.092
Total BV-anaerobes	NA	4.55 (4.15 – 4.95)	4.78 (4.17 – 5.39)	4.50 (3.77 – 5.24)	3.36‡‡ (2.43 – 4.29)	5.62‡‡ (4.99 – 6.25)	0.002
Total pathobionts	NA	2.01 (1.48 – 2.54)	2.36 (1.28 – 3.44)	2.08 (1.00 – 3.17)	1.34 (0.29 – 2.39)	2.30 (0.98 – 3.62)	0.447
Total other bacteria	NA	1.46 (1.01 – 1.92)	1.80 (0.84 – 2.76)	1.30 (0.36 – 2.24)	0.57 (-0.10 – 1.24)	2.20 (1.07 – 3.34)	0.066
VMB types (n %):							
- Li		35 (52.2)	7 (41.2)	9 (52.9)	14 (82.4)	5 (31.3)	
- Lcr		0	0	0	0	0	
- Lo		2 (3.0)	1 (5.9)	1 (5.9)	0	0	
- LA	NA	18 (26.9)	3 (17.7)	5 (29.4)	2 (11.8)	8 (50.0)	0.048
- BV_GV		2 (3.0)	1 (5.9)	0	0	1 (5.9)	
- BV_noGV		0	0	0	0	0	
- GV		4 (6.0)	2 (11.8)	0	0	2 (12.5)	
- PB		6 (9.0)	3 (17.7)	2 (11.8)	1 (5.9)	0	

BV bacterial vaginosis-like, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *CI* confidence interval, *conc* concentration, *CT Chlamydia trachomatis*, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GV G. vaginalis*-dominated, *GynLP* Gynophilus LP, *HSV2* herpes simplex virus type 2, *IQR* inter-quartile range, *LA* lactobacilli and anaerobes, *Lcr Lactobacillus crispatus*-dominated, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *Metro* metronidazole, *Mo* month, *NA* not applicable, *NG Neisseria gonorrhoeae*, *PB* pathobionts-containing, *Scr* screening visit, *TV Trichomonas vaginalis*, *UTI* urinary tract infection, *VMB* vaginal microbiota.
*Fisher's exact test for binary/categorical outcomes and Kruskall Wallis test for continuous outcomes, between randomisation groups. †Total numbers are slightly lower due to 1-4 missing values. ‡Seven women reported using oral contraception, 18 hormonal injections, and 17 hormonal implants. §n=144; women at the screening visit that were HIV-positive and/or pregnant, or were otherwise ineligible, did not undergo testing for this urogenital disease. ¶The modified Amsel criteria were considered positive if two or three of the following criteria were positive: 1) >20% clue cells on wet mount 2) a positive KOH (amine) test; 3) vaginal pH>4.5. ‖*n* = 146; women at the screening visit who were HIV-positive and/or pregnant, or were otherwise ineligible, did not undergo testing for this urogenital disease. **All enrolled participants were negative for HIV and pregnancy, and were treated for UTI and syphilis if positive at screening. Twenty-three enrolled participants (six controls, seven in the metronidazole group, four in the EF+ group, and six in the GynLP group) were positive for CT and/or NG at screening and had not received treatment by the enrolment visit. ††Total numbers are slightly lower due to invalid qPCR results: overall enrolment population is n=63, control group *n* = 16, and metronidazole group *n* = 14. ‡‡p<0.05 by Mann Whitney U test, compared to control group. §§p<0.01 by Mann Whitney U test, compared to control group.

VMB compositions at baseline

By design, all randomised women at screening had BV (Nugent score 7-10 and/or by modified Amsel criteria) and/or TV (by wet mount and/or culture): 82.4% had BV alone, 14.7% had BV and TV, and 2.9% had TV alone. Therefore, as expected, most women had dysbiotic VMB types by 16S rRNA gene sequencing at screening: BV_GV (41.8%), LA (17.9%), BV_noGV (11.9%), GV (11.9%), and PB (1.5%). However, 14.9% had a lactobacilli-dominated Li VMB type, of whom 60% were TV-negative, and these women would not have needed metronidazole treatment. Also by design, all randomised women were BV-negative (by modified Amsel criteria) and TV-negative (by wet mount and culture) at the time of randomisation. However, almost half of the women (44.8%) were not lactobacilli-dominated by sequencing at the time of randomisation, and none of them were *L. crispatus*-dominated (which is considered the most optimal VMB state): their VMB types were Li (52.2%), Lo (3.0%), LA (26.9%), PB (9.0%), GV (6.0%), and BV_GV (3.0%) (table 3.1; see Chapter 2).

At randomisation, the mean total bacterial concentration ranged from 5.54-6.34 $\log_{10}$ cells/µl in the four randomisation groups, and these ranges were 5.22-5.97 for lactobacilli, 3.36-5.62 for BV-anaerobes, 1.34-2.36 for pathobionts, and 0.57-2.20 for 'other bacteria' (table 3.1; relative abundance data in Appendix tables C.1 and C.4). The mean richness ranged from 7.5-17.8, and the mean Simpson diversity from 0.15-0.38. Randomisation did not completely balance the baseline VMB compositions of the four groups: the concentrations total bacteria and BV-anaerobes were higher in the GynLP group than the control group (Mann Whitney U test $p<0.05$), and the BV-anaerobes concentration ($p<0.01$) and Simpson diversity ($p<0.05$) were lower in the EF+ group than the control group (table 3.1).

Variables that were associated with at least one VMB composition variable in unadjusted mixed effects models (Appendix table C.5) included currently using hormonal contraception or being pregnant (associated with a higher pathobionts concentration), above-average sexual risk taking based on reported numbers of partners and condom use (higher pathobionts concentration), aged 30 years or older (lower BV-anaerobes and pathobionts concentrations), and managing menses with a sanitary pad compared to other methods (higher Nugent score). Reporting current urogenital symptoms was also associated with VMB composition but this likely represents reverse causality. We could not exclude

women with ongoing chlamydia and/or gonorrhoea infections at randomisation because the turn-around time of diagnostic testing was slow, but the VMB compositions after metronidazole treatment of women with and without ongoing chlamydia and/or gonorrhoea infection were similar (Appendix figure C.1). As mentioned earlier, short course antibiotic use that was not part of the study interventions was not associated with VMB composition either (Appendix tables C.2 and C.5; figure C.1).

Safety

Two serious adverse events occurred but these were judged not to be related to study participation: one woman in the oral metronidazole group was hospitalised for typhoid fever and one woman in the EF+ group for malaria during pregnancy. Both events occurred after the intervention period and both women recovered completely. Two women reported non-serious social harms that were judged related to study participation. One woman in the control group was beaten by her partner because she engaged in self-sampling; she withdrew from self-sampling but continued participation in the study. One woman in the GynLP group suffered verbal harassment from her partner and her sister for taking part in the study and elected to withdraw.

During the intervention period, urogenital symptoms (mostly genital itching, burning, and pain during sex, but no vaginal discharge) were reported by 13.4% of participants with no differences between groups, and only two speculum exam, and no bimanual exam, findings were reported by the physician (table 3.2). After product cessation, urogenital symptom reporting was similar compared to the intervention period (10.8%), but the number of speculum exam findings increased (32.8%), likely reflecting the high urogenital infection incidence in this cohort. A total of 41 adverse events were spontaneously reported between enrolment and M6. All of them were judged definitely not or unlikely to be related to trial participation, and they were evenly distributed among groups.

Table 3.2: Safety endpoints

	Total (*n* = 68)	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 17)**	**p***
Patient-reported symptoms and clinician-observed signs at Scr and Enr						
Any (current or in last 2 weeks) self-reported urogenital symptoms, at Scr (n %)	49 (72.1)†	11 (64.7)	13 (76.5)	13 (76.5)	12 (70.6)	0.933
Any (current) symptoms at Enr (n %)	0	0	0	0	0	NA
Any abnormal pelvic exam findings at Scr, clinician-observed (n %)‡	3 (4.4)§	1 (5.9)	1 (5.9)	0	1 (5.9)	1.00
Any abnormal pelvic exam findings at Enr, clinician-observed (n %)‡	1 (1.5)¶	0	0	0	1 (5.9)	1.00
AEs – Structurally assessed between Enr and M2 (during product use)						
Any current urogenital symptoms (n %)‖	9 (13.4)**	2 (11.8)	2 (11.8)	2 (11.8)	3 (18.8)	0.894
Any abnormal pelvic exam findings, clinician-observed (n %)‖	2 (3.0)††	0	0	0	2 (12.5)	0.054
Any abnormal bimanual exam findings, clinician-observed (n %)‖	0	0	0	0	0	NA

	Total (*n* = 68)	Controls (*n* = 17)	Metro (*n* = 17)	EF+ (*n* = 17)	GynLP (*n* = 17)	p*
AEs – Structurally assessed between M2 and M6 (after product cessation)						
Any current urogenital symptoms (n %)‖	7 (10.8)‡‡	3 (17.7)	1 (5.9)	1 (6.3)	2 (13.3)	0.700
Any abnormal pelvic exam findings, clinician-observed (n %)‖	21 (32.8) §§	6 (35.3)	5 (31.3)	6 (37.5)	4 (26.7)	0.951
Any abnormal bimanual exam findings, clinician-observed (n %)‖	1 (1.6)¶¶	0	0	0	1 (6.7)	0.234
AEs – Not structurally assessed	**Total (N)**	**Controls (N)**	**Metro (N)**	**EF+ (N)**	**GynLP (N)**	**p***
Number of women with reported AEs‖‖	27	8	4	8	7	0.439
Total number reported AEs	41	13	6	9	13	0.324
Between Enr - M2	32	12	4	5	11	NA
Between M2 - M6***	9	1	2	4	2	NA
AEs – Not structurally assessed	**Total AEs (*n* = 41)**	**Controls (*n* = 13)**	**Metro (*n* = 6)**	**EF+ (*n* = 9)**	**GynLP (*n* = 13)**	**p***
Severity of reported AE (n %):						
- Mild	2 (4.9)	0	0	1 (11.1)	1 (7.7)	
- Moderate	36 (87.8)	13 (100)	5 (83.3)	6 (66.7)	12 (92.3)	NA
- Severe	3 (7.3)	0	1 (16.7)	2 (22.2)	0	
- Life-threatening	0	0	0	0	0	
Deemed related to study by physician (n %):						
- Definitely not related	10 (24.4)	3 (23.1)	2 (33.3)	3 (33.3)	2 (15.4)	NA
- Unlikely	31 (75.6)	10 (76.9)	4 (66.7)	6 (66.7)	11 (84.6)	
- Possible/probable/definitely related	0	0	0	0	0	
Outcome of reported AE (n %):						
- Fully recovered	40 (97.6)	12 (92.3)	6 (100)	9 (100)	13 (100)	
- Not fully recovered/deteriorated/ permanent damage/death	0	0	0	0	0	NA
- Ongoing	1 (2.4)	1 (7.7)†††	0	0	0	
Action taken by physician (n %):						
- None	6 (14.6)	1 (7.7)	0	2 (22.2)	3 (23.1)	
- Medication given	35 (85.4)	12 (92.3)	6 (100)	7 (77.8)	10 (76.9)	NA
- Study discontinuation	0	0	0	0	0	
- Hospitalisation‡‡‡	0	0	0	0	0	

AE adverse event, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M2/6* Month 2/6 visit, *Metro* metronidazole, *NA* not applicable, *Scr* screening visit.

*Fisher's exact test for binary outcomes and Kruskall Wallis test for continuous outcomes, between groups. †Most common symptom (89.8%) is genital itching. ‡No abnormal findings observed during bimanual exams. §Includes vaginal discharge (*n*=2) and cervical polyps (*n*=1). ¶Unusual cervical discharge. ‖Total numbers are slightly lower due to loss to follow-up (Fig. 1). No missing values. **Includes genital itching (*n*=8) and burning (*n*=3), pain during sex (*n*=4), and burning when urinating, lower abdominal pain, unusual vaginal discharge, and genital/anal sores (all *n*=1). ††One had ulcers/blisters in the vagina, one had lesions on the perineum and labia majora. ‡‡Includes genital itching (*n*=5) and burning (*n*=3), lower abdominal pain (*n*=4), pain during sex, burning when urinating, urinary frequency/urgency (all *n*=2), and unusual vaginal discharge (*n*=1). §§Includes unusual vaginal (*n*=4) and cervical (*n*=13) discharge, and cervicitis (*n*=10). ¶¶Cervical motion tenderness. ‖‖Most common AE according to MedDRA coding was "Infections and infestations" (*n*=14), followed by "Reproductive system and breast disorders" (*n*=9) and "Gastrointestinal disorders" (*n*=7). Sexually transmitted infections were not considered AEs but secondary outcomes. ***No AEs were reported after the M6 visit. †††Case of dental caries. ‡‡‡Both serious AEs involved hospitalisations that were not initiated by the study physician.

Preliminary efficacy: primary microscopy endpoints

In modified intent-to-treat (ITT) analyses that excluded women who had negative modified Amsel criteria but a Nugent score of 7-10 at baseline (n=17), the BV incidence rate by Nugent score during the intervention period was 10.18 per person-year at risk (PY) in the control group, and lower in the metronidazole (1.41/PY; p=0.004), EF+ (3.58/PY; p=0.043), and GynLP groups (5.36/PY; p=0.220) (table 3.3). Mean Nugent scores during the intervention period were highest in the control group, lowest in the metronidazole group, and in between in the two vaginal probiotics groups (figure 3.3a). By the end of the intervention period, many women had developed microbiological BV without symptoms. In line with standard practice, they were not treated, but they were also no longer included in the 'person-

years at risk' denominator because they had already developed the endpoint. BV incidence rates were therefore much lower (1.26/PY overall; table 3.3) after cessation of the intervention, and similar between groups. The results for BV incidence by modified Amsel criteria were similar to those for Nugent scores, and no vulvovaginal candidiasis was diagnosed after randomisation.

Table 3.3: Preliminary efficacy – primary endpoints (modified ITT analyses)

BV IR Enr – M2	**Controls**		**Metronidazole**		**EF+**		**GynLP**	
	n*/N	**IR (95% CI)†**	***n*/N***	**IR (95% CI)†**	***n*/N***	**IR (95% CI)†**	***n*/N***	**IR (95% CI)†**
Nugent 7-10	9/11	10.18 (5.48 – 18.92)	2/10	1.41 (0.35 – 5.62)	5/12	3.58 (1.61 – 7.96)	6/10	5.36 (2.41 – 11.93)
Modified Amsel	11/15	7.53 (4.28 – 13.26)	3/11	2.04 (0.66 – 6.31)	6/13	3.36 (1.51 – 7.48)	4/9	3.35 (1.26 – 8.92)
BV IR M2 – M6								
Nugent 7-10	4/11	0.91 (0.34 – 2.41)	5/8	1.86 (0.78 – 4.48)	6/9	1.58 (0.71 – 3.52)	2/7	0.76 (0.19 – 3.03)
Modified Amsel	3/12	2.96 (0.33 – 3.15)	5/10	2.25 (0.94 – 5.40)	6/8	3.84 (1.72 – 8.54)	3/8	1.83 (0.59 – 5.67)
BV IRR Enr – M2	**Controls**		**Metronidazole**		**EF+**		**GynLP**	
			IRR (95% CI)‡		**IRR (95% CI)‡**		**IRR (95% CI)‡**	
Nugent 7-10	NA		0.14 (0.01 – 0.65)		0.35 (0.10 – 1.07)		0.53 (0.16 – 1.60)	
Modified Amsel	NA		0.27 (0.05 – 1.00)		0.45 (0.14 – 1.28)		0.44 (0.10 – 1.47)	
BV IRR M2 – M6								
Nugent 7-10	NA		2.06 (0.44 – 10.37)		1.74 (0.41 – 8.41)		0.84 (0.08 – 5.85)	
Modified Amsel	NA		2.22 (0.43 – 14.27)		3.78 (0.81 – 23.35)		1.80 (0.24 – 13.46)	

BV bacterial vaginosis, CI confidence interval, EF+ Ecologic Femi+, Enr enrolment visit, GynLP Gynophilus LP, IR incidence rate IRR incidence rate ratio, *ITT* intent to treat *M2/6* Month 2/6 visit, *NA* not applicable.
There were no cases of vulvovaginal candidiasis during follow-up. Participants with a Nugent score of 7-10 at the enrolment visit were omitted from modified ITT analyses.
*Number of women (n) who developed at least one incident infection during the specified time period as a proportion of the women who had at least one follow-up visit in that time period (N). †Incident infections divided by person-years at risk. Three participants in the Enr – M2 BV by modified Amsel model, seven in the Enr – M2 BV by Nugent model, 10 in the M2 – M6 BV by modified Amsel model, and 11 in the M2 – M6 BV by Nugent model were omitted due to having been at risk for less than 10 person-days. ‡Compared to the control group.

Preliminary efficacy: secondary sequencing endpoints

We took the baseline VMB composition imbalances into account by not only showing bacterial group means at each visit but also mean differences with enrolment (table 3.4 and figure 3.3c-l for concentrations, and Appendix figure C.2 and table C.4 for relative abundances), and by using mixed effects models including participant identification number as the random effect to determine if any changes were statistically significant (table 3.5).

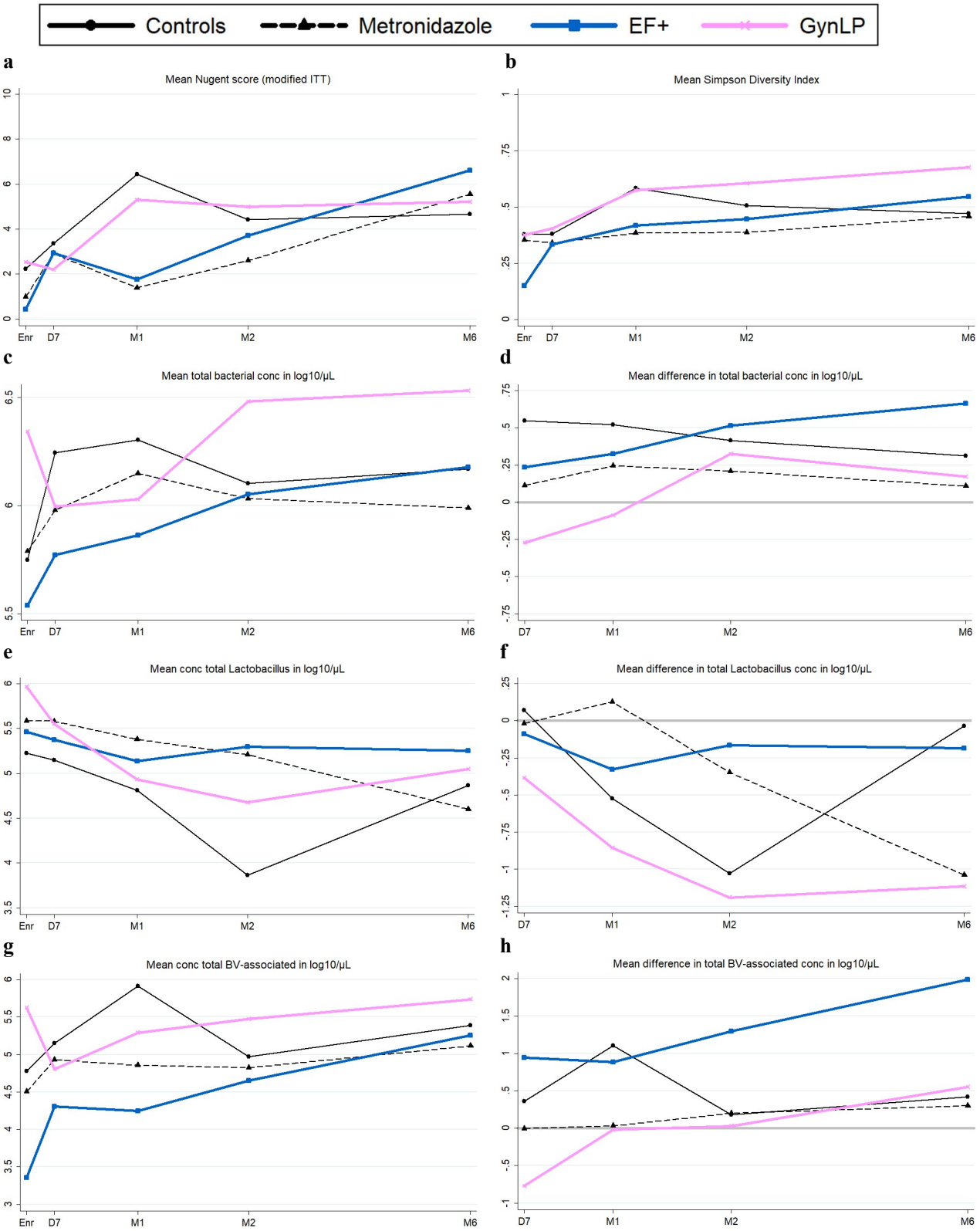
Table 3.4: Preliminary efficacy – bacterial group concentrations

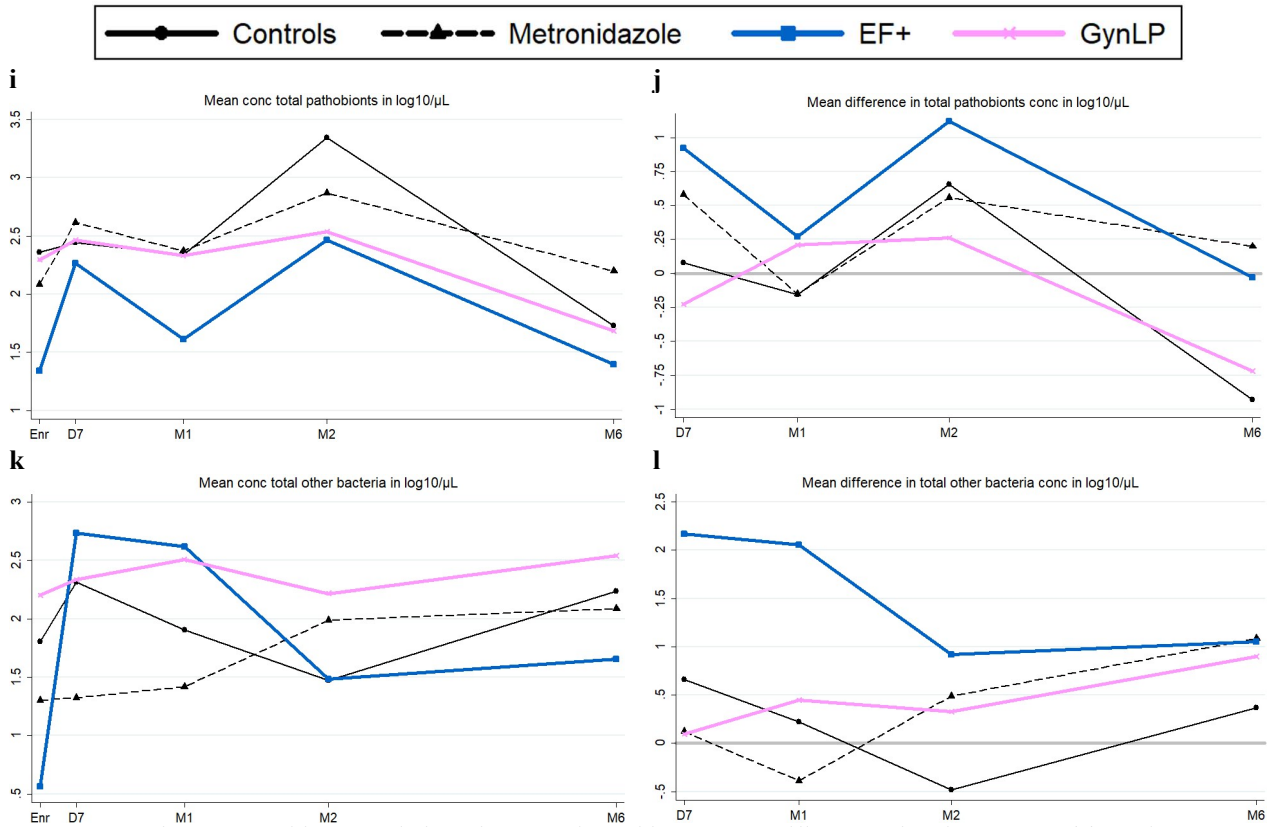
VMB outcome	Groups	Enr (*n* = 63)*	Products used			Ceased
			D7 (*n* = 62)*	M1 (*n* = 65)*	M2 (*n* = 63)*	M6 (*n* = 60)*
Total 16S rRNA conc in log_{10} cells/µL, mean (95% CI)	Control	6.29 (5.87 – 6.72)	6.80 (6.38 – 7.23)	6.76 (6.23 – 7.30)	6.63 (6.05 – 7.21)	6.65 (6.05 – 7.25)
	Metro	6.34 (5.95 – 6.73)	6.52 (6.16 – 6.87)	6.69 (6.25 – 7.12)	6.58 (6.15 – 7.01)	6.50 (5.95 – 7.05)
	EF+	6.12 (5.86 – 6.39)	6.35 (5.93 – 6.76)	6.42 (6.03 – 6.82)	6.58 (6.26 – 6.90)	6.65 (6.28 – 7.02)
	GynLP	6.86 (6.46 – 7.26)	6.54 (6.01 – 7.06)	6.55 (5.94 – 7.16)	6.97 (6.54 – 7.40)	7.02 (6.45 – 7.60)
Total bacterial conc in log_{10} cells/µL, mean (95% CI)	Control	5.75 (5.30 – 6.20)	6.24 (5.80 – 6.69)	6.30 (5.78 – 6.83)	6.10 (5.51 – 6.69)	6.18 (5.57 – 6.77)
	Metro	5.79 (5.39 – 6.19)	5.98 (5.63 – 6.33)	6.15 (5.70 – 6.60)	6.03 (5.59 – 6.47)	5.99 (5.34 – 6.58)
	EF+	5.54 (5.28 – 5.80)	5.77 (5.34 – 6.21)	5.86 (5.44 – 6.28)	6.05 (5.70 – 6.40)	6.18 (5.78 – 6.58)
	GynLP	6.34 (5.95 – 6.73)	6.00 (5.45 – 6.53)	6.03 (5.38 – 6.68)	6.48 (6.02 – 6.94)	6.53 (5.93 – 7.13)
Total *Lactobacillus* conc in log_{10} cells/µL, mean (95% CI)	Control	5.22 (4.63 – 5.82)	5.15 (4.25 – 6.05)	4.81 (3.94 – 5.68)	3.86 (2.53 – 5.19)	4.86 (3.82 – 5.91)
	Metro	5.59 (5.20 – 5.97)	5.58 (5.15 – 6.01)	5.38 (4.47 – 6.29)	5.21 (4.27 – 6.14)	4.60 (3.58 – 3.62)
	EF+	5.46 (5.19 – 5.73)	5.37 (4.96 – 5.79)	5.14 (4.40 – 5.87)	5.30 (4.97 – 5.63)	5.25 (4.80 – 5.70)
	GynLP	5.97 (5.44 – 6.49)	5.55 (4.92 – 6.18)	4.93 (4.43 – 5.43)	4.68 (3.55 – 5.81)	5.05 (4.45 – 5.65)
Total BV-associated conc in log_{10} cells/µL, mean (95% CI)	Control	4.78 (4.17 – 5.39)	5.15 (4.21 – 6.09)	5.92 (5.28 – 6.55)	4.97 (3.79 – 6.15)	5.39 (5.56 – 6.22)
	Metro	4.50 (3.77 – 5.24)	4.93 (4.23 – 5.63)	4.85 (3.81 – 5.90)	4.82 (4.11 – 5.54)	5.11 (4.16 – 6.07)
	EF+	3.36 (2.43 – 4.29)	4.31 (3.51 – 5.10)	4.25 (3.26 – 5.23)	4.65 (3.47 – 5.84)	5.26 (4.18 – 6.33)
	GynLP	5.62 (4.99 – 6.25)	4.81 (3.82 – 5.79)	5.29 (4.30 – 6.28)	5.48 (4.29 – 6.67)	5.74 (4.38 – 7.09)
Total pathobionts conc in log_{10} cells/µL, mean (95% CI)	Control	2.36 (1.28 – 3.45)	2.44 (1.08 – 3.81)	2.34 (1.13 – 3.54)	3.35 (2.21 – 4.48)	1.73 (0.69 – 2.77)
	Metro	2.09 (1.00 – 3.17)	2.62 (1.32 – 3.91)	2.37 (1.18 – 3.56)	2.87 (1.79 – 3.95)	2.20 (0.99 – 3.40)
	EF+	1.34 (0.29 – 2.39)	2.26 (1.18 – 3.35)	1.61 (0.51 – 2.72)	2.46 (1.39 – 3.54)	1.40 (0.50 – 2.30)
	GynLP	2.30 (0.98 – 3.62)	2.46 (1.30 – 3.63)	2.33 (0.99 – 3.68)	2.54 (1.24 – 3.93)	1.68 (0.25 – 3.12)
Total other bacteria conc in log_{10} cells/µL, mean (95% CI)	Control	1.80 (0.84 – 2.76)	2.31 (1.34 – 3.29)	1.91 (0.82 – 2.99)	1.47 (0.49 – 2.46)	2.24 (1.21 – 3.26)
	Metro	1.30 (0.36 – 2.24)	1.32 (0.35 – 2.30)	1.42 (0.23 – 2.61)	1.99 (1.08 – 2.90)	2.09 (1.04 – 3.13)
	EF+	0.57 (-0.10 – 1.24)	2.73 (1.94 – 3.53)	2.62 (1.56 – 3.68)	1.48 (0.51 – 2.46)	1.66 (0.56 – 2.75)
	GynLP	2.20 (1.07 – 3.34)	2.34 (1.04 – 3.63)	2.51 (1.26 – 3.75)	2.22 (1.09 – 3.34)	2.54 (1.17 – 3.91)
Total EF+ strains conc in log_{10} cells/µL, mean (95% CI)	Control	0.17† (-0.19 – 0.53)	0.41† (-0.19 – 1.00)	0.12† (-0.14 – 0.38)	0.16† (-0.19 – 0.51)	0.24† (-0.27 – 0.74)
	Metro	0.21† (-0.11 – 0.53)	0.39† (-0.18 – 0.97)	0.16† (-0.18 – 0.49)	0.25† (-0.28 – 0.77)	0.31† (-0.35 – 0.97)
	EF+	0.30† (-0.14 – 0.74)	1.92 (0.92 – 2.91)	1.51 (0.41 – 2.62)	0.48 (-0.22 – 1.18)	0 (0 – 0)

	GynLP	0.45† (-0.21 – 1.10)	0.79† (-0.18 – 1.76)	0.64† (-0.13 – 1.40)	0.26† (-0.30 – 0.82)	0 (0 – 0)
VMB Outcome	**Groups**	**Enr**	**D7**	**M1**	**M2**	**M6**
Total GynLP strain conc in log_{10} cells/µL, mean (95% CI)	Control	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	Metro	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	EF+	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	GynLP	0 (0 – 0)	1.05 (-0.22 – 2.31)	0.88 (-0.18 – 1.94)	0.25 (-0.28 – 0.78)	0 (0 – 0)
Difference in total bacterial conc in log_{10} cells/µL – compared to Enr visit, mean (95% CI)	Control	0	0.55 (-0.02 – 1.11)	0.52 (-0.02 – 1.06)	0.41 (-0.11 – 0.94)	0.31 (-0.35 – 0.97)
	Metro	0	0.11 (-0.12 – 0.35)	0.24 (-0.39 – 0.88)	0.21 (-0.21 – 0.62)	0.11 (-0.57 – 0.79)
	EF+	0	0.23 (-0.09 – 0.55)	0.32 (-0.08 – 0.73)	0.51 (0.06 – 0.97)	0.66 (0.26 – 1.07)
	GynLP	0	-0.27 (-0.72 – 0.18)	-0.09 (-0.65 – 0.48)	0.32 (0.01 – 0.63)	0.17 (-0.31 – 0.65)
Difference in total *Lactobacillus* conc in log_{10} cells/µL – compared to Enr visit, mean (95% CI)	Control	0	0.07 (-0.52 – 0.66)	-0.52 (-1.21 – 0.17)	-1.03 (-2.14 – 0.09)	-0.04 (-0.72 – 0.64)
	Metro	0	-0.02 (-0.44 – 0.40)	0.13 (-0.62 – 0.88)	-0.35 (-1.46 – 0.77)	-1.03 (-2.42 – 0.34)
	EF+	0	-0.09 (-0.58 – 0.41)	-0.33 (-1.15 – 0.50)	-0.16 (-0.58 – 0.25)	-0.18 (-0.73 – 0.36)
	GynLP	0	-0.38 (-1.12 – 0.35)	-0.86 (-1.16 – -0.04)	-1.19 (-2.52 – 0.15)	-1.11 (-1.78 – -0.45)
Difference in total BV-associated conc in log_{10} cells/µL – compared to Enr visit, mean (95% CI)	Control	0	0.36 (-0.71 – 1.42)	1.10 (0.17 – 2.04)	0.18 (-0.96 – 1.32)	0.42 (-0.62 – 1.47)
	Metro	0	0 (-1.04 – 1.03)	0.03 (-1.04 – 1.11)	0.20 (-0.56 – 0.95)	0.30 (-1.09 – 1.69)
	EF+	0	0.95 (-0.04 – 1.93)	0.89 (-0.05 – 1.82)	1.30 (0.15 – 2.44)	1.98 (0.64 – 3.33)
	GynLP	0	-0.77 (-1.59 – 0.04)	-0.02 (-0.70 – 0.65)	0.03 (-1.02 – 1.08)	0.55 (-0.21 – 1.31)
Difference in total pathobionts conc in log_{10} cells/µL – compared to Enr visit, mean (95% CI)	Control	0	0.07 (-1.87 – 2.03)	-0.16 (-1.46 – 1.15)	0.66 (-0.34 – 1.66)	-0.93 (-2.32 – 0.46)
	Metro	0	0.58 (-1.18 – 2.34)	-0.15 (-1.63 – 1.33)	0.56 (-0.90 – 2.01)	0.20 (-1.20 – 1.60)
	EF+	0	0.92 (-0.29 – 2.13)	0.27 (-0.54 – 1.80)	1.12 (-0.03 – 2.27)	-0.03 (-1.30 – 1.24)
	GynLP	0	-0.23 (-1.59 – 1.13)	0.21 (-1.38 – 1.80)	0.26 (-1.58 – 2.10)	-0.72 (-2.99 – 1.54)
Difference in total other bacteria conc in log_{10} cells/µL – compared to Enr visit, mean (95% CI)	Control	0	0.66 (-0.62 – 1.94)	0.22 (-1.09 – 1.54)	-0.48 (-1.88 – 0.92)	0.37 (-0.86 – 1.60)
	Metro	0	0.12 (-1.87 – 2.11)	-0.39 (-1.73 – 0.96)	0.49 (-0.84 – 1.81)	1.09 (-0.29 – 2.47)
	EF+	0	2.17 (1.27 – 3.06)	2.05 (1.00 – 3.11)	0.92 (-0.10 – 1.94)	1.05 (-0.22 – 2.33)
	GynLP	0	0.09 (-1.35 – 1.54)	0.45 (-0.77 – 1.67)	0.33 (-1.30 – 1.95)	0.90 (-0.39 – 2.18)

BV bacterial vaginosis, *CI* confidence interval, *Conc* concentration, *D7* Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M1/2/6* Month 1/2/6 visit, *Metro* metronidazole group, *VMB* vaginal microbiota.
*Total numbers are slightly lower than enrolled women (and not lost to follow-up) per time point due to invalid results. Numbers missing per group is at most two at Enr, D7, M1, or M2 visits, and four at the M6 visit. †These are naturally occurring EF+ strains with 100% identity with the EF+ probiotic strains.

Figure 3.3: Preliminary efficacy by Nugent score, alpha diversity, and bacterial group concentrations





Conc concentration, *D7* Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *ITT* intention to treat, *M1/2/6* Month 1/2/6 visit, *Scr* screening visit, *VMB* vaginal microbiota.

Changes in VMB outcomes over time per randomisation group. See table 3.4 for 95% confidence intervals.

a Mean Nugent scores over time, only including women (n=51) with Nugent scores 0-6 at enrolment (modified ITT analysis). **b** Mean alpha diversity over time. **c** Mean bacterial cell concentration over time. **d** Difference in mean bacterial cell concentration with enrolment, over time. **e** Mean lactobacilli concentration over time. **f** Difference in mean lactobacilli concentration with enrolment, over time. **g** Mean BV-associated anaerobes concentration over time. **h** Difference in mean BV-associated anaerobes concentration with enrolment, over time. **i** Mean pathobionts concentration over time. **j** Difference in mean pathobionts concentration with enrolment, over time. **k** Mean other bacteria concentration over time. **l** Difference in mean other bacteria concentration with enrolment, over time.

Immediately after BV treatment completion, the VMBs of most women gradually worsened (lactobacilli declined and BV-anaerobes expanded) due to the high-risk nature of the cohort. The mean lactobacilli concentration declined to a low of 3.86 $\log_{10}$ cells/µl at M2 in the control group, but less so in the intervention groups (ranging from 4.60-5.58 $\log_{10}$ cells/µl at follow-up visits). In unadjusted mixed effects models using data from the intervention period only (table 3.5), metronidazole users had a higher lactobacilli concentration (p=0.043) and relative abundance (p=0.006) than controls, EF+ users had a higher relative abundance (p=0.014) but not concentration than controls, and GynLP users had trends in the same directions that were not statistically significant. The expansion of BV-anaerobes was significantly lower in oral metronidazole users (relative abundance; p=0.023), and in EF+ users (concentration; p=0.041), compared to controls. Mean pathobionts concentrations were low in all groups throughout, ranging from 1.61-3.35 $\log_{10}$ cells/µl at follow-up visits. Mixed effects models did not identify any significant associations between randomisation groups and pathobionts concentrations or relative abundances, but showed trends ($0.05<p<0.1$) towards lower pathobionts relative abundances in the two vaginal probiotics groups compared to controls (table 3.5). Mean concentrations of 'other

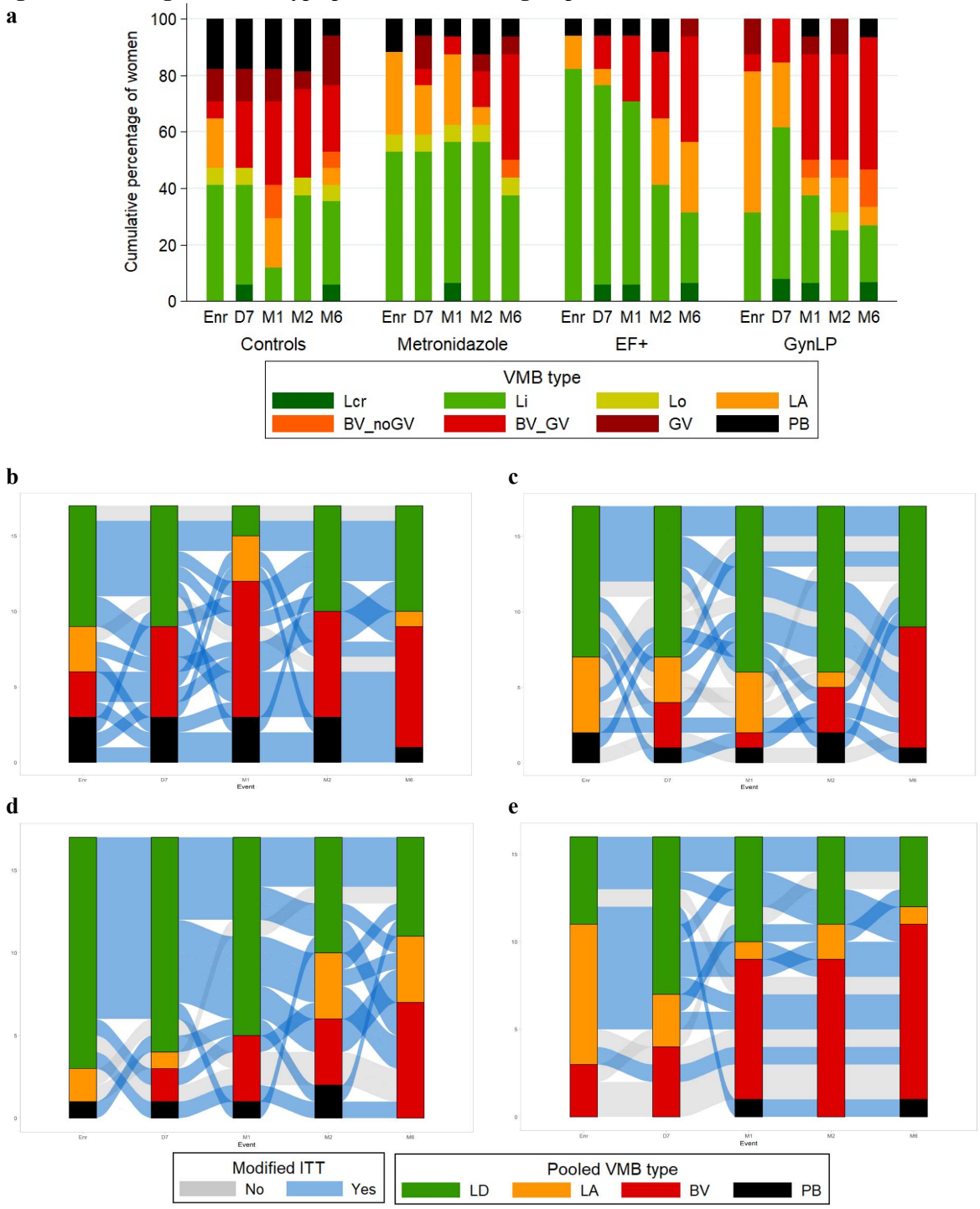
bacteria' were also low throughout, but highest in the EF+ group at the D7 and M1 visits (EF+ contains a *Bifidobacterium* strain). This was significant in unadjusted mixed effects models for relative abundances (p=0.023) but not concentrations. The proportions of women in each group at each visit having a particular VMB type corresponded with the concentration and relative abundance data, and additionally showed that – in lactobacilli-dominated women – the Li VMB type was far more common than the Lcr and Lo VMB types throughout (figure 3.4). Among women with dysbiosis, the LA and BV_GV VMB types continued to be the most common dysbiosis types during follow-up. In unadjusted mixed effects models using intervention period data only, metronidazole users and EF+ users, each compared to controls, were significantly less likely to have dysbiotic VMB types (BV_GV, BV_noGV, and GV combined; p=0.012 and p=0.029, respectively) (table 3.5).

Table 3.5: Preliminary efficacy – mixed effects models

Unadjusted mixed effects models	**Metro**		**EF+**		**GynLP**	
	OR (95% CI)*	**p***	**OR (95% CI)***	**p***	**OR (95% CI)***	**p***
Nugent score†	0.16 (0.02 – 1.09)	0.062	0.25 (0.04 – 1.66)	0.151	1.68 (0.24 – 11.56)	0.599
Nugent score categories†						
- 4-6 vs 0-3	1.04 (0.20 – 5.50)	0.960	0.36 (0.05 – 2.43)	0.293	2.16 (0.38 – 12.31)	0.387
- 7-10 vs 0-3	0.08 (0.01 – 0.80)	0.032	2.16 (0.38 – 12.31)	0.148	1.12 (0.15 – 8.28)	0.913
Total bacterial conc†	0.85 (0.53 – 1.36)	0.497	0.72 (0.45 – 1.14)	0.160	0.96 (0.60 – 1.55)	0.877
Total *Lactobacillus* conc†	2.14 (1.02 – 4.49)	0.043	1.86 (0.90 – 3.86)	0.095	1.43 (0.67 – 3.04)	0.352
Total BV-associated conc†	0.58 (0.23 – 1.49)	0.260	0.38 (0.15 – 0.96)	0.041	0.89 (0.34 – 2.31)	0.812
Total pathobionts conc†	0.91 (0.30 – 2.75)	0.865	0.57 (0.19 – 1.71)	0.318	0.79 (0.25 – 2.43)	0.676
Total other bacteria conc†	0.72 (0.27 – 1.90)	0.507	1.42 (0.55 – 3.70)	0.471	1.60 (0.60 – 4.29)	0.349
Total *Lactobacillus* RA‡	1.36 (1.09 – 1.70)	0.006	1.32 (1.06 – 1.64)	0.014	1.10 (0.88 – 1.38)	0.408
Total BV-associated RA‡	0.79 (0.65 – 0.97)	0.023	0.83 (0.68 – 1.01)	0.067	1.00 (0.81 – 1.22)	0.973
Total pathobionts RA‡	0.93 (0.84 – 1.03)	0.159	0.92 (0.83 – 1.01)	0.093	0.92 (0.83 – 1.02)	0.100
Total other bacteria RA‡	1.00 (1.00– 1.01)	0.739	1.01 (1.00 – 1.01)	0.023	1.00 (1.00– 1.01)	0.671
Pooled VMB type‡						
- LA vs LD	1.17 (0.24 – 5.78)	0.849	1.20 (0.24 – 5.92)	0.823	1.72 (0.33 – 9.04)	0.520
- BV vs LD	0.02 (0.00 – 0.43)	0.012	0.04 (0.00 – 0.73)	0.029	0.39 (0.03 – 5.50)	0.487
- PB vs LD	0.06 (0.00 – 1.54)	0.090	0.08 (0.00 – 1.77)	0.109	0.14 (0.01 – 3.34)	0.225
Simpson diversity index‡	0.90 (0.77 – 1.06)	0.200	0.94 (0.80 – 1.10)	0.454	1.07 (0.91 – 1.26)	0.429
Adjusted mixed effects models§						
Nugent score†	0.19 (0.03 – 1.31)	0.092	0.26 (0.04 – 1.85)	0.178	2.05 (0.30 – 13.92)	0.464
Nugent score categories†						
- 4-6 vs 0-3	1.24 (0.20 – 7.82)	0.821	0.36 (0.04 – 3.13)	0.353	2.42 (0.36 – 16.26)	0.362
- 7-10 vs 0-3	0.06 (0.00 – 0.77)	0.031	0.19 (0.02 – 1.90)	0.156	1.42 (0.17 – 11.81)	0.747
Total bacterial conc†	0.75 (0.47 – 1.18)	0.208	0.64 (0.40 – 1.02)	0.061	0.90 (0.57 – 1.43)	0.666
Total *Lactobacillus* conc†	1.74 (0.83 – 3.67)	0.142	1.47 (0.69 – 3.16)	0.319	1.26 (0.60 – 2.68)	0.541
Total BV-associated conc†	0.57 (0.22 – 1.47)	0.243	0.37 (0.14 – 0.98)	0.046	0.91 (0.35 – 2.36)	0.848
Total pathobionts conc†	0.99 (0.35 – 2.77)	0.980	0.66 (0.23 – 1.81)	0.445	0.98 (0.36 – 2.90)	0.965
Total other bacteria conc†	0.65 (0.25 – 1.69)	0.374	1.31 (0.49 – 3.50)	0.591	1.79 (0.68 – 4.71)	0.239
Total *Lactobacillus* RA‡	1.32 (1.06 – 1.65)	0.014	1.30 (1.03 – 1.64)	0.025	1.06 (0.85 – 1.33)	0.601
Total BV-associated RA‡	0.81 (0.66 – 1.00)	0.049	0.83 (0.67 – 1.03)	0.098	1.02 (0.83 – 1.26)	0.855
Total pathobionts RA‡	0.93 (0.84 – 1.03)	0.180	0.92 (0.83 – 1.02)	0.120	0.93 (0.84 – 1.03)	0.147
Total other bacteria RA‡	1.00 (1.00 – 1.01)	0.436	1.01 (1.00 – 1.01)	0.009	1.00 (1.00 – 1.01)	0.439
Pooled VMB type‡						
- LA vs LD	1.46 (0.26 – 8.11)	0.665	1.59 (0.27 – 9.55)	0.610	2.11 (0.37 – 12.15)	0.401
- BV vs LD	0.02 (0.00 – 0.48)	0.017	0.02 (0.00 – 0.64)	0.027	0.44 (0.02 – 7.84)	0.575
- PB vs LD	0.08 (0.00 – 1.49)	0.090	0.11 (0.01 – 2.02)	0.136	0.21 (0.01 – 3.71)	0.284
Simpson diversity index‡	0.93 (0.78 – 1.09)	0.359	0.96 (0.81 – 1.13)	0.597	1.10 (0.93 – 1.30)	0.272

BV bacterial vaginosis, *CI* confidence interval, *conc* concentration, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *LA* lactobacilli and anaerobes, *LD Lactobacillus*-dominated, *Metro* metronidazole group, *OR* odds ratio, *PB* pathobionts, *VMB* vaginal microbiota.
*Compared to the control group. †Including all valid samples during product use (D7, M1, and M2 visits). Self-sampled samples were also taken during product use, but were not Gram stained nor tested by 16S rRNA gene qPCR (see Methods). ‡Including all valid samples during product use (D7, M1, and M2 visits, and self-sampled samples). §Model adjusted for hormonal/pregnancy status, sexual risk taking, and age (see Materials and Methods).

Figure 3.4: Changes in VMB type per randomisation group over time



BV bacterial vaginosis-like, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *D7* Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GV G. vaginalis*-dominated, *GynLP* Gynophilus LP, *ITT* intent-to-treat, *LA* lactobacilli and anaerobes, *Lcr Lactobacillus crispatus*-dominated, *LD Lactobacillus*-dominated, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *M1/M2/M6* month 1/2/6 visit, *PB* pathobionts-containing, *VMB* vaginal microbiota.

a Changes in VMB type membership per randomisation group over time. **b-e** Alluvial diagrams of changes in pooled VMB type over time per group: control group (**b**), metronidazole group (**c**), EF+ group (**d**), and GynLP group (**e**). Missing pooled VMB types at D7, M1, M2, and M6 were imputed with the types at the preceding visit, and at enrolment with the type at D7. Alluvial diagrams also show whether the participant is in the modified ITT population (Nugent score 0-6 at enrolment) or not.

The associations in unadjusted mixed effects models persisted after adjustment for hormonal contraception use/pregnancy, sexual risk taking, and age, except for the association with *Lactobacillus* concentration among metronidazole users. Metronidazole users compared to controls had a significantly higher *Lactobacillus* relative abundance (p=0.014), a significantly lower BV-associated bacteria relative abundance (p=0.049), and were significantly less likely to have BV by Nugent scoring (p=0.031) or by VMB types (BV_GV, BV_noGV, and GV combined; p=0.017) (table 3.5). EF+ users compared to controls had a significantly higher *Lactobacillus* relative abundance (p=0.025), a significantly lower BV-associated bacteria concentration (p=0.046), a significantly higher relative abundance of 'other bacteria' (p=0.009), and were significantly less likely to have a BV-like VMB type (p=0.027).

Detection of probiotic strains

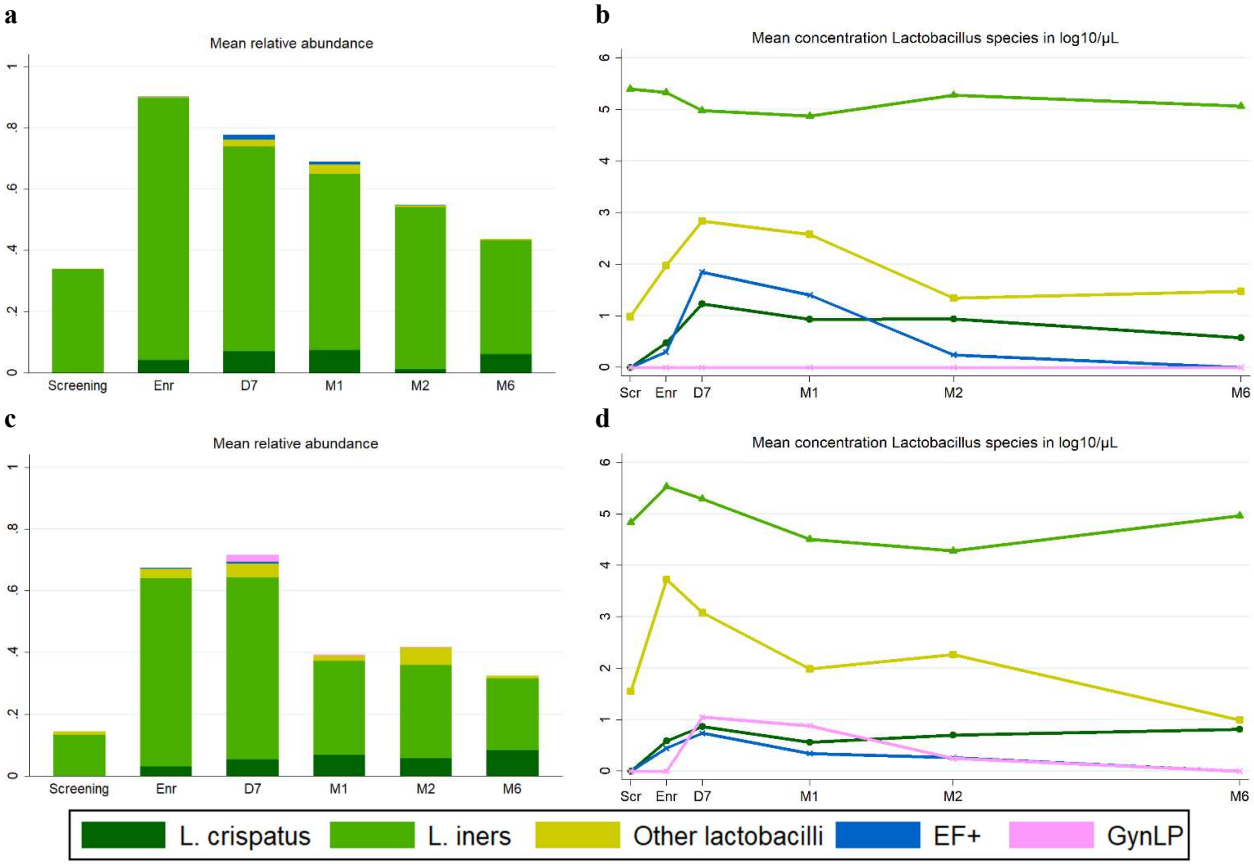
During the intervention period, relevant probiotic strains were detected in 39% of samples from EF+ users and 20% of samples from GynLP users (all swabs combined, including self-sampled swabs). The detection percentages were 58% and 31%, respectively, in sensitivity analyses using non-rarefied sequencing data. Some of the EF+ strains cannot be differentiated from naturally occurring strains, and EF+-like strains were therefore detected (at low levels) in all groups at most time points (table 3.4, figure 3.5). However, the mean concentrations were highest in the EF+ group during the intervention period (mean concentrations 0.48-1.92 $\log_{10}$ cells/µl per visit for all women combined, and 3.62-4.28 $\log_{10}$ cells/µl per visit for women who did have EF+ strains detected using rarefied data). The GynLP strain was only detected in the GynLP group during the intervention period (mean concentrations 0.25-1.05 $\log_{10}$ cells/µl per visit for all women combined, and 3.72-4.55 $\log_{10}$ cells/µl per visit for women who did have GynLP detected using rarefied data). Inter- and intra-individual differences between participants were high: the highest vaginal probiotic concentration detected in an individual EF+ user was 5.51 $\log_{10}$ cells/µl, and in an individual GynLP user was 6.17 $\log_{10}$ cells/µl. During the intervention period, the mean relative abundances of the probiotic strains were 0.03 in both EF+ and GynLP users, and 0.08 and 0.15, respectively, if only samples in which any strains were detected were included.

VMB transitions

The stacked graph and alluvial diagrams (figure 3.4) show that transitions from one VMB type to another were common. As expected, most transitions during the intervention period were from *Lactobacillus*-dominated states to dysbiotic states, but the reverse also occurred. Transitions between VMB types were common in all randomisation groups. The percentage of actual transitions divided by potential transitions between D7 and M6 were 29/66 (43.9%) in the control group, 32/64 (50.0%) in the

metronidazole group, 28/67 (41.8%) in the EF+ group and 24/56 (42.9%) in the GynLP group (Fisher's exact p=0.792).

Figure 3.5: Detection of probiotic strains during the trial



D7 Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M1/2/6* Month 1/2/6 visit, *Scr* screening visit.
a,b Mean relative abundance (**a**) and mean concentration (**b**) of *Lactobacillus* species over time in the EF+ group. **c,d** Mean relative abundance (**c**) and mean concentration (**d**) of *Lactobacillus* species over time in the GynLP group. The length of bars in (**a**) and (**c**) depicts total relative abundance of all *Lactobacillus* species combined.

Incidence of sexually transmitted and urinary tract infections

As expected, the incidences of HIV (n=2), herpes simplex type 2 (n=1), syphilis (n=1), gonorrhoea (n=5), chlamydia (n=6), TV (n=5) and urinary tract infection (n=7) were too low to determine differences between randomisation groups (Appendix table C.6).

3.4 Discussion

This trial showed that all three interventions were safe. Our preliminary efficacy results confirm that intermittent use of metronidazole reduces BV recurrence,[132–134] and suggest that intermittent use of lactobacilli-containing vaginal probiotics may also reduce BV recurrence. We also found that vaginal probiotic use is acceptable and feasible in African settings (to be reported elsewhere). These findings are important because many women and clinicians would prefer safe and efficacious vaginal probiotics

to metronidazole as they are not expected to negatively affect other body niche microbiotas or cause antimicrobial resistance, and may have fewer side effects.

Our trial was funded as a pilot study and therefore had a modest sample size. Despite this, many of the preliminary efficacy associations for intermittent metronidazole and EF+ use reached statistical significance. While this was not the case for intermittent GynLP use, all of the trends in this group were in the same directions. We believe that the GynLP group suffered a few disadvantages compared to the other randomisation groups, which may explain the lack of statistical significance. Randomisation imbalances commonly occur in small trials,[149] and in our trial, this led to women in the GynLP group being more dysbiotic at baseline than controls. We ameliorated this disadvantage in our analysis strategy, but we may not have been able to eradicate it. In addition, GynLP users were less adherent on average than metronidazole and EF+ users. Differences in adherence using triangulated data did not reach statistical significance, but probiotic strain detection rates (58% for EF+ samples and 31% for GynLP samples using non-rarefied data) support this claim. Biose has since simplified the dosing regimen of next generation Gynophilus products to two fixed days a week to boost adherence. Finally, GynLP dosing (once every four days) was similar to metronidazole dosing (twice weekly) but less frequent than EF+ dosing (once per day for the first five days followed by thrice weekly for the remainder of the intervention period).

Probiotic detection rates were 58% for EF+ samples and 31% for GynLP samples, and inter- and intra-individual variabilities were high (with probiotic concentrations ranging from zero to 6.17 $\log_{10}$ cells/µl). This detection variability is consistent with most other vaginal probiotic studies that used sampling at non-daily intervals and molecular assessment methods.[76,142,150,151] A major drawback of all of those studies, including ours, is that product use was not directly observed but self-reported, and precise information about the time period between last product insertion and sample collection was lacking. The average total bacterial load of a healthy vagina is currently not known.[152] The average vaginal surface area was estimated to be 87.5 cm^2.[153] One Dacron swab head in this study absorbed about 10^6/µL bacteria. If we assume that one swab head absorbs on average 200 µL,[154] and that this covers about 1 cm^2 of a total of about 100 cm^2 vaginal surface, the total bacterial load in the vagina would be in the order of $2x10^{10}$ bacteria. The vaginal probiotic strain(s) in our trial were both applied at about $1.5x10^9$ CFU per dose, which would be about 7.5% of the total vaginal load after application if all probiotic bacteria were to remain in the vagina. We detected mean relative abundances of 7.7% for EF+ strains and 15.1% for GynLP when only samples with any relevant probiotic strains detected during product use were included (thereby eliminating any potential non-adherence). A recently published study of Gynophilus Slow Release tablet (which is almost identical to GynLP) in which women self-sampled every day showed that mean vaginal concentrations of Lcr35 by qPCR were between 10^4 and 10^6 CFU/µl in women who used the tablet once every four or five days.[155] Using our estimated

concentration data, we detected a similar concentration range in samples with any GynLP detected. This consistency is reassuring, but the question remains whether probiotic concentrations of this order of magnitude optimally prevent BV recurrence in the long-term. Furthermore, all studies referenced in this paragraph, including ours, have shown that probiotic strains do not persist in the vagina after dosing has ceased. The second question then is whether the colonisation capacity of probiotic bacteria should be improved. Our data suggest that probiotic lactobacilli may boost 'natural' lactobacilli indirectly, which may be sufficient to establish vaginal eubiosis. Indirect effects may include increased localised lactic acid production, modulation of cervicovaginal mucosal immune responses, and/or inhibition of biofilm formation, by probiotic bacteria.[12,74,6]

Additional limitations of our study include the high urogenital infection risk of this cohort (which makes prevention more challenging), and our inability to fully control for potential confounders. However, the mixed effects models were controlled for some of the best known VMB determinants (hormonal contraception, pregnancy, sexual risk taking, and age).[4,27,127,156] We were not able to exclude women with chlamydia and/or gonorrhoea infection at the time of randomisation due to the slow laboratory turn-around time, but the VMB compositions of women with and without infection were similar, and we therefore think that this did not negatively affect our results.

With the development of better genomic and culturing methods, we are now on the cusp of a new era in vaginal probiotic research. Past vaginal probiotic studies have shown mixed results,[72,135–145] but almost all of these studies used imprecise VMB assessments based on clinical symptoms and microscopy. The addition of sequencing methods showed that many more women than previously thought are not lactobacilli-dominated after standard antibiotic BV treatment, that host responses to antibiotic and probiotic treatment are highly variable, and that it is possible to differentiate between probiotic strains and 'natural' lactobacilli. Furthermore, others have shown that quantifying relative abundance data in the same manner as we have done in this study correlates well with species-specific quantitative PCRs of non-minority species.[129,130] This then allows for microbiota data reduction into quantitative variables that can be analysed in mixed effects models that adjust for repeated measures and confounding. We recommend that future trials incorporate these or other rigorous methods, optimise dosing and timing of product insertion versus sample collection, and enrol women with various urogenital risk profiles. Ideally, these trials would also evaluate the effects of interventions on vaginal biofilm formation, and – eventually – the impact on pregnancy complications, HIV epidemics, and other adverse outcomes.

Chapter 4 - Vaginal Probiotic Adherence and Acceptability in High-Risk Rwandan Women Participating in a Pilot Randomised Controlled Trial: A Mixed-Methods Approach

This chapter has been submitted to an international peer-reviewed journal, has been reviewed by three reviewers of the journal, but has not yet been accepted for publication: Verwijs MC, Agaba SK, Umulisa MM, Uwineza M, Nivoliez A, Lievens E, van de Wijgert JHHM. Vaginal probiotic adherence and acceptability in high-risk Rwandan women participating in a pilot randomised controlled trial: a mixed-methods approach. 2019; (*submitted for publication*). The version presented here is the author-approved second submission with only minor modifications (numbering of figures, tables, and references).

The data described in this chapter were also collected within the Rwanda VMB trial. I reviewed the survey and social science data that had been collected by the Rinda Ubuzima team, and conducted the site close-out visit, in Kigali, Rwanda, under the supervision of Professor Janneke van de Wijgert (my primary supervisor). I developed the analytical approach, performed the statistical analyses, wrote the first draft of the manuscript, and coordinated the submission process and responses to reviewers. All authors commented on and approved the final manuscript.

Abstract

Introduction: Bacterial vaginosis (BV) recurrence is common. We evaluated the adherence and acceptability of intermittent use of two vaginal probiotics and one antibiotic to prevent recurrence.

Materials and methods: We performed repeated adherence and acceptability assessments using mixed methods within a pilot randomised controlled trial, conducted at a research clinic in Kigali, Rwanda. We included high-risk Rwandan women (n=68) with BV and/or trichomoniasis. Women were randomised to four groups (n=17 each) after completing metronidazole treatment: behavioural counselling only, or behavioural counselling plus two-month intermittent use of oral metronidazole, Ecologic Femi+ (EF+) vaginal capsule, or Gynophilus LP (GynLP) vaginal tablet. Adherence and acceptability data from randomised women were collected in structured face-to-face interviews, semi-structured focus group discussions and in-depth interviews, daily diaries, and counting of used/unused study products. Randomised women and women attending recruitment sessions (n=131) were surveyed about vaginal infection knowledge.

Results: Most women (93%) were sex workers. At baseline, they were unfamiliar with BV, and had never used probiotics. All probiotic users reported that insertion became easier over time. Triangulated adherence data showed that 100% of EF+ users and 81.3% of GynLP users used ≥80% of required doses. Younger age, asking many questions at enrolment, having menses, and reporting urogenital symptoms showed non-significant trends towards a lower perfect adherence likelihood. Qualitative data suggested that women believed that the probiotics reduced BV recurrence, but that partners were sometimes unsupportive of study participation. Self-reported vaginal washing practices decreased during follow-up, but sexual risk behaviours did not. Most women (80%) with an uncircumcised steady partner discussed penile hygiene with him, but many women found this difficult, especially with male clients.

Discussion: High-risk women require education about vaginal infections. Vaginal probiotic acceptability and adherence were high in this cohort. Our results can be used to inform future product development and to fine-tune counselling messages in prevention programs.

4.1 Introduction

Bacterial vaginosis (BV) is a vaginal condition in which fastidious anaerobes such as *Gardnerella vaginalis* increase while beneficial, lactic acid-producing lactobacilli decrease.[4] Often asymptomatic, it is associated with increased risks of sexually transmitted infections (STIs) and HIV transmission, pelvic inflammatory disease, and adverse pregnancy outcomes.[9,18,20,157] Although BV is treatable with antibiotics, the risk of recurrence is high.[46,52] The prevalence of BV varies among regions and ethnic groups but is highest in sub-Saharan Africa, where it is estimated at 30-50%.[37]

Vaginally-administered probiotics containing lactobacilli are considered a promising new strategy to restore a lactobacilli-dominated vaginal microbiota during and/or after antibiotic treatment, or to prevent BV.[47] While some probiotics have been available on the market for several years, clinical trials to support beneficial effects have only recently been initiated for most products.[128,137,139,141] Acceptability is an important component of these trials, to maximise future uptake and adherence of vaginal probiotics should they be proven efficacious. The acceptability of a novel vaginal product depends on factors such as the characteristics of the population studied, characteristics of and experiences with the product, types of sexual relationships and partner support, and community perceptions.[158,159]

We conducted a clinical trial of intermittent use of two vaginal probiotics and oral metronidazole to prevent BV recurrence in Rwandan women who had been treated for BV and/or *Trichomonas vaginalis* (TV). We used qualitative and quantitative research methods to assess adherence and acceptability with vaginal probiotic use. We triangulated various sources of adherence data to obtain adherence estimates per woman for each period of intermittent product use in between study visits, and determined correlates of adherence.

4.2 Materials and Methods

The pilot clinical trial took place from June 2015 to February 2016 at the Rinda Ubuzima research clinic in Kigali, Rwanda. Women who had been successfully treated for BV/TV with a seven-day course of oral metronidazole (Tricozole, Laboratory & Allied Ltd, Nairobi, Kenya) were randomised to four intervention groups (n=17 each) to prevent BV recurrence: behavioural counselling only (controls), or behavioural counselling plus intermittent use of two different vaginal probiotics or oral metronidazole for two months. Women were seen at screening, enrolment (product use initiation, if applicable), Day 7, Month 1, Month 2 (product use cessation, if applicable), and Month 6. Product efficacies were not known during the trial, and preliminary efficacy results are reported elsewhere.[160] The behavioural counselling focussed on safer sex practices, cessation of vaginal practices, and increasing male penile hygiene to prevent BV.[161]

Study population

Women aged 18-45 at risk of HIV/STIs (defined as having had more than one sex partner and/or having been treated for an STI and/or BV in the last 12 months) were eligible for enrolment if they were confirmed HIV-negative, non-pregnant, diagnosed with BV and/or TV, and cured after seven-day oral metronidazole treatment. Other clinical exclusion criteria were applied but were rare.[160] Women were recruited by study staff with the assistance of Community Mobilisers who had strong ties with local high-risk women (particularly sex workers).

Study products and dosing

Ecologic Femi+ (EF+; Winclove Probiotics, Amsterdam, Netherlands) is a vaginal capsule containing lyophilised lactic acid-producing bacteria. EF+ was used once per day for five days followed by thrice weekly, for two months. Gynophilus LP (GynLP; Biose, Aurillac, France) is a tablet containing the *Lactobacillus rhamnosus* Lcr35 strain. The tablet disintegrates in the vagina and forms a gel that slowly releases the probiotic bacteria. GynLP was used once every four days for two months. The first dose was inserted at the clinic under direct observation of a clinician, and remaining doses were self-administered at home. Women were asked not to cleanse or insert other products into the vagina after probiotic insertion to allow the probiotics to dissolve. They were also told that they were allowed to cease probiotic use during menses, but were encouraged to continue. Intermittent metronidazole use was chosen as a positive control intervention because studies conducted in the U.S. and Kenya have shown a 30-40% reduction in BV recurrence.[132,134] Metronidazole users took 500 mg generic oral metronidazole (Laboratory & Allied ltd, Nairobi, Kenya) twice weekly for two months. Participants and clinicians were not blinded.

Acceptability, adherence, behavioural, and vaginal infection knowledge assessments

Acceptability was assessed at the enrolment visit prior to product use initiation and at the Month 2 visit after the full two months of use. Adherence was assessed during the intervention period, at the Day 7, Month 1, and Month 2 visits. Sexual and other behaviours were assessed at all study visits. Participants were interviewed face-to-face in Kinyarwanda by a trained study nurse using structured questionnaires with multiple-choice questions, questions requiring a number or date, and an adherence self-rating scale (from 0-10). In between visits, participants used pictorial diary cards (figure 4.1) to record daily episodes of product use, vaginal sex, condom use, and vaginal practices. Those using study products returned the product packaging and unused products (if applicable) to their clinic visits, where they were counted by study staff. Any discrepancies between data sources were discussed with participants, and consensus, triangulated assessments of adherence were recorded on the questionnaires. Additionally, 131 women were interviewed about their knowledge of vaginal infections (such as BV and STIs) using a structured questionnaire during recruitment sessions (n=61; regardless of eligibility) and at enrolment visits (n=70; this included the 68 randomised women, and two women who attended

enrolment visits but turned out to be ineligible). Women were interviewed before being counselled at study visits or before receiving information at recruitment sessions. This questionnaire contained multiple-choice and open-ended questions. Responses to the open-ended questions were categorised and discussed by two different researchers until consensus about the answer categories was reached.

Figure 4.1: Pictorial diary card

Date/Month	Descriptions	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Indicate each time you used study product								
	Used study product							
Indicate each sex act								
	Sex with condom							
	Sex without condom							
Indicate each time you washed/inserted something inside the vagina other than study product								
By washing inside, we mean inserting an entire finger inside the vaginal canal								
	Washed inside vagina with water only							
	Washed inside vagina with soap and water							
	Inserted something else (herbs, powders, etc.)							
Indicate each day of menstrual bleeding								
	Had menstrual bleeding							

The picture provided is the English translation of the pictorial card; participants received a version in Kinyarwanda.

Four semi-structured focus group discussions (FGDs) with 7-11 participants per group (total n=38), and four semi-structured individual in-depth interviews (IDIs) were held with enrolled participants, about their experiences with and opinions of the products, sexual behaviour, and vaginal practices. Women randomised to the behavioural counselling only group were not included in the FGDs and IDIs. All had completed their product use period. The interviews were unlinked anonymous, and women used pseudonyms to enable them to talk freely despite the fact that the discussions and interviews were taped. All interviews took place between November 2015 and March 2016, were held in Kinyarwanda, recorded on tape, transcribed verbatim, and translated into English.

Data analysis

Questionnaire data were analysed using Stata 13 (StataCorp, College Station, TX, USA). The proportion of women with ≥80%/≥90%/100% adherence in the probiotic groups were compared by Fisher's exact tests. Changes in self-reported vaginal practices and sexual behaviours over time were tested using McNemar's test for binary outcomes, and Wilcoxon's signed-rank test for continuous outcomes. To study associations of participant characteristics with triangulated adherence, we used bivariable mixed effects models, with perfect adherence (defined as having used all doses as instructed)

per interval between study visits during the intervention period as the outcome, participant identification numbers as the random effect, and one participant characteristic at the time as the fixed effect. We could not determine correlates of acceptability due to limited variation in the acceptability data (reported acceptability was high throughout the trial).

The FGD and IDI transcripts were read and discussed by three researchers (MV, MU, and JvdW). The Chief Investigator (JvdW) decided that data saturation had been met when the fourth FGD and the fourth IDI transcript had become available in March 2016. The transcripts were then coded using NVivo 10.0 (QSR International, Melbourne, Australia) by one single researcher (MV). The discussions and interviews were semi-structured, with themes and associated codes prepared a priori, as well as new elements that emerged from the data. The codes were derived from an acceptability framework that has been used in studies of vaginal products for contraception or HIV prevention.[158,159,162] Components of the framework include study population characteristics, product attributes, sexual encounter and relational attributes, and the contextual environment (e.g. community perceptions of product use).

Ethical statement

All participants provided written consent for study participation, and separate consent for participation in FGDs/IDIs. All non-married participants aged 18-20 also required parental/guardian consent per Rwandan law at the time of the study. The participants received 3 GBP per visit (in local currency) as a reimbursement for time and transport costs. Care was taken to protect participant privacy and confidentiality. The study was sponsored by the University of Liverpool, approved by the Rwanda National Ethics Committee and the University of Liverpool Research Ethics Subcommittee for Physical Interventions, and registered on ClinicalTrials.gov (NCT02459665).

Participant and public involvement

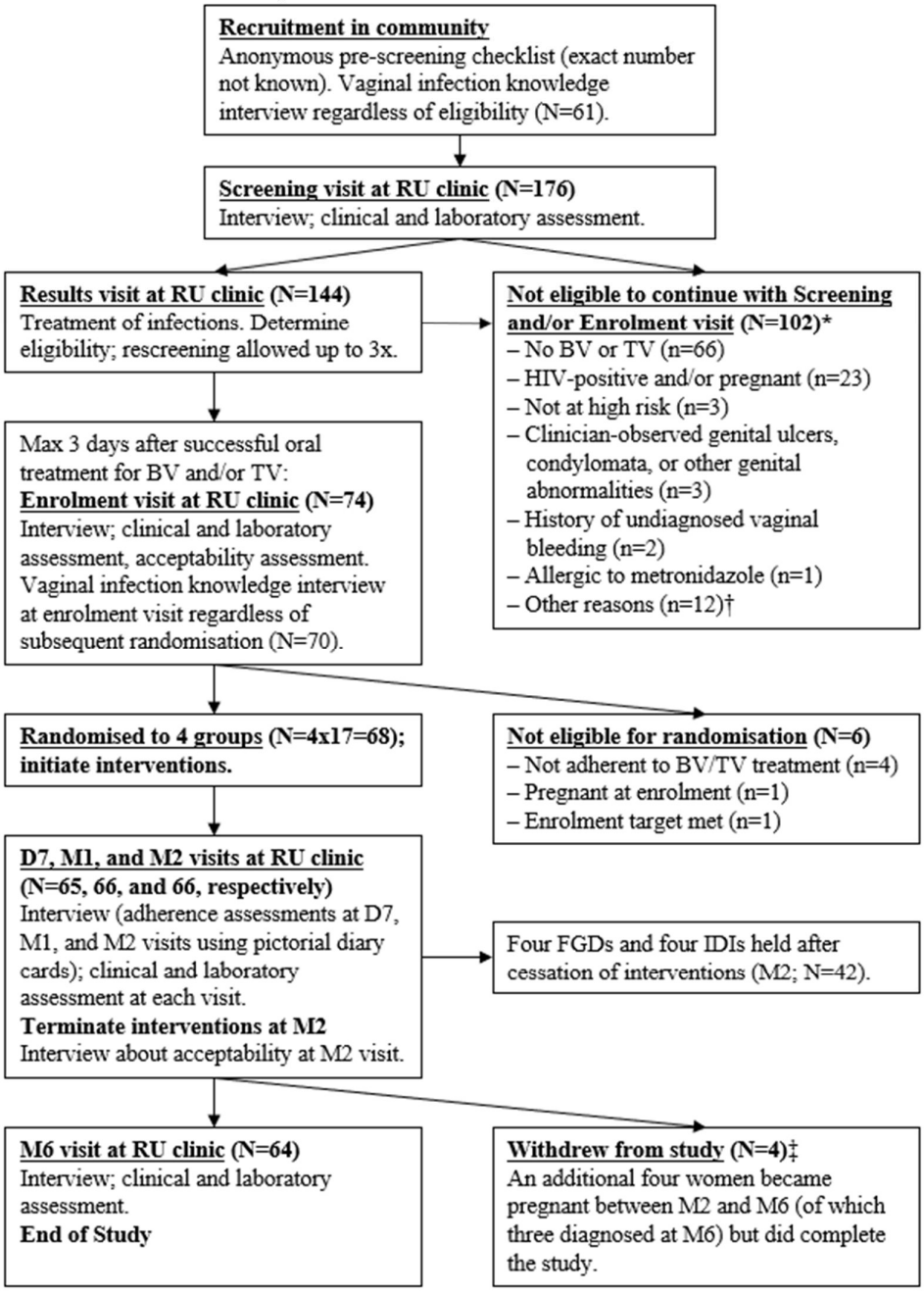
A subset of the enrolled participants were invited to comment on study design and experiences with the interventions during the FGDs/IDIs. Participants were not invited to develop outcomes, interpret the results, or to contribute to the writing or editing of this document for readability or accuracy. The preliminary results of this study were discussed with 32 stakeholders during a workshop held at the Ministry of Health in Kigali, Rwanda, in December 2017. These stakeholders included representatives of the Ministry of Health, the National University of Rwanda, the National Ethics Committee, local hospitals and clinics, and local non-governmental and women's organisations.

4.3 Results

Baseline characteristics

We screened 176 women: bacterial STI prevalence was 31.3% and BV prevalence by Gram stain Nugent scoring was 47.9%. All 68 randomised women were at risk of STI/HIV transmission, with 93.1% reporting having exchanged sex for money and/or goods in the previous month (figure 4.2, table 4.1). We collected 29.93 person-years of data. Four women withdrew their informed consent during the study (for reasons unrelated to study product acceptability). None were lost to follow-up.

Figure 4.2: Flowchart of the study



BV bacterial vaginosis, *D7* day 7 visit, *FGD* focus group discussion, *IDI* in-depth interview, *M1/2/6* Month 1/2/6 visit, *RU* Rinda Ubuzima, *TV Trichomonas vaginalis*.
Acceptability assessments were made at enrolment and at the M2 visit. Adherence assessments were made using self-rated assessments, pictorial diary cards, and returned packaging at the D7, M1, and M2 visits (after which product use was ceased). The vaginal infection knowledge survey was held at recruitment sessions in the community and at the enrolment visit. Changes in sexual risk-taking and vaginal practices were assessed at each follow-up visits and compared to answers given during the enrol visit. All this themes were discussed during the eight FGDs and IDIs.
*Totals to 110 reasons among 102 women because there could be more than one reason per woman. †Reasons: outside of metronidazole treatment window (n=5), enrolment target already met (n=4), has a mental disorder (n=1), did not complete screening procedures and was subsequently lost to follow=up (n=1), withdrew consent during the screening visit because she thought the reimbursement was too low (n=1). ‡Reasons: moved away from Kigali (n=2), lost interest because symptoms resolved (n=1), and was verbally harassed by partner and sister about study participation (n=1).

Table 4.1: Baseline characteristics of enrolled population

	Controls (n=17)	Metronidazole (n=17)	EF+ (n=17)	GynLP (n=17)
Median age (IQR)	29 (24–36)	30 (27–34)	33 (28–35)	30 (27–35)
Marital status (n %)				
- Never married	16 (94.1)	11 (64.7)	10 (58.8)	13 (76.5)
- Married	1 (5.9)	1 (5.9)	2 (11.8)	1 (5.9)
- Divorced	0	5 (29.4)	4 (23.5)	3 (17.6)
- Widowed	0	0	1 (5.9)	0
Education level (n %)				
- No schooling	5 (29.4)	3 (17.6)	3 (17.6)	3 (17.7)
- Primary school not completed	7 (41.2)	7 (41.2)	13 (76.5)	4 (23.5)
- Primary school completed	4 (23.5)	5 (29.4)	1 (5.9)	7 (41.2)
- At least some secondary school	1 (5.9)	2 (11.8)	0	3 (17.7)
Median number of sex partners last month (IQR)	5 (3–20)	5 (2–10)	3 (2–15)	3 (2–20)
Exchanged sex for money/goods past month (n %)	17 (100)	14 (82.4)	15 (88.2)	17 (100)
At least one laboratory-confirmed STI* (n %)	8 (47.1)	8 (47.1)	4 (23.5)	9 (52.9)
Median weekly frequency of washing body (IQR)	7 (7–7)	7 (7–7)	7 (7–7)	7 (4–7)
Ever washing the genitalia (n %)				
- Yes, outside only	12 (70.7)	14 (82.4)	15 (88.3)	14 (82.3)
- Yes, both inside and outside	5 (29.4)	3 (17.6)	2 (11.7)	3 (17.7)
- Yes, inside only	0	0	0	0
If reports washing inside, median weekly frequency (IQR)	14 (7–16)	14 (14–14)	11 (7–14)	7 (3–12)

EF+ Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *IQR* inter-quartile range, *M2* Month 2 visit, *STI* sexually transmitted disease.
*Chlamydia, gonorrhoea, and/or syphilis.

Adherence

Triangulated adherence was high: 100% of EF+ users and 81.3% of GynLP users used ≥80% of required doses (Fisher's exact p=0.103; table 4.2), and these percentages were 88.2% and 68.8% for ≥90% (p=0.225), and 58.8% and 50% for 100% of required doses (p=0.732), respectively. In comparison, these percentages were 88.2%, 82.4%, and 70.6%, respectively, for oral metronidazole users. Reported reasons of non-adherence to vaginal probiotics during face-to-face interviews were 'simply forgetting' (n=9), experiencing side-effects (n=2), menses (n=2), and being away from home and having left products at home (n=1). Additional reasons for missing doses mentioned during FGDs/IDIs were being drunk (n=2) and being confused about the dosing schedule (n=2). Only one woman in the metronidazole arm reported missing doses due to experiencing side-effects. Most women in FGDs reported using all doses as instructed and finding it easy to adhere, and thought that the diary cards served as a useful reminder to use the products.

Table 4.2: Adherence to study interventions

Adherence to study products	**Metronidazole (n=17)**	**EF+ (n=17)**	**GynLP (n=16)**
Adherence Enr–D7, median % (IQR)	100 (100–100)	100 (100–100)	100 (100–100)
Adherence D7–M1, median % (IQR)	100 (100–100)	100 (100–100)	100 (91.7–100)
Adherence M1–M2, median % (IQR)	100 (100–100)	100 (100–100)	100 (92.3–100)
Overall adherence Enr–M2, median % (IQR)	100 (96.3–100)	100 (100–100)	98.3 (89.3–100)
Overall adherence Enr–M2 (n %)			
- Perfect*	12 (70.6)	10 (58.8)	8 (50.0)
- Adherence ≥90%	14 (82.4)	15 (88.2)	11 (68.8)
- Adherence ≥80%	15 (88.2)	17 (100)	13 (81.3)
Number of times menses Enr–M2 (n %)†			
- Never	7 (41.2)	4 (23.5)	2 (12.5)
- Once	6 (35.3)	5 (29.4)	4 (25.0)
- Twice	4 (23.5)	8 (47.1)	10 (62.5)
Did not use product during menses at least once (n %)			
- Yes	4 (23.5)	3 (17.6)	5 (31.3)
- NA (never had menses)	7 (41.2)	4 (23.5)	2 (12.5)
Self-reported reasons for non-adherence‡	**Metronidazole**	**EF+**	**GynLP**
D7: Self-reported reasons why not able to use all doses as instructed (n %)§			
- Simply forgot	0	2 (11.8)	0
- Product had side effects	0	0	1 (6.7)¶
M1: Self-reported reasons why not able to use all doses as instructed (n %)§			
- Simply forgot	1 (6.3)	1 (5.9)	1 (6.3)
- Product had side effects	1 (6.3)‖	0	1 (6.3)‡‡
- Did not like product for another reason	1 (6.3)‖	0	0
- Other	1 (6.3)**	1 (5.9)††	2 (12.5)§§
M2: Self-reported reasons why not able to use all doses as instructed (n %)§			
- Simply forgot	1 (6.3)	2 (11.8)	3 (18.8)
- Travelled and forgot to take product	1 (6.3)	0	1 (6.25)
- Other	0	1 (5.9)¶¶	1 (6.3)‖‖
D7: Participant thinks she used product correctly most of the time (n %)	17 (100)	16 (94.1)	14 (93.3)
M1: Participant thinks she used product correctly most of the time (n %)	13 (86.7)	17 (100)	11 (68.8)
M2: Participant thinks she used product correctly most of the time (n %)	15 (93.7)	16 (94.1)	14 (87.5)

D7 Day 7, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *IQR* inter-quartile range, *M1/2* Month 1/2, *NA* not applicable. *Defined as 100% of the prescribed doses used at the prescribed times after nurse review of the participant's diary card and returned used packaging and unused product. †Number of times menses in the control group: never 2 (11.8%), once 3 (17.8%), twice 11 (64.7%), and thrice 1 (5.9%). ‡Numbers of participants per randomisation group may very slightly due to loss to follow-up. Participants with ≥90% adherence not shown. §Multiple answers possible. ¶Participant reported vulval itching and burning when passing urine. ‖Participant reported mild gastritis and wanting to withdraw from the study anyway. **Participant reported receiving oral metronidazole therapy for 7 days due to infection. ††Participant reported having menses twice in one month; decided to use less of her product until the next study visit. ‡‡Participant reported genital itching, genital burning, and pain during sex. §§One participant reported missing the D7 study visit and therefore running out of supplies. Another participant reported not to have used the study product during menses (which she was allowed to do). ¶¶Participant reported being drunk and therefore forgetting to take the study product. ‖‖Participant reported taking the study product correctly but that the product came out during menses

Acceptability: ease-of-use

No participants reported having heard about probiotics before study participation. After product use, all vaginal probiotic users reported feeling very comfortable with insertion and that insertion became easier over time. All but one woman reported inserting while lying down (table 4.3).

Table 4.3: Acceptability of interventions

Acceptability of study products at Enr	Controls (n=17)	Metronidazole (n=17)	EF+ (n=17)	GynLP (n=17)
Nurse reports having explained intervention to participant in detail (n %)	17 (100)	17 (100)	17 (100)	17 (100)
Nurse reports participant asked questions (n %)* - Yes, a few - Yes, many	 6 (35.3) 0	 2 (11.8) 0	 11 (64.7) 0	 11 (64.7) 2 (11.8)
First dose applied† under supervision (n %)	NA	17 (100)	17 (100)	17 (100)
Median number of attempts participant made until successful application (IQR)	NA	NA	1 (1–1)	1 (1–1)
Participant seemed comfortable with the insertion after these attempts, according to study nurse (n %) - Yes, very - Yes, somewhat	NA	NA	 17 (100) 0	 16 (94.1) 1 (5.9)
Acceptability of study products at M2				
Self-reported usual time of insertion (n %) - Before going to sleep - After bathing in the morning	NA	NA	17 (100) 0	15 (100)‡ 0
Level of comfort with vaginal insertion after 2 months of use, self-reported (n %) - Very comfortable - Somewhat comfortable	NA	NA	 17 (100) 0	 15 (100)‡ 0
Reported insertion becoming easier over time (n %)	NA	NA	17 (100)	15 (100)‡
Reported manner of insertion§ (n %) - While lying down - While squatting	NA	NA	17 (100) 1 (5.9)	14 (93.3)‡ 1 (6.7)
Acceptability of penile hygiene intervention at M2				
Reports having told main sex partner to regularly clean the penis, including underneath the foreskin (n %)¶ - Yes - No, because he is circumcised - No, other reason	 3 (17.7) 10 (58.8) 1 (5.9)	 3 (18.8) 9 (56.2) 0	 3 (17.6) 6 (35.3) 1 (5.9)	 3 (18.8) 5 (31.3) 1 (6.3)
If yes, response by the main partner (n %)‖ - He said that he would do so in the future - He said that he already does this - He said that he is not interested	 2 (66.7) 1 (33.3) 0	 1 (33.3) 1 (33.3) 1 (33.3)	 1 (33.3) 0 2 (66.7)	 1 (33.3) 1 (33.3) 1 (33.3)

EF+ Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *IQR* inter-quartile range, *M2* Month 2 visit, *NA* Not applicable.
*One missing value. †Whether oral insertion (oral metronidazole group) or vaginal insertion (Ecologic Femi+ and Gynophilus LP groups). ‡N=15 due to participants withdrawing informed consent. §Multiple answers possible; hence totals can be more than 100%. ¶Women with no main sex partner not included. ‖N=3 in all four groups.

Acceptability: bodily changes and product perception

During FGDs, several women using either vaginal probiotic reported the product (partially) "*coming out*" during the first few uses, but that this decreased after having gained experience. Many EF+ and GynLP users reported an increase in vaginal wetness, which was considered a positive attribute by most. Some women reported increased libido. For example, one EF+ user said: "*I felt a great desire to* [have] *sex again and again.*" In contrast, one metronidazole user reported a decrease in libido. Most women believed that the vaginal probiotics decreased the recurrence of symptomatic BV (our preliminary efficacy data suggest that BV incidence had in fact decreased),[160] and a few believed that they also prevented STI acquisition (the trial had insufficient statistical power to assess this).

Acceptability: support

One social harm related to vaginal probiotic use was reported: a GynLP user was verbally harassed by her partner and her sister because of her study participation, and opted to withdraw her informed consent. Reports of partner, family, and community support during the FGDs/IDIs were mixed: some women reported problems with loved ones. Negative reactions from male partners were more often based on suspicions about study participation than the products themselves. One EF+ user said: "*He* [her partner] *did not accept that. He asked me to go together with him to the clinic* [a local health centre] *and check if I am not HIV-positive*." Another participant using metronidazole mentioned wanting to join the study to her husband, who forbade her to participate. However, she decided to join anyway: "*he did not know that I was using the study product, because he had refused me to join* [the] *study before… I used them* [the study products] *without informing him.*" All sex workers except one stated that they had not discussed study participation with male clients.

Acceptability: worries and concerns

In the FGDs, one woman reported hearing rumours prior to enrolling that vaginal products "*can damage the uterus or cause tumours in the womb.*" However, most participants thought that vaginal probiotics would be acceptable to Rwandan women. One GynLP user argued: "*They* [already] *give us vaginal pills*", by which she meant vaginal medications for yeast infections. Some women were concerned about future product availability and pricing. They hoped that probiotics would be distributed cheaply through the Rwandan *Mutuelle* public health insurance because they would otherwise be inaccessible to many women. One metronidazole user was concerned about a limited applicability of probiotics because BV is not diagnosed by laboratory testing in Rwanda: "*They do not have adequate medical instruments to test diseases, you tell the physician how* […] *you feel and by guessing the disease, he gives you at least four medications, saying that you may have trichomonas, you may have syphilis, you may have gonorrhoea* [she refers to syndromic management.[21,24]] *At health centre-level they do not have medical equipment to test diseases, meaning that they will not know who to give that* [probiotic/antibiotic maintenance therapy] *medication*."

Vaginal practices and sexual risk-taking

At enrolment, 49.3% of the women reported to never use products inside the vagina, and at Month 6, this increased to 81.5% (OR 5.2, 95% CI 1.96-17.34; table 4.4). During FGDs, some women understood that vaginal washing practices may increase the risk of vaginal infection, but others did not. A participant stated: "*You get them* [i.e., vaginal diseases] *anyway… whether you wash or not*". In one FGD, 10 of 11 participants stated having ceased vaginal practices thanks to the study counselling. It should be noted that in contrast to many other African populations, Rwandan women use vaginal practices to increase rather than reduce vaginal lubrication. Women mentioned the use of herbs (*umushishiro*), Vaseline, and oils for this purpose. Self-reported sexual risk taking by face-to-face

interview did not change over time, except for a significant reduction in reported numbers of sex partners in the previous month at Month 6 compared to enrolment. No women in FGDs/IDIs mentioned adopting safer sex practices (such as consistent condom use) in response to the counselling messages. During face-to-face interviews at the Month 2 visit, 12 of 15 women (80%) who had an uncircumcised main sex partner reported asking him to regularly clean his penis in the future (table 4.3). While most women in FGDs understood that using condoms and improved penile hygiene could reduce BV rates, some mentioned that they found it difficult to discuss these topics with male partners. One participant stated that this is especially difficult being a sex worker: "*a man gives you his own money and you start educating him to wash!*" However, another sex worker reported refusing sex with uncircumcised clients: "*you leave him, because he has a lot* [of] *germs*". Several women reported discussing circumcision with their partners; one participant reported telling her husband: "*It is better that you do circumcision because it is a good thing… you would get a chance of not contracting diseases.*"

Table 4.4: Changes in reported vaginal cleansing practices and (sexual) behaviour between the enrolment and the M6 visit

Self-reported sociodemographic characteristics	Enr (n=71)	M6 (n=65)	OR (95% CI)*
			P value*
Reports using no products inside the vagina (other than for managing menses; all participants) (n %)	35 (49.3)	53 (81.5)	5.2 (1.96–17.34)
			<0.001
Reports using no products inside the vagina (other than for managing menses; controls and metronidazole users only)† (n %)	15 (44.1)	27 (79.4)	13.0 (1.95–552.5)
			0.002
Reports using water only (n %)	23 (32.4)	10 (15.4)	0.37 (0.13–0.92)
			0.029
Reports using water and soap (n %)	3 (4.2)	2 (3.1)	0.67 (0.06–5.82)
			1.00
Reports using paper, cloth or cotton wool (n %)	9 (12.7)	0 (0)	0.13 (0.00–0.93)‡
			0.008
Reports using traditional herbs, stones, powders as vaginal cleansing practice (n %)	1 (1.4)	1 (1.5)	1.00 (0.01–78.5)‡
			1.00
Mean weekly frequency of vaginal practices (95% CI)	2.15 (0.97–3.34)	0.64 (0.18–1.11)	NA
			0.328
Median number of sex partners in last month at baseline or per month during follow-up period (IQR)	5 (3–16)	2 (1–4)	NA
			<0.001
Any condom use reported in past two weeks (Enr) or since last study visit (M6), versus none (n %)	64 (90.1)	60 (92.3)	1.67 (0.32–10.7)
			0.727
Reports exchanging sex for money/goods in past month (Enr) or since last study visit (M6) (n %)	65 (91.5)	58 (89.2)	0.80 (0.16–3.72)
			1.00

CI confidence interval, *Enr* enrolment visit, *IQR* inter-quartile range, *M6* Month 6 visit, *NA* not applicable, *OR* odds ratio.
*McNemar's OR and p-value for binary variables and Wilcoxon signed-rank test p-value for continuous variables, comparing the response at M6 with the response at Enr. ORs with 95% CI were also calculated for binary pre/post data. †N=34. ‡To enable calculation of effect measures, a zero value was replaced by 1.

Correlates of adherence

In bivariable mixed effects models including the probiotic groups only, no participant characteristics were significantly associated with perfect adherence (table 4.5). However, non-significant trends were observed. Younger age, asking many questions at enrolment, having menses during the previous study interval, and reporting urogenital symptoms were associated with a lower likelihood of perfect adherence. When including oral metronidazole users, menses was significantly associated with a lower

likelihood of perfect adherence (p=0.008). There were no significant associations between randomisation group and perfect adherence.

Table 4.5: Participant characteristics associated with perfect adherence

Participant characteristics	EF+ and GynLP users		EF+, GynLP and oral metronidazole users	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Randomisation group: GynLP versus EF+	0.68 (0.22–2.11)	0.505	ND	ND
Randomisation group: - EF+ versus metronidazole - GynLP versus metronidazole	ND	ND	0.53 (0.15–1.81) 0.36 (0.11–1.23)	0.308 0.103
Age in years: ≥30 years versus <30	2.66 (0.90–7.82)	0.076	1.60 (0.61–4.15)	0.336
Marital status: - Married versus never married - Divorced versus never married - Widowed versus never married	 0.97 (0.14–6.58) 1.18 (0.29–4.79) ND	 0.976 0.912 0.991	 1.17 (0.20–6.99) 1.39 (0.42–4.57) ND	 0.865 0.586 0.990
At least some schooling versus no schooling	1.20 (0.59–2.45)	0.619	0.80 (0.22–2.95)	0.740
Number of sex partners last month: five or more versus four or less.	0.58 (0.18–1.83)	0.351	0.49 (0.17–1.37)	0.173
Exchanged sex for money/goods past month	ND	0.990	ND	0.986
Nurse reported participant asked questions at Enr - Yes, many versus none - Yes, a few versus none	 0.19 (0.02–1.52) 0.83 (0.24–2.83)	 0.116 0.761	 0.15 (0.02–1.19) 0.83 (0.27–2.57)	 0.072 0.744
Had menses during study visit interval	0.41 (0.14–1.20)	0.104	0.26 (0.09–0.70)	0.008
Reported alcohol consumption during study: - Once or twice per week versus never - More than twice per week versus never	 0.54 (0.14–2.12) 0.92 (0.18–4.81)	 0.373 0.920	 0.34 (0.11–1.08) 0.81 (0.19–3.49)	 0.068 0.774
Reported at least one urogenital symptom during study interval versus none	0.11 (0.01–1.56)	0.103	0.30 (0.04–2.16)	0.231
Reported at least one adverse event during study visit interval (excluding urogenital symptoms) versus none	0.43 (0.10–1.83)	0.253	0.55 (0.15–2.05)	0.371

CI confidence interval, *D7* Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M1/2* Month 1/2 visit, *ND* non-determinable, *OR* odds ratio.
Sociodemographic characteristics associated with perfect adherence in bivariable mixed effects models, in the enrolment–D7, D7–M1, and M1–M2 study visit intervals.

Vaginal infection knowledge

Almost all participants reported having heard of 'diseases of the vagina' and STIs before, but only 4.6% knew what bacteria were (table 4.6). The STIs most often spontaneously named (in numerical order) were HIV, gonorrhoea, and syphilis; only one participant reported having heard of BV. After having received an explanation about what BV is, only one woman reported ever having been diagnosed with BV. Most participants could name at least one cause or potential consequence of vaginal infections. Consequences wrongfully attributed to vaginal infections were death (4.6% of women), infant malformations (3.9%), and cervical cancer/tumours (3.1%).

Table 4.6: Vaginal infection knowledge

	Recruitment (n=61)	Enrolment (n=70)	Total (n=131)
Median age (IQR)	32 (27–35)*	31 (27–35)	31 (27–35)
Has heard of diseases of the vagina before (n %)	60 (98.4)	70 (100)	130 (99.2)
Reports knowing what bacteria are before study (n %)	5 (8.2)	1 (1.4)	6 (4.6)
Reports having heard about STIs before study (n %)	61 (100)	70 (100)	131 (100)
If yes, spontaneously named, without probing† (n %)			
- HIV	58 (95.1)	65 (92.9)	123 (93.9)
- Gonorrhoea	58 (95.1)	65 (92.9)	123 (93.9)
- Syphilis	44 (72.1)	59 (84.3)	103 (78.7)
- Trichomoniasis	38 (62.3)	48 (68.6)	86 (65.7)
- Hepatitis	3 (4.9)	3 (4.3)	6 (4.6)
- Yeast infection	0	3 (4.3)	3 (2.3)
- BV	0	2 (2.9)	2 (1.5)
- Urinary tract infection	1 (1.6)	1 (1.4)	2 (1.5)
- Chlamydia	0	1 (1.4)	1 (0.8)
- Herpes	0	1 (1.4)	1 (0.8)
- Human papillomavirus / cervical cancer	1 (1.6)	0	1 (0.8)
Reports having heard about BV before this study (n %)	1 (1.6)	0	1 (0.8)
Spontaneously reported reasons why women get vaginal disease, without probing† (n %)			
- Poor toilet hygiene	37 (60.7)	40 (57.1)	77 (58.8)
- Multiple sex partners	28 (45.9)	36 (51.4)	64 (48.9)
- After sex	25 (41.0)	30 (43.0)	55 (42.0)
- Dirty underwear	19 (31.2)	35 (50.0)	54 (41.2)
- Poor vaginal hygiene	26 (42.6)	22 (31.4)	48 (36.6)
- Poor penile hygiene of male partner(s)	4 (6.6)	17 (24.3)	21 (16.0)
- Traditional vaginal practices and washing	3 (4.9)	12 (17.1)	15 (11.5)
- New sex partner	6 (9.8)	3 (4.3)	9 (6.9)
- Use of contraception	1 (1.6)	3 (4.3)	4 (3.1)
- (Improper) use of sanitary pads or tampons	1 (1.6)	3 (4.3)	4 (3.1)
- Other	3 (4.9)‡	1 (1.4)§	4 (3.1)
- Cannot name any reasons	1 (1.6)	0	1 (0.8)
Spontaneously reported negative consequences of vaginal disease being named, without probing† (n %)			
- Foul smell from the vagina	30 (49.2)	39 (56.5)	69 (53.1)
- Difficulty getting pregnant	18 (29.5)	33 (47.8)	51 (39.2)
- Miscarriage	16 (26.2)	33 (47.8)	49 (37.7)
- Abnormal vaginal discharge	12 (19.7)	28 (40.6)	40 (30.8)
- Baby born too early	16 (26.2)	22 (31.9)	38 (29.2)
- Severe infection / fever of the woman	7 (11.5)	7 (10.1)	14 (10.8)
- Infection / fever of the newborn baby	5 (8.2)	3 (4.4)	8 (6.2)
- Itching	4 (6.6)	4 (5.8)	8 (6.2)
- Other consequences to the baby	3 (4.9)	3 (4.4)	6 (4.6)
- Cervical cancer or tumours	2 (3.3)	3 (4.4)	5 (3.9)
- Death	4 (6.6)	0	4 (3.1)
- HIV/STIs	1 (1.6)	3 (4.4)	4 (3.1)
- Pain during intercourse	0	3 (4.4)	3 (2.3)
- Cannot name any consequence	17 (27.9)	19 (27.5)	36 (27.7)

BV bacterial vaginosis, *IQR* interquartile range, *STI* sexually transmitted infection.

*One missing value. †Open-ended question. Totals may be more than 100%. ‡Participants report: "If you are infected with STIs", sharing underwear, and unprotected sex. §Participant reports: vaginal medicine.

4.4 Discussion

Several studies of different vaginal probiotics have been conducted, some of them in sub-Saharan Africa.[128,137,139,141] However, none reported in-depth acceptability and adherence data. Our study suggests high vaginal probiotic acceptability and adherence in high-risk Rwandan women. We found no statistically significant correlates of perfect adherence, partially due to limited statistical power, but younger age, asking many questions about product use at enrolment, current menses, and reporting urogenital symptoms showed trends towards a lower likelihood of perfect adherence. Vaginal probiotics are currently unavailable on the market in most African countries, and it is important to study acceptability in different target populations to inform product development and future marketing strategies.

We could not evaluate the impact of self-reported acceptability aspects on adherence because almost all women reported very high acceptability in face-to-face interviews throughout the trial. Such interviews are known to suffer from social desirability bias. However, women seemed to speak freely in the FGDs, and those data indicate that they did not have major issues with product attributes or insertion. However, some women reported difficulties due to lack of male partner support. The reported increase in vaginal wetness after probiotic insertion was not considered problematic, as lubrication during sex is preferred by most Rwandan men and women.[163] This might be different in other countries where dry sex is preferred.[164] We did find a non-significant lower adherence to GynLP compared to EF+, which might be explained by differences in formulation: GynLP forms a gel in the vagina whereas EF+ capsules merely release lyophilised bacteria. Previous research indicated high adherence to GynLP.[155] Unfortunately, the impact of these formulation differences was insufficiently probed during the FGDs. Participants indicated that they found the diary cards helpful in reminding them to use their products, and we believe that self-monitoring tools might indeed be helpful in maximising adherence.[165]

Our data suggest that counselling was partially effective in changing behaviours that increase BV risk. Significantly more women reported not engaging in vaginal practices at the end of the study, and most women with uncircumcised steady male partners reported having discussed penile hygiene with them. However, many women mentioned in FGDs that they found it difficult to discuss condom use and penile hygiene with male partners, especially clients. Women reduced their sexual risks only to a limited extent during follow-up, reporting a reduction in numbers of sex partners but no differences in engaging in sex work and condom use in face-to-face interviews. While these results are encouraging, it is difficult to assess to what extent they were influenced by social desirability bias.

Our survey with women at recruitment sessions and enrolment visits showed that high-risk Rwandan women had heard of several STIs, but were generally unaware of BV, its causes and potential consequences, and what they can do to prevent it. Experiences with HIV show that public health

interventions can only succeed if health care professionals and the public have sufficient knowledge of causes and consequences of disease.[166–168] High-risk Rwandan women (and health care professionals) should therefore be educated about BV.

Limitations

Our study had limited statistical power, and social desirability bias may have affected some of our results, as is often the case in studies of this nature. Additionally, it should be noted that product efficacy, availability and cost are important determinants of acceptability, and were not evaluated in our study, although preliminary efficacy results in this study were promising.[160] We could not directly compare experiences with, and opinions about, the two different vaginal probiotics because each woman used only one product and qualitative data depth was suboptimal. In the FGDs/IDIs, it was sometimes difficult to ascertain whether participants were referring to personal experiences, or to wider community perceptions. Strengths of our study include the use of a mixed-methods approach and triangulated adherence data.

Conclusions

The prevention of BV recurrence will likely have to include several components to be successful, such as improved diagnostics, treatments, and prophylactic products (for example probiotics), but also improved information, education, and counselling messages targeted to at-risk women and their partners. The results of this study can be used to inform future product development, and to fine-tune counselling messages in future trials.

Chapter 5 - Impact of Vaginal Probiotics on the Vaginal Microbiota: a Systematic Review

This systematic review was conducted with my primary supervisor Professor van de Wijgert (JvdW) and a medical student (Connie Rees, CR) as a team effort in accordance with the PRISMA guidelines for systematic reviews.[169] I was the medical student's day-to-day supervisor on this project. I wrote the first draft of the systematic review protocol, and submitted it to the PROSPERO registration database for systematic reviews after it had been approved by my primary supervisor. The medical student and I performed the database searches, and independently selected eligible papers, with my primary supervisor acting as a tiebreaker when we did not reach consensus. The medical student and I each extracted data from the selected papers independently, I consolidated the data extraction results, and my primary supervisor reviewed and approved the consolidated data extraction database. I wrote this chapter based on the approved consolidated data extraction database. My primary supervisor then used this chapter to write a much shortened manuscript, which is currently in press at the *British Journal of Obstetrics and Gynaecology*: van de Wijgert JHHM, Verwijs MC. Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs. *BJOG* 2019; doi:10.1111/1471-0528.15870. [Epub ahead of print]. The shortened manuscript is not presented in this thesis.

Abstract

Introduction: Probiotics or live biotherapeutic products consisting of beneficial lactobacilli are often prescribed in clinical practice or bought over-the-counter to cure or prevent common types of vaginal dysbiosis such as BV or VVC. However, it is unclear whether vaginally-delivered probiotics are effective in promoting an optimal, *Lactobacillus*-dominated VMB.

Materials and Methods: We conducted a systematic review to assess the impact of vaginal probiotics on the VMB. Outcomes included BV cure and/or recurrence by Nugent or Ison-Hay Gram stain scoring, VVC cure and/or incidence by fungal culture or KOH wet mount, and bacterial VMB composition as characterised by modern molecular techniques. We also assessed the risk of bias of all studies.

Results: Our review of 34 eligible studies showed that trial designs were highly heterogeneous, evaluating vaginal probiotics containing different *Lactobacillus* strains used as main, adjuvant, and/or maintenance therapy. All studies were judged medium- or high-risk; no articles had a low overall risk of bias. All of the six results of five medium-risk of bias studies with BV and molecular VMB outcomes results were promising: four results were statistically significant and two were non-significant. The eight high-risk BV studies showed mixed results: five studies showed significant beneficial effects of probiotic use, one study showed non-significant Nugent results but the VMB results were significantly beneficial, and two studies did not show any beneficial effects. Most of the beneficial outcomes were in studies that used probiotics to prevent the recurrence of BV. Positive effects were limited to the period of probiotic use and, in the two studies that compared these directly, less efficacious than antibiotic use. The studies with molecular VMB outcomes showed significantly increased relative abundance or concentration of lactobacilli or increased prevalences of a *Lactobacillus*-dominated VMB during probiotic use. In contrast, most of the twelve studies with VVC as an outcome were single-arm pre/post intervention studies of high overall risk of bias. One medium-risk VVC study found no benefit of using probiotics. Of the eleven high-risk VVC studies, five showed significantly beneficial effects of vaginal probiotic use, three studies showed non-significant beneficial effects or had mixed results, and three studies showed no beneficial effects of probiotic use. Small-scale studies among healthy women reporting the detectability of probiotic strains in the VMB showed a generally low proportion of users with detectable probiotic strains. Many of the included studies had methodological weaknesses: most had a limited sample size and therefore lacked statistical power and could not compare responders to non-responders, only a few studies reported adherence measures in a comprehensive manner, and many had some degree of ascertainment bias due to their (clinical and/or laboratory) methods. Furthermore, few controlled for known confounding factors such as hormonal contraception use, pregnancy status, menses, and sexual behaviour.

Discussion: Evidence that vaginal probiotics have a beneficial effect on the VMB is conflicting although some promising studies exist, mainly those using probiotic therapy to prevent BV recurrence. Well-powered clinical trials that incorporate in-depth molecular analyses, adjust for known confounding factors, and compare responders to non-responders are needed.

5.1 Introduction

Vaginal dysbiosis is a condition in which the vaginal microbiota (VMB) deviates from its optimal, low-diversity, and *Lactobacillus*-dominated state. The most common types of vaginal dysbiosis are bacterial vaginosis (BV), in which anaerobic BV-associated bacteria overgrow lactobacilli, and vulvovaginal candidiasis (VVC), caused by *Candida albicans* or other yeast species.[4] Modern molecular techniques such as quantitative polymerase chain reaction (qPCR) and 16S rRNA gene sequencing permit in-depth VMB assessment.[4] However, most molecular VMB studies have been descriptive only and next generation sequencing data have not often been incorporated as outcomes in clinical trials and other interventional studies thus far.[170]

Vaginal dysbiosis is often asymptomatic and is associated with important sequelae such as pelvic inflammatory disease, increased acquisition of HIV and other sexually transmitted infections (STIs), and adverse pregnancy outcomes.[18–20,87] BV can be treated with oral and vaginal antibiotics such as metronidazole. Treatment is often effective but recurrence rates are high.[46,47] Additional interventions may be needed to decrease the risk of recurrence: behavioural interventions decrease BV incidence by decreasing engagement in sexual risk taking (e.g. consistent condom use) and vaginal hygiene practices, and hormonal (particularly oestrogen-containing) contraception use decreases BV incidence by promoting lactobacilli.[26,46,47,171] It has been hypothesised that disrupting recurrence-inducing biofilms formed by BV-anaerobes may be a promising future strategy to reduce BV incidence.[26,46,47,172]

Another intervention of particular interest is the use of beneficial exogenous bacteria, most often lactobacilli, to restore or maintain a healthy VMB as probiotics or live biotherapeutic products (LBPs; also known as next-generation probiotics).[71] Probiotics can be delivered both vaginally and orally. A clear advantage of probiotic lactobacilli is that they can be used for a prolonged period of time without risking the development of antibiotic resistance. Possible mechanisms in which probiotic strains exert positive effects include replacing non-optimal bacterial species and dominating the VMB, the increased production of localised lactic acid, modulation of local cervicovaginal mucosal immune responses, inhibition of biofilm formation, and/or inhibition of *C. albicans* hyphae formation.[6,12,72–74,173] While probiotics have been available on the market for decades, scientific effectiveness studies have only been conducted recently.[46,47] It is unknown whether probiotic strains need to dominate the VMB to exert positive effects, or if they can also restore or maintain an optimal VMB at (relatively) low concentrations. It is also unclear what the best treatment strategy is: should probiotics be used to restore an optimal *Lactobacillus*-dominated VMB, as main treatment or as adjuvant treatment together with antibiotic or antifungal therapy, or should probiotics be used as maintenance therapy to prevent vaginal dysbiosis when a woman's VMB is *Lactobacillus*-dominated? Therefore, we performed a systematic review to assess how vaginal probiotics impact the VMB.

5.2 Materials and Methods

In this systematic review, we examined the impact of vaginal probiotics on the VMB, focussing on BV and VVC cure and/or incidence/recurrence as outcomes, and on VMB composition as measured by modern molecular methods. Two primary research questions (1, 2) as well as one secondary research question (3) were posed:

1) In sexually active women (regardless of menopausal, pregnancy, or vaginal dysbiosis status), what is the effect of a vaginal probiotic (used as a main, adjuvant, or maintenance therapy) on the VMB composition as measured by Nugent[78] or Ison-Hay[174] Gram stain scoring, or molecular methods?
2) In sexually active women (regardless of menopausal, pregnancy, or vaginal dysbiosis status), what is the effect of a vaginal probiotic (used as a main, adjuvant, or maintenance therapy) on the incidence of VVC as measured by wet mount, culture, or molecular methods?
3) In sexually active women (regardless of menopausal or pregnancy status) suffering from vaginal dysbiosis, which treatment (vaginal probiotic, vaginal probiotic dosing strategy, oral probiotic, or oral or vaginal probiotic) is more effective when directly compared to one another?

The protocol of the systematic review is available upon request, and was registered on PROSPERO under the identifier CRD42017075717.[175] We conducted a systematic review according to the PRISMA 2009 guidelines.[169] The bibliographic databases searched were MEDLINE[176] and Embase.[177] The search was performed on 15 January 2019 (search strategy in table 5.1). No filters were used except for 'English language'. The search results of the databases were compared, and duplicates were manually removed in Microsoft Excel. Two researchers (MV, CR) independently screened the resulting list based on title and abstract to identify articles that were thought to be relevant for research questions 1 and 2 (relevant articles for research question 3 were to be selected from the articles already selected for questions 1 and 2). Further articles were identified by screening the reference lists of the relevant articles, as well as those of relevant reviews or opinion pieces.

We included studies performed in sexually active women regardless of menopausal, pregnancy, or vaginal dysbiosis status. Studies using (modified) Amsel criteria, bacterial culture, other forms of Gram stain scoring (e.g. reporting *Lactobacillus* counts only), BV rapid testing, or symptom-based diagnoses were not included, and neither were studies in which the method of diagnosing BV was unclear. Studies reporting VVC cases based on reported symptoms and/or observed signs were not included, and neither were studies in which the method of diagnosing VVC was unclear. The intervention(s) used had to include at least one vaginal probiotic as main or adjuvant therapy (as treatment of vaginal dysbiosis) or maintenance therapy (to prevent vaginal dysbiosis). Any years of study publication and all research settings were permitted. Articles were excluded if the intervention(s) consisted of an oral probiotic only, if the mode of administration of the probiotics was unclear, if outcomes were based on clinical

signs/symptoms only, or if the probiotic administered was yoghurt (without any specification of the bacteria present in the yoghurt given). Discrepancies between the two screeners were discussed by full-text screening of the article, with JvdW acting as a tie-breaker when necessary. Corresponding authors of articles with no available full-text were contacted by e-mail to ask for copies; articles with no available full-text were excluded.

Table 5.1: Search strategy of the systematic review

Pubmed version (all fields): Vagin* OR Vaginal AND ("bacterial vaginosis" OR vaginosis OR vaginitis OR dysbiosis OR flora OR microflora OR microbiome OR microbiota OR atopobium vaginae OR gardnerella OR streptococc* OR candid* OR mobiluncus OR bacteroides OR bifidobacteri* OR enterobacteri* OR staphylococc* OR trichomona* OR urinary tract infection OR UTI OR urogenital infection) AND (probio* OR yogurt OR yoghurt OR lactobacill* OR lactococc*) Filter: English language
Embase version (all fields): Vagin* OR Vaginal AND ("bacterial vaginosis" OR vaginosis OR vaginitis OR dysbiosis OR flora OR microflora OR microbiome OR microbiota OR "atopobium vaginae" OR gardnerella OR streptococc* OR candid* OR mobiluncus OR bacteroides OR bifidobacteri* OR enterobacteri* OR staphylococc* OR trichomona* OR "urinary tract infection" OR UTI OR "urogenital infection") AND (probio* OR yogurt OR yoghurt OR lactobacill* OR lactococc*) Filter: English language

The search strategy used in this systematic review, performed on 15 January 2019. No filters were used except for 'English language'

Three researchers (MV, CR, JvdW) performed source article data extraction and discussed discrepancies. Each article was assessed for risk of bias as described in the Cochrane Handbook for Systematic Reviews of Interventions.[178] An overall risk of bias was designed by us and assessed per study. Articles for which all potential biases (selection, performance, detection/ ascertainment, attrition, reporting, and 'other' bias, with the latter including confounding) were assessed as 'low risk' were judged as 'overall low risk of bias', those with a maximum of two 'high or 'unclear risk' of bias as 'overall medium risk of bias', and those with three or more 'high or unclear risk' of bias as 'overall high risk of bias'. Articles were not excluded based on risk of bias assessments.

Risks of bias plots and risk of bias summaries were made using RevMan 5.3.5 (The Cochrane Collaboration, Copenhagen, Denmark). Probiotic therapy types were classified as 'main therapy' if the probiotic was used as sole therapy to treat a positive case of BV and/or VVC; 'adjuvant therapy' if the probiotic was given in combination with an antibiotic or antifungal drug to treat a positive case of BV and/or VVC; and 'maintenance therapy' if the probiotic was given to maintain an optimal VMB and prevent incident vaginal dysbiosis. Pilot trials or phase I studies among healthy women with no vaginal dysbiosis at baseline that were solely designed to detect the presence of probiotic strains in the VMB of users were classified as vaginal detection studies. If no statistical analyses were reported in the article but absolute numbers of cases per exposure group were available, we performed Chi-squared testing

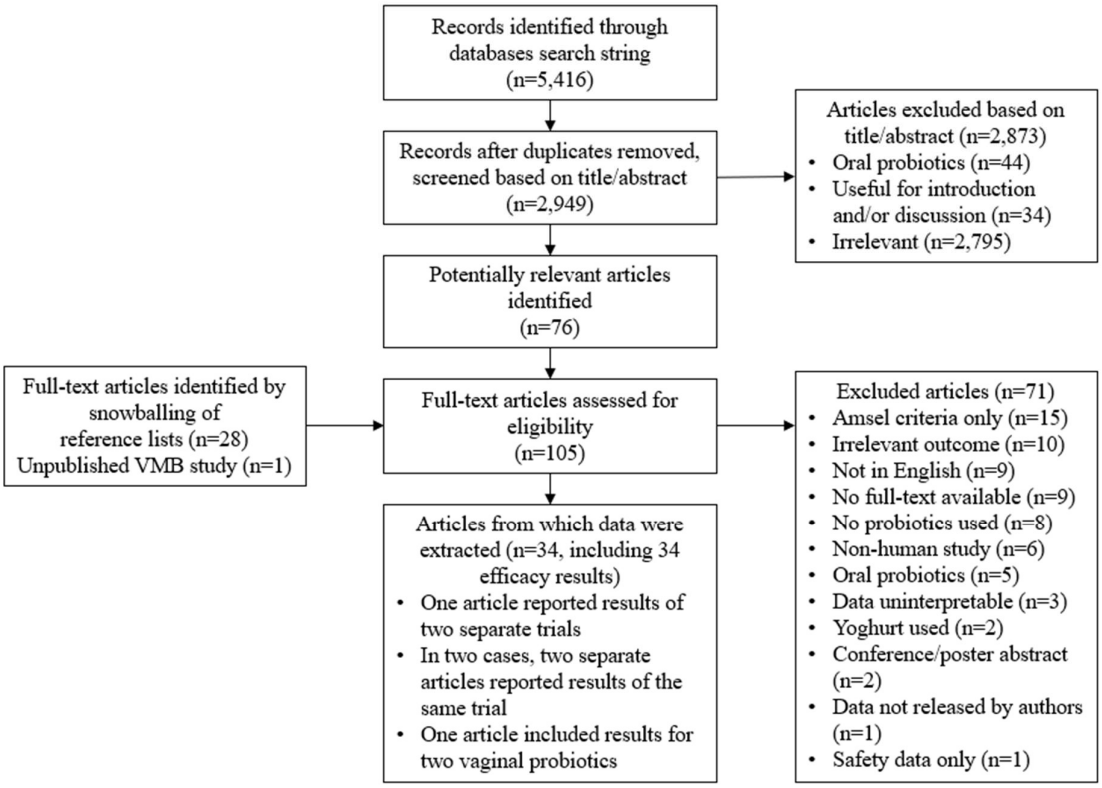
(for cross-sectional comparisons between arms) and/or McNemar's test (for pre/post within-arm comparisons) using Stata 13 (StataCorp, College Station, USA).

In this systematic review, we also included the results of the Rwanda VMB Study ("Preparing for a clinical trial of interventions to maintain normal vaginal microbiota for preventing adverse reproductive health outcomes in Africa") which was conducted in Rwanda in 2015-2016.[160] This study tested two different vaginal probiotics in parallel. The results of this pilot randomised clinical trial (RCT), reported in Chapter 3, have been submitted to a journal and the co-author-reviewed primary data analysis report based on Nugent scoring, VVC by KOH wet mount, and 16S rRNA gene sequencing is available.

5.3 Results

We identified 5,416 articles using the search criteria, 2,949 unique articles after removal of duplicates, and identified 34 relevant articles reporting 34 separate efficacy results, including the two products from the aforementioned Rwanda VMB study (flow diagram in figure 5.1). Three articles were excluded during the data extraction stage because the data were uninterpretable, and one article was a safety study reporting only three VVC endpoints.[179–182] We found two cases of two articles reporting distinct results from the same trial,[128,183–185] while one publication reported results of two closely related but separately conducted trials.[186] Of the 34 studies identified, 13 had BV and/or in-depth molecular VMB composition as an outcome (relating to our first primary research question)[72,135–145,160] with a total of BV 14 efficacy results, and 12 studies reported 12 results on VVC (relating to our second primary research question).[67,145,184–194] A total of 14 studies reported 15 results that incorporated molecular outcomes reported vaginal detection of vaginal probiotics, or other outcomes related to the retrievability of probiotic strains.[76,135,142,143,150,151,155,160,183,186,187,195–197] Only one study comprehensively reported BV and VVC efficacy rates as well as molecular VMB outcomes (the Rwanda VMB study), but however did not have any VVC cases during follow-up.[160] Of the 16 studies that incorporated any type of molecular VMB (composition and/or vaginal detection) outcomes, 13 performed sequencing on bacterial culture isolates and five used direct sequencing of DNA extracted from vaginal swabs (two studies used a combination of techniques). These 16 molecular VMB studies used a wide variety of molecular techniques: random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), denaturing gradient gel electrophoresis (DGGE) followed by Sanger sequencing, repetitive element sequence-PCR (rep-PCR), 16S rRNA gene sequencing, and species-specific qPCRs. Most of the former techniques did not permit to measure the relative abundances of the probiotics studied, and only measured the (non-quantifiable) presence of the probiotic strains. These studies do not describe the impact of the vaginal probiotics on the VMB but have been classified as vaginal detection studies, as explained in the methods section.

Figure 5.1: PRISMA flow diagram



VMB vaginal microbiota.
The PRISMA flow diagram of the studies screened and included in this systematic review.

The 34 studies evaluated 22 different probiotic products (see Appendix table D.1 for characteristics of included studies and products used, and Appendix table D.2 for summary of evidence per product). *L. crispatus* CTV-05 (Lactin-V; four studies including 132 participants),[76,128,150,183,187] Gynoflor (three studies including 359 participants)[137,184,185,188] and *L. reuteri* RC-14 with *L. rhamnosus* GR-1 (four studies with 47 participants)[139,142,196,197] were studied most often. Most probiotics contained between 10^8 and 10^{10} colony-forming units (CFU) of bacteria per dose. All products contained lactobacilli as active agents. One probiotic (Gynoflor) contained *L. acidophilus* KS400 in combination with 30 µg oestradiol, while several products contained acidifying agents: lactic acid (Kramegin, Estromineral Probiogel, and one unnamed *L. acidophilus* LA14-containing probiotic),[145,189,194] citric acid (ActiCand 30),[191] and a combination of ascorbic acid, adipic acid and stearic acid (Florisia).[72,140] Two probiotic products contained *Streptococcus thermophilus* (Lactagyn, Femilac),[67,193] one product contained *Pediococcus acidilactici* (Ellen capsules),[195] and another *Bifidobacterium bifidum* (Ecologic Femi+).[160]

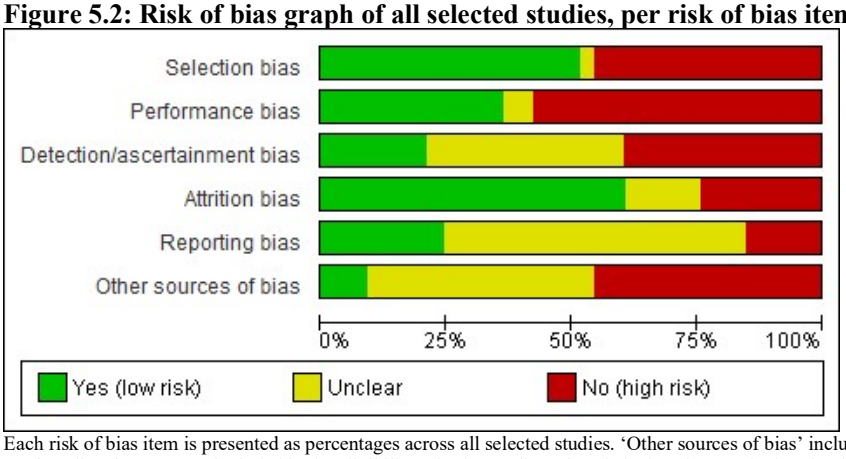
The included studies were very heterogeneous (Appendix table D.1). The majority of the studies took place in Europe or North America, with studies elsewhere conducted in Turkey,[188] Australia,[67,137] Nigeria,[139] India,[140] China,[141] and Rwanda.[160] Most studies recruited in STI clinics or health centres and general practices, and included pre-menopausal women only, with only two studies conducted among

post-menopausal women.[142,184,185] No studies focussed on pregnant women exclusively. The total duration of probiotic use varied between three days to changing dosage schemes spanning six months, while the duration of follow-up ranged from seven days to over one year. Of the studies that were not solely vaginal detection studies but reported BV/VMB or VVC efficacy rates, ten studies evaluated probiotics as main therapy, three as adjuvant therapy, and 14 as maintenance therapy. It should be noted that some studies stated that they used probiotics as maintenance therapy, but the authors did not report whether the participants were successfully cured of BV or VVC at the start of probiotic therapy.[67,137,138,192,193] We did however classify these studies as maintenance therapy studies.

Quality assessments

Most included studies had small sample sizes (ranging from 14 to 450 in BV/VMB or VVC efficacy studies) and were therefore underpowered, and 14 out of 34 studies did not include formal statistical power assessments. In only five studies responders were comprehensively compared to non-responders. Most BV/molecular VMB studies (11 out of 13) were RCTs but eight of the 12 VVC studies were non-randomised, e.g. pre/post interventional studies[76,143,145,184–186,188,189,191,192,194,196,197] rather than RCTs, and exposure groups were sometimes poorly defined.

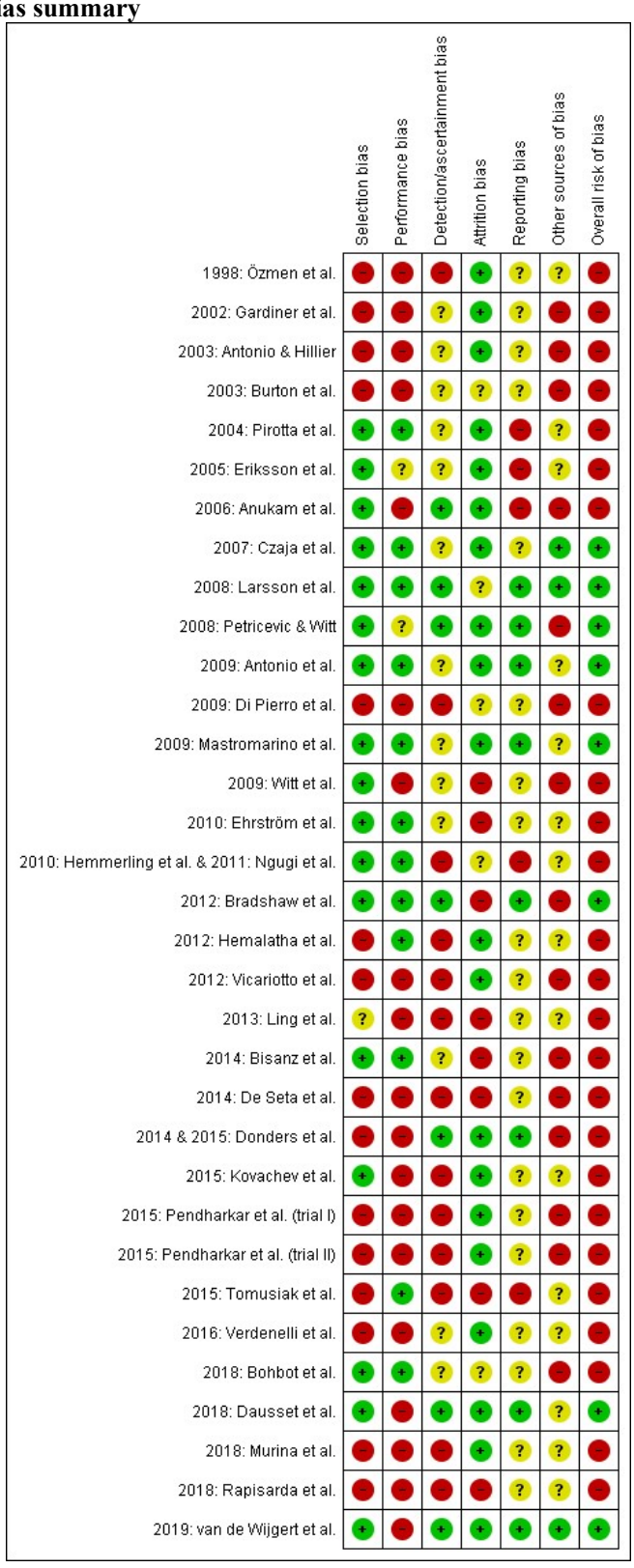
Figure 5.2: Risk of bias graph of all selected studies, per risk of bias item



Each risk of bias item is presented as percentages across all selected studies. 'Other sources of bias' included assessments on whether confounding factors were included in the eligibility criteria or adjusted statistical analyses, whether the 'intent-to-treat' results were also published, and whether the statistical testing used was appropriate.

Only twelve studies reported adherence data, of which only two included adherence data that were not self-reported.[160,183] Four studies reported the impact of probiotics on molecular VMB composition in additional to BV (Nugent or Ison-Hay scoring) or VVC rates.[141–143,160] Three studies with VMB/BV outcomes also included vaginal detection data,[142,143,160] whereas two studies with VVC outcomes included vaginal detection outcomes.[186,187] Few studies reported incorporating quality controls, for example ensuring probiotic viability,[67,137] inter-user reliability of Nugent scoring,[137–139,160] or incorporating negative and/or positive controls in molecular studies.[160]

Figure 5.3: Risk of bias summary



The authors' judgements about each risk of bias item per study; the studies are ordered by publication year. 'Other sources of bias' included assessments on whether confounding factors were included in the eligibility criteria or statistical analyses, whether the 'intent-to-treat' results were reported, and whether appropriate statistical was used. Studies with high overall risk of bias (seventh column) have a minus sign symbol, while studies with medium overall risk of bias have a plus sign symbol. No studies had low overall risk of bias.

In many studies the microbiological inclusion criteria differed from the microbiological outcome criteria (Appendix table D.1), and many reported results without detailing the statistical analyses performed. Others used statistical techniques that did not take confounding or repeated measures into account, such as the McNemar's test for within-arm pre/post comparisons, or Fisher's exact test to compare treatment arms at one timepoint. We also observed multiple instances in which erroneous statistical testing was performed: for example, in six studies statistical tests for independent data were used for pre/post comparisons, which are dependent data.[145,151,185,189,191,193] Other studies did not statistically compare results in the intervention group to those in the control group. Twenty-six studies were judged to have a high overall risk of bias, eight a medium overall risk of bias, and none a low overall risk of bias (figures 5.2 and 5.3). Most studies were at risk of selection and performance bias; only ten studies were double-blinded RCTs.[67,72,128,135,137,142,144,150,183,187,195] The risk of detection/ascertainment bias was common because outcome assessments were not done in a blinded fashion, or differed per exposure group. Attrition and reporting bias risk were also common: some studies had high loss to follow-up and multiple articles did not (fully) report intent-to-treat (ITT) analysis results, or misidentified the ITT population (e.g. excluding non-adherent, non-responsive or symptomatic participants from all analyses). The most common bias among studies was the lack of consideration of potential confounding factors such hormonal contraception use, pregnancy status, menses, vaginal practices, and sexual behaviour.

Studies with BV/VMB composition as an outcome

Thirteen studies had BV and/or molecular VMB composition as an outcome (table 5.2), resulting in 14 BV/VMB results. One study used Ison-Hay criteria,[135] whereas the other BV studies used Nugent criteria. Four studies used molecular methods to characterise VMB composition before, during, and after probiotic use in more detail.[141–143,160] Five BV/VMB trials (including one study that incorporated molecular methods) were assessed as having an overall medium risk of bias, and the remaining eight were assessed as having an overall high risk of bias. Eleven out of thirteen studies were RCTs. The two remaining studies (both high-risk) were pre/post interventional studies with women using a vaginal probiotic only.[143,145]

Table 5.2: Summary of BV/VMB efficacy studies

Reference	Probiotic	Study design	Results
Articles with a medium overall risk of bias			
Larsson[135]	EcoVag (*L. gasseri* EB01-DSM 14869 + *L. rhamnosus* Lbp PB01-DSM 14870) as adjuvant and maintenance tx after clindamycin for BV.	RCT: placebo control	10 days of EcoVag after clindamycin did not improve BV cure (by Ison-Hay; 32/50 cured) compared to placebo (37/50 cured) but significantly reduced the cumulative incidence of BV/intermediate microbiota within 4 menstrual cycles when used for 10 days after each menses (13/37 versus 21/39 incident cases, respectively).
Petricevic[136]	Gynophilus (*L. casei rhamnosus* Lcr35) as maintenance tx after clindamycin for BV.	RCT: no-intervention control	7 days of Gynophilus after clindamycin resulted in significantly improved BV cure (by Nugent 0-3) compared to women receiving clindamycin only (69/83 versus 31/88, respectively) assessed 5 weeks after cessation of the intervention period.
Mastro-marino[72]	Florisia *(L. brevis* CD2 + *L. salivarius* subsp. s*alicinius* FV2 + *L. plantarum* FV9) as main tx for BV.	RCT: placebo control	7 days of Florisia (without any antibiotic) was significantly more efficacious in curing BV (15/18 Nugent 0-3, 3/18 Nugent 4-6) than placebo (2/16 Nugent 4-6; 14/16 persistent Nugent 7-10) and was associated with significantly lower BV (Nugent 7-10) cumulative incidence in the 2 weeks after Florisia cessation (7/18 and 13/16, respectively).
Bradhaw[137]	Gynoflor *(L. acidophilus* KS400 + 0.03mg oestradiol) as maintenance tx to prevent BV recurrence.	RCT: placebo and clindamycin cream controls	12 days of Gynoflor after oral metronidazole treatment for BV (Nugent 7-10) resulted in a borderline (p=0.13) lower BV recurrence (9/133) at M1 compared to 12 days of placebo (13/135) but higher compared to 7 days of vaginal clindamycin (5/140). At M6, BV recurrence was comparable between the three arms (37/133, 36/135, and 42/140, respectively).
van de Wijgert[160]	EF+ (*B. bifidum* W28 + *L. acidophilus* W70 + *L. helveticus* W74 + *L. brevis* W63 + *L. plantarum* W21 + *L. salivarius* W24) as maintenance tx to prevent BV recurrence.	RCT: no-intervention and oral metronidazole controls	Intermittent EF+ use after oral metronidazole BV treatment resulted in significantly lower BV (Nugent 7-10) recurrence than no-intervention (incidence 3.58/PY versus 10.18/PY, respectively), and significantly reduced BV-anaerobes concentration by sequencing, during the 2 months of use, but the effect disappeared by 4 months after cessation of use.
van de Wijgert[160]	GynLP (*L. rhamnosus* Lcr35 *regenerans*) as maintenance tx to prevent BV recurrence	RCT: no-intervention and oral metronidazole controls	Intermittent GynLP use after oral metronidazole BV treatment resulted in a non-significant lower BV (Nugent 7-10) recurrence than controls (incidence 5.36/PY versus 10.18/PY, respectively), and non-significantly reduced BV-anaerobes concentration by sequencing, during the 2 months of use, but the effect disappeared by 4 months after cessation of use.
Articles with a high overall risk of bias			
Eriksson[138]	*L. gasseri* + *L. casei* var *rhamnosus* + *L. fermentum* (impregnated tampons) as main-tenance tx after clindamycin to prevent BV.	RCT: placebo control	Use of lactobacilli-impregnated tampons for 5 or more days during the first and second menses after clindamycin treatment was not efficacious in reducing BV recurrence (by Nugent 4-10) by the end of the two menstrual cycles (41/91) compared to placebo tampons (34/96).
Anukam[139]	GR-1/RC-14 *(L. fermentum* RC-14 + *L. rhamnosus* GR-1) as main tx for BV.	RCT: metronidazole gel control	5 days of GR-1+RC-14 capsules (without any antibiotic) was significantly more efficacious in curing BV (12/20 Nugent 0-3) than 5 days of metronidazole gel (6/20) when assessed directly after the intervention, and also in reducing BV recurrence (Nugent 7-10) up to 25 days post-intervention (2/17 versus 9/18, resp.).

Reference	Probiotic	Study design	Results
Hemalatha[140]	Florisia (*L. brevis* CD2 + *L. salivarius* subsp. *salicinius* + *L. plantarum*) as tx in women with variable BV status.	RCT: pH lowering tablet control	8 days of Florisia (without any antibiotic) was not efficacious in curing BV (by Nugent; 36/75 cured) compared to pH-lowering tablet (29/73 cured).
Ling[141]	*L. delbrueckii* subsp. *lactis* DM8909 as main tx for BV.	RCT: metronidazole control	7 days of unnamed *L. delbrueckii*-containing probiotic (without any antibiotic) was equally effective as vaginal metronidazole gel in curing BV (assessed 5 days after treatment completion; 22/25 and 25/30 cured, respectively) and was associated with a significantly lower recurrence 30 days after treatment completion than metronidazole gel (0/25 versus 5/30 recurrences). The molecular VMB data also suggest benefits of using the probiotic.
Bisanz[142]	GR-1/RC-14 (*L. rhamnosus* GR-1 + *L. reuteri* RC-14) as main tx for Nugent 4-6.	Randomised controlled cross-over trial: placebo control	In women with Nugent 4-6, 3 days of GR-1/RC-14 use (without any antibiotic) did not result in more women with Nugent score improvement compared to placebo use (2/10 and 0/10 improved one day after use, and 2/9 and 1/8 improved 8 days after use). However, the *Lactobacillus* relative abundance was significantly increased, and *Atopobium* relative abundance significantly decreased, one day after use.
Verdenelli[143]	SYNBIO gin (*L. rhamnosus* IMC 501 + *L. paracasei* IMC 502) as maintenance tx to prevent BV and VVC.	Pre-/post-intervention study	In women with Nugent 0-6, 7 days of SYNBIO gin (without any antibiotic) resulted in a significantly higher proportion of women with Nugent 0-3 directly after therapy (28/35 versus 21/35 at baseline), and this persisted for 21 days after cessation of therapy (28/35 still had Nugent 0-3; insufficient data for McNemar testing). SYNBIO gin use also resulted in a significant increase in *Lactobacillus* concentration ($p<0.01$).
Bohbot[144]	Physioflor (*L. crispatus* IP 174178) as maintenance tx to prevent BV recurrence.	RCT: placebo control	Physioflor for 14 days immediately after metronidazole therapy, plus 14 days in three subsequent menstrual cycles, resulted in significantly lower BV cumulative incidence (Nugent 7-10), and time to BV recurrence, compared to placebo (8/39 and 16/39 had at least one recurrence, respectively). However, the effects disappeared 84 days after product cessation ($p=0.922$, absolute numbers not given).
Rapisarda[145]	*L. acidophilus* LA14 as main tx for Nugent 4-10.	Pre-/post-intervention study	In women with Nugent 4-10, 14 days of *L. acidophilus* LA14 use (without any antibiotic) resulted in 46/60 women cured (Nugent 0-3) at the end of therapy, and this persisted for 28 days after product cessation (50/60).

BV bacterial vaginosis, *EF*+ Ecologic Femi+, *GynLP* Gynophilus LP, *M1/6* Month 1/6, *PY* person-years at risk, *RCT* randomised controlled trial, *tx* therapy, *VMB* vaginal microbiota.
Studies examining the efficacy of vaginal probiotics on BV (by Nugent or Ison-Hay scoring of Gram stains) rates and/or proportions and/or molecular VMB outcomes were selected for inclusion in this table. See Appendix table D.1 for in-depth study characteristics.

All five BV/VMB studies with medium overall risk of bias were RCTs (table 5.2). Of the five BV/VMB studies (yielding a total of six BV/VMB results), four results showed significant beneficial effects, and two results showed non-significant but beneficial effects of vaginal probiotics use. Three of the five studies included placebo control groups,[72,135,136] and two included both controls with no intervention and antibiotic controls.[137,160] The three studies with placebo controls focussed on BV cure proportions: two of them showed significantly beneficial effects,[72,136] and while the other did not show beneficial effects

of adjuvant probiotic use on BV cure proportions, the study did show significantly lower BV recurrence after use of the probiotic during the next four menstrual cycles.[135] The other two BV/VMB studies focussed on BV recurrence: one showed a non-significantly lower BV recurrence among probiotic users after metronidazole compared to no-intervention controls,[137] and the other study showed significantly lower BV recurrence, and beneficial VMB effects, for one probiotic product and non-significant lower BV recurrence and non-significant beneficial VMB effects for the other product.[160] Both of these studies also included a positive antibiotic use arm which showed that the antibiotic maintenance users had lower BV recurrence than the probiotic maintenance users.[137,160]

Eight BV studies (including three studies that incorporated molecular methods) were assessed as having an overall high risk of bias, and these studies showed comparable results to the medium-risk studies (table 5.2). Five studies showed significant beneficial effects of probiotic use, one study showed non-significant Nugent results but the VMB results were significantly beneficial, and two studies did not show any beneficial effects. Six studies were RCTs whereas two studies were pre/post intervention studies in women receiving a vaginal probiotic without any antibiotic.[143,145] Both pre/post intervention studies showed that probiotic use significantly increased the proportion of women with Nugent 0-3, and that these effects lasted beyond the product use period. Three of the six RCTs included placebo controls,[138,142,144] two included antibiotic controls,[139,141] and one included controls using a vaginal pH-lowering tablet.[140] Four studies evaluated BV cure: two studies showed a significant benefit of probiotic use compared to metronidazole use,[139,141] one study was underpowered to show an effect on BV cure but did show beneficial VMB outcomes,[142] and one showed no significant effect on BV cure compared to a pH-lowering tablet.[140] The four studies evaluating BV recurrence showed significant reductions among probiotic users in three studies[139,141,144] and no reduction in another.[138] It should be noted that women enrolled in the latter study used the vaginal probiotic during menses only.

Studies with VVC as an outcome

Twelve studies had VVC as an outcome (table 5.3), using wet mount or fungal culture to detect *Candida*. None of the studies used (q)PCR-based methods. Only one study was assessed as having a medium overall risk of bias, the other eleven had a high overall risk of bias.

The study with a medium overall risk of bias was an RCT conducted in the USA which compared five-day Lactin-V use to placebo in BV- and VVC-negative, healthy women with a history of recurrent urinary tract infections.[187] No women used antifungals (nor antibiotics for other indications) and the proportion of women with incident VVC cases up to 25 days after product cessation was similar in the Lactin-V group (4/15 women) compared to the placebo group (2/15).

Table 5.3: Summary of VVC efficacy studies

Reference	Probiotic	Study design	Results
Articles with a medium overall risk of bias			
Czaja[187]	*L. crispatus* CTV-05 (not Lactin-V) as recurrent UTI maintenance tx. VVC as part of safety assessments.	RCT: placebo control	In women with a history of recurrent UTI but without current dysuria, 5 days of *L. crispatus* CTV-05 use resulted in a similar VVC cumulative incidence (4/15) compared to placebo use (2/15) up to 25 days after cessation of use.
Articles with a high overall risk of bias			
Özmen[188]	Gynoflor (*L. acidophilus* KS400 + 0.03mg oestradiol) as main BV tx and as adjuvant to metronidazole tx. BV and VVC outcomes but BV ineligible (Amsel).	Pre-/post-intervention study in three separate, non-randomised groups	12 days of Gynoflor with or without metronidazole, compared to metronidazole alone (to treat BV), resulted in significantly lower cumulative VVC incidence (3/96 and 2/97, versus 14/114, respectively) in the 10-13 days after cessation of use.
Pirotta[67]	Femilac *(L. rhamnosus + L. delbrueckki + L. acidophilus + S. thermophilus*) as maintenance tx after non-metronidazole antibiotic to prevent VVC. Also Lactobac oral powder but results not reported here.	2x2 factorial design RCT: placebo control	10 days of Femilac during (6 days) and after (4 days) non-metronidazole antibiotic use, compared to placebo controls, was not effective in reducing VVC incidence within 18 days after cessation of Femilac use (17/59 versus 9/54, respectively).
Di Pierro[189]	Kramegin *(L. acidophilus* + *Krameria triandra* plant extract + 15mg lactic acid) as main tx of VVC.	Pre-/post-intervention study	10 days of Kramegin (without antifungal) cured 75/75 women with acute VVC, and 20/30 women with recurrent VVC (both significant compared to baseline). Cure was assessed 7 days after last administration.
Witt[190]	*L. gasseri* as (additional) maintenance tx together with itraconazole to prevent VVC recurrence.	RCT: probiotic + itraconazole versus itraconazole only	In women with acute VVC and a history of recurrent VVC, after induction with itraconazole twice weekly for one month, itraconazole (once per month) with *L. gasseri* (6 consecutive days per month) during 6 months resulted in a similar VVC recurrence rate as itraconazole (once per month) only (15/45 versus 12/31, respectively). Results were also similar 6 months after product cessation (6/25 versus 5/23, respectively).
Vicariotto[191]	ActiCand *(L. fermentum* LF10 + *L. acidophilus* LA02) as main tx for VVC.	Pre-/post-intervention study	In women with acute VVC and a history of recurrent VVC, ActiCand (without antifungal; 7 consecutive nights, followed by 3 nights per week for 3 weeks, and one night per week for 4 weeks) resulted in a significant reduction in VVC cases to 7/30 women after cessation of therapy at Day 56.
De Seta[192]	Gyno-Canesflor (*L. plantarum* P17630) as maintenance tx to prevent VVC recurrence.	Non-randomised prospective cohort study	In women with acute VVC but no history of recurrent VVC, 34 days of Gyno-Canesflor after clotrimazole treatment, compared to placebo use, resulted in a (non-significant, Fisher's p=0.095) lower cumulative VVC incidence (1/40 versus 5/40, respectively).
Donders[184] & Donders[185]	Gynoflor *(L. acidophilus* KS400 + 0.03mg oestradiol) as maintenance tx to prevent VVC.	Pre-/post-intervention study	A 84-day single-arm Gynoflor treatment scheme (once daily for 4 weeks followed by thrice weekly for 8 weeks) for atrophic vaginitis in postmenopausal women with breast cancer temporarily increased asymptomatic VVC prevalence after two weeks of use (from 2/14 to 7/16) but returned to baseline values after one (3/16), two (3/16), and three (3/13) months of use.

Reference	Probiotic	Study design	Results
Pendharkar - Trial II[186]	EcoVag (*L. gasseri* DSM 14869 + *L. rhamnosus* DSM 14870) as adjuvant/ maintenance tx to prevent BV and VVC recurrence.	Non-randomised prospective cohort study	In women with recurrent VVC, fluconazole (7 days in cycle 1 followed by once weekly in cycles 2+3, and biweekly in cycles 4-6) with EcoVag (10 days in cycle 2, and once weekly in cycles 2-6) did not significantly improve the VVC cure fraction 6 months after treatment cessation compared to the fluconazole regimen without EcoVag (8/9 versus 7/10, resp.) but almost all women in both groups were cured.
Kovachev[193]	Lactagyn (*L. acidophilus, L. rhamnosus, S. thermophilus, L. delbrueckii* subsp. *bulgaricus*) as maintenance tx to prevent VVC recurrence.	RCT: no-intervention control	In women with acute VVC, antifungal treatment (fluconazole and fenticonazole) followed by 10-day Lactagyn therapy resulted in lower VVC recurrence (10/209) at the final follow-up visit 25-30 days after product cessation than antifungal treatment alone (76/207). The authors report this as two non-significant pre/post results, but these are significant when the appropriate test (McNemar) is used, and when the two groups are compared directly.
Rapisarda[145]	*L. acidophilus* LA14 as main tx for BV. VVC also assessed.	Pre-/post-intervention study	In women with NS 4-10 and 21/60 with acute VVC, 14 days of *L. acidophilus* LA14 without antifungals or antibiotics resulted in a significant reduction of VVC cases to 9/60 one day after cessation of use, and 6/60 4 weeks after cessation of use. Mean *Candida* culture counts also decreased significantly.
Murina[194]	Estromineral Probiogel *(L. fermentum* LF10 + *L. plantarum* LP02) as main tx for BV and VVC.	Pre-/post-intervention study	Estromineral Probiogel (without antifungal) cured 51/82 women with acute VVC and 27/27 in women with recurrent VVC between 20-30 days after therapy initiation (both significant compared to baseline), but therapy longer than the intended 6 days was required in 57.3% and 63.0% women, respectively, due to persistence of symptoms.

BV bacterial vaginosis, *M1/6* Month 1/6, *tx* therapy, *UTI* urinary tract infection, *VMB* vaginal microbiome, *VVC* vulvovaginal candidiasis. Studies examining the efficacy of vaginal probiotics on VVC (by wet mount or fungal culture) rates were selected for inclusion in this table. There were no studies with *Candida* by molecular methods outcomes. See Appendix table D.1 for in-depth study characteristics. The Rwanda VMB Study is not shown in this overview as this study had no VVC cases during follow-up (see Chapter 3).

We found eleven VVC articles with high overall risk of bias; six of these were pre/post interventional studies rather than RCTs (table 5.3).[145,184,185,188,189,191,194] Two studies were non-randomised cohort studies with parallel comparison groups (one included a placebo comparison group,[192] and one compared women using fluconazole together with probiotic to women using fluconazole only[186]), and three studies were RCTs (one with a placebo group,[67] one study with a group without interventions,[193] and one study compared women using itraconazole together with probiotic to women using itraconazole only[190]). Of these eleven high-risk VVC studies, five showed significantly beneficial effects of vaginal probiotic use, three studies showed non-significant beneficial effects or had mixed results, and three studies showed no beneficial effects of probiotic use. Four studies focussed on VVC cure: all were pre/post intervention studies in which no antifungals were given to the participants.[145,189,191,194] Cure rates were high (57-100%) but all women received the interventions, and none of the studies reported long-term VVC recurrence. Four other studies assessed VVC recurrence after combined probiotic/antifungal treatment:[186,190,192,193] the two studies examining antifungal treatment combined with probiotic use showed no differences in VVC recurrence,[186,190] whereas the two studies that compared probiotic after antifungals with placebo[192] or no intervention[193] showed significantly lower VVC

recurrence. Two other studies assessed VVC incidence as secondary outcomes after therapy for BV[188] or non-gynaecological infections[67]: the first study showed that Gynoflor with or without metronidazole resulted in a lower VVC incidence than when using metronidazole alone,[188] whereas the second study showed no benefit of probiotic maintenance therapy to prevent post-antibiotic VVC.[67] A study in which postmenopausal women atrophic vaginitis received probiotics without antifungals showed no difference in VVC prevalence before and after use.[184,185]

Probiotic strain detection studies

Fourteen studies assessed the vaginal detection of probiotic strains and/or their relative abundance, concentrations, or colonisation capacity (with the Rwanda VMB study reporting results for two probiotics; Appendix table D.3), using a variety of molecular techniques as described previously. Only three studies had probiotic strains detected in all probiotic users.[143,196,197] Overall, probiotic strains were detected in 59-100% of users or in 20-39% of collected swabs during use or directly after product cessation. None of the vaginal detection studies accurately assessed the timing between last probiotic insertion and vaginal sample collection for detection measurement. In eleven products, probiotic strain detection was assessed more than a week after cessation of use: these studies showed that detection rates decreased after ceasing the product.[142,143,150,151,160,186,195–197] Most studies only assessed this at one single timepoint, and the assessment varied widely, between two weeks to six months after product cessation. Three studies associated adherence to probiotic strain retrievability and found that probiotic strains were not always detected in women using all products according to schedule,[160,183,186] but two of these studies only assessed adherence by self-report. There were two studies that reported individual cases in which probiotic strains dominated the VMB at a relative abundance of 50% or more,[155,160] but none of the studies that combined probiotic detection with a measure of absolute concentration or relative abundance found that probiotic strains dominated the vagina in all users.[142,143,155,160,186] Three studies reported probiotic concentrations: an Italian study using SYNBIO gin showed mean concentrations of the probiotic strains of around 9 $\log_{10}$ cells/µl (the article reports this as 6 $\log_{10}$ cells/g; I presumed one gram of DNA buffer equals a volume of around 1,000 µl) directly after administration.[143] The Rwanda VMB study reported mean concentrations between 0.48-1.92 $\log_{10}$ cells/µl of Ecologic Femi+ strains and mean concentrations between 0.25-1.05 $\log_{10}$ cells/µl of Gynophilus LP strains at the study visits during product use.[160] These concentrations were however much higher in women when any probiotic strains were detected, of around 4 $\log_{10}$ cells/µl. A French phase I safety study found that Gynophilus strain concentrations were around or above 10^7 CFU/ml,[155] which is comparable to the concentrations found in the Rwanda VMB study. Interestingly, two studies found that *L. crispatus* CTV-05 was less often detected in women who had sexual intercourse during the product use period, regardless of whether the intercourse was protected or not,[128,150] and four studies (of which three in women using *L. crispatus* CTV-05) found that detection was less likely if women had

native lactobacilli prior to probiotic use (*L. crispatus* in the case of the CTV-05 studies and any lactobacilli in the case of an EcoVag study).[76,128,150,186]

Studies comparing multiple, differing treatment strategies

A Turkish study in women with BV compared Gynoflor as main therapy, metronidazole with Gynoflor as adjuvant therapy, and metronidazole only: it was found that the cumulative incidence of VVC was comparable between the former two groups (3/96 and 2/97, respectively), and lower than when using metronidazole alone (14/114).[188] A Norwegian study found that EcoVag probiotic was not effective as adjuvant therapy for BV compared to placebo (32/50 versus 37/50, respectively), but was effective as maintenance therapy as BV/intermediate microbiota recurrence during maintenance therapy was significantly lower in the probiotic group than in the placebo group (13/37 versus 21/39, respectively).[135] The same probiotic but in different doses or formulations was compared in one *L. crispatus* CTV-05 study[150] and in one Gynophilus study,[155] but none of these studies reported efficacy outcomes (see Appendix table D.3 for outcomes of these two vaginal detection studies). An Australian study compared a vaginal (Femilac) and an oral (Lactobac) probiotic to prevent VVC in women using antibiotics for non-gynaecological infections, showing no benefit for either probiotic compared to placebo.[67] Two studies compared vaginal probiotics directly with each other:[160,196] one study only reported detection results[196] (Appendix table D.3), while the Rwanda VMB study showed significantly lower BV recurrence, and higher *Lactobacillus* and lower BV-associated anaerobes relative abundance in intermittent Ecologic Femi+ users but not in intermittent Gynophilus LP users. However, this study was a pilot study with preliminary efficacy outcomes only, and all effect sizes of Gynophilus LP users were in the same direction as Ecologic Femi+ users.[160]

5.4 Discussion

We examined the impact of vaginal probiotics on the VMB in this systematic review and showed that published clinical efficacy studies have shown promise for BV cure and prevention but much less so for VVC cure and prevention. The five BV studies (with six results) with medium overall risk of bias suggested that vaginal probiotic use may have benefits: four results were significantly positive, and two were positive but non-significant. The effect of probiotics, in most cases, seemed to be greatest when used to prevent BV recurrence, and was limited to the period of use and disappeared after product cessation. The eight BV studies with a high overall risk of bias often lacked the statistical power to detect any robust effect, but five out of eight studies also showed positive effects. Overall, most results were moderate in size and probiotics were usually less effective than antibiotic use when comparisons of this nature were made. We also showed that vaginal probiotics may have positive effects on the VMB, for example by increasing the presence of *Lactobacillus* relative abundances and/or concentrations or by decreasing the prevalence of BV-like VMB types. Unfortunately, the studies with molecular VMB outcomes were very heterogenous, reported results were sparse, and most of these

trials had major methodological issues such as loss to follow-up;[141] this make it difficult to interpret the significance of the molecular VMB results. All but one VVC efficacy studies were judged to have a high overall risk of bias, and many of these were pre/post interventional studies rather than RCTs. Five of twelve VVC efficacy studies showed positive effects, but it should be noted that four of these were pre/post studies with often limited follow-up time and no comparison groups. As it is known that *C. albicans* is highly inflammatory and predominantly occurs in and favours an optimal *Lactobacillus*-dominated environment,[4,9,87] it can be doubted whether *Lactobacillus*-based probiotics could be efficacious in reducing VVC rates.

Most of the vaginal detection studies showed that the probiotic strains are not detected in all users or in all vaginal swabs taken during use. The concentrations or relative abundances of vaginal probiotics were often low when measured, and the strains generally did not persist in the vagina after product cessation. This is most likely due to issues regarding adherence (social desirability bias playing an important role), infrequent or low dosing and formulation problems, strain selection and strain survivability in the vagina, and/or study designs in which it was difficult to accurately associate probiotic insertion timing with vaginal swab sampling timing. While it could be argued that probiotics do not need to dominate the VMB in order to exert their beneficial effects, the findings of this review do beg the question whether the dose contained in vaginal probiotics, which in these studies never exceeded 10^{10} CFU/dose and was around 10^{8} CFU/dose in most products, should be increased further to guarantee a higher relative abundance of probiotic strains in the VMB. Another possibility is that dosages should be given more frequently to ensure proper delivery in the vaginal niche. We could not comprehensively answer our third research question on the best treatment strategy to use probiotics. Antibiotic treatment of BV is often effective but recurrence rates are high.[46,47] Given that we found BV studies using probiotics to prevent BV were successful the most often, it may be more effective to use vaginal probiotics either as adjuvant BV therapy together with antibiotics, or as maintenance therapy to prevent BV, and not as main BV therapy without antibiotic treatment. However, this point of view is a recommendation only and evidence in the form of studies directly comparing different treatment strategies is lacking. None of the probiotic studies included reported major safety concerns for vaginal probiotics use.

The studies that we included were very heterogeneous, with little consistency when it comes to the products/strains used, the dosage and formulation types, the indication, the dosing strategy, or outcomes. Probiotics received little regulation by regulatory bodies until fairly recently and therefore many different products exist;[71] we opted to conduct a review including all *Lactobacillus*-containing vaginal probiotics and not limit ourselves to one probiotic product. Due to heterogeneity in study design and a lack of studies comparing multiple types of probiotics, it is difficult to ascertain which probiotic strains or products were the most successful. It has previously been shown that oestrogen favours a

healthy VMB dominated by lactobacilli.[26,171] This would make combinations of low-dose oestradiol and lactobacilli such as used in Gynoflor promising, especially in post-menopausal women, but there is no epidemiological evidence available to prove this. *L. crispatus* is the most favourable *Lactobacillus* species,[4,8] and therefore would make a sensible probiotic candidate, but several studies show *L. crispatus* CTV-05 detectability is low in women who have native *L. crispatus*.[76,128,150] *L. crispatus* may however be a good option for women whose native optimal VMB is composed of *L. iners*, e.g. in African or African American women, being at greatest risk of vaginal dysbiosis-related sequelae. However, there is no current evidence for this. In high-resource settings, stratified or personalised probiotic recommendations based on a woman's individual (VMB) characteristics may be promising.

The vast majority of included studies had a high overall risk of bias and many methodological flaws, and the interpretability of the efficacy results was therefore limited. Most studies lacked the statistical power to compare responders to non-responders. Future studies should be adequately powered to detect differences in BV/VVC cure or recurrence rates between study arms. Trials should ideally include negative control arms, and positive control arms with antibiotic or antifungal therapy. If probiotic therapy is initiated after antibiotic/antifungal treatment, laboratory and microbiological testing should be performed prior to initiation of probiotics to ensure a healthy VMB at baseline and allow for proper examination of BV/VVC recurrence. Adherence assessments should be included at regular intervals and reported clearly. Furthermore, RCTs using vaginal probiotics should include assessments of the efficacy of the products at regular intervals during product use (including precise timings of product insertion and vaginal swab sampling), directly after stopping study products, and at a time point after product cessation spanning multiple menstrual cycles. In at-risk populations HIV/STI incidence during follow-up should be recorded. The Amsel criteria[77] should not be used in clinical studies due to its low sensitivity and specificity. Studies should use either Nugent[78] or Ison-Hay[174] Gram stain scoring (both at baseline as part of the eligibility criteria and during follow-up), and should incorporate molecular methods as outcomes by (16S rRNA or cpn60 gene) sequencing and/or species-specific qPCRs. Using molecular methods permits to describe the VMB more in-depth than Gram stain scoring methods, and precision and statistical power increase when the outcomes are expressed as continuous relative abundances and/or concentrations rather than Gram stain scoring categories. Quantification of the probiotic strains and main bacterial species present in the VMB, instead of merely reporting relative abundances, is now obtainable and highly advisable.[129,130] Future studies should incorporate more robust statistical analyses such as incidence rate ratios (comparing incidence in intervention groups to control groups), Cox regression combined with Kaplan-Meier curves in the case of BV or VVC recurrence rates, and/or mixed effects models. Sub-analyses (for example comparing responders to non-responders) or per protocol analyses should also be performed, as well as statistical analyses with adjustments for confounding factors known to affect the VMB, such as sexual behaviour (including condom use), hormonal contraception, pregnancy status, and menses,[4,26,156,198] if sample size permits: while

confounding factors or baseline imbalances can be accounted for without statistical adjustments in studies with a high number of participants, this is not the case in trials with a low sample size.[199] Acceptability assessments of the vaginal products (by surveys and/or qualitative in-depth interviews) should be performed and reported when unknown in that specific population to ensure proper adherence. Finally, (serious) adverse events should be reported systematically in accordance with international guidelines.[200,201]

The strength of this systematic review is that we evaluated the use of vaginal probiotics in the broadest way possible, assessing the impact of vaginal probiotics from several viewpoints, such as VMB outcomes, probiotic products/strains used, and treatment strategy of the trial. The limitation of this systematic review is that many of the included studies had a small number of participants, used differing *Lactobacillus* species and strains, had differing and/or no control groups, had a short follow-up, and were of (very) low methodological quality. Conducting a meta-analysis on BV and VVC rates/proportions was not possible due to the high heterogeneity of the studies and the often-unclear presentation of results. As well as the other risk of bias items mentioned, there is a risk of publication bias as studies with positive and significant results are more likely to be submitted, and to be accepted, for publication. We did not examine the impact of oral probiotics on the VMB in this systematic review. The rationale is that we thought it would be likely that the effect of oral probiotics is more diluted than that of vaginal probiotics as the former are not delivered directly at the intended target site; the impact of oral probiotics on the VMB could be the subject of a separate systematic review.

To conclude, the currently available evidence that vaginal probiotics are helpful in improving VMB composition is limited but most studies with BV rates as an outcome showed a decrease in BV recurrence, and most studies with molecular VMB outcomes showed an increase in lactobacilli. The evidence to use vaginal probiotics to cure or prevent VVC is very limited. The interpretability of the results in the studies was severely hampered by their poor methodological quality. Well-powered and well-conducted clinical trials that incorporate in-depth molecular analyses, adjust for known confounding factors, and compare responders to non-responders are needed.

Chapter 6 - Targeted Point-of-Care Testing Compared to Syndromic Management of Urogenital Infections in Women: a Cross-Sectional Screening and Diagnostic Accuracy Study

This chapter has been published in *The Lancet Infectious Diseases*. The version presented here is the author-approved and Lancet-accepted version with only minor modifications (title of the chapter, numbering of figures, tables, and references). The version that has since appeared in *The Lancet Infectious Diseases* was edited by the Lancet editorial team to conform to Lancet style: Verwijs MC, Agaba SK, Sumanyi JC, Umulisa MM, Mwambarangwe L, Musengamana V, Uwineza M, Cuylaerts V, Crucitti T, Jespers V, van de Wijgert JHHM. Targeted point-of-care testing compared with syndromic management of urogenital infections in women (WISH): a cross-sectional screening and diagnostic accuracy study. *Lancet Infect Dis* 2019; 19(6):658-69.

I oversaw the implementation of the approved study protocol by having weekly teleconference calls with the study team at the Rinda Ubuzima research clinic in Kigali, Rwanda, and by conducting three monitoring visits to that clinic during and immediately after completion of the data collection. I developed the analytical approach and performed the statistical analyses under the supervision of my primary supervisor Professor Janneke van de Wijgert. My primary supervisor and I wrote the manuscript together. All authors commented on and approved the final manuscript.

Abstract

Introduction: Sexually transmitted and urogenital infections are typically managed by World Health Organisation (WHO)-recommended syndromic algorithms in resource-poor countries, and presumptively in Europe. Vaginal discharge (VDS) and lower abdominal pain (LAP) algorithms in women perform poorly. The main aim of the WISH study was to compare the performances of VDS/LAP algorithms incorporating point-of-care tests (POCTs), and of WHO syndromic algorithms, with gold standard test results.

Materials and Methods: At-risk Rwandan women (N=705) underwent POCTs for bacterial vaginosis (BV; vaginal pH≥5.0) and *Trichomonas vaginalis* (TV; OSOM) regardless of symptom-reporting. Women with a positive risk score were POC-tested for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (CT/NG; GeneXpert). Vulvovaginal candidiasis (VVC) was treated presumptively. Nucleic acid amplification tests (NAATs) were done for CT/NG, TV, BV, and VVC on everyone and were used as gold standards.

Results: NAAT-based prevalences were: 60/705 (8.5%) CT, 50/705 (7.1%) NG, 111/690 (16.1%) TV, 125/690 (18.1%) BV, and 59/690 (8.6%) VVC. Infection-specific sensitivities of the WHO VDS/LAP algorithms ranged from 58.3-74.6%, and specificities from 44.7-50.6%. WISH POCT-based algorithms had good sensitivity (68.5-76.0%) and specificity (97.4-100%) for CT, NG, and TV but low specificity for BV (41.2%; sensitivity 95.2%), and modest sensitivity (64.4%) and specificity (69.4%) for VVC. Sensitivity (73.6%) and specificity (100%) for BV improves by screening all women for vaginal pH, and confirmatory testing of those with pH≥5.5 (n=275). Staff and participants considered POC testing feasible and acceptable.

Discussion: POC testing for urogenital infections improves performance and is feasible in resource-poor settings. POCT development should continue, including POCTs targeting multiple conditions. Additional studies in other populations, including low prevalence populations, are warranted.

6.1 Introduction

Sexually transmitted infections (STIs) and other urogenital infections cause a major burden of disease worldwide.[40] The World Health Organisation (WHO) estimated that 357 million new curable infections caused by *Chlamydia trachomatis* (CT), *Neisseria gonorrhoea* (NG), *Trichomonas vaginalis* (TV), and *Treponema pallidum* (syphilis) occurred in 2012.[38] Furthermore, in women, bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and urinary tract infections (UTIs) are common.[87,202] Long-term sequelae include increased risk of HIV acquisition and transmission, pelvic inflammatory disease, pregnancy complications, and invasive neonatal infections.[17,87,203]

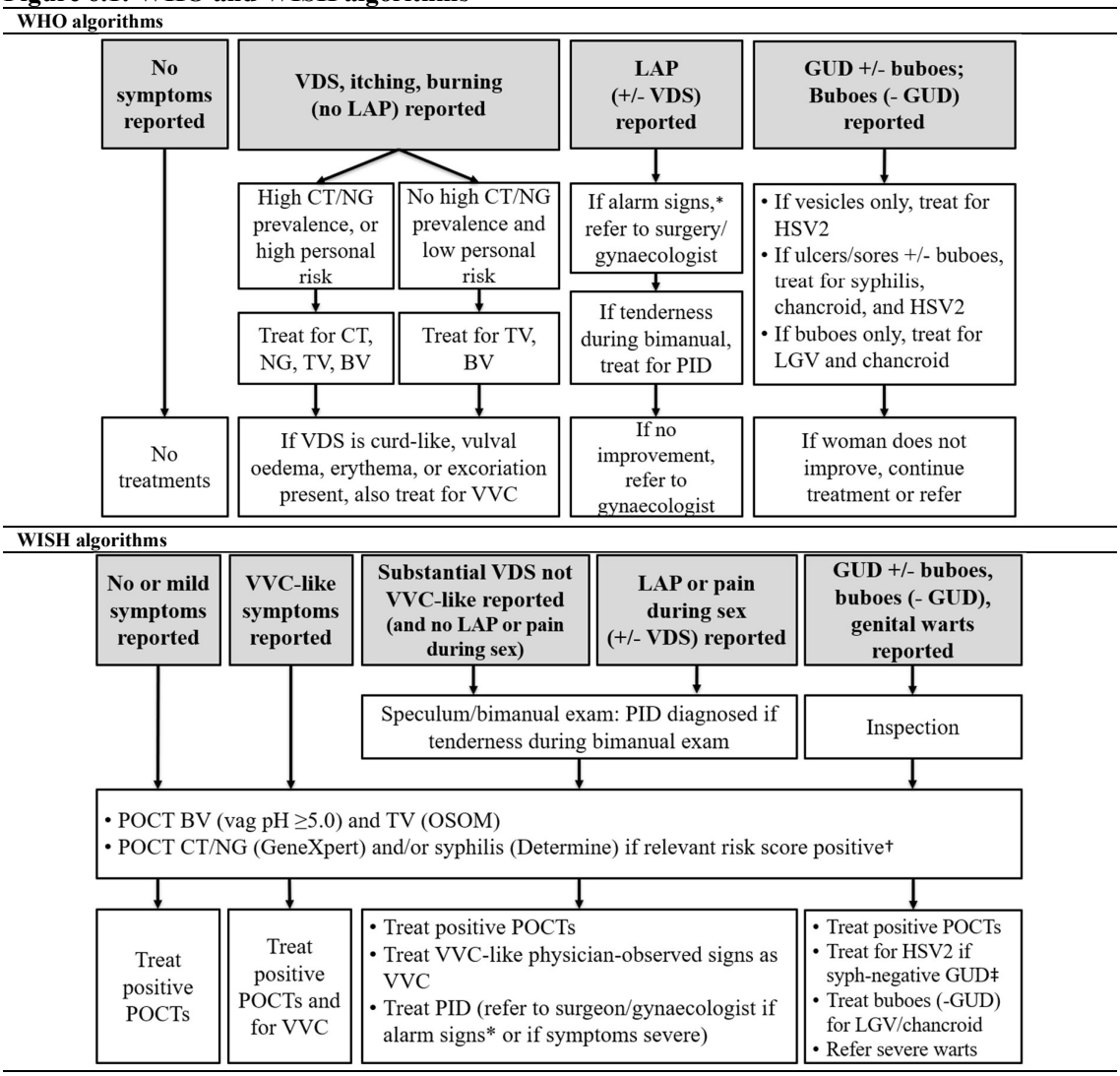
Most resource-poor countries diagnose genital infections syndromically, using local guidelines that are based on the WHO Guidelines for the Management of Sexually Transmitted Infections (figure 6.1).[21,93] Each patient-reported symptom, potentially augmented by clinician-observed signs during physical examination, is treated for all organisms that might cause that symptom.[21] In women, the four main syndromes on which the WHO algorithms are based are vaginal discharge syndrome (VDS) without lower abdominal pain (LAP), LAP with or without VDS, genital ulcers with or without inguinal buboes, and inguinal buboes without genital ulcers (figure 6.1).[21] The VDS syndrome is the most common.[93,94] The WHO recommends that VDS is always treated for BV and TV, is treated for CT and NG if local prevalence is high and/or locally designed risk assessments are positive, and is also treated for VVC if the discharge is curd-like and vulval oedema, erythema, and/or excoriations are present (figure 6.1). In Europe, most STIs and urogenital infections in women are treated presumptively by primary care physicians.

Syndromic and presumptive approaches miss all asymptomatic infections by definition. Asymptomatic infections in women are common, and are associated with the complications outlined above.[9,87] Furthermore, many studies in different countries have shown that the performance of VDS and LAP algorithms in symptomatic women are suboptimal, leading to under-, over-, and inadequate treatment of patients.[94,97,98,100]

The Women's Improvement of Sexual and reproductive Health Study (WISH) in Kigali, Rwanda, sought to improve case-finding and infection management in women by introducing point-of-care tests (POCT).[105] Our aim was to use POCTs that comply with the WHO ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end users) as much as possible.[108,106] Participants were first asked about urogenital symptoms as if we were to provide them with syndromic care, but were then offered POCT-based WISH algorithms (see Methods). Stored samples from all women were also tested by nucleic acid amplification (NAAT) gold standard tests. This design allowed us to compare the performance of the WISH algorithms with the WHO algorithms

and with gold standard results, to evaluate the feasibility and acceptability of the WISH algorithms, to recommend optimal algorithms given currently available POCTs, and to identify POCT development gaps.

Figure 6.1: WHO and WISH algorithms



POCT descriptions

POCTs included in algorithms:

- CT/NG: GeneXpert CT/NG assay (Cepheid, Sunnyvale, USA).
- TV: TV OSOM assay (Sekisui Diagnostics, Lexington, USA).
- BV: EcoCare vaginal pH swab (Merete Medical, Luckenwalde, Germany) with pH ≥5.0 considered positive.
- Syphilis: Alere Determine Syphilis rapid test (Alere, Waltham, USA) with confirmation of active infection by the rapid plasma reagin test (SpinReact, Girona, Spain).

POCTs not included in algorithms, and not evaluated in the WISH study, but offered to participants:

- UTI: urinalysis dipstick test (Acon, San Diego, USA). Any nitrite and/or leukocytes were considered UTI-positive as recommended by the Rwandan Ministry of Health.
- HIV: Determine HIV-1/2 rapid test (Alere, Waltham, USA), followed by Trinity Biotech Uni-gold HIV rapid test (Trinity Biotech, Bray, Ireland) if reactive, and Vironostika ELISA (Biomerieux, Marcy, France; performed at the National Reference Laboratory in Rwanda) as a tie-breaker if needed.
- Pregnancy: Nova human chorionic gonadotropin urine dipstick test (Atlas Link Technology Co., Beijing, China).

BV bacterial vaginosis, *CT Chlamydia trachomatis, GUD* genital ulcer disease, *HSV2* Herpes simplex virus type 2, *LAP* lower abdominal pain, *LGV* lymphogranuloma venereum, *NG Neisseria gonorrhoeae, PID* pelvic inflammatory disease, *POCT* point-of-care test, *TV Trichomonas vaginalis, UTI* urinary tract infection, *Vag* vaginal, *VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis.
The top rows are symptoms reported by participants. For details about the syndromic management algorithms, see WHO guidelines.[21]

*LAP (WHO) and LAP or pain during sex (WISH) required examination. Alarm signs: missed/overdue period, recent delivery/abortion/miscarriage, abdominal guarding and/or rebound tenderness, abnormal vaginal bleeding, and abdominal mass. Treated for PID if no alarm signs but cervical motion, uterine, and/or adnexal tenderness present, or when lower abdominal tenderness and VDS present. PID treatment covered CT/NG/TV/BV. The VDS algorithm is followed if no alarm signs or PID. †One or more of the following: currently pregnant, exchanged sex for money or goods in the past 12 months, new sexual partner in the past three months, or relevant clinical signs observed by a physician. For the CT/NG score, these were VDS with an offensive smell and/or PID. For the syphilis score, these were GUD and/or inguinal buboes observed by a physician. ‡Syphilis-negative GUD to be treated for *H. ducreyi* if no response to HSV2 treatment.

6.2 Materials and Methods

This cross-sectional study was carried out at the Rinda Ubuzima research clinic and laboratory in Kigali by staff with multiyear experience in sexual and reproductive health care. Our aim was to recruit women at risk of HIV and STIs but at varying degrees of risk and not exclusively sex workers. Recruitment activities were implemented by study staff with the help of 'community mobilisers.' Two of them were women who had taken part in previous Rinda Ubuzima studies, and one was a community organiser. They organised recruitment meetings, and distributed flyers at health centres, pharmacies, markets, and at women's organisation and 'umuganda' community meetings. Participants were encouraged to refer their friends. Women aged 18 or older at risk of STIs (defined as having had more than one sex partner and/or having been treated for at least one STI in the past year) with or without urogenital symptoms were enrolled between July 2016 and March 2017. HIV-positive and pregnant women were not excluded. Women were told that they would be screened for urogenital infections free of charge, that they could only be screened once, and that they would not receive a monetary reimbursement for participation.

Clinic visit procedures

At the study visit, participants underwent a face-to-face interview that included questions about current (including past two weeks) urogenital symptoms. Participants were first asked if they experienced any symptoms without prompting ('spontaneously reported'), followed by questions about 14 specific symptoms ('structurally reported'). All women were offered comprehensive counselling and could select topics themselves.

Next, the WISH algorithms were implemented (figure 6.1, which includes POCTs details, and Appendix E, pages 174-175, for reasons why these POCTs were selected). All women were offered HIV, pregnancy, TV, and BV testing. We used ASSURED POCTs to diagnose HIV, pregnancy, and TV (OSOM), and a vaginal pH swab to diagnose BV (EcoCare; pH≥5.0 was considered BV). Since ASSURED POCTs for CT and NG with adequate performance are not yet available,[204,205] we used the GeneXpert CT/NG assay. This POC NAAT assay is more than 95% sensitive and specific for both organisms,[91] but requires equipment, is expensive (we paid 18.25 USD for consumables per test), and takes 90 minutes to return results. We therefore only offered GeneXpert testing to women who had a positive CT/NG risk score (figure 6.1). We used an ASSURED POCT for syphilis (Determine, with a confirmatory rapid plasma reagin test), but only offered testing to women who had a positive syphilis

risk score (figure 6.1) because the syphilis prevalence was expected to be low.[25,206]

The WISH algorithms called for mild VDS to be ignored if none of the POCTs were positive and for VVC-like symptoms to be treated for VVC presumptively. Women reporting substantial VDS not typical for VVC and/or LAP (including pain during sex) were to be offered a speculum and bimanual examination by a physician. Physician-observed substantial VDS or pelvic inflammatory disease resulted in a positive CT/NG score followed by CT/NG testing, and pelvic inflammatory disease was to be treated even if the test was negative. Women with genital ulcers/buboes would be offered inspection by a physician. Any visible lesions resulted in a positive syphilis risk score followed by syphilis testing, and would be diagnosed as syphilis, syphilis-negative genital ulcer disease, inguinal buboes without genital ulcers, and/or genital warts. Urinalysis testing was only offered to women reporting urinary symptoms and the presence of any nitrite and/or leukocytes in urine was considered a UTI.

Blood (4.5 ml EDTA), urine, and TV OSOM and EcoCare vaginal swabs were collected as required to implement the WISH algorithms. In addition, a GeneXpert swab and two polyester vaginal swabs were collected from all women for gold standard testing (see below). Treatment, partner notification, and referral procedures are described in Appendix E, pages 178-179 and table E.1.

Participants could opt out of each service offered and the reasons were recorded. Our aim was to deliver all services during the main visit within half a day. However, women could choose to leave before having received all results, and either return for a scheduled additional visit or receive instructions regarding the need for follow-up via letter or mobile phone. Care was taken to preserve participant confidentiality throughout the study.

Gold standard testing

The CT/NG GeneXpert assay was considered a gold standard because of its excellent performance compared to other validated CT/NG NAAT tests.[91] While only women who had a positive CT/NG score were tested that same day, GeneXpert swabs were also taken from all other women and tested in batches. All other gold standard NAAT tests were conducted in the STI reference laboratory of the Institute of Tropical Medicine in Antwerp, Belgium: TV, *Mycoplasma genitalium* (MG), *Candida albicans*, *Lactobacillus* genus, *Gardnerella vaginalis*, and *Atopobium vaginae* on vaginal swabs, and *Escherichia coli* on urine (Appendix E, pages 175-177).[83] *C. albicans* qPCR is not a true gold standard test for VVC, but we will refer to it as such in this paper (Appendix E, page 176). True BV cases were determined by a validated vaginal qPCR score [$\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*) below -2] (Appendix E, page 177).[85] HIV, syphilis, pregnancy, and UTI POCTs were offered as a service to participants and their performances were not evaluated.[109,110]

Acceptability and feasibility

A subset of participants (n=107) were interviewed about their experiences with WISH procedures by an interviewer with no previous relationship with the interviewee using a semi-structured 'client satisfaction survey' (Appendix E, page 180). Another subset of participants (n=20) was observed and timed throughout their clinic trajectory.

Statistical analysis

The target sample size was 500 to 1,000 participants, depending on resources. With a sample size of 500, we expected to identify 50-175 true cases of each relevant infection.[25,206] Statistical analyses were performed using Stata 13 (StataCorp, College Station, USA). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with 95% confidence intervals were calculated for proportions of women that would have been or were treated for a specific VDS/LAP-associated organism (CT, NG, TV, BV, and VVC; table 6.1) in accordance with the WHO and WISH algorithms, respectively, each compared to gold standard cases. We used the WHO VDS algorithm in the absence of physical examination and assumed that all women with VDS would have been treated for CT and NG (in addition to TV and BV) due to high prevalences in our study population (Appendix E).[25,206]

Table 6.1: Conditions under which organism-specific treatments were dispensed, or would have been dispensed, in each algorithm

Organism	WHO algorithms	WISH algorithms	Gold standard
CT	– VDS and high risk or high local prevalence – PID (after bimanual for LAP)	Positive CT GeneXpert, after positive risk-scoring‡	Positive CT GeneXpert
NG	– VDS and high risk or high local prevalence – PID (after bimanual for LAP)	Positive NG GeneXpert, after positive risk-scoring‡	Positive NG GeneXpert
TV	– VDS – PID (after bimanual for LAP)	Positive TV OSOM, on everyone	Positive TV PCR
BV	– VDS – PID (after bimanual for LAP)	Vaginal pH ≥5.0 on EcoCare pH swab, on everyone	BV by vaginal qPCR score§
VVC	– VDS that is curd-like, vulval edema, erythema, or excoriations present*	VVC-like symptoms reported (see WHO algorithm) or observed	Positive *Candida albicans* qPCR
Syphilis	– GUD (after visualisation by provider)†	Positive syphilis POCT confirmed by RPR, after positive risk-scoring‡	Not available

BV bacterial vaginosis, *CT Chlamydia trachomatis*, *GUD* genital ulcer disease, *LAP* lower abdominal pain, *NG Neisseria gonorrhoeae*, *PID* pelvic inflammatory disease, *(q)PCR* (quantitative) polymerase chain reaction, *RPR* rapid plasma reagin, *TV Trichomonas vaginalis*, *VDS* vaginal discharge syndrome, *WHO* World Health Organisation.
See Appendix E for rationale behind choice for gold standard tests.
*The latter three WHO-specified criteria (vulval edema, erythema, or excoriations) seem inappropriate for the algorithm without examinations. We therefore assumed that a woman would have been treated for VVC if she reported VDS, and in addition, this VDS was curd-like and/or occurred with genital itching or burning. †Only women with a positive RPR and no recent treatment should be treated. However, in the absence of this information, we assumed that all women with GUD would have been treated for syphilis. ‡See figure 6.1 for CT/NG and syphilis risk scores. §See methods for the vaginal qPCR score.

Ethical statement

All participants provided written informed consent. The study was sponsored by the University of Liverpool, approved by the Rwanda National Ethics Committee and the University of Liverpool

Research Ethics Subcommittee for Physical Interventions, and registered in ClinicalTrials.gov (NCT03045809).

6.3 Results

All 705 enrolled participants completed a study visit, and 51 women attended 53 additional visits to receive results and/or treatment (n=40 women), because of persistent or new symptoms (n=11), because of a mild allergic reaction to metronidazole (n=2; no other adverse events or social harms reported), for a speculum examination (n=1), and/or to obtain information (n=1). Participants were a median of 32.9 years (range: 18-62), 65.4% were never married, 32.2% reported a new sex partner in the past three months, and 35.5% had engaged in sex work in the past 12 months (table 6.2). The majority of women reported to ever have been tested for HIV (99.0%) or to ever have been treated for an STI (71.2%), with 19.0% reporting to be living with HIV. Most women reported at least one urogenital symptom (85.7%), and more symptoms were reported structurally than spontaneously (Appendix, table E.2). Of the women structurally reporting symptoms, 7.1% had already sought medical care for these symptoms, and 17.1% had used traditional medications.

Algorithm performances and optimal algorithms

Table 6.3 shows the percentages of women who were treated, or would have been treated, for specific infections based on WHO, WISH, and gold standard algorithms. Almost half (306/690, 44.3%) of the women had at least one VDS/LAP-associated infection by gold standard testing: 60/705 (8.5%) CT, 50/705 (7.1%) NG, 111/690 (16.1%) TV, 125/690 (18.1%) BV, and 59/690 (8.6%) VVC; 229/690 (33.2%) had one VDS/LAP-associated infection and 77/690 (11.2%) had two or more infections (correlation matrix of gold standard infections shown in Appendix, table E.3).

Table 6.2: Characteristics of study participants

Demographic data	N (% of 705) or median (IQR)
Age, years	32.9 (28.2-38.0)
Marital status	
- Never married	461 (65.4)
- Married	209 (29.7)
- Divorced	18 (2.6)
- Widowed	17 (2.4)
Highest educational level attained	
- No schooling	113 (16.0)
- Primary school not completed	159 (22.6)
- Primary school completed	176 (25.0)
- Secondary school not completed	135 (19.2)
- Secondary school completed	90 (12.8)
- More than secondary school	32 (4.5)
Sexual history	
Male sex partners in lifetime	4 (2-8)
Male sex partners in past 12 months	2 (1-3)
New sex partner in the past three months	227 (32.2)
Currently has a main sex partner	618 (87.7)
Number of vaginal sex acts in the past two weeks	4 (2-10)
Has had anal sex in the past two weeks	7 (1.0)
Condom use during vaginal sex in past 2 weeks (N=704)	
- Always	24 (3.4)
- Sometimes but not always	114 (16.2)
- Never	566 (80.4)
Used condom during last vaginal sex act (N=704)	83 (11.2)
Exchanged sex for money or goods in past 12 months	250 (35.5)
Reproductive and contraceptive history	
Pregnancies in lifetime	3 (2-4)
Ever used a product to prevent pregnancy	527 (74.8)
Currently using a product to prevent pregnancy	222 (31.5)
If yes, product currently using (N=222):	
- Combined estrogen/progestin pills*	38 (17.1)
- Progestin injections†	61 (27.5)
- Progestin implant‡	77 (34.7)
- Copper IUD	37 (16.7)
- Participant is sterilised	9 (4.1)
General medical history	
Is currently taking an antibiotic or antifungal§	134 (19.0)
Tested for HIV in the past	698 (99.0)
Number of times tested for HIV in lifetime	4 (3-6)
Known to be HIV-positive prior to main visit	135 (19.2)
Treated for an STI in the past	502 (71.2)
Treated for BV in the past	35 (5.0)
Treated for VVC in the past	129 (18.3)
Treated for UTI in the past	100 (14.2)
Participant-reported symptoms	
Any urogenital symptom – structurally reported	604 (85.7)
VDS curd-like	265 (37.6)
VDS offensive-smelling	119 (16.9)
LAP	245 (34.8)
Genital ulcers/blisters/sores	41 (5.8)
Inguinal buboes	1 (0.1)
Genital warts	0

BV bacterial vaginosis, *IQR* interquartile range, *IUD* intra-uterine device, *LAP* lower abdominal pain, *STI* sexually transmitted infection, *UTI* urinary tract infection, *VDS* vaginal discharge syndrome.
All data are self-reported by participants.

*All reported ethinylestradiol/levonorgestrel use. None reported progestin-only pills. †48 reported using depot medroxyprogesterone acetate, 13 reported norethisterone use. ‡All reported using a levonorgestrel-releasing implant. §Includes 124 HIV-positive women using trimethoprim/sulfamethoxazole prophylaxis.

Table 6.3: Infections treated, or would have been treated, using different algorithms

VDS-associated infections treated	Gold standard testing n (% of 705)	WHO algorithms*† n (% of 705)	WISH algorithms* n (% of 705)
At least one	306 (44.3)‡	392 (55.6)	608 (86.2)
CT	60 (8.5)	392 (55.6)	43 (6.1)
NG	50 (7.1)	392 (55.6)	38 (5.4)
TV	111 (16.1)‡	392 (55.6)	92 (13.1)
BV	125 (18.1)‡	392 (55.6)	466 (66.1)
VVC	59 (8.6)‡	366 (51.9)	235 (33.3)
Other conditions treated	**Gold standard testing n (% of 705)**	**WHO algorithms*† n (% of 705)**	**WISH algorithms* n (% of 705)**
MG	26 (3.8)‡	NA	NA
Syphilis	NA	16 (2.3)	21 (3.0)
PID	NA	29 (4.1)	32 (4.5)
HIV	NA	NA	55 (7.8)§
UTI	NA	NA	161 (22.8)
Pregnancy	NA	NA	33 (4.7)
Vaginal pH results	**Gold standard testing n (% of 705)**	**WHO algorithms*† n (% of 705)**	**WISH algorithms* n (% of 705)**
4.0	NA	NA	68 (9.7)
4.5	NA	NA	171 (24.3)
5.0	NA	NA	180 (25.5)
5.5	NA	NA	107 (15.2)
6.0	NA	NA	137 (19.4)
6.5	NA	NA	28 (4.0)
7.0	NA	NA	11 (1.6)
7.5	NA	NA	3 (0.4)

BV bacterial vaginosis, *CT Chlamydia trachomatis, MG Mycoplasma genitalium*, *NA* not applicable, *NG Neisseria gonorrhoeae, PID* pelvic inflammatory disease, *TV Trichomonas vaginalis, VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis, *UTI* urinary tract infection. *Algorithms are explained in figure 6.1 and table 6.1. †WHO published two VDS algorithms: one that incorporates speculum examinations and one that does not. We used the algorithm without speculum examination but with differentiation between not VVC-like (treated for CT, NG, TV, and BV) and VVC-like (also treated for VVC) based on structural reporting. ‡N=690 due to 15 invalid PCR results. §Only includes participants who tested positive for HIV at RU. Includes 24 known HIV-positive women who wanted to be retested.

An additional 26/690 (3.8%) tested MG-positive, but this infection was not included in the WHO or WISH algorithms. If the WHO VDS/LAP algorithms had been used, 392/705 (51.9%) of the women would have received treatment for all five VDS/LAP-associated infections and an additional 26/705 (3.7%) for all except VVC. The WISH algorithms identified similar numbers of CT, NG, and TV cases as gold standard testing, but much higher numbers of BV and VVC cases (table 6.3). Compared to gold standard results, the WHO VDS/LAP algorithms had moderate sensitivity (58.3-74.6%) and poor specificity (44.7-50.6%) for all infections (table 6.4). The WISH algorithms had good sensitivity (68.5-76.0%) and high specificity (97.4-100%) for CT, NG, and TV but low specificity for BV (41.2%; sensitivity 95.2%), and moderate sensitivity (64.4%) and specificity (69.4%) for VVC.

Table 6.4: Performance of the WHO syndromic algorithms, WISH algorithms, and optimal BV algorithm compared to gold standard testing

Conditions* (N=705)	GS		WHO VDS/LAP algorithms									
	Neg n	Pos n	n POC† tested	n GS† tested	TP‡ n	FP‡ n	FN‡ n	TN‡ n	Sens‡ % (95% CI)	Spec‡ % (95% CI)	PPV‡ % (95% CI)	NPV‡ % (95% CI)
CT	645	60	0	0	35	357	25	288	58.3 (45.5-70.2)	44.7 (40.8-48.5)	8.9 (6.5-12.2)	92.0 (88.4-94.6)
NG	655	50	0	0	33	359	17	296	66.0 (51.8-77.8)	45.2 (41.4-49.0)	8.4 (6.0-11.6)	94.6 (91.4-96.6)
CT and/or NG	605	100	0	0	61	331	39	274	61.0 (51.1-70.1)	45.3 (41.4-49.3)	15.6 (12.3-19.5)	87.5 (83.4-90.8)
TV	579	111	0	0	67	315	44	264	60.4 (50.9-69.1)	45.6 (41.6-49.7)	17.5 (14.0-21.7)	85.7 (81.3-89.2)
BV	565	125	0	0	77	305	48	260	61.6 (52.7-69.7)	46.0 (41.9-50.2)	20.2 (16.4-24.5)	84.4 (79.9-88.1)
BV and/or TV	481	209	0	0	124	258	85	223	59.3 (52.5-65.8)	46.4 (41.3-50.8)	32.5 (27.9-72.1)	72.4 (67.1-77.1)
VVC	631	59	0	0	44	312	15	319	74.6 (61.9-84.1)	50.6 (46.6-54.5)	12.4 (9.3-16.2)	95.5 (92.7-97.3)
	GS		**WISH algorithms**									
CT	645	60	396	0	43	0	17	645	71.7 (58.3-81.7)	100 (100-100)	100 (100-100)	97.4 (95.9-98.4)
NG	655	50	396	0	38	0	12	655	76.0 (62.2-85.9)	100 (100-100)	100 (100-100)	98.2 (96.9-99.0)
CT and/or NG	605	100	396	0	75	0	25	605	75.0 (65.5-82.6)	100 (100-100)	100 (100-100)	96.0 (94.2-97.3)
TV	579	111	690	0	76	15	35	564	68.5 (59.2-76.5)	97.4 (95.7-98.4)	83.5 (74.4-89.8)	94.2 (92.0-95.8)
BV	565	125	690	0	119	332	6	233	95.2 (89.7-97.8)	41.2 (37.2-45.4)	26.4 (22.5-30.7)	97.5 (94.5-98.9)
BV and/or TV	481	209	690	0	194	274	15	207	92.8 (88.4-95.6)	43.0 (38.7-47.5)	41.5 (37.1-46.0)	93.2 (89.1-95.9)
VVC	631	59	0	0	38	193	21	438	64.4 (51.4-75.6)	69.4 (65.7-72.9)	16.5 (12.2-21.8)	95.4 (93.1-97.0)
	GS		**Optimal BV and VVC algorithms§**									
BV	565	125	690	275	92	0	33	565	73.6 (65.1-80.6)	100 (100-100)	100 (100-100)	94.5 (92.3-96.1)
BV and/or TV	481	209	690	223	152	10	57	471	72.7 (66.3-78.4)	97.9 (96.2-98.9)	93.8 (88.9-96.7)	89.2 (86.2-91.6)
VVC	631	59	690	279	35	0	24	631	59.3 (46.3-71.1)	100 (100-100)	100 (100-100)	96.3 (94.6-97.5)

BV bacterial vaginosis, *CI* confidence interval, *CT Chlamydia trachomatis*, *FN* false negative, *FP* false positive, *GS* gold standard, *LAP* lower abdominal pain, *NG Neisseria gonorrhoeae*, *Neg* negative, *NPV* negative predictive value, *POC* point-of-care, *Pos* positive, *PPV* positive predictive value, *Sens* sensitivity, *Spec* specificity, *TN* true negative, *TP* true positive, *TV Trichomonas vaginalis*, *VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis.

*See table 6.1 for definitions. Performance statistics were also calculated for CT and NG combined and BV and TV combined, because the former are assessed by one assay and the latter require the same treatment. †These are the numbers that would require POCT and gold standard testing if each respective algorithm were to be implemented in a real-life situation. This number is lower for BV and/or TV than for BV alone because women who had a positive TV POCT would already be treated with metronidazole, which also covers BV. ‡Compared to gold standard testing. For TV, BV, and VVC, N=690 due to 15 invalid PCR results. §All women would have a vaginal pH determined and those with pH ≥5.5 would also have a vaginal qPCR score done (see methods). Only women with pH ≥5.5 and a positive vaginal qPCR score would be treated for BV. Women would only be tested for VVC if they had VVC-like symptoms and had tested negative for CT, NG, TV, and BV (by optimal algorithm), or were pregnant (regardless of symptoms).

We used the GeneXpert CT/NG assay in WISH and for gold standard testing, but in WISH only women with a positive CT/NG risk score were tested. This resulted in 56.2% of the women being tested, and 25.0% of true infections missed (Appendix, table E.4). The TV OSOM POCT was offered to all women and had moderate sensitivity (68.5%). The main problem with the WISH algorithms was the high number of false-positive BV and VVC diagnoses. We therefore designed post-hoc 'optimal' BV and

VVC algorithms using WISH study data (Appendix E, pages 179-180). We achieved the best balance between reducing BV false-positives and numbers of women requiring testing by determining vaginal pH in all women (as had previously been done), but adding a confirmatory test when pH≥5.5 (275 confirmatory tests required). Sensitivity, specificity, PPV and NPV were 73.6%, 100%, 100%, and 94.5%, respectively, when using the vaginal qPCR score as the confirmatory test (table 6.4), and were only slightly reduced when using the *Lactobacillus* qPCR as a stand-alone confirmatory test (with $<10^5$ geq/ml considered BV; Appendix, table E.5). The optimal VVC algorithm was based on the following observations (Appendix E, pages 179-180): 1) no vaginal pH cut-off could adequately predict VVC (data not shown); and 2) pregnant women were much more likely to have VVC than BV (19.4% and 6.5%, respectively). In the optimal VVC algorithm, women would only be tested for VVC if they had VVC-like symptoms and had tested negative for CT, NG, TV, and BV (using the optimal algorithm) or were pregnant. The sensitivity, specificity, PPV, and NPV were 59.3%, 100%, 100%, and 96.3%, respectively.

Of note, neither participant-reported symptoms nor clinician-observed signs correlated with the presence of any infection (table 6.5). Furthermore, performance of WISH CT, NG, TV, optimal BV, and optimal VVC algorithms were similar or slightly worse when restricted to a subgroup of women who had been seeking care or were taking traditional medications for their current symptoms (Appendix, table E.5). Also of note, 161/705 (22.8%) of WISH participants were treated for a UTI because urinalysis detected any nitrite and/or leukocytes in their urine whereas only 41/161 (25.5%) of their urines had an *E. coli* concentration of $\geq 10^5$ geq/ml by qPCR (Appendix, table E.6 and page 180).

Other clinical observations

In WISH, VDS/LAP-associated POCT-confirmed infections and VVC were by far the most common diagnoses made, followed by urinalysis-based UTI. POCT-confirmed syphilis (n=21), and syndromic diagnoses of pelvic inflammatory disease (n=32), non-syphilis genital ulcers (n=16), inguinal buboes without genital ulcers (n=0), and genital warts (n=3) were much less common. Study physicians performed speculum/bimanual examinations on 56.6% of participants, which was more than had been anticipated (Appendix, table E.7 and page 177). Treatments and referrals were delivered as required with few treatment failures, but the uptake of partner notification was suboptimal: 782 identified partners of 201 women (28.5%) required partner notification but only 61 (7.8%) of them were treated at the study clinic (Appendix, table E.7 and pages 179).

Table 6.5: Associations between symptoms/signs and infections diagnosed by gold standard testing

Symptom structurally reported (N=705)	N	CT/NG (N=100)	TV (N=111)	BV* (N=125)	VVC (N=59)	Syphilis (N=21)	No infection† (N=377)
No symptoms reported	136	14 (10.3)	17 (12.7)	22 (16.4)	3 (2.2)	3 (4.6)	84 (62.7)
Any unusual VDS	386	57 (14.8)	65 (17.3)	76 (20.2)	44 (11.7)	11 (5.3)	193 (51.3)
Curd-like VDS	265	36 (13.6)	41 (15.7)	41 (15.7)	31 (11.9)	8 (5.6)	142 (54.4)
Any unusual VDS without other symptoms	25	9 (36.0)	6 (25.0)	6 (25.0)	0	0	9 (37.5)
Genital itching and/or burning	470	68 (14.5)	80 (17.5)	85 (18.6)	51 (11.1)	16 (6.4)	239 (52.2)
Genital itching and/or burning without other symptoms	80	17 (21.3)	18 (22.8)	14 (17.7)	6 (7.6)	3 (7.5)	35 (44.3)
LAP and/or pain during sex	308	42 (13.6)	42 (14.0)	48 (16.0)	26 (8.6)	8 (5.0)	173 (57.5)
LAP and/or pain during sex without other symptoms	31	3 (9.7)	0	4 (12.9)	2 (6.5)	1 (4.8)	22 (71.0)
GUD (no-one reported inguinal buboes)	41	4 (9.8)	3 (7.7)	4 (10.3)	4 (10.3)	3 (10.3)	25 (64.1)
GUD without other symptoms	3	0	0	0	0	0	3 (100)
Sign observed during examination (N=399)	**N**	**CT/NG (N=59)**	**TV (N=61)**	**BV* (N=77)**	**VVC (N=42)**	**Syphilis (N=15)**	**No infection† (N=203)**
Any abnormal vaginal/cervical discharge/pus	139	44 (31.7)	34 (24.6)	31 (22.5)	25 (18.1)	5 (6.0)	47 (34.1)
Abnormal vaginal/cervical discharge/pus, curd-like	66	5 (7.6)	9 (13.6)	7 (10.6)	22 (33.3)	1 (2.8)	31 (47.0)
Abnormal genital odour	34	6 (17.7)	3 (9.1)	10 (30.3)	3 (9.1)	1 (5.3)	16 (48.5)
Cervicitis, vaginitis and/or vulvitis	84	18 (21.4)	13 (15.9)	21 (25.6)	20 (24.4)	1 (2.4)	28 (34.2)
Uterine, adnexal or cervical motion tenderness (=PID)	31	16 (51.6)	8 (26.7)	7 (23.3)	3 (10.0)	0	6 (20.0)
GUD +/- inguinal buboes, any location	40	5 (12.5)	5 (13.5)	10 (27.0)	6 (16.2)	6 (15.0)	14 (37.8)
Inguinal buboes (without GUD)	0	0	0	0	0	0	0

BV bacterial vaginosis, *CT Chlamydia trachomatis*, *GUD* genital ulcer disease, *LAP* lower abdominal pain, *NG Neisseria gonorrhoeae*, *PID* pelvic inflammatory disease, *POCT* point-of-care test, *TV Trichomonas vaginalis*, *VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis.
Data are n/(N %), where % are row percentages. The denominator N is the number of women reporting that specific symptom (with or without other symptoms as indicated), or women who underwent a speculum examination and had that specific sign (multiple signs allowed). The actual denominator per cell may vary slightly due to the 15 invalid gold standard results. Women could have multiple infections.
*BV defined as vaginal qPCR score [$\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*Gardnerella vaginalis* + *Atopobium vaginae*)] <-2. †Defined as no CT/NG, TV, BV, and VVC by gold standard test, and no syphilis by POCT.

Feasibility and acceptability of integrating POCTs

Participants accepted all testing services offered to them with the exception of HIV testing (rejected by 15.3%, mostly due to known HIV-positive status) and pregnancy testing (rejected by 17.0%, mostly because of reliable contraceptive use or known pregnancy) (Appendix, table E.8). Most women (86.9%) who were offered a CT/NG POCT chose to wait for the results, 10.4% elected to be contacted by phone or letter, and only 1.3% elected to schedule a follow-up appointment. All women accepted counselling, and a variety of topics were chosen, but only 6.2% were interested in a condom demonstration (Appendix, table E.9). Women who did not have to wait for a GeneXpert CT/NG result spent a median of 98 minutes at the clinic whereas women who did have to wait a median of 212 minutes (Appendix, table E.10).

The 107 participants who completed a client satisfaction survey liked all WISH procedures (Appendix, table E.11). The main point of criticism was the visit duration: 42.1% of the women thought the visit was long but everyone thought that the services received were worth it. Study staff reported that the POCTs were easy to perform and interpret, and did not identify any major testing or clinic flow

problems (data not shown).

6.4 Discussion

In the WISH study, VDS (including genital itching and burning) and LAP (including pain during sex) were by far the most common symptoms reported. The majority of the women reporting these symptoms did not require immediate treatment because they either had no infection, or had mild vaginal dysbiosis (defined here as above-normal vaginal concentrations of inflammatory micro-organisms that did not reach diagnostic thresholds), which may have resolved without treatment.[4,9] When a condition was present, patient-reported symptoms and clinician-observed signs did not accurately predict which one, as has been shown in other studies.[94,97,98,100] We therefore believe that the WHO VDS/LAP algorithms should be revised and that the revised algorithms should incorporate POCTs to improve diagnostic accuracy. The WISH study showed that POCTs are implementable and highly acceptable in resource-poor settings. An additional advantage may be that clinicians improve their diagnostic skills over time by comparing their symptoms/signs-based diagnoses with POCT test results. However, major barriers include the lack of ASSURED POCTs for some infections, time constraints, and costs. The goal for the coming years therefore should be to continue POCT development, and design algorithms that maximise appropriate treatments delivered; minimise complications, overtreatment, and drug resistance; and minimise the number of POCTs required to achieve this. Some of the time and human resources required to conduct POCTs might be recuperated by integrating sexual and reproductive health services for women, and by minimising the number of speculum examinations. In the WISH study, these did not have much added value, other than identifying some cases of (suspected) pelvic inflammatory disease. The number of POCTs required might be reduced by risk scoring based on local epidemiology to decide who is tested for which infections, as we did in WISH, but risk scores would have to be locally validated.

The WHO algorithms were designed for women seeking care for urogenital symptoms. The WISH study tested women at risk of urogenital infections regardless of symptoms, recognising the fact that asymptomatic infections are common, continue to fuel HIV/STI epidemics, and can cause complications.[9,87] Moving forward, we recommend that guidelines address both types of case-finding. Women who proactively seek care for VDS/LAP symptoms because their quality of life is negatively affected would benefit from testing for CT, NG, TV, BV, and VVC. Pregnant women are at risk of the most severe complications (such as preterm birth and neonatal sepsis),[87] and we therefore believe that they should be comprehensively screened for HIV, STIs, BV, VVC, and vaginal pathobiont carriage (e.g. *Streptococcus agalactiae*), regardless of symptoms. Women at high risk of HIV/STIs (to be defined locally) should be tested for HIV/STIs regardless of symptoms, and possibly also BV and VVC, in an effort to control HIV/STI epidemics and minimise infertility and pelvic inflammatory disease. We

believe that these recommendations should not just apply to resource-poor countries but also to primary care settings in Europe and elsewhere.

ASSURED POCTs would improve feasibility, performance, and cost-effectiveness of both types of case-finding.[106,108] HIV, syphilis, and pregnancy POCTs have already been successfully integrated in many clinics and screening programmes.[109,110] We and others obtained good results with the GeneXpert CT/NG and TV OSOM POCTs,[91] but the former could be improved by reducing turnaround time and costs, and a next generation of TV POCTs with improved performance would be welcomed. Furthermore, better POCTs for BV and VVC should be developed. The WISH study showed that vaginal pH can be used as an initial screening test for BV but would require confirmatory testing. We used three qPCR assays to identify true BV cases (*Lactobacillus*, *G. vaginalis*, and *A. vaginae*), but we also showed that a qPCR for *Lactobacillus* genus suffices. Gram stain Nugent scoring[78] could also be used as a confirmatory test. Unfortunately, POCTs based on detecting *G. vaginalis* enzymes or metabolites, or *C. albicans* antigens/antibodies, have shown inadequate sensitivity and specificity thus far.[46,207] A combined NAAT-based POCT for BV (*Lactobacillus* concentration), TV (presence), and VVC (*Candida* genus presence) may provide the best balance between optimising diagnostic accuracy and minimising required resources. Such a POCT could be used in combination with a CT/NG POCT (e.g. in women seeking care for VDS/LAP symptoms) and/or other POCTs (e.g. to screen pregnant women and women at high risk of HIV/STIs, as outlined above). The WISH data also suggest that better UTI POCTs should be developed.[208]

The WISH study was implemented in a high prevalence population by highly trained and experienced staff who had access to adequate clinic and laboratory resources. Additional studies are required in low prevalence settings and in public primary care clinics. Even though we tried to recruit women with and without symptoms, our recruitment strategies mostly attracted women with symptoms, and it was not always clear which women would have sought care for their symptoms in real life. Furthermore, the 95% confidence intervals of our sensitivity estimates were wide. Future studies should enrol women who are seeking care for urogenital symptoms, as well as women not seeking care, in sufficient numbers to achieve high precision performance estimates in both groups. As with most observational studies, our data collection likely suffered from selection and social-desirability bias despite our efforts to minimise these. Finally, the WISH study did not include a cost-effectiveness component. Cost-effectiveness studies of different POCT-based algorithms in different settings will be essential.

We conclude that POCTs should be integrated into urogenital infection algorithms directed at women seeking care for symptoms and vulnerable women regardless of symptoms. However, improved availability of ASSURED POCTs is required, and we particularly recommend the development of a NAAT-based POCT combining BV, TV, and VVC diagnoses.

Chapter 7 - Synthesis, Conclusions, and Recommendations

The final chapter summarises, discusses, and synthesises the key findings of this thesis.

7.1 Rationale and Aim of The Thesis

The aim of this thesis was to inform potential improvements in the sexual and reproductive health of Rwandan women, with an emphasis on vaginal dysbiosis. Urogenital infections, including vaginal dysbiosis, are often associated with urogenital symptoms such as vaginal discharge and lower abdominal pain, which can cause great discomfort to women. Better diagnostics and therapeutics are therefore needed to cure women who present at medical practices or clinics with symptoms. However, better identification and treatment are also necessary to decrease the incidence of the many potential complications of urogenital infections, such as increased risk of HIV transmission (both male-to-female and female-to-male), pelvic inflammatory disease, and adverse pregnancy outcomes such as stillbirth, invasive neonatal infections, and preterm birth.[9,17–20] Pelvic inflammatory disease can be caused by sexually transmitted infections (STIs) such as *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), and *Trichomonas vaginalis* (TV), but also by bacterial vaginosis (BV)-anaerobes and pathobionts;[19] it is associated with sequelae such as ectopic pregnancies, infertility, and chronic pelvic pain.[87] BV is also associated with infertility in its own right, as well as with a lower success rate of in vitro fertilisation.[209,210]

Urogenital infections are common worldwide. Prevalences of STIs and BV have been shown to be highest in resource-poor settings, particularly in sub-Saharan Africa.[36,87] Improved urogenital care in sub-Saharan Africa is therefore urgently needed, and could be achieved by introducing better diagnostic and treatment options. This thesis investigated several such options. Specifically, we investigated the impact of oral metronidazole and two different vaginal lactobacilli-containing probiotics on the vaginal microbiota (VMB) and on the cure and recurrence of BV, and we studied the feasibility, acceptability and diagnostic accuracy of integrating point-of-care testing (POCTs) for urogenital infections into urogenital care.

7.2 Interventions to Cure BV and/or to Reduce BV Recurrence

VMB impact of oral metronidazole

Chapter 2 showed that oral metronidazole use not only decreased the mean BV-anaerobes concentration as expected, but also increased the mean *Lactobacillus* concentration. However, it is important to note that treatment was often only partially successful: only 16.4% of women had a BV-anaerobes concentration reduction of more than 50%, and only 5.5% of women had complete eradication. Furthermore, treatment was unsuccessful (as defined by Nugent scoring) in 45.5% of women. Treatment failure was associated with high *Gardnerella vaginalis* relative abundance, potentially due to

biofilm presence, and with higher pathobionts concentration (its mean concentration in women with unsuccessful treatment was 2.30 $\log_{10}$ cells/µl, and in women with successful treatment 1.09 $\log_{10}$ cells/µl). It is possible that metronidazole is capable of eliminating dispersed *G. vaginalis* at modest concentrations, but is less able to do so when high-concentration *G. vaginalis* is present and/or when *G. vaginalis* is present in a biofilm. The findings also confirm that most pathobionts are not sensitive to metronidazole, and that this hampers the intended cure of women with high pathobionts concentration.

Chapter 3 discussed the safety and preliminary efficacy of intermittent oral metronidazole use to prevent BV recurrence. Metronidazole was effective in decreasing BV recurrence (at an incidence rate ratio [IRR] of 0.14 compared to negative controls), and had positive effects on the VMB, such as increasing the relative abundance of lactobacilli by 32% and reducing the relative abundance of BV-anaerobes by 19%. However, all beneficial effects disappeared after cessation of metronidazole use, possibly due to the fact that our study enrolled women at very high-risk of urogenital infections.

VMB impact of lactobacilli-containing vaginal probiotics

Chapter 3 discussed the safety and preliminary efficacy of intermittent use of two lactobacilli-containing vaginal probiotics (Ecologic Femi+ and Gynophilus LP) to prevent BV recurrence. Ecologic Femi+ was effective in decreasing BV recurrence (IRR 0.35 compared to negative controls), and had positive effects on the VMB, such as increasing the relative abundance of lactobacilli by 30%. Gynophilus LP also seemed to have positive VMB effects although these did not reach statistical significance, possibly due to insufficient statistical power. All effects disappeared after product use cessation, and this may again have been due to the high-risk nature of our cohort. We also showed that the intermittent use of vaginal probiotics was acceptable to the Rwandan participants, and that adherence to the interventions was high, with 100% of EF+ users and 81.3% of GynLP users using ≥80% of required doses. Our survey data showed that behavioural counselling is needed to better inform women about what they can do themselves to prevent BV and STIs.

We also conducted a systematic review on the impact of lactobacilli-containing vaginal probiotics on the VMB. None of the 34 studies included in the review reported major safety issues. Most studies were of poor methodological quality and were underpowered, but the overall conclusion was that lactobacilli-containing vaginal probiotics hold promise for BV cure and prevention. All of the six results contained in five medium-risk of bias studies with BV and molecular VMB outcomes results were promising: four results were statistically significant and two were non-significant. The eight high-risk BV studies showed mixed results: five studies showed significant beneficial effects of probiotic use, one study showed non-significant Nugent results but the VMB results were significantly beneficial, and two studies did not show any beneficial effects. Vaginal probiotics are however less promising for vulvovaginal candidiasis (VVC) cure and prevention: one medium-risk VVC study found no benefit of

using probiotics, and of the eleven high-risk VVC studies, five showed significantly beneficial effects of vaginal probiotic use, three studies showed non-significant beneficial effects or had mixed results, and three studies showed no beneficial effects of probiotic use. Only two studies were able to differentiate between probiotic and autologous lactobacilli strains, including our own study.[155,160] Our systematic review could not identify which *Lactobacillus* product/strain and which vaginal probiotic dosing strategy was the most successful.

Alternative interventions, overall conclusions and future studies

The question remains whether vaginal probiotics are sufficiently promising to warrant large clinical trials investigating their efficacy, or whether the focus should be on alternative interventions for treating or preventing BV. These could include the use of hormonal contraception, lactic acid and other acidifying agents, biofilm-disrupting treatment (such as vaginal boric acid, retrocyclins, and DNases), phage therapy, immune modifiers (such as lactoferrin), and behavioural interventions such as medical male circumcision and treatment of male sexual partners.[26,46,47,73,116,161,211,212] In some women, it may be useful to alter the dose and/or formulation of metronidazole or clindamycin to achieve higher cure rates.[213,214] Nevertheless, most of these interventions have only been studied in small studies and need to be assessed in well-powered clinical trials to be proven worthwhile.

This thesis shows that oral metronidazole was effective in curing BV (metronidazole was effective in 54.5% of women, although complete eradication of BV-anaerobes only occurred in 5.5%) and preventing BV (intermittent use of metronidazole reduced BV recurrence by 86%). However, oral metronidazole may not lend itself to long-term chronic use. Earlier studies had also shown that intermittent metronidazole was efficacious in preventing BV recurrence (by 45-57% in those studies)[132,134,137] but this did not lead to its widespread use in clinical practice. Metronidazole use – and especially chronic use - likely leads to gut microbiota dysbiosis,[215] which in turn causes the gastro-intestinal side-effects that have been associated with metronidazole (particularly when used in combination with alcohol).[50] Moreover, although resistance to metronidazole is currently not common,[53] long-term preventive use of antibiotics is not favoured in the current context of the large-scale introduction of antibiotic stewardship programmes. Therefore, in practice, intermittent metronidazole use may play a role in the management of women with severe recurrent BV, but will most likely not be widely introduced.

This thesis also showed that vaginal lactobacilli-containing probiotics are promising in reducing BV recurrence (intermittent use of Gynophilus LP and Ecologic Femi+ decreased BV by Nugent recurrence by 47 and 65%, respectively). When direct comparisons were made with similar intermittent use of an antibiotic, the effectiveness of the vaginal probiotics was inferior to metronidazole (our study)[160] or clindamycin (Bradshaw et al).[137] However, all studies conducted with vaginal lactobacilli-containing

probiotics to date have shown that these products are safe. Furthermore, vaginal application would not interfere with the gut microbiota. They could therefore be sold over-the-counter without physician-involvement. However, the regulatory landscape of probiotics will change considerably in the near future (see Chapter 1), with the EMA and local drug regulatory agencies requiring human drug approval when health claims are made. This could be considered a positive change, because in the current situation, many vaginal and oral lactobacilli-containing probiotics claiming BV cure and/or prevention of recurrence are on the market in the absence of an evidence-base. However, the majority of companies producing these probiotics operate in the food and cosmetics industries and do not have the capacity to develop human drugs. As far as we know, only one American company (Osel Inc, Mountainview, CA, USA) is currently developing a vaginal probiotic as a human drug (Lactin-V, containing a *L. crispatus* isolated from a healthy woman).[216]

Expanding the evidence-base for lactobacilli-containing vaginal probiotics not only requires large investments by these companies and others, it also requires improved trial designs that incorporate molecular techniques. Randomised controlled clinical trials are far superior to observational cohort or pre-post treatment studies. All studies should include detailed VMB assessments at baseline just prior to randomisation, and as often as possible during follow-up over several menstrual cycles, to track changes over time at the molecular level, and to compare responders with non-responders. They should include at least one follow-up visit after product use cessation to assess whether the intervention(s) had any lasting effects beyond the period of product use. Probiotic and autologous *Lactobacillus* strains should be differentiated by molecular testing. The quantities of all key *Lactobacillus* strains (probiotic and autologous), BV-anaerobes, and pathobionts should be assessed at each time point. Ideally, the presence of cervicovaginal inflammation and biofilm should also be assessed at each time point. The exact timings of product use and sample collection, adherence, and known confounders should be assessed and taken into account in the statistical analyses. Eventually, the impact of product use on the incidence of vaginal dysbiosis-related complications (STI/HIV transmission, pelvic inflammatory disease, and neonatal infections whenever applicable) should also be studied.

As stated earlier, our study showed that pathobionts are not sensitive to metronidazole. Other studies have shown that pathobiont presence in the vagina is common. For example, the prevalence in pregnant women of *Streptococcus agalactiae*, the best-studied vaginal pathobiont, is 17.9% worldwide.[45] Vaginal pathobionts and their (epidemiological and microbiological) behaviour should therefore be investigated more thoroughly than has been done to date. Women with a high concentration of pathobionts who are symptomatic or at risk of complications (e.g. pregnant women) might benefit from antibiotic therapy with an antibiotic other than metronidazole; the choice of antibiotic may depend on the specific pathobiont genera/species that are present. Our study also showed that the presence of a high *G. vaginalis* concentration decreases metronidazole treatment success. Other studies have shown that the

prevalence of VMBs dominated by a single BV-anaerobe, most commonly *G. vaginalis*, is not as high as the prevalence of traditional highly diverse BV, but is sufficiently substantial to warrant further research.[5] For example, from a therapeutic point of view, it would be useful to know what proportion of these women have a *G. vaginalis*-containing biofilm. Currently, these women are treated in the same manner as traditional high-diversity BV patients, but they may require higher antibiotic dosing, and may benefit from biofilm-disrupting agents in combination with antibiotic treatment. Potent anti-anaerobe antibiotics targeting all *G. vaginalis* clades may be useful but are currently not in development.

7.3 Introduction of POC Testing to Improve Urogenital Disease Case-Finding

Introduction of POC testing in standard urogenital care

The WISH study showed that the introduction of POCTs in standard urogenital care for women in Kigali, Rwanda, was feasible, and had superior performance to the use of WHO syndromic management algorithms. Infection-specific sensitivities of the WHO algorithms compared to the gold standards ranged from 58.3-74.6%, and specificities from 44.7-50.6%. The WISH POCT-based algorithms that we introduced had good sensitivity (68.5-76.0%) and specificity (97.4-100%) for CT, NG, and TV but low specificity for BV (41.2%; sensitivity 95.2%), and modest sensitivity (64.4%) and specificity (69.4%) for VVC. The study also showed that POCT integration was acceptable to the Rinda Ubuzima staff and to the study participants. We therefore believe that the WHO syndromic management algorithms should be revised to incorporate the possibility of POC testing. The current syndromic management algorithms were designed for women seeking care for their symptoms. We showed, however, that urogenital symptoms reported by participants, but also physician-observed signs during pelvic examinations, did not correlate well with gold standard laboratory test-confirmed urogenital infections (see table 6.5). Therefore, the WHO sexually transmitted infections guidelines should move from a merely symptom-based system to a system that includes screening of at-risk populations, and women at risk of complications (such as pregnant women) ought to be offered testing even if they are asymptomatic. Nevertheless, there are major barriers that make the introduction of POC testing in resource-poor settings difficult. These include the current lack of ASSURED ('Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end users') POCTs for some common infections, uncertainties about the cost-effectiveness of POCT introduction, and increased health care system demands, especially availability of adequately trained personnel. These potential barriers should be addressed in future studies.

Future POCT development

As described in the introduction, ASSURED POCTs are available and widely used for HIV, syphilis, and pregnancy, including in resource-poor settings.[109,110] However, no ASSURED POCTs are available for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). The sensitivity and specificity of

POCTs for BV, VVC, and TV should be improved to fully adhere to the ASSURED criteria. Symptomatic women seeking care for their symptoms, or pregnant women, may benefit from combined NAAT-based tests for BV (e.g. *Lactobacillus* abundance), TV, and VVC. Multiplex tests may be less expensive than using multiple POCTs for the same clinic visit (as discussed in Appendix E). Optimised algorithms for selecting women to be tested for BV and VVC by traditional non-ASSURED laboratory testing (Chapter 6), such as Nugent scoring, culture, or qPCR, could provide an immediate way forward but are likely to be too complex and expensive to be routinely introduced in urogenital care in resource-poor settings. Finally, POCTs for pathobiont carriage (e.g. *Streptococcus agalactiae*, staphylococci, and *Escherichia coli*) should also be developed, for example for testing pregnant women. However, the first priority is to improve the pathobiont evidence-base because we currently know much less about vaginal pathobionts than about STIs or traditional BV and VVC, and its consequences are severe.

Methodology issues in the WISH study

The reasons behind the selection of POCTs for evaluation in the WISH study are discussed in Chapter 6. We intended to choose POCTs that complied with the ASSURED criteria, and specifically assays that could be conducted and interpreted by primary care clinic staff in African settings. Unfortunately, many of the selected POCTs did not fully adhere to these criteria as outlined in Appendix E and in the previous section.

Appendix E also outlines the selection of clinical reference or gold standard testing in the WISH study. We chose nucleic acid amplification testing for CT/NG (by GeneXpert), VVC (by *Candida albicans* quantitative PCR), TV (by qualitative *T. vaginalis* PCR), and BV (by the vaginal qPCR score[85] based on concentrations of *Lactobacillus* genus, *G. vaginalis*, and *A. vaginae*). The *C. albicans* qPCR is not a true gold standard test as VVC can be caused by other yeasts/fungi. However, this test was chosen as *C. albicans* is the principal causative organism of VVC,[30] and due to the pro-inflammatory nature of *C. albicans*: asymptomatic carriage is only likely when present at low concentrations. Future research is needed to better determine at which concentration threshold *C. albicans* causes urogenital symptoms and/or sequelae. Similarly, the vaginal qPCR score used was not a true gold standard test; the score was based on comparisons with the Nugent criteria, in which [$\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*)] below -2 had the highest diagnostic accuracy compared with a Nugent score of 7-10.[85] A cut-off of -2 means that a BV diagnosis is made when the *Lactobacillus* concentration is at least 100 times lower than the combined *G. vaginalis* and *A. vaginae* concentration; this cut-off is quite stringent, and we cannot discard the possibility that women with a higher vaginal qPCR score may still need treatment in some cases. As is the case for VVC, the lactobacilli concentration threshold below which treatment is needed and sequelae are prevented is currently not known. It should be noted that the vaginal qPCR score is by no means a POCT due to the laboratory infrastructure needed to perform the qPCR tests, but we do think that the vaginal health qPCR score

could serve as a starting point for the development of future ASSURED BV assays. It may even be possible to use the *Lactobacillus* concentration on its own instead of the combination of the three concentrations; our WISH data suggest that the performance would decline minimally.

Future studies

We show in this thesis that POC testing for urogenital infections improves case-finding and is feasible in resource-poor settings. Additional studies should be conducted to further investigate these findings, including in populations with lower prevalences of urogenital infections. In our view, and as outlined in the discussion of Chapter 6, the two most important future steps in this field are optimisation of diagnostic criteria (particularly for BV, pathobionts, and other poorly characterised types of vaginal dysbiosis), and the development of ASSURED POCTS, including POCTs targeting multiple conditions.

To determine the cost-effectiveness of POC-testing for urogenital infections, large randomised clinical trials should be conducted and should only take place when ASSURED POCTs for all main urogenital infections (CT/NG, TV, BV, and VVC; potentially also pathobionts when targeting pregnant women) are available as outlined in the section 'Future POCT development'. A good example would be a cluster randomised clinical trial comparing clinics providing WHO syndromic management (such as STI clinics and general practices) with clinics offering women POC-testing regardless of symptom-reporting. However, such a design poses ethical concerns because we now have ample evidence that POC-testing will outperform syndromic management; the research question would not be whether POC-testing will perform better but by how much, and what this means in terms of the incidence of complications and cost-effectiveness. A stepped-wedge cluster randomised clinical trial with phased introduction of POC testing might be a good way of dealing with these ethical concerns.[217]

Final conclusions on POCT integration

The uncertainties about POCTs and their cost-effectiveness should not deter the reproductive health care community from the fact that the status quo of urogenital infection management is unsustainable. This thesis clearly shows that syndromic management algorithms have poor performance, and that reported symptoms and observed signs do not adequately predict the presence of urogenital infections. It is both possible and necessary to improve urogenital disease case-finding by working towards introducing the currently available POCTs for CT, NG, and TV in urogenital care, including in resource-poor settings.

7.4 General Methodological Limitations of the Studies

In this thesis, the high-dimensional 16S rRNA gene sequencing data were reduced in both conventional and non-conventional ways. The most novel data reduction method that we used was the categorisation of amplicon sequence variants (ASVs) into four bacterial groups based on existing biological

knowledge of the pathogenicity of VMB bacteria. Lactobacilli were considered optimal and were grouped together into one group.[4,8] Anaerobic bacteria that have been described in the scientific literature as being associated with BV were grouped together into another group that we referred to as 'BV-anaerobes'. Bacteria known to have higher pathogenicity than BV anaerobes, and that have been associated with invasive disease in vulnerable people (such as neonates and hospitalised patients) were categorised as 'pathobionts'. The ASVs that could not be placed in any of these three groups formed the fourth group (called 'other bacteria'), which ended up consisting of normal, harmless skin bacteria (e.g. Corynebacteria), Bifidobacteria, and a few other minority species. Our VMB types were not solely based on hierarchical clustering, as is usually the case, but also took the relative abundance of these four bacterial groups into account. We did this because hierarchical clustering is based on relative abundances only and does not take pathogenicity into account. For example, women with 70% lactobacilli and 30% *S. agalactiae* may end up in a lactobacilli-dominated cluster together with women who have 95-100% lactobacilli, whereas we believe that the 30% *S. agalactiae* may matter from a clinical point of view. These methods have never been used by others in the VMB field. We did present them to VMB experts in international conferences, and initial reactions were positive.

In this thesis, we estimated bacterial group concentrations using a combination of relative abundances and the total 16S gene concentration of each sample while correcting for 16S copy numbers per taxa. This method has been used by other research groups, including research groups in the VMB field.[118,218,219] Recently, two groups have shown that the concentrations generated with this method correlate well with species-specific qPCR-based concentrations for key VMB bacteria, but it should be noted that the method has not yet been validated for most minority species.[129,130] The most important advantage of using estimated concentrations instead of relative abundances is that the data are no longer compositional; they are therefore easier to interpret and can be modelled in conventional biostatistical models. For example, we reported in Chapter 2 that the relative abundance of pathobionts was higher after metronidazole therapy compared to before therapy. This could be interpreted to mean that metronidazole kills anaerobes, thereby providing an opportunity for pathobionts to occupy the niche. However, the estimated pathobionts concentrations before and after treatment were similar; the relative abundance of pathobionts increased after treatment because the total bacterial concentration decreased.

The discussion sections of Chapters 2-6 described the strengths and limitations of the VMB and WISH studies in detail. An overarching limitation is that both studies were conducted at one single study site in an urban setting in Kigali, Rwanda. This study site had a long history of working with women at high risk of STIs, which may have introduced a selection bias. This is particularly true for the Rwanda VMB study (Chapters 2-4); the study team did make a large effort to recruit a more diverse group of women in terms of STI risk for the WISH study (Chapter 6). The results described in this thesis may therefore not be generalisable to general populations of women, not even in Rwanda.

7.5 Final Recommendations

To conclude, it is clear that improvements in both the diagnosis and treatment of vaginal dysbiosis and STIs are urgently needed, particularly in sub-Saharan Africa and in other resource-poor settings where the burden of these infections is highest.

Based on this thesis, we make the following recommendations:

- Definitions and clinical relevance of various VMB states should be improved and standardised. The microbiological knowledge that has been accumulated in recent years should be incorporated in these definitions. Microbiological treatment thresholds should be determined.
- The portfolio of therapies available for vaginal dysbiosis treatment and prevention should be expanded, and could include (combinations of) novel/improved antibiotics, biofilm-disrupting agents, acidifying agents, vaginal probiotics, and female sex hormones.
- All interventions should be evidence-based, and this evidence should include microbiological assessments. In the case of probiotics, the molecular techniques used should be able to differentiate between probiotic strains and autologous strains.
- The WHO syndromic management guidelines should be amended to allow for, or even promote, POC-testing in all settings where this is possible. This should also include POC-testing of asymptomatic women who are at risk of urogenital infections or the complications thereof (e.g. pregnant women).
- ASSURED POCTs should be developed for all urogenital infections: a combined POCT for BV, TV, and VVC is essential, as well as POCTs for various pathobionts to be used in pregnant women.
- In the context of clinical research, researchers should never solely rely on symptoms and/or signs for urogenital infection case identification, but should also test for them, preferably using highly sensitive and specific gold standard tests.

Appendix A

Supplementary Methods (corresponding to Chapters 2 and 3)

We conducted a randomised pilot clinical trial to determine safety, preliminary efficacy, acceptability, and feasibility of one antibiotic and two vaginal probiotic interventions to prevent BV recurrence in women diagnosed and treated for bacterial vaginosis (BV) and/or *Trichomonas vaginalis* (TV) in Kigali, Rwanda. We chose to include both women with BV and/or TV as the treatment to these two conditions is the same, and BV and TV are closely interlinked.[9] This trial was carried out at the Rinda Ubuzima research clinic and laboratory in Kigali, Rwanda, from June 2015 until February 2016. The study was funded as a pilot study and the sample size was therefore determined by budget availability. In Chapter 2, the vaginal microbiota (VMB) effects of the initial metronidazole treatment for BV/TV prior to randomisation are reported. In Chapter 3, the VMB effects of the antibiotic and probiotic interventions after randomisation are assessed. A flow diagram of the entire trial is shown in figure 3.1.

Recruitment, eligibility criteria, and informed consent procedures

The target population was women at high risk of urogenital infections living in Kigali, Rwanda. Recruitment activities were implemented by study staff with the help of Community Mobilisers, who were selected due to their strong connections with high-risk women in Kigali. They helped staff organise recruitment meetings in relevant communities. At these meetings, an anonymous pre-screening checklist containing the most important eligibility criteria was used, but no information recorded, and potentially eligible women were referred to the Rinda Ubuzima clinic for screening.

The main eligibility criteria are described in Chapter 2 and 3. We focussed on BV patients, but also included women diagnosed with TV because we expected most of them to also have BV (which was indeed the case), and because the treatment for BV and TV is identical. We chose to treat women with BV and/or TV with seven days of 500 mg generic oral metronidazole (Tricozole; Laboratory & Allied ltd, Nairobi, Kenya) twice daily. Previous studies suggest that oral and vaginal metronidazole of similar dose and duration of use have similar efficacy for BV,[48,49] but none of the vaginal metronidazole gels that were available on the market when we designed our study had proven stability at 30 °C. Additional exclusion criteria included physician-observed genital ulcers, condylomata or other genital abnormalities; having had an invasive gynaecological procedure in the three months prior to screening; history of significant urogenital prolapse, undiagnosed vaginal bleeding, urine or faecal incontinence, or blood clotting disorders; allergy to metronidazole or any other components of the study drugs; not willing to terminate use of other oral or vaginal probiotics; or participating in another health intervention study. These criteria applied to only six women (figures 2.1 and 3.1). HIV-positive and pregnant women were referred to local HIV and antenatal care clinics for care.

All participants provided written informed consent. The age of majority for Rwandan women was 21 at the time of study implementation, and we therefore also obtained parent/guardian consent for non-married participants aged 18-20 years. Participants and/or parents/guardians with insufficient literacy could sign by thumbprint but the informed consent process was observed by an independent witness who co-signed the informed consent form. The witness could not be a Rinda Ubuzima staff member, but could be another participant. Participants received the equivalent of three GBP per visit in local currency as a reimbursement for time spent at the clinic and transport costs.

Clinic procedures

An overview of visit procedures is presented in figures 2.1 and 3.1. At the screening visit (referred to in Chapter 2 as "pre-treatment visit"), participants underwent a face-to-face interview, speculum examination, real-time testing for HIV, pregnancy, urinary tract infection, BV, and TV, and collection of samples for sexually transmitted infection and future molecular testing (see below). At the end of this visit, preliminary eligibility was assessed, and initial treatments and referrals were given based on available test results. Testing for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum* (syphilis) and herpes simplex virus type 2 was conducted on stored samples after the participant had left the clinic. Results of the syphilis and herpes simplex type 2 tests were shared with participants at a results visit one to two weeks after the screening visit, additional treatments were given as required, and

eligibility was reassessed. Positive herpes simplex type 2 serology was not a reason for exclusion. Chlamydia and gonorrhoea results were shared with participants as soon as they were available. We did not use them to determine eligibility as originally intended because the turn-around time of polymerase chain reaction (PCR) testing at the National Reference Laboratory in Kigali was slow. At the end of the screening and results visits cascade, women were either declared ineligible, or diagnosed with BV (by Nugent score[78] and/or modified Amsel criteria,[77] defined as two of three positive: vaginal pH>4.5, positive whiff test, or ≥20% clue cells) and/or TV (by culture and/or wet mount) and treated with 500 mg oral metronidazole twice per day for seven days. An enrolment visit (referred to in Chapter 2 as "post-treatment visit"), was scheduled within three days after treatment completion, and only women whose treatment had been successful (no BV by modified Amsel criteria and no TV on wet mount), and who were still not pregnant and free of urinary tract infection and syphilis, were randomised to the four groups described in Chapter 3. Women could be rescreened a maximum of three times.

We considered Nugent scores a more accurate marker of vaginal dysbiosis than Amsel criteria.[4] However, we used Amsel criteria to determine eligibility because results are available immediately. We deliberately disregarded the vaginal discharge criterion, because many studies have shown that the presence of vaginal discharge does not correlate well with the presence of vaginal dysbiosis.[220] Studies have also shown that the presence of vaginal dysbiosis in the absence of vaginal discharge can be harmful (e.g. is associated with increased vaginal inflammation, HIV acquisition, preterm birth, etc.).[87] Finally, our intention was to use vaginal probiotics to prevent microbiological dysbiosis and not to cure symptoms. For all of these reasons, we decided to use modified Amsel criteria, defined as at least two of three laboratory criteria positive (vaginal pH>4.5, amine smell when adding KOH to the smear, and presence of clue cells when evaluating the smear under a microscope).

The Chief Investigator (my primary supervisor, Prof. Janneke van de Wijgert) based in Liverpool used a random number generator to assign participant identification numbers to groups in blocks of four. At the Rinda Ubuzima clinic, each eligible woman was assigned the next available participant identification number, and the corresponding sealed envelope was opened to reveal her randomisation group. The laboratory technicians were blinded but it was not possible to blind the clinicians and participants. Behavioural counselling was offered to all participants in all randomisation groups. The counselling focussed on reducing known risk factors for BV such as unprotected sex, vaginal hygiene practices, male partner penile hygiene practices, and alcohol use (which can lead to unprotected sex and can cause severe side effects when used in combination with metronidazole). At follow-up visits, participants underwent a face-to-face interview, counselling, speculum examination, laboratory assessments (BV by modified Amsel criteria and Nugent scoring, TV by wet mount and culture, vulvovaginal candidiasis by wet mount, and additional testing if clinically indicated), and sampling for future molecular testing. HIV, sexually transmitted infection, urinary tract infection, and pregnancy tests were repeated at Month 6 (M6) only. All participants were offered male condoms free of charge at each study visit. Forty-six participants made unscheduled visits to collect treatment for an infection that was diagnosed by laboratory testing after the participant had left the clinic (n=35 women), because of new symptoms (n=14), and/or to withdraw informed consent (n=1).

Adherence to the three antibiotic and probiotic interventions described in Chapter 3 and 4 was assessed at the Day 7 (D7), Month 1 (M1), and Month 2 (M2) visits by structured interviewer-administered questionnaire, review of a diary card that the participant completed in between study visits, review of returned used packaging and unused product, and by asking the participant to complete a self-rating adherence scale. These different sources of adherence data were triangulated to arrive at an overall level of adherence (between 0-100%) for each participant between visits. Women were allowed to cease product use during menstrual bleeding (done by 12 women), and the adherence data were not adjusted for this.

Diagnostic testing

All diagnostic testing was conducted onsite at the Rinda Ubuzima clinic or at the National Reference Laboratory in Kigali using validated procedures. Vaginal swab, blood, and urine specimens were processed on the collection day and either tested immediately or stored at -80°C until testing. Dacron

vaginal swabs were used for wet mounts, Gram stains, and TV InPouch culture (Biomed Diagnostics, White City, OR, USA) at all study visits, as described in the Chapter 2 and 3. All other diagnostic tests were only done at screening ("pre-treatment visit"), M6, and when judged clinically necessary by the physician, with the exception of pregnancy and urinalysis tests, which were repeated at enrolment ("post-treatment visit") prior to randomisation. Whole blood was tested for HIV 1/2 using the Kehua HIV Rapid Test (Kehua Bio-engineering, Shanghai, China), followed by the Alere Determine HIV-1/2 Rapid Test (Abbott Laboratories, Tokyo, Japan) for confirmation of positive results and the Unigold HIV Rapid Test (Trinity Biotech, Bray, Ireland) as tie-breaker (if applicable). Urine was tested for pregnancy using a Human chorionic gonadotropin, and for urinary tract infection using a urinalysis, dipstick test (both by Nova, Atlast Link Technology, Beijing, China). Endocervical swabs were tested for *Chlamydia trachomatis* (CT)/ *Neisseria gonorrhoeae* (NG) by real-time Polymerase Chain Reaction (PCR; Presto, Beek, The Netherlands).[221] A sub-sample of 49 M6 samples were tested by GeneXpert CT/NG assay (Cepheid, CA, USA) after study completion. The sensitivities and specificities of the two real-time PCR assays are high and comparable.[91] Plasma was tested for herpes simplex virus type 2 serology (Kalon, Guildford, UK; using an optical density cut-off of >1.1 for a positive result and <0.9 for a negative result) and syphilis by Rapid Plasma Reagin test followed by *T. pallidum* Hemagglutination Assay (both by Spinreact, Girona, Spain).

Molecular laboratory methods

Rationale for 16S rRNA gene sequencing of the VMB

It has long been recognised that BV is a polybacterial condition. However, it has been difficult to define its precise microbiological nature due to the limitations of microscopy and culture.[4,9] 16S rRNA sequencing has revolutionised the field. The 16S gene is unique to bacteria, and contains highly preserved regions that can be used to quantify and amplify all 16S genes present in a vaginal sample, as well as variable regions that can be sequenced and then used to determine which bacteria are present.[222] Many sequencing studies have been conducted since the turn of the century, and these revealed that VMBs are usually dominated by five main *Lactobacillus* species (of which *L. crispatus* and *L. iners* are the most common).[4] The most common type of vaginal dysbiosis is BV, and the long list of anaerobes that are typically associated with BV have now been well characterised (referred to as 'BV-anaerobes'; see list in table A.1).[4] Occasionally, bacteria other than lactobacilli or BV-anaerobes are identified, including pathobionts, skin bacteria, and Bifidobacteria.[4] These bacteria have not yet been very well characterised epidemiologically and clinically. Pathobionts are bacteria that have a higher pathogenicity than BV-anaerobes, and are often associated with hospital and neonatal infections, including Proteobacteria, streptococci, staphylococci, and enterococci.[87] We assessed pathobionts separately from BV-anaerobes, because they seem to behave differently (although more research is needed to evaluate this properly) and are not typically treated with metronidazole when they cause hospital or neonatal infections.

DNA extraction

For molecular testing, the physician collected two Dacron vaginal swabs per woman during speculum examinations at screening, enrolment, and each scheduled follow-up visit (N=1,016 swabs). The swab heads were stored dry at Rinda Ubuzima at -80°C on the collection day. The 12 women in the self-sampling group (see Chapter 3 for details) self-sampled two flocked swabs (Copan Diagnostics, Murrieta, CA, USA) every Monday, Wednesday, and Friday of the first month after randomisation (N=258 swabs). The swab heads were initially stored at room temperature in the participant's home in cryovials containing 1 ml RNALater (Thermo Scientific, Paisley, UK), and then at the Rinda Ubuzima laboratory at -80°C within seven days after collection. Frozen samples were shipped to Liverpool on dry ice. DNA extraction and sequencing were done at the University of Liverpool Centre for Genomic research.[117] DNA was extracted from one sample per participant per time point (N=639 swabs, including the swabs self-sampled by twelve women). The samples were thawed, and DNA was extracted from each sample by adding 180 µl of enzymatic lysis buffer containing lysozyme (Sigma-Aldrich, Dorset, UK); incubation for 30 minutes at 37 ^{0}C; adding 25 µl of proteinase K and 200 µl of buffer AL using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Manchester, UK); incubation for 30 minutes at 56^0 C; and bead-beating after adding 200 mg of 0.1 mm zirconia/silica beads (Thistle Scientific, Glasgow, UK)

on a Qiagen TissueLyser II (Qiagen, Manchester, UK) for 5 minutes at 25 Hz. Next, 200 µl of 100% ethanol was added, the sample was centrifuged, the swab head was discarded, and the pellet was purified in four subsequent centrifugation steps after adding one-by-one 200 µl 100% ethanol, 500 µl buffer AW1, 500 µl buffer AW2 and 75 µl buffer AE as per manufacturer's instructions (Qiagen, Manchester, UK). We included one negative control (an empty tube) with each DNA extraction round of 24 study samples to be able to detect contaminants in extraction reagents downstream. The DNA concentration of randomly selected samples was measured by Qubit (Invitrogen, Thermo Scientific, Paisley, UK) and the DNA quality of all samples by Nanodrop (Thermo Scientific, Paisley, UK).

PCR amplification and 16S rRNA gene sequencing

Each of the 667 DNA samples (639 study samples and 28 negative controls) underwent two PCR rounds for 16S rRNA gene amplification and barcoding. In the first PCR round, the V3-V4 region of the 16S rRNA gene was amplified as described previously.[223] DNA was amplified in a 25 µl reaction volume using 1.25 µl of a 10 µM concentration of 319F 5'-ACTCCTACGGGAGGCAGCAG-3' forward primer and 1.25 µl of a 10 µM concentration of 806R 5'-GGACTACHVGGGTWTCTAAT-3' reverse primer, 12.5 µl NEB Next HF 2x PCR Master Mix (New England Biolabs, Hitchin, UK), 9 µl of nuclease-free water and 1 µl of DNA extraction product. The first denaturation cycle took 30 seconds at 98 ^{0}C and was followed by 10 cycles consisting of a denaturation cycle of 10 seconds (at 98 ^{0}C), an annealing cycle of 30 seconds (at 58 ^{0}C), an extension cycle of 30 seconds (at 72 ^{0}C), and a final extension cycle of 5 minutes at 72 ^{0}C. PCR products were then purified and size-selected using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK) in a 0.8:1.0 bead-to-sample ratio. The second PCR round was to barcode V3-V4 sequences by a dual-index approach using standard Illumina Nextera XT index kit v2 (Illumina, San Diego, CA, USA), permitting multiplexing of up to 384 samples. The barcoding was performed in a 25 µl reaction volume using 2.5 µl of Index 1 primer, 2.5 µl of Index 2 primer, 12.5 µl NEB Next HF 2x PCR Master Mix and 7.5 µl sample. The first denaturation cycle took 3 minutes at 98 ^{0}C and was followed by 15 cycles consisting of a denaturation cycle of 30 seconds (at 98 ^{0}C), an annealing cycle of 30 seconds (at 55 ^{0}C), an extension cycle of 30 seconds (at 72 ^{0}C), and a final extension cycle of 5 minutes at 72 ^{0}C. PCR products were then purified using AMPure beads as explained above, again in a 0.8:1.0 bead-to-sample ratio. We added a negative control to each PCR run (10 µl of nuclease-free water instead of 9 µl of nuclease-free water and 1 µl of DNA) to identify contaminants, as well as a commercially available positive control (10 µl of 0.2 ng/µl ZymoBiomics Microbial Community DNA standard; Zymo Research Corp, Irvine, CA, USA). The DNA extraction negative controls were also included in the PCR runs. DNA from samples collected at different visits but belonging to the same participant were included in the same PCR run to control for inherent differences between PCR runs. PCR product DNA concentrations of each sample (N=683, including negative and positive controls: 639 study samples, eight positive PCR controls, eight negative PCR controls, and 28 negative DNA extraction controls) were measured using the Qubit Fluorometer with the dsDNA HS Assay kit (Invitrogen, Thermo Scientific, Paisley, UK). All samples, including the positive and negative controls, were successfully amplified and used for subsequent steps.

Amplicons from samples were evenly pooled into sequencing libraries at a mass of 0.8 ng DNA per amplicon. To achieve this, Qubit DNA concentrations were used to calculate the volumes of each study sample to be added. Samples with a DNA concentration of <0.30 ng/µl (e.g., the negative controls) were added in a fixed volume of 1 µl. The libraries were sequenced on an Illumina HiSeq instrument (Illumina, San Diego, CA, USA), run in rapid mode, 2x300bp using a 250PE and 50PE kit. DNA from samples collected at different visits but belonging to the same participant were included in the same library to control for inherent differences between sequencing runs.

Panbacterial 16S rRNA gene qPCR

The panbacterial 16S rRNA gene copy concentrations of all samples collected at study visits and containing at least 1,111 reads by Illumina HiSeq sequencing (N=393; see 'Further data processing' below for rationale of rarefaction) were determined at the Institute for Genome Sciences of the University of Maryland (Baltimore, MD, USA) using the BactQuant qPCR assay. This assay was developed based on an analyses of 4,938 16S rRNA gene sequences in the Greengenes database.[118,224] The DNA samples were tested as described previously.[118,225] Briefly, 1.5 µl of template (1:10 diluted

DNA) was added to 3.5 µl of reaction mix, with the final reaction containing 1.8 µM each of the forward (341F) and reverse (806R) primer targeting the 16S V3-V4 region, 225 nM of the TaqManW probe, 1X Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Thermo Scientific, Waltham, MA, USA) and molecular-grade water. Each experiment included an in-run standard curve (ranging from 10 to 10^8, with 10^2–10^8 in 10-fold serial linear dilutions) and no-template controls performed in triplicate. Amplification and real-time fluorescence detections were performed on the Bio-Rad CFX 384 instrument (Bio-Rad Inc., Hercules, CA, USA) using the following PCR conditions: 3 minutes at 50 °C for UDG treatment, 10 minutes at 95 °C for Taq activation, 15 seconds at 95 °C for denaturation and 1 minute at 60 °C for annealing and extension, times 40 cycles. Cycle threshold (Ct) value for each 16S qPCR reaction were obtained using a manual Ct threshold of 0.05 and automatic baseline. The 16S rRNA gene concentration was reported in copies/µL for each sample.

Molecular data processing

We obtained a mean raw unpaired read count of 374,543 reads per study sample (95% confidence interval (CI): 305,845 – 443,242) in run 1 and 302,431 reads per study sample (95% CI: 266,423 – 338,440) in run 2. Reads were first demultiplexed, and primer sequences were removed from forward and reverse reads using Cutadapt 1.2.1.[146] All subsequent steps were performed in the DADA2 version 1.4.0 package for large paired end datasets in R version 3.4.3 (R core team, 2015).[119] We chose DADA2 because of its ability to resolve reads differing by only one nucleotide, thereby maximising our chances of differentiating probiotic reads from naturally occurring *Lactobacillus* species reads. Error correction was performed using the *fastqFilter* command with parameter settings aiming to maximise read retention. For forward and reverse reads, respectively, the minimum read lengths (*truncLen*) were set to 260 and 210 based on the quality plots, *maxEE* to a maximum of 5 and 8 expected errors, *maxN* to zero ambiguous bases allowed, and *truncQ* to zero. Around 10% of reads were discarded after error correction. Next, error rates of forward and reverse reads were determined using the *learnErrors* command. Forward and reverse reads were dereplicated (assigned to unique amplicon sequence variants (ASVs)) using the *derepFastq* command, and denoised (ASVs with higher than average error rates discarded) using the *dada* command.[119,226] Forward and reverse reads were then merged into overlapping reads using the *mergePairs* command. Bimeras (chimeric compositions of two separate parent ASVs) were removed using the *removeBimeraDenovo* command with the Silva version 128 database as the reference database;[120] 10.1% of ASVs were identified as bimeric and removed. Overall, a median of 16% of the raw reads per study sample was removed during these DADA2 clean-up steps.

Taxonomic assignment was also done in DADA2 in two steps: *assignTaxonomy* to map ASVs to taxa at genus level or above using the RDP classifier with a minimum bootstrap value of 50% and the Silva v128 database as the reference database,[120,227] followed by *addSpecies* to map ASVs to species level, allowing only ASVs with exact (100%) identity matches with species in the Silva database to be assigned to that species, and allowing assignment of one ASV to multiple species.

Further data processing

Further data processing was performed in Microsoft Excel 2013 (starting with a spreadsheet containing the sequences, taxonomic assignments, and read counts for each ASV per sample) and STATA version 13 (StataCorp, College Station, TX, USA). We removed all rare ASVs (defined as a read count in all samples combined of less than 100), four non-bacterial ASVs, and two likely contaminant ASVs (a *Rhodanobacter glycinis/terrae* and a *Sneathia* genus) that were present in more than one negative control at relative abundances higher than in any study sample. The vaginal taxa BV-associated bacterium 1 (BVAB1), BVAB2, *Mageeibacillus indolicus* (BVAB3), BVAB TM7 and *Fenollaria massiliensis* are not included in the Silva v128 database but their sequences have been published elsewhere. Similarly, the *Lactobacillus* and *Bifidobacterium* species included in the vaginal probiotic Ecologic Femi+ (EF+; Winclove Probiotics, Amsterdam, Netherlands) are not included in the Silva database, but their sequences were provided to us by Winclove. The vaginal probiotic Gynophilus LP (GynLP; Biose, Aurillac, France) contains *Lactobacillus rhamnosus* (Lcr strain 35), and the NCBI database contains a reference sequence for this probiotic strain.[228] We identified all of the above species in our ASV spreadsheet using the *Needleman-Wunsch Global Align Nucleotide Sequences* function on the National Center for Biotechnology Information (NCBI) website,[229] requiring 100% matches

between reference sequences and uploaded DADA2-derived ASVs of interest. The Silva-based taxonomic assignment of 146 ASVs with a relative abundance of at least 0.05% of the read count of all samples combined (out of a total of 1,797 ASVs) were double-checked using the *Microbial Nucleotide BLAST (BLASTn)* function on the NCBI website.[147,230] Using the non-redundant V3-V4 version of the Vaginal 16S rRNA Reference Database as a tiebreaker,[148] three discordances were resolved, 24 *Lactobacillus* genus ASVs were reassigned to various *Lactobacillus* species, six *Gardnerella* genus ASVs were reassigned to *G. vaginalis*, and one *Atopobium* genus ASV was reassigned to *A. vaginae*. Next, read counts for ASVs assigned to the exact same taxonomy were summed for each sample. Finally, we rarefied at a depth of 1,111 reads (the lowest total read count for a specific sample above 1,000 reads) using the *GUniFrac* 1.0 package in R.[121] The rarefied ASV table contained 629 samples and 401 unique ASVs (10/639 samples became invalid due to rarefaction), with 255 (63.6%) mapping to species level, 116 (28.9%) to genus level, and 30 (7.5%) to higher taxonomic levels. Rarefied read counts were transformed into relative abundances using the *prop.table* function in R. Of the 401 ASVs, 177 ASVs were present at a relative abundance of at least 1% in at least one sample; the other 224 ASVs were minority species.

Panbacterial 16S rRNA gene data

Of the 393 samples that were tested by BactQuant assay, fourteen samples did not amplify in two of three, or all three, of the triplicate reactions, or had skewed low 16S rRNA gene concentration results of <1,000 copies/µl and were considered outliers. A total of 14/393 samples (=3.6%) were therefore excluded from all concentration analyses. We estimated the ASV-specific concentrations per sample using the sample-specific 16S rRNA gene concentration data. First, we identified the 16S rRNA gene copy number per ASV in the NCBI version of the rrnDB database,[231] and in the case of missing data, in the RDP version of the rrnDB database (which only contains information at genus level and above) and the Greengenes database.[224] If an ASV was mapped to multiple species at genus level, the mean of the mean 16S gene copy number for each individual species was used. If the mean 16S gene copy number of a species was not present in the database, we used the mean copy number of the corresponding genus. BVAB1 and BVAB2 belong to the *Clostridiales* order and lower level taxonomic information is not available. We therefore used the *Clostridiales* order copy number (=4.62). Concentrations in cells/µl per ASV per sample were estimated by multiplying the ASV-specific copy-normalised rarefied relative abundance by the sample-specific 16S rRNA gene copies concentration. This method has been shown by others to correlate well with species-specific quantitative PCR results for non-minority species.[129,130] It yielded concentrations for 401 ASVs in 379 samples in cells/µl, which were $\log_{10}$-transformed. Concentrations between zero and one cell/µl were set to one prior to $\log_{10}$-transformation to prevent skewed negative values.

Number of samples available for analyses in Chapter 2

We had vaginal swabs available for all 68 enrolled women prior and after metronidazole treatment (N=136; these are included in the 639 vaginal swabs described above). Of these vaginal swabs, 134 study samples had at least 1,111 reads after Illumina sequencing and therefore underwent the bacterial 16S rRNA gene qPCR. The rarefied ASV table used for the analyses in Chapter 2 contained 134 samples and 204 unique ASVs (2/136 samples became invalid due to rarefaction), with 133 (65.2%) mapping to species level, 55 (27.0%) to genus level, and 16 (7.8%) to higher taxonomic levels. Of the 134 samples that were tested by BactQuant assay, five samples did not amplify in two of three, or all three, of the triplicate reactions, or had skewed low 16S rRNA gene concentration results of <1,000 copies/µl and were considered outliers. A total of 5/134 samples (= 3.7%) were therefore excluded from all concentration analyses. This yielded concentrations for 204 ASVs in 129 samples in cells/µl, which were $\log_{10}$-transformed. Concentrations between zero and one cell/µl were set to one prior to $\log_{10}$-transformation to prevent skewed negative values.

Molecular endpoints: richness, diversity, VMB types, and bacterial groups

Data reduction was required for molecular efficacy analyses, and was done in three different ways (see Chapters 2 and 3 for further details). First, we calculated richness and alpha diversity for each sample using the rarefied relative abundance data. Richness was defined as the total number of ASVs per sample. Alpha diversity was determined by Simpson diversity index (1-D) using the *phyloseq* package

version 1.14.0 in R.[232] Second, given that much VMB composition knowledge has become available since the turn of the century,[4] we used this existing knowledge to assign all 401 ASVs to four bacterial groups (table A.1): 1) lactobacilli (with a further subdivision into EF+ strains, the GynLP strain, and 'natural' lactobacilli); 2) BV-anaerobes (all Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Tenericutes except those included in the other three groups; these have consistently been associated with BV); 3) pathobionts (most Proteobacteria, and streptococci, staphylococci, enterococci, Spirochaetaceae, *Listeria*, and *Chlamydia trachomatis*; these are considered to have higher intrinsic pathogenicity than BV-anaerobes and have not consistently been associated with BV[9]); and 4) 'other bacteria' (a rest group, containing Actinobacteria that are known to be (facultative) aerobic skin bacteria, *Bifidobacterium* species, and three difficult to classify minority species). Within each sample, read counts of ASVs belonging to the same bacterial group were summed. This resulted in four relative abundances (one for each bacterial group) per sample, which sum to 1.0 for each sample. Third, we clustered the rarefied relative abundance data using the *phyloseq* package in R using Euclidean distance with complete linkage.[232] The generated clusters were used to assign samples to eight VMB types, as described in Chapter 2 and 3 (note that the dataset of 134 samples in Chapter 2 only yielded seven VMB types in total; there were no samples assigned to VMB type *Lcr*). To improve the statistical power of the mixed effects models, and to facilitate visualisations of VMB transitions in alluvial diagrams (Chapters 2 and 3), the eight VMB types were condensed further into four 'pooled VMB types' as follows: 1) lactobacilli-dominated (LD; which combined original VMB types 1-3 dominated by *L. iners*, *L. crispatus* or other lactobacilli; n=292), 2) lactobacilli and anaerobes (LA; original VMB type 4; n=86); 3) BV-like (BV; original VMB types 5-7 combined, which were all characterised by anaerobes other than lactobacilli with different proportions of *G. vaginalis*; n=202); and pathobionts (PB; original VMB type 8, which include all VMBs with ≥20% pathobionts; n=49).

For further clarification, a sample containing 30% *L. iners*, 30% other lactobacilli, 20% *G. vaginalis*, and 20% other BV-anaerobes, would have been assigned to the LA (lactobacilli and anaerobes) VMB type, as the total relative abundance of lactobacilli was 0.60. If this sample contained one million 16S rRNA genes per µl, and each of the species included in the sample only contained one 16S rRNA gene copy, it would contain a lactobacilli concentration of [30%+30%] x one million = 600,000/µl, a BV-anaerobes concentration of [20%+20%] x one million = 400,000/µl, a pathobionts concentration of 0/µl, and a 'other bacteria' concentration of 0/µl. Therefore, each sample was assigned to a mutually exclusive VMB type, and had relative abundance data (and, if applicable, estimated concentration data) for each of the four bacterial groups.

All VMB types, except for the three *Lactobacillus*-dominated VMB types, were considered dysbiotic. The bacterial groups BV-anaerobes and pathobionts were also considered dysbiotic.

Statistical analysis and figures in Chapter 2

Concentration changes were expressed as percentages per individual participant as follows: [concentration at the enrolment/post-treatment visit] – [concentration at the screening/pre-treatment visit] divided by [concentration at the screening/pre-treatment visit]. When the pre-treatment concentration was zero and the post-treatment concentration was greater than zero, the increase was set to 100% or the highest value among the other participants, whichever was greatest.

Bar graphs, bar charts, and scatter plots were made in STATA. Other data visualisations were made in R: three-dimensional plots of the three main non-metric multidimensional scaling (NMDS) vectors were made using *vegan* and *plotly* packages,[233,234] heatmaps showing the twenty ASVs with highest median relative abundance were made using the *gplots* package,[235] and alluvial diagrams were made using the *ggalluvial* package to compare (pooled) VMB types before and after treatment.[236]

Trial endpoints and hypotheses (Chapter 3)

The primary aims of the trial were to determine the safety and preliminary efficacy of the interventions, each compared to the no intervention group. We hypothesised that all interventions would be safe. The primary preliminary efficacy endpoints were the incidence of BV by Nugent score and modified Amsel criteria (which were hypothesised to decrease), and symptomatic vulvovaginal candidiasis (VVC) by

wet mount (which was hypothesised not to increase). The secondary preliminary efficacy endpoints included membership of specific VMB clusters/types and bacterial group concentrations over time as determined by Illumina HiSeq sequencing.

Safety endpoints in Chapter 3
The main safety endpoints were self-reported solicited and unsolicited (serious) adverse events (AEs) and social harms, and clinician-observed speculum exam findings. Adverse events were coded using the Medical Dictionary for Regulatory Activities (medDRA version 19.1, McLean, VA, USA). Laboratory test results were not considered AEs, but primary and secondary endpoints.

Primary preliminary efficacy endpoints in Chapter 3
For the BV by Nugent 7-10 and modified Amsel criteria and VVC endpoints, see the 'diagnostic testing' section above. Analyses were also conducted using a Nugent score of 4-10 and the full Amsel criteria as the definition of BV but the results are not shown in Chapter 3 because they are similar to the Nugent 7-10 and modified Amsel criteria results, and less informative than the molecular data.

Statistical analyses (Chapter 3)
Statistical analyses were performed using STATA version 13 (StataCorp, College Station, TX, USA). We used the *phyloseq* and *gplots* packages in R to make heatmaps of the 20 ASVs with the highest mean relative abundance (figure 3.2a) or concentration (figure 3.2b) across all study samples.[235] Bar graphs and line graphs were made using the *catplot* and *scatter* functions in STATA and alluvial diagrams were made using the *ggalluvial* package in R.[236]

Most statistical comparisons were between randomisation groups (each intervention group compared to the no-intervention group) at screening, enrolment, and longitudinally over time. For cross-sectional analyses, we used Fisher's exact test to compare binary and categorical variables, Kruskal-Wallis test to compare continuous variables, and Mann Whitney U test for pairwise comparisons of continuous variables if the Kruskal-Wallis test was significant at $p<0.05$. For longitudinal analyses, we used incidence rates (IRs) and incidence rate ratios (IRRs) with 95% confidence intervals (CI; primary endpoints only), and mixed effects models. IRs were defined as the number of new infections during follow-up divided by the person-years at risk for that infection using the *ststet* function in STATA. Women who had BV recurrence within 10 days that persisted until M6 were considered persistent infections to prevent inflation of the IRs. IRRs were calculated by dividing the IR of each intervention group by the IR of the control group. Mixed effects models were done in STATA using the *xtmelogit* function for categorical endpoints and the *mixed* function for continuous endpoints. All models included one VMB endpoint at a time as the outcome, the participant identification number as the random effect, and randomisation group as the main fixed effect. Three variables were added as additional fixed effects in adjusted mixed effects models because they were associated at $p<0.05$ in mixed effects models with at least one of four a priori selected VMB endpoints (Nugent score, or concentrations of lactobacilli, BV-associated anaerobes, or pathobionts; table C.5), and data were available at all study visits. They were 'current use of hormonal contraception or being pregnant' (versus not using contraception and not being pregnant; six samples from copper intrauterine device users were excluded), a 'sexual risk' composite variable (with lower risk defined as having reported condom use at each vaginal sex act since the last study visit and fewer than the median of five sex partners in the past month), and 'age' (30 years or older versus younger than 30 years; the median age of the screened population was 30 years). One additional variable was associated with one of the a priori selected VMB outcomes at $p<0.05$: managing menses with sanitary pads versus other methods (table C.5). However, this variable was not added as an additional fixed effect in the adjusted mixed effects models due to only having been recorded at enrolment visits and not at follow-up visits.

All analyses were conducted on the intent-to-treat (ITT) population (n=68), and IR and IRR analyses were also conducted on a modified ITT population (n=51). Women who did not have a Nugent score 0-6 (n=17 women) at the time of randomisation were excluded from the modified ITT population. Another 17 women had ongoing chlamydia and/or gonorrhoea infection at the time of randomisation, but the molecular analyses did not identify substantial differences in the VMB compositions of women

with and without ongoing infection and we therefore did not exclude them (figure C.1). In accordance with local treatment guidelines, BV and VVC were treated when laboratory-confirmed and symptoms and/or clinician-observed signs were present. TV and other sexually transmitted infections were always treated when identified by laboratory testing. Some women received antimicrobial drugs for other ailments from external clinics. Antimicrobial use during the study is shown in table C.1. We took antimicrobial use into account to determine whether cases were likely incident or persistent, but we did not remove users from the modified ITT population because they were evenly distributed between the randomisation groups and we expected the short course treatments to have less of an effect on the VMB than the longer-term interventions.

IR and IRR analyses were conducted for the product use period (between enrolment and M2, including samples from D7, M1, and M2) as well as the period between M2 and M6 (M6 samples). Mixed effects models were done for the product use period only (D7, M1, M2, and self-collected samples) to determine if any of the observed effects were statistically significant; we did not run mixed effects models for the period after product cessation because the IR/IRR analyses and graphs had already clearly shown that the observed effects during the intervention period had disappeared after cessation of the interventions.

Table A.1: List of amplicon sequence variants detected and classification into bacterial groups

List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
Abiotrophia defectiva	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Actinobacillus genus	Pathobionts	Gammaproteobacteria (Pasteurellales)	Yes
Actinomyces family	BV	Actinobacteria/Actinobacteria (Actinomycetales)	No
Actinomyces genus	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Actinomyces europaeus	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Actinomyces funkei	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Actinomyces neuii	BV	Actinobacteria/Actinobacteria (Actinomycetales)	No
Actinomyces odontolyticus	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Actinomyces turicensis	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Actinomyces urogenitalis	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Aerococcus genus	BV	Firmicutes/Bacilli (Lactobacillales)	No
Aerococcus christensenii	BV	Firmicutes/Bacilli (Lactobacillales)	No
Aeromonas caviae/dhakensis/enteropelogenes/ hydrophila/jandaei/taiwanensis/veronii	Pathobionts	Gammaproteobacteria (Aeromonadales)	Yes
Alistipes finegoldii/onderdonkii	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Alloiococcus genus	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Alloprevotella genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Alloprevotella rava	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Alloscardovia omnicolens	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	Yes
Anaerococcus genus	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus hydrogenalis	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus lactolyticus	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus murdochii	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus obesiensis	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus prevotii	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Anaerococcus prevotii/tetradius	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus provenciensis	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaerococcus vaginalis	BV	Firmicutes/Tissierellia (Tissierellales)	No
Anaeroplasma genus	BV	Tenericutus/Mollicutus (Anaeroplasmatales)	Yes
Anaerostipes hadrus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Anaerotruncus genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Anaerovibrio genus	BV	Firmicutes/Negativicutes (Selenomonadales)	Yes
Arcanobacterium genus	BV	Actinobacteria/Actinobacteria (Actinomycetales)	No
Asteroleplasma genus	BV	Tenericutus/Mollicutus (Anaeroplasmatales)	Yes
Atopobium genus	BV	Actinobacteria/Coriobacteria (Coriobacteriales)	No
Atopobium deltae	BV	Actinobacteria/Coriobacteria (Coriobacteriales)	Yes
Atopobium vaginae	BV	Actinobacteria/Coriobacteria (Coriobacteriales)	No
Bacillus altitudinis/amyloliquefaciens/firmus/ licheniformis/mojavensis/subtilis/tequilensis/timonensis/ velezensis	Other	Firmicutes/Bacilli (Bacillales)	Yes
Bacteroidetes phylum	BV	Bacteroidetes	Yes
Bacteroides genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroidales S24-7 group family	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Bacteroides caccae	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroides dorei/vulgatus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroides fragilis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Bacteroides fragilis/xylanisolvens	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroides pleibeius	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroides thetaiotaomicron	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroides uniformis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Bacteroides vulgatus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Betaproteobacteria class	Other	Betaproteobacteria	Yes
Bifidobacterium adolescentis/faecale/stercoris	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	Yes
Bifidobacterium breve	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	No
Bifidobacterium catenulatum/kashiwanohense/ pseudocatenulatum	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	No
Bifidobacterium kashiwanohense	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	Yes
Bifidobacterium longum	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	No
Blautia genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Blautia faecis	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Blautia obeum	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Blautia obeum/wexlerae	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Blautia stercoris	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Brevibacterium genus	Other	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Brevibacerium luteolum	Other	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Brevibacterium massiliense/ravenspurgense	Other	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Brevibacterium paucivorans	Other	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Brevundimonas albigilva/nasdae/vesicularis	Other	Alphaproteobacteria (Caulobacterales)	Yes

List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
Bulleidia genus	BV	Firmicutes/Erysipelotrichi (Erysipelotichales)	Yes
Butyricicoccus genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Butyrivibrio genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Butyrivibrio crossotus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
BVAB TM7	BV	Unclassified (TM7 division)	No
BVAB1	BV	Firmicutes/Clostridia (Clostridiales)	No
BVAB2	BV	Firmicutes/Clostridia (Clostridiales)	No
Campylobacter genus	Pathobionts	Epsilonproteobacteria (Campylobacterales)	No
Campylobacter hominis	Pathobionts	Epsilonproteobacteria (Campylobacterales)	Yes
Campylobacter ureolyticum	Pathobionts	Epsilonproteobacteria (Campylobacterales)	Yes
Caproiciproducens genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Carnobacteriaceae family	Other	Firmicutes/Bacilli (Lactobacillales)	Yes
Catenibacterium genus	BV	Firmicutes/Erysipelotrichi (Erysipelotichales)	Yes
Catenibacterium mitsuokai	BV	Firmicutes/Erysipelotrichi (Erysipelotichales)	Yes
Catonella morbi	BV	Firmicutes/Clostridia (Clostridiales)	No
Chlamydia trachomatis	Pathobionts	Chlamydiae (Chlamydiales)	Yes
Christensenellaceae_R-7_group genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Clostridiales order	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Clostridiales_vadinBB60_group family	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Clostridium_sensu_stricto_1 genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Clostridium celatum/disporicum	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Clostridium_sensu_stricto_1perfringens/thermophilus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Collinsella genus	BV	Actinobacteria/Coriobacteria (Coriobacteriales)	Yes
Collinsella aerofaciens	BV	Actinobacteria/Coriobacteria (Coriobacteriales)	No
Coprococcus genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Coprococcus_1 catus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Coprococcus_2 eutactus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Coprococcus_3 comes	BV	Firmicutes/Clostridia (Clostridiales)	No
Coriobacteriaceae family	BV	Actinobacteria/Coriobacteria (Coriobacteriales)	Yes
Corynebacteriaceae family	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium genus	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 genus	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	No
Corynebacterium atypicum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium glucuronolyticum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	No
Corynebacterium jeikeium	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium minutissimum/singulare	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium pyruviciproducens	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 afermentans/coyleae	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 amycolatum/jeikeium/urealyticum/ vitaeruminis	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	No
Corynebacterium_1 aurimucosum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	No
Corynebacterium_1 aurimucosum/minutissimum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 aurimucosum/pseudogenitalium/ tuberculostearicum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 aurimucosum/simulans/striatum/ xerosis	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 aurimucosum/striatum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 coyleae	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 freneyi/xerosis	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 genitalium	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 imitans	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 minutissimum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 pseudogenitalium	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 riegelii	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 tuscaniense	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Corynebacterium_1 urealyticum	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Delftia acidovorans/lacustris/tsuruhatensis	Other	Betaproteobacteria (Burkholderiales)	Yes
Dermabacter hominis	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Dermabacter hominis/vaginalis	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Dermabacter jinjuensis	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Desulfovibrio genus	BV	Deltaproteobacteria (Desulfovibrionales)	Yes
Dialister genus	BV	Firmicutes/Negativicutes (Veillonellales)	No
Dialister micraerophilus/microaerophilus	BV	Firmicutes/Negativicutes (Veillonellales)	No
Dialister propionicifaciens	BV	Firmicutes/Negativicutes (Veillonellales)	No
Dorea genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Dorea formicigenerans	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Dorea longicatena	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Enterobacter genus	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Enterococcus genus	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Enterococcus azikeevi/durans/faecalis/faecium/hirae/	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No

List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
lactis/mundtii/raffinosus/ratti/rivorum/thailandicus/ villorum			
Enterococcus durans/faecalis/faecium	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Enterococcus durans/faecium/phoeniculicola/ thailandicus	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	Yes
Enterococcus faecalis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Enterococcus faecium	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Eremococcus genus	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Eremococcus colecola	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Erysipelotrichaceae family	BV	Firmicutes/Erysipelotrichia (Erysipelotrichales)	Yes
Erysipelotrichaceae UCG-003 genus	BV	Firmicutes/Erysipelotrichia (Erysipelotrichales)	Yes
Erysipelatoclostridium ramosum	BV	Firmicutes/Erysipelotrichia (Erysipelotrichales)	Yes
Escherichia/Shigella genus	Pathobionts	Gammaproteobacteria (Enterobacterales)	No
Ezakiella genus	BV	Firmicutes/Tissierellia (unclassified Tissierellia)	No
Ezakiella peruensis	BV	Firmicutes/Tissierellia (unclassified Tissierellia)	Yes
Facklamia genus	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Facklamia hominis	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Facklamia languida	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Faecalibacterium genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Faecalibacterium cf./prausnitzii	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Faecalibacterium prausnitzii	BV	Firmicutes/Clostridia (Clostridiales)	No
Family XI family	Other	Cyanobacteria (order not described)	Yes
Family XIII family	Other	Cyanobacteria (order not described)	Yes
Family XIII UCG-001 genus	Other	Cyanobacteria (order not described)	Yes
Fastidiosipila genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Fenollaria massiliensis strain *DNF00604*	BV	Firmicutes/Clostridia (Clostridiales)	No
Filifactor genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Finegoldia genus	BV	Firmicutes/Tissierellia (Tissierellales)	No
Finegoldia magna	BV	Firmicutes/Tissierellia (Tissierellales)	No
Fusicatenibacter genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Fusicatenibacter saccharivorans	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Fusobacterium genus	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Fusobacterium equinum/gonidiaformans	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Fusobacterium nucleatum	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Gallicola genus	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Gammaproteobacteria class	Pathobionts	Gammaproteobacteria	Yes
Gardnerella genus	BV	Actinobacteria/Actinobacteria (Bifidobacteriales)	No
Gardnerella vaginalis	BV	Actinobacteria/Actinobacteria (Bifidobacteriales)	No
Gastranaerophilales order	Other	Cyanobacteria Melainabacteria Group (Candidatus Melainabacteria)	Yes
Gemella genus	BV	Firmicutes/Bacilli (Bacillales)	No
Gemella asaccharolytica	BV	Firmicutes/Bacilli (Bacillales)	No
Gemella haemolysans/sanguinis/taiwanensis	BV	Firmicutes/Bacilli (Bacillales)	No
Gemella parahaemolysans	BV	Firmicutes/Bacilli (Bacillales)	No
Globicatella sanguinis	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Granulicatella genus	BV	Firmicutes/Bacilli (Lactobacillales)	No
Granulicatella adiacens/para-adiacens	BV	Firmicutes/Bacilli (Lactobacillales)	Yes
Granulicatella elegans	BV	Firmicutes/Bacilli (Lactobacillales)	No
Haemophilus genus	Pathobionts	Gammaproteobacteria (Pasteurellales)	No
Haemophilus haemolyticus	Pathobionts	Gammaproteobacteria (Pasteurellales)	Yes
Haemophilus haemolyticus/influenzae	Pathobionts	Gammaproteobacteria (Pasteurellales)	Yes
Haemophilus haemolyticus/influenzae/quentini	Pathobionts	Gammaproteobacteria (Pasteurellales)	Yes
Haemophilus influenzae/parainfluenzae	Pathobionts	Gammaproteobacteria (Pasteurellales)	Yes
Haemophilus parainfluenzae	Pathobionts	Gammaproteobacteria (Pasteurellales)	No
Helcobacillus genus	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Helcococcus genus	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Holdemanella genus	BV	Firmicutes/Erysipelotrichia (Erysipelotrichiales)	Yes
Holdemanella biformis	BV	Firmicutes/Erysipelotrichia (Erysipelotrichiales)	Yes
Howardella genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Intestinimonas genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Intestinibacter bartletti	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Janibacter genus	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Johnsonella genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Jonquetella anthropi	BV	Synergistetes/Synergistia (Synergistales)	Yes
Klebsiella granulomatis/oxytoca/pneumoniae/ quasipneumoniae/variicola	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Klebsiella oxytoca/pneumoniae	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Klebsiella oxytoca/pneumoniae/variicola	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Kocuria marina	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Lachnoclostridium genus	BV	Firmicutes/Clostridia (Clostridiales)	No

List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
Lachnospiraceae family	BV	Firmicutes/Clostridia (Clostridiales)	No
Lachnospiraceae_FCS020_group genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospiraceae_FE2018_group genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospiraceae_ND3007_group genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospiraceae_NK3A20_group genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospiraceae_NK4A136_group genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Lachnospiraceae_UCG-001 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospiraceae_UCG-004 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospiraceae_UCG-010 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lachnospira genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Lachnospira pectinoschiza	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Lactobacillus genus	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus agilis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus coleohominis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus crispatus	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus crispatus/acidophilus/casei/gallinarum	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus crispatus/gallinarum/acidophilus/ kitasatonis/ultunensis/helveticus/amylovorus/ kefiranofaciens/hamsteri	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus crispatus/gasseri/helveticus/johnsonii/ kefiranofaciens	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus delbrueckii	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus faecis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	Yes
Lactobacillus fermentum	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	Yes
Lactobacillus fermentum/curieae/delbrueckii/ingluviei/ oris/plantarum	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus fermentum/gasseri/reuteri/vaginalis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus fermentum/mucosae	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus fornicalis/jensenii	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus gasseri	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus iatae/johnsonii/taiwanensis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus iners	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus jensenii	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus jensenii/fornicalis/psittaci	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus johnsonii/prophage/taiwanensis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus johnsonii/taiwanensis/gasseri	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus mucosae	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	Yes
Lactobacillus oris	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus plantarum	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus pontis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus reuteri	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus ruminis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus saerimneri/sakei	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	Yes
Lactobacillus vaginalis	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactobacillus vaginalis/reuteri	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Lactococcus formosensis/garvieae/lactis	Other	Firmicutes/Bacilli (Lactobacillales)	Yes
Lawsonella genus	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	Yes
Lawsonella clevelandensis	Other	Actinobacteria/Actinobacteria (Corynebacteriales)	No
Listeria innocua/ivanovii/marthii/monocytogenes/phage/ seeligeri/welshimeri	Pathobionts	Firmicutes/Bacilli (Bacillales)	Yes
*Mageeibacillus indolicus (*formerly BVAB3*)*	BV	Firmicutes/Clostridia (Clostridiales)	No
Marvinbryantia genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Megasphaera genus	BV	Firmicutes/Negativicutes (Veillonellales)	No
Micrococcaceae family	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Micrococcus alkanovora/aloeverae/antarcticus/ endophyticus/indicus/luteus/yunnanensis	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Micrococcus aloeverae/luteus/lylae/yunnanensis	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Micrococcus lylae	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Mitsuokella genus	BV	Firmicutes/Negativicutes (Veillonellales)	No
Mobiluncus genus	BV	Actinobacteria/Actinobacteria (Actinomycetales)	No
Mobiluncus curtisii	BV	Actinobacteria/Actinobacteria (Actinomycetales)	No
Mobiluncus mulieris	BV	Actinobacteria/Actinobacteria (Actinomycetales)	No
Moryella genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Moryella indoligenes	BV	Firmicutes/Clostridia (Clostridiales)	No
Murdochiella genus	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Murdochiella asaccharolytica	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Mycoplasmataceae family	BV	Tenericutes/Mollicutes (Mycoplasmatales)	No
Mycoplasma genus	BV	Tenericutes/Mollicutes (Mycoplasmatales)	No
Mycoplasma genitalium	BV	Tenericutes/Mollicutes (Mycoplasmatales)	Yes
Mycoplasma hominis	BV	Tenericutes/Mollicutes (Mycoplasmatales)	Yes

List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
Mycoplasma spermatophilum	BV	Tenericutes/Mollicutes (Mycoplasmatales)	Yes
Negativicoccus genus	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Negativicoccus succinicivorans	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Neisseriaceae family	Pathobionts	Betaproteobacteria (Neisseriales)	Yes
Neisseria genus	Pathobionts	Betaproteobacteria (Neisseriales)	Yes
Neisseria cinerea/meningitidis	Pathobionts	Betaproteobacteria (Neisseriales)	Yes
Neisseria flava/lactamica/macacae/meningitidis/ mucosa/perflava/sicca	Pathobionts	Betaproteobacteria (Neisseriales)	Yes
Neisseria flavescens	Pathobionts	Betaproteobacteria (Neisseriales)	Yes
Neisseria gonorrhoeae/meningitidis	Pathobionts	Betaproteobacteria (Neisseriales)	No
Neisseria mucosa	Pathobionts	Betaproteobacteria (Neisseriales)	Yes
Nesterenkonia lacusekhoensis	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Nosocomiicoccus genus	Other	Firmicutes/Bacilli (Bacillales)	Yes
Odoribacter splanchnicus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Olsenella genus	BV	Actinobacteria/Coriobacteriia (Coriobacteriales)	Yes
Paeniglutamicibacter genus	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Parabacteroides genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Parabacteroides distasonis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Parabacteroides merdae	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Paraprevotella genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Parvimonas genus	BV	Firmicutes/Tissierellia (Tissierellales)	No
Parvimonas micra	BV	Firmicutes/Tissierellia (Tissierellales)	No
Peptococcus genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Peptococcus niger	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Peptoniphilus genus	BV	Firmicutes/Tissierellia (Tissierellales)	No
Peptoniphilus asaccharolyticus	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Peptoniphilus asaccharolyticus/grossensis/harei	BV	Firmicutes/Tissierellia (Tissierellales)	No
Peptoniphilus coxii	BV	Firmicutes/Tissierellia (Tissierellales)	No
Peptoniphilus duerdenii	BV	Firmicutes/Tissierellia (Tissierellales)	No
Peptoniphilus gorbachii/rhinitidis	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Peptoniphilus harei	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Peptoniphilus lacrimalis	BV	Firmicutes/Tissierellia (Tissierellales)	No
Peptoniphilus massiliensis	BV	Firmicutes/Tissierellia (Tissierellales)	Yes
Peptostreptococcaceae family	BV	Firmicutes/Clostridia (Clostridiales)	No
Peptostreptococcus genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Peptostreptococcus anaerobius	BV	Firmicutes/Clostridia (Clostridiales)	No
Phascolarctobacterium genus	BV	Firmicutes/Negativicutes (Acidaminococcales)	Yes
Phascolarctobacterium faecium	BV	Firmicutes/Negativicutes (Acidaminococcales)	Yes
Phascolarctobacterium succinatutens	BV	Firmicutes/Negativicutes (Acidaminococcales)	Yes
Porphyromonas genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Porphyromonas asaccharolytica	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Porphyromonas somerae	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Porphyromonas uenonis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotellaceae family	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Prevotellaceae_NK3B31_group genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Prevotellaceae_UCG-003 genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Prevotella genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella amnii	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella bergensis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Prevotella bivia	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella bivia/denticola	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella buccalis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella colorans	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Prevotella disiens	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella intermedia	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	Yes
Prevotella melaninogenica	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella timonensis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella_2 stercorea	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella_6 corporis	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Prevotella_9 copri	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Propionibacterium acnes/avium	Other	Actinobacteria/Actinobacteria (Propionibacteriales)	Yes
Propionimicrobium genus	Other	Actinobacteria/Actinobacteria (Propionibacteriales)	Yes
Propionimicrobium lymphophilum	Other	Actinobacteria/Actinobacteria (Propionibacteriales)	Yes
Proteobacteria phylum	Other	Proteobacteria	Yes
Pseudomonadaceae family	Pathobionts	Gammaproteobacteria (Pseudomonales)	Yes
Pyramidobacter genus	BV	Synergistetes/Synergistia (Synergistales)	Yes
Raoultella genus	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Raoultella ornithinolytica	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Raoultella ornithinolytica/planticola	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Rickettsiales_Incertae_Sedis family	Pathobionts	Alphaproteobacteria (Rickettsiales)	Yes

List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
Rikenellaceae_RC9_gut_group genus	BV	Bacteroidetes/Bacteroidia (Bacteroidales)	No
Romboutsia genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Roseburia genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Roseburia faecis	BV	Firmicutes/Clostridia (Clostridiales)	No
Roseburia intestinalis	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Roseburia inulinivorans	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Rothia genus	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Rothia mucilaginosa	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Ruminiclostridium genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcaceae family	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcaceae_UCG-002 genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Ruminococcaceae_UCG-003 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcaceae_UCG-005 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcaceae_UCG-010 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcaceae_UCG-013 genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcaceae_UCG-014 genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Ruminococcaceae_NK4A214_group genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcus genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Ruminococcaceae_UCG-002 bacterium	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ruminococcus_2 bromii	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Saccharibacteria phylum	BV	Unclassified (TM7 division)	No
Sarcina genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Senegalimassilia genus	BV	Actinobacteria/Coriobacteriia (Coriobacteriales)	No
Senegalimassilia anaerobia	BV	Actinobacteria/Coriobacteriia (Coriobacteriales)	Yes
Serratia entomophila/marcescens/nematodiphila	Pathobionts	Gammaproteobacteria (Enterobacterales)	Yes
Shuttleworthia genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Slackia exigua	BV	Actinobacteria/Coriobacteriia (Eggerthellales)	Yes
Slackia isofavoniconvertens	BV	Actinobacteria/Coriobacteriia (Eggerthellales)	Yes
Sneathia genus	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Sneathia amnii	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Sneathia amnii/sanguinegens	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Sneathia sanguinegens	BV	Fusobacteria/Fusobacteriia (Fusobacteriales)	No
Solobacterium genus	BV	Firmicutes/Erysipelotrichia (Erysipelotrichales)	Yes
Solobacterium moorei	BV	Firmicutes/Erysipelotrichia (Erysipelotrichales)	Yes
Spirochaetaceae family	Pathobionts	Spirochaetes/Spirochaetia (Spirochaetales)	Yes
SR1_(Absconditabacteria) phylum	BV	Unclassified (Absconditabacteria division)	Yes
Staphylococcus genus	Pathobionts	Firmicutes/Bacilli (Bacillales)	Yes
Staphylococcus argenteus/aureus/equorum/phage/ schweitzeri/simiae	Pathobionts	Firmicutes/Bacilli (Bacillales)	Yes
Staphylococcus aureus/capitis/caprae/epidermidis/ haemolyticus/warneri	Pathobionts	Firmicutes/Bacilli (Bacillales)	No
Staphylococcus aureus/devriesei/epidermidis/ haemolyticus	Pathobionts	Firmicutes/Bacilli (Bacillales)	No
Staphylococcus epidermidis	Pathobionts	Firmicutes/Bacilli (Bacillales)	Yes
Staphylococcus epidermidis/haemolyticus	Pathobionts	Firmicutes/Bacilli (Bacillales)	No
Staphylococcus epidermidis/haemolyticus/hominis	Pathobionts	Firmicutes/Bacilli (Bacillales)	No
Staphylococcus haemolyticus	Pathobionts	Firmicutes/Bacilli (Bacillales)	No
Staphylococcus haemolyticus/petrasii	Pathobionts	Firmicutes/Bacilli (Bacillales)	No
Staphylococcus hominis	Pathobionts	Firmicutes/Bacilli (Bacillales)	Yes
Stenotrophomonas maltophilia	Pathobionts	Gammaproteobacteria (Xanthomonadales)	Yes
Stenotrophomonas maltophilia/rhizophila	Pathobionts	Gammaproteobacteria (Xanthomonadales)	Yes
Streptococcus genus	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus agalactiae/pyogenes	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus alactolyticus/equinus/gallolyticus/ macedonicus/pasteuri/pasteurianus	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus anginosus/constellatus/intermedius	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	Yes
Streptococcus anginosus/intermedius/sanguinis/suis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus anginosus/milleri	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus australis/infantis/mitis/oralis/sanguinis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	Yes
Streptococcus dentisani/infantis/mitis/oligofermentans/ oralis/pneumoniae/pseudopneumoniae/sanguinis/ tigurinus	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus equinus/infantarius/lutetiensis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus gordonii/mitis/oligofermentans/sanguinis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	Yes
Streptococcus mitis/parasanguinis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus oralis/parasanguinis/sanguinis	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	No
Streptococcus pneumoniae	Pathobionts	Firmicutes/Bacilli (Lactobacillales)	Yes
Subdoligranulum genus	BV	Firmicutes/Clostridia (Clostridiales)	No
Succinivibrio genus	Other	Gammaproteobacteria (Aeromonadales)	Yes
Sutterella genus	Other	Betaproteobacteria (Burkholderiales)	No

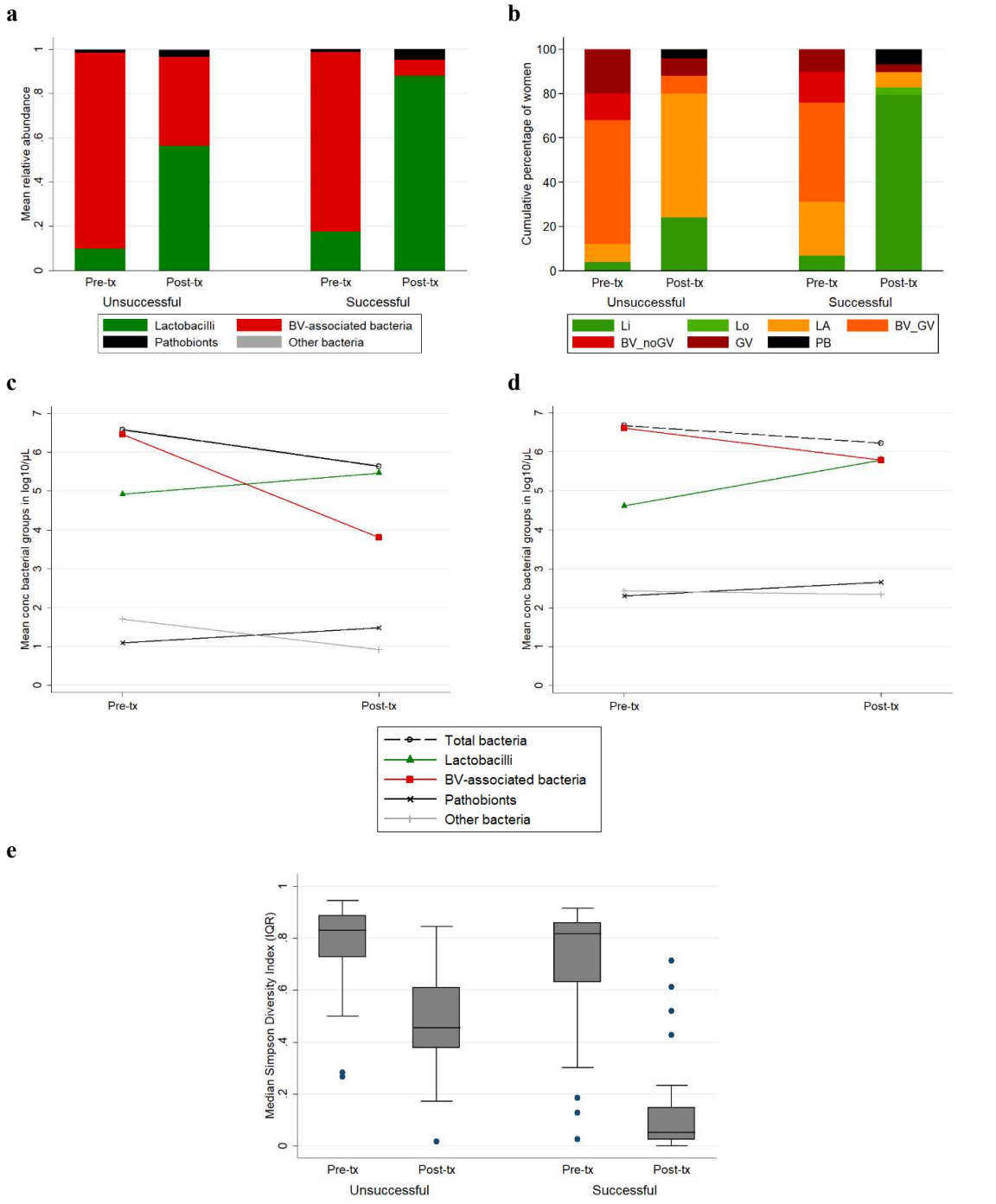
List of taxa (alphabetical order)	Classification	Phylum/Class (Order)*	Minority taxon†
Sutterella morbirenis/sanguinus	Other	Betaproteobacteria (Burkholderiales)	Yes
Sutterella wadsworthensis	Other	Betaproteobacteria (Burkholderiales)	Yes
Terrisporobacter genus	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Treponema_2 genus	Pathobionts	Spirochaetes/Spirochaetia (Spirochaetales)	Yes
Trueperella bernardiae	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Tyzzerella_4 nexilis	BV	Firmicutes/Clostridia (Clostridiales)	Yes
Ureaplasma genus	BV	Tenericutes/Mollicutes (Mycoplasmatales)	No
Ureaplasma parvum/urealyticum	BV	Tenericutes/Mollicutes (Mycoplasmatales)	No
Ureaplasma urealyticum	BV	Tenericutes/Mollicutes (Mycoplasmatales)	No
VadinBE97 family	Other	Lentisphaerae: Candidate division VadinBE97	Yes
Varibaculum genus	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Varibaculum cambriense	BV	Actinobacteria/Actinobacteria (Actinomycetales)	Yes
Veillonellaceae family	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Veillonella genus	BV	Firmicutes/Negativicutes (Veillonellales)	No
Veillonella atypica	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Veillonella dispar	BV	Firmicutes/Negativicutes (Veillonellales)	No
Veillonella dispar/parvula	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Veillonella montpellierensis	BV	Firmicutes/Negativicutes (Veillonellales)	No
Veillonella parvula	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Veillonella parvula/rogosae	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Veillonella ratti/seminalis	BV	Firmicutes/Negativicutes (Veillonellales)	No
Veillonella rogosae	BV	Firmicutes/Negativicutes (Veillonellales)	Yes
Weissella cibaria/confusa/koreensis	BV	Firmicutes/Bacilli (Lactobacillales)	No
Zimmermannella genus	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
Zimmermannella bifida	Other	Actinobacteria/Actinobacteria (Micrococcales)	Yes
EF+ BBW28	Other	Actinobacteria/Actinobacteria (Bifidobacteriales)	No
EF+ LAW70	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
EF+ LBW63	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
EF+ LHW74	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
EF+ LPW21	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
EF+ LSW24	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No
Gynophilus	Lactobacilli	Firmicutes/Bacilli (Lactobacillales)	No

BV bacterial vaginosis, *BVAB* BV-associated bacterium, *EF+* Ecologic Femi+.
This list contains all 425 amplicon sequence variants of Chapter 2 (containing 204 amplicon sequence variants in all 136 samples) and Chapter 3 (containing 425 amplicon sequence variants in all 639 samples) of this thesis, including the 24 amplicon sequence variants that were deleted due to rarefaction.
*Based on NCBI taxonomy browser. †<1% relative abundance in all study samples.

Appendix B

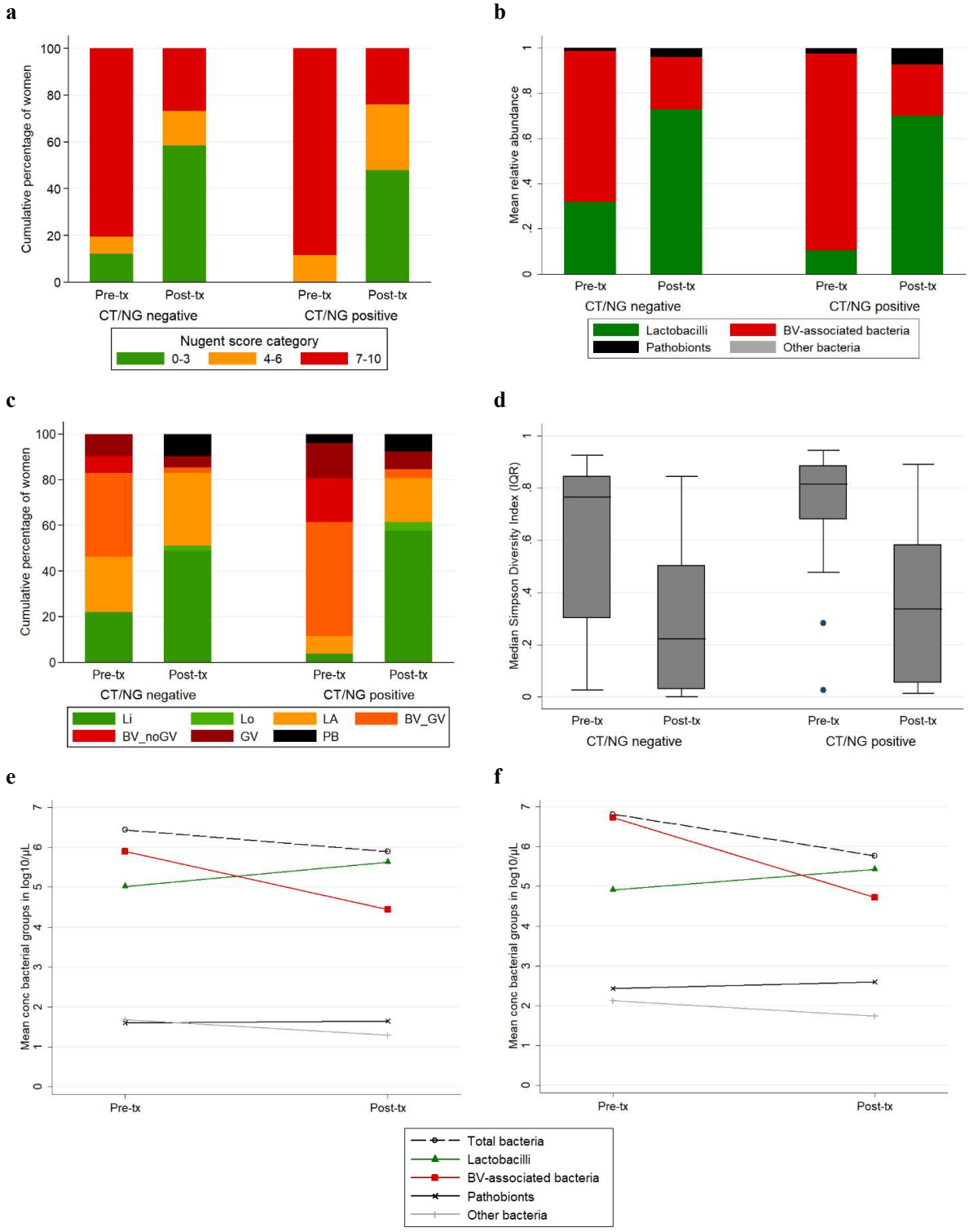
This appendix corresponds to Chapter 2.

Figure B.1: VMB outcomes pre- and post-treatment, stratified by metronidazole treatment success



BV bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *Conc* concentration, *GV G. vaginalis*-dominated, *IQR* inter-quartile range, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts-containing, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit, *VMB* vaginal microbiota.
a-e Figures show changes in VMB characteristics before and after metronidazole treatment: bacterial group mean relative abundances (**a**), VMB types (**b**), bacterial groups concentrations of women with successful treatment by Nugent scoring (n=28 at pre-tx, n=29 at post-tx; see table 2.2 for 95% confidence intervals) (**c**). **d** bacterial group concentrations of women with unsuccessful treatment by Nugent scoring (n=25 at pre-tx, n=22 at post-tx). **e** median inverse Simpson diversity index before and after metronidazole treatment, stratified by treatment success.

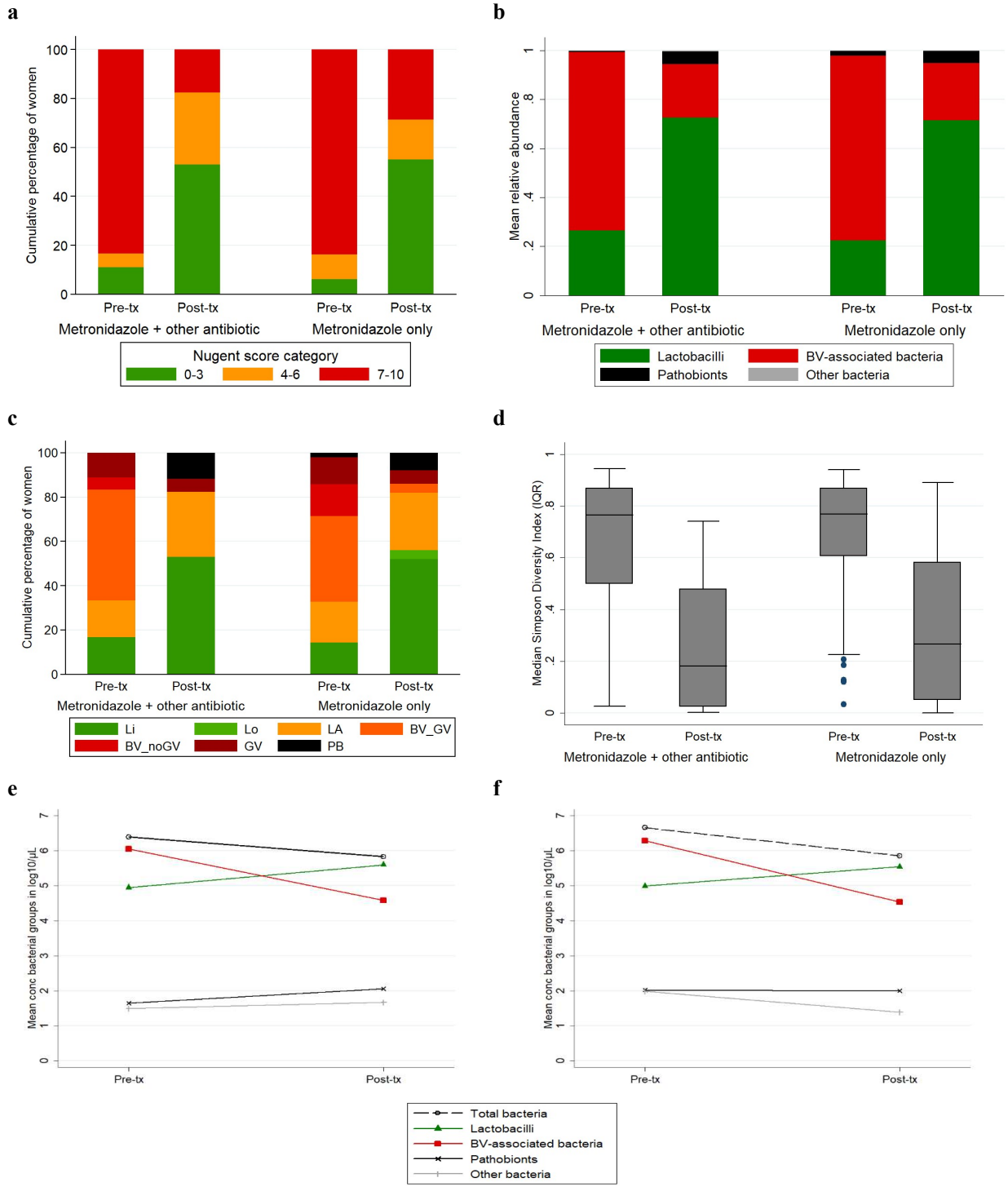
Figure B.2: VMB outcomes pre- and post-treatment, stratified by ongoing CT/NG infection



BV bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *Conc* concentration, *CT Chlamydia trachomatis*, *GV G. vaginalis*-dominated, *IQR* inter-quartile range, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *NG Neisseria gonorrhoeae*, *PB* pathobionts-containing, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit, *VMB* vaginal microbiota.

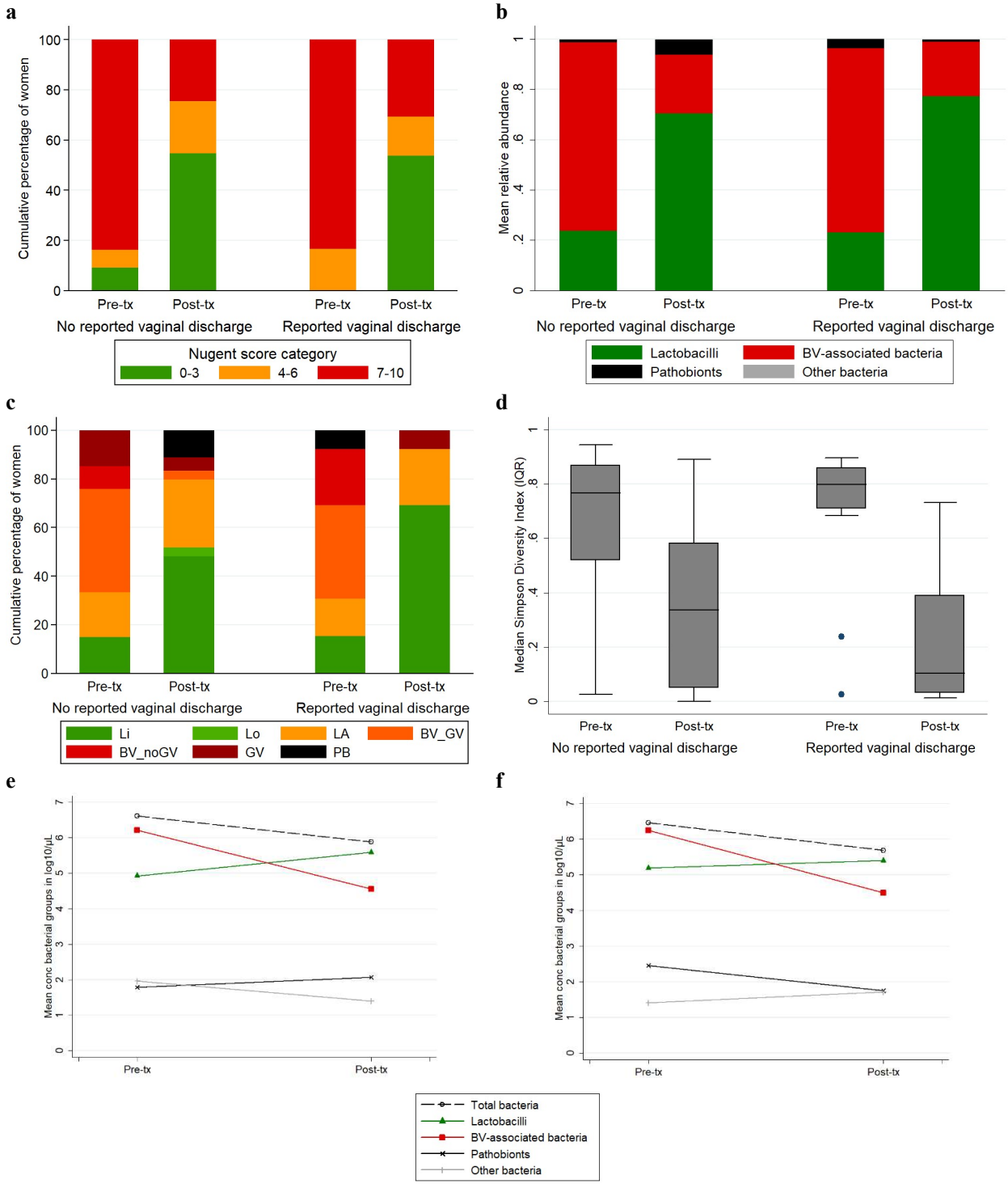
a-f Figures show changes in VMB characteristics before and after metronidazole treatment, stratified by CT/NG status at baseline: Nugent score categories (**a**), bacterial group mean relative abundances (**b**), VMB types (**c**), and median inverse Simpson diversity index (**d**). **e** Bacterial group concentrations of CT/NG-negative participants at baseline (n=40 at pre-tx, n=39 at post-tx; see table B.1 for 95% confidence intervals). **f** Bacterial group concentrations of CT/NG-positive participants at baseline (n=26 at pre-tx, n=24 at post-tx).

Figure B.3: VMB outcomes pre- and post-treatment, stratified by additional antibiotic use



BV bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *Conc* concentration, *GV G. vaginalis*-dominated, *IQR* inter-quartile range, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts-containing, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit, *VMB* vaginal microbiota.
a-f Figures show changes in VMB characteristics before and after metronidazole treatment, stratified by use of another antibiotic in addition to metronidazole: Nugent score categories (**a**), bacterial group mean relative abundances (**b**), VMB types (**c**), and median inverse Simpson diversity index (**d**). **e** Bacterial group concentrations of participants who received another antibiotic in addition to metronidazole at baseline (n=18 at pre-treatment visit, n=17 at post-treatment visit; see table B.2 for 95% confidence intervals). **f** Bacterial group concentrations of participants who only received metronidazole at baseline (n=49 at pre-treatment visit, n=50 at post-treatment visit).

Figure B.4: VMB outcomes pre- and post-treatment, stratified by pre-treatment vaginal discharge



BV bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *Conc* concentration, *GV G. vaginalis*-dominated, *IQR* inter-quartile range, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts-containing, *Pre-tx* pre-treatment visit, *Post-tx* post-treatment visit, *VMB* vaginal microbiota.
a-f Figures show changes in VMB characteristics before and after metronidazole treatment, stratified by unusual vaginal discharge symptoms at the pre-treatment visit: Nugent score categories (**a**), bacterial group mean relative abundances (**b**), VMB types (**c**), and median Simpson diversity 1-D (**d**). **e** Bacterial group concentrations of participants who did not report unusual vaginal discharge at the pre-treatment visit (n=13; see table B.3 for 95% confidence intervals). **f** Bacterial group concentrations of participants who reported unusual vaginal discharge at the pre-treatment visit (n=55).

Table B.1: VMB characteristics pre- and post-treatment, stratified by ongoing CT/NG infection

VMB Outcomes	All participants			CT/NG negative at baseline†			CT/NG positive at baseline†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 41)	Post-treatment (n = 41)	p*	Pre-treatment (n = 26)	Post-treatment (n = 26)	p*
Nugent categories (n %)‡ – 0-3 – 4-6 – 7-10	 5 (7.5) 6 (9.0) 56 (83.6)	 36 (54.6) 13 (19.7) 17 (25.8)	<0.001	 5 (12.2) 3 (7.2) 33 (80.5)	 24 (58.5) 6 (14.6) 11 (26.8)	<0.001	 0 3 (11.5) 33 (88.5)	 12 (48.0) 7 (28.0) 6 (24.0)	0.001
Mean inverse Simpson diversity index (95% CI)§	0.67 (0.60 – 0.73)	0.31 (0.25 – 0.38)	<0.001	0.63 (0.53 – 0.72)	0.30 (0.21 – 0.39)	<0.001	0.74 (0.65 – 0.82)	0.33 (0.22 – 0.45)	<0.001
VMB type (n %)§: – Li – Lo – LA – BV_GV – BV_noGV – GV – PB	 10 (14.9) 0 12 (17.9) 28 (41.8) 8 (11.9) 8 (11.9) 1 (1.5)	 35 (52.2) 2 (3.0) 18 (26.9) 2 (3.0) 0 4 (6.0) 6 (9.0)	<0.001	 9 (22.0) 0 10 (24.4) 15 (36.6) 3 (7.3) 4 (9.8) 0	 20 (48.8) 1 (2.4) 13 (31.7) 1 (2.4) 0 2 (4.9) 4 (9.8)	0.001	 1 (3.9) 0 2 (7.7) 13 (50.0) 5 (19.2) 4 (15.4) 1 (3.9)	 15 (57.7) 1 (3.9) 5 (19.2) 1 (3.9) 0 2 (7.7) 2 (7.7)	0.003
Vaginal pH, median (IQR)	5.3 (5.0 – 5.6)	4.4 (3.6 – 4.6)	<0.001	5.3 (4.7 – 5.6)	4.3 (3.6 – 4.4)	<0.001	5.5 (5.0 – 5.6)	4.4 (3.6 – 4.7)	<0.001
Vulvovaginal candidiasis (n %)	6 (8.8)	4 (5.9)	0.527	5 (11.9)	2 (4.8)	0.257	1 (3.9)	2 (7.7)	0.564
Bacterial group relative abundances: mean (95% CI)§									
Total lactobacilli	0.24 (0.15 – 0.32)	0.72 (0.64 – 0.80)	<0.001	0.32 (0.20 – 0.43)	0.73 (0.63 – 0.83)	<0.001	0.10 (0.02 – 0.19)	0.70 (0.56 – 0.84)	<0.001
Total BV-associated anaerobes	0.75 (0.67 – 0.83)	0.23 (0.16 – 0.30)	<0.001	0.67 (0.55 – 0.79)	0.23 (0.14 – 0.33)	<0.001	0.87 (0.79 – 0.96)	0.23 (0.11 – 0.34)	<0.001
Total pathobionts	0.02 (0.01 – 0.03)	0.05 (0.02 – 0.09)	0.050	0.01 (0 – 0.02)	0.04 (0.01 – 0.07)	0.821	0.02 (0 – 0.05)	0.07 (-0.01 – 0.15)	0.015
Total other bacteria	0 (0 – 0)	0 (0 – 0)	0.674	0 (0 – 0)	0 (0 – 0)	0.354	0 (0 – 0)	0 (0 – 0.01)	0.590
Bacterial group concentrations in log_{10} cells/µL: mean (95% CI)¶									
Total bacteria	6.59 (6.39 – 6.78)	5.85 (5.66 – 6.04)	<0.001	6.44 (6.20 – 6.68)	5.90 (5.66 – 6.13)	0.002	6.82 (6.49 – 7.14)	5.78 (5.44 – 6.11)	<0.001
Total lactobacilli	4.98 (4.61 – 5.35)	5.56 (5.34 – 5.78)	0.017	5.02 (4.52 – 5.52)	5.63 (5.39 – 5.88)	0.112	4.92 (4.33 – 5.50)	5.43 (4.99 – 5.88)	0.072
Total BV-anaerobes	6.23 (5.88 – 6.57)	4.55 (4.14 – 4.95)	<0.001	5.89 (5.40 – 6.39)	4.44 (3.87 – 5.01)	<0.001	6.73 (6.36 – 7.09)	4.73 (4.17 – 5.28)	<0.001
Total pathobionts	1.92 (1.36 – 2.48)	2.01 (1.48 – 2.54)	0.939	1.59 (0.91 – 2.27)	1.65 (0.96 – 2.32)	0.464	2.43 (1.43 – 3.42)	2.60 (1.73 – 3.47)	0.474

VMB Outcomes	All participants			CT/NG negative at baseline†			CT/NG positive at baseline†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 41)	Post-treatment (n = 41)	p*	Pre-treatment (n = 26)	Post-treatment (n = 26)	p*
Total other bacteria	1.85 (1.36 – 2.35)	1.46 (1.01 – 1.92)	0.176	1.68 (1.06 – 2.30)	1.29 (0.74 – 1.84)	0.216	2.12 (1.27 – 2.97)	1.74 (0.90 – 2.58)	0.554
Individual bacterial species/genera concentrations in log_{10} cells/µL: mean (95% CI)¶									
L. iners	4.81 (4.38 – 5.24)	5.28 (4.94 – 5.62)	0.072	4.89 (4.32 – 5.46)	5.33 (4.89 – 5.76)	0.394	4.69 (4.00 – 5.39)	5.21 (4.63 – 5.80)	0.072
L. crispatus‖	0.15 (-0.02 – 0.33)	0.51 (0.16 – 0.85)	0.089	0.25 (-0.04 – 0.55)	0.54 (0.06 – 1.02)	0.388	0 (0 – 0)	0.45 (-0.07 – 0.97)	0.084
Other lactobacilli**	1.46 (0.97 – 1.94)	3.03 (2.57 – 3.48)	<0.001	1.74 (1.11 – 2.37)	3.17 (2.60 – 3.74)	0.002	1.03 (0.23 – 1.82)	2.79 (1.99 – 3.85)	0.002
Gardnerella vaginalis	5.62 (5.20 – 6.03)	4.12 (3.63 – 4.61)	<0.001	5.23 (4.60 – 5.86)	3.96 (3.27 – 4.65)	0.009	6.22 (5.85 – 6.59)	4.37 (3.68 – 5.07)	<0.001
Atopobium vaginae	4.58 (4.00 – 5.16)	1.54 (1.06 – 2.02)	<0.001	4.13 (3.31 – 4.94)	1.73 (1.10 – 2.37)	<0.001	5.27 (4.50 – 6.03)	1.22 (0.45 – 1.99)	<0.001
Prevotella species	4.67 (4.18 – 5.16)	1.35 (0.90 – 1.79)	<0.001	4.29 (3.60 – 4.98)	1.41 (0.85 – 1.98)	<0.001	5.25 (4.60 – 5.90)	1.24 (0.46 – 2.01)	<0.001
Sneathia species	4.18 (3.63 – 4.73)	1.08 (0.63 – 1.54)	<0.001	3.79 (3.05 – 4.52)	0.85 (0.28 – 1.43)	<0.001	4.79 (3.97 – 5.61)	1.44 (0.66 – 2.21)	<0.001
Megasphaera species	3.17 (2.56 – 3.79)	0.22 (-0.01 – 0.44)	<0.001	2.74 (1.91 – 3.56)	0.13 (-0.06 – 0.33)	<0.001	3.84 (2.92 – 4.76)	0.36 (-0.16 – 0.88)	<0.001
Veillonella species	2.37 (1.75 – 3.00)	0.28 (0.01 – 0.56)	<0.001	2.22 (1.46 – 2.99)	0.45 (0.01 – 0.99)	0.001	2.60 (1.46 – 3.73)	0 (0 – 0)	0.001
BVAB1	1.76 (1.11 – 2.42)	0.46 (0.15 – 0.77)	<0.001	0.99 (0.29 – 1.69)	0.24 (0.01 – 0.47)	0.072	2.95 (1.77 – 4.13)	0.83 (0.09 – 1.57)	0.002
Fusobacterium species	0.53 (0.17 – 0.89)	0 (0 – 0)	0.008	0.33 (-0.05 – 0.70)	0 (0 – 0)	0.083	0.85 (0.12 – 1.57)	0 (0 – 0)	0.046
Streptococcus species	1.47 (0.92 – 2.02)	1.34 (0.84 – 1.85)	0.453	1.50 (0.82 – 2.17)	1.14 (0.53 – 1.75)	0.056	1.43 (0.44 – 2.43)	1.67 (0.73 – 2.60)	0.350
Staphylococcus species	0.26 (0.05 – 0.47)	0.60 (0.27 – 0.93)	0.655	0.29 (0.01 – 0.57)	0.31 (0 – 0.61)	0.317	0.22 (-0.10 – 0.53)	1.07 (0.36 – 1.79)	0.317
Escherichia/*Shigella*	0.10 (-0.04 – 0.25)	0.86 (0.45 – 1.27)	0.317	0.17 (-0.07 – 0.41)	0.70 (0.20 – 1.20)	0.317	0 (0 – 0)	1.12 (0.39 – 1.85)	Not determinable

BV bacterial vaginosis, *BVAB1* BV-associated bacterium type 1, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial low *G. vaginalis*, *CI* confidence interval, *CT Chlamydia trachomatis, GV G. vaginalis*-dominated, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *NG Neisseria gonorrhoeae, PB* pathobionts-containing, *VMB* vaginal microbiota.
*Stuart-Maxwell test for matched categorical data, and Wilcoxon signed-rank test for matched continuous data. †Participants included regardless of Nugent score at pre-treatment and post-treatment visits.
‡Valid Nugent data available for 67 participants at the pre-treatment visit and 66 participants at the post-treatment visit. §Relative abundance, Simpson inverse diversity indices, and VMB type data available for 67 participants at each visit. ¶Concentration data may contain at most five missing values (see Appendix A Supplementary Methods). ‖Includes all amplicon sequence variants attributed to *L. crispatus*, also those with multiple species assignments. **Includes amplicon sequence variants attributed to *L. jensenii*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, and *Lactobacillus* genus, as well as 11 other minority amplicon sequence variants.

Table B.2: VMB characteristics pre- and post-treatment, stratified by antibiotic use

VMB Outcomes	All participants			Used metronidazole only†			Used metronidazole plus another antibiotic†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 50)	Post-treatment (n = 50)	p*	Pre-treatment (n = 18)	Post-treatment (n = 18)	p*
Nugent categories (n %)‡ – 0-3 – 4-6 – 7-10	 5 (7.5) 6 (9.0) 56 (83.6)	 36 (54.6) 13 (19.7) 17 (25.8)	<0.001	 3 (6.1) 5 (10.2) 41 (83.7)	 27 (55.1) 8 (16.3) 14 (28.6)	<0.001	 2 (11.1) 1 (5.6) 15 (83.8)	 9 (52.9) 5 (29.4) 3 (17.7)	0.005
Mean inverse Simpson diversity index (95% CI)§	0.67 (0.60 – 0.73)	0.31 (0.25 – 0.38)	<0.001	0.68 (0.61 – 0.75)	0.32 (0.24 – 0.40)	<0.001	0.64 (0.49 – 0.79)	0.29 (0.15 – 0.43)	0.005
VMB type (n %)§: – Li – Lo – LA – BV_GV – BV_noGV – GV – PB	 10 (14.9) 0 12 (17.9) 28 (41.8) 8 (11.9) 8 (11.9) 1 (1.5)	 35 (52.2) 2 (3.0) 18 (26.9) 2 (3.0) 0 4 (6.0) 6 (9.0)	<0.001	 7 (14.3) 0 9 (18.4) 19 (38.8) 7 (14.3) 6 (12.2) 1 (2.0)	 26 (62.0) 2 (4.0) 13 (26.0) 2 (4.0) 0 3 (6.0) 4 (8.0)	<0.001	 3 (16.7) 0 3 (16.7) 9 (50.0) 1 (5.6) 2 (11.1) 0	 9 (52.9) 0 5 (29.4) 0 0 1 (5.9) 2 (11.8)	0.054
Vaginal pH, median (IQR)	5.3 (5.0 – 5.6)	4.4 (3.6 – 4.6)	<0.001	5.3 (5.0 – 5.6)	4.4 (3.6 – 5.6)	<0.001	5.5 (5.0 – 5.6)	4.4 (4.1 – 4.7)	<0.001
Vulvovaginal candidiasis (n %)	6 (8.8)	4 (5.9)	0.527	3 (6.0)	4 (8.0)	0.706	3 (16.7)	0	0.083
Bacterial group relative abundances: mean (95% CI)§									
Total lactobacilli	0.24 (0.15 – 0.32)	0.72 (0.64 – 0.80)	<0.001	0.22 (0.13 – 0.32)	0.71 (0.62 – 0.81)	<0.001	0.26 (0.08 – 0.45)	0.72 (0.55 – 0.89)	0.006
Total BV-associated anaerobes	0.75 (0.67 – 0.83)	0.23 (0.16 – 0.30)	<0.001	0.75 (0.66 – 0.85)	0.23 (0.15 – 0.32)	<0.001	0.73 (0.54 – 0.91)	0.22 (0.07 – 0.37)	0.001
Total pathobionts	0.02 (0.01 – 0.03)	0.05 (0.02 – 0.09)	0.050	0.02 (0.01 – 0.04)	0.05 (0.01 – 0.09)	0.218	0 (0 – 0.01)	0.05 (-0.01 – 0.11)	0.092
Total other bacteria	0 (0 – 0)	0 (0 – 0)	0.674	0 (0 – 0)	0 (0 – 0)	0.323	0 (0 – 0_	0.01 (0 – 0.01)	0.416
Bacterial group concentrations in log_{10} cells/µL: mean (95% CI)¶									
Total bacteria	6.59 (6.39 – 6.78)	5.85 (5.66 – 6.04)	<0.001	6.66 (6.44 – 6.88)	5.86 (5.64 – 6.08)	<0.001	6.40 (5.96 – 6.84)	5.83 (5.41 – 6.25)	0.114
Total lactobacilli	4.98 (4.61 – 5.35)	5.56 (5.34 – 5.78)	0.017	4.99 (4.51 – 5.47)	5.54 (5.29 – 5.80)	0.087	4.95 (4.42 – 5.48)	5.60 (5.09 – 6.10)	0.109
Total BV-anaerobes	6.23 (5.88 – 6.57)	4.55 (4.14 – 4.95)	<0.001	6.29 (5.89 – 6.69)	4.54 (4.04 – 5.03)	<0.001	6.05 (5.34 – 6.77)	4.59 (3.86 – 5.31)	0.008
Total pathobionts	1.92 (1.36 – 2.48)	2.01 (1.48 – 2.54)	0.939	2.02 (1.35 – 2.70)	1.99 (1.36 – 2.63)	0.725	1.65 (0.54 – 2.75)	2.06 (0.98 – 3.14)	0.545

VMB Outcomes	All participants			Used metronidazole only†			Used metronidazole plus another antibiotic†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 50)	Post-treatment (n = 50)	p*	Pre-treatment (n = 18)	Post-treatment (n = 18)	p*
Total other bacteria	1.85 (1.36 – 2.35)	1.46 (1.01 – 1.92)	0.176	1.99 (1.41 – 2.57)	1.39 (0.88 – 1.90)	0.093	1.49 (0.50 – 2.49)	1.67 (0.59 – 2.76)	0.704
Individual bacterial species/genera concentrations in log_{10} cells/µL: mean (95% CI)¶									
L. iners	4.81 (4.38 – 5.24)	5.28 (4.94 – 5.62)	0.072	4.84 (4.31 – 5.37)	5.25 (4.84 – 5.65)	0.291	4.74 (3.96 – 5.51)	5.39 (4.71 – 6.08)	0.109
L. crispatus‖	0.15 (-0.02 – 0.33)	0.51 (0.16 – 0.85)	0.089	0.13 (-0.06 – 0.32)	0.37 (0 – 0.75)	0.180	0.21 (-0.24 – 0.67)	0.90 (0.03 – 1.76)	0.271
Other lactobacilli**	1.46 (0.97 – 1.94)	3.03 (2.57 – 3.48)	<0.001	1.40 (0.82 – 1.98)	2.92 (2.38 – 3.47)	<0.001	1.18 (0.45 – 1.91)	3.31 (2.45 – 4.17)	0.046
Gardnerella vaginalis	5.62 (5.20 – 6.03)	4.12 (3.63 – 4.61)	<0.001	5.65 (5.15 – 6.16)	4.18 (3.61 – 4.74)	<0.001	5.52 (4.71 – 6.34)	3.95 (2.83 – 5.06)	0.070
Atopobium vaginae	4.58 (4.00 – 5.16)	1.54 (1.06 – 2.02)	<0.001	4.52 (3.80 – 5.24)	1.87 (1.29 – 2.46)	<0.001	4.74 (3.72 – 5.76)	0.55 (-0.11 – 1.21)	0.001
Prevotella species	4.67 (4.18 – 5.16)	1.35 (0.90 – 1.79)	<0.001	4.74 (4.20 – 5.29)	1.31 (0.77 – 1.85)	<0.001	4.48 (3.29 – 5.66)	1.45 (0.59 – 2.30)	0.001
Sneathia species	4.18 (3.63 – 4.73)	1.08 (0.63 – 1.54)	<0.001	4.05 (3.37 – 4.73)	1.17 (0.61 – 1.73)	<0.001	4.51 (3.54 – 5.48)	0.81 (0.02 – 1.61)	<0.001
Megasphaera species	3.17 (2.56 – 3.79)	0.22 (-0.01 – 0.44)	<0.001	3.23 (2.50 – 3.96)	0.26 (-0.04 – 0.55)	<0.001	3.02 (1.75 – 4.29)	0.11 (-0.12 – 0.34)	0.004
Veillonella species	2.37 (1.75 – 3.00)	0.28 (0.01 – 0.56)	<0.001	2.37 (1.63 – 3.12)	0.38 (0.01 – 0.75)	<0.001	2.36 (1.08 – 3.64)	0 (0 – 0)	0.005
BVAB1	1.76 (1.11 – 2.42)	0.46 (0.15 – 0.77)	<0.001	1.97 (1.17 – 2.78)	0.56 (0.16 – 0.96)	0.001	1.20 (0.05 – 2.36)	0.17 (-0.19 – 0.52)	0.144
Fusobacterium species	0.53 (0.17 – 0.89)	0 (0 – 0)	0.008	0.47 (0.06 – 0.89)	0 (0 – 0)	0.046	0.69 (-0.10 – 1.48)	0 (0 – 0)	0.084
Streptococcus species	1.47 (0.92 – 2.02)	1.34 (0.84 – 1.85)	0.453	1.64 (0.96 – 2.31)	1.47 (0.85 – 2.09)	0.420	1.04 (0.03 – 2.04)	0.95 (0.07 – 1.84)	0.911
Staphylococcus species	0.26 (0.05 – 0.47)	0.60 (0.27 – 0.93)	0.655	0.18 (-0.03 – 0.39)	0.41 (0.07 – 0.76)	0.317	0.47 (-0.07 – 1.00)	1.14 (0.29 – 1.99)	0.317
Escherichia/*Shigella*	0.10 (-0.04 – 0.25)	0.86 (0.45 – 1.27)	0.317	0.14 (-0.06 – 0.34)	0.74 (0.27 – 1.20)	0.317	0 (0 – 0)	1.21 (0.28 – 2.13)	Not determinable

BV bacterial vaginosis, *BVAB1* BV-associated bacterium type 1, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *CI* confidence interval, *GV G. vaginalis*-dominated, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts-containing, *VMB* vaginal microbiota.

*Stuart-Maxwell test for matched categorical data, and Wilcoxon signed-rank test for matched continuous data. †The other antibiotic were ciprofloxacin for urinary tract infection and penicillin for syphilis. Both groups include three women each (total n=6) who received antifungal treatment for vulvovaginal candidiasis. ‡Valid Nugent data available for 67 participants at the pre-treatment visit and 66 participants at the post-treatment visit. §Relative abundance, Simpson inverse diversity indices, and VMB type data available for 67 participants at each visit. ¶Concentration data may contain at most five missing values (see Appendix A Supplementary Methods). ‖Includes all amplicon sequence variants attributed to *L. crispatus*, also those with multiple species assignments. **Includes amplicon sequence variants attributed to *L. jensenii*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, and *Lactobacillus* genus, as well as 11 other minority amplicon sequence variants.

Table B.3: VMB characteristics pre- and post-treatment, stratified by reported vaginal discharge symptoms at the pre-treatment visit

VMB Outcomes	All participants			Reported vaginal discharge at pre-treatment visit†			Dit not report any vaginal discharge at pre-treatment visit†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 13)	Post-treatment (n = 13)	p*	Pre-treatment (n = 55)	Post-treatment (n = 55)	p*
Nugent categories (n %)‡ – 0-3 – 4-6 – 7-10	 5 (7.5) 6 (9.0) 56 (83.6)	 36 (54.6) 13 (19.7) 17 (25.8)	<0.001	 0 2 (16.7) 10 (83.3)	 7 (53.9) 2 (15.4) 4 (30.8)	0.030	 5 (9.1) 4 (7.3) 46 (83.6)	 29 (54.7) 11 (20.8) 13 (24.5)	<0.001
Mean inverse Simpson diversity index (95% CI)§	0.67 (0.60 – 0.73)	0.31 (0.25 – 0.38)	<0.001	0.70 (0.54 – 0.86)	0.24 (0.08 – 0.39)	0.006	0.66 (0.59 – 0.74)	0.33 (0.26 – 0.41)	<0.001
VMB type (n %)§: – Li – Lo – LA – BV_GV – BV_noGV – GV – PB	 10 (14.9) 0 12 (17.9) 28 (41.8) 8 (11.9) 8 (11.9) 1 (1.5)	 35 (52.2) 2 (3.0) 18 (26.9) 2 (3.0) 0 4 (6.0) 6 (9.0)	<0.001	 2 (15.4) 0 2 (15.4) 5 (38.5) 3 (23.1) 0 1 (7.7)	 9 (69.2) 0 3 (23.1) 0 0 1 (7.7) 0	0.109	 8 (14.8) 0 10 (18.5) 23 (42.6) 5 (9.3) 8 (14.8) 0	 26 (48.2) 2 (3.7) 15 (27.8) 2 (3.7) 0 3 (5.6) 6 (11.1)	<0.001
Vaginal pH, median (IQR)	5.3 (5.0 – 5.6)	4.4 (3.6 – 4.6)	<0.001	5.3 (5.3 – 5.6)	4.1 (3.6 – 4.4)	0.001	5.3 (4.7 – 5.6)	4.4 (4.1 – 4.7)	<0.001
Vulvovaginal candidiasis (n %)	6 (8.8)	4 (5.9)	0.527	0	1 (7.7)	0.317	6 (10.9)	3 (5.5)	0.317
Bacterial group relative abundances: mean (95% CI)§									
Total lactobacilli	0.24 (0.15 – 0.32)	0.72 (0.64 – 0.80)	<0.001	0.23 (0.03 – 0.43)	0.77 (0.58 – 0.96)	0.005	0.24 (0.14 – 0.33)	0.70 (0.62 – 0.79)	<0.001
Total BV-associated anaerobes	0.75 (0.67 – 0.83)	0.23 (0.16 – 0.30)	<0.001	0.73 (0.54 – 0.93)	0.22 (0.03 – 0.41)	0.007	0.75 (0.66 – 0.84)	0.23 (0.15 – 0.31)	<0.001
Total pathobionts	0.02 (0.01 – 0.03)	0.05 (0.02 – 0.09)	0.050	0.04 (-0.01 – 0.08)	0.01 (0 – 0.02)	0.597	0.01 (0 – 0.02)	0.06 (0.02 – 0.10)	0.015
Total other bacteria	0 (0 – 0)	0 (0 – 0)	0.674	0 (0 – 0)	0 (0 – 0)	0.405	0 (0 – 0)	0 (0 – 0)	0.386
Bacterial group concentrations in log_{10} cells/µL: mean (95% CI)¶									
Total bacteria	6.59 (6.39 – 6.78)	5.85 (5.66 – 6.04)	<0.001	6.47 (6.17 – 6.77)	5.69 (5.43 – 5.95)	0.003	6.62 (6.38 – 6.85)	5.89 (5.66 – 6.12)	<0.001
Total lactobacilli	4.98 (4.61 – 5.35)	5.56 (5.34 – 5.78)	0.017	5.19 (4.70 – 5.69)	5.41 (5.03 – 5.79)	0.433	4.93 (4.48 – 5.38)	5.59 (5.33 – 5.86)	0.026
Total BV-anaerobes	6.23 (5.88 – 6.57)	4.55 (4.14 – 4.95)	<0.001	6.25 (5.77 – 6.72)	4.50 (3.83 – 5.18)	0.006	6.22 (5.81 – 6.63)	4.56 (4.08 – 5.04)	<0.001
Total pathobionts	1.92 (1.36 – 2.48)	2.01 (1.48 – 2.54)	0.939	2.46 (0.96 – 3.95)	1.75 (0.51 – 2.99)	0.428	1.79 (1.17 – 2.41)	2.07 (1.46 – 2.68)	0.618

VMB Outcomes	All participants			Reported vaginal discharge at pre-treatment visit†			Dit not report any vaginal discharge at pre-treatment visit†		
	Pre-treatment (n = 68)	Post-treatment (n = 68)	p*	Pre-treatment (n = 13)	Post-treatment (n = 13)	p*	Pre-treatment (n = 55)	Post-treatment (n = 55)	p*
Total other bacteria	1.85 (1.36 – 2.35)	1.46 (1.01 – 1.92)	0.176	1.41 (0.26 – 2.56)	1.72 (0.68 – 2.75)	1.00	1.96 (1.40 – 2.52)	1.40 (0.88 – 1.92)	0.138
Individual bacterial species/genera concentrations in log_{10} cells/µL: mean (95% CI)¶									
L. iners	4.81 (4.38 – 5.24)	5.28 (4.94 – 5.62)	0.072	5.14 (4.64 – 5.63)	4.85 (3.71 – 5.99)	0.638	4.73 (4.21 – 5.26)	5.39 (5.04 – 5.73)	0.062
L. crispatus‖	0.15 (-0.02 – 0.33)	0.51 (0.16 – 0.85)	0.089	0 (0 – 0)	0.43 (-0.51 – 1.36)	0.317	0.19 (-0.03 – 0.41)	0.53 (0.14 – 0.91)	0.149
Other lactobacilli**	1.46 (0.97 – 1.94)	3.03 (2.57 – 3.48)	<0.001	1.02 (-0.17 – 2.21)	2.32 (1.12 – 3.53)	0.077	1.57 (1.02 – 2.11)	3.19 (2.70 – 3.69)	<0.001
Gardnerella vaginalis	5.62 (5.20 – 6.03)	4.12 (3.63 – 4.61)	<0.001	5.59 (5.05 – 6.12)	4.33 (3.62 – 5.05)	0.023	5.62 (5.11 – 6.13)	4.07 (3.48 – 4.66)	<0.001
Atopobium vaginae	4.58 (4.00 – 5.16)	1.54 (1.06 – 2.02)	<0.001	4.15 (2.64 – 5.65)	1.53 (0.30 – 2.76)	0.016	4.68 (4.04 – 5.33)	1.54 (1.00 – 2.08)	<0.001
Prevotella species	4.67 (4.18 – 5.16)	1.35 (0.90 – 1.79)	<0.001	4.87 (4.25 – 5.50)	1.64 (0.51 – 2.76)	0.004	4.62 (4.02 – 5.22)	1.28 (0.78 – 1.77)	<0.001
Sneathia species	4.18 (3.63 – 4.73)	1.08 (0.63 – 1.54)	<0.001	4.08 (2.92 – 5.24)	1.24 (0.01 – 2.47)	0.008	4.20 (3.56 – 4.84)	1.05 (0.54 – 1.55)	<0.001
Megasphaera species	3.17 (2.56 – 3.79)	0.22 (-0.01 – 0.44)	<0.001	3.22 (1.83 – 4.61)	0 (0 – 0)	0.006	3.16 (2.45 – 3.87)	0.27 (-0.01 – 0.55)	<0.001
Veillonella species	2.37 (1.75 – 3.00)	0.28 (0.01 – 0.56)	<0.001	2.17 (0.60 – 3.73)	0.41 (-0.50 – 1.33)	0.132	2.42 (1.72 – 3.13)	0.25 (-0.04 – 0.54)	<0.001
BVAB1	1.76 (1.11 – 2.42)	0.46 (0.15 – 0.77)	<0.001	2.94 (1.18 – 4.70)	0.76 (-0.14 – 1.66)	0.016	1.47 (0.77 – 2.17)	0.39 (0.06 – 0.73)	0.007
Fusobacterium species	0.53 (0.17 – 0.89)	0 (0 – 0)	0.008	0.66 (-0.32 – 1.63)	0 (0 – 0)	0.158	0.50 (0.11 – 0.90)	0 (0 – 0)	0.025
Streptococcus species	1.47 (0.92 – 2.02)	1.34 (0.84 – 1.85)	0.453	2.17 (0.64 – 3.69)	0.78 (-0.38 – 1.93)	0.139	1.30 (0.71 – 1.90)	1.47 (0.90 – 2.05)	0.996
Staphylococcus species	0.26 (0.05 – 0.47)	0.60 (0.27 – 0.93)	0.655	0.20 (-0.24 – 0.64)	0.30 (-0.36 – 0.95)	Not determinable	0.27 (0.04 – 0.51)	0.67 (0.28 – 1.06)	0.655
Escherichia/*Shigella*	0.10 (-0.04 – 0.25)	0.86 (0.45 – 1.27)	0.317	0 (0 – 0)	0.49 (-0.25 – 1.23)	Not determinable	0.13 (-0.05 – 0.31)	0.95 (0.47 – 1.43)	0.317

BV bacterial vaginosis, *BVAB1* BV-associated bacterium type 1, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *CI* confidence interval, *GV G. vaginalis*-dominated, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *PB* pathobionts-containing, *VMB* vaginal microbiota.

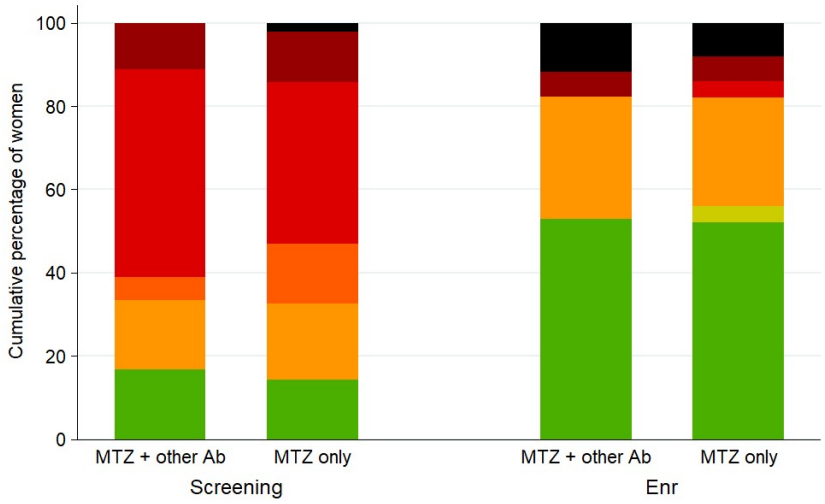
*Stuart-Maxwell test for matched categorical data, and Wilcoxon signed-rank test for matched continuous data. †Current or in the past two weeks. ‡Valid Nugent data available for 67 participants at the pre-treatment visit and 66 participants at the post-treatment visit. §Relative abundance, Simpson inverse diversity indices, and VMB type data available for 67 participants at each visit. ¶Concentration data may contain at most five missing values (see Appendix A Supplementary Methods). ‖Includes all amplicon sequence variants attributed to *L. crispatus*, also those with multiple species assignments. **Includes amplicon sequence variants attributed to *L. jensenii*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, and *Lactobacillus* genus, as well as 11 other minority amplicon sequence variants.

Appendix C

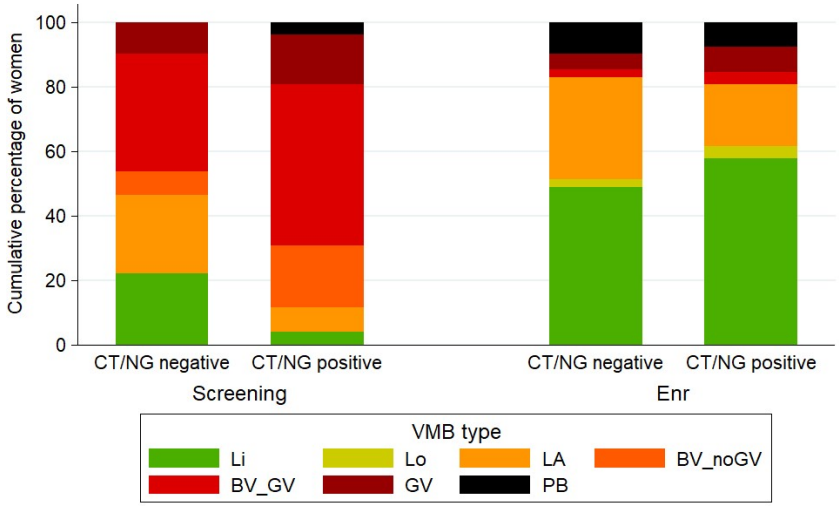
This appendix corresponds to Chapter 3.

Figure C.1: VMB comparisons between women who received metronidazole and another antibiotic at screening or metronidazole only, and between women with and without *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* at screening and enrolment

a



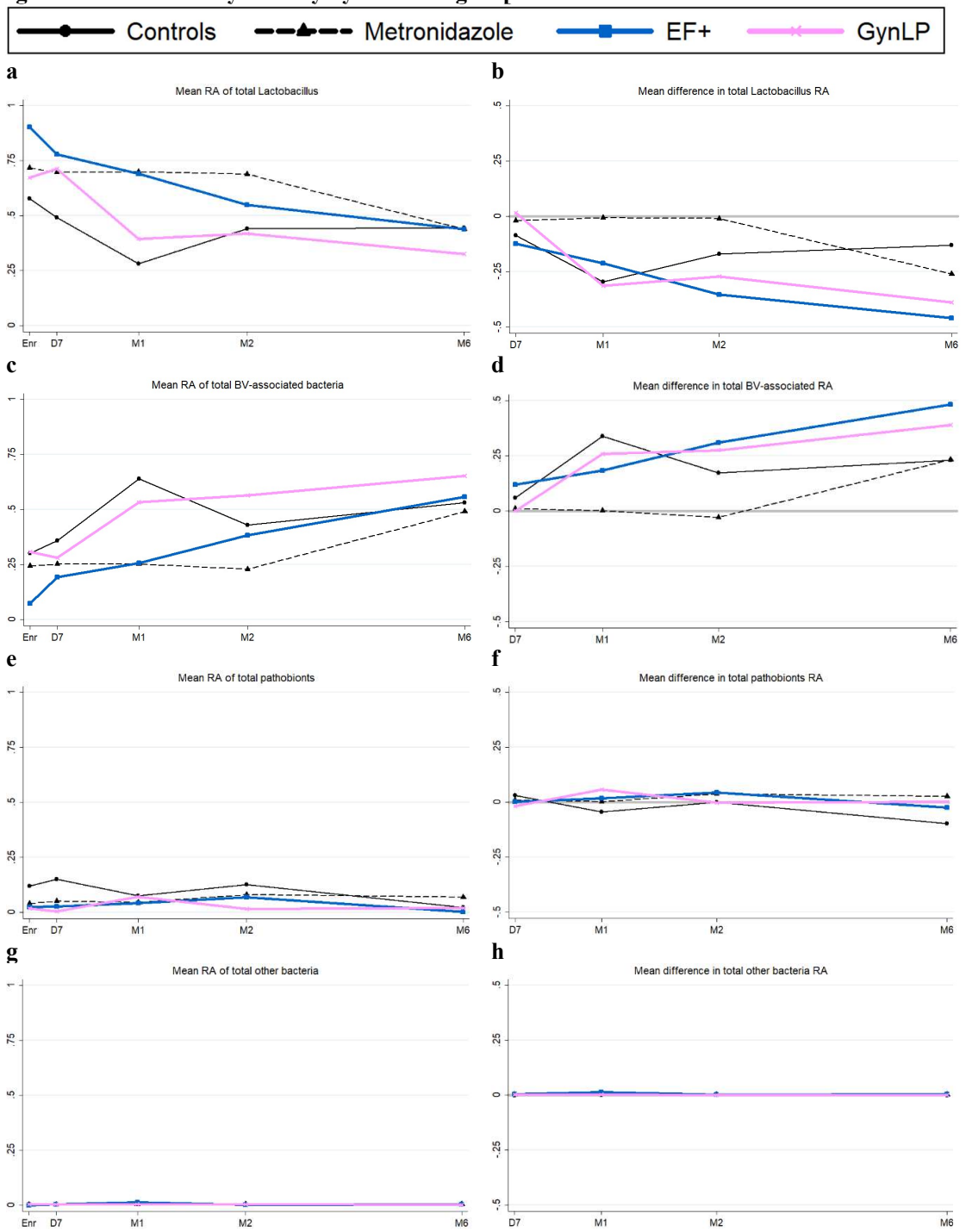
b



Ab antibiotics, *BV* bacterial vaginosis, *BV_GV* polybacterial *Gardnerella vaginalis*-containing, *BV_noGV* polybacterial but low *G. vaginalis*, *CT Chlamydia trachomatis*, *D7* Day 7 visit, *Enr* enrolment visit, *GV G. vaginalis*-dominated, *MTZ* metronidazole, *LA* lactobacilli and anaerobes, *Li L. iners*-dominated, *Lo* other lactobacilli-dominated, *NG Neisseria gonorrhoeae*, *PB* pathobionts-containing, *VMB* vaginal microbiota.

a Comparison of VMB type membership of enrolled participants who had used metronidazole with another antibiotic (n=18) or metronidazole only (n=50) between the screening and enrolment visits. **b** Comparison of VMB type membership of enrolled participants who were positive (n=26) or negative (n=41) for CT/NG at the screening visit, but received CT/NG treatment after the enrolment visit (if applicable). All women did use oral metronidazole for seven days between these two visits.

Figure C.2: Preliminary efficacy by bacterial group relative abundance



BV bacterial vaginosis, *D7* Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M1/M2/M6* month 1/2/6 visit, *RA* relative abundance, *Scr* screening visit.

Changes in relative abundances of bacterial groups over time per randomisation group. See table C.4 for 95% confidence intervals. **a** Mean lactobacilli relative abundance over time. **b** Difference in mean lactobacilli relative abundance with enrolment, over time. **c** Mean BV-associated anaerobes relative abundance over time. **d** Difference in mean BV-associated anaerobes relative abundance with enrolment, over time. **e** Mean pathobionts relative abundance over time. **f** Difference in mean relative abundance with enrolment, over time. **g** Mean other bacteria relative abundance over time. **h** Difference in mean other bacteria relative abundance with enrolment, over time.

Table C.1: Additional baseline characteristics

Sociodemographic characteristics at Scr/Enr	Screened (*n* = 175)	Enrolled (*n* = 68)	Controls (*n* = 17)	Metro (*n* = 17)	EF+ (*n* = 17)	GynLP (*n* = 17)	p*
Marital status (n %)							
- Married	8 (4.6)	5 (7.4)	1 (5.9)	1 (5.9)	2 (11.8)	1 (5.9)	
- Never married	127 (72.6)	50 (73.5)	16 (94.1)	11 (64.7)	10 (58.8)	13 (76.5)	0.199
- Divorced	34 (19.4)	12 (17.6)	0	5 (29.4)	4 (23.5)	3 (17.6)	
- Widowed	6 (3.4)	1 (1.5)	0	0	1 (5.9)	0	
Educational level (n %)							
- No schooling	37 (21.1)	14 (20.6)	5 (29.4)	3 (17.6)	3 (17.6)	3 (17.7)	
- Primary school uncompleted	72 (41.1)	31 (45.6)	7 (41.2)	7 (41.2)	13 (76.5)	4 (23.5)	0.102
- Primary school completed	40 (22.9)	17 (25.0)	4 (23.5)	5 (29.4)	1 (5.9)	7 (41.2)	
- Beyond primary school	26 (14.9)	6 (8.8)	1 (5.9)	2 (11.8)	0	3 (17.7)	
Number of sex partners last 12 months, median (IQR)	15 (4 – 144)	11 (4 – 152)	15 (4 – 160)	20 (5 – 106)	8 (3 – 50)	6 (4 – 240)	0.838
Vaginal sex frequency last 2 weeks, median (IQR)	13 (8 – 20)	12 (8 – 18)	12 (8 – 18)	12 (8 – 16)	12 (7 – 18)	11 (8 – 30)	0.975
Exchanged sex for money/ goods in past month (n %)†	62 (93.1)	63 (92.6)	17 (100)	14 (82.4)	15 (88.2)	17 (100)	0.155
Any condom use past two weeks (n %)							
- Always	44 (25.1)	14 (20.6)	4 (23.5)	3 (17.6)	2 (11.8)	5 (29.4)	0.671
- Sometimes but not always	120 (68.6)	51 (75.0)	13 (76.5)	13 (76.5)	13 (76.5)	12 (70.6)	
- Never	11 (6.3)	3 (4.4)	0	1 (5.9)	2 (11.8)	0	
Pregnancies in lifetime, Median (IQR)†	3 (2 – 4)	3 (2 – 4)	3 (2 – 5)	3 (2 – 4)	3 (3 – 4)	3 (2 -4)	0.722
Currently breastfeeding (n %)†	38 (22.1)	14 (21.2)	4 (23.5)	3 (18.8)	4 (23.5)	3 (18.8)	1.00
Ever washing genitalia (n %)							
- Yes, outside only	NA	55 (80.9)	12 (70.7)	14 (82.4)	15 (88.3)	14 (82.3)	0.704
- Yes, both inside and outside‡		13 (19.1)	5 (29.4)	3 (17.6)	2 (11.7)	3 (17.7)	
Practices to manage menstrual blood or spotting in the past 12 months (n %)	NA						0.695
- Sanitary pad		57 (83.8)	13 (76.5)	15 (88.2)	13 (76.5)	16 (94.1)	
- Others§		16 (23.5)	4 (23.5)	3 (17.6)	4 (23.5)	5 (29.4)	
- Nothing/no menses		3 (4.4)	1 (5.9)	1 (5.9)	1 (5.9)	0	
Frequency eating yoghurt (n %)†,¶	NA						
- Never		34 (50.7)	8 (47.1)	7 (41.2)	9 (52.9)	10 (62.5)	
- Less than once per week		14 (20.9)	5 (29.4)	3 (17.6)	4 (23.5)	2 (12.5)	0.792
- More than once per week		-19 (28.4)	4 (23.5)	7 (41.2)	4 (23.5)	4 (25.0)	
VMB outcomes at Enr	**Screened (*n* = 175)**	**Enrolled (*n* = 67)**	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 16)**	**p***
RA total *Lactobacillus*, mean (95% CI)	NA	0.72 (0.64 – 0.80)	0.58 (0.38 – 0.78)	0.72 (0.56 – 0.88)	0.90‖ (0.82 – 0.98)	0.67 (0.51 – 0.83)	0.017
RA total BV-associated, mean (95% CI)	NA	0.23 (0.16 – 0.30)	0.30 (0.12 – 0.48)	0.24 (0.10 – 0.39)	0.07‖ (-0.01 – 0.15)	0.31 (0.14 – 0.47)	0.015
RA total pathobionts, mean (95% CI)	NA	0.05 (0.02 – 0.09)	0.12 (-0.01 – 0.25)	0.04 (-0.01 – 0.09)	0.02 (-0.01 – 0.06)	0.02 (0 – 0.04)	0.303
RA total other bacteria mean (95% CI)	NA	0 (0 – 0)	0 (0 – 0.01)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0.01)	0.034

BV bacterial vaginosis, *CI* confidence interval, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *Metro* metronidazole, *RA* relative abundance.

*Kruskall Wallis test comparing randomisation groups. †Contains between 1-4 missing values. ‡No washing or washing inside only was never reported. Twenty-seven women washed outside of menses, of whom 23 used water, three water/soap, and one 'western vaginal medicine'. §Multiple responses possible. Other practices reported were tissue, toilet paper, paper, cloth or cotton wool put inside the vagina (*n*=8) or inside the underwear (*n*=8). ¶No participants had ever used or heard of probiotics before. ‖p<0.05 by Mann Whitney U test, compared to the control group.

Table C.2: Antimicrobial use during the trial

Between Enr and M2	**Controls *n* women**	**Metronidazole *n* women**	**EF+ *n* women**	**GynLP *n* women**	**p***
Metronidazole†	1	2	1	3	
Tinidazole‡	1	0	0	0	
Both 'azoles' combined	2	2	1	3	0.688
Other antibiotic§	3	2	3	4	0.781
Antifungals	0	0	0	0	1.000
Between M2 and M6	**Controls**	**Metronidazole**	**EF+**	**GynLP**	**p***
Metronidazole¶	4	1	1	2	0.439
Other antibiotic‖	9	6	5	6	0.634
Antifungals	0	0	0	0	1.000
Mixed effects models using data between Enr and M2	**Total *Lactobacillus* conc**		**Total BV-anaerobes conc**		
	OR (95% CI)	**p**	**OR (95% CI)**	**p**	
Antibiotic use for a non-study indication in the previous 14 days	0.94 (0.40 – 2.21)	0.879	0.68 (0.23 – 1.94)	0.467	

BV bacterial vaginosis, *CI* confidence interval, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M1/2* Month 1/2 visit, *OR* odds ratio, *TV Trichomonas vaginalis*.

*Fisher's exact test comparing all four groups. †Prescribed for BV, TV, or amoebiasis (the latter prescribed by an external clinic). ‡Prescribed for amoebiasis/ dysentery (externally). §Includes amoxicillin prescribed for abortion prophylaxis, tonsillitis, dental caries, tooth extraction, cough, and trauma (all externally), chloramphenicol prescribed for an upper respiratory tract infection (externally), ciprofloxacin prescribed for urinary tract infection (at study clinic) and typhoid fever (externally), cloxacillin prescribed for a traumatic wound (externally), and doxycycline prescribed for chlamydia (at study clinic) and an unspecified sexually transmitted infection (externally). ¶Prescribed for BV, TV and amoebiasis (all at the study clinic). ‖Includes amoxicillin prescribed for tonsillitis and contusion (both externally), ciprofloxacin prescribed for gonorrhoea or urinary tract infection (of which 8/12 at the study clinic), and doxycycline for chlamydia (all but one at the study clinic).

Table C.3: Adherence with interventions during the trial

Adherence	**Metronidazole (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 16)**
Adherence Enr – D7, median % (IQR)	100 (100 – 100)	100 (100 – 100)	100 (100 – 100)
Adherence D7 – M1, median % (IQR)	100 (100 – 100)	100 (100 – 100)	100 (91.7 – 100)
Adherence M1 – M2, median % (IQR)	100 (100 – 100)	100 (100 – 100)	100 (92.9 – 100)
Overall adherence Enr-M2, median % (IQR)	100 (96.3 – 100)	100 (100 – 100)	98.3 (89.3 – 100)
Overall adherence Enr – M2 (n %)			
- Perfect adherence*	12 (70.6)	10 (58.8)	8 (50.0)
- Non-perfect adherence	5 (29.4)	7 (41.2)	8 (50.0)
- Adherence ≥90%	14 (82.4)	15 (88.2)	11 (68.8)
- Adherence ≥80%	15 (88.2)	17 (100)	13 (81.3)
Number of times menses Enr – M2 (n %)†			
- Never	7 (41.2)	4 (23.5)	2 (12.5)
- Once	6 (35.3)	5 (29.4)	4 (25.0)
- Twice	4 (23.5)	8 (47.1)	10 (62.5)
- Thrice	0	0	0
Did not use product during menses at least once (n %)			
- Yes	4 (23.5)	3 (17.6)	5 (31.3)
- No	6 (35.3)	10 (58.8)	9 (56.2)
- NA (never had menses)	7 (41.2)	4 (23.5)	2 (12.5)

D7 day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment visit, *GynLP* Gynophilus LP, *M1/2* Month 1/2 visit, *NA* not applicable, *IQR* inter-quartile range.

*Defined as 100% of the prescribed doses used at the prescribed times after nurse review of the participant's diary card and returned used packaging and unused product. †Number of times menses in the control group: Never 2 (11.8%), once 3 (17.8%), twice 11 (64.7%), and thrice 1 (5.9%).

Table C.4: Preliminary efficacy – bacterial group relative abundances

VMB Outcome	Groups	Enr (*n* = 67)*	Products used			Ceased
			D7 (*n* = 64)*	M1 (*n* = 66)*	M2 (*n* = 65)*	M6 (*n* = 64)*
RA total *Lactobacillus*, mean (95% CI)	Control	0.58 (0.38 – 0.78)	0.49 (0.25 – 0.73)	0.28 (0.08 – 0.48)	0.44 (0.18 – 0.70)	0.45 (0.21 – 0.68)
	Metro	0.72 (0.56 – 0.88)	0.70 (0.51 – 0.88)	0.70 (0.51 – 0.89)	0.69 (0.48 – 0.90)	0.44 (0.19 – 0.69)
	EF+	0.90 (0.82 – 0.98)	0.78 (0.60 – 0.96)	0.69 (0.47 – 0.90)	0.55 (0.32 – 0.77)	0.44 (0.22 – 0.66)
	GynLP	0.67 (0.51 – 0.83)	0.71 (0.49 – 0.93)	0.39 (0.15 – 0.63)	0.42 (0.17 – 0.67)	0.33 (0.09 – 0.57)
RA total BV-associated bacteria, mean (95% CI)	Control	0.30 (0.12 – 0.48)	0.36 (0.14 – 0.57)	0.64 (0.44 – 0.84)	0.43 (0.19 – 0.67)	0.53 (0.30 – 0.76)
	Metro	0.24 (0.10 – 0.39)	0.25 (0.09 – 0.42)	0.25 (0.10 – 0.40)	0.23 (0.04 – 0.42)	0.49 (0.24 – 0.74)
	EF+	0.07 (-0.01 – 0.15)	0.19 (0.02 – 0.36)	0.26 (0.05 – 0.46)	0.38 (0.18 – 0.58)	0.56 (0.34 – 0.78)
	GynLP	0.31 (0.14 – 0.47)	0.28 (0.07 – 0.49)	0.53 (0.29 – 0.77)	0.56 (0.32 – 0.81)	0.65 (0.41 – 0.90)
RA total pathobionts, mean (95% CI)	Control	0.12 (-0.01 – 0.25)	0.15 (-0.01 – 0.31)	0.08 (0 – 0.15)	0.13 (-0.02 – 0.28)	0.02 (-0.02 – 0.06)
	Metro	0.04 (-0.01 – 0.09)	0.05 (-0.01 – 0.11)	0.05 (-0.03 – 0.12)	0.08 (-0.04 – 0.20)	0.07 (-0.04 – 0.18)
	EF+	0.02 (-0.01 – 0.06)	0.03 (0 – 0.05)	0.04 (-0.02 – 0.11)	0.07 (-0.03 – 0.16)	0 (0 – 0)
	GynLP	0.02 (0 – 0.04)	0 (0 – 0.01)	0.07 (-0.06 – 0.20)	0.01 (0 – 0.03)	0.02 (-0.02 – 0.06)
Mean RA total other bacteria, mean (95% CI)	Control	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0)
	Metro	0 (0 – 0)	0 (0 – 0.01)	0.01 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)
	EF+	0 (0 – 0)	0 (0 – 0.01)	0.01 (0 – 0.03)	0 (0 – 0)	0 (0 – 0.01)
	GynLP	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0)
Difference in RA total *Lactobacillus* compared to Enr, mean (95% CI)	Control	0	-0.09 (-0.29 – 0.12)	-0.29 (-0.55 – -0.04)	-0.17 (-0.38 – 0.05)	-0.13 (-0.40 – 0.14)
	Metro	0	-0.02 (-0.24 – 0.20)	-0.01 (-0.20 – 0.19)	-0.01 (-0.21 – 0.19)	-0.26 (-0.57 – 0.05)
	EF+	0	-0.12 (-0.31 – 0.06)	-0.21 (-0.43 – 0.00)	-0.35 (-0.58 – -0.13)	-0.46 (-0.72 – -0.21)
	GynLP	0	0.02 (-0.22 – 0.25)	-0.32 (-0.60 – -0.04)	-0.27 (-0.57 – 0.02)	-0.39 (-0.68 – -0.10)
Difference in RA total BV-associated bacteria compared to Enr, mean (95% CI)	Control	0	0.06 (-0.15 – 0.27)	0.34 (0.09 – 0.59)	0.17 (-0.04 – 0.38)	0.23 (-0.03 – 0.49)
	Metro	0	0.01 (-0.17 – 0.19)	0 (-0.18 – 0.19)	-0.03 (-0.24 – 0.18)	0.23 (-0.08 – 0.54)
	EF+	0	0.12 (-0.06 – 0.30)	0.18 (-0.02 – 0.39)	0.31 (0.10 – 0.52)	0.48 (0.23 – 0.73)
	GynLP	0	0 (-0.23 – 0.23)	0.26 (-0.01 – 0.52)	0.28 (-0.02 – 0.57)	0.39 (0.10 – 0.68)

VMB Outcome	Groups	Enr	D7	M1	M2	M6
Difference in RA total pathobionts compared to Enr, mean (95% CI)	Control	0	0.03 (-0.13 – 0.19)	-0.04 (-0.15 – 0.06)	0 (-0.10 – 0.09)	-0.10 (-0.19 – 0)
	Metro	0	0.01 (-0.08 – 0.09)	0 (-0.07 – 0.08)	0.04 (-0.08 – 0.15)	0.03 (-0.10 – 0.15)
	EF+	0	0 (-0.03 – 0.03)	0.02 (-0.05 – 0.09)	0.04 (-0.02 – 0.11)	-0.02 (-0.06 – 0.01)
	GynLP	0	-0.02 (-0.05 – 0.01)	0.06 (-0.08 – 0.20)	0 (-0.02 – 0.02)	0 (-0.05 – 0.05)
Difference in RA total other bacteria compared to Enr, mean (95% CI)	Control	0	0 (-0.01 – 0)	0 (-0.01 – 0.01)	0 (-0.01 – 0.01)	0 (-0.01 – 0)
	Metro	0	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0)	0 (0 – 0.01)
	EF+	0	0 (0 – 0.01)	0.01 (0 – 0.03)	0 (0 – 0)	0 (0 – 0.01)
	GynLP	0	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0)	0 (0 – 0)
RA total EF+ strains, mean (95% CI)	Control	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	Metro	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	EF+	0 (0 – 0)	0.02 (0 – 0.04)	0.01 (0 – 0.03)	0 (0 – 0.01)	0 (0 – 0)
	GynLP	0 (0 – 0.01)†	0.01 (0 – 0.01)†	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
RA total GynLP strains, mean (95% CI)	Control	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	Metro	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	EF+	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
	GynLP	0 (0 – 0)	0.02 (-0.01 – 0.06)	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0)

BV bacterial vaginosis, *CI* confidence interval, *Conc* concentration, *D7* Day 7 visit, *EF+* Ecologic Femi+, *Enr* enrolment, *GynLP* Gynophilus LP, *M1/2/6* Month 1/2/6 visit, *Metro* oral metronidazole group, *RA* relative abundance, *VMB* vaginal microbiota.
*Total numbers are slightly lower than enrolled women (and not lost to follow-up) per time point due to invalid results. Numbers missing per group is at most two at Enr, M1, M2, and M6 visits, and four at the D7 visit (GynLP group only, due to two missed visits). †These are naturally occurring EF+ strains with 100% identity with the EF+ probiotic strains.

Table C.5: Characteristics associated with VMB composition

	Outcome variables							
	Total *Lactobacillus* conc		Total BV-anaerobes conc		Total pathobionts conc		Nugent score	
Characteristics	**OR (95% CI)***	**p**	**OR (95% CI)***	**p**	**OR (95% CI)***	**p**	**OR (95% CI)***	**p**
Currently uses hormonal contraception or is pregnant†	0.91 (0.60 – 1.37)	0.638	1.03 (0.61 – 1.74)	0.921	2.50 (1.36 – 4.63	0.003	0.96 (0.40 – 2.28)	0.920
Sample taken within 7 days after menses‡	0.91 (0.65 – 1.27)	0.576	1.30 (0.87 – 1.94)	0.201	0.80 (0.47 – 1.34)	0.387	2.26 (0.93 – 5.52)	0.072
Reports urogenital symptoms	0.88 (0.62 – 1.25)	0.475	3.05 (2.03 – 4.60)	<0.001	1.07 (0.63 – 1.81)	0.802	14.81 (6.64 – 33.08)	<0.001
Reports unusual vaginal discharge	0.89 (0.45 – 1.75)	0.734	4.45 (1.97 – 10.07)	<0.001	1.65 (0.59 – 4.61)	0.339	69.60 (15.14 – 320.04)	<0.001
Has taken antibiotics in the previous 14 days§	0.94 (0.40 – 2.21)	0.879	0.68 (0.23 – 1.94)	0.467	1.17 (0.87 – 11.67)	0.080	1.22 (0.10 – 14.88)	0.875
Currently breastfeeding	1.01 (0.99 – 1.03)	0.259	1.00 (0.98 – 1.02)	0.871	1.00 (0.97 – 1.03)	0.963	0.99 (0.95 – 1.04)	0.775
Uses condoms consistently	1.26 (0.93 – 1.69)	0.134	0.79 (0.55 – 1.15)	0.218	0.82 (0.52 – 1.29)	0.389	0.49 (0.24 – 0.99)	0.048
Reports five or more sex partners in the past month¶	1.24 (0.84 – 1.82)	0.276	1.00 (0.62 – 1.60)	0.986	1.94 (1.11 – 3.72)	0.019	1.01 (0.44 – 2.36)	0.973
Reports 'below average' sexual risk taking‖	1.14 (0.78 – 1.67)	0.506	0.98 (0.61– 1.56)	0.918	0.52 (0.29 – 0.92)	0.026	0.61 (0.24 – 1.55)	0.299
Exchanged sex for money and/or goods¶	1.42 (0.76 – 2.66)	0.271	0.63 (0.29 – 1.37)	0.243	0.77 (0.30 – 2.00)	0.598	0.53 (0.12 – 2.33)	0.401
Is 30 years or older**	1.20 (0.77 – 1.89)	0.423	0.46 (0.27 – 0.79)	0.005	0.47 (0.24 – 0.92)	0.027	0.60 (0.24 – 1.49)	0.271
Manages menses with sanitary pad (versus other methods)††	1.32 (0.75 – 2.31)	0.331	1.87 (0.61 – 5.73)	0.274	0.69 (0.16 – 1.19)	0.616	10.69 (1.13 – 101.07)	0.039
Consumes yoghurt <1/week or more (versus never)††	0.90 (0.60 – 1.34)	0.596	1.00 (0.45 – 2.26)	0.993	0.43 (0.15 – 1.19)	0.103	3.98 (0.79 – 20.07)	0.095
Reports ever washing inside the vagina	0.92 (0.60 – 1.41)	0.692	0.74 (0.45 – 1.24)	0.253	0.99 (0.52 – 1.88)	0.964	0.47 (0.16 – 1.40)	0.174

BV bacterial vaginosis, *CI* confidence interval, *Conc* concentration, *OR* odds ratio.
*Covariates were tested in mixed effects models with participant identification number as a random effect, the potential predictor as fixed effects, and the four vaginal microbiota outcomes as separate outcomes. All possible time points were included in the models. The potential predictor variables were not correlated with each other. †Women using a copper intra-uterine device were not included in this model. ‡Versus remainder of the cycle. §Only antibiotics given for reasons not associated with the primary/secondary outcomes of the study were included. ¶Or in the past two months (when asked at the M2 visit), or in the past four months (when asked at the M6 visit). ‖Composite variable of condom use consistency and number of sexual partners: women were considered low risk when they reported fewer than five sexual partners in the past month plus consistent condom use. The variable 'exchanging sex for money and/or goods' (next row) was not added to this composite variable because the great majority of women reported this behaviour (table C.1). **Versus 29 years old or younger. ††Asked at the enrolment visit only.

Table C.6: Incidence of sexually transmitted and urinary tract infections

	Controls		Metronidazole		Ecologic Femi+		Gynophilus LP		Total	
	n/N*	IR (95% CI)	n/N*	IR (95% CI)	n/N*	IR (95% CI)	n/N*	IR (95% CI)	n/N*	IR (95% CI)
TV *InPouch:* Enr-M2	2/17	1.10 (0.35 – 3.41)	0/17	0	0/17	0	0/16	0	2/67	0.27 (0.09 – 0.84)
TV *InPouch*: M2-M6	1/17	0.20 (0.03 – 1.42)	2/16	0.42 (0.11 – 1.70)	0/16	0	1/15	0.23 (0.03 – 1.65)	4/64	0.21 (0.08 – 0.57)
CT: Enr-M6	1/17	0.16 (0.09 – 1.42)	2/16	0.36 (0.09 – 1.42)	2/16	0.29 (0.07 – 1.15)	1/15	0.18 (0.03 – 1.29)	6/64	0.25 (0.11 – 0.55)
NG: Enr-M6	1/17	0.14 (0.02 – 0.98)	2/16	0.28 (0.07 – 1.14)	2/16	0.29 (0.07 – 1.18)	0/15	0	5/64	0.18 (0.08 – 0.44)
UTI†: Enr-M6	2/17	0.39 (0.13 – 1.20)	2/16	0.27 (0.07 – 1.09)	1/16	0.14 (0.02 – 0.97)	2/16	0.29 (0.07 – 1.14)	7/65	0.27 (0.14 – 0.54)

CI confidence interval, *CT Chlamydia trachomatis*, *Enr* enrolment visit, *IR* incidence rate, *M2/6* month 2/6 visit, *NG Neisseria gonorrhoeae*, *STI* sexually transmitted infection, *TV Trichomonas vaginalis*, *UTI* urinary tract infection.

Incidence rates of STIs and UTIs during study follow-up. Incidence rate ratios were not calculated due to low number of incident cases.

*Number of women (n) who developed at least one incident infection during the specified time period as a proportion of the women who completed all follow-up visits in that time period (N). †One participant in the GynLP group was tested for UTI at an unscheduled visit between M2 and M6 and subsequently withdrew her informed consent.

Appendix D

This appendix corresponds to Chapter 5.

Table D.1: Characteristics of the included studies, ordered by publication year

Reference	Setting, country of study	Study design	VMB status at baseline	Vaginal probiotics and other intervention(s) (strategy: main therapy, adjuvant, maintenance, vaginal detection study)	Total N of participants, comparison groups (n)	Age range in years	Pb use, follow-up in days	Outcome: BV/VVC/ molecular
Özmen[188]	Gyn. clinic, Turkey	NR PPIS in three groups	BV (Amsel) + GV (BC) pos. VVC (FC) neg.	MTZ po 500 mg bid daily, for 7d + Gynoflor Pb[a] daily, for 12d vs. Gynoflor Pb[a] only daily, for 12d vs. CG: MTZ po 500 mg twice daily, for 7d. Strategy: main/adjuvant.	N=307. Ab + adjuvant Pb (96) vs. Pb only (97) vs. Ab only CG (114).	18-53	12, 22-35	VVC
Gardiner[196]	Unclear setting, Canada	NB, RCT	NS ND, all high lactobacilli by BC. VVC ND.	*L. fermentum* RC-14 + *L. rhamnosus* GR-1 Pb[b] for 3d vs. *L. rhamnosus* GG Pb[c] for 3d. Both given immediately after menses. Strategy: detection study only.	N=10. RC-14/GR-1 Pb (5) vs. GG Pb (5).	21-51	3, 21	Molecular
Antonio & Hillier[76]	Unclear setting, USA	PPIS	BV (NS: 0-3 or 0-6?) neg.; VVC ND.	*L. crispatus* CTV-05 Pb[d] twice daily for 3d. Strategy: detection study only.	N=9. All women used Pb, pre/post results given.	18-40	3, 9-11	Molecular
Burton[197]	Unclear setting, Canada	PPIS	BV/VVC tests ND; BV/VVC considered neg.	*L. fermentum* RC-14 + *L. rhamnosus* GR-1 Pb[b] for 3d vs. CG: controls, but of unclear nature. Strategy: detection study only.	N=19. Pre/post Pb users (10) vs. CG (9).	Not clear[e]	3, 6 mo	Molecular
Pirotta[67]	GPs/ pharma-cies, Australia	DB, PC, 2x2 factorial RCT	All asympto-matic, 21.2% VVC (FC) pos. BV testing ND.	All used Ab for non-gynaecological infections. 2x2 factorial design of four products: used 1/2 vag. and 1/2 oral products: Femilac vag pessary Pb[f] daily for 10d; Pb po (Lactobac), vag. placebo, and po placebo. Strategy: maintenance.	N=278. Both Vag. + po Pb (67) vs. vag. placebo + po Pb (73) vs. vag. Pb + po placebo (70) vs. vag. + po placebo (68).	18-50	10, 28	VVC
Eriksson[138]	STI clinic, Scandi-navia	DB, PC, RCT	BV (Amsel) pos., 69.7% NS 7-10. All VVC pos. excluded.	Ab (clindamycin 100 mg vag. ovules once daily, 3d) for all. Pb-impregnated tampons,[g] to use during menses (length of use depended on menses, but >4 days) vs. CG: placebo tampons during menses. Strategy: maintenance.	N=255. Ab + Pb (127) vs. Ab + placebo CG (128).	18-53	>5, ~56	BV
Anukam[139]	Clinics, Nigeria	NB, RCT	BV pos. (NS 7-10); VVC neg.	*L. reuteri* RC-14 + *L. rhamnosus* GR-1 Pb[h] 1x/d for 5d vs. CG: 0.75% MTZ vag. gel twice daily, 5d. Strategy: main.	N=40. Pb (20) vs. MTZ vag. gel CG (20).	18-50	5, 30	BV
Czaja[187]	Student HC, USA	Phase I DB, PC, RCT	BV & VVC neg. at baseline.	*L. crispatus* CTV-05 suppositories[i] inserted daily for 5d vs. CG: placebo vag. suppository used identically, for 5d. Strategy: maintenance (also detection data).	N=30. Pb (15) vs. placebo CG (15).	18-35	5, 26-34	VVC, Molecular

Reference	Setting	Study design	VMB status at baseline	Vaginal probiotics and other intervention(s), strategy	Total N, comparison groups (n)	Age range	Follow-up	Outcome
Larsson[135]	Gyn. clinic, Norway	DB, PC, RCT	BV (by Amsel) pos.; VVC (WM?) pos. excluded.	Ab (2% vag. clindamycin cream, 7d) for all, before baseline. EcoVag Pb[j] capsule for 10d, and then for 10d for 3 menstrual cycles vs. CG: placebo vag. capsules, identical use as Pb. Strategy: adjuvant/maintenance.	N=100. Ab + Pb (50) vs. Ab + CG (50).	18-53	~120,[j] ~6mo	BV
Petricevic[136]	Gyn. clinic, Austria	RCT	BV pos. (by NS); VVC not mentioned.	Ab (clindamycin 300 mg po twice daily, 7d) for all. Gynophilus Pb[k] capsule for 7d vs. CG of unclear nature but seems non-interventional. Strategy: maintenance.	N=190. Ab + Pb (95) vs. CG: Ab only (95).	18-45	7, 42	BV
Antonio[150]	Clinics, USA	DB, RCT	BV neg. (NS, unclear), VVC (WM?) neg.	All used *L. crispatus* CTV-05 capsules, twice a day for 3d; two groups: 10^6 vs. 10^8 CFU/dose. Strategy: detection study only.	N=90. CTV-05 10^6 (45) vs. CTV-05 10^8 CFU/dose (45).	14-21	3, 28	Molecular
Di Pierro[189]	Unclear, Italy (?)	PPIS	Partially (r) VVC (FC) pos.	All received Kramegin[l] vag. tablet, inserted daily for 10d. Strategy: main.	N=105. All women used Pb.	18-42	10, 17	VVC
Mastro-marino[72]	Gyn. clinic, Italy	DB, PC, RCT	BV (Amsel) pos., VVC (FC) neg.	Florisia Pb tablet[m] inserted daily for 7d vs. CG: placebo used identically, used for 7d. Strategy: main.	N=39. Florisia Pb (20) vs. CG (19).	≥18	7, 21	BV
Witt[190]	Gyn. clinic, Austria	NB, RCT	VVC (FC) pos. BV status not specified.	*L. gasseri* Pb[n] tablet given in six-month dosage scheme, with Af tx[n] vs. Af only, given for +/- 6mo[o] vs. classic homeopathy group (no Af). Strategy: maintenance.	N=150. Pb + Af (50) vs. Af only (50) vs. homeopathy (50).	17-56	6 mo,[n] 1y	VVC
Ehrström[195]	Gyn. clinic, Sweden	DB, PC, RCT	BV (Amsel) neg. VVC (FC) neg.	Ab or Af for all.[p] LN40/LN99/LN113/LN23 Pb capsule[q] used for 5d vs. CG: placebo group, identical use as Pb. Strategy: maintenance (mainly detection results).	N=95. Pb (60) vs. CG (35).	18-45	5, 6mo	Molecular
Hemmer-ling[183] & Ngugi[128]	Research clinic, USA	Phase II DB, PC, RCT	BV (NS 7-10) pos. VVC status unclear.	Ab (0.75% MTZ gel before baseline, 5d) for all. Lactin-V Pb[r] for 5d, and again at D12 and D19 vs. CG: placebo group, identical use as Pb. Strategy: detection study only.	N=24. Ab + Pb (18) vs. Ab + placebo CG (6).	18-50	19, 28	Molecular
Bradshaw[137]	Sex. health clinic, Australia	DB, PC, RCT	Amsel pos. + NS 4-6, or NS 7-10. VVC (FC): 11.6%.	Ab (MTZ po 400 mg twice daily, for 7d) for all. Gynoflor Pb[s] daily, for 12d vs. Ab group: 2% vag. clindamycin cream, 7d vs. CG: placebo pessary, identical use as Pb. Strategy: maintenance.	N=450. Clindamycin (150) vs. Pb (150) vs. placebo CG (150).	18-50	12, 6mo	BV
Hema-latha[140]	Unclear, India	DB, RCT	117/159 NS 4-10. VVC?	Florisia Pb[t] daily, for 8d vs. CG: pH-lowering vag. tablet used identically as Pb. Strategy: main.	N=159. Pb (82) vs. pH-lowering CG (77).	20-40	8, 9	BV
Vicariotto[191]	Clinic, Italy	Pilot PPIS	'Severe' VVC (by WM, FC). BV ND.	ActiCand 30 (*L. fermentum* LF10 and *L. acidophilus* LA02 Pb[u]) for 7d, and then every 3d for 21d, then every week for 28d. Strategy: main.	N=30. All used Pb.	23-64	56,[v] 56	VVC

Reference	Setting	Study design	VMB status at baseline	Vaginal probiotics and other intervention(s), strategy	Total N, comparison groups (n)	Age range	Follow-up	Outcome
Ling[141]	Gyn. clinic, China	NB, RCT	BV pos. (NS 7-10). VVC ND.	*L. delbrueckii* subsp. *lactis* DM8909 Pb[v] for 10d vs. CG: vag. MTZ 500 mg (unknown formulation: most likely gel?) once daily, 7d. Strategy: main.	N=121. Pb (53) vs. MTZ CG (68).	>17,[w] 19-50	10, 37	BV, Molecular
Bisanz[142]	Clinic, Canada	Cross-over, PC DB RCT	NS 4-6. VVC ND.	*L. rhamnosus* GR-1 and *L. reuteri* RC-14 Pb,[x] twice a day for 3d vs. CG: placebo, identical use. Strategy: main (also detection results).	N=14. Pb (7) vs. placebo CG (7), then cross-over.	40-80	3, 114	BV, Molecular
De Seta[192]	Gyn. clinic, Italy	NR NB PPIS or RCS?	VVC pos. (by WM). BV 0-6.	Af (2% clotrimazole vag. cream daily, 3d), for all. *L. plantarum* P17630 Pb[y] for 6d, then weekly for 28d vs. CG: vag. lubricant, identical use. Strategy: maintenance.	N=89. Af + Pb (45) vs. Af + vag. lubricant (44).	18-45	~34, ~124	VVC
Donders[184] & Donders[185]	Clinics, Belgium/ Germany	PPIS	All atrophic vaginitis. 2/ 14 VVC pos.	All used non-steroidal aromatase inhibitors. Gynoflor Pb[z] once daily for 28d, then thrice weekly for 56d for all participants (no CG). Strategy: main (mainly intended as safety study).	N=16. Pb for all.	52-63	84, 84	VVC
Pendharkar - Trial I[186]	Gyn. clinic, Norway	NR prospective cohort study	VVC (WM) neg. BV pos. (Amsel).[aa]	Ab: vag. 0.75% MTZ gel, vag. and po clindamycin[cc] for all. EcoVag Pb[bb] 1x daily for 5d, and for 5d on days 5-10 after next menstruation. Strategy: detection study only.	N=10. All used Ab+Pb.	26-49	~60, 1y	Molecular
Pendharkar - Trial II[186]	Gyn. clinics, Sweden	NR prospective cohort study	Some BV pos. (Amsel), some VVC (WM) pos.[bb]	BV-pos. women received Ab and EcoVag Pb[cc]; rVVC pos. women received Af tx scheme and Ecovag Pb[dd]; other VVC (seemingly rVVC) pos. women received Af tx scheme[ee] with no Pb. Strategy: adjuvant/maintenance (also detection results).	N=30. BV pos.: Ab + Pb (11); rVVC pos.: Af + Pb (9); (r)VVC (?) pos.: Af + placebo (10).	22-43	6 mo,[dd] 1y	VVC, Molecular
Tomusiak[151]	Gyn. clinics, Poland	DB, PC, RCT	Low lactos and /or NS 4-6. FC VVC neg.	InVag Pb[ff] given once daily for 7d vs. CG: placebo vag. capsule, identical use as Pb. Strategy: main (only detection results eligible).	N=160. Pb (86) vs. placebo CG (74).	18-40	7, ~21	Molecular
Kovachev[193]	Gyn. clinic, Bulgaria	NB, RCT	>99.5% VVC (FC) pos. BV?	Af (single dose 150mg po fluconazole and 600 mg vag. fenticonazole) for all. Lactagyn Pb[gg] 5d after finishing Af tx scheme, for 10d vs. CG: received Af tx only. Strategy: maintenance.	N=416. Af+Pb (209) vs. Af only CG (207).	17-50	10, 35-40	VVC
Verdenelli[143]	Gyn. clinic, Italy	PPIS	NS 0-6, VVC (FC?) neg.	SYNBIO gin Pb[hh] given once daily for 7d. Strategy: maintenance (also detection results).	N=35. Pb for all.	18-48	7, 28	BV, Molecular
Bohbot[144]	Clinics/ GPs, France	DB, PC, RCT	BV neg. (mod. Amsel). VVC ND.	Ab (MTZ po 500 mg 2x daily, 7d) before baseline Physioflor Pb[ii] given daily for 14d, for 4 menstrual cycles vs. CG: placebo capsule. Strategy: maintenance.	N=100. Pb (52) vs. CG (48).	≥18, mean 35.7	+/- 56, 196	BV

Reference	Setting	Study design	VMB status at baseline	Vaginal probiotics and other intervention(s), strategy	Total N, comparison groups (n)	Age range	Follow-up	Outcome
Rapisarda[145]	Gyn. clinic, Italy	PPIS	All NS 4-10, some VVC pos. (FC).	*L. acidophilus* LA14 Pb (unknown dose), given daily for 14d. Strategy: main.	N=106. All used Pb.	18-45	14, 42	BV, VVC
Murina[194]	Unclear setting, Italy	PPIS	Some BV (Amsel) pos., VVC (WM) pos.	All received EPB Pb[ii] daily, for 6d, then twice weekly for 21d if symptoms still present.[ii] Strategy: main.	N=209. BV pos. (100) vs. symp. VVC pos. (82) vs. rVVC (27).	≥18, mean 35.8	6, ~32	VVC
Dausset[155]	Clinics, France	NB, RCT	BV/VVC neg. (unclear)	Gynophilus Pb[kk] 350 mg immediate release, once daily vs. Pb 1000 mg slow-release (SR) once every 3d vs. Pb 1000 mg SR once every 4d vs. Pb 1000 mg SR once every 5d. Strategy: detection study only.	N=33. 350mg daily (9) vs. 1g SR every 3d (8) vs. 1g SR every 4d (9) vs. 1g SR every 5d (7).	21-52	21, ~25	Molecular
van de Wijgert[160]	STI clinic, Rwanda	NB, pilot RCT	Neg. for BV (Amsel), VVC (WM).	MTZ po 500 mg twice daily (7d) before baseline. CG (behavioural counselling) vs. 500mg po MTZ twice weekly, for 60d vs. EF+ Pb[ll] daily for 5d, then thrice weekly for 55d vs. Gynophilus LP Pb[mm] once every 4d for 60d. Strategy: maintenance (also detection results).	N=68. CG (17) vs. MTZ Ab (17) vs. EF+ Pb (17) vs. Gynophilus LP Pb (17).	18-45	60, 6mo	BV, VVC, Molecular

Ab antibiotics, *Af* antifungals, *BC* bacterial culture, *BV* bacterial vaginosis, *CFU* colony-forming units, *CG* control group, *DB* double-blind, *EF+* Ecologic Femi+, *EPB* Estromineral Probiogel, *FC* fungal culture, *GP* general practitioner centres, *GV Gardnerella vaginalis*, Gyn gynaecological, *HC* health centre, *mo* months, *MTZ* metronidazole, *NB* not blinded, *ND* not done, *Neg*. negative, *NR* non-randomised, *NS* Nugent score, *Pb* probiotics, *PC* placebo-controlled, *PPIS* pre-/post- interventional study, *po* per os (oral), *Pos*. positive, *RCS* retrospective cohort study, *RCT* randomised controlled trial, *STI* sexually transmitted infections, *symp* symptomatic, *tx* therapy, *UTI* urinary tract infection, *vag*. vaginal, *(r)VVC* (recurrent) vulvovaginal candidiasis, *WM* wet mount.

The table includes all included studies in the systematic review, including studies with probiotic detection outcomes only. The days of probiotic use is the total timespan over which probiotic products were used, and may be part of treatment schemes in which probiotics were not used every single day. See footnotes per study for in-depth information. Follow-up days has been calculated in days after initiation of probiotic products, whenever possible.

a. Gynoflor (Medinova AG, Zurich, Switzerland) vaginal suppository of 10^7 to $7x10^8$ viable micro-organisms from a lyophilised culture of *L. acidophilus*, 600mg lactose and 30 µg estriol.
b. *L. fermentum* RC-14 and *L. rhamnosus* GR-1 were given in freeze-dried form, in a total of 10^9 CFU/dose.
c. *L. rhamnosus* GG (ConAgra Foods, Omaha, USA) was given in vaginal capsules, containing 10^{10} CFU/dose.
d. *L. crispatus* CTV-05 (not Lactin-V, which is given as a vaginal applicator), given as a vaginal capsule containing 10^8 CFU/dose. Product was inserted twice daily for 3 days.
e. Age of participants is not reported, but all participants are premenopausal.
f. Contains *L. rhamnosus*, *L. delbrueckii*, *L. acidophilus* and *S. thermophilus*; doses not specified.
g. Tampons were impregnated with freeze-dried *L. gasseri*, *L. casei* var *rhamnosus* and *L. fermentum*, used as maintenance therapy. Each lactobacilli-impregnated tampon contained 10^8 living bacteria. Women using at least 5 tampons were included in efficacy analyses.
h. *L. reuteri* (formerly *fermentum*) RC-14 and *L. rhamnosus* GR-1, given as vaginal gelatin capsules, 10^9 CFU/dose of each spp., two capsules per night. Different dosage than Gardiner, 2002 & Burton 2003.
i. Containing *L. crispatus* CTV-05 at 5 x 10^8 CFU/dose, inserted daily for 5 days. Not the commercially available Lactin-V, this product was developed later.
j. Containing freeze-dried *L. gasseri* (Lba EB01-DSM 14869) and *L. rhamnosus* (Lbp PB01-DSM 14870) at a combined minimum of 10^{8-9} CFU/capsule.
k. Gynophilus vaginal capsule (Biose, Aurillac, France) contains at least 10^9 CFU/dose of *L. rhamnosus* 35.
l. Kramegin vaginal tablet (containing 1 mg plant extract *Krameria triandra* root extract, *L. acidophilus* (1 billion UFC - presumably meant CFU.) and 15 mg lactic acid.
m. Containing at least 10^9 CFU/dose of viable lactobacilli (*L. brevis* (CD2), *L. salivarius* subsp. *salicinius* (FV2), and *L. plantarum* (FV9).
n. Lactobacilli ($2x10^8$-$2x10^9$ *L. gasseri* lyophilisates) in a vaginal tablet (Gebro Pharma, Fieberbrunn, Austria), as adjuvant therapy. First, women received an induction regimen of single-day itraconazole 200 mg bid. After their next menses, participants continued with 200 mg bid itraconazole twice weekly for 4 weeks. Then (within 1 week of their next menstruation) they received maintenance therapy of itraconazole 200 mg bid once a month, for 6 months, as well as vaginal lactobacilli daily for 6 consecutive days, monthly, for 6 months.

o. Participants received an induction regimen of single-day itraconazole 200 mg bid. After their next menses, participants continued with 200 mg bid itraconazole twice weekly for 4 weeks. Then (within 1 week of their next menses) they received maintenance therapy of itraconazole 200 mg bid once a month, for 6 months.
p. Before baseline, BV-pos. women were treated for BV with local clindamycin 100 mg ovules, and VVC-pos. were treated with clotrimazole 200 mg vag. tablets for 3 consecutive nights.
q. Vaginal hydroxypropyl methylcellulose capsules (Ellen AB, Sweden) containing an inert carrying matrix (maltodextrin and magnesium stearate) and between 10^8 - 10^{10} viable cells of a probiotic substance, which was a mixture of freeze-dried *L. gasseri* LN40 (36% of weight), *L. fermentum* LN99 (27%) *L. casei* subsp. *rhamnosus* LN113 (27%), and *P. acidilactici* LN23 (10%)
r. Containing *L. crispatus* CTV-05, $2x10^9$ CFU/mL in a vaginal applicator.
s. Gynoflor vaginal pessaries (Medinova AG, Zurich, Switzerland) containing 10^7 CFU/dose of live *L. acidophilus* KS400, 30 µg oestriol and excipients.
t. Blend of at least 10^9 CFU of viable *L. brevis* CD2, *L. salivarius* subsp. *salicinius*, and *L. plantarum*; presumably same strains as article by Mastromarino et al. (2009),[72] but not clear.
u. Vaginal tablets containing > 0.4 billion live cells of both probiotic *L. fermentum* LF10 and *L. acidophilus* LA02 used as a main therapy, as part of a treatment scheme.
v. Suppository/capsule containing at least 10^9 CFU/dose of live *L. delbrueckii* subsp. *lactis* DM8909, once daily at bedtime for 10 days.
w. Ages of individual participants is only available in the supplementary materials and only available for participants who completed both FU visits (N=55).
x. Vaginal capsules of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (2.56×10^9 CFU/dose each).
y. Vaginal capsules of *L. plantarum* P17630 (Gyno-Canesflor, Bayer Canesten), $>10^8$ CFU/dose.
z. Gynoflor (Medinova AG, Zurich, Switzerland) contains 100 million viable *L. acidophilus* KS400 and an dose of 30 µg E3/oestriol.
aa. The study of Pendharkar et al. (2015) consisted of two separate but related clinical trials. We reported these separately for clarity.
bb. All used EcoVag capsules (contain *L. gasseri* (DSM 14869) and *L. rhamnosus* (DSM 14870) at 1×10^8 CFU of each strain per capsule) given as adjuvant therapy. Women in the BV group were given a seven-day course of daily 2% vaginal clindamycin cream (Dalacin cream 2%) together with oral clindamycin 300 mg bid for 7 days. After this, therapy was started with EcoVag capsules for 5 days, as adjuvant therapy. After the next menstruation, women were given a 5-day course of vaginal 0.75% metronidazole gel followed by 5 more days with EcoVag. Oral clindamycin treatment was also given to sexual partners.
cc. In this group, women used EcoVag capsules contain *L. gasseri* (DSM 14869) and *L. rhamnosus* (DSM 14870) at 1×10^8 CFU of each strain per capsule, given as adjuvant therapy. Women in the BV group were given a 7-day course of daily 2% vaginal clindamycin cream (Dalacin 2%) together with oral clindamycin 300 mg BID for 7 days. After this, therapy was started with EcoVag capsules for 10 days, as adjuvant therapy. After the next menstruation, women were given a 5-day course of vaginal 0.75% metronidazole gel followed by 10 more days with EcoVag. After the second menstruation, EcoVag capsules were given once every week for the next 4 months. Oral clindamycin treatment was also given to the patients' sexual partners.
dd. Women were given 50 mg (oral?) fluconazole per day for 28 days, and EcoVag capsules for 10 days (days 18-28). After the first menses, they were given EcoVag capsules for 10 days along a weekly course of fluconazole 200 mg for 2 months. This was followed by a third course of fluconazole 200 mg once every 2 weeks for the next 3 months. After the second menses, EcoVag was given once weekly for 4 months.
ee. Women with VVC (unclear whether these are also women with recurrent VVC or just normal VVC) were given the same anti-fungal treatment as in the Af+EcoVag group.
ff. InVag probiotic capsule contains 3 viable bacterial strains present at $>10^9$ CFU/ml. InVag contains 25% *L. fermentum* 57A, 25% *L. plantarum* 57B, 50% *L. gasseri* 57C, and excipients.
gg. All women first received single dose fluconazole po (150 mg) and vag. fenticonazole (600 mg). Af+Pb followed this by vag. capsules of Lactagyn, containing live *Lactobacillus* spp: *L. acidophilus*, *L. rhamnosus, S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*. Strains and doses unknown. 10 Pb applications were given on the 5th day after anti-fungal treatment (for 10 days?).
hh. Probiotic suppository SYNBIO gin, consisting of a Witepsol H15 matrix containing total of at least 10^9 CFU of viable lactobacilli (1:1 of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502).
ii. *L. crispatus* IP 174178 (Physioflor) vaginal capsules (10^9 CFU/gram; total grams unclear); to be taken for 14 days, daily administration, for four consecutive menstrual cycles.
jj. All received a vaginal gel containing lactic acid (among others) in combination with a vag. capsule (EPB) of *L. fermentum* LF10 (presumably 0.5 billion CFU/dose) and *L. plantarum* LP02 (0.5 billion) and prebiotic galacto-oligosaccharides for six evenings; a telephone consultation was made 14d after baseline; if "troublesome symptoms" (not specified) were present, EPB was administered twice weekly for three more weeks.
kk. Contains *L. rhamnosus* regenerans 35 (Lcr35).
ll. Ecologic Femi+ (Winclove Probiotics, Amsterdam, The Netherlands) is a vaginal capsule containing lactoferrin and $>1.5x10^9$ CFU/capsule of lactic acid-producing bacteria. These bacteria include *B. bifidum* W28, *L. acidophilus* W70, *L. helveticus* W74, *L. brevis* W63, *L. plantarum* W21, and *L. salivarius* W24.
mm. Gynophilus LP vaginal tablets (Biose, Aurillac, France) contain 876.9 mg *L. rhamnosus* regenerans 35 (Lcr35).

Table D.2: Summary of evidence per vaginal probiotic product, ordered by number of users

Probiotic (brand name; manufacturer)	Summary of evidence
Lactobacillus acidophilus KS400; also contains 30 µg oestriol (Gynoflor; Medinova)	3 studies (total N=359). A Turkish study showed Gynoflor given for BV, with metronidazole or alone, resulted in significantly lower VVC cumulative incidence, compared to metronidazole alone.[188] An Australian study showed Gynoflor therapy after metronidazole resulted in lower BV recurrence than placebo, but higher than vaginal clindamycin after metronidazole.[137] A Belgian study in which women used Gynoflor for atrophic vaginitis, showed an increase in VVC rates right after initiation; the increase was temporary only, decreasing after one month of use.[184,185]
L. acidophilus, *L. rhamnosus*, *Streptococcus thermophilus*, *L. delbrueckii* subsp. *bulgaricus* (Lactagyn; Palcare Enterprises)	1 study (N=209). A Bulgarian study showed Lactagyn therapy after antifungal treatment resulted in lower VVC recurrence at the final follow-up visit (25-30 days after product cessation) than antifungal treatment alone.[193]
L. fermentum LF10, *L. plantarum* LP02 (Estromineral Probiogel; Meda Pharma)	1 study (N=209). In an Italian pre/post study women received Estromineral Probiogel without antifungals for incidental VVC or rVVC for six days, which resulted in high cure rates.[194] However, more than 50% of women in both groups required prolonged therapy due to persistence of symptoms.
L. rhamnosus Lcr35 (Gynophilus; Biose)	3 studies (total N=145). An Austrian study showed that Gynophilus after clindamycin reduced BV recurrence, compared to placebo after clindamycin.[136] A French study comparing four formulations/dosages found a high conc. of probiotic strains in all cases and greater acceptability when using slow-release products.[155] A Rwandan study in women using intermittent Gynophilus after metronidazole resulted in non-significant lower BV recurrence compared to no-intervention controls.[160] Probiotic detectability was low. There were no significant VMB changes.
L. rhamnosus, *L. delbrueckii, L. acidophilus, S. thermophilus* (Femilac; now Lallemand Inc)	1 study (N=137). An Australian study showed that ten days of Femilac during and after (non-metronidazole) antibiotic use, compared to placebo controls, was not effective in preventing VVC within 18 days after cessation of use.[67]
L. crispatus CTV-05; (named Lactin-V when in vaginal applicator; Osel)	4 studies (total N=132). An American study showed high CTV-05 detection in the vagina of healthy women without native *L. crispatus*, but not in those with native *L. crispatus*.[76] A study in women with recurrent urinary tract infections showed a similar VVC cumulative incidence after CTV-05 initiation. It showed low CTV-05 strain detection.[187] Another study showed that CTV-05 was only detected a few weeks after use, and that protected/unprotected sex and *L. crispatus* colonisation at baseline were associated with detection failure.[150] A trial with 18 BV-positive CTV-05 users showed that BV-anaerobes at baseline were not associated with a lower likelihood of detection; having native *L. crispatus* or having protected/unprotected sex was associated with lower detection odds.[128,183]
L. gasseri, L. casei var *rhamnosus, L. fermentum* (unnamed; Medipharm AB)	1 study (N=127). A Scandinavian study using *Lactobacillus*-impregnated tampons after clindamycin treatment during menses was not efficacious in reducing BV recurrence compared to placebo tampons.[138]
L. acidophilus LA14; also contains lactic acid (unknown brand and manufacturer)	1 study (N=106). A single-arm Italian study in women with Nugent 4-10 showed that 14-day LA14 use (without antibiotics or antifungals) resulted in 46/60 women cured (Nugent 0-3), which persisted after product cessation.[145] LA14 use also significantly decreased VVC rates and *Candida* fungal culture counts.
L. acidophilus; including lactic acid (Kramegin; PharmExtracta)	1 study (N=105). A single-arm Italian study among women with incidental or rVVC showed that ten-day Kramegin use (without antifungals) was successful in curing VVC.[189]
L. brevis CD2, *L. salivarius* subsp. *salicinius* FV2, *L. plantarum* FV9 (Florisia; VSL3 Pharmaceuticals)	2 studies (total N=102). In an Italian study, Florisia (without antibiotics) was more successful in treating BV than placebo.[72] This effect persisted two weeks after product cessation. In an Indian study, Florisia was not efficacious in curing BV, compared to pH-lowering tablet users.[140]

Probiotic	Summary of evidence
L. gasseri LN40, *L. fermentum* LN99, *L. casei* subsp. *rhamnosus* LN113, *Pediococcus acidilactici* LN23 (Ellen capsules; Ellen AB)*	1 study (N=95). In a Swedish study, five-day probiotic use resulted in probiotic strains detectability in a majority of women.[195] At next visits, the number of women with presence of one or more strains gradually decreased.
L. fermentum 57A, *L. plantarum* 57B, *L. gasseri* 57C (InVag; IBBS BioMed)*	1 study (N=86). In a Polish study there was detection of probiotic strains in most of the users, but this decreased quickly after product cessation.[151]
L. gasseri Lba EB01-DSM 14869, *L. rhamnosus* Lbp PB01-DSM 14870 (EcoVag; Bifodan A/S)	3 studies (total N=80). A Norwegian study showed that EcoVag (without antibiotics) was not better at curing BV than placebo, but EcoVag did significantly reduce the cumulative incidence of BV/intermediate microbiota when used after each menses, for four consecutive cycles.[135] A Norwegian study among BV-positive (by Amsel) women showed that strains could be isolated in most users.[186] A Swedish study in women with rVVC showed that fluconazole with EcoVag did not significantly improve VVC cure, compared to fluconazole alone. EcoVag strains were detected in a minority of women (more often in women without native lactobacilli).[186]
L. delbrueckii subsp. *lactis* DM8909 (unnamed; unknown company)	1 study (N=53). In a Chinese study, *L. delbrueckii* was not better than vaginal metronidazole as treatment for BV, but there were significantly fewer BV relapses 30 days after product use in the women using probiotics (compared to metronidazole users).[141] The molecular VMB assessments also showed benefits.
L. crispatus IP 174178 (Physioflor; IPRAD Pharma)	1 study (N=52). A French study showed 14-day Physioflor therapy after metronidazole resulted in significantly lower BV cumulative incidence and longer time to BV recurrence, compared to placebo, but the effects disappeared after use. [150]
L. gasseri (unnamed; Gebro Pharma)	1 study (N=50). An Austrian study showed no effect of using itraconazole with *L. gasseri* to prevent VVC, compared to women using itraconazole alone.[190]
L. reuteri RC-14 (formerly *L. fermentum* RC-14), *L. rhamnosus* GR-1 (unnamed; Chr. Hansen A/S)	4 studies (total N=47). A Canadian study showed high strain detection directly after use, but a decrease during follow-up; GR-1 was detected more often than RC-14.[196] A Canadian study showed 100% detection during use but detectability decreased after use; RC-14 disappeared quicker than GR-1.[197] A Nigerian study showed a higher BV cure rate of five-day RC-14/GR-1 therapy than five-day vaginal metronidazole gel therapy; BV recurrence after use was also lower in probiotic users.[139] A Canadian trial in post-menopausal women with Nugent 4-6 showed no significant changes in Nugent scores when using RC-14/GR-1, but there were significant increases in *Lactobacillus* spp. and decreases in *Atopobium* spp.[142]
L. plantarum P17630 (Gyno-Canesflor, Bayer Canesten)	1 study (N=45). An Italian study showed 34-day Gyno-Canesflor use after clotrimazole treatment resulted in non-significant lower VVC recurrence than placebo users.[192]
L. rhamnosus IMC 501, *L. paracasei* IMC 502 (SYNBIO gin; Synbiotec)	1 study (N=35). A single-arm Italian study in women with Nugent 0-6 and seven-day SYNBIO use (no antibiotics) resulted in an increase of Nugent 0-3 proportions and a significant *Lactobacillus* conc. increase.[143] Strains were detected in all users.
L. fermentum LF10, *L. acidophilus* LA02 (ActiCand 30; ItalFarmaco)	1 study (N=30). In a single-arm Italian study in women with severe rVVC, ActiCand use without antifungals resulted in a significant reduction of VVC cases, with 23 out of 30 women being cured.[191]
B. bifidum W28, *L. acidophilus* W70, *L. helveticus* W74, *L. brevis* W63, *L. plantarum* W21, *L. salivarius* W24 (Ecologic Femi+; Winclove)	1 study (N=17). A Rwandan study using Ecologic Femi+ intermittent therapy after metronidazole for BV resulted in significantly lower BV incidence during use.[160] Use also resulted in significant higher *Lactobacillus* abundances, lower BV-anaerobes conc., and lower likelihood of having a BV-like VMB type, compared to no-intervention controls. All effects disappeared after product use. The detectability and conc. of probiotic strains was low.
L. rhamnosus GG (unnamed; ConAgra Foods)*	1 study (N=5). A Canadian colonisation study showed high detection of GG strains, which gradually decreased over time after product cessation.[196]

BV bacterial vaginosis, *conc.* concentration, *spp.* species, *VMB* vaginal microbiota, *(r)VVC* (recurrent) vulvovaginal candidiasis.
*Only vaginal detection studies available for this product; no comprehensive BV/VMB or VVC efficacy results available.

Table D.3: Detection and colonisation rates of probiotic strains

Reference	Probiotic(s)	Short methods overview	Vaginal detection	Abundance/ concentration	Other results of interest
Gardiner[196]	RC-14/GR-1 *(L. fermentum* RC-14 + *L. rhamnosus* GR-1) and *L. rhamnosus* GG; vaginal detection in healthy women only.	RAPD of vaginal culture isolates; PFGE on selected samples to distinguish *L. rhamnosus* GR-1 from *L. rhamnosus* GG.	5/5 users had at least one strain detected directly after the 3-day use period. Decreased to 1-2/10 users per strain at 18 days after use.	NA	NA
Antonio & Hillier[76]	*L. crispatus* CTV-05 (not Lactin-V); vaginal detection in healthy women only.	DNA hybridisation with whole-chromosome probes to identify *Lactobacillus* spp.; rep-PCR to identify CTV-05.	CTV-05 detected in 5/9 users directly after the 3-day use period. This increased to 6/9 users at 6-8 days after use.	NA	CTV-05 detected in 5/5 users without native *L. crispatus* at baseline and in 0/4 without.
Burton[197]	RC-14/GR-1 *(L. fermentum* RC-14 + *L. rhamnosus* GR-1); vaginal detection in healthy women only.	RAPD DNA fingerprinting of culture isolates. VMB changes after RC-14/GR-1 use determined by DGGE analysis and Sanger sequencing of excised gel bands.	10/10 users had at least one strain detected directly after the 3-day use period. Decreased to 0-2/10 users per strain at 18 days after use.	NA	NA
Czaja[187]	*L. crispatus* CTV-05 (not Lactin-V) as maintenance tx to prevent UTIs.	Rep-PCR for *L. crispatus* CTV-05, on culture isolates. Rep-PCR to identify *Lactobacillus* spp.	CTV-05 detected in 4/15 users during follow-up (one or two days after 5 days of use and at 4 weeks).	NA	NA
Antonio[150]	*L. crispatus* CTV-05 (not Lactin-V) vaginal detection in healthy women.	Rep-PCR for *L. crispatus* CTV-05, on culture isolates. Rep-PCR to identify *Lactobacillus* spp.	After 3 days of use, CTV-05 detected in 69% of 45 women at one or more D7 or D21 visits, and 59% at the D28 visit.	NA	28/31 without native *L. crispatus* had CTV-05 detected, and 24/47 (51%) with (Fisher's exact $p<0.001$). Sexual intercourse (with condoms, $p=0.02$; without condoms, $p<0.001$) and native *L. crispatus* ($p=0.001$) at baseline associated with lack of CTV-05 detection.

Reference	Probiotic(s)	Short methods overview	Vaginal detection	Abundance/ concentration	Other results of interest
Ehrström[195]	Ellen capsules (*L. gasseri* LN40 + *L. fermentum* LN99 + *L. casei* subsp. *rhamnosus* LN113 + *Pediococcus acidilactici* LN23) as maintenance tx after clotrimazole.	qPCR to identify *Lactobacillus* and *Pediococcus* culture isolates. Strain identification of vaginal culture isolates using RAPD.	At least one probiotic strain detected in 47/53 users directly after the 5-day use period; 27/51 after first menses; 3/50 at 6 months.	NA	NA
Hemmerling[183] & Ngugi[128]	*L. crispatus* CTV-05 (Lactin-V) as maintenance tx after vaginal metronidazole.	Rep-PCR to identify CTV-05 strains, 16S qPCR of specific vaginal bacteria.	CTV-05 detected in 11/18 users during and/or up to 7 days after the 19-day use period; this was 7/9 in women who had used all seven applicators.	NA	Lack of CTV-05 detection associated with sexual intercourse with or without condoms and with having native *L. crispatus* at baseline; no association with baseline presence of anaerobes.
Bisanz[142]	RC-14/GR-1 (*L. reuteri* RC-14 + *L. rhamnosus* GR-1) as main tx for Nugent 4-6.	16S rRNA gene V6 sequencing.	At least on probiotic strain detected in 7/12 users directly after the 3-day use period, but only in one user within the 17 days after cessation.	GR-1 was usually far more abundant than RC-14, but never dominated the VMB.	NA
Pendharkar – Trial I[186]	EcoVag (*L. gasseri* DSM 14869 + *L. rhamnosus* DSM 14870) as adjuvant/maintenance tx to prevent BV recurrence.	Gram-positive bacilli on culture used for rep-PCR, DNA profiles were compared with those of EcoVag strains. Identification was confirmed by using RAPD with primers for both EcoVag spp.	At least one probiotic strain detected in 9/10 users during use, which persisted for two weeks after cessation in 8/10, two months in 3/9, and three months in 2/9.	NA	The proportion of women with EcoVag strains detected was non-significantly higher in those cured for BV compared to those experiencing a relapse (Fisher's exact p=0.16).

Reference	Probiotic(s)	Short methods overview	Vaginal detection	Abundance/ concentration	Other results of interest
Pendharkar - Trial II[186]	EcoVag *(L. gasseri* DSM 14869 + *L. rhamnosus* DSM 14870) as adjuvant/ maintenance tx to prevent BV and VVC recurrence (different participant groups).	Gram-positive bacilli on culture used for rep-PCR, DNA profiles were compared with those of EcoVag strains. Identification was confirmed by using RAPD with primers for both the EcoVag spp.	Probiotic strains detected in 5/7 adherent women using EcoVag and antibiotics, and 8/9 using EcoVag and antifungals, during the 6-month intervention period. Detection decreased from two weeks after tx cessation onwards (data not reported).	NA	Natural lactobacilli more common than probiotic strains in 73% of the samples. EcoVag strains detected more often among women who did not have natural lactobacilli at baseline.
Tomusiak[151]	InVag (*L. fermentum* 57A + *L. plantarum* 57B + *L. gasseri* 57C) as main tx for NS 4-6 or "low lacto count".	PCR for spp. identification, PFGE/multilocus sequence-typing to confirm InVag spp. presence.	At least one probiotic strain detected in 82% of 86 users directly after use, and in 47.5% 14 days after cessation.	NA	NA
Verdenelli[143]	SYNBIO Gin (*L. rhamnosus* IMC 501 + *L. paracasei* IMC 502) as maintenance tx to prevent BV and VVC.	Real-time qPCR for total lactobacilli and SYNBIO strains using specific primers, and RAPD on culture isolates for SYNBIO strains presence.	At least one probiotic strain detected in 35/35 users directly after 7 days of use. Decreased to 21/35 users 21 days after use.	Proportion of total lactobacilli: 22.9% for IMC501 and 23.8% for IMC502 directly after use. Decreased to 14.3% and 9.4% 21 days after use.	Significant increase in total *Lactobacillus* after SYNBIO use.
Dausset[155]	Gynophilus (*L. rhamnosus* Lcr35 regenerans) in two formulations: immediate release and slow-release. Vaginal detection in healthy women only.	Lcr35 by qPCR.	Data are presented as daily mean concentrations per regimen (a total of 35 women used the probiotic for 21 days every 3, 4 or 5 days, and sampled daily) but the means suggest that all women at all visits had Lcr35 detected.	Mean concentration of 0.48-1.92 $\log_{10}$ cells/µl per FU visit during the 2-month intervention period. Mean relative abundance of 3% (7.7% if any strains detected).	Concentrations were much higher (up to 4 $\log_{10}$ cells/µl) in women with any probiotic strain detected. There was no clear association with self-reported adherence.

Reference	Probiotic(s)	Short methods overview	Vaginal detection	Abundance/ concentration	Other results of interest
van de Wijgert[160]	Ecologic Femi+ *(B. bifidum* W28 + *L. acidophilus* W70 + *L. helveticus* W74 + *L. brevis* W63 + *L. plantarum* W21 + *L. salivarius* W24) as maintenance tx to prevent BV recurrence.	16S rRNA gene V3V4 sequencing, total 16S rRNA copies concentration by BactQuant, and estimated concentrations per taxon using both.	At least one probiotic strain detected in 39.3% of samples during the 2-month intermittent use period. No longer detected 4 months after cessation of use.	Mean concentration of 0.48-1.92 log_{10} cells/µl per follow-up visit during the 2-month intervention period. Mean relative abundance of 3% (7.7% if any strains detected).	Concentrations were much higher (up to 4 log_{10} cells/µl) in women with any probiotic strain detected. There was no clear association with self-reported adherence.
van de Wijgert[160]	Gynophilus LP (*L. rhamnosus* Lcr35 *regenerans*) as maintenance tx to prevent BV recurrence.	16S rRNA gene V3V4 sequencing, total 16S rRNA copies concentration by BactQuant, and estimated concentrations per taxon using both.	The probiotic strain detected in 19.8% of samples during the 2-month intermittent use period. No longer detected 4 months after cessation of use.	Mean concentration of 0.25-1.05 log_{10} cells/µl per follow-up visit during the 2-month intervention period. Mean relative abundance of 3% (15.1% if any strain detected).	

CFU colony-forming unit, *conc*. concentration, *DGGE* denaturing gradient gel electrophoresis, *EF+* Ecologic Femi+, *GynLP* Gynophilus LP, *Lcr35 L. rhamnosus* Lcr35, *NA* not applicable, *Pb* probiotic, *PFGE* pulsed-field gel electrophoresis, *qPCR* quantitative polymerase chain reaction, *RAPD* random amplification of polymorphic DNA, *rep-PCR* repetitive element sequence-PCR, *VMB* vaginal microbiota.

Appendix E

This appendix corresponds to Chapter 6.

Informed consent procedures
All participants provided written informed consent. The age of majority for Rwandan women was lowered from 21 to 18 years in November 2016, and we also obtained parent/guardian consent for non-married participants aged 18-20 years until this was no longer required. Participants and/or parents/guardians with insufficient literacy could sign by thumbprint but the informed consent process was observed by an independent witness who co-signed the informed consent form. The witness could not be a Rinda Ubuzima staff member, but could be another participant.

Selection of POCTs for evaluation in WISH
We chose POCTs that comply with the WHO ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end users) as much as possible,[106,108] and that are feasible to conduct and interpret by primary care clinic staff in African settings. For example, some might argue that microscopy is an 'almost ASSURED' diagnostic method for diagnosing bacterial vaginosis (BV). Microscopy is not equipment-free, but microscopes are much more readily available than other types of equipment. However, our experience is that microscopy is not 'user-friendly'. While the acts of preparing a slide and viewing it under a microscope might be feasible after sufficient practice, the actual recognition of human cell types and micro-organisms requires a much higher level of biology training than primary care clinic staff in African settings have typically received. Also, Gram staining requires a laboratory with dedicated sinks and running water for safety reasons, and it is not practical to stain one slide at a time.

The development of ASSURED POCTs for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) has been ongoing for years. Unfortunately, lateral flow tests based on CT/NG-specific antibody, antigen, or enzyme detection to date have had low sensitivity.[92,237] The GeneXpert CT/NG assay (Cepheid, Sunnyvale, USA) was the first CT/NG POCT based on nucleic acid amplification testing (NAAT) and has excellent performance compared to other validated CT/NG NAATs.[91,238] However, it is not ASSURED because it requires equipment, is expensive (we paid 18.25 USD for consumables per test), and takes 90 minutes to return results. We chose to use this test anyway because it was the best CT/NG POCT available at the time of WISH study implementation, and because GeneXpert machines are widely available in Rwanda for tuberculosis testing. The machines are modular and can accommodate different types of test cartridges. Cepheid is currently considering developing next-generation GeneXpert CT/NG assays with shorter turn-around times but development has not yet been initiated (personal communication with representatives of Cepheid Europe in July 2018). Furthermore, additional NAAT-based POCTs for CT and/or NG are under development and prices might decline as a result of market competition.[239–241] In the WISH study, the Rwandan laboratory technicians were trained by a Cepheid representative based in Nairobi, Kenya. The representative visited the Rinda Ubuzima laboratory, assisted the team with equipment set-up, and conducted a one-day training using this equipment so that any start-up problems could be resolved immediately. The University of Liverpool investigators also attended this training to be able to assist the Rwandan technicians with potential future problems. However, we did not experience any problems during the study. Also, the percentage of invalid test results throughout the study was in line with those expected by Cepheid (fewer than 5% of the tests overall).

For *Trichomonas vaginalis* (TV), we chose the TV OSOM test (Sekisui Diagnostics, Lexington, USA) because experts consider it ASSURED and best in its class of lateral flow tests.[242] It is easy and quick to perform, has sensitivities of 83-86% compared with NAATs,[242] and is cheaper than NAATs (we paid 6.35 USD for consumables per test). POC NAATs that will likely have improved sensitivities are available or under development, including a module that can be used on the GeneXpert machine, but those are not ASSURED and more expensive.[242]

BV and vulvovaginal candidiasis (VVC) are not infections by a single pathogen but changes from an immuno-tolerant lactobacilli-dominated vaginal microbiological ecology towards a proinflammatory ecology. Knowledge about the vaginal ecology has significantly increased in recent years.[4] From a microbiological perspective, BV is a bacterial dysbiosis, and VVC is a dysbiosis characterised by an overgrowth of yeasts/fungi. BV is characterised by a persistent depletion of lactobacilli and an increased concentration of other bacteria, most commonly – but not exclusively – a highly diverse mixture of anaerobes including *Gardnerella vaginalis* and *Atopobium vaginae*. From a clinical perspective, the gold standard BV diagnosis is a Gram stain Nugent score of 7-10,[78] but the Amsel criteria are also often used.[77] Currently, only symptomatic BV in women seeking care is treated, even though asymptomatic BV has been associated with pregnancy complications and other complications.[17] There is no consensus yet about which types and levels of vaginal bacterial dysbiosis should be treated to prevent complications.[9] The Amsel criteria include a high vaginal pH as one of the criteria, and in a recent study at Rinda Ubuzima, we found that a vaginal pH≥5.0 predicted a Nugent score of 7-10 with 71.0% sensitivity and 76.0% specificity (unpublished data). Vaginal pH can be measured easily and cheaply. We therefore decided to use the EcoCare vaginal pH swab (Merete Medical, Luckenwalde, Germany) with pH ≥5.0 as the cut-off to diagnose BV in the WISH study. Some BV POCTs detecting enzymes or metabolites produced by *G. vaginalis* and/or other anaerobic bacteria (e.g. sialidase, proline aminopeptidase, or amines) have been developed,[46] but did not significantly outperform the vaginal pH results that we obtained in our previous study (unpublished data). VVC POCTs detecting *Candida* antigens/antibodies have either shown inadequate sensitivity and specificity,[243,244,207] or have not been compared to gold standard NAAT or culture assays.[245] Molecular tests targeting lactobacilli and/or key BV-associated bacteria and/or *Candida* species are available or being developed, but are expensive and not ASSURED.[84,246–249]

Other POCTs offered to WISH participants
We wanted to offer WISH participants a complete sexual health screening package and therefore offered them HIV, syphilis, pregnancy, and urinary tract infection (UTI) POCTs as a service. We did not evaluate the performance of these POCTs because they are either known to have good performance (HIV, syphilis, pregnancy), and/or we did not have suitable gold standard test results available to us to compare the POCT results to (syphilis, UTI).

Validated ASSURED[106,108] POCTs with good performance for HIV, syphilis, and pregnancy have been available for many years, including in low and middle-income countries.[109,110] In WISH, we used locally available POCTs that were recommended by the Rwanda Ministry of Health at the time of the study (see legend of figure 6.1).

UTIs in women can be caused by multiple pathogens. *Escherichia coli* is the most common cause (>70% of cases[208]), but other causes include *Enterococcus* species, *Klebsiella pneumoniae*, *Proteus mirabilis*, and STI pathogens such as NG. By far the most commonly used UTI POCTs are lateral flow tests detecting leukocytes, nitrites and other bacterial metabolites. These are readily available and cheap, but have modest performance when compared to urine culture.[208] Lateral flow tests to detect *E. coli* in urine are being developed.[250] The WISH study focussed on genital infections and we therefore elected to use a locally available urinalysis dipstick assessing leukocytes and nitrites for UTI POC testing (figure 6.1). While we did not formally evaluate the performance of this urinalysis dipstick, we compared dipstick findings with urine *E. coli* qPCR findings (see 'gold standard testing' below) in this Appendix (table E.6).

Gold standard testing

CT/NG GeneXpert at Rinda Ubuzima, Kigali, Rwanda
The CT/NG GeneXpert assay was considered a gold standard because of its high performance compared to other validated CT/NG NAATs (see above). While only women who had a positive CT/NG score were tested that same day, GeneXpert swabs were also taken from all other women and tested in batches. All GeneXpert assays were performed in the onsite Rinda Ubuzima laboratory in Kigali, Rwanda.

Assays conducted at the Institute of Tropical Medicine in Antwerp, Belgium
All other NAATs were conducted in the STI reference laboratory of the Institute of Tropical Medicine in Antwerp, Belgium.

All women were asked to self-sample two polyester swabs during the main visit. This was optional but none of the women refused. Each swab head was stored in a 2 ml cryovial containing 1 ml RNA*later* (ThermoFisher Scientific, Paisley, UK) at -80°C during study implementation. Urine samples from women who underwent pregnancy and/or UTI testing (N=641) were centrifuged at 3,000g and the pellet was stored at -80°C in 2 ml cryovials. After all main visits had been completed, swab heads in RNA*later* were defrosted and shipped to Belgium at room temperature. Urine pellets were transported to Belgium on dry ice. DNA was extracted from vaginal swab heads and urine pellets using the Abbott m2000sp automated extraction platform (Abbott Laboratories, Chicago, USA) and an elution volume of 200 µL, incorporating an extra lysis step.[251]

Validated in-house polymerase chain reaction (PCR) assays were performed targeting the following organisms: TV, *Mycoplasma genitalium* (MG), *Candida albicans*, *Lactobacillus* genus, *G. vaginalis*, and *A. vaginae* on vaginal swabs, and *E. coli* on urine samples. The real time TV PCR targeted a fragment of 92 base pairs of a TV-specific repeat gene. In brief, the 25 µL PCR mixture contained 12.5 µL of Platinum Q, PCR SuperMix UDG (Invitrogen, California, USA), 0.9 µL of 25 µM Primers TV001 (5'AAAGATGGGTGTTTTAAGCTAGATAAGG-3') and of 25 µM Primers TV002 (5' TCTGTGCCGTCTTCAAGTATGC-3'), 0.25 µL Probe TV003 (5' /56 FAM/AGTTCATGT/ZEN/CCTCTCCAAGCGTAACT/3IABkFQ/-3'), 10 µL DNA extract and 0.45 µL RNAse-free water. Primers and probes were described by Pillay et al.[252] The amplification comprised an initial heating of 50°C for 2 minutes and of 95°C for 3 minutes followed by 45 cycles of 95°C for 20 seconds and 60°C for 60 seconds. MG was detected by applying a previously published in house real time PCR assay.[253] The concentrations of *Lactobacillus* genus, *G. vaginalis* and *A. vaginae* were determined by quantitative (q)PCRs as previously described.[83] The concentrations of *C. albicans* were determined by qPCR with primers previously published by Cools et al (CA_rRNA R 5'-TTGAAGATATACGTGGTGG-3' and CA_rRNA F 5'-TTTGCTTGAAAGACGGTA-3').[254] The 25µL PCR mixture contained 12.5µL Rotor-Gene SYBR Green (Qiagen, Hilden, Germany), 2.5µL of 10 µM of each primer, 2.5µL RNAse-free water and 5µL of DNA extract. The concentrations of *E. coli* were determined by qPCR with primers previously published by Chern et al. (EC23S857 assay, *E. coli*_rRNA R 5'-TGTCTCCCGTGATAACtTTCTC-3, *E. coli*_rRNA F 5'-GGTAGAGCACTGTTTtGGCA-3', *E. coli*_rRNA probe 5'-TCATCCCGACTTACCAACCCG-3').[255] The 25µL PCR mixture contained 12.5µL Platinum Q PCR SuperMix-UDG (2x) (Invitrogen, California, USA, 0.9µL of 25µM of each primer, 0.7µL of 5µM probe and 10µL of DNA extract. The amplification protocols of *C. albicans* and *E. coli* were the same as previously described for *Lactobacillus* genus.[83] All primers and probes were synthesised by Integrated DNA Technologies (IDT, Illinois, USA), and all amplification reactions were performed using the Corbett Life Science Rotor-Gene™ 6000 (Qiagen, Venlo, the Netherlands).

The qPCRs for TV and MG were qualitative. The other qPCRs were quantitative and run in duplicate. Organism concentrations were expressed as genome equivalents per ml (geq/ml; the mean of duplicates) and $\log_{10}$-transformed. The qualitative PCR results for TV and MG were considered gold standards. A gold standard BV diagnosis was made when the vaginal qPCR score was below -2 (see next paragraph). The *C. albicans* qPCR was not considered a true gold standard for VVC because VVC can be caused by multiple yeasts/fungi. We have, however, referred to it as a gold standard test in this paper for convenience and readability, and would also like to point out the following: 1) it is estimated that 70-95% of VVC cases are caused by *C. albicans*;[30] and 2) *Candida* species are known to be proinflammatory; asymptomatic 'carriage' is therefore only likely when present in low concentrations. In the WISH study, *C. albicans* was only detected by qPCR in 8.6% of the women, and in 68% of those women, it was present at concentrations $\geq 10^5$ geq/ml. *C. albicans* therefore does not seem to qualify as a 'harmless commensal' in the vagina. However, it is currently not known above which concentration threshold on average *C. albicans* (plus other yeasts/fungi) causes

symptoms and/or complications, and more research is needed to develop an optimal gold standard test. Similarly, the *E. coli* qPCR was not considered a true gold standard for UTI because UTIs can be caused by multiple organisms (see above). Because UTIs are not included in the WHO syndromic guidelines, and we did not have optimal gold standard test results, we opted to compare the urinalysis dipstick and *E. coli* qPCR results in this Appendix (table E.6), but to not formally evaluate the urinalysis dipstick performance.

Vaginal qPCR score to identify true BV cases

As described above, from a clinical perspective, the gold standard BV diagnosis is a Nugent score of 7-10.[78] We followed Kenyan, Rwandan and South African women over two menstrual cycles and assessed their vaginal microbiota by Gram stain Nugent scoring and by conducting qPCRs of relevant vaginal bacteria on vaginal swabs.[85] About a third of the 387 women (35.7%) had a Nugent score of 7-10 (BV). Individual and combinations of qPCR assay results were compared to a Nugent score of 7-10, and the highest diagnostic accuracy was achieved with what we subsequently termed the vaginal qPCR score [$\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*)] below -2. The sensitivity was 93.4% and specificity 83.6%. We wanted to keep the number of research procedures in the WISH study to a minimum in order to mimic a real life clinic setting, and we therefore elected not to conduct Gram stain Nugent scoring. Instead, we collected two extra vaginal swabs to allow for all required gold standard testing after study completion, and used the vaginal qPCR score as the gold standard for BV. Please note that the use of such a continuous score acknowledges that the vaginal microbiota exist as a continuum from completely lactobacilli-dominated (a typical concentration in the order of 10^6 lactobacilli per vaginal swab, which would translate into a vaginal qPCR score of 6) to completely dysbiotic (0 lactobacilli but high concentrations of other bacteria, which would translate into a vaginal qPCR score below 0) and everything in between. Some women may not reach the BV threshold of -2, but could still have too many inflammatory bacteria compared to immuno-tolerant lactobacilli, and even develop symptoms and/or complications because of this. We have termed this 'mild dysbiosis'. It is currently not known above which concentration threshold on average vaginal bacterial dysbiosis causes symptoms and/or complications.

Additional information about the WHO and WISH algorithms

The WHO and WISH algorithms are described in detail in the text and figure 6.1. We offer additional clarifications here. WHO published two vaginal discharge syndrome (VDS) algorithms: one that incorporates speculum examinations and one that does not.[40] We used the algorithm without speculum examination but with differentiation between not VVC-like (treated for CT, NG, TV, and BV because prevalences in our study population were expected to be high) and VVC-like (also treated for VVC) based on structural reporting instead of speculum examination findings. The WHO and WISH lower abdominal pain (LAP) algorithms aim to identify patients with pelvic inflammatory disease, which can be life-threatening: if LAP is reported, a bimanual exam is done, and pelvic inflammatory disease is diagnosed if there is adnexal or cervical motion tenderness during the bimanual exam. The WHO algorithm does not specifically mention pain during sex as a reason to do a bimanual exam, but the WISH study team decided that pain during sex may also be indicative of pelvic inflammatory disease. We therefore included it in the WISH algorithm. This resulted in only three additional patients being diagnosed with pelvic inflammatory disease compared to the WHO algorithm (32 and 29 cases, respectively; see table 6.3). Finally, the genital ulcer disease (GUD) algorithm included genital warts in the WISH study but not in the WHO guidelines. However, no genital warts were diagnosed during the study.

As is described in Chapter 6, study physicians performed speculum/bimanual examinations on 399/705 (56.6%) participants, which was more than we had anticipated. The data show that they did examinations in all cases of LAP and pain during sex as required, but also in almost all cases of participant-reported VDS that were not VVC-like (which was only recommended in cases of substantial VDS), and all cases of GUD/buboes (whereas genital inspection may have sufficed). Furthermore, we question the added value of speculum/bimanual examinations other than for

diagnosing pelvic inflammatory disease: participant and physician judgments on whether VDS was VVC-like or not were not accurate (table 6.5).

Treatment, partner notification, and referral

Urogenital infections were managed in accordance with the Republic of Rwanda National Guidelines for Prevention and Management of HIV, STIs & Other Blood Borne Infections (2013).[24] Only medically qualified physicians were allowed to dispense treatments (commonly used drugs were stocked in the study clinic) or prescriptions for treatments, and they were instructed to refer complications to a qualified specialist in a referral hospital in Kigali. First and second choice treatment recommendations at the time of the study for the most common infections are listed in the table E.1 below. In some cases, etiologic diagnoses became available after the participant had already left the study clinic (e.g. after completion of the gold standard testing). In the case of curable STIs (NG, CT, TV, and syphilis), women were contacted and asked to attend the study clinic as soon as possible for treatment and partner notification. Pregnant women were referred to antenatal care, HIV-positive women to HIV care, and all women were informed where they could obtain contraceptive methods and screening for cervical cancer.

Table E.1: Treatments options for main urogenital infections

Infection	First choice treatment	Second choice treatment	Window periods for partner notification
CT	Doxycycline 100mg twice per day for 7 days	Erythromycin 1g twice per day for 7 days	4 weeks if symptomatic; 6 months if asymptomatic or PID.
NG	Ciprofloxacin 1g single oral dose	Ceftriaxone 250mg in one single IM dose [After discussion with Rwandan MoH: 500mg IM allowed in case of suspected resistance]	3 months, or 6 months if PID.
Syphilis (primary)	Benzathine benzyl penicillin 2.4 million IU IM single dose	Erythromycin 1g orally 2x per day x 14 days OR Doxycycline 100mg orally twice per day x 14 days	3 months
TV	Metronidazole 2g single oral dose OR 400 or 500mg orally twice per day for 7 days	Tinidazole 2g single oral dose OR 500 mg twice per day for 7 days	4 weeks
BV	Metronidazole 400 or 500mg orally twice per day for 7 days OR 2g single oral dose.	Tinidazole 2g single oral dose once a day for 2 days or 1g per day for 5 days. Alternative: Clindamycin 300mg orally twice per day for 7 days	NA
VVC	Fluconazole 150mg single oral dose	Clotrimazole 200mg pessaries every night for 3 nights	NA
UTI	Ciprofloxacin 500mg orally twice per day x 7 days.	Ceftriaxone IM 125mg twice per day for 5 days.	NA

BV bacterial vaginosis, *CT Chlamydia trachomatis, IM* intramuscular, *IU* international units, *MoH* Ministry of Health, *NA* not applicable. *NG Neisseria gonorrhoeae, PID* pelvic inflammatory disease, *TV Trichomonas vaginalis*, *UTI* urinary tract infection, *VVC* vulvovaginal candidiasis.

Partner notification can cause social harms and was therefore only done in the case of laboratory-confirmed curable STIs or pelvic inflammatory disease and when the participant consented to a certain partner to be notified. Women with HIV were referred to health centres that provided comprehensive HIV care, including partner notification for HIV. The window periods in the table E.1 above were used to identify partners requiring notification. Partners were not tested but received treatment for the infection that was diagnosed in the index case with one exception: partners of women with pelvic inflammatory disease were tested for CT and NG and only treated for these infections if one or both tests were positive.

Women were offered partner notification choices for each partner as follows:

1. She could give a partner notification card to the partner. This card listed the address, contact information, and opening hours of the studyclinic, and a request to come to the clinic as soon as possible for 'medical follow-up'.
2. She could allow study staff to notify the partner by telephone, mail or home visit. This could be done anonymously if preferred by the participant.

As described in Chapter 6, treatments and referrals were delivered as required with few treatment failures, but the uptake of partner notification was suboptimal: 782 identified partners of 201 women (28.5%) required partner notification but only 61 (7.8%) of them were treated at the study clinic (table E.7). The main reasons were that many women did not consent to notifying some or all partners, or insisted on notifying partners themselves but likely did not follow through (we only have anecdotal evidence for the latter). These are well-known hurdles to partner notification, especially in understaffed and underresourced clinics. Partner notification results might be improved by improving index case privacy and confidentiality (e.g. mobile phone or internet-based notification with the option to notify anonymously),[256] or by improving convenience (e.g. providing the index case with multiple treatment courses, and asking her/him to deliver these to relevant partners).[257] We did not investigate these options in the WISH study.

Steps undertaken to optimise the BV algorithm

We first determined the optimal vaginal pH cut-off for diagnosing BV in both symptomatic (defined as structurally reporting genital itching/burning, any unusual vaginal discharge, lower abdominal pain and/or pain during sex) and asymptomatic women. This was pH 5.5, with similar performance in both groups (data not shown). The negative predictive value (NPV) was 92%, but the positive predictive value (PPV) was only 34%, in both groups. A confirmatory test in those with pH≥5.5 regardless of symptoms therefore seemed required to improve the PPV. We achieved the best balance between reducing BV false-positives and numbers of women requiring testing by determining vaginal pH in all women (as had previously been done), but adding a confirmatory test (the vaginal qPCR score) when pH≥5.5. This resulted in a sensitivity of 73.6% and specificity of 100% (table 6.4), and would require 275/705 (39.0%) confirmatory tests (223 if women already being treated with metronidazole for TV are subtracted). In the performance calculations of this new optimal BV algorithm, we used the vaginal qPCR score as the confirmatory test (see explanation above). However, this score may not be practical in real life because it requires three separate qPCR assays. We therefore assessed the performance of *Lactobacillus* genus concentration on its own as a confirmatory test. We found that a *Lactobacillus* concentration of $<10^5$ geq/ml only slightly reduced algorithm performance (table E.5). Finally, we determined the performance of *Lactobacillus* genus concentration done on all women (compared to the vaginal qPCR score on all women as the gold standard) and achieved a sensitivity of 78.4% and specificity of 95.0% (table E.5). Unfortunately, we did not have Nugent scores available to us (see explanation in 'vaginal qPCR score to identify true BV cases' above), but it is important to note that it would also be possible to screen all women for vaginal pH and use Gram stain Nugent scoring as the confirmatory test (see 'selection of POCTs for evaluation in WISH' for a more detailed discussion about the advantages and disadvantes of Gram stain Nugent scoring).

Steps undertaken to optimise the VVC algorithm

We did not conduct any tests for VVC in the WISH study but evaluated several potential ways to improve the VVC algorithm with the *C. albicans* qPCR data generated with stored swabs after completion of the WISH study. Clinicians often claim that they can recognise VVC during a speculum exam but our data show many false positives and low PPV both in the presence and absence of a speculum exam (PPV was 17.5% and 14.3%, respectively; data not shown). We also found that neither participant-reported symptoms nor clinician-observed signs were correlated with the presence of VVC or any other infection (table 6.5). We then investigated whether the presence of clinical signs and/or self-reported symptoms among women who tested qPCR positive for *C. albicans* depended on the *C. albicans* concentration. However, the median concentrations in women reporting different symptoms or exhibiting different signs were similar (ranging from 5.3 to 5.8 $\log_{10}$ geq/ml) with overlapping interquartile ranges (data not shown). We concluded that VVC cannot be accurately

diagnosed based on symptoms and signs. Next, we assessed the relationship between vaginal pH and VVC but found a wide pH range in women with a positive qPCR for *C. albicans* with and without symptoms (data not shown). We did observe, however, that pregnant women were more likely to have VVC than BV (19.4% and 6.5%, respectively). This led to the following optimal VVC algorithm: women would only be tested for VVC if they had VVC-like symptoms and had tested negative for CT, NG, TV, and BV (using the optimal algorithm) or were pregnant (regardless of symptoms). The sensitivity, specificity, positive predictive value and negative predictive value were 59.3%, 100%, 100% and 96.3%, respectively. This is, however, a complex algorithm 'by exclusion' and better POCTs are therefore desirable as discussed in Chapter 6.

Comparison of urinalysis dipstick and urine E. coli qPCR results
As was mentioned in Chapter 6, 161/705 (22.8%) of WISH participants were treated for an UTI because urinalysis detected any nitrite and/or leukocytes in their urine. However, only 41/161 (25.5%) of these women had a urine *E. coli* concentration of $\geq 10^5$ geq/ml by qPCR, and an additional 12/161 (7.5%) had a urine *E. coli* concentration of >0 and $<10^5$ geq/ml (table E.6). We used a cut-off of $\geq 10^5$ geq/ml because many clinical guidelines use a cut-off of $\geq 10^5$ colony forming units in culture per ml urine for UTI diagnosis.[258] As mentioned earlier, it has been estimated that about 70% of UTIs are caused by *E. coli*,[208] but this 30% gap cannot fully explain the positive urinalysis results in the 108/161 (67.1%) of women without any *E. coli* in their urine. Table E.6 also shows that 19.8% of women without symptoms had some *E. coli* in their urine by qPCR, and that the correlation between urinalysis results and *E. coli* qPCR results in symptomatic women was generally poor (Pearson's correlations range from -0.0423 to 0.4562; we did not have urinalysis results for asymptomatic women).

Feasibility and acceptability
As part of our feasibility and acceptability procedures, physicians who were not part of the day-to-day Rwandan study implementation team observed study staff and participants during monitoring visits to Rwanda. The observers were Dutch physicians with STI management and gynaecology experience. Participants to be observed were selected as follows: they had scheduled visits while the observers were in Rwanda and roughly equal proportions had a positive versus a negative CT/NG risk score (this was done because the CT/NG testing procedures took 90 min). In addition, care was taken to observe both study physicians and both study nurses roughly an equal number of times. Participants were observed from when they entered the study clinic until they left, and all of the procedures that they underwent were timed (table E.10).

In addition, we conducted client satisfaction surveys with 107 participants (table E.11). We aimed to interview about 100 women because we estimated that we would have reached data saturation of the open-ended survey questions by then. We deliberately started the client satisfaction interviews after study staff had settled into a comfortable routine with the study procedures, about six weeks into study enrolment. We selected about five participants per week, and they were selected based on the availability of a study clinician to conduct their interview: the interviewer had to be a study clinician who had not personally implemented study procedures with the interviewee.

One of the external observers also conducted interviews with all eight Rwandan study team members soon after data collection had been completed, but the information obtained during these interviews only confirmed the findings described in Chapter 6 and this appendix and did not add any new insights. These data are therefore not shown.

Table E.2: Spontaneously versus structurally reported symptoms

Urogenital symptoms n (% of 705) in each column*	Spontaneous total	Structural total	Spontaneous, not structural	Structural, not spontaneous
Any reported	575 (81.6)	604 (85.7)†	0	29 (4.1)
Any unusual VDS	247 (35.0)	386 (54.8)	2 (0.3)	141 (20.0)
Unusual VDS, curd-like	176 (25.0)	265 (37.6)	3 (0.4)	92 (13.0)
Unusual VDS, offensive smell	52 (7.4)	119 (16.9)	1 (0.1)	68 (9.6)
Unusual VDS, other	21 (3.0)‡	26 (3.7)§	11 (1.6)	16 (2.3)
Any genital itching and/or burning	384 (54.5)	470 (66.7)	1 (0.1)	87 (12.3)
Genital itching	344 (48.8)	409 (58.0)	5 (0.7)	70 (9.9)
Genital burning	65 (9.2)	212 (30.1)	0	147 (20.9)
Any LAP and/or pain during sex	167 (23.7)	308 (43.7)	0	141 (20.0)
LAP	144 (20.4)	245 (34.8)	0	101 (14.3)
Pain during sex	33 (4.7)	142 (20.1)	0	109 (15.5)
Any UTI symptoms	179 (25.4)	348 (49.4)	1 (0.1)	170 (24.1)
Burning when passing urine	133 (18.9)	262 (37.2)	6 (0.9)	135 (19.1)
Frequent urination/urge	54 (7.7)	176 (25.0)	0	122 (17.3)
Blood in urine	1 (0.1)	5 (0.7)	0	4 (0.6)
Other: smelly odour in urine	2 (0.3)	0	2 (0.3)	0
Ulcers/blisters/sores genital/anal	10 (1.42)	41 (5.8)	0	31 (4.4)
Swelling/bubo inguinal area	0	1 (0.1)	0	1 (0.1)
Warts genital/anal	0	0	0	0
Postcoital/intermenstrual bleeding	1 (0.1)	14 (2.0)	0	13 (1.8)

LAP lower abdominal pain, *UTI* urinary tract infection, *VDS* vaginal discharge syndrome.
*May total to more than 100% because the participant could report multiple symptoms. †We did not assess participant-reported severity of the symptoms but 43 of the 604 participants with structurally reported symptoms (7.1%) had been seeking medical care for their symptoms and 103 (17.1%) had used traditional medications. ‡Five participants described the VDS as having a yellow color; two as "pus-like"; and 14 as unusual without specifying further. §18 participants described the VDS as having a yellow color; three as "pus-like"; two as "chocolate-like"; and three as unusual without specifying further.

Table E.3: Gold standard infections correlation matrix

Outcome n (r)* (N=690)	HIV†	CT	NG	TV	BV	VVC	Syphilis‡	MG
HIV†	162	7 (-0.08)	24 (0.16)	39 (0.12)	31 (0.01)	16 (0.03)	14 (0.18)	11 (0.09)
CT	7 (-0.08)	58	10 (0.12)	13 (0.05)	14 (0.05)	3 (-0.04)	0 (-0.05)	4 (0.05)
NG	24 (0.16)	10 (0.12)	50	15 (0.11)	8 (-0.01)	4 (0)	5 (0.11)	4 (0.06)
TV	39 (0.12)	13 (0.05)	15 (0.11)	111	27 (0.07)	7 (-0.04)	7 (0.08)	7 (0.06)
BV	31 (0.01)	14 (0.05)	8 (-0.01)	27 (0.07)	125	9 (-0.03)	5 (0.03)	8 (0.07)
VVC	16 (0.03)	3 (-0.04)	4 (0)	7 (-0.04)	9 (-0.03)	59	1 (-0.02)	3 (0.02)
Syphilis‡	14 (0.18)	0 (-0.05)	5 (0.11)	7 (0.08)	5 (0.03)	1 (-0.02)	21	0 (-0.04)
MG	11 (0.09)	4 (0.05)	4 (0.06)	7 (0.06)	8 (0.07)	3 (0.02)	0 (-0.04)	26

BV bacterial vaginosis, *CT Chlamydia trachomatis, MG Mycoplasma genitalium*, *NG Neisseria gonorrhoeae, TV Trichomonas vaginalis, VVC* vulvovaginal candidiasis.
*Pearson correlation coefficient. †Either newly diagnosed, known infection confirmed, or known infection not tested in the WISH study but reported by participant. ‡Syphilis by WISH procedures: women who were positive for the syphilis risk score were tested by Syphilis Determine assay (with confirmation of active infection by rapid plasma reagin if needed). Women negative for the risk score were considered negative for syphilis.

Table E.4: Risk scoring results

CT/NG risk score	**n (% of 705)**
Currently pregnant	62 (8.8)
Exchanged sex for money or goods in the past 12 months*	250 (35.5)
New sex partner in the past three months*	224 (31.8)
Abnormal cervicovaginal discharge during speculum and/or cervical motion/adnexal tenderness during bimanual examination (if speculum/bimanual examination not done, the answer was no)†	80 (11.4)
Final CT/NG risk score positive‡	396 (56.2)
Syphilis risk score	**n (% of 705)**
Currently pregnant	62 (8.8)
Exchanged sex for money or goods in the past 12 months*	250 (35.5)
New sex partner in the past three months*	224 (31.8)
Genital ulcers/blisters/sores visible during speculum examination (if not done, the answer was no)§	40 (5.7)
Final syphilis risk score positive‡	378 (53.6)

CT Chlamydia trachomatis, *NG Neisseria gonorrhoeae*.

*157 women reported both sex work and a new sex partner, 93 women reported sex work only, and 67 women reported a new sex partner only. Therefore, the risk in our population was predominanty via sex work. †75 of these 80 women reported VDS or LAP during structural questioning. However, 376 and 239 women in total reported VDS and/or LAP, respectively; the risk score would not have been feasible if we had included all women reporting VDS and/or LAP symptoms. ‡The risk score was positive if at least one of the four criteria was positive. §16 of these 40 women reported GUD during structural questioning. In total, 25 women reported GUD but did not actually have any upon speculum examination, and 24 women did not report any but turned out to have them during a speculum examination that was done for another reason. Therefore, GUD self-reporting is unreliable.

Table E.5: Performance of WISH and optimal BV/VVC algorithms compared to gold standard testing among women who sought care

	GS*		WISH (CT, NG, TV)* and optimal (BV, VVC)† algorithms in women who sought care‡								
Women who sought care (N=141)§	**Neg n**	**Pos n**	**n POC¶ tested**	**n GS¶ tested**	**TP n**	**FP n**	**FN n**	**TN n**	**Sens % (95% CI)**	**Spec % (95% CI)**	**PPV % (95% CI)**	**NPV % (95% CI)**
CT	131	10	74	0	6	0	4	131	60.0 (28.1-85.2)	100 (100-100)	100 (100-100)	97.0 (92.3-98.9)
NG	124	17	74	0	10	0	7	124	58.8 (34.4-79.6)	100 (100-100)	100 (100-100)	94.7 (89.1-97.5)
CT and/or NG	118	23	74	0	14	0	9	118	60.9 (39.6-78.7)	100 (100-100)	100 (100-100)	92.9 (86.8-96.3)
TV	127	12	139	0	7	0	5	127	58.3 (29.5-82.4)	100 (100-100)	100 (100-100)	96.2 (91.1-98.4)
BV	110	29	139	56	21	0	8	119	72.4 (53.2-85.8)	100 (100-100)	100 (100-100)	93.2 (86.9-96.6)
BV and/or TV	103	36	139	49	23	0	13	103	63.9 (46.9-78.0)	100 (100-100)	100 (100-100)	88.8 (81.5-93.4)
VVC	121	18	139	72	11	0	7	121	61.1 (37.0-80.8)	100 (100-100)	100 (100-100)	94.5 (88.9-97.4)
All women (N=690)	**GS**		**Optimal BV/VVC algorithms but using *Lactobacillus* qPCR as confirmatory test‖**								
BV	565	125	690	275	72	19	53	546	57.6 (48.7-66.0)	96.6 (94.8-97.8)	79.1 (69.5-86.3)	91.2 (88.6-93.2)
BV and/or TV	481	209	690	223	136	25	73	456	65.1 (58.3-71.3)	94.8 (92.4-96.5)	84.5 (78.0-89.3)	86.2 (83.0-88.9)
VVC	631	59	690	281	37	0	22	631	62.7 (49.7-74.1)	100 (100-100)	100 (100-100)	96.6 (94.9-97.8)
Women who sought care (N=139)§												
BV	110	29	139	56	18	1	11	109	62.1 (43.1-77.9)	99.1 (93.7-99.9)	94.7 (69.1-99.3)	90.8 (84.1-94.9)
BV and/or TV	103	36	139	49	21	1	15	102	58.3 (41.5-73.4)	99.0 (93.3-99.9)	95.5 (72.6-99.4)	87.2 (79.7-92.2)
VVC	121	18	139	72	12	0	6	121	66.7 (42.0-84.7)	100 (100-100)	100 (100-100)	95.3 (89.8-97.9)
All women (N=690)	***Lactobacillus* qPCR on all women to diagnose BV (with <10^5 geq/ml treated for BV)**											
BV	565	125	690	0	98	28	27	537	78.4 (70.3-84.8)	95.0 (92.9-96.6)	77.8 (69.6-84.2)	95.2 (93.1-96.7)

BV bacterial vaginosis, *CI* confidence interval, *CT Chlamydia trachomatis*, *FN* false negative, *FP* false positive, *GS* gold standard, *NG Neisseria gonorrhoeae*, *Neg* negative, *NPV* negative predictive value, *POC* point-of-care, *Pos* positive, *PPV* positive predictive value, *Sens* sensitivity, *Spec* specificity, *TN* true negative, *TP* true positive, *TV Trichomonas vaginalis*, *VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis.

*See table 6.1 for definitions. Performance statistics were also calculated for CT, NG combined, and BV and TV combined, because the former are assessed by one assay and the latter require the same treatment. †All women would have a vaginal pH determined and those with pH ≥5.5 would also have a vaginal qPCR score done (see methods). Only women with pH ≥5.5 and a positive vaginal qPCR score would be treated for BV. Women would only be tested for VVC if they had VVC-like symptoms and had tested negative for CT, NG, TV, and BV (by optimal algorithm), or were pregnant (regardless of symptoms). ‡Defined as women who had visited a clinic and/or women who had taken traditional medications for symptoms reported to be current or recent (last two weeks). §Performance measures are compared to gold standard testing. For TV, BV, and VVC, N=139 due to 2 invalid PCR results. ¶These are the numbers that would require POCT and gold standard testing if each respective algorithm were to be implemented in a real-life situation. ‖Same as †, but instead of using the vaginal qPCR score as the confirmatory test after vaginal pH ≥5.5, we used the *Lactobacillus* qPCR only (with <10^5 geq/ml treated for BV). This same definition of BV was used in the VVC algorithm. The vaginal qPCR score on everyone was used as the gold standard BV result.

Table E.6: Urinalysis compared to *Escherichia coli* qPCR test results

All women with urine samples (N=641)*	Total n	*E. coli* qPCR†			OR (95% CI)§ Chi-squared p
		0 geq/ml (n %)‡	>0 to <10^5 geq/ml (n %)‡	≥10^5 geq/ml (n %)‡	
With UTI symptoms	363	262 (72.2)	32 (8.8)	69 (19.0)	2.67 (1.60-4.45)
Without symptoms	278	223 (80.2)	33 (11.9)	22 (7.9)	p<0.0001
Women reporting UTI symptoms only (N=363)*	**Total n**	***E. coli* qPCR†**			**Correlation r¶ (95% CI)**
		0 geq/ml (n %)‡	**>0 to <10^5 geq/ml (n %)‡**	**≥10^5 geq/ml (n %)‡**	
≥1+ leukocytes and/or nitrite-positive‖	161	108 (67.1)	12 (7.5)	41 (25.5)	NA
Nitrite negative, 0 leukocytes	202	154 (76.2)	20 (9.9)	28 (13.9)	-0.042 (-0.149-0.066)
Nitrite negative, 1+ leukocytes	64	52 (81.3)	6 (9.4)	6 (9.4)	
Nitrite negative, 2+ leukocytes	46	36 (78.3)	5 (10.9)	5 (10.9)	
Nitrite negative, 3+ leukocytes	21	17 (80.9)	1 (4.8)	3 (14.3)	
Nitrite positive, 0 leukocytes	1	1 (100)	0	0	0.186 (-0.187-0.512)
Nitrite positive, 1+ leukocytes	6	0	0	6 (100)	
Nitrite positive, 2+ leukocytes	14	2 (14.3)	0	12 (85.7)	
Nitrite positive, 3+ leukocytes	9	0	0	9 (100)	

CI confidence interval, *Geq* genome-equivalent units, *ml* milliliter, *NA* not applicable, *OR* odds ratio, *qPCR* quantitative polymerase chain reaction, *UTI* urinary tract infection.

*363/641 women underwent urinalysis testing after reporting UTI-related symptoms. Urine was collected from the other 278/641 women for pregnancy testing, but these women did not undergo urinalysis testing because they did not report symptoms. All 641 urine samples were tested for *E. coli* concentration by qPCR. †*E. coli* qPCR concentration levels, devided into three categories: 0 geq/ml, >0 and <10^5 geq/ml, and ≥10^5 geq/ml. ‡Listed percentages are row percentages. §Chi-squared test for trend. The OR compares *E. coli* qPCR ≥10^5 geq/ml with 0 geq/ml in women with and without UTI symptoms. ¶Pearson's correlation of leukocyte level as an ordinal variable with *E. coli* concentration by qPCR in $\log_{10}$ geq/ml as a continuous variable. The overall correlation of the 8 nitrite/leukocyte level rows as one ordinal variable with *E. coli* concentration is r=0.4562 (95% CI 0.371-0.534). ‖This is the algorithm that was used in the WISH study. All 161 women who were positive for this algorithm were treated for a UTI.

Table E.7: Clinical findings and actions by study clinicians

Speculum/bimanual exam results at main visits (study physicians)	**n (% of 399)**
Any abnormalities observed during speculum exam	216 (54.1)
Abnormalities*:	
- Enlarged/tender inguinal lymph nodes	0
- Abnormal (genital) odour	34 (8.5)
- Warts or condylomata (any location genitalia)	5 (1.3)†
- Ulcers/blisters/sores suggestive of STI in vulva	22 (5.5)
- Vulvitis	21 (5.3)
- Any other lesion on vulva	0
- Vaginal mass (polyp, myoma, etc.)	4 (1.0)
- Ulcers/blisters/sores suggestive of STI in vagina	3 (0.8)
- Vaginitis	39 (9.8)
- Any other lesion on vaginal epithelium	0
- Cervicitis	42 (10.5)
- Any other lesion on cervical epithelium	1 (0.2)
- Abnormal vaginal or cervical discharge/pus	138 (34.6)
- Other‡	14 (3.2)
Any abnormalities observed during bimanual exam	41 (10.3)
Abnormalities*:	
- Any uterine or adnexal mass	4 (9.8)
- Any uterine, adnexal or cervical motion tenderness	31 (4.4)
- Other§	12 (29.3)
Syndromic diagnoses within WISH algorithms at main visits (study physicians)	**n (% of 705)**
None	262 (37.2)
VVC	238 (33.8)
VDS – not VVC, tested negative for BV/TV/CT/NG	12 (1.7)
LAP (with or without VDS) - no tenderness during bimanual	204 (28.9)
LAP (with or without VDS) - tenderness during bimanual (=PID)	29 (4.1)
Suspected PID for other reasons¶	3 (0.4)
GUD with or without inguinal buboes - tested negative for syphilis	16 (2.3)
Inguinal buboes without GUD	0
Genital warts/condylomata	3 (0.4)
UTI symptoms testing negative for UTI	134 (19.0)
Other: cervical tumor/mass	1 (0.1)
Delivery of positive POCT results (study physicians or nurses)‖	**n (% of 705)**
Had no positive results	164 (23.3)
Received all her positive results at main visit; no pending results when leaving	505 (71.6)
Received all her positive results at main visit; all pending results negative and received by phone/texts	26 (3.7)
Received all her positive results at main visit; all pending results negative and received at additional visit	1 (0.1)
A pending result came back positive and received result and/or treatment at additional visit	7 (1.0)
A pending result came back positive and received result by phone/text	1 (0.1)
Received all her positive results at an additional visit	1 (0.1)
Received all positive results by phone/text	0
Treatment failures (study physicians)**	**n (% of 705)**
Had at least one suspected ongoing untreated infection	4 (0.6)
Had at least one suspected re-infection after having received appropriate treatment	2 (0.3)
Had at least one suspected treatment failure	4 (0.6)
Active referrals made (study physicians)	**n (% of 705)**
No referrals needed	626 (88.8)
Because of new HIV diagnosis	34 (4.8)
Because of new pregnancy	25 (3.6)
For further gynecological evaluation/treatment	18 (2.6)
Because of wish to start/change family planning method	0
Other: For other medical specialist diagnosis & treatment	1 (0.1)
Other: For starting antiretroviral therapy of previously known HIV infection	4 (0.6)
At least one referral offered but declined	2 (0.3)
Partner notification and treatment (study physicians or nurses)	**n (% of 705)**
Had at least one partner requiring notification during the study††	201 (28.5)
Total number of partners requiring notification during the study	782
Median number of partners requiring notification per woman with an infection [IQR]	2 (1 – 3)
Total numbers of partners that the women consented to being notified	238
Number of women who agreed to:	
- All of the identified partners being notified	111 (15.7)
- Some of the identified partners being notified	59 (8.4)
- None of the identified partners being notified	31 (4.4)
Number of women who chose to:	
- Notify all partners herself	150 (21.3)
- Have RU staff notify all partners (in agreed-upon manner)	13 (1.8)
- Notify some partners herself and some by RU staff	2 (0.3)
- Other: Notification by RU staff with both partners present	2 (0.3)
Total number of eligible partners treated (% out of the total number of partners identified)	61 (7.8)

BV bacterial vaginosis, *CT Chlamydia trachomatis*, *GUD* genital ulcer disease, *LAP* lower abdominal pain, *NG Neisseria gonorrhoeae*, *PID* pelvic inflammatory disease, *POCT* point-of-care test, *TV Trichomonas vaginalis*, *UTI* urinary tract infection, *VDS* vaginal discharge syndrome, *VVC* vulvovaginal candidiasis.
*May total to more than 100% because one woman could have multiple signs/diagnoses. †Two of these cases were a small number of warts that the study physician found difficult to reliably differentiate from ulcers. These participants were not treated for warts. ‡Includes menstrual blood from cervical os (8), Bartholin's cyst (1), dry skin at external genitalia (1), uterine prolapse (1), acne due to shaving (1), depigmentation of the vulva (1), and satellite lesions suggestive of VVC (1). §Includes hard, indured cervix (4), large volume of uterus consistent with early pregnancy(2), abnormal pain during exam (5), and absence of cervix consistent with hysterectomy (1). ¶Two participants underwent a bimanual exam for reasons other than LAP, had tenderness, and were treated for PID. One participant reported severe LAP in the past two weeks but not during the main visit, and did not have tenderness during the bimanual exam. However, her CT and NG results both came back positive, and the study physician decided to treat her for PID. Her LAP disappeared after completion of the treatment. ||GeneXpert CT/NG counts as one result and was considered positive if the test was positive for at least one organism.
**Treatment was in accordance with the Rwanda national treatment guidelines at the time of the study (see above). Drugs dispensed included metronidazole (BV, TV, PID), tinidazole (BV), fluconazole (VVC), clotrimazole (VVC), ciprofloxacin (NG, PID, UTI), ceftriaxone (NG), doxycycline (CT), erythromycin (CT, PID), benzyl penicillin (syphilis), acyclovir (herpes simplex virus type 2), pivmecillinam (UTI), and amoxicillin/clavulanic acid (UTI). ††Based on both POCT and gold standard testing results.

Table E.8: WISH services offered and accepted

Services	Offered n (% of 705)	Accepted (% offered)	Reasons for declining[a]	Sample taken (% offered)	Received result same day	Received tx or referral same day
HIV test	700[b] (99.3%)	Yes: 593 (84.7%) No: 107 (15.3%)	- Known HIV+: 106 - Other, was tested few months ago: 1 - Missing: 0	- EDTA blood: 591 (99.7%) - Fingerstick: 2 (0.3%) - Missing: 0	- Yes: 590 - No, other time: 3 - Not at all: 0 - Missing: 0 **55 positive**	- Yes, newly diagnosed: 31 - Yes, known infection[c]: 3 - No, other time: 0 - Not at all[d]: 21 - Missing: 0
Pregnancy test	702[e] (99.6%)	Yes: 583 (83.0%) No: 119 (17.0%)	- Known pregnant: 26 - Uses reliable contraception: 52 - Other[f]: 38 - Missing[f]: 3	- Urine: 581 (99.7%) - Missing: 2 (0.3%)	- Yes (offered): 579 - Yes (mistake)[g]: 2 - No, other time: 1 - Not at all: 0 - Missing: 3 **33 positive**	- Yes: 24 - No, other time: 0 - Not at all[h]: 9 - Missing: 0
UTI test	352 (49.9%)	All	NA	- Urine: 351 (99.7%) - Missing[i]: 1 (0.3%)	- Yes (offered): 351 - Yes (mistake)[j]: 12 - No, other time: 0 - Not at all: 0 - Missing[i]: 1 **161 positive**	- Yes: 160 - No, other time: 1 - Not at all: 0 - Missing: 0
Vaginal pH for BV	705 (100%)	All	NA	- pH swab: 705 (100%) - NA: 0 - Missing: 0	- Yes: 705 - No, other time: 0 - Not at all: 0 - NA: 0 - Missing: 0 **466 positive**	- Yes: 464 - No, other time: 1 - Not at all: 0 - Missing[k]: 1
TV test	705 (100%)	All	NA	- Kit swab: 705 (100%) - NA: 0 - Missing: 0	- Yes: 703 - No, other time: 2 - Not at all: 0 - Missing: 0 **92 positive**	- Yes: 92 - No, other time: 0 - Not at all: 0 - Missing: 0
CT/NG test[l]	396 (56.2%)	All	NA	- Kit swab: 396 (100%) - Urine[m]: 1 (0.3%) - Missing: 0	- Yes: 354 - No, other time: 42 - Not at all: 0 - Missing: 0 **75 positive**[n]	- Yes: 66 - No, other time: 9 - Not at all: 0 - Missing: 0
Syphilis test	378 (53.6%)	All	NA	- EDTA blood: 377 (99.7%) - Fingerstick[o]: 2 (0.5%) - Missing: 0	- Yes (offered): 375 - Yes (mistake)[p]: 1 - No, other time: 2 - Not at all: 0 - Missing[q]: 1 **21 positive**	- Yes: 21 - No, other time: 0 - Not at all: 0 - Missing: 0

Services	Offered n (% 705)	Accepted (% offered)	Reasons for declining[a]	Done the same day?
Speculum/ bimanual examination	397 (56.3%)	Yes: 395 (99.5%) No[r]: 2 (0.5%)	- Refused out of fear: 1 - Does not tolerate speculum: 1	- Yes: 391 - Yes, but not offered[s]: 8 - No, other time: 0 - Missing[t]: 4
Counselling	705 (100%)	All	NA	All
Male condoms (MCs)	705 (100%)	Yes: 386 (54.8%) No: 319 (45.2%)	- Never uses MCs: 247 - Still has MCs at home: 35 - Partner(s) refuse(s) MCs: 20 - Partner(s) bring(s) MCs: 10 - Wants to get pregnant: 1 - Missing: 9	All
Willing to wait for CT/NG results (women whose risk score was positive and who did not opt-out of testing)			*n=396* - Yes, wanted to wait for the results: 344 (86.9%) - No, wanted to come back for results later: 5 (1.3%) - No, wanted to receive results by text/phone/letter: 41 (10.4%) - Missing: 6 (1.5%) - Never received results: 0	

ART antiretroviral treatment, *BV* bacterial vaginosis, *CT Chlamydia trachomatis*, *NA* not applicable, *NG Neisseria gonorrhoeae*, *PID* patient identification number, *TV Trichomonas vaginalis*, *UTI* urinary tract infection.

a. May total to more than 100% because multiple answers possible.
b. Five women were not offered HIV testing because they were known HIV-positive.
c. These participants were known HIV-positive but had not yet been referred for ART.
d. These participants were known HIV-positive and were in care, with some already receiving ART and others not yet. At the time of the WISH study, the Rwandan government was still rolling out the latest WHO recommendations of starting all HIV-positive people on ART regardless of CD4 count. These women opted in for HIV testing because they wanted to be retested.
e. Three women were not offered a pregnancy test because they were visibly pregnant.

f. Other reasons included: is sterilised or menopausal (12), states that last sexual encounter was a long time ago (7), had menses recently (4), believes that she cannot get pregnant due to having given birth recently (1), recently had a pregnancy test (1). Two women who opted out refused to give a reason. All three women with missing reasons used an IUD.
g. Two women who had opted out of a pregnancy test were mistakenly tested anyway.
h. Seven women were already in antenatal care. Two women did not want to be referred.
i. One woman was offered a urinalysis test but the urine sample was not taken.
j. 12 women had a urine sample taken for a UTI test despite not being offered a UTI test. Of these, 5 were mistakenly ordered from the laboratory, and 7 were not ordered but accidentally performed (because the urine was available for pregnancy testing). 2 of the 12 women should have been offered a UTI test because they reported relevant symptoms.
k. Treatment information for one woman is missing.
l. This does not include samples taken from women who had a negative risk score for testing later on in the study. That was done to enable test performance calculations but was not part of the WISH clinical algorithms.
m. One woman had both a vaginal swab and a urine sample taken because swab testing came back invalid twice.
n. 75 women had a positive CT and/or NG result: 43 positive for CT and 38 for NG (6 were positive for both CT and NG).
o. One woman had both EDTA blood and a fingerstick sample taken for syphilis testing.
p. One woman had a syphilis test performed despite not being offered one; the laboratory performed the test by mistake on blood available for an HIV test.
q. One woman had a positive risk score and was offered syphilis testing. However, the test was not requested from the lab.
r. One woman accepted the examination initially, which was postponed because of time contraints. However, the woman opted out of the exam at the additional visit because of fear.
s. Two of these eight women requested an exam without having relevant symptoms. The other six women were symptomatic and it is likely that the study physician neglected to document that an exam was offered.
t. Four women were offered an examination but not results are reported. One of these only reported "frequent urination or urgent need to urinate" and the physician therefore likely erroneously reported that an examination was offered.

Table E.9: Counselling topics chosen by participants

Counsellors and counselling topics	Main visit n (% of 705)	Additional visit n (% of 4)
General counselling performed by:		
- Nurse/counsellor	704 (99.9)	0
- Physician	1 (0.1)	4 (100)
Topics that were discussed during general counselling*:		
– HIV basic facts	56 (7.9)	1 (25.0)
– STIs basic facts	130 (18.4)	4 (100)
– HIV & STI treatment	330 (46.8)	4 (100)
– HIV & STI prevention	652 (92.5)	3 (75.0)
– HIV & STIs: Condom use demonstration	44 (6.2)	1 (25.0)
– BV and VVC basic facts	594 (84.3)	1 (25.0)
– BV and VVC treatment	553 (78.4)	0
– BV and VVC prevention	694 (98.4)	0
– UTIs: what it is, consequences if not treated, prevention	244 (34.6)	1 (25.0)
– Family planning	295 (41.8)	0
– Domestic violence: including referrals	116 (16.5)	1 (25.0)
– Other, specify: hepatitis	1 (0.1)	0
– Other, specify: condylomata	1 (0.1)	0
HIV post-test counselling performed:	*(N=588†)*	*(N=3‡)*
- Nurse/counsellor	30 (5.1)	0
- Physician§	558 (94.9)	3 (100)
Topics that were discussed during HIV post-test counselling:	*(N=588†)*	*(N=3‡)*
– Negative result for HIV test	534 (90.8)	3 (100)
– Positive or equivocal result for HIV test	54 (9.2)	0

BV bacterial vaginosis, *STI* sexually transmitted infection, *UTI* urinary tract infection.
*May total to more than 100% because women could choose multiple topics. †Numbers are lower than 705 due to women opting out of HIV testing. ‡No participants were tested for HIV during additional visits; these counselling sessions were provided to women who came with their partners for partner treatment and testing. §The study physicians often performed the HIV post-test counselling at the same time as giving women their other test results. This fitted better into the clinic flow and enabled the nurse/counsellors to see new patients.

Table E.10: Timing of procedures at Main Visits

Procedures	n	Duration in median min (IQR)
Duration of procedures at reception	14	5 (3-12)
Duration of informed consent procedures and obtaining contact details	20	25.5 (21-32.5)
Duration of face-to-face interview	20	23 (21.5-27)
Duration of counselling	20	7 (6-9.5)
Duration of blood collection	19	4 (3-5)
Duration of vaginal swab collection	20	6 (5.5-7.5)
Duration of urine collection	18	2 (2-3)
Duration of speculum examination	7	4 (3-8)
Duration of bimanual examination	6	2 (1-2)
Duration between delivering the last sample to the lab and being called for results	20	95.5 (14-104)
Duration of diagnosing and counselling by physician	20	6.5 (5-12.5)
Duration of treatment and partner notification procedures by physician	18	3.5 (2-6)
Overall trajectories	**n**	**Duration**
Total duration spent at RU with nurse/counsellor	20	82 (73-93)
Total duration spent at RU with physician	20	14 (10-24.5)
Total duration spent on laboratory testing	21	104 (26-115)
Total duration spent at RU	20	222.5 (138-237.5)
Total duration spent at RU without research procedures*	20	182.5 (111-216)
Total duration without research procedures with CT/NG testing+results†	13	212 (190-219)
Total duration without research procedures and without CT/NG testing+results‡	7	98 (78-123)

CT Chlamydia trachomatis, *IQR* inter-quartile range, *NG Neisseria gonorrhoeae*, *RU* Rinda Ubuzima research clinic.
*Duration excludes time for informed consent procedures and client satisfaction survey. †The participant was positive for the CT/NG risk score and elected to wait for the results. ‡The participant was either negative for the CT/NG risk score or chose not to wait for the results.

Table E.11: Client satisfaction survey results

Questions asked in face-to-face interview	n (% of 107)
Agreed with the following statements:	
- "I felt welcome at RU"	107 (100)
- "The study staff were friendly"	107 (100)
- "The instructions I received along the way were clear"	107 (100)
- "The medical services I received were of good quality"	107 (100)
- "The medical services I received were useful"	107 (100)
- "The counselling/information I received was of good quality"	107 (100)
- "The counselling/information I received was useful"	107 (100)
Time spent at RU, estimated by the participant: median minutes (IQR)	209 (150-251)
Feelings about the clinic visit duration:	
- Thought it was fine	62 (57.9)
- Was bothered by it but not much	4 (3.8)
- Thought it was very long, but worth it due to all the services received	41 (38.3)
- Thought it was much too long and would not do it again	0
Comparison of experience at RU during study visit, compared to other places where HIV/STI/women's issues-related services are given:	
- Liked RU better	104 (97.2)
- All services are similar	0
- Liked the other services better	0
- Has never been to other places	3 (2.8)
Reasons for preferring RU over other clinics*:	*(N = 104)*
- Friendly staff / attention was paid to participants†	58 (55.8)
- High number of tests performed / more useful or better-quality tests than elsewhere‡	56 (53.8)
- Thought that the counselling and information obtained were useful	28 (26.9)
- Quick and well-organised services	28 (26.9)
- Services free-of-charge	24 (23.0)
- Unclear / very general reasons given	7 (6.7)
Is willing to be tested in future, even when asymptomatic	100 (93.5)
Is willing to pay for services such as those offered at RU	*(N=106)* 95 (89.6)

IQR interquartile range, *RU* Rinda Ubuzima research clinic, *STIs* sexually transmitted infections.
*May total to more than 100% because the participant could give multiple answers. The question was open-ended and categories were created during the data analysis stage. †Includes answers such as "the staff is friendly", and comments about refreshments being offered to participants. ‡Includes quotes suggesting that more testing was done in WISH than at local clinics, such as "you test all the diseases", "you take many samples", "you test before treatment", or "you examine deeply".

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