MONASH University

Investigating the pathogenesis of bacterial vaginosis

Erica Louise Plummer
MSc (Bioinformatics), BMedSc/BBiotech

A thesis submitted for the degree of Doctor of Philosophy at Monash University in 2021
Melbourne Sexual Health Centre & Central Clinical School
Faculty of Medicine, Nursing and Health Sciences

Copyright notice

Abstract

Bacterial vaginosis (BV) is a highly prevalent vaginal condition that negatively impacts on a woman's overall quality of life and is associated with serious clinical sequelae. BV is characterised by a non-optimal vaginal microbiota that typically has low abundance of lactic acid producing lactobacilli, and increased abundance of anaerobic bacteria including *Gardnerella* species (collectively termed BV-associated bacteria). BV recurrence following first-line treatment with antibiotics is unacceptably high, and there have been few improvements in BV cure for decades. Difficulties with improving BV cure include its complex aetiology and pathogenesis. Through five research chapters (**Chapters 2-6**), this thesis aimed to investigate the pathogenesis of BV, with a focus on evidence for sexual transmission, and to determine what factors influence the composition and stability of the vaginal microbiota.

Chapters 2 and **3** investigated the relationship between sexual and behavioural practices, vaginal microbiota composition and *Gardnerella vaginalis* clade distribution in a cohort of Australian women who have sex with women (WSW). **Chapter 2** found that sex with a new partner was associated with a significant change in the vaginal microbiota, marked by an increase in bacterial diversity and an increase the abundance of BV-associated bacteria. **Chapter 3** explored the distribution of four *G. vaginalis* clades in WSW and found that *G. vaginalis* clade 1 was positively associated with both BV and non-optimal vaginal microbiota composition, whereas *G. vaginalis* clade 4 was not associated with either BV or non-optimal vaginal microbiota composition. Furthermore, the detection of *G. vaginalis* clades was associated with a range of different sexual practices; for example, clade 1 was associated with increasing number of recent sexual partners. These data suggest that *G. vaginalis* clades have varying levels of pathogenicity in WSW, and are acquired via sexual activity. Collectively, the findings from **Chapters 2** and **3** provide microbiological evidence for the sexual exchange of bacteria between women, and suggest that sexual transmission plays a role in BV acquisition. Importantly, these findings indicate that partner treatment may be an appropriate strategy to improve BV cure.

Chapter 4 was a prospective, open-label pilot study of concurrent male partner treatment for BV that utilised combination oral and topical antimicrobial therapy for men. Concurrent partner treatment reduced the abundance of BV-associated bacteria in the genital

microbiota of both partners immediately post-treatment and this reduction was sustained in the majority of women throughout the 12-week follow-up period. There were only five cases of BV-recurrence (5/29; 17%) during follow up, lower than what is observed following standard female only treatment in this population (>50%). Importantly, partner treatment was adhered to and well tolerated by men. These data provide microbiological evidence and preliminary efficacy estimates that support continued investigation of partner treatment by randomised controlled trial as a strategy to improve BV cure.

Chapter 5 was a cross-sectional study of 1,272 women attending a sexual health service that investigated the contribution of *Mycoplasma hominis* and *Ureaplasma* spp. to symptoms and clinical signs in nonpregnant women in order to inform testing indications and treatment guidelines for these bacteria. *Ureaplasma* spp. were not associated with any symptom or sign. *M. hominis* was associated with vaginal malodour and abnormal vaginal discharge in women with BV but was not associated with symptoms/signs in women without BV. These data provide evidence against the routine testing and screening of nonpregnant women for *M. hominis*, *Ureaplasma* spp. in a sexual health setting and support the need to focus testing efforts on BV rather than *M. hominis* and *Ureaplasma* spp.

Chapter 6 was a systematic review of the use of lactic acid-containing products for BV cure and their impact on the vaginal microbiota. Seven studies were eligible, and the review identified a lack of high-quality evidence to support the use of lactic acid for BV treatment and modification of the vaginal microbiota. Importantly, the review highlighted that adequately powered and rigorous randomised trials with accompanying vaginal microbiota data are needed to evaluate the efficacy of lactic acid as a BV treatment strategy.

Overall, this thesis provides insights into the pathogensis of BV and specifically provides evidence to support a role of sexual transmission in the acquisition and recurrence of BV. Additionally, this thesis adds to our understanding of what factors influence the composition of the vaginal microbiota, and what factors modify the vaginal microbiota to a non-optimal state. This information is highly relevant to the development of new strategies to prevent and treat BV, and improve sexual and reproductive health outcomes for women globally.

Publications during candidature

Publications that contribute to this thesis

1. **Plummer EL***, Vodstrcil LA*, Fairley CK, Tabrizi SN, Garland SM, Law MG, Hocking JS, Fethers KA, Bulach DM, Murray GL^, Bradshaw CS^ (2019). Sexual practices have a significant impact on the vaginal microbiota of women who have sex with women. ***Sci Rep*** 9 (1):19749. doi:10.1038/s41598-019-55929-7 (* joint first authors; ^ joint senior authors). **(Chapter 2)**

2. **Plummer EL,** Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Garland SM, Chow EPF, Bulach DM, Fethers KA, Hocking JS, Bradshaw CS (2020). *Gardnerella vaginalis* clade distribution is associated with behavioral practices and Nugent score in women who have sex with women. ***J Infect Dis*** 221 (3):454-463. doi:10.1093/infdis/jiz474. **(Chapter 3)**

3. **Plummer EL,** Vodstrcil LA, Bodiyabadu K, Murray GL, Doyle M, Latimer RL, Fairley CK, Payne M, Chow EPF, Garland SM, Bradshaw CS (2021). Are *Mycoplasma hominis, Ureaplasma urealyticum* and *Ureaplasma parvum* associated with specific genital symptoms and clinical signs in non-pregnant women? ***Clin Infect Dis.*** doi:10.1093/cid/ciab061. **(Chapter 5)**

4. **Plummer EL**, Bradshaw CS, Doyle M, Fairley CK, Murray GL, Bateson D, Masson L, Slifirski J, Tachedjian G, Vodstrcil LA (2021). Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: A systematic review. ***PLoS One*** 16 (2):e0246953. doi:10.1371/journal.pone.0246953. **(Chapter 6)**

5. **Plummer EL**, Vodstrcil LA, Doyle M, Danielewski JA, Murray GL, Fehler G, Fairley CK, Bulach DM, Garland SM, Chow EPF, Hocking JS, Bradshaw CS. A prospective, open-label pilot study of concurrent male partner treatment for bacterial vaginosis. Submitted to *mBio* in June 2021. **(Chapter 4)**

Co-author publications undertaken during my candidature that did not directly contribute to this thesis

1. Vodstrcil LA, **Plummer EL**, Doyle M, Fairley CK, McGuiness C, Bateson D, Hocking JS, Law MG, Petoumenos K, Donovan B, Chow EPF, Bradshaw CS (2020) Treating male partners of women with bacterial vaginosis (StepUp): a protocol for a randomised controlled trial to assess the clinical effectiveness of male partner treatment for reducing the risk of BV recurrence. ***BMC Infect Dis*** 20 (834). doi:https://doi.org/10.1186/s12879-020-05563-w

2. Ratten LK, **Plummer EL**, Murray GL, Danielewski J, Fairley CK, Garland SM, Hocking JS, Tachedjian G, Chow EPF, Bradshaw CS, Vodstrcil LA (2020) Sex is associated with the persistence of non-optimal vaginal microbiota following treatment for bacterial

vaginosis: a prospective cohort study. ***BJOG.*** doi: https://doi.org/10.1111/1471-0528.16430

3. Doyle M, Vodstrcil LA, **Plummer EL**, Aguirre I, Fairley CK, Bradshaw CS (2020) Nonquinolone options for the treatment of *Mycoplasma genitalium* in the era of increased resistance. ***Open Forum Infect Dis*** 7 (8):ofaa291. doi:10.1093/ofid/ofaa291
4. Vodstrcil LA, **Plummer E**, Fairley CK, Tachedjian G, Law MG, Hocking JS, Worthington K, Grant M, Okoko N, Bradshaw CS (2019) Combined oral contraceptive pill-exposure alone does not reduce the risk of bacterial vaginosis recurrence in a pilot randomised controlled trial. ***Sci Rep*** 9 (1):3555. doi:10.1038/s41598-019-39879-8

Oral presentations, poster presentations and awards and recognitions during candidature

Oral presentations

1. **Plummer EL**, Vodstrcil LA, Fairley CK, Danielewski JA, Murray GL, Doyle M, Fehler G, McGuinness C, Bulach D, Garland SM, Chow EPF, Hocking JS, Bradshaw CS. *The impact of concurrent partner treatment for bacterial vaginosis on the genital microbiota of heterosexual couples: a pilot study.* Virtual STI & HIV 2021 World Congress, 14 – 17 Jul 2021. **(Chapter 4)**
2. **Plummer EL**, Vodstrcil LA, Fairley CK, Danielewski JA, Murray GL, Doyle M, Fehler G, McGuinness C, Bulach D, Garland SM, Chow EPF, Hocking JS, Bradshaw CS. *The impact of partner treatment for bacterial vaginosis on the genital microbiota of heterosexual couples.* 2020 Virtual Impact AMR Research Colloquium, 25 Nov 2020. **(Chapter 4)** ***[Invited speaker]***
3. **Plummer EL**, Vodstrcil LA, Bodiyabadu K, Murray GL, Doyle M, Latimer RL, Fairley CK, Chow EPF, Garland SM, Bradshaw CS. *The contribution of common genital mycoplasmas to genital symptoms and clinical signs in women.* 2020 Virtual Joint HIV&AIDS and Sexual Health Conferences, 16 – 20 Nov 2020. **(Chapter 5)**
4. **Plummer EL**, Vodstrcil LA, Fairley CK, Danielewski JA, Murray GL, Doyle M, Fehler G, McGuinness C, Bulach D, Garland SM, Chow EPF, Hocking JS, Bradshaw CS. *The impact of partner treatment for bacterial vaginosis on the genital microbiota of heterosexual couples.* 2020 Virtual Joint HIV&AIDS and Sexual Health Conferences, 16 – 20 Nov 2020. **(Chapter 4)**
5. **Plummer EL**, Vodstrcil LA, Bodiyabadu K, Murray GL, Doyle M, Latimer RL, Fairley CK, Chow EPF, Garland SM, Bradshaw CS. *The contribution of Mycoplasma hominis, Ureaplasma urealyticum and Ureaplasma parvum to genital symptoms and clinical signs in women.* Central Clinical School Postgraduate Symposium, Melbourne, Australia, 4 – 5 Nov 2020. **(Chapter 5)**

6. **Plummer EL** (2019). Bacterial vaginosis (BV) and the vaginal microbiota**.** Invited talk at the Australian and New Zealand Vulvovaginal Society 2019 Vulva Education Day, State Library Victoria, Melbourne, Australia, 9 – 10 Nov 2019. ***[Invited speaker]***
7. **Plummer EL**, Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women.* Royal Women's Hospital Research Week, Melbourne, Australia, 19 Nov – 21 Nov 2018. **(Chapter 2)**
8. **Plummer EL**, Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women***.** Keystone Symposia on the "Role of the Genital Tract Microbiome in Sexual and Reproductive Health" in Cape Town, Western Cape, South Africa, 11 Dec – 15 Dec 2018. **(Chapter 2)**
9. **Plummer EL,** Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women.* IUSTI Asia Pacific Sexual Health Congress Auckland, New Zealand, 1 Nov – 3 Nov 2018. **(Chapter 2)**
10. **Plummer EL,** Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women.* Central Clinical School Postgraduate Symposium, Melbourne, Australia, 12 Nov 2018. **(Chapter 2)**
11. **Plummer EL,** Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women***.** VIIN Young Investigator Symposium, Parkville, Australia, 18 October 2018. **(Chapter 2)**

Poster presentations

1. **Plummer EL**, Bradshaw CS, Doyle M, Fairley CK, Murray GL, Bateson D, Masson L, Slifirski J, Tachedjian G, Vodstrcil LA. *Lactic acid for the treatment of bacterial vaginosis and the impact of lactic acid-containing products on the vaginal microbiota: a systematic review.* 2020 Virtual Joint HIV&AIDS and Sexual Health Conferences, 16 – 20 Nov 2020. **(Chapter 5)**
2. **Plummer EL**, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Murray GL, Doyle M, Fehler G, Kaiser MJ, McGuinness C, Garland SM, Bulach DM, Chow EPF, Hocking JS, Bradshaw CS. *A pilot study of male partner treatment in women with bacterial vaginosis.* Royal Women's Hospital Research Week, Melbourne, Australia, 18 – 19 Nov 2019. **(Chapter 4)**
3. **Plummer EL**, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Garland SM, Chow EPF, Bulach DM, Fethers KA, Hocking JS, Bradshaw CS. Gardnerella vaginalis *clade distribution is associated with behavioural practices and Nugent score in women who*

have sex with women. Central Clinical School Postgraduate Symposium, Melbourne, Australia, 7 Oct 2019. **(Chapter 3)**

4. **Plummer EL**, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Garland SM, Chow EPF, Bulach DM, Fethers KA, Hocking JS, Bradshaw CS. Gardnerella vaginalis *clade distribution is associated with behavioural practices, Nugent score and vaginal microbiota in women who have sex with women.* STI & HIV 2019 World Congress, Vancouver Canada, 14 – 17 Jul 2019. **(Chapter 3)**
5. **Plummer EL**, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Murray GL, Doyle M, Fehler G, Kaiser MJ, McGuinness C, Garland SM, Bulach DM, Chow EPF, Hocking JS, Bradshaw CS. *Combined oral and topical antibiotic therapy for male partners of women with bacterial vaginosis: A pilot study.* STI & HIV 2019 World Congress, Vancouver Canada, 14 – 17 Jul 2019. **(Chapter 4)**
6. **Plummer EL**, Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women.* Royal Women's Hospital Research Week, Melbourne, Australia, 19 – 21 Nov 2018. **(Chapter 2)**
7. **Plummer EL**, Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women***.** Keystone Symposia on the "Role of the Genital Tract Microbiome in Sexual and Reproductive Health" in Cape Town, Western Cape, South Africa, 11 – 15 Dec 2018. **(Chapter 2)**

Awards and recognitions

1. **American STD Association – Best oral presentation (Young Investigator) awarded at Virtual STI & HIV 2021 World Congress, 14 – 17 Jul 2021**. Plummer EL, Vodstrcil LA, Fairley CK, Danielewski JA, Murray GL, Doyle M, Fehler G, McGuinness C, Bulach D, Garland SM, Chow EPF, Hocking JS, Bradshaw CS. *The impact of concurrent partner treatment for bacterial vaginosis on the genital microbiota of heterosexual couples: a pilot study.* **(Chapter 4)**
2. **First prize poster award – Young investigator category – STI & HIV 2019 World Congress, Vancouver Canada, 14 – 17 Jul 2019.** Plummer EL, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Garland SM, Chow EPF, Bulach DM, Fethers KA, Hocking JS, Bradshaw CS. Gardnerella vaginalis *clade distribution is associated with behaviour and Nugent score in women who have sex with women.* **(Chapter 3)**
3. **Third place poster presentation award, 2019 Central Clinical School postgraduate symposium.** Plummer EL, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Garland SM, Chow EPF, Bulach DM, Fethers KA, Hocking JS, Bradshaw CS. Gardnerella vaginalis *clade distribution is associated with behaviour and Nugent score in women who have sex with women.* **(Chapter 3)**

4. **Post-graduate student oral presentation winner, Royal Women's Hospital Research Week, Melbourne, Australia, 19 Nov – 21 Nov 2018.** Plummer EL, Vodstrcil LA, Tabrizi SN, Garland SM, Fairley CK, Tan A, Law MG, Hocking JS, Bulach DM, Murray GL, Bradshaw CS. *Sexual Behaviours Impact the Vaginal Microbiota of Women Who Have Sex with Women.* **(Chapter 2)**
5. **Recipient of a VIRTUAL Registration Scholarship to attend the 2020 Joint Australasian HIV&AIDS + Sexual Health Conferences: VIRTUAL, 16 Nov – 20 Nov 2020.** Awarding Body: Australasian Society for HIV, Viral Hepatitis and Sexual Health Medicine.
6. **Recipient of a Keystone Symposia Scholarship to attend the Keystone Symposium on Role of the Genital Tract Microbiome in Sexual and Reproductive Health, Cape Town, Western Cape, South Africa 11 Dec – 15 Dec, 2018.** Awarding Body: Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, USA, Grant #1R13HD097894-01
7. **Recipient of an Australian Government Research Training Program (RTP) scholarship.**

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes four original papers published in peer reviewed journals and one manuscript that has been submitted for publication in a peer reviewed journal. The core theme of the thesis is the pathogenesis of BV and the vaginal microbiota. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Melbourne Sexual Health Centre, Alfred Health and Central Clinical School under the supervision of Professor Catriona Bradshaw.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of the original research chapters including published works (**Chapters 2-6**) my contribution to the work involved the following:

Chapter	Publication title	Status	% and nature of student contribution	% and nature of co-author contribution	Co-author Monash student Y/N
2	Sexual practices have a significant impact on the vaginal microbiota of women who have sex with women	Published	50% (joint first author): contributed to the data analysis plan, performed the bioinformatics and statistical analysis, contributed to data interpretation, wrote the first draft of the manuscript, revised it for submission	1) L Vodstrcil (25% joint first author): Conceived of, and designed the study, contributed to the original cohort study from which this microbiota analysis arose, contributed to the data analysis plan, contributed to, and supervised the statistical analysis, contributed to data interpretation, manuscript preparation and review. 2) C Fairley (1%): assisted with study conception and design, contributed to the original cohort study, provided manuscript input and review 3) S Tabrizi (1%): Conceived and designed the study, supervised the laboratory component, manuscript input and review 2% 4) S Garland (1%): provided manuscript input and review 5) M Law (1%): provided statistical advice, manuscript input and review 6) J Hocking (1%): assisted with study conception and design, contributed to the original cohort study, provided manuscript input and review 7) K Fethers (1%): contributed to the original cohort study, provided manuscript input and review 8) D Bulach (1%): provided manuscript input and review 9) G Murray (6%): contributed to the data analysis plan, and provided input into data interpretation, manuscript preparation and review.	N for all

Chapter	Publication title	Status	% and nature of student contribution	% and nature of co-author contribution	Co-author Monash student Y/N
				10) C Bradshaw (12%): Conceived of, designed and funded the study, contributed to the original cohort study, assisted with, and supervised the statistical analysis and data interpretation, provided manuscript input and review.	
3	*Gardnerella vaginalis* clade distribution Is associated with behavioral practices and Nugent score in women who have sex with women	Published	65% (first author): contributed to the data analysis plan, performed the bioinformatics and statistical analysis, contributed to data interpretation, wrote the first draft of the manuscript, revised it for submission	1) L Vodstrcil (11%): Conceived of, and designed the study, contributed to the original cohort study, contributed to the data analysis plan, and supervised the statistical analysis, contributed to data interpretation, provided manuscript input and review 2) G Murray (2%): provided manuscript input and review 3) C Fairley (2%): contributed to the original cohort study, provided manuscript input and review 4) J Danielewski (2%): provided manuscript input and review 5) S Garland (1%): provided manuscript input 6) E Chow (1%): provided manuscript input 7) D Bulach (2%): provided manuscript review and supervised the bioinformatics analysis 8) K Fethers (1%): contributed to the original cohort study, provided manuscript input 9) J Hocking (2%): contributed to the original cohort study, provided manuscript input and review	N for all

Chapter	Publication title	Status	% and nature of student contribution	% and nature of co-author contribution	Co-author Monash student Y/N
				10) C Bradshaw (11%): Conceived of, designed and funded the study, contributed to the original cohort study, contributed to the data analysis plan and supervised the statistical analysis, contributed to data interpretation, provided manuscript input and review	
4	A prospective, open-label pilot study of concurrent male partner treatment for bacterial vaginosis	Submitted	65% (first author): contributed to the study protocol, prepared, and submitted the ethics modification, assisted with developing participant recruitment and study pack materials, assisted with recruitment, set up the study database, developed the data analysis plan, performed the bioinformatics and statistical analysis, wrote the first draft of the manuscript, revised and prepared it for submission.	1) L Vodstrcil (8%): Conceived of, and designed the study, contributed to the study protocol, supervised the ethics submission, design of study materials, data analysis and interpretation, provided manuscript input and review 2) M Doyle (5%): Recruited participants and conducted participant follow-up, performed data entry, provided manuscript input and review 3) J Danielewski (5%): Developed laboratory methods protocol, performed DNA extraction, provided manuscript input and review 4) G Murray (1%): Supervised laboratory work, provided manuscript input and review 5) G Fehler (1%): Performed Nugent scoring, reviewed the manuscript 6) C Fairley (1%): provided manuscript input and review 7) S Garland (1%): provided manuscript input and review 8) E Chow (1%): provided manuscript input and review 9) D Bulach (1%): provided manuscript input and review 10) J Hocking (1%): provided manuscript input and review	N for all

Chapter	Publication title	Status	% and nature of student contribution	% and nature of co-author contribution	Co-author Monash student Y/N
				11) C Bradshaw (10%): Conceived of, designed and funded the study, contributed to the study protocol, supervised the ethics submission, design of study materials, data analysis and interpretation, provided manuscript input and review	
5	Are *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* associated with specific genital symptoms and clinical signs in non-pregnant women?	Published	65% (first author): contributed to the data analysis plan, performed the statistical analysis, contributed to data interpretation, wrote the first draft of the manuscript, revised it for submission	1) L Vodstrcil (10%): Contributed to the design and protocol of the original study from which this manuscript arose, contributed to the data analysis plan, contributed to data interpretation, provided manuscript input and review 2) K Bodiyabadu (5%): performed the laboratory work, provided manuscript input and review 3) G Murray (2%): supervised the laboratory work, provided manuscript input and review 4) M Doyle (2%): contributed to the original study including recruitment, participant follow-up, data entry and making study packs, provided manuscript input and review 5) R Latimer (2%): Contributed to the design and protocol of the original study from which this manuscript arose, performed data cleaning of study database, provided manuscript input and review 6) C Fairley (1%): provided statistical input and reviewed the manuscript 7) M Payne (1%): provided manuscript input and review	Y (R Latimer) N for all others

Chapter	Publication title	Status	% and nature of student contribution	% and nature of co-author contribution	Co-author Monash student Y/N
				8) E Chow (1%): provided manuscript input and review 9) S Garland (1%): provided manuscript input and review 10) C Bradshaw (10%): Conceived of, designed and funded the study, contributed to the data analysis plan, contributed to data interpretation, provided manuscript input and review	
6	Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: A systematic review	Published	65% (first author): Conceived and designed the study, wrote the PROSPERO protocol, performed literature search, reviewed articles for eligibility, performed data extraction, bias assessment and data analysis, wrote the first draft of the manuscript, revised it for submission	1) C Bradshaw (10%): Conceived and designed the study, contributed to the study protocol, provided clinical interpretation of findings, provided manuscript input and review 2) M Doyle (8%): reviewed articles for eligibility, checked data extraction and bias assessment, provided manuscript input and review 3) C Fairley (1%): provided manuscript input and review 4) G Murray (1%): provided manuscript input and review 5) D Bateson (1%): provided manuscript input and review 6) L Masson (1%): provided manuscript input and review 7) J Slifirski (2%): reviewed articles for eligibility, checked data extraction and bias assessment, provided manuscript input and review 8) G Tachedjian (1%): provided manuscript input and review 9) L Vodstrcil (10%): Conceived and designed the study, contributed to the study protocol, supervised the data	N for all

Chapter	Publication title	Status	% and nature of student contribution	% and nature of co-author contribution	Co-author Monash student Y/N
				extraction and analysis, provided manuscript input and review	

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Erica Plummer

Student signature: **Date:** 29/07/2021

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Professor Catriona Bradshaw

Main Supervisor signature: **Date:** 29/07/2021

Acknowledgements

This thesis is the product of the collaboration, guidance and support that I received from many individuals and I would like to express my gratitude to them.

I firstly acknowledge my supervisory team: Professor Catriona Bradshaw, Dr Lenka Vodstrcil, Dr Dieter Bulach, Dr Gerald Murray, Professor Christopher Fairley, and Professor Suzanne Garland.

I cannot thank Cat and Lenka enough for their academic insight, support, and encouragement over the course of my PhD. Over the past few years Cat and Lenka have dedicated so much time and enthusiasm to teaching and training me. They have always made time to meet with me, answer my questions and provide feedback, knowledge and advice. From the first day I met them, Lenka and Cat made me feel welcome and a valued member of the team. I have thoroughly enjoyed being part of the *Genital Microbiota and Mycoplasma Group* at Melbourne Sexual Health Centre and look forward to continuing our work together. These few sentences do not reflect just how grateful I am to have had Cat and Lenka as my supervisors. It has been a privilege to be their student and I can honestly say that I would not be the researcher I am today without their help. I had a great set of projects to work on and they were more enjoyable because Lenka and Cat were supporting and motivating me every step of the way.

Thank you to Dieter, Gerald, Kit and Suzanne for the guidance, knowledge, mentorship and support you have provided me throughout my PhD. I feel very fortunate to have had such an experienced and multidisciplinary group of supervisors. Thank you to Kit for welcoming me to the Melbourne Sexual Health Centre, and for always providing quick, thoughtful and critical feedback on publication drafts. Thank you to Dieter and Suzanne for taking me on as a master's student all those years ago and giving me my first real taste of research, and to Gerald and Suzanne for allowing me the workplace flexibility to pursue a PhD.

Dr Jennifer Danielewski and Michelle Doyle have been instrumental to my PhD and I would not have made it to the end of my PhD without the help and support they gave me. Thank you to Jen for the endless hours put towards developing laboratory protocols and performing experimental work, and for answering all my lab questions. Thank you to Michelle for all the time spent recruiting and following StepUp-2 couples, for saving the day

and jumping in as the second reviewer on the systematic review, and for answering all my questions about how the clinic works.

Thanks also to the following people who have contributed a lot of time to different aspects of my PhD: Glenda Fehler, Kaveesha Bodiyabadu, Dr Rosie Latimer, Colette McGuiness, Marti Kaiser, Karen Worthington, Mark Chung, James Unger, Josephine Slifirski, Professor Sepehr Tabrizi, among others. Sincere thanks also to the numerous co-authors - there are too many to name individually but I thank them for the time spent reviewing manuscripts and for the constructive comments and advice.

I would also like to extend my thanks to all the clinicians at Melbourne Sexual Health Centre who referred participants to the studies included in this thesis, particularly StepUp-2. And of course, thank all the women and couples who participated in the various studies that contributed to my PhD. Well over a thousand people provided time, samples and data, and without their valuable contribution none of this research would be possible.

Finally, I would like to thank Aaron, my family and friends. My PhD has been made easier because of their love, laughter and encouragement.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship.

Table of contents

List of abbreviations

AHR	Adjusted hazard ratio
ALDEX	ANOVA-like differential expression tool
ANCOM	Analysis of composition of microbiomes
ANI	Average nucleotide identity
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ANZCTR	Australian New Zealand clinical trials registry
AOR	Adjusted odds ratio
aRRR	Adjusted relative risk ratio
ARDRA	Amplified ribosomal DNA restriction analysis
ASV	Amplicon sequence variant
BID	Twice daily
BV	Bacterial vaginosis
BVAB	Bacterial vaginosis associated bacteria
CI	Confidence interval
CLR	Centred-log ratio
COC	Combined oral contraceptive
CONSORT	Consolidated standards of reporting trials
CST	Community state type
DADA2	Divisive amplicon denoising algorithm 2
dDDH	Digital DNA-DNA hybridisation
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FDA	Food and drug administration
FDR	False discovery rate
FPU	First pass urine
FSP	Female sexual partner
FUSS	Female university student study
GEE	Generalised estimating equations
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	Hazard ratio
IQR	interquartile range
IUD	intrauterine device
LTFU	Loss to follow up
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MSP	Male sexual partner
MTZ	Metronidazole
NCBI	National center for biotechnology information
NMDS	Non-metric multidimensional scaling
NP	New sexual partner
NS	Nugent score
OMLA	Oligomeric lactic acid
OR	Odds ratio
OTU	Operational taxonomic unit

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pES	Pooled effect size
PID	Pelvic inflammatory disease
PMNL	Polymorphonuclear leukocytes
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
qPCR	Quantitative polymerase chain reaction
RCT	Randomised controlled trial
RR	Risk ratio
rRNA	Ribosomal ribonucleic acid
RRR	Relative risk ratio
RTP	Research training program
SP	Sexual partner
SRA	Short read archive
TREND	Transparent reporting of evaluations with nonrandomised designs
USA	United States of America
USD	US dollar
VALENCIA	Vaginal community state type nearest centroid classifier
VVC	Vulvovaginal candidiasis
WSW	Women who have sex with women

List of tables

List of figures

Thesis structure and chapter outline

This thesis comprises seven chapters which are briefly outlined below.

Chapter 1 presents a detailed literature review that broadly discusses the vaginal microbiota and bacterial vaginosis (BV), and sets the scene for the subsequent original research chapters. The literature review covers themes including 1) optimal and non-optimal organisms that are present in the vagina, 2) the prevalence, diagnosis and treatment of BV, and 3) key topics concerning the pathogenesis of BV.

Chapters 2-6 present original research including four manuscripts that have been published in peer reviewed journals and one manuscript is currently being revised for resubmission to a peer reviewed journal.

- **Chapter 2** presents an analysis of the impact of behavioural and sexual practices on the vaginal microbiota composition of Australian women who have sex with women (WSW).
- **Chapter 3** presents an analysis of the association of *Gardnerella vaginalis* clade distribution with sexual and behavioural practices and Nugent score among Australian WSW.
- **Chapter 4** presents a pilot study of concurrent partner treatment for BV that 1) assessed the impact of concurrent male partner treatment on the genital microbiota over a 12-week period, 2) determined the adherence to, and acceptability and tolerability of concurrent male partner treatment, and 3) determined preliminary estimates of the efficacy of concurrent male partner treatment.
- **Chapter 5** presents an analysis of the association of *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* with specific symptoms and clinical signs in nonpregnant women.
- **Chapter 6** presents a systematic review that investigated 1) the effect of intravaginal lactic acid-containing products for BV cure, and 2) the impact of intravaginal lactic acid-containing products on the vaginal microbiota as assessed using molecular methods.

The final chapter **(Chapter 7)** presents an integrated discussion of the key learnings from this thesis, implications for clinical practice and recommendations for future areas of research.

Chapter 1. Introduction and literature review

1.1. Introduction

The vaginal microbiome has an important role in maintaining vaginal health, and dysbiosis of the vaginal microbiome has been associated with a number of adverse sexual and reproductive outcomes. It is well established that the vaginal microbiota composition can be influenced by a range of different factors and may vary greatly between women and within a woman over her lifetime. Thus, defining what constitutes a normal or healthy vaginal microbiota is difficult. McKinnon *et al.* (2019) proposed the use of the term 'optimal', in place of 'healthy' and 'normal', to describe vaginal microbiota that is "associated with no vaginal symptoms, lack of genital inflammation and favourable sexual and reproductive health outcomes, including decreased risk of HIV acquisition". Conversely, a 'non-optimal' vaginal microbiota is a microbiota that is "associated with vaginal symptoms, and/or genital inflammation and/or adverse sexual and reproductive health outcomes, including increased risk of HIV acquisition" (McKinnon *et al.*, 2019).

The optimal vaginal microbiota of reproductive aged women is typically characterised by a dominance of lactic acid producing *Lactobacillus spp.* including *Lactobacillus crispatus, Lactobacillus gasseri* and *Lactobacillus jensenii* (Boskey *et al.*, 2001; Boskey *et al.*, 1999; Fredricks *et al.*, 2005; McKinnon *et al.*, 2019; Nugent *et al.*, 1991; Ravel *et al.*, 2011).

Bacterial vaginosis (BV) is the most common vaginal condition of reproductive aged women. The aetiology of BV is unknown, but BV is characterised microbiologically by a non-optimal vaginal microbiota composition with reduced abundance of lactic acid producing *Lactobacillus* spp. and increased prevalence and abundance of facultative and strict anaerobic bacteria (Fredricks *et al.*, 2005; Ravel *et al.*, 2011). Although approximately 50% of women with BV are asymptomatic, women with symptomatic BV may experience vaginal discharge and vaginal odour. Women with BV are at increased risk of a range of adverse clinical sequelae including miscarriage, preterm birth and pelvic inflammatory disease (PID). BV is also associated with increased risk of acquisition of sexually transmitted infections (STIs) and increased risk of acquisition and transmission of HIV (Brotman *et al.*, 2010; Brotman *et al.*, 2014b; Cohen *et al.*, 2012; Gupta *et al.*, 1998; Hawes *et al.*, 1996; Kaul *et al.*,

2007; Koumans *et al.*, 1999; Martin *et al.*, 1999; Myer *et al.*, 2005; Ness *et al.*, 2005; Taha *et al.*, 1998). In 2019, the global cost of treating symptomatic BV was estimatd to be 4.8 billion USD (95% CI 3.7-6.1 billion USD) per year, and this does not include costs arising from associated sequelae (Peebles *et al.*, 2019).

Current first line treatment for BV involves antibiotic treatment with 7 days of oral metronidazole or intravaginal clindamycin or 5 days of intravaginal metronidazole (Australian Sexual Health Alliance, 2018). These treatments both achieve similar one month cure of approximately ~70-85% (Oduyebo *et al.*, 2009); however up to 50% of women experience BV recurrence within three months of treatment (Bradshaw *et al.*, 2006a; Sobel *et al.*, 1993). BV recurrences result in repeat presentations to clinical services and repeated antibiotic use. Furthermore, the sequelae associated with BV have significant clinical and healthcare expenditure implications. For example, preterm birth is a leading cause of neonatal mortality globally and BV has been reported to account for as much as 82% of the attributable risk for preterm birth (Purwar *et al.*, 2001).

New treatments for BV are clearly needed and a thorough understanding of the pathogenesis of BV and the impact of behavioural and sexual practices on vaginal microbiota composition is essential for informing prevention strategies and improving BV treatment outcomes. The following literature review will discuss key topics concerning the vaginal microbiota as well as the treatment, aetiology, and epidemiology of BV.

1.2. The vaginal microbiota

1.2.1. Analysing the vaginal microbiota

Culture and microscopy were used as early tools for qualitative and quantitative investigation of the vaginal microbiota. More recently, molecular methods have been used to study vaginal microbiota composition, including but not limited to quantitative polymerase chain reaction (qPCR), high-throughput amplicon sequencing, and whole metagenome sequencing. Amplification of short region/s of the 16S rRNA gene followed by sequencing is one of the most commonly used methods for characterising the bacterial composition of the vaginal microbiota; targeted sequencing of the *cpn60* gene has also been used (Albert *et al.*, 2015).

The function and structure of the 16S rRNA gene makes it a useful target for microbiota studies. The 16S rRNA gene encodes the 16S ribosomal RNA which is one of three ribosomal RNAs that make up the bacterial ribosome. The ribosome has a fundamental role in protein synthesis and is present in all bacteria (Srinivasan & Fredricks, 2008). The 16S rRNA gene is ~1540 base pairs in length and is comprised of nine hypervariable regions (V1-V9) that are flanked by highly conserved regions (Figure 1). The sequence of the hypervariable regions varies between different bacteria, whereas the conserved regions are typically identical or highly similar across most bacteria (Chakravorty *et al.*, 2007). Because of this structure, universal PCR primers complementary to conserved regions can be used to amplify the hypervariable regions and allow taxonomic identification of bacteria present by matching to a database of classified sequences.

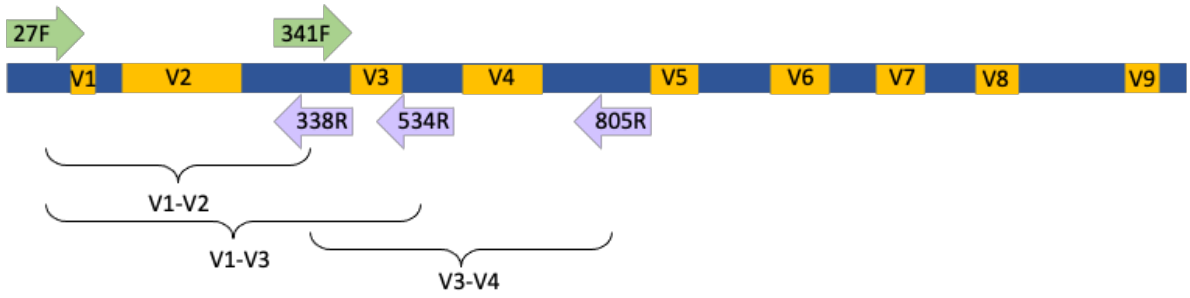


Figure 1. Schematic representation of the 16S rRNA gene

Conserved regions are represented in blue, hypervariable regions (V1-V9) are represented in yellow. Locations of commonly used forward (in green) and reverse (in purple) primers are depicted. Adapted from "Research techniques made simple: bacterial 16S ribosomal RNA gene sequencing in cutaneous research" by Jo et al. (2016). Journal of Investigative Dermatology, 136(3), e23-e27; and "Assessment of in vitro *and* in silico *protocols for sequence-based characterization of the human vaginal microbiome" by Hugerth et al. (2020). mSphere, 5(6). doi:10.1128/mSphere.00448-20*

Different regions of the 16S rRNA gene have varying ability to discriminate certain taxa and it is important to have an understanding of the organisms that may be present in a sample in order to optimise experimental design (Chakravorty *et al.*, 2007). For example, previous studies of the vaginal microbiota have used PCR primers targeting the V1-V2 regions (Gajer *et al.*, 2012; Gottschick *et al.*, 2017a; Ravel *et al.*, 2011), which is now known to

underrepresent *Gardnerella vaginalis*, an important vaginal bacterium. Additionally, an *in silico* evaluation identified that key vaginal genera including *Chalmydia* and *Mycoplasma* are poorly covered by a number of different variable regions including the V3-V5 regions and the V4 region (Hugerth *et al.*, 2020). Although primers targeting the V3-V4 regions are commonly used to characterise the composition of the vaginal microbiota, many different regions of the 16S rRNA gene have been used (van de Wijgert *et al.*, 2014), and there is currently no consensus on what region/s is best. Van Der Pol *et al.* (2019) presented an *in silico* and experimental comparison of different 16S rRNA gene primer sets for characterisation of the vaginal microbiota. They reported that V4 universal primers were more effective than universal primers for V1-V3 and V3-V4 regions for the detection of 13 clinically relevant vaginal bacterial species when using an in-house extended version of the Greengenes 16S rRNA gene reference database. A recent study however, reported that relative abundance data generated using the V1-V3 (utilising 27F/515R primers) or V3-V4 (utilisiting 341F/805R primers) showed good agreement with quantitative data from qPCR for key vaginal bacteria (*L. iners*, *L. crispatus* and *G. vaginalis*). The study however did not examine the agreement of the V4 region with qPCR data (Hugerth *et al.*, 2020). As discussed at the *Vaginal microbiome Pre-Congress Symposium* held at the STI & HIV 2019 World Congress, every step in a microbiota study (i.e. sample collection, DNA extraction, primer choice, PCR amplification, bioinformatics and statistical analysis) can introduce bias (Balkus *et al.*, 2019a; Balkus *et al.*, 2019b), and as no method is optimal, it is important that researchers have an understanding of the biases associated with their methodology and that such biases are clearly stated in publications. These biases underscore the dilligence required to effectively compare and appropriately conclude from amplicon sequencing studies using 16S rRNA gene.

1.2.2. The vaginal microbiota of reproductive aged women

Albert Döderlein identified and cultured Gram-positive bacilli (Döderlein's bacilli) from vaginal secretions of healthy women in 1892. Döderlein's bacilli were later renamed to *Lactobacillus,* and early in the twentieth century it was recognised that lactobacilli were important for vaginal health, and that absence or reduction of lactobacilli was associated with abnormal vaginal discharge (Bautista *et al.*, 2016; Donders, 2007; Martin, 2012). The first molecular investigation of the vaginal microbiota was performed in 2002 (Burton &

Reid, 2002), and since then, molecular studies have greatly expanded our understanding of the vaginal microbiota. In 2010, Ravel *et al.* (2011) presented a landmark analysis of the vaginal microbiota composition of 394 asymptomatic women of reproductive age from the United States, using pyrosequencing of the V1-V2 regions of the 16S rRNA gene. They described five community state types (CSTs; Figure 2), four of which were dominated by *Lactobacillus* species (CST I-*Lactobacillus crispatus* dominated, CST II-*Lactobacillus gasseri* dominated, CST III-*Lactobacillus iners* dominated, and CST V-*Lactobacillus jensenii* dominated) and one was deficient in *Lactobacillus* (CST IV- diversity group with low relative abundance of lactic acid bacteria and high abundance of anaerobic bacteria).

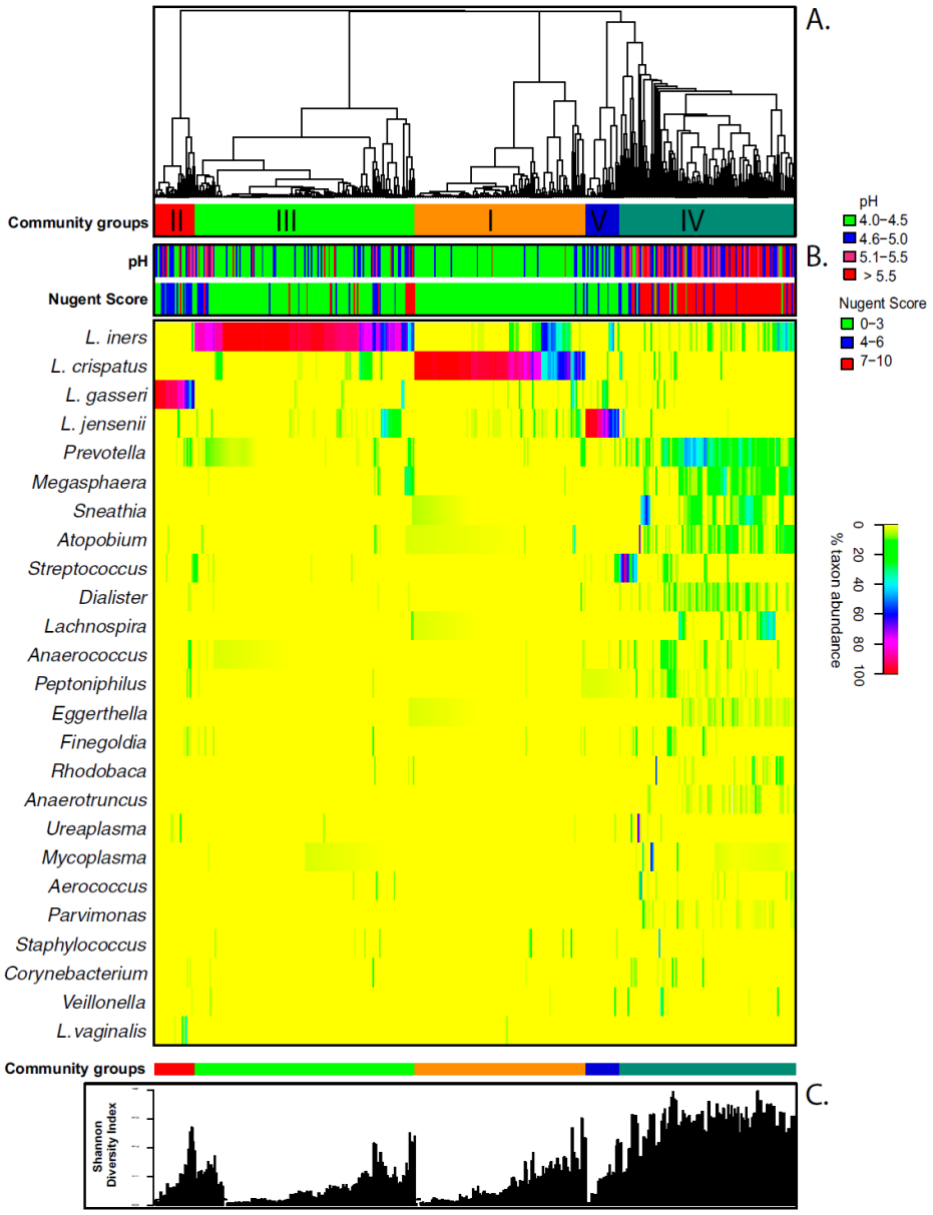


Figure 2. The vaginal microbiota composition of women of reproductive age

Panel A – Complete linkage hierarchical clustering was used to determine five CSTs: I-L. crispatus dominated, II-L. gasseri dominated, III-L. iners dominated, IV- diversity group and V- L. jensenii dominated. Panel B- The heatmap displays the relative abundance of 25 bacterial taxa detected in 394 women in the United States. Each vertical line represents the bacterial composition of one vaginal specimen. The vaginal pH and Nugent score of each specimen is indicated above the heatmap. Panel C – the bacterial diversity of each specimen (as calculated using the Shannon Diversity Index). Adapted from "Vaginal microbiome of reproductive-age

women" by Ravel et al. (2011). Proceedings of the National Academy of Sciences of the United States of America, 108 Suppl 1, 4680-4687.

Molecular studies have highlighted that although the vaginal microbiota of reproductive aged women is often low diversity and dominated by *Lactobacillus* spp., significant variability in the diversity and composition of the vaginal microbiota exists between women (Albert *et al.*, 2015; Borgdorff *et al.*, 2017; Chaban *et al.*, 2014; Fettweis *et al.*, 2014; Gajer *et al.*, 2012; Romero *et al.*, 2014; Srinivasan *et al.*, 2012; Vodstrcil *et al.*, 2017; Zhou *et al.*, 2007; Zhou *et al.*, 2010). Indeed, the composition of the vaginal microbiota composition is thought to be influenced by a number of factors including, but not limited to, sexual practices (Gajer *et al.*, 2012; Muzny *et al.*, 2013; Schwebke *et al.*, 1999; Vodstrcil *et al.*, 2017; Wessels *et al.*, 2017), douching (Sabo *et al.*, 2019; van der Veer *et al.*, 2019), smoking (Bradshaw *et al.*, 2014; Brotman *et al.*, 2014a; Nelson *et al.*, 2018), contraceptive practices (Joesoef *et al.*, 2001), diet (Tuddenham *et al.*, 2019), menses (Gajer *et al.*, 2012; van der Veer *et al.*, 2019), and oestrogen levels (Wessels *et al.*, 2018).

Previous studies have also demonstrated an association between vaginal microbiota composition and race or ethnicity (Anahtar *et al.*, 2015; Borgdorff *et al.*, 2017; Fettweis *et al.*, 2014; Ravel *et al.*, 2011; Vodstrcil *et al.*, 2017; Zhou *et al.*, 2010). For example, African-American and Hispanic women are more likely to have a *Lactobacillus*-depleted vaginal microbiota compared to Caucasian and Asian women (Fettweis *et al.*, 2014; Ravel *et al.*, 2011; Zhou *et al.*, 2010). Studies have reported that *L. crispatus* dominated communities are more common in Caucasian women (Fettweis *et al.*, 2014; Ravel *et al.*, 2011; Vodstrcil *et al.*, 2017), and Asian, African-American and Hispanic women are more likely to be colonised by *L. iners* than other *Lactobacillus* species (Fettweis *et al.*, 2014; Ravel *et al.*, 2011). A Dutch study found that *L. crispatus* was more prevalent in ethnically Dutch women compared to other ethnic groups in Amsterdam (Borgdorff *et al.*, 2017). Following adjustment for contraceptive use and sexual practices, women of African Surinamese ethnicity or Ghanaian ethnicity were more likely to have a diverse *G. vaginalis* vaginal microbiota compared to Dutch women (Borgdorff *et al.*, 2017). African Surinamese ethnicity was also associated with *L. iners* dominated microbiota in adjusted analysis (Borgdorff *et al.*, 2017). Although differences in vaginal microbiota composition across ethnic or racial groups may suggest

that host genetics or immune function may have an important role in driving vaginal microbiota composition (Ravel *et al.*, 2011; Zhou *et al.*, 2010), the association between ethnicity and microbiota composition is likely to be confounded by cultural practices, sexual networks or other factors listed above that impact vaginal microbiota composition (Kenyon & Osbak, 2015; Kenyon *et al.*, 2019; Wessels *et al.*, 2018).

As stated in the introduction, a vaginal microbiota that is abundant in lactic acid producing lactobacilli (specifically non-*L. iners Lactobacillus* spp.) is considered optimal (McKinnon *et al.*, 2019), and contributes to favourable reproductive and sexual health outcomes. The role and importance of lactobacilli in the vagina is discussed in Section 1.2.4 of this review. Conversely, a deviation from an optimal state is defined as a non-optimal vaginal microbiota composition. Non-optimal vaginal microbiota composition has been associated with genital symptoms, and adverse reproductive and sexual health outcomes including increased risk of STI and HIV acquisition (Brotman *et al.*, 2010; Gosmann *et al.*, 2017), genital inflammation (Lennard *et al.*, 2018; Muzny *et al.*, 2019a), increased risk of PID (Ness *et al.*, 2005), as well as preterm labour and delivery (Fettweis *et al.*, 2019; Tabatabaei *et al.*, 2019). BV is the most common vaginal condition of reproductive aged women and microbiologically it is characterised by a non-optimal vaginal microbiota composition.

1.2.3. The vaginal microbiota in bacterial vaginosis

In 1953, Leopold first isolated small Gram-negative rods from cervical samples collected from women with cervicitis that appeared to be closely related to *Haemophilus* (Leopold, 1953). Two years later, Gardner and Dukes reported that *Haemophilus vaginalis* (later renamed as *Gardnerella vaginalis*), was the aetiological agent responsible for the majority of cases of 'non-specific' bacterial vaginitis (Gardner & Dukes, 1955). Gardner and Dukes studied vaginal secretions from 370 women and cultured *G. vaginalis* from 92% of women diagnosed with BV (127/138). Eleven women with a primary diagnosis of BV were not positive for *G. vaginalis* but had grade II (partially replaced *Lactobacillus* spp., n=8) or grade III (completely replaced *Lactobacillus* spp., n=3) vaginal microbial composition. *G. vaginalis* was also detected in 12 women with a primary diagnosis of *Trichomonas vaginalis* and two women with a primary diagnosis of clinical moniliasis. *G. vaginalis* was not present in any of the 78 women who were determined to have clinically normal vaginal discharge.

Gardner and Dukes conducted a highly unethical study where they inoculated women without BV with pure cultures of *G. vaginalis*. They reported that only one of 13 inoculated women developed BV symptoms and had *G. vaginalis* recovered by culture, an additional two women had *G. vaginalis* recovered by culture but did not develop BV-symptoms (Gardner & Dukes, 1955). A subsequent unethical study found that only seven of 29 pregnant women inoculated with pure cultures of *G. vaginalis* developed clinical signs of BV and had *G. vaginalis* recovered (Criswell *et al.*, 1969). Five of nine women who were inoculated with *G. vaginalis* that had been incubated for 12 hours (i.e. in the late logarithmic stage of growth) developed BV, compared to two of 20 women who were inoculated with *G. vaginalis* that had been incubated for 24 hours (i.e. in the stationary stage of growth). The authors concluded that failure of *G. vaginalis* to colonise healthy volunteers was likely due to its stage of growth and non-viability of the inoculum, or its fastidious nature (Criswell *et al.*, 1969; Gardner & Dukes, 1955).

Gardner and Dukes (Gardner & Dukes, 1955) conducted an additional unethical experiment where they inoculated 15 women who were negative for *G. vaginalis* with vaginal discharge from women with clinical BV who were positive for *G. vaginalis*. Eleven of the 15 inoculated women developed clinical symptoms of BV and had *G. vaginalis* recovered by culture. Based on these findings, Gardner and Dukes concluded that *G. vaginalis* fulfilled all four of Koch's postulates for BV and could be considered the aetiological agent responsible for BV.

As discussed in a review by Martin (2012), one of the first reports of the polymicrobial nature of BV was made the early 1900s, prior to the discovery of *G. vaginalis*. Arthur Hale Curtis described a vaginal discharge syndrome (which he termed 'white-discharge' syndrome) and cultured black-pigmented anaerobes, curved anaerobic motile rods, anaerobic cocci and Gram-variable diphtheroid rods from women with 'white-discharge'. Curtis also described that women with 'white-discharge' lacked *Lactobacillus*. Culture and microscopy studies conducted in the 1980s and 1990s built on these early observations and compared the vaginal microbiology of women with and without BV. In these studies, *G. vaginalis* and anaerobes (including *Bacteroides*, *Peptostreptococcus, Mobiluncus spp., Prevotella spp., Fusobacterium nucleatum, Mycoplasma hominis* and others) were more frequently detected in women with BV than women without BV (Hillier, 1993; Spiegel *et al.*, 1983).

Molecular methods have considerably expanded our understanding of the vaginal microbiota composition in women with BV, particularly regarding uncultivable bacteria. In 2005 Fedricks and colleagues identified bacteria in vaginal samples collected from women with without BV, using broad range PCR of the 16S rRNA gene with clone analysis, and bacteria specific PCR assays (Fredricks *et al.*, 2005). They found that women with BV had more bacterial phylotypes detected compared to women without BV (range of 9-17 phylotypes *vs* 1-6 phylotypes), and that organisms including *G. vaginalis*, *Atopobium vaginae, Prevotella* spp. and *Megasphaera* spp. were frequently detected in women with BV. In addition, they described three newly identified *Clostridia*-like bacteria that were commonly detected in women with BV: BVAB1 (more recently designated *Candidatus Lachnocurva vaginae),* BVAB2 and BVAB3 (more recently designated *Mageeibacillus indolicus*). In contrast, women without BV were dominated by *Lactobacillus* species, most commonly *L. crispatus* and *L. iners*. Based on the findings from Fredricks *et al.* (2005) and findings from numerous other molecular studies, one can conclude that the vaginal microbiota of women with BV is characterised by a reduction in the abundance of lactic acid producing *Lactobacillus* spp. and an increase in the abundance of *G. vaginalis* and/or other strict and facultative anaerobes, collectively termed BV-associated bacteria. BV-associated bacteria include *A. vaginae*, *Mobiluncus* spp., *Sneathia* spp., *Prevotella* spp., *Megasphaera* spp., *Dialister* spp., *Peptoniphilus* spp., among others (Biagi *et al.*, 2009; Datcu *et al.*, 2013; Dols *et al.*, 2011; Dumonceaux *et al.*, 2009; Gajer *et al.*, 2012; Haggerty *et al.*, 2009; Jespers *et al.*, 2017; Jespers *et al.*, 2012; Ling *et al.*, 2010; Ling *et al.*, 2013; Mitchell *et al.*, 2009; Oakley *et al.*, 2008; Pepin *et al.*, 2011; Ravel *et al.*, 2011; Santiago *et al.*, 2012; Schellenberg *et al.*, 2009; Shipitsyna *et al.*, 2013; Srinivasan *et al.*, 2012; Srinivasan *et al.*, 2010; Vodstrcil *et al.*, 2017; Wertz *et al.*, 2008; Yoshimura *et al.*, 2011; Zozaya-Hinchliffe *et al.*, 2010).

Molecular characterisation of BV has also highlighted that the microbial composition, bacterial diversity and dominant species can vary greatly between women diagnosed with BV. While some women with BV have highly diverse vaginal microbiota (i.e. presence of a number of different bacterial species, none of which is dominant), other women may be dominated by a single anaerobic species (McKinnon *et al.*, 2019; Ratten *et al.*, 2021; Ravel *et al.*, 2013; Srinivasan *et al.*, 2012; van de Wijgert, 2017).

1.2.4. The common genital Mollicutes

Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum and *Ureaplasma parvum* belong to the Mollicutes class of bacteria and are detected in the urogenital tract of reproductive aged men and women (Taylor-Robinson, 2017). This section of the literature review will briefly discuss the prevalence and detection of these organisms in the vaginal microbiota.

Mycoplasma genitalium is a recognised STI that has been associated with cervicitis (Latimer *et al.*, 2019a; Lillis *et al.*, 2018; Lusk *et al.*, 2011), as well as increased risk of PID, preterm birth, spontaneous abortion and infertility by meta-analysis (Lis *et al.*, 2015). In 2018, a meta-analysis estimated the prevalence of *M. genitalium* among women in the general population to be 1.39% (95% CI 0.81, 2.38), and 0.92 (95% CI 0.60, 1.40) among pregnant women attending antenatal clinics (Baumann *et al.*, 2018). In contrast to *M. genitalium*, the common genital Mollicutes (i.e. *M. hominis*, *U. urealyticum* and *U. parvum*) are not considered 'true' STIs. Although there is evidence supporting an association between vaginal colonisation of *Ureaplasma* spp. (specifically *U. parvum*) and obstetric complications, including preterm birth (Kataoka *et al.*, 2006; Payne *et al.*, 2016; Rittenschober-Bohm *et al.*, 2018), there is limited evidence to suggest that *M. hominis* and the ureaplasmas have a causal role in symptoms and/or disease in nonpregnant women (Horner *et al.*, 2018). These organisms are commonly recovered from symptomatic and asymptomatic individuals (Taylor-Robinson, 2017), and their acquisition in adults is thought to primarily occur via sexual activity (McCormack *et al.*, 1972; McCormack *et al.*, 1973; Taylor-Robinson & McCormack, 1980), with colonisation frequency increasing with increasing number of sexual partners (McCormack *et al.*, 1972; McCormack *et al.*, 1973; Payne *et al.*, 2016; Silva *et al.*, 2018; Taylor-Robinson & McCormack, 1980). The prevalence of common genital Mollicutes (as determined using molecular methods) among reproductive aged nonpregnant women ranges from 3.2-26.2% for *M. hominis*, 7.6-28.4% for *U. urealyticum* and 22.4-67.3% for *U. parvum* (Cox *et al.*, 2016; Foschi *et al.*, 2018; Kim *et al.*, 2014; Leli *et al.*, 2018; Lobao *et al.*, 2017; Ouzounova-Raykova *et al.*, 2011; Silva *et al.*, 2018). The wide range in prevalence for these organisms may be a result of differences between the populations studied, including differences in sexual practices, as well as differences in the prevalence of concurrent genital infections. Of note, the common genital Mollicutes, particularly *M. hominis,* have been

associated with BV (Cox *et al.*, 2016; Foschi *et al.*, 2018; Keane *et al.*, 2000; Rumyantseva *et al.*, 2019; Taylor-Robinson, 2017; Zozaya-Hinchliffe *et al.*, 2010). For example, *M. hominis* is more often detected in women with BV compared to women without BV, and often at higher bacterial load (Cox *et al.*, 2016; Keane *et al.*, 2000; Rumyantseva *et al.*, 2019). However, BV can occur in the absence of *M. hominis, U. urealyticum* and *U. parvum*, and these organisms have also been detected in women with *Lactobacillus*-dominated microbiota (Cox *et al.*, 2016; Malaguti *et al.*, 2015; Taylor-Robinson, 2017).

With increasing use of multiplex PCR assays for STI testing and screening, laboratories are increasingly reporting on the detection of *M. hominis*, *U. urealyticum* and *U. parvum*. This routine reporting can lead clinicians, particularly those in primary care, to assume that antimicrobial treatment of these organisms is required. This increasing practice is a cause for concern, particularly given rising rates of antibiotic resistance among bacterial STIs (Horner *et al.*, 2018). Further evidence is needed to understand the role of *M. hominis*, *U. urealyticum* and *U. parvum* in causing symptoms and disease to inform testing guidelines for these organisms.

1.2.5. The importance of lactobacilli in the vaginal microbiota

The composition of the vaginal microbiota changes over a woman's lifetime, and as stated above, several factors may influence its composition (Lewis *et al.*, 2017). The high levels of oestrogen and vaginal glycogen during the reproductive years are thought to contribute to lactobacilli dominance of the vaginal microbiota (Amabebe & Anumba, 2018; Smith & Ravel, 2017; Witkin & Linhares, 2017). Glycogen is catabolised by α-amylase to produce maltose, maltotriose and α-dextrines (Nasioudis *et al.*, 2015; Spear *et al.*, 2014), which are metabolised by lactobacilli to produce lactic acid (Amabebe & Anumba, 2018; Witkin & Linhares, 2017). Lactic acid acidifies the vaginal environment (Godha *et al.*, 2018; Tachedjian *et al.*, 2017) which supports further growth of lactobacilli (Aldunate *et al.*, 2015), and lactic acid has antimicrobial properties that are independent to acidification. Using vaginal lavage samples, Mirmonsef *et al.* (2014) determined the concentration of free glycogen, the vaginal pH and the vaginal microbiota composition of 20 African American women of reproductive age. Mirmonsef *et al.* (2014) found that high free glycogen levels were associated with both a high relative abundance of *Lactobacillus* spp. as well as a low vaginal pH. Notably, while

the relative abundance of *L. crispatus* was significantly increased in women with high free glycogen levels, the abundance of *L. iners* was not significantly increased in samples with high free glycogen levels.

During the pre-pubertal years when oestrogen levels are low, the vaginal microbiota has correspondingly low *Lactobacillus* abundance (Hill *et al.*, 1995; Smith & Ravel, 2017) and *Lactobacillus* abundance appear to increase during the transition to puberty (Hickey *et al.*, 2015). Additionally, oestrogen levels increase during pregnancy resulting in increased glycogen (Witkin, 2015), and pregnant women have been shown to have higher relative abundance of *Lactobacillus* in their vaginal microbiota compared to nonpregnant women (Romero *et al.*, 2014). Furthermore, following menopause, oestrogen levels decline and the abundance of vaginal *Lactobacillus* decreases in some women (Hillier & Lau, 1997; Mirmonsef *et al.*, 2015). Additionally, postmenopausal women receiving hormone replacement therapy have been shown to have higher *Lactobacillus* abundance compared to those not receiving hormone therapy (Gliniewicz *et al.*, 2019; Heinemann & Reid, 2005). Taken together, these findings suggest a link between oestrogen, glycogen and the vaginal microbiota composition. However, serum oestrogen levels do not always directly correlate with free glycogen levels or vaginal *Lactobacillus* abundance/concentration (Mirmonsef *et al.*, 2016; Mirmonsef *et al.*, 2015). The mechanism by which oestrogen supports lactobacilli dominance is not fully understood but may be dependent on how the vaginal tissue responds to oestrogen (Mitchell *et al.*, 2017). A study of menopausal and post-menopausal women found no correlation between free glycogen levels and vaginal microbiota composition (Mitchell *et al.*, 2017), which contrasts to the findings presented by Mirmonsef *et al.* (2014), and this may in part be due to differences in the vaginal epithelium of menopausal women compared to women of reproductive age.

Not all *Lactobacillus* spp. are considered equal in their ability to maintain vaginal and reproductive health. Although *L. iners* is commonly detected in the vaginal microbiota of women without BV, its presence in the vaginal microbiota of women with BV has been well documented (Fredricks *et al.*, 2005; Hummelen *et al.*, 2010; Lehtoranta *et al.*, 2020; Ravel *et al.*, 2013; Ravel *et al.*, 2011; Srinivasan *et al.*, 2012; Tamrakar *et al.*, 2007; Verwijs *et al.*, 2019; Zozaya-Hinchliffe *et al.*, 2010). *L. iners* is also commonly detected in women following antibiotic treatment for BV (Ferris *et al.*, 2007; Gottschick *et al.*, 2017a; Lehtoranta *et al.*,

2020; Mayer *et al.*, 2015; Plummer *et al.*, 2018a; Verwijs *et al.*, 2019). Additionally, *L. iners* is more likely than other lactobacilli to exist in the vaginal microbiota in the presence of pathogens including HIV, human papillomavirus (HPV) and *Chlamydia trachomatis* (Borgdorff *et al.*, 2014; Brotman *et al.*, 2014b; van de Wijgert, 2017; van de Wijgert *et al.*, 2014; van der Veer *et al.*, 2017), and an *L. iners* dominated vaginal microbiota has been associated with increased risk of infection with *C. trachomatis* (Edwards *et al.*, 2019; van Houdt *et al.*, 2018). Furthermore, women with a *L. iners* vaginal microbiota are more likely to transition to a *Lactobacillus* deficient microbiota compared to women with *L. crispatus* dominated microbiota (Gajer *et al.*, 2012; Verstraelen *et al.*, 2009).

L. iners exhibits several features unique amongst *Lactobacillus* spp. For example, *L. iners* genomes uniquely and consistently contain a gene for a cholesterol-dependent-cytolysin called inerolysin, which belongs to a family of pore-forming toxins (Kwak *et al.*, 2020; Rampersaud *et al.*, 2011). A number of bacterial species encode cytolysins, and a comparative genomic analysis demonstrated that the gene sequence for inerolysin is closely related to the gene sequence for vaginolysin (France *et al.*, 2016) (a well described virulence factor of *G. vaginalis* discussed in Section 1.6.1 that has cytotoxic activity towards vaginal epithelial cells, cervical cells and human erythrocytes (Gelber *et al.*, 2008)). The unique presence of the inerolysin gene in *L. iners*, and its relatedness to vaginolysin indicate that inerolysin may have been acquired through horizontal transfer (France *et al.*, 2016). Interestingly, inerolysin has been shown to be upregulated in BV (Macklaim *et al.*, 2013). This, combined with its relatedness to vaginolysin, has led to the hypothesis that *L. iners* (and inerolysin) may contribute to the pathogenesis of BV (Macklaim *et al.*, 2013). In fact, *L. iners* has been shown to differentially express over 10% of its genes in women with BV compared to women without BV (Macklaim *et al.*, 2013).

Another important characteristic of *L. iners* is its ability to strongly adhere to vaginal epithelial cells. For example, the vaginal strain *L. iners* AB-I has been shown to more strongly adhere to human fibronectin (a binding glycoprotein present on the surface of vaginal epithelial cells) compared to other *Lactobacillus* species (McMillan *et al.*, 2013). Additionally, Castro *et al.* (2013) demonstrated that a *G. vaginalis* strain isolated from a woman with BV was able to displace *L. crispatus* that was adherent to HeLa cells but was not able to displace *L. iners*.

Collectively, these data suggest that *L. iners* may be able to effectively adapt to and persist in non-optimal vaginal microbiota states such as BV. However, whether *L. iners* causes vaginal dysbiosis is not known (Petrova *et al.*, 2017). It is possible that *L. iners* acts as a transitional species and can exist in both an optimal state and non-optimal state. It is also possible that both beneficial and pathogenic variants of *L. iners* exist and are differentially abundant in women with BV *vs* women without BV, but this has not been explored in detail. *L. iners* is clearly well adapted to the vaginal environment (Petrova *et al.*, 2017), however it appears to lack some of the key characteristics of other *Lactobacillus* species that are thought to promote or contribute to vaginal health. Key beneficial characteristics of *Lactobacillus* species will be discussed below.

1.2.5.1. Production of lactic acid and other antimicrobial and inhibitory compounds

Lactobacillus spp. produce a number of antimicrobial compounds including lactic acid, hydrogen peroxide, bacteriocins and biosurfactants (Amabebe & Anumba, 2018). The beneficial effect of *Lactobacillus* bacteria in the vagina was previously attributed to the production of hydrogen peroxide (Klebanoff *et al.*, 1991). This is now considered implausible because 1) the cervicovaginal environment is hypoxic, and oxygen is needed to produce hydrogen peroxide in sufficient quantities to have an antimicrobial effect, 2) cervicovaginal fluid has antioxidant properties that prevents the antimicrobial effect of hydrogen peroxide, and 3) hydrogen peroxide has higher toxicity to vaginal lactobacilli than to BV-associated bacteria (O'Hanlon *et al.*, 2013; Tachedjian *et al.*, 2018). It is now recognised that a key beneficial characteristic of lactobacilli is lactic acid production (Tachedjian *et al.*, 2018).

A study of women with *Lactobacillus* dominated vaginal microbiota demonstrated that the pH of cervicovaginal fluid decreases as the concentration of lactate increases, and suggesting that lactic acid is primarily responsible for vaginal acidification in a lactobacillus dominated vaginal microbiota (O'Hanlon *et al.*, 2013). Lactic acid exists in two isomers: L-lactic acid and D-lactic acid. While L-lactic acid is produced in small amounts by vaginal epithelial cells, the majority of L-lactic acid (>85%) is produced by bacteria (Boskey *et al.*, 2001), and D-lactic acid is almost exclusively produced by bacteria (Witkin *et al.*, 2013). As a result, the majority of lactic acid present in the vagina is produced by bacteria, and the ratio

of D:L isomers has been shown to be characteristic of the particular *Lactobacillus* species that dominates the vaginal microbiota. For example, the ratio of D:L-lactic acid is higher among women with an *L. crispatus* dominated vaginal microbiota and conversely, the ratio of D:L-lactic acid is lower among women with an *L. iners* dominated vaginal microbiota (Boskey *et al.*, 2001). This highlights that *Lactobacillus* spp. differ in their ability to produce each isomer. A study of pure cultures of *Lactobacillus* spp. demonstrated that while *L. crispatus* and *L. gasseri* produced both the L- and D-isomers, *L. jensenii* only produced the D-isomer and *L. iners* only produced the L-isomer in quantities above the limit of detection (Witkin *et al.*, 2013). In addition, *L. iners* produces lower concentrations of lactic acid *in vitro* compared to other *Lactobacillus* spp. (Witkin *et al.*, 2013). It has been hypothesised that D-lactic acid is more protective against upper genital tract infections compared to L-lactic acid (Witkin *et al.*, 2013), and the protective effects of *L. crispatus* compared to *L. iners* have been partly attributed to its ability to produce D-lactic acid (Tachedjian *et al.*, 2017).

In vitro, lactic acid inactivates *C. trachomatis* (Gong *et al.*, 2014; Nardini *et al.*, 2016), *Neisseria gonorrhoeae* (Breshears *et al.*, 2015; Graver & Wade, 2011) and *E. coli* (Juarez Tomas *et al.*, 2003), and suppresses BV-associated bacteria (Breshears *et al.*, 2015; O'Hanlon *et al.*, 2011). Lactic acid also inactivates HIV in both culture and cervicovaginal secretions (Aldunate *et al.*, 2013). Aldunate *et al.* (2013) demonstrated that a pH of 3.8, lactic acid more potently inactivated HIV compared to acetic acid and hydrochloric acid, suggesting that lactic acid has antiviral properties that extend beyond acidification. Furthermore, lactobacilli promote autophagy via lactic acid, which is an important process that removes potentially toxic molecules as well as intracellular microorganisms (Witkin & Linhares, 2017), and it has been noted that autophagy activity is higher when *L. crispatus* dominates the vaginal microbiota compared to *L. iners* (Leizer *et al.*, 2018). Additionally, lactic acid has anti-inflamatory properties (Delgado-Diaz *et al.*, 2019). Manhanzva and colleagues (Manhanzva *et al.*, 2020) recently showed that D-lactate production by *Lactobacillus* isolates was inversely associated with cytokine production. This is consistent with an *in vitro* study that showed lactic acid induces an anti-inflammatory response from cervicovaginal epithelial cells and prevents production of inflammatory cytokines and chemokines (Hearps *et al.*, 2017).

Bacteriocins are molecules sectreted by a bacterium that have bactericidal or bacteriostatic

activity on neighbouring non-isogenic bacteria (Alvarez-Sieiro *et al.*, 2016). Several bacteriocins from *Lactobacillus* spp. have been described. For example, Lactocin 160 is a peptide produced by a vaginal culture of *Lactobacillus rhamnosus* that has been shown to inhibit *G. vaginalis in vitro* through forming pores in the target bacterial cell membrane (Turovskiy *et al.*, 2009). *Lactobacillus acidophilus* KS400, the species used in the vaginal probiotic Gynoflor, produces bacteriocins that have *in vitro* antimicrobial activity towards *G. vaginalis*, *Streptococcus agalactiae*, *P. aeruginosa* and *Lactobacillus delbrueckii* (Gaspar *et al.*, 2018). Additionally, Fermenticin HV6b, produced by the vaginal strain *Lactobacillus fermentum* HV6b MTCC10770, has antimicrobial activity against a number of microorganisms *in vitro* including *G. vaginalis*, *N. gonorrhoeae* and *Candida albicans* (Kaur *et al.*, 2013).

Some lactobacilli also secrete biosurfactants, which may have antimicrobial properties and/or prevent potential pathogens from adhering to host epithelium and forming a biofilm (Satpute *et al.*, 2016a; Satpute *et al.*, 2016b).

1.2.5.2. Immune system interactions

However, optimal *Lactobacillus* spp. have consistently been associated with a non-inflammatory vaginal environment in both *in vivo* and *in vitro* studies (Anahtar *et al.*, 2015; Rose *et al.*, 2012; Sakai *et al.*, 2004; Shannon *et al.*, 2017). For example, Anahtar *et al.* (2015) found that in a cohort of 94 asymptomatic South African women, pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, IFN-γ, IL-10, IL-8, IL-12p70, IL-4, and FLT-3L) were higher among women with *Lactobacillus* deficient vaginal microbiota compared to women with non-*iners Lactobacillus* vaginal microbiota. IFN-γ was also higher in women with *L. iners* microbiota compared to women dominated by other *Lactobacillus* species. Similarly, a study of 51 black women explored the association between vaginal microbiota composition, genital immunology and herpes simplex virus type 2 (HSV-2) seropositivity, and found that genital inflammation was associated with decreased relative abundance of *L. crispatus* (Shannon *et al.*, 2017). Women with a vaginal microbiota deficient in *Lactobacillus* spp. and abundant in diverse anaerobic bacteria had increased levels of pro-inflammatory cytokines IL-1α and IL-1β. Additionally, the proportion of women with increased pro-inflammatory cytokines was higher among women with *L. iners* (n=4/22, 18.2%) or *Lactobacillus* deficient

microbiota (9/20, 45%) than women with a *L. crispatus* or *L. gasseri* dominated microbiota (n=0/9, 0%) (Shannon *et al.*, 2017). No associations were observed between microbiota composition and CD4+ levels, or microbiota and HSV-2 seropositivity. It should be noted that in both studies, the number of women with a non-*iners Lactobacillus* vaginal microbiota was small (n=8 and n=9, respectively). Interestingly, *Lactobacillus* isolates recovered from women with a non-optimal vaginal microbiota composition (i.e. Nugent score 4-10) have been shown to be significantly more inflammatory compared to *Lactobacillus* isolates recovered from women with lactobacilli dominated microbiota (Nugent score 0-3) (Manhanzva *et al.*, 2020).

1.2.5.3. Vaginal adherence, competitive exclusion

Bacterial adhesion to epithelial cells is important for colonisation of mucous membranes. *Lactobacillus* spp. demonstrate high affinity for vaginal epithelial receptor sites and can adhere to vaginal epithelium and prevent adhesion of potential pathogens including *G. vaginalis, Escherichia coli and Streptococcus agalactiae* through competitive exclusion (Boris *et al.*, 1998; Chan *et al.*, 1985).

In summary, lactobacilli have key characteristics that are thought to promote or contribute to optimal vaginal health. As discussed briefly in Section 1.2.2, a reduction of *Lactobacillus* spp. in the vagina, as seen in BV, is associated with non-optimal outcomes for women. The remaining sections of this literature review will discuss BV.

1.3. Bacterial vaginosis: prevalence, clinical symptoms, and associated sequelae

1.3.1. Prevalence of bacterial vaginosis

The prevalence of BV varies widely across countries and between different population groups. For example, within Australia, the prevalence of BV amongst women aged 16-25 is approximately 12% (Bradshaw *et al.*, 2013b), but prevalence estimates of 27% were reported in a cohort of Australian women who have sex with women (WSW) (Bradshaw *et al.*, 2014).

In a recent systematic review and meta-analysis, the global prevalence of BV among reproductive-aged women in the general population was found to range from 23% (95% CI 18, 28%) in Europe and Central Asia, to 29% (95% CI 21, 37%) in South East Asia (Figure 3) (Peebles *et al.*, 2019). This meta-analysis excluded studies of populations not representative of the general population or where BV prevalence was expected to be different from the general population (i.e. STI clinic populations, populations selected on the basis of symptomology or presence of a BV-associated condition, sex workers, incarcerated women, studies exclusively of HIV-1 negative women in countries with a high HIV prevalence). The review found BV prevalence varied with ethnicity within specific geographical regions. For example, BV prevalence was 27% (95% CI 24, 31) in North America, with prevalence estimates of 33.2% in black women, 30.7% in Hispanic women, and 22.7% in white women and 11.1% in Asian women (Peebles *et al.*, 2019).

1.3.2. Symptoms of bacterial vaginosis

Women with BV report symptoms including a profuse vaginal discharge and an unpleasant vaginal malodour, and generally do not report symptoms of dysuria, dyspareunia, or vaginal itching, burning or pain (Amsel *et al.*, 1983; Klebanoff *et al.*, 2004). Up to 50% of women with BV are asymptomatic (Amsel *et al.*, 1983; Klebanoff *et al.*, 2004), however it is important to note that adverse outcomes of BV may be present regardless of a woman's symptom status (Muzny & Schwebke, 2020). There is minimal research into the psychological impact of BV; however, women with recurrent BV often report feelings of shame and embarrassment (Payne *et al.*, 2010) and a qualitative study found recurrent-BV to have a moderate to severe negative impact on the self-esteem, sex life and overall quality of life (Bilardi *et al.*, 2013).

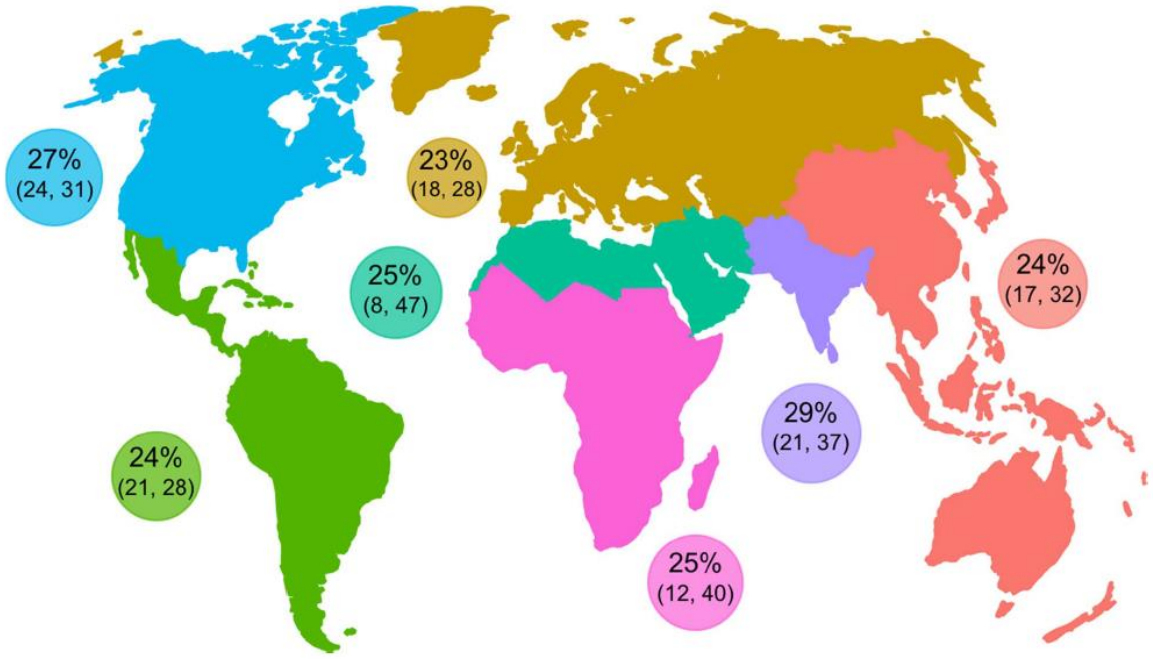


Figure 3. The global prevalence of bacterial vaginosis in the general population

The prevalence of BV amongst women of reproductive age by World Bank region (presented with 95% confidence intervals). Reprinted from "High global burden and costs of bacterial vaginosis: a systematic review and meta-analysis" by Peebles et al. (2019). Sexually Transmitted Diseases, 46(5), 304-311.

1.3.3. Adverse sequelae of bacterial vaginosis

BV, regardless of whether or not a woman is symptomatic, is associated with serious obstetric and gynaecologic sequelae. For example, BV and BV-associated bacteria have been associated with premature labour, preterm birth, low birthweight, miscarriage, and increased risk of infertility (Donders *et al.*, 2009; Hay *et al.*, 1994; Honest *et al.*, 2004; Isik *et al.*, 2016; Nelson *et al.*, 2009; Ralph *et al.*, 1999). A meta-analysis conducted in 2003 of 18 studies found BV to be associated with a two-fold increase in the risk of preterm birth (OR 2.19; 95% CI 1.54-3.12), and ten-fold increased risk of spontaneous abortion (OR 9.91; 95% CI 1.99-49.34), though only three studies contributed to this outcome (Leitich *et al.*, 2003). A meta-analysis of 32 studies of women with asymptomatic BV confirmed the previous results; asymptomatic BV was associated with a two-fold increased risk of preterm delivery (OR 2.16; 95% CI 1.56-3.00), and a six-fold increased risk of late miscarriage (OR 6.32; 95% CI 3.65-10.94) (Leitich & Kiss, 2007).

BV, and the detection of BV-associated bacteria, has been associated with increased risk of acquisition (and detection) of *C. trachomatis*, *N. gonorrhoeae, T. vaginalis* and HSV-2, as well as increased risk of acquisition and persistence of HPV (Brotman *et al.*, 2012; Brotman *et al.*, 2014b; Cherpes *et al.*, 2003; Hillier *et al.*, 1992; Martin *et al.*, 1999; Stoner *et al.*, 2012; Wiesenfeld *et al.*, 2003). A longitudinal study of 3,620 nonpregnant women reported that BV was associated with increased risk of incident STI (*C. trachomatis*, *N. gonorrhoeae* and/or *T. vaginalis* infection) after adjustment for age, ethnicity, antibiotic and antifungal use, douching and sexual risk factors (adjusted hazard ratio [AHR] 1.73, 95% CI 1.42-2.11) (Brotman *et al.*, 2010). BV, including the detection of BV-associated bacteria, has also been associated with increased risk of PID (Haggerty *et al.*, 2004; Haggerty *et al.*, 2016; Ness *et al.*, 2005; Peipert *et al.*, 1997; Wiesenfeld *et al.*, 2002). However, although BV-associated bacteria are commonly recovered from women with PID (Brunham *et al.*, 2015), no causal link between BV and PID has been established (Taylor *et al.*, 2013).

BV is also associated with increased risk of both acquisition and transmission of HIV (Cohen *et al.*, 2012; Sewankambo *et al.*, 1997; Taha *et al.*, 1998). A meta-analysis of 23 HIV incidence studies found that BV was associated with increased risk of HIV acquisition (pooled relative risk estimate 1.6, 95% CI 1.2-2.1) (Atashili *et al.*, 2008). The same study reported higher HIV seroprevalence in women with BV compared to women without BV in 20 of 21 included studies, however heterogeneity across the studies precluded calculation of a pooled estimate (Atashili *et al.*, 2008). A study of 2,236 HIV-1 seropositive women and their HIV-1 uninfected male partners found that HIV-1 incidence was higher in male partners of women with BV (2.91/100 person years) compared to male partners of women without BV (0.76/100 person years; hazard ratio [HR] 3.62, 95% CI 1.74-7.52) (Cohen *et al.*, 2012). BV has also been associated with HIV-1 shedding in the genital tract (Cu-Uvin *et al.*, 2001), which supports the hypothesis that BV may increase the risk of HIV transmission.

1.4. Bacterial vaginosis: diagnostic approaches

Although commercial PCR assays are commonly used by diagnostic laboratories for BV diagnosis (Muzny & Kardas, 2020), the two gold-standard diagnostic methods are the Amsel criteria (Amsel *et al.*, 1983) and the Nugent score method (Nugent *et al.*, 1991).

1.4.1. Amsel criteria

In clinic practice, three or more of the following Amsel criteria must be met for a diagnosis of BV to be made (Amsel *et al.*, 1983):

i. thin white-grey homogeneous vaginal discharge,
ii. vaginal pH≥4.5,
iii. positive whiff test - release of an amine (fishy) odour after 10% potassium hydroxide is added to vaginal secretions, and
iv. presence of clue cells >20 % of total epithelial cells in vaginal discharge on saline wet mount. Clue cells are epithelial cells that are coated with adherent Gram-variable coccobacilli and are considered the most reliable indicator for BV out of the four Amsel criteria (Sodhani *et al.*, 2005; Thomason *et al.*, 1990).

In general practice settings where onsite laboratory services are not available, a diagnosis of BV can be made when the clinician notes the presence of homogenous vaginal discharge, vaginal pH≥4.5 and vaginal malodour.

1.4.2. Nugent score method

BV is also commonly diagnosed microscopically using the Nugent score (Nugent *et al.*, 1991), a Gram-stain based scoring method of wet mount preparations of vaginal discharge. The Nugent score ranges from 0-10 and is calculated based on the sum of observed numbers of Gram-positive rods (*Lactobacillus* morphotypes), short Gram-negative to Gram-variable rods (*G. vaginalis* and *Bacteroides* morphotypes) and curved Gram-negative rods (*Mobiluncus* morphotypes, Table 1) (Nugent *et al.*, 1991). A score of 0-3 is considered non-BV, a score of 4-6 is considered intermediate-BV and a score of 7-10 is considered Nugent-BV (Figure 4).

Table 1 - Nugent Scoring criteria for diagnosis of bacterial vaginosis

	Number of morphotypes per field		
Score assigned[a]	***Lactobacillus* spp. morphotypes**	***G. vaginalis* and *Bacteroides* spp. morphotypes**	***Mobiluncus* spp. morphotypes**
0	>30	0	0
1	5-30	<1	<1 to 1-4

2	1-4	1-4	5-30 to >30
3	<1	5-30	
4	0	>30	

This table was adapted from "Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation" by Nugent *et al*. (1991). *Journal of Clinical Microbiology,* 29(2), 297-301.

[a] The score assigned is the average number of morphotypes seen per field of view. The Nugent score is the sum of scores assigned for *Lactobacillus, G. vaginalis* and *Mobiluncus* spp. morphotypes.

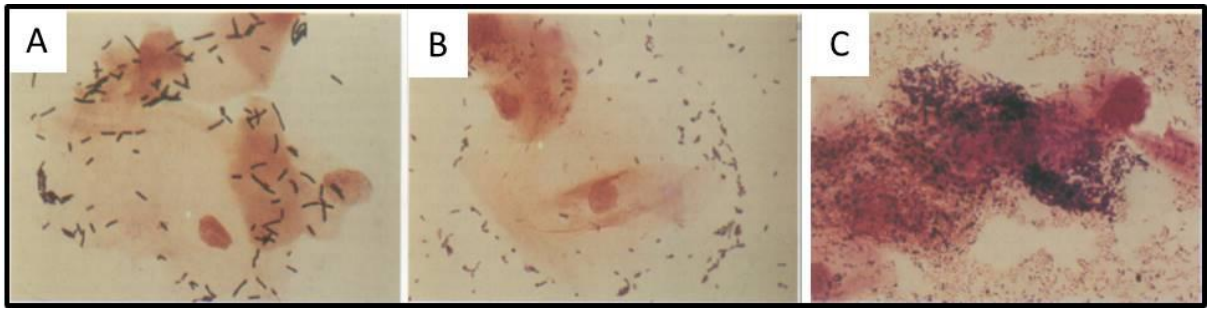


Figure 4. Gram-stained vaginal smears

Figure 4A depicts a vaginal smear with Nugent score=0 (>30 Lactobacillus morphotypes and no G. vaginalis or Mobiluncus morphotypes), representing 'normal' or non-BV vaginal microbiota. Figure 4B depicts a vaginal smear with Nugent score=4 (5-30 Lactobacillus morphotypes and 5-30 G. vaginalis morphotypes), representing intermediate-BV. Figure 4C depicts a vaginal smear with Nugent score=10 (no Lactobacillus morphotypes, >30 G. vaginalis morphotypes, >30 Mobiluncus morphotypes and a clue cell is evident in the centre of the figure), representing Nugent-BV. Adapted from "Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation" by Nugent et al. (1991). Journal of Clinical Microbiology, 29(2), 297-301.

In a research setting, the Nugent score has some advantages over the Amsel criteria. The Nugent score provides a crude measure of the vaginal microbiota composition and importantly removes the requirement for a clinical examination and can be applied to vaginal smears that have been self-collected by participants at home. However, the combination of ≥3 Amsel criteria and a Nugent score of 4-10 is often used in clinical trials that evaluate new treatments for BV to ensure women who are recruited have symptomatic BV. Using the Nugent score alone provides no measure of symptoms, and restricting the Nugent score to 7-10 can miss cases who have clinically relevant BV (i.e. ≥3 Amsel criteria)

but have an intermediate Nugent score (i.e. 4-6) due to the presence of lactobacilli.

1.5. Bacterial vaginosis: current treatments

Current first line treatments for BV include oral metronidazole 400mg or 500mg twice daily for 7-days, intravaginal 2% clindamycin cream once at night for 7-days, and 5-day course of 0.75% intravaginal metronidazole gel (Workowski & Bolan, 2015). Metronidazole is a nitroimidazole antibiotic that has activity against protozoans and anaerobic bacteria including *G. vaginalis, Bacteroides* spp., *Clostridium* spp., *Prevotella* spp., *Porphyromonas* spp., and *Fusobacterium* spp., among others. *Lactobacillus* spp. have reduced susceptibility to metronidazole (Freeman *et al.*, 1997; Lofmark *et al.*, 2010; Sobel & Sobel, 2015). Metronidazole enters the bacterial cell by passive diffusion and is metabolised to form metronidazole radicals which degrade DNA, prevent DNA synthesis and cause cell lysis (Edwards, 1993). Clindamycin is a lincosamide antibiotic that has broad spectrum activity against Gram-positive cocci as well as anaerobic Gram positive and Gram-negative bacteria and works by binding the 50S subunit of the ribosome and preventing protein synthesis (Smieja, 1998).

Oral metronidazole and clindamycin have equivalent 4-week cure rates of approximately 70-85% (Oduyebo *et al.*, 2009), and vaginal metronidazole has been reported to achieve cure in between 61-94% of women at 4-weeks post treatment (Koumans *et al.*, 2002). However, recurrence following standard treatment is common. In a randomised double blind trial in 1993, Sobel *et al.* (1993) compared BV recurrence rates in women randomised to receive either oral metronidazole (500mg twice daily for 7-days) or intravaginal clindamycin 2% cream (5g vaginal applicator for 7-days). Authors reported a high attrition rate, with 32 women dropping out over the follow-up period. At 3-months post treatment, 69% of women treated with oral metronidazole and 54% of women treated with topical clindamycin experienced symptomatic recurrence. Bradshaw *et al.* (2006a) enrolled 139 women into a 12-month study to determine factors associated with BV recurrence following treatment with oral metronidazole. Of the 130 women to attend at least one follow-up visit, 43% (95% CI 34, 52%) experienced BV recurrence (defined as a Nugent score=7-10) within 3-months post-treatment, and this increased to 52% (95% CI 43, 61%) by 6-month post-treatment and 58% (95% CI 49, 66%) by 12-months post-treatment.

1.6. Bacterial vaginosis: key topics in BV pathogenesis

Various investigators have recently captured the current state of knowledge surrounding BV:

> *'Bacterial vaginosis is one of the great conundrums in sexual and reproductive health'* (Unemo *et al.*, 2017)

> *'Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused?'* (Marrazzo, 2011).

These phrases are used because after more than 60 years of research, much about BV remains unknown, including its aeitiology. It is not clear if BV results from the acquisition of a single organism or a polymicrobial consortium, or from overgrowth of BV-associated bacteria in response to specific host or behavioural factors. Additionally, the mechanism/s that drive the high rates of recurrence seen following standard antibiotic therapy are incompletely understood. However, current reviews indicate that the following factors are likely to be involved: 1) reinfection from an untreated sexual partner, 2) persistence of disease, and 3) failure to re-establish an optimal vaginal microbiota after treatment (Bradshaw & Brotman, 2015; Bradshaw & Sobel, 2016; Unemo *et al.*, 2017). Key topics related to the pathogenesis of BV will be discussed below.

1.6.1. *Gardnerella vaginalis* mechanisms of pathogenesis

Gardnerella vaginalis is a Gram-positive facultative anaerobic short bacillus that has a thin cell wall and often stains Gram-variable (Catlin, 1992). As discussed in Section 1.2.3, in 1955 *G. vaginalis* was proposed as the sole aetiological agent responsible for BV (Gardner & Dukes, 1955). Although it is now accepted that BV is associated with a polymicrobial dysbiosis of the vaginal microbiota, *G. vaginalis* is still thought to play a key role in BV pathogenesis, potentially as a founder or initiating organism (Coleman & Gaydos, 2018; Muzny & Schwebke, 2013; Muzny *et al.*, 2019c; Schwebke *et al.*, 2014b). *G. vaginalis* is almost always recovered from the vagina of women who have BV, with studies reporting the prevalence of *G. vaginalis* to be as high as 100% in women with BV (Bradshaw *et al.*, 2006b; Fethers *et al.*, 2012; Fredricks *et al.*, 2005; Janulaitiene *et al.*, 2017; Srinivasan *et al.*, 2012).

G. vaginalis has several characteristics that are important for BV pathogenesis. *G. vaginalis* can adhere to vaginal epithelium and form a biofilm, which is a community of adherent bacteria and their extracellular matrices (Patterson *et al.*, 2010; Swidsinski *et al.*, 2005). BV-biofilms comprised *G. vaginalis* and other BV-associated bacteria including *A. vaginae*, have been shown to persist following antibiotic treatment for BV (Swidsinski *et al.*, 2008). *G. vaginalis* is often the predominant species present in BV-biofilms and is thought to be more effective at initiating and forming biofilms than other BV-associated bacteria (Alves *et al.*, 2014; Patterson *et al.*, 2010). For example, a study conducted in 2013 reported that *G. vaginalis* was able to better adhere to an inert surface covered in epithelial cells in the presence of *L. crispatus* than other BV-associated bacteria (Machado *et al.*, 2013). In 2010, Patterson *et al.* (2010) investigated the virulence properties eight BV-associated bacteria isolates: *G. vaginalis, A. vaginae, Prevotella bivia, Peptostreptococcus* spp.*, Peptoniphilus* spp.*, Veillonella* spp.*, Mobiluncus mulieris,* and *F. nucleatum*. Researchers demonstrated that both *G. vaginalis* and *Peptoniphilus* were able to strongly adhere to vaginal epithelial cells in culture, whereas other isolates examined exhibited limited or no adherence to vaginal epithelial cells. The researchers also reported that only *G. vaginalis* and *F. nucleatum* were able to form a biofilm *in* vitro, and while the *G. vaginalis* biofilm was thick and strongly adherent, the *F. nucleatum* biofilm was able to be partially dislodged by washing (Patterson *et al.*, 2010). Interestingly, *G. vaginalis* can also form biofilms in acidic environments (Udayalaxmi *et al.*, 2012), which may contribute to its ability to grow in the presence of *Lactobacillus*.

Gardnerella vaginalis also produces virulence factors including vaginolysin and sialidase (Briselden *et al.*, 1992; Gelber *et al.*, 2008; Hardy *et al.*, 2017). Vaginolysin is a cholesterol-dependent-cytolysin that lyses vaginal epithelial cells (Gelber *et al.*, 2008). Cholesterol-dependent-cytolysins are essential virulence factors for a number of Gram-positive bacteria, including *Listeria monocytogenes*, *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Tweten, 2005). Vaginolysin has been shown to bind to cholesterol receptors on the surface of vaginal epithelial cells, lyse human erythrocytes and vaginal epithelial cells and activate the p38 MAPK and IL-8 immune pathways (Gelber *et al.*, 2008; Zvirbliene *et al.*, 2010). Although the exact mechanism by which vaginolysin contributes to *G. vaginalis* pathogenicity is not known, it is thought to be important in the immunopathology of BV.

Sialidase is an enzyme that degrades the cervicovaginal mucus by cleaving sialic acid from glycans (Lewis *et al.*, 2013). Sialidase may assist *G. vaginalis* with adhering to vaginal epithelium and biofilm formation (Hardy *et al.*, 2017) and may also assist *G. vaginalis* to evade the host immune system through incorporating cleaved sialic acids into the surface of bacterial cells, similar to what has been demonstrated for other pathogens that possess sialidase, e.g. *Trypanosoma cruzi* (Freire-de-Lima *et al.*, 2015).

Gardnerella vaginalis can tolerate the high oxidation-reduction (redox) potential of the vaginal environment and may act to lower the redox potential to facilitate growth of strict anaerobic bacteria including *P. bivia* and *A. vaginae* (Muzny *et al.*, 2019c; Schwebke *et al.*, 2014b). In support of this, *A. vaginae* is a common constituent of *G. vaginalis* dominated biofilms (Hardy *et al.*, 2015), but *A. vaginae* is rarely detected in the vagina in the absence of *G. vaginalis* (Bradshaw *et al.*, 2006b). Furthermore, *G. vaginalis* has been shown to have a synergistic relationship with other BV-associated bacteria including *P. bivia* (Chen *et al.*, 1979; Pybus & Onderdonk, 1997). *G. vaginalis* produces amino acids which enhance the growth of *P. bivia*, and *P. bivia* produces ammonia which stimulates growth of *G. vaginalis* (Pybus & Onderdonk, 1997). *P. bivia* also produces sialidase (Briselden *et al.*, 1992) which further contributes to degradation of the cervicovaginal mucosa and biofilm formation. Muzny *et al.* (2019c) proposed a conceptual model on the BV pathogenesis that centres around the initiation of BV by specific pathogenic strains of *G. vaginalis* and the synergistic relationship between *G. vaginalis* and *P. bivia* and *A. vaginae*.

1.6.1.1. *Gardnerella vaginalis* genetic variation and typing schemes

Gardnerella vaginalis can also be present in the vaginal microbiota of women who do not have BV. For example, *G. vaginalis* prevalence has frequently reported to be around 40-50% in women without BV by Nugent score (Bradshaw *et al.*, 2006b; Fethers *et al.*, 2012; Menard *et al.*, 2008; Schwebke *et al.*, 2014a), but prevalence upwards of 75% has also been reported in BV-negative women (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019; Shipitsyna *et al.*, 2013). This finding has led to the hypothesis that both commensal and pathogenic variants of *G. vaginalis* may exist.

Several different classification schemes have been used for typing *G. vaginalis* strains. Piot *et al.* (1984) identified eight *G. vaginalis* biotypes based on results from a series of biochemical tests (i.e. identification of hippurate, lipase, β-galactosidase activity), but found no differences in the biotypes present in women with BV versus women without BV. Additional *G. vaginalis* biotypes were also identified in subsequent studies (Benito *et al.*, 1986; Pedraza-Aviles *et al.*, 1995). In 1997, Ingianni *et al.* (1997) used amplified ribosomal DNA restriction analysis (ARDRA) with eight different restriction enzymes to study *G. vaginalis* genotypes and identified 3 or 4 different genotypes, depending on the enzyme used. Similar to what was found in biotyping studies, no association between BV and *G. vaginalis* genotype was observed.

Recent studies have shown that substantial genetic diversity exists within the *G. vaginalis* species (Ahmed *et al.*, 2012; Cornejo *et al.*, 2018; Harwich *et al.*, 2010; Paramel Jayaprakash *et al.*, 2012; Schellenberg *et al.*, 2016). Ahmed *et al.* (2012) performed a comparative genomics analysis of 17 *G. vaginalis* isolates and found that the genetic diversity among the 17 isolates was high for a bacterial species. Using neighbour-grouping analyses of both distributed gene possession data and core gene allelic data, Ahmed *et al.* (2012) defined two genetically distinct sets of *G. vaginalis* strains, each of which was comprised of two genetically distinct subgroups. The level of genomic diversity within each subgroup was within the range of diversity expected for a bacterial species, which provided a strong argument that each of the four subgroups (or 'clades') of *G. vaginalis* should be classified as individual species.

Building on the work by Ahmed *et al*, Balashov *et al.* (2014) designed a multiplex clade-specific qPCR assay for detecting each of the four *G. vaginalis* clades, and what followed was a number of studies that described the distribution of *G. vaginalis* clades among women with and without BV (Hilbert *et al.*, 2017; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019; Vodstrcil *et al.*, 2017). The findings from these studies regarding the association between BV and the detection of individual *G. vaginalis* clades is summarised in Table 2, and briefly discussed below.

Table 2 - Reported associations between bacterial vaginosis and the detection of individual *Gardnerella vaginalis* clades

	Clade 1	Clade 2	Clade 3	Clade 4
Balashov *et al.* (2014)	Nugent BV Amsel BV	Intermediate Nugent score	Amsel BV	
Janulaitiene *et al.* (2017)	Nugent + Amsel BV	Nugent + Amsel BV		
Vodstrcil *et al.* (2017)				Nugent BV
Shipitsyna *et al.* (2019)	Nugent BV, Intermediate Nugent score	Nugent BV, Intermediate Nugent score		Nugent BV

Nugent BV defined as Nugent Score= 7-10; Intermediate Nugent Score defined as Nugent score= 4-6; Amsel BV defined as ≥3 Amsel criteria; Nugent + Amsel BV defined as Nugent score=7-10 & ≥3 Amsel criteria.

Balashov *et al.* (2014) reported that clade 1 was associated with both Nugent-BV and Amsel-BV, and clade 3 was associated with Amsel-BV only, whereas clade 2 was associated with intermediate BV by Nugent score, and no associations were identified between clade 4 and Nugent score or Amsel-BV. Janulaitiene *et al.* (2017) analysed *G. vaginalis* clade distribution in 109 Lithuanian women and found that while clades 1 and 2 were associated with Nugent-BV, clades 3 and 4 were not associated with Nugent score. Vodstrcil *et al.* (2017) reported that detection of clade 4 was associated with Nugent-BV in a cohort of 52 sexually inexperienced women, and Shipitsyna *et al.* (2019) reported that clades 1, 2 and 4 were associated with Nugent-BV. Hilbert *et al.* (2017) determined the abundance of each of the four clades in a longitudinal study of women with and without BV and found that the abundance of each clade was significantly increased in women with BV (by both Nugent score and Amsel criteria) compared to women without BV. One finding that is consistent across studies is that the presence of multiple *G. vaginalis* clades is strongly associated with both Nugent-BV and Amsel-BV (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019; Vodstrcil *et al.*, 2017).

Other research groups have used different methodologies to report/define *G. vaginalis* subgroups. For example, four *G. vaginalis* subgroups have been identified by sequence analysis of the cpn60 gene, which encodes the universal 60 kDa chaperonin protein (Paramel Jayaprakash *et al.*, 2012; Schellenberg *et al.*, 2016). The cpn60 subgroups

correspond to the four clades identified by Ahmed *et al.* (2012) (Table 3). In addition, a phylogenetic and gene enrichment analysis of 35 *G. vaginalis* strains identified three *G. vaginalis* ecotypes, each of which was split into two subgroups (Cornejo *et al.*, 2018). Ecotype 1A/B corresponds to clade 1, ecotype 2A/B corresponds to clade 2 and ecotype 3A/B corresponds to clades 3 and 4, Table 3 (Cornejo *et al.*, 2018).

Table 3 - Relationship between *G. vaginalis* groupings and novel *Gardnerella* species

Clade[a]	Cpn60 subgroup[b]	Ecotype[c]	Novel *Gardnerella* species[d]
1	C	1A	***G. vaginalis*[e]**
		1B	Undefined *Gardnerella* species 2[f]
2	B	2A/2B	***G. piotii*,** Undefined *Gardnerella* species 3
3	D	3A	Undefined *Gardnerella* species 8, 9, and 10
NA[g]	NA[g]		Undefined *Gardnerella* species 7
4	A	3B	***G. leopoldii*, *G. swidsinskii***
NA[h]	NA[h]	NA[h]	Undefined *Gardnerella* species 11, 12, 13 and 14

Information from this table was adapted from "Resolution and cooccurrence patterns of *Gardnerella leopoldii, G. swidsinskii, G. piotii,* and *G. vaginalis* within the vaginal microbiome." By Hill et al. (2019). *Infection and Immunity, 87*(12).

[a] *G. vaginalis* clades as determined by (Ahmed *et al.*, 2012) or as identified by (Balashov *et al.*, 2014)

[b] *G. vaginalis* cpn60 subgroups determined by Schellenberg *et al.* (2016)

[c] *G. vaginalis* ecotypes determined by Cornejo *et al.* (2018)

[d] *Gardnerella* species described by Vaneechoutte *et al.* (2019). Named *Gardnerella* spp. have been bolded. *Gardnerella* species #14 was proposed by Putonti *et al.* (2021)

[e] One *G. vaginalis* genome was identified as ecotype 1B

[f] Three *Gardnerella* species 2 genomes did not belong to any previously defined clade or cpn60 subgroup.

[g] Clade and cpn60 subgroup unknown for undefined *Gardnerella* species 7

[h] Clade, cpn60 subgroup and ecotype unknown for undefined *Gardnerella* species 11, 12, 13 and 14

Interestingly, some *G. vaginalis* isolates have been found to not belong to any *G. vaginalis* clade by clade-specific qPCR or cpn60 *G. vaginalis* subgroup (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017; Schellenberg *et al.*, 2016), which suggests that additional clades may exist or that there is diversity within the four identified clades (Cornejo *et al.*, 2018). In support of this, Vaneechoutte *et al.* (2019) analysed 81 publicly available sequenced complete genomes belonging to the *Gardnerella* genus using digital DNA-DNA hybridisation (dDDH) and average nucleotide identity (ANI). Based on established cut-offs for species

delineation using dDDH and ANI, 13 genomic species were identified in the *Gardnerella* genus, five of which were comprised of only one *Gardnerella* genome. Vaneechoutte *et al.* (2019) next determined protein composition profiles (using MALDI-TOF mass spectrometry) and biochemical properties (sialidase and β-galactosidase activity) and based on findings, proposed an emended description to the *Gardnerella* genus to include a revised description of *G. vaginalis* and description of three novel species *G. leopoldii*, *G. swidsinskii* and *G. piotii*. A subsequent study published in 2021 analysed 113 *Gardnerella* genomes and using ANI analysis identified an additional *Gardnerella* species that was comprised of only one isolate (Putonti *et al.*, 2021). It is possible that as we continue to sequence and analyse *Gardnerella* isolates additional species will be revealed. Studies suggest that although horizontal gene transfer (HGT) is common among *Gardnerella* spp. (Devault *et al.*, 2017; Yeoman *et al.*, 2010), it appears to occur more frequently within a clade/species than between clades/species (Ahmed *et al.*, 2012; Bohr *et al.*, 2020). However, our understanding of the degree to which HGT occurs between *Gardnerella* spp. is limited by the number of available isolates that represent each individual species.

An important consideration for vaginal microbiota studies is that the 13 *Gardnerella* species cannot be discriminated based on the 16S rRNA gene sequence (Vaneechoutte *et al.*, 2019), Additionally, the 13 species cannot be uniquely delineated by previous subgrouping methods (Table 3) or culture methods. However, Hill *et al.* (2019) demonstrated that the 13 species can be resolved by phylogenetic analysis of a 552bp sequence of the cpn60 gene. Using cpn60 analysis, Hill *et al.* (2019) also reported a significant association between the relative abundance of and Nugent-BV and each of *G. vaginalis, G. swidsinskii* and *G. piotii*, but found no association between *G. leopoldii* and BV. Interestingly, *G. swidsinskii* and *G. leopoldii* were previously considered together as clade 4 and this may account for some of the inconsistencies in previous clade association studies.

The different *Gardnerella* spp. are thought to possess different virulence potential (Cornejo *et al.*, 2018; Janulaitiene *et al.*, 2018; Santiago *et al.*, 2011; Schellenberg *et al.*, 2016; Schuyler *et al.*, 2015). For example, ecotype 1 (i.e. *G. vaginalis* and *Gardnerella* species 2) uniquely encodes glycosidases that may aid degradation of cervicovaginal mucus (Cornejo *et al.*, 2018). Additionally, isolates from *Gardnerella* clades 1, 2 and 3 (*G. vaginalis*, *G. piotii* and *Gardnerella* species 2, 3, 8, 9, 10, 11 and 14) have been shown to possess the sialidase gene,

though not all demonstrate sialidase activity (Cornejo *et al.*, 2018; Janulaitiene *et al.*, 2018; Putonti *et al.*, 2021; Schellenberg *et al.*, 2016). Conversely, clade 4 isolates (i.e. *G. leopoldii* and *G. swidsinskii*) mostly lack the sialidase gene and have no sialidase activity (Cornejo *et al.*, 2018; Janulaitiene *et al.*, 2018; Schellenberg *et al.*, 2016). *Gardnerella* species 7, 12 and 13 also lack the gene that encodes sialidase (Putonti *et al.*, 2021). Furthermore, there are six genes involved in the mucin degradation pathway, and interestingly, only isolates belonging to *G. vaginalis* and *Gardnerella* species 2 have been shown to have all six genes present (Putonti *et al.*, 2021).

The gene that encodes vaginolysin, *vly*, is not part of the *Gardnerella* core genome (Bohr *et al.*, 2020) and given the hypothesised role of vaginolysin in BV pathogenesis, one may expect *vly* to be present in virulent *Gardnerella* species and absent from commensal/less virulent *Gardnerella* species. A recent comparative analysis of *Gardnerella* genomes demonstrated that the coding region of *vly* was present in 95 of 113 analysed genomes (Putonti *et al.*, 2021). The *vly* gene was absent from *Gardnerella* species 11 (represented by only one genome) and was present in only one of nine *G. piotii* strains. For all other *Gardnerella* species the *vly* gene was present in most genomes (Putonti *et al.*, 2021). As mentioned above, HGT appears to occur less frequently between *Gardnerella* species than within *Gardnerella* species. However, the limited evidence available suggests that *vly* may not adhere to this and may in fact be transferred between *Gardnerella* species/clades (Ahmed *et al.*, 2012; Bohr *et al.*, 2020). Interestingly, an analysis of 91 publicly available *Gardnerella* genomes identified five distinct vaginolysin types (Type 1A, 1B, 1C, 2 and 3) based on differences in the amino acid sequence (Garcia *et al.*, 2021). The five vaginolysin types were differentially distributed among *Gardnerella* spp., with clade 4 (i.e. *G. leopoldii* and *G. swidsinskii*) uniquely possessing vaginolysin type 2. Furthermore, vaginolysin type 2 was shown to induce a stronger IL-8 response compared to type 1 (Garcia *et al.*, 2021). Based on these data, the authors hypothesised that these amino acid differences may impact how vaginolysin interacts with the host, the subsequent host immune response and the development of symptoms. Further studies are needed to understand the role of vaginolysin in the pathogenesis of BV.

1.6.1.2. A final comment about *Gardnerella* taxonomy

As discussed above, several different typing/classification schemes have been applied to *G. vaginalis* in order to better understand the relationship between *G. vaginalis* and BV. More recently the taxonomy for the genus *Gardnerella* has been reorganised (Vaneechoutte *et al.*, 2019) and thirteen species have been proposed: three new species have been given official taxonomic standing (*G. swidsinskii*, *G. piotii* and *G. leopoldii*) and the description of *G. vaginalis* has been amended and narrowed. These changes to *Gardnerella* taxonomy occurred during this thesis, and for the purpose of consistency, the older broader definition of *G. vaginalis* has been used throughout this thesis, except where specified. There is still much to learn about the genetic diversity of the *Gardnerella* genus, as well as the relationship between groups of isolates and BV.

1.6.2. The role of sexual transmission in bacterial vaginosis

BV has a similar epidemiological profile to STIs. For example, a meta-analysis of 43 studies reporting sexual risk factors of prevalent, incident and/or recurrent BV, found that inconsistent condom use, as well as new and increased number of sexual partners, were associated with increased risk of BV (Fethers *et al.*, 2008). Other sexual practices have been associated with increased risk of BV including penile-vaginal sex (Cherpes *et al.*, 2008; Fethers *et al.*, 2009), increased frequency of sex (Bradshaw *et al.*, 2013a; Schwebke & Desmond, 2005; Vodstrcil *et al.*, 2019), vaginal sex after anal sex (Cherpes *et al.*, 2008), digital-vaginal sex (Muzny *et al.*, 2019b; Vodstrcil *et al.*, 2015) and digital-anal sex (Muzny *et al.*, 2019b). In addition, consistent condom use has been associated with decreased risk of BV (Calzolari *et al.*, 2000; Hutchinson *et al.*, 2007a; Schwebke & Desmond, 2005; Smart *et al.*, 2004). Furthermore, studies that have followed women longitudinally post antibiotic treatment for BV have shown that individuals who have the same sexual partner before and after antibiotic treatment have two to three fold increased risk of BV recurrence following treatment (Bradshaw *et al.*, 2006a; Bradshaw *et al.*, 2013a; Vodstrcil *et al.*, 2019).

Early age of sexual debut has been associated with BV (Fethers *et al.*, 2009; Larsson *et al.*, 1991), and the Female University Student Study (the FUSS cohort) of 528 young women found no cases of prevalent BV amongst 83 sexually inexperienced women (i.e. women without a history of coital or noncoital sexual contact) (Fethers *et al.*, 2009). Furthermore, longitudinal analysis of the FUSS cohort reported no cases of incident BV in women who did

not report having vaginal sex during the study period (0 [95% CI 0-3.2]/100 person years), and incident BV only occurred in women who were vaginally sexually active during follow-up (2.2 [95% CI 0.8-4.9]/100 person years) (Fethers *et al.*, 2011). Previous reports of BV and *G. vaginalis* in sexually inexperienced females (Bump & Buesching, 1988; Vaca *et al.*, 2010; Yen *et al.*, 2003) have been used as evidence against sexual transmission of BV; however these studies defined sexual inexperience as no self-report of penile-vaginal sex, which can be subject to miss-reporting, and did not capture information about non-coital sexual practices.

Evidence from female partnerships also provide support for sexual transmission of BV. High concordance of Nugent score categories (or BV) has been shown between female sexual partners (ranging from 70 to 95% concordance across studies) (Berger *et al.*, 1995; Bradshaw *et al.*, 2014; Evans *et al.*, 2007; Marrazzo *et al.*, 2002; Vodstrcil *et al.*, 2015). Longitudinal studies of WSW have shown that risk factors for incident BV include having a sexual partner with a history of BV, BV symptoms or microbiologically confirmed BV (Marrazzo *et al.*, 2010b; Vodstrcil *et al.*, 2015). Additionally, sharing of vaginal insertive sex toys (Marrazzo *et al.*, 2002; Marrazzo *et al.*, 2010a; Vodstrcil *et al.*, 2015) and receptive oral sex with a female partner have both been associated with an increased risk of BV (Marrazzo *et al.*, 2010b; Vodstrcil *et al.*, 2015). Furthermore, a PCR based study of 31 monogamous female couples found that both women in a couple harboured identical vaginal *Lactobacillus* strains in 77% of cases, which further supports sharing of vaginal bacteria between female sexual partners (Marrazzo *et al.*, 2009). Additionally, a recent study of 36 WSW found that incident-BV occurred at a median of four days following sexual activity, suggesting BV has an incubation period of four days which is consistent with bacterial STIs including *N. gonorrhoeae* and *C. trachomatis* (Muzny *et al.*, 2019b).

Sexual activity has also been shown to impact the vaginal microbiota composition. For example, Mitchell *et al.* (2011) investigated the impact of sexual activity on the vaginal microbiota in WSW using culture methods and found that sharing of sex toys was associated with reduced concentration of *Lactobacillus*; and conversely, digital-vaginal sex and sex toy use was associated with increased colonisation and concentration of *G. vaginalis*. Among adolescent girls in Tanzania, the presence of *A. vaginae*, *G. vaginalis* and *P. bivia* was associated with self-report of penile-vaginal sex (Francis *et al.*, 2020). Fethers *et al.* (2012) examined associations between eight BV-associated bacteria (assessed by qPCR) and sexual

practices among sexually experienced and sexually inexperienced women. The study reported that six of the eight BV-associated bacteria (*Sneathia* spp., *Leptotrichia*, BVAB1, BVAB2, BVAB3, *Megasphaera* Type 1) were rare or absent among sexually inexperienced women. Additionally, after adjusting for BV status, all eight BV-associated bacteria (except for BVAB1) were associated with increased numbers of lifetime sexual partners, and *G. vaginalis*, *Sneathia* spp., *Leptotrichia* spp. and BVAB were more commonly detected in women reporting unprotected vaginal sex compared to women not reporting this practice (Fethers *et al.*, 2012). Longitudinal studies of young women have reported changes in the vaginal microbiota composition following sexual debut, including increased prevalence of *G. vaginalis* and *A. vaginae* (Jespers *et al.*, 2016; Mitchell *et al.*, 2012). A 16S rRNA gene sequencing study of 52 women from the FUSS cohort found that women engaging in penile-vaginal sex were more likely than women not engaging in penile-vaginal sex to have a vaginal microbiota dominated by *L. iners* or *G. vaginalis* relative to a *L. crispatus* dominated microbiota (Vodstrcil *et al.*, 2017). Additionally, women reporting penile-vaginal sex were more likely to have multiple *G. vaginalis* clades rather than a single *G. vaginalis* clade present in their vaginal microbiota (Vodstrcil *et al.*, 2017). Together these data indicate that sexual practices influence the composition of the vaginal microbiota.

1.6.2.1. The male genital microbiota and a male reservoir of BV-associated bacteria

In 1955 Gardner and Dukes hypothesised that *G. vaginalis* could be sexually transmitted between heterosexual couples (Gardner & Dukes, 1955). They reported that 45 of 47 husbands of women with BV were culture positive for *G. vaginalis*, whereas of none of 20 male medical students tested were culture positive for *G. vaginalis*. Gardner and Dukes then followed 41 women longitudinal for six-months' post BV treatment, and nine women experienced BV recurrence. Six of the nine husbands were tested for *G. vaginalis* and all were culture positive. Based on these data, and the data discussed in Section 1.2.3, Gardner and Dukes hypothesised the following "while being easy to cure, [BV] can be expected to recur if husbands are not treated simultaneously." (Gardner & Dukes, 1955) Since the experimental work by Gardner and Dukes, evidence supporting a male reservoir of BV associated bacteria has continued to grow.

While there have been numerous studies investigating the microbiota composition of the female genital tract, there are fewer published studies concerning the male genital microbiota (Onywera *et al.*, 2020). *Corynebacterium* and *Staphylococcus* are considered major constituents of the penile skin microbiota and have been identified in samples collected from the coronal sulcus, glans penis, foreskin, and penile shaft (Carda-Dieguez *et al.*, 2019; Liu *et al.*, 2015; Nelson *et al.*, 2012; Price *et al.*, 2010; Zozaya *et al.*, 2016). Additionally, anaerobic BV-associated bacteria including *Finegoldia*, *Prevotella*, *Peptoniphilus*, *Anaerococcus*, *G. vaginalis*, *Porphyromonas* and *Dialister* are commonly detected in penile skin specimens (Eren *et al.*, 2011; Liu *et al.*, 2015; Liu *et al.*, 2013; Liu *et al.*, 2017; Nelson *et al.*, 2012; Plummer *et al.*, 2018a; Price *et al.*, 2010; Prodger *et al.*, 2021; Schwebke *et al.*, 2009; Zozaya *et al.*, 2016). Microbial communities have also been identified in samples from other male genital sites including the urine, urethra/meatus and semen (Dong *et al.*, 2011; Eren *et al.*, 2011; Gottschick *et al.*, 2017b; Mandar *et al.*, 2015; Mehta *et al.*, 2020a; Nelson *et al.*, 2012; Nelson *et al.*, 2010; Schwebke *et al.*, 2009; Zozaya *et al.*, 2016). Genera including *Streptococcus, Lactobacillus, Staphylococcus, Veillonella* and *Corynebacterium* are commonly recovered from male urinary and urethral/meatal samples (Dong *et al.*, 2011; Mehta *et al.*, 2020a; Nelson *et al.*, 2012; Nelson *et al.*, 2010; Pohl *et al.*, 2020; Zozaya *et al.*, 2016), as are BV-associated bacteria including *G. vaginalis*, *Prevotella* (including *P. amnii* and *P. bivia*), *Anaerococcus*, *Sneathia spp.*, *Aerococcus* and *A. vaginae* (Dong *et al.*, 2011; Eren *et al.*, 2011; Gottschick *et al.*, 2017b; Nelson *et al.*, 2012; Nelson *et al.*, 2010; Schwebke *et al.*, 2009; Zozaya *et al.*, 2016).

Interestingly, the genital microbiota composition of circumcised males has been shown to be different to that of uncircumcised males (Liu *et al.*, 2013; Mehta *et al.*, 2020b; Nelson *et al.*, 2012; Price *et al.*, 2010). For example, a large randomised controlled trial (RCT) of prospective male circumcision that was conducted in Rakai, Uganda found that the total bacterial load and bacterial diversity of the coronal sulcus significantly decreased one year after circumcision (Liu *et al.*, 2013). Additionally, the prevalence and abundance of anaerobic BV-associated bacteria (including *Peptoniphilus*, *Anaerococcus, Prevotella* and *Porphyromonas*) decreased following circumcision, whereas the prevalence of both *Corynebacterium* and *Staphylococcus* increased (Liu *et al.*, 2013). Additional studies have reported similar changes in the penile microbiota composition post circumcision (Price *et*

al., 2010), or have reported lower abundance/prevalence of anaerobic bacteria (including BV-associated bacteria) and higher abundance/prevalence of *Corynebacterium* and *Staphylococcus* among circumcised males compared to uncircumcised males (Liu *et al.*, 2016; Mehta *et al.*, 2012; Nelson *et al.*, 2012). This is of particular importance as incident-BV has been associated with having an uncircumcised male sexual partner in an observational study (Cherpes *et al.*, 2008), and a large RCT of prospective male circumcision conducted in Rakai, Uganda, found that circumcision significantly reduced the risk of BV in female sexual partners (adjusted prevalence risk ratio 0.60; 95% CI 0.38, 0.94) (Gray *et al.*, 2009).

There is evidence that the vaginal microbiota composition (and/or BV-status of a woman) may influence or be influenced by the genital microbiota composition of her male sexual partner. For example, Liu *et al.* (2015) demonstrated that male partners of women with Nugent-BV were more likely to have BV-associated bacteria present on the coronal sulcus compared to male partners of women without BV. Conversely, *Corynebacterium* and *Staphylococcus* were more prevalent in male partners of women without BV compared to male partners of women with BV. Additionally, in a recent prospective study of 168 heterosexual couples, Mehta *et al.* (2020b) reported that the baseline composition of the male urethral and cutaneous penile microbiota both accurately predicted incident Nugent-BV in female sexual partners up to six to 12 months later. Given the temporal nature of their findings, the authors hypothesised that the genital microbiota composition of men may not simply be reflective of their female partner's vaginal microbiota composition, but instead may contribute to her vaginal microbiota composition and subsequent risk of BV.

There have been a number of studies to describe the concordance and/or sharing of genital microbiota between sexual partners (Carda-Dieguez *et al.*, 2019; Eren *et al.*, 2011; Mandar *et al.*, 2015; Mehta *et al.*, 2020a; Mehta *et al.*, 2020b; Piot *et al.*, 1984; Plummer *et al.*, 2018a; Swidsinski *et al.*, 2010; Zozaya *et al.*, 2016). For example, the same *G. vaginalis* biotype was isolated from the vagina and male urethra of 11 of the 12 heterosexual couples when specimens were collected on the same or following day (Piot *et al.*, 1984). A study characterising the distribution of adherent and dispersed *G. vaginalis* in urine samples detected a *G. vaginalis* biofilm in the urine of 100% of men where their female sexual partner was positive for a *G. vaginalis* biofilm (Swidsinski *et al.*, 2010). Furthermore, a study

of 53 monogamous heterosexual couples found significant correlation in *G. vaginalis* oligotype composition between sexual partners, regardless of BV status (Eren *et al.*, 2011).

Zozaya *et al.* (2016) described the penile skin, male urethral and vaginal microbiota of 65 monogamous heterosexual couples with and without BV and reported positive correlation of specific taxa between the vagina and both the penile skin and male urethra. Of note, *Megasphaera* Type 1, *Prevotella spp.* (*Prevotella* 123-f2-42, *Prevotella* 123-f-110) and BVAB1 were highly positively correlated between the vagina and penile skin of BV-couples (ρ>0.477 and p-value<0.0001), and *Prevotella* 123-f2-42 and *P. bivia* were highly positively correlated between the vagina and male urethra of BV-couples (ρ>0.510 and p-value<0.0001). Positive correlations were also observed between bacterial communities from non-BV couples, including *Prevotella* 123-f-82, *Dialister* and both *L. crispatus* and *L. jensenii*, however the strength of these correlations was lower than what was observed in BV-couples. Similarly, a study investigating the seminal and vaginal microbiota of couples found that a large number of identified taxa (74 of 189 operational taxonomic units [OTUs]) were shared between couples and included taxa from the *Lactobacillus*, *Streptococcus*, *Veillonella, Porphyromonas* and *Atopobium* genera (Mandar *et al.*, 2015). Furthermore, a preliminary analysis of 45 couples explored the genital microbiota of women (vaginal samples) with BV and their regular male partner (pooled coronal sulcus, urethral and urine samples) found that *Sneathia* spp. and *A. vaginae* were positively correlated between partners (Muzny *et al.*, 2018).

Plummer *et al.* (2018a) investigated the vaginal and penile microbiota of 22 monogamous heterosexual couples undergoing partner treatment for BV (study described in Section 1.6.3.1), and in contrast to the findings outlined above, found no correlation of taxa between the vaginal and penile microbiota of couples prior to antibiotic treatment or immediately following treatment. However, both *Prevotella* and *Dialister* were positively correlated (ρ = 0.72 and ρ = 0.71, respectively) between the female and male genital microbiota of sexual couples at the one-month follow-up visit.

Collectively, these data suggest that multiple genital sites in men are a reservoir for BV-associated bacteria and that genital bacteria are shared or exchanged between sexual partners. These findings also highlight the role of sexual activity in BV-acquisition and

support the hypothesis that reinfection from an untreated sexual partner likely contributes to the high rates of BV recurrence seen in women.

1.6.3. Treating sexual partners of women with bacterial vaginosis

Despite the strong evidence for sexual transmission of BV, STI treatment guidelines do not recommend antibiotic treatment of male sexual partners of women who have BV (Australian Sexual Health Alliance, 2018; Hay *et al.*, 2012; Sherrard *et al.*, 2018; Workowski & Bolan, 2015), and current BV treatments are aimed solely at affected women. This is likely due to failure of prior RCTs of partner treatment for BV, as well as the absence of an equivalent clinical condition in men. Completed and ongoing partner treatment trials will be discussed below.

1.6.3.1. Male partner treatment trials

Seven RCTs of male partner treatment for BV have been completed to date (Colli *et al.*, 1997; Mengel *et al.*, 1989; Moi *et al.*, 1989; Schwebke *et al.*, 2021; Swedberg *et al.*, 1985; Vejtorp *et al.*, 1988; Vutyavanich *et al.*, 1993). Each of the RCTs competed to date including the interventions used, study findings and key limitations have been summarised in Table 4. Of the seven trials, only one reported a reduction in BV symptoms at 8-weeks post treatment among women whose partner was treated, however the study did not report point estimates (Mengel *et al.*, 1989). The remaining six trials did not find any reduction in BV recurrence following partner treatment. The first six partner treatment trials were conducted in the 1980s-1990s and have been extensively reviewed (Amaya-Guio *et al.*, 2016; Mehta, 2012; Potter, 1999). A systematic review from 2012 (Mehta, 2012) highlighted that there were significant methodological limitations with the six earliest partner treatment trials, including limited power, suboptimal treatment regimens, and lack of treatment adherence data. Further the review deemed it unlikely that the results of these trials would be considered conclusive by current standards for conducting RCTs, and that high quality, adequately powered RCTs of partner treatment RCTs are needed to accurately determine the true impact of partner treatment on BV recurrence (Mehta, 2012). Additionally, a 2016 Cochrane review of these trials concluded that partner treatment does not reduce BV recurrence; however, the review rated the quality of evidence from the past trials as being very low (Amaya-Guio *et al.*, 2016). It is important to note that the most

recently completed partner treatment trial, a well-designed trial and the first to treat both women and men with a current first-line BV-treatment (oral metronidazole 500mg twice a day for 7-days), also failed to reduce BV recurrence (Schwebke *et al.*, 2021) (Table 4). Schwebke *et al.* (2021) reported recurrence rates of 81% in women whose partners received metronidazole and 80% in women whose partners received placebo. Post-hoc analyses demonstrated significantly lower recurrence among women whose partners were 100% adherent to treatment compared to women whose partners were non-adherent, suggesting that treatment did have a benefit among adherent couples. However, even among fully adherent couples, recurrence was approximately 75%. Women had to have ≥2 BV episodes in the prior 12 months to be eligible to participate in the study by Schwebke *et al.* (2021) and authors partially attributed the high levels of recurrence to the 'heavily BV-experienced' nature of the population. They hypothesise that women likely had a BV-biofilm that was unable to be effectively cleared with metronidazole. Additionally, the authors indicated that recruited women were also heavily antibiotic-exposed, and resistance to metronidazole likely contributed to treatment failure (J. Sobel, personal communication, 21 May 2021).

Table 4 - Summary of male partner treatment trials completed to date

Author, Year	Female treatment	Male treatment	Comparator	Primary outcome and result if available	Key limitations
Swedberg *et al.* (1985)	Oral MTZ 2g single dose OR oral MTZ 500mg BID x 7d	Same treatment as female partner	No treatment	Culture negative for *G. vaginalis* and improved vaginal symptoms at 3 weeks: 68% [17/25] v 64% [25/39] RR=1.06; 95% CI 0.74, 1.52	Small sample size Un-even randomisation groups Non-standard evaluation of BV at endpoint Suboptimal treatment regimen used in one randomisation arm Adherence not reported Power/sample size calculation not reported Did not control for sexual practices in analyses
Vejtorp *et al.* (1988)	Oral MTZ 2g on d1 and d3	Oral MTZ 2g on d1 and d3	Placebo	Clinically diagnosed BV at 5 weeks: 25% [13/53] vs 29% [15/52] RR=0.85; 95% CI 0.45, 1.61	Suboptimal treatment regimen Adherence not reported Power/sample size calculation not reported Randomisation methods not reported Did not control for sexual practices in analyses
Mengel *et al.* (1989)	Oral MTZ 500mg BID x 7d OR oral	Oral MTZ 2g single dose	Placebo	Symptoms and clinical cure of BV at 2, 5, and 8 weeks. BV on vaginal Gram stain at 2 and 5 weeks.	Suboptimal treatment regimen used in one randomisation arm Incomplete reporting of endpoint results

	MTZ 2g single dose			No point estimates reported, and unable to be calculated.	Adherence not reported Power/sample size calculation not reported Did not control for sexual practices during treatment period
Moi *et al.* (1989)	Oral MTZ 2g d1 and d3	Oral MTZ 2g on d1 and d3	Placebo	Relapse of clinically diagnosed BV at 12 weeks: 21% [20/95] vs 16% [15/95] RR=1.33; 95% CI 0.73, 2.44	Suboptimal treatment regimen Adherence not reported Power/sample size calculation not reported Did not control for sexual practices in analyses
Vutyavanich *et al.* (1993)	Oral tinidazole 2g single dose	Oral tinidazole 2g single dose	Placebo	Clinical cure of BV at 4 weeks: 72% [83/116] vs 63% [74/117] RR=1.13; 95% CI 0.95-1.35	Suboptimal treatment regimen Power/sample size calculation not reported
Colli *et al.* (1997)	2% intravaginal clindamycin cream once per night x 7d	Oral clindamycin 150mg QID x 7d	Placebo	Clinically diagnosed BV recurrence at 12 weeks: 31.9% [22/69] vs 30.0% [21/70] RR= 1.06; 95% CI 0.65, 1.75	Suboptimal treatment regimen used in males Adherence was not reported for women Did not control for sexual practices in analyses
Schwebke *et al.* (2021)	Oral MTZ 500mg BID x 7d	Oral MTZ 500mg BID x 7d	Placebo	BV recurrence at 16-weeks: 81% [87/107] vs 80% (86/107) RR= 1.01; 95% CI 0.89, 1.15	Did not control for sexual practices during treatment period

MTZ, metronidazole; BID, twice per day; QID; four times per day, RR, risk ratio; CI, confidence interval; BV, bacterial vaginosis; d, days
Table adapted from "Systematic review of randomized trials of treatment of male sexual partners for improved bacteria vaginosis outcomes in women." by Mehta (2012). *Sexually Transmitted Diseases, 39*(10), 822-830; and "Should partners of women with bacterial vaginosis be treated?" by Muzny (2018). *Contemporary OB/GYN, August 2018*.

There is an obvious disconnect between the outcome of partner treatment trials conducted to date and the epidemiological and microbiological evidence (presented in Section 1.6.2) for a role of sexual transmission in the pathogenesis of recurrent BV. Factors including persistence of an established and adherent BV-biofilm, antimicrobial resistant organisms, and failure to re-colonise the vagina with beneficial lactobacilli also likely contribute to treatment failure, and these will be briefly discussed in Section 1.6.4 of this review. However, a common and important characteristic of past male partner treatment RCTs completed to date is that they have all used oral therapy only for men (Colli *et al.*, 1997; Mengel *et al.*, 1989; Moi *et al.*, 1989; Schwebke *et al.*, 2021; Swedberg *et al.*, 1985; Vejtorp *et al.*, 1988; Vutyavanich *et al.*, 1993), none have evaluated topical antibiotic therapy for males. As discussed in Section 1.6.2.1, BV-associated bacteria have been detected in multiple genitourinary sites in men, including the coronal sulcus/glans penis, the urine and distal urethra (Mehta *et al.*, 2020b; Nelson *et al.*, 2012; Zozaya *et al.*, 2016). Therefore, one may hypothesise that concurrent topical and oral antibiotic therapy would be more effective at addressing multisite carriage of BV-associated bacteria in men than oral therapy alone, and that the use of oral therapy alone may have contributed to the failure of past male partner RCTs.

There have been no published RCTs of topical penile antibiotics for male partners of women with BV. However, there has been one RCT of a topical penile microbicide (62% ethanol gel) (Bukusi *et al.*, 2011). In this study, women received metronidazole 400 mg three times daily for 7-days and male partners were randomised to receive either the intervention (62% ethanol gel applied to the penile skin at least once daily, and before and after vaginal sex) or a control group (no treatment). This study reported a non-significant increase in BV recurrence following metronidazole treatment in the intervention arm (AHR 1.39; 95% CI 0.98, 1.99), but was limited by poor adherence to the microbicide and lack of metronidazole adherence data. It should also be noted that a systematic review concluded there is insufficient evidence to support the use of antiseptics in women for BV (Verstraelen *et al.*, 2012), so perhaps it is not surprising that a topical penile microbicide did not reduce BV recurrence.

In 2018, Plummer *et al.* (2018a) presented a pilot study of 22 couples that investigated the acceptability and tolerability of combined oral and topical antimicrobial therapy of male

partners of women being treated for BV. All women received a first-line treatment for their BV and all men received both oral metronidazole 400mg twice a day *(BID)* for 7-days and 2% topical clindamycin cream which was applied to the head of the penis and upper shaft (under the foreskin if uncircumcised) *BID* for 7-days. The study reported that combined oral and topical antibiotic therapy was well tolerated and acceptable to men; minimal side effects were reported and treatment adherence was high. Couples were followed for three weeks post treatment completion and the study reported that suppression of BV-associated bacteria was sustained in 14 of 16 women, all of whom were having unprotected sex with a male partner. This study had important limitations including the single arm design, small sample size and short duration of follow-up. Additionally, suboptimal genital samples from males greatly limited the authors ability to assess the impact of antimicrobials on the microbiota of males at both the cutaneous and urethral site.

Recruitment to a RCT of combined oral and topical antimicrobial therapy of male partners of women with BV commenced in 2019 (ANZCTR: ACTRN12619000196145) (Vodstrcil *et al.*, 2020). In this trial all women receive a first-line treatment for their BV, and male partners are randomised to receive either combined oral and topical antimicrobial therapy (i.e. the same intervention utilised in Plummer *et al.* (2018a)) or no treatment (i.e. standard of care where only the female is treated). The primary outcome of this trial is BV recurrence defined as 3 or 4 Amsel criteria and a Nugent score of 4–10 within 12 weeks following randomisation. Recruitment to this study is currently ongoing.

1.6.3.2. Female partner treatment trials

Due to high concordance for BV between female sexual partners, Australian STI management guidelines recommend the assessment of female partners for BV (Australian Sexual Health Alliance, 2018). Despite this, there have been no trials of antimicrobial treatment for female partners of women with BV. This is potentially due to the ethical implications of randomising a female partner to no treatment when it is highly likely that they have BV. There has however been one study to investigate the efficacy of a behavioural intervention in preventing BV recurrence in WSW (Marrazzo *et al.*, 2011). Women with BV were randomised to either an intervention (an interview educating women about BV, BV concordance between WSW, and methods to reduce transfer of vaginal fluids to female

partners, and provision of a safe sex kit including condoms for shared vaginal sex-toy use, nitrile gloves for digital-vaginal sex and water-based lubricant) or control group (information on the importance of adhering to routine cervical screening). The study found that while women in the control group were more likely than the intervention group to report practicing receptive digital-vaginal sex without gloves, there was no difference in BV recurrence between the two groups in unadjusted analyses (HR 1.12; 95% CI 0.62, 2.01) or following adjustment for a history of BV, race and baseline report of genital-to-genital contact (HR 1.03; 95% CI 0.54, 1.97).

1.6.4. Persistence of disease and failure to recolonise with optimal *Lactobacillus*

Several studies have reported that women with a past history of BV have increased risk of BV recurrence (Bradshaw *et al.*, 2006a; Vodstrcil *et al.*, 2019; Xiao *et al.*, 2019). While this may reflect exisiting behavioural or sexual practices that place a woman at risk of recurrence (including reinfection with BV-associated bacteria following exposure to an untreated sexual partner), it may also indicate persistence of BV-bacteria or failure to recolonise the vaginal microbiota with optimal *Lactobacillus* species following treatment. Antibiotic resistance (Eschenbach, 2007), persistence or re-emergence of a dense BV-biofilm (Machado & Cerca, 2015; Swidsinski *et al.*, 2005), auto-inoculation from an endogenous source (i.e. gastrointestinal tract) (Holst, 1990; Marrazzo *et al.*, 2012), and the influence of other factors such as host genetics or immune function (Murphy & Mitchell, 2016), diet (Tuddenham *et al.*, 2019), contraceptive practices (Joesoef *et al.*, 2001), smoking (Bradshaw *et al.*, 2014; Brotman *et al.*, 2014a; Hellberg *et al.*, 2000; Mehta *et al.*, 2015) or douching (Brotman *et al.*, 2008b; Hutchinson *et al.*, 2007b; Ness *et al.*, 2002; van der Veer *et al.*, 2019) may all contribute to persistence of BV or impede recolonisation with favourable bacteria. Some of these factors will be discussed below.

There are limited data available regarding the antimicrobial resistance of BV-associated bacteria as many of these organisms are not culturable. However, intrinsic resistance to metronidazole has been documented for both *G. vaginalis* and *A. vaginae* (De Backer *et al.*, 2006; Ferris *et al.*, 2004; Kharsany *et al.*, 1993; Knupp de Souza *et al.*, 2016; Nagaraja, 2008; Schuyler *et al.*, 2015; Simoes *et al.*, 2001). One study tested the antibiotic sensitivity of 50 *G.*

vaginalis strains isolated from women and found that while 68% of the 50 strains were metronidazole resistant, 76% were sensitive to clindamycin (Nagaraja, 2008). Another study investigated the antimicrobial susceptibility of 93 clinical *G. vaginalis* isolates and reported that the majority of isolates were sensitive to metronidazole, but one isolate demonstrated high level metronidazole resistance. All 93 isolates were sensitive to clindamycin (Kharsany *et al.*, 1993). A subsequent study reported that of 204 *G. vaginalis* isolates, 93% were susceptibility to clindamycin (7% were resistant), and 26% were susceptible to metronidazole (60% were resistant and 14% demonstrated intermediate results) (Knupp de Souza *et al.*, 2016). Additionally, Schuyler *et al.* (2015) determined the metronidazole susceptibility of 87 *G. vaginalis* isolates and found resistance to be significantly associated with clade type; all clade 3 (n=14) and clade 4 (n=22) isolates analysed were intrinsically resistant to metronidazole, whereas only a small number of clade 1 (n=16/37) and clade 2 (n=1/14) isolates were metronidazole resistant. Resistance of anaerobic organisms to clindamycin has also been documented. In a study by Beigi *et al.* (2004), 119 women with BV were randomised to receive either intravaginal metronidazole or intravaginal clindamycin. Although the authors reported no difference in BV-cure between treatment arms, they found that 16% of anaerobic organisms isolated from women pre-treatment were resistant to clindamycin as were 59% of organisms isolated post-treatment. In contrast, resistance to metronidazole was uncommon. Furthermore, the authors noted that at 70-90 days post-treatment, clindamycin resistant anaerobic bacteria were more common in women who were treated with clindamycin *vs* metronidazole (approximately 80% and 30%, respectively) (Beigi *et al.*, 2004). An additional study from this cohort reported that of 865 isolates of anaerobic Gram-negative rods from women pre- and post-treatment, 0.3% were resistant to metronidazole (Austin *et al.*, 2005). Organisms isolated in this study were *Prevotella* spp. (*P. bivia* and both non-pigmented and black-pigmented *Prevotella* spp.), *Porphyromonas* spp. and *Bacteroides* spp. Together, these data highlight that antimicrobial resistance to both first-line treatment options for BV is not insignificant, and that it may be a contributing factor to treatment failure.

Adherent vaginal biofilms comprised predominately *G. vaginalis* (and other BV-associated bacteria including *A. vaginae*) are present in women with BV and absent from women without BV (Swidsinski *et al.*, 2005), and likely contribute to treatment failure. Dense

biofilms act as a barrier to reduce antibiotic penetration, and quorum sensing between organisms in the biofilm can result in activation of resistance mechanisms (i.e. expression of efflux pumps) (Muzny & Schwebke, 2015; Zhao *et al.*, 2020). As discussed in Section 1.6.1, BV-biofilms have been shown to persist following metronidazole treatment (Swidsinski *et al.*, 2008). Additionally, although *G. vaginalis* biofilms can be temporarily inactivated by moxifloxacin, they reappear 10-12 weeks post-treatment (Swidsinski *et al.*, 2011). An *in vitro* study determined the antimicrobial susceptibility of planktonic and biofilm-forming *G. vaginalis* isolates and found that the minimum inhibitory concentration of both metronidazole and clindamycin was higher for biofilm-forming isolates compared to planktonic isolates (Li *et al.*, 2020). Consistent with this, although metronidazole has been shown to prevent *G. vaginalis* biofilm formation *in vitro*, it has limited ability to disrupt established *G. vaginalis* biofilms (Gottschick *et al.*, 2016). Therefore, antibiofilm agents (i.e. biofilm disrupters and quorum sensing inhibitors) may be required to effectively treat BV (Muzny & Schwebke, 2015), particularly in the setting of highly persistent disease.

Contraceptive practices may influence to the risk of post-treatment BV recurrence in a number of ways. It is plausible that BV-associated bacteria form a biofilm on intrauterine devices (IUD), much in the same way that bacteria adhere to and form biofilms on other medical devices such as catheters and prosthetic joints (Donlan, 2001; Wu *et al.*, 2015). A study investigating the microbiological profile of IUDs removed from 100 women found that 76% of removed IUDs were positive for at least one BV-associated bacteria (*G. vaginalis, A. vaginae, Mobiluncus* spp., or *Ureaplasma urealyticum*) using PCR (Adam *et al.*, 2018). *G. vaginalis* was the most frequently detected BV-associated bacteria (present on 62% of removed IUDs), followed by *A. vaginae* (present on 32%). However, published studies have failed to consistently identify an association between IUD use and BV, with some studies reporting increased risk of BV and/or increased abundance of BV-associated bacteria in IUD users (Achilles *et al.*, 2018; Avonts *et al.*, 1990; Brooks *et al.*, 2017b; Joesoef *et al.*, 2001; Moi, 1990; Peebles *et al.*, 2020), and others finding no association (Bassis *et al.*, 2017; Donders *et al.*, 2011; Lessard *et al.*, 2008). Inconsistencies across studies may be due to the type of IUD used. Achilles *et al.* (2018) described the impact of contraception initiation on the vaginal microbiota of 266 women over a six-month period. Women selected their method of contraception from six options: copper IUD, hormonal injection (depot

medroxyprogesterone acetate, norethisterone enanthate, or medroxyprogesterone acetate and ethinylestradiol) and hormonal implant (levonorgestrel or etonogestrel). While the prevalence of BV increased significantly overtime in women using copper IUDs, it did not change overtime in hormonal contraception users. Furthermore, the log concentration of *G. vaginalis* and *A. vaginalis* significantly increased in copper IUD users at 30, 90 and 180 days following IUD insertion, but the concentration of BV-associated bacteria was not affected by hormonal methods of contraception. In contrast, a small longitudinal study of 11 women reported a modest increase in the relative abundance of *L. crispatus* after placement of levonorgestrel IUD (Jacobson *et al.*, 2014).

It has been hypothesised that bleeding may mediate the association between IUD use and BV (Madden *et al.*, 2012). Heavy menstrual bleeding is a frequently reported side effect following copper IUD insertion (Bateson *et al.*, 2016; Rezk *et al.*, 2019) and unscheduled bleeding is also commonly reported following insertion of hormonal IUDs (Donders *et al.*, 2017a; Grunloh *et al.*, 2013; Zigler & McNicholas, 2017). However, unscheduled bleeding typically resolves after 3-6 months of hormonal IUD use (Zigler & McNicholas, 2017), and after 6–12 months of copper IUD use (Sanders *et al.*, 2018). Additionally, most hormonal IUD users report reduced duration and volume of menstrual bleeding 12 months after device insertion (Zigler & McNicholas, 2017). The composition of the vaginal microbiota has been shown to vary over the menstrual cycle (Gajer *et al.*, 2012; Keane *et al.*, 1997; Srinivasan *et al.*, 2010; van der Veer *et al.*, 2019), with higher concentration/abundance of non-*Lactobacillus* spp. during menses (Eschenbach *et al.*, 2000; Schwebke *et al.*, 1997; Srinivasan *et al.*, 2010). Additionally, BV has been reported to occur more frequently during the first week of the menstrual cycle when there is bleeding and oestrogen levels are reduced (Holzman *et al.*, 2001). Menstrual blood increases the vaginal pH (Eschenbach *et al.*, 2000) which may favour growth of bacteria other than *Lactobacillus*, and lactobacilli adhere to red blood cells which may result in loss of lactobacilli during menstruation (Madden *et al.*, 2012). In addition, some BV-associated bacteria including *G. vaginalis* utilise blood for growth (Onderdonk *et al.*, 2016). As such, it is possible that IUDs may encourage growth of BV-associated bacteria via increased vaginal bleeding. However, the mechanisms underlying the association between IUD use and BV risk needs further investigation.

Hormonal contraception may have a beneficial effect on the vaginal microbiota. A systematic review and meta-analysis found that hormonal contraceptive use was associated with a reduced risk of prevalent BV (pooled effect size [pES] 0.68; 95% CI 0.63, 0.73), incident BV (pES = 0.82; 95% CI 0.72, 0.92), and recurrent BV (pES 0.69; 95% CI 0.59, 0.91) (Vodstrcil *et al.*, 2013). Interestingly, a positive effect of hormonal contraception was observed for both combined oral contraceptives (COC) and progesterone only containing contraceptives (Vodstrcil *et al.*, 2013). Oestrogen containing contraceptives may support a *Lactobacillus* dominated microbiota and reduce the risk of BV recurrence through increasing vaginal glycogen levels (due to increased levels of oestrogen), which may subsequently increase *Lactobacillus* levels, as described in Section 1.4. However, a recent pilot RCT investigating the efficacy of COC for reducing BV recurrence following antibiotic treatment reported that COC use did not significantly reduce BV recurrence (Vodstrcil *et al.*, 2019); although the study was impacted by a small sample size and uneven attrition. Interestingly, having the same sexual partner pre/post-treatment and having a history of BV were both associated with an increased risk of BV recurrence, suggesting that these well-known risk-factors for BV may overshadow any beneficial effect of COC use on the vaginal microbiota composition (Vodstrcil *et al.*, 2019).

Together, these data suggest that the pathogenesis of BV is complex and likely to be multifactorial, and that a combination of treatment approaches involving partner treatment, biofilm disruption and restoration and support of an optimal *Lactobacillus* dominated vaginal microbiota (through the use of hormonal contraceptives, *Lactobacillus* containing probiotics, vaginal acidifying agents i.e. lactic acid and/or prebiotics) may collectively be required to effectively treat BV and prevent post-treatment recurrence (Bradshaw & Brotman, 2015; Bradshaw & Sobel, 2016; Unemo *et al.*, 2017).

1.7. Literature review summary

As outlined in the literature review, new and more effective treatments for BV are needed to reduce the serious sequelae associated with BV and the repeated antibiotic use that result from multiple recurrences. The evidence suggests that while the pathogenesis of post-treatment BV recurrence is likely to be multifactorial, sexual transmission of BV-associated bacteria has an important role in incident and recurrent BV. Importantly, the

contribution of sexual transmission to BV incidence and recurrence may vary between women. This thesis aimed to investigate the pathogenesis of BV with a focus on sexual transmission. Additional aims of this thesis were to investigate how behavioural, sexual, and microbiological factors influence the composition and stability of the vaginal microbiota.

Chapter 2. The impact of sexual practices on the composition of the vaginal microbiota of women who have sex with women

2.1. Overview

Studies consistently report a higher prevalence of BV among WSW compared to women who exclusively report sex with men (Bradshaw *et al.*, 2013b; Evans *et al.*, 2007; Koumans *et al.*, 2007; Smart *et al.*, 2004). It is not known if the high prevalence of BV in WSW is due to specific sexual practices, or other factors. Section 1.6.2 of the literature review discusses the impact of sexual practices on the composition of the vaginal microbiota. However, there have been limited studies investigating how sexual practices influence or alter the vaginal microbiota composition of WSW. Given the burden of BV among WSW, it is important to understand how sexual and behavioural practices alter the vaginal microbiota to inform prevention and treatment approaches for non-optimal microbiota in this population.

Chapter 2 aims to address this gap in the literature and presents a detailed analysis of the impact of sexual practices on the composition of the vaginal microbiota of 100 WSW. Women in this study were participants in a two-year longitudinal cohort study of Australian WSW designed investigate epidemiological and microbiological factors associated with incident BV (the Women on Women's [WOW] Health Cohort Study) (Vodstrcil *et al.*, 2015).

Chapter 2 is presented as the accepted version of the following peer reviewed manuscript. No alterations to the text have been made, except that abbreviations, and figure and table numbers have been adjusted to generate a consistent presentation within this thesis. Additionally, the methods section has been moved to appear before the results to be consistent with other chapters within this thesis.

Publication:

> **Plummer EL***, Vodstrcil LA*, Fairley CK, Tabrizi SN, Garland SM, Law MG, Hocking JS, Fethers KA, Bulach DM, Murray GL^, Bradshaw CS^ (2019). Sexual practices have a significant impact on the vaginal microbiota of women who have sex with women. ***Sci Rep*** 9 (1):19749.

(* joint first authors; ^ joint senior authors)

Findings from this manuscript have also been presented at the following national and international conferences:

- Keystone Symposia on the "Role of the Genital Tract Microbiome in Sexual and Reproductive Health" in Cape Town, Western Cape, South Africa, 11 Dec – 15 Dec 2018. [Oral and poster]
- IUSTI [International Union against Sexually Transmitted Infections] Asia Pacific Sexual Health Congress Auckland, New Zealand, 1 Nov – 3 Nov 2018. [Oral]
- Royal Women's Hospital Research Week, Melbourne, Australia, 19 Nov-21 Nov 2018. [Oral/poster] **Post graduate student oral presentation winner**
- Central Clinical School Postgraduate Symposium, Melbourne, Australia, 12 Nov 2018. [Oral]
- VIIN Young Investigator Symposium, Parkville, Australia, 18 October 2018. [Oral]

All supplementary files referred to in this chapter have been appended to this thesis in Appendix A.

A PDF of the published manuscript is in Appendix E.

2.2. Abstract

Women-who-have-sex-with-women (WSW) are at increased risk of BV. We investigated the impact of practices and past BV on the vaginal microbiota within a two-year longitudinal cohort of Australian WSW. Self-collected vaginal swabs were used to characterise the vaginal microbiota using 16S rRNA gene sequencing. Hierarchical clustering defined CSTs. Bacterial diversity was calculated using the Shannon diversity index and instability of the vaginal microbiota was assessed by change of CST and Bray-Curtis dissimilarity. Sex with a new partner increased the bacterial diversity (adjusted-coefficient 0.41; 95% CI 0.21, 0.60; p<0.001) and instability of the vaginal microbiota, in terms of both change of CST (adjusted-OR 2.65; 95% CI 1.34, 5.22; p=0.005) and increased Bray-Curtis dissimilarity (adjusted-coefficient 0.21; 95% CI 0.11, 0.31; p<0.001). Women reporting sex with a new partner were

more likely than women reporting no new partner to have a vaginal microbiota characterised by *Gardnerella vaginalis* (adjusted-relative-risk-ratio[aRRR] 3.45; 95% CI 1.42, 8.41; p=0.006) or anaerobic BV-associated bacteria (aRRR 3.62; 95% CI 1.43, 9.14; p=0.007) relative to a *Lactobacillus crispatus* dominated microbiota. Sex with a new partner altered the vaginal microbiota of WSW by increasing the diversity and abundance of BV-associated bacteria. These findings highlight the influence of practices on the development of a non-optimal vaginal microbiota and provide microbiological support for the sexual exchange of bacteria between women.

2.3. Introduction

The vaginal microbiota has an important role in protecting against a range of adverse obstetric and gynaecological outcomes including miscarriage, preterm birth, and transmission and acquisition of STIs and HIV (Brotman *et al.*, 2010; Cohen *et al.*, 2012; Koumans *et al.*, 1999; Myer *et al.*, 2005). The optimal vaginal microbiota of reproductive aged women is typically characterised by low bacterial diversity and high relative abundance of *Lactobacillus* spp., commonly *Lactobacillus crispatus* (Fredricks *et al.*, 2005; Ravel *et al.*, 2011; Verhelst *et al.*, 2005).

BV is the most common vaginal dysbiosis and is characterised by a decrease in lactobacilli and increase in the diversity and abundance of facultative and strict anaerobic bacteria including *G. vaginalis* (Fredricks *et al.*, 2005; Nugent *et al.*, 1991; Srinivasan *et al.*, 2012). The pathogenesis of BV is complex and mounting epidemiological and microbiological evidence suggests that sexual activity has a role in both BV incidence and recurrence. Inconsistent condom use and new or increased numbers of sexual partners have been shown by meta-analysis to increase BV risk (Fethers *et al.*, 2008). Other sexual practices associated with increased risk of BV include penile-vaginal sex (Cherpes *et al.*, 2008; Fethers *et al.*, 2009), vaginal sex after anal sex (Cherpes *et al.*, 2008), receptive oral sex with a female partner (Marrazzo *et al.*, 2010b; Vodstrcil *et al.*, 2015), and shared use of sex toys between women (Marrazzo *et al.*, 2002; Marrazzo *et al.*, 2010a; Vodstrcil *et al.*, 2015). BV prevalence is high amongst WSW with estimates ranging from 25-52% (Bailey *et al.*, 2004; Berger *et al.*, 1995; Bradshaw *et al.*, 2014; Evans *et al.*, 2007; Marrazzo *et al.*, 2010a; McCaffrey *et al.*, 1999).

Whether increased prevalence of BV in WSW is due to sexual practices or other factors is not known.

A number of studies have found sexual activity is associated with disturbance of the vaginal microbiota (Gajer *et al.*, 2012; Pepin *et al.*, 2011; Schwebke *et al.*, 1999; Vodstrcil *et al.*, 2017), however there are limited data describing how specific sexual practices influence the vaginal microbiota in WSW. Mitchell *et al.* (2011) used culture methods and found that sharing of sex toys with female partners was associated with reduced concentration of *Lactobacillus,* and digital-vaginal sex and sex toy use was associated with increased colonization of *G. vaginalis*.

Understanding how specific sexual practices influence the composition of the vaginal microbiota and contribute to vaginal dysbiosis and BV is important in order to develop effective treatment and prevention strategies. The primary objective of our study was to describe the impact of sexual practices on the vaginal microbiota of a subset of women participating in a cohort of Australian WSW.

2.4. Methods

2.4.1. Participant and specimen selection

Participants were selected from the WOW Health study, a two-year cohort of 298 WSW designed to examine epidemiological and microbiological factors associated with incident BV (Bradshaw *et al.*, 2014; Vodstrcil *et al.*, 2015). Women reported a female sexual partner (FSP) within 18 months prior to enrolment and were BV negative (Nugent score <7 (Nugent *et al.*, 1991)) on three consecutive baseline vaginal smears collected one week apart. Women self-collected a vaginal swab and smear, and completed a detailed questionnaire every three months until study endpoint (diagnosis of incident BV [Nugent score =7-10] or 24 months without BV). Women were instructed to avoid specimen collection on the heaviest days of their menstrual cycle (Vodstrcil *et al.*, 2015).

For the microbiota sub-study, we included all women who developed incident BV (n=51) and an equal number of women who did not (initially controls were over-selected using a random sort command in Stata/IC (v14.2, StataCorp LP, College Station, USA)). Seven of the 51 women to develop incident BV co-enrolled in the original cohort with their FSP (Vodstrcil

et al., 2015). As such, controls were then frequency matched on co-enrolment status and age to ensure a similar distribution of both variables (for example, the last non co-enrolled control was replaced with the next randomly selected co-enrolled control). Each woman contributed a baseline specimen and an endpoint specimen (BV-specimen from women with incident BV or the 24 month specimen from women without BV). Up to three interim specimens were included for each woman (typically the last two specimens collected prior to the endpoint specimen). If a specimen could not be used/located, an earlier specimen from that participant was used.

Ethical approval was obtained from the Human Research Ethics Committees of Alfred Hospital, Melbourne, Australia and the University of Melbourne. All research was performed in accordance with the National Statement on Ethical Conduct in Human Research. Informed written consent was obtained from all participants for the use of their specimens in the current study.

2.4.2. Laboratory methods

Swabs were agitated in 1mL RNAlater (Thermo Fisher Scientific, Waltham, USA) and stored at -80°C prior to DNA extraction using the MagNA Pure 96 instrument and the DNA and Viral NA small volume kit (Roche Diagnostics, Mannheim, Germany). Dual index primers 341F/805R with heterogeneity spacers (Fadrosh *et al.*, 2014; Shipitsyna *et al.*, 2013; Sinclair *et al.*, 2015) were used for PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene. Libraries were sequenced by Micromon Genomics (Micromon, Monash University, Victoria, Australia) on the MiSeq platform (Illumina, San Diego, CA, USA). Sequence reads are available in the NCBI Sequence Read Archive under Bioproject PRJNA434520.

2.4.3. Sequence data analysis

Barcodes were extracted using QIIME v1.9.0 (Caporaso *et al.*, 2010) and demultiplexing was performed using idemp (https://github.com/yhwu/idemp). Primers and heterogeneity spacers were removed using TagCleaner standalone version 0.16 (Schmieder *et al.*, 2010). Reads were processed using DADA2 v1.6.0 (Callahan *et al.*, 2016). Reads were truncated based on quality profiles (at 250 bases for read 1 and 220 bases for read 2) and were

discarded if they had ambiguous bases or exceeded the number of expected errors based on quality scores. Chimeras were identified and removed. Taxonomy was assigned using the default RDP Classifier implemented in DADA2 and the Silva reference database (v128) (Quast *et al.*, 2013). Species level assignment was performed using exact matching in the DADA2 package and taxonomy for *Lactobacillus* spp. was confirmed by a BLAST search against a database of 16S rRNA gene sequences from 158 type strains. Not all ASVs were able to be assigned to species level.

BVAB1 has previously been misclassified as *Shuttleworthia* (Oakley *et al.*, 2008) and BVAB3 is named as *Fastidiosiplia* in the Silva database (Van Der Pol *et al.*, 2019). We aligned *Shuttleworthia* and *Fastidiosiplia* ASVs (amplicon sequence variants) against BVAB1 (NCBI GenBank AY724739.1), BVAB2 (AY724740.1) and BVAB3 (AY724741.1) using Clustal Omega (EMBL-EBI) (Chojnacki *et al.*, 2017; Sievers *et al.*, 2011). *Shuttleworthia* ASV had 100% identity to BVAB1. Two *Fastidiosiplia* ASVs had high identity to BVAB2 (99.50 and 100% identity, respectively), and a third *Fastidiosiplia* ASV had 100% identity to BVAB3. The ASVs were reclassified accordingly.

ASVs were removed if they had a total abundance of less than 0.001% or were present in only one specimen. The ASV table was screened for contaminants previously identified in negative controls (Plummer *et al.*, 2018a), as well as common sequencing contaminants (removed ASVs belonging to *Facklamia* and *Shewanella* genera and Halomonadaceae family) (Jervis-Bardy *et al.*, 2015; Salter *et al.*, 2014). Specimens with fewer than 1000 reads were excluded from analysis. Participants were excluded if they did not have an enrolment specimen or did not have any follow-up specimens.

Diversity metrics and CST were generated using the Vegan package (Oksanen *et al.*, 2020) and R Studio [V 1.1.419, Boston, USA] employing R v3.4.3. Alpha diversity was calculated using the Shannon Diversity Index using ASV data. ASVs assigned to the same taxonomy were merged and the relative abundance of each taxon was used for CST identification. Hierarchical clustering of Euclidean distances with Ward linkage was performed on the relative abundance of each taxon and a scree plot of within cluster distances was used to inform the number of CSTs. Bray-Curtis dissimilarity scores were calculated between consecutive paired specimens from each participant. The heatmap was generated using the

ComplexHeatmap package (Gu *et al.*, 2016) and the same metrics used to identify CSTs. Change of CST was defined as change or no change in CST between consecutive paired specimens.

2.4.4. Statistical analysis

Statistical models that accounted for repeated measures within individuals were fitted using generalised estimating equations (GEE) to investigate the impact of characteristics and practices on the diversity (Shannon-Diversity Index) and instability (change of CST or Bray-Curtis dissimilarity) of the vaginal microbiota. GEE linear regression analyses were used when the outcome was Shannon-Diversity Index or Bray-Curtis dissimilarity, with the regression coefficient representing the mean difference of each outcome between the reference and comparison group/s for each characteristic/practice investigated. GEE logistic regression was used when change of CST was the outcome. Characteristics and practices deemed significant in univariate analyses ($p<0.05$) were included in multivariable analyses.

We also analysed the type of CST change observed. Specimens were allocated one of four change type between sequential specimens: 1) no change; 2) change from one *Lactobacillus* CST to another *Lactobacillus* CST; 3) change from one *Lactobacillus* CST to a non-*Lactobacillus* CST; or 4) change from a non-*Lactobacillus* CST to any other CST. Multinomial regression was used to investigate the relationship between practices and type of CST change relative to the risk of no change, generating relative risk ratios and 95% confidence intervals.

Multinomial regression was also used to assess associations between characteristics and microbiota composition (i.e. CST-classification of a sample). CST1-*L. crispatus* was the reference group for all analyses. This analysis calculated the risk of having a vaginal microbiota of a specific CST (details of CSTs provided in results below) compared to the risk of a vaginal microbiota of CST1, clustering for multiple samples from individual participants.

Characteristics and practices deemed significant in univariate analyses ($p<0.05$) were included in multivariable analyses. Statistical analyses were performed using STATA v14.2, unless otherwise specified.

2.4.5. Availability of data and materials

The raw sequencing data are publicly available in the NCBI Sequence Read Archive under the Bioproject number PRJNA434520.

2.5. Results

2.5.1. Description of participants at baseline and longitudinally

Baseline characteristics and sexual practices of participants are summarised in Table 5. Specimens from 102 women were initially selected for inclusion in the study; however, two were removed post quality control of sequencing data (as described below), leaving 100 women in the study population. The median age of participants at enrolment was 28 years (IQR 24-37 years). Most women were Australian born (86%), had tertiary level education (81%) and had a FSP at enrolment (72%). Twenty-two percent of women reported a past history of BV.

Table 5 - Characteristics of study participants at baseline

Characteristic	Total (N=100)
Age[a]	
≤28	52
>28	48
Country of Birth[b]	
Australia	86
Other	14
Self-reported past history of BV	
No	78
Yes	22
Douching (ever)[c]	
No	79
Yes	20
Baseline sexual practices	
Current regular FSP	
No[d]	28
Yes	72
Number of FSPs in previous 12 months[a]	
≤1	60
>1	40

Ever had vaginal sex with a man	
No	26
Yes[e]	74
Community State Type at baseline	
CST1-*L. crispatus*	41
CST2-*Lactobacillus* mixed	19
CST3-*L. iners*	30
CST4-*G. vaginalis* and diverse	3
CST5- anaerobic and diverse[f]	7

Abbreviations: BV, bacterial vaginosis; FSP, female sexual partner;
[a] Continuous variables dichotomised at median value
[b] n=81 women reported Australian or English ethnicity, n=11 reported a European ethnicity, n=8 reported a non-European ethnicity (other ethnicities reported were Chinese, Malaysian/Indian, Indian, Israeli, Chilean).
[c] Data missing from one participant
[d] n=6 women reported a current MSP (male sexual partner) at baseline
[e] n=47 women reported ≥1 MSP in previous 12 months
[f] The top five most prevalent taxa in CST5: *Dialister* spp., *Prevotella* spp., *G. vaginalis*, *L. iners* and *Peptoniphilus* spp.

Longitudinally, most women reported receiving oral sex from an FSP (85%) and use of sex toys with an FSP (72%). Fourteen women (14%) reported vaginal sex with a male during the study period. Forty women (40%) reported sex with a new partner in one or more interval (25 women reported one new sexual partner and 15 women two or more new partners over the study period). New partners were predominantly female; 28 women reported a female new partner/s, three women reported having a male new partner/s and nine women reported both female and male new partners.

A total of 372 specimens from 102 women underwent sequencing and 5, 061, 171 sequence reads were generated. Following quality control, 4, 942, 634 reads representing 393 ASVs remained. Nine specimens had <1000 reads and were excluded; consequently, two participants were excluded from analysis as one did not have an enrolment specimen and one did not have longitudinal specimens post quality control. Thus, a total of 360 specimens from 100 women were included in analyses. This included 100 enrolment specimens and 260 longitudinal specimens, 47 of which represented incident BV. The median number of reads per specimen was 12, 504 (IQR 7, 460-18, 344).

2.5.2. Vaginal community state types

Hierarchical clustering identified eight CSTs, Figure 5. Five CSTs were characterised by *Lactobacillus*: CST1-*L. crispatus* (n=152 specimens), CST2-*Lactobacillus* mixed (comprised of *L. crispatus* and *L. iners;* n=29), and CST3-*L. iners* (n=93), CST6-*L. gasseri* (n=5), CST8-*L. jensenii/L. fornicalis* (n=10). The remaining three CSTs were: CST4-*G. vaginalis* and diverse (n=40 specimens), CST5-anaerobic and diverse (n=28), CST7-*Bifidobacterium longum* (n=3). The five most prevalent taxa identified in specimens in CST5 were BV-associated bacteria *Dialister* spp., *Prevotella* spp., *G. vaginalis*, *L. iners* and *Peptoniphilus* spp. For analysis purposes, the two small *Lactobacillus* CSTs (CST6 and CST8) were combined with CST2-*Lactobacillus* mixed, and CST7-*B. longum* was combined with other anaerobic dominated specimens in CST5-anaerobic and diverse.

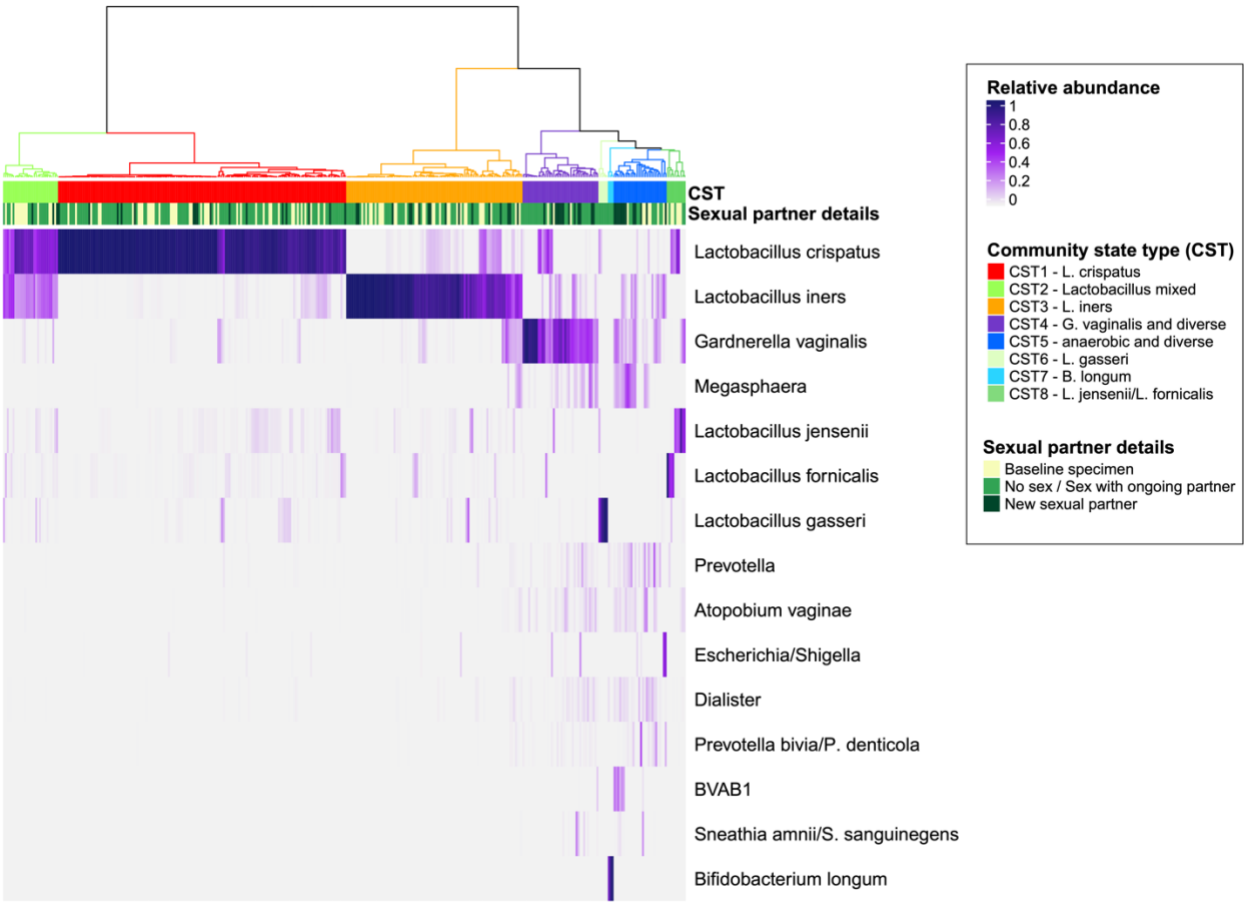


Figure 5. The vaginal microbiota of women who have sex with women

The heatmap displays the relative abundance of the 15 most abundant bacteria detected in women in this study. Hierarchical clustering of Euclidean distances with Ward linkage was

used to determine eight CSTs: CST1-L. crispatus, CST2-Lactobacillus mixed, and CST3-L. iners (n=93), CST4-G. vaginalis and diverse, CST5-anaerobic and diverse, CST6-L. gasseri, CST7-Bifidobacterium longum, CST8-L. jensenii/L. fornicalis. Exposure to a new sexual partner in the preceding 90 days is indicated above the heatmap.

All women at baseline had normal (Nugent score=0-3, n=92/100) or intermediate microbiota (Nugent score =4-6, n=8/100) by the Nugent score method (Nugent *et al.*, 1991). Most women (n=90) clustered into a *Lactobacillus* dominated CST (CST1-*L. crispatus* (n=41), CST2-*Lactobacillus* mixed (n=19) and CST3-*L. iners* (n=30)).

Of the longitudinal specimens with normal (Nugent score=0-3, n=204) and intermediate (Nugent score=4-6, n=9) microbiota, most (89%) clustered to a *Lactobacillus* CST [CST1-*L. crispatus* (n=111/213, 52%), CST2-*Lactobacillus* mixed (n=24/213; 11%) and CST3-*L. iners* (n=55/213; 26%)]. The majority of incident BV specimens (Nugent score=7-10, n=47) clustered with CST4-*G. vaginalis* and diverse (n=26/47; 55%) and CST5-anaerobic and diverse (n=12/47; 26%).

2.5.3. Factors associated with vaginal microbiota diversity

In univariate analyses, sex with a new partner compared with no sex or sex in an ongoing relationship (defined as relationship for >3 months) was significantly associated with increased bacterial diversity of the vaginal microbiota (coefficient 0.49, 95% CI 0.30-0.68, p<0.001; Table 6). Other characteristics associated with increased microbiota diversity included self-reported past history of BV, smoking, having two or more sexual partners in a study interval (i.e. the period of time between two specimen collections), frequent sexual activity (several times/week), receptive oral sex from any FSP and self-report of BV symptoms (abnormal vaginal odour and/or vaginal discharge; Table 6). Douching and sharing of sex toys had a borderline association with increased diversity.

We included sex with a new partner, frequency of sex, smoking, oral sex and past history of BV in a multivariable model (Table 6). Digital anal sex was not included in adjusted analyses to limit over-fitting the model. Sex with a new partner (adjusted coefficient 0.41, 95% CI 0.21-0.60, p<0.001), frequent sex (adj. coefficient 0.20, 95% CI 0.00-0.41, p=0.049) and past history of BV (adj. coefficient 0.26, 95% CI 0.04-0.48, p=0.018) were significantly associated

with increased diversity of the vaginal microbiota. Smoking and receptive oral sex with an FSP were not associated with diversity adjusted analyses.

To explore the relationship between oral sex, exposure to a new sexual partner, and microbiota diversity, we investigated 1) the impact of new partner exposure on diversity stratified by receptive oral sex, and 2) investigated the interaction between new partner exposure and receptive oral sex. Although new partner exposure was significantly associated with microbiota diversity in women reporting oral sex and not in women who did not practice oral sex (Supplementary Table A1), the 95% confidence intervals overlapped, suggesting no statistical difference in the effect of new partner by oral sex, and furthermore there was no evidence of interaction when formally tested (p=0.110).

Table 6 - Characteristics and sexual practices associated with bacterial diversity of the vaginal microbiota

Characteristic	n women reporting exposure, n intervals exposure reported (N=100 women and 360 intervals)	Coeff. (95% CI) [a]	*P* value[a]	Adj Coeff. (95% CI) [b]	*P* value[b]
Self-reported past history of BV					
No	78, 285	ref		ref	
Yes	22, 75	0.28 (0.06, 0.50)	**0.013**	0.26 (0.04, 0.48)	**0.018**
Longitudinal characteristics[c]					
Any smoking[d, e]					
No	47, 210	ref		ref	
Yes	53, 149	0.18 (0.01, 0.35)	**0.036**	0.08(-0.09, 0.25)	0.352
Any douching[e]					
No	94, 351	ref			
Yes	6, 8	0.50 (-0.02, 1.01)	0.060		
Number of SP					
0	3, 38[f]	ref			
1	67, 270[f]	0.03 (-0.23, 0.29)	0.818		
≥2	30, 52[f]	0.36 (0.04, 0.67)	**0.027**		
Frequency of sex					
Once/month or less	14, 118[g]	ref		ref	
Several times/month	36, 131[g]	0.17 (-0.02, 0.36)	0.074	0.13 (-0.05, 0.31)	0.164
Several times/week	50, 111[g]	0.30 (0.10, 0.51)	**0.003**	0.20 (0.00, 0.41)	**0.049**
Sex with NP[h]					

No	60, 300	ref		ref	
Yes	40, 60	0.49 (0.30, 0.68)	**<0.001**	0.41 (0.21, 0.60)	**<0.001**
Sexual practices with an FSP[i]					
Any receptive oral vaginal sex					
No[j, e]	15, 138	ref		ref	
Yes	85, 221	0.24 (0.08, 0.40)	**0.003**	0.15 (-0.02, 0.31)	0.076
Any digital anal sex					
No[j, e]	77, 317	ref			
Yes	23, 41	0.32 (0.08, 0.57)	**0.010**		
Sharing of sex toys[e]					
No toys/washed/condoms used[j]	58, 272	ref			
Unwashed	42, 87	0.18 (-0.00, 0.37)	0.051		
Sexual practices with an MSP[k]					
Any vaginal sex					
No[l, e]	86, 321	ref			
Yes	14, 38	0.07 (-0.21, 0.35)	0.637		
Self-reported symptoms					
Self-reported abnormal vaginal discharge and/or odour					
No	72, 317	ref			
Yes	28, 43	0.32 (0.09, 0.55)	**0.007**		

Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to FSP or MSP); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner

Missing data for variables included in this analysis occurred in <0.5% of intervals.

[a] Univariate GEE linear regression, where participant ID is panel variable. The regression coefficient represents the mean difference of Shannon diversity between the reference and comparison group/s for each characteristic/practice investigated.
[b] Multivariable GEE linear regression, where participant ID is panel variable.
[c] Longitudinal characteristics were measured as any exposure over the prior study interval (~90 days). No significant associations were identified between Shannon diversity and either hormonal contraceptive use or recent menses.
[d] There was no dose-response relationship between smoking and Shannon diversity
[e] Missing data from a maximum of two intervals for some variables
[f] For women reporting different numbers of sexual partners in two or more intervals, the category representing the highest number of sexual partners has been used to calculate n women reporting exposure.
[g] For women reporting different frequencies of sexual activity in two or more intervals, the most frequent category has been used to calculate n women reporting exposure.
[h] Sex with a new partner with who first sexual contact was within 90 days
[i] No significant associations were identified between Shannon diversity and the following sexual practices with an FSP: Any digital-vaginal sex from an FSP, any receptive oral anal sex from an FSP and current FSP with BV symptoms. These practices have been left out to simplify the table.
[j] Or did not have a FSP
[k] No significant associations were identified between Shannon diversity and the following sexual practices with an MSP: Condoms used for vaginal sex, vaginal sex after anal sex, any receptive oral vaginal sex from an MSP, any digital-vaginal sex from an MSP, any anal sex from an MSP. These practices have been left out to simplify the table.
[l] Or did not have a MSP

2.5.4. Factors associated with instability of the vaginal microbiota

Compositional change (instability) was measured by change of CST and Bray-Curtis dissimilarity score, calculated between consecutive longitudinal specimens.

Eighty-three women (83%) experienced at least one change of CST during the study period, accounting for 138 instances of CST change. Interestingly, changing between different *Lactobacillus* CSTs (n=66/138, 48% of all CST changes) was as common as changing from a *Lactobacillus* CST to CST4-*G. vaginalis* and diverse or CST5-anaerobic and diverse (n=50/138, 36%).

Practices significantly associated with change of CST by univariate analysis (smoking and sex with a new partner) were included in a multivariable model with CST of index specimen (i.e. the first specimen of each consecutive pair; Table 7). In the adjusted analysis, sex with a new partner (adjusted odds ratio [AOR] 2.65, 95% CI 1.34, 5.22, p=0.005) and smoking (AOR 1.79, 95% CI 1.03, 3.11, p=0.039) were both associated with an increased odds of change of CST when adjusted for CST of index specimen. Additionally, women with a vaginal microbiota classified as CST2-*Lactobacillus* mixed (AOR 6.65, 95% CI 2.81, 15.76, p<0.001), CST3-*L. iners* (AOR 3.13, 95% CI 1.67, 5.87, p<0.001) or CST5-anaerobic and diverse (AOR 13.18, 95% CI 2.83, 61.31, p<0.001) were more likely to change CST in the next interval compared with women with a vaginal microbiota of CST1-*L. crispatus*. Having a CST4-*G. vaginalis* and diverse microbiota was not significantly associated with change of CST, likely because the majority of CST4 samples represented an endpoint specimen i.e. incident BV (n=26/40, 65%).

Table 7 -Characteristics and sexual practices associated with instability of the vaginal microbiota as measured by change of CST between consecutive specimens

Characteristic	OR (95% CI)	*P* value[a]	AOR (95% CI)	*P* value[b]
Self-reported past history of BV				
No	1			
Yes	1.21 (0.64, 2.29)	0.553		
Longitudinal practices[cd]				
Any smoking[e]				
No	1		1	

Yes	2.10 (1.25, 3.54)	**0.005**	1.79 (1.03, 3.11)	**0.039**
No. of cigarettes smoked				
0/non-smoker	1			
1-7	1.98 (0.87, 4.49)	0.104		
8+	1.61 (0.78, 3.33)	0.198		
Number of SP				
0	1			
1	0.74 (0.33, 1.67)	0.464		
≥2	2.12 (0.73, 6.14)	0.165		
Frequency of sex				
Once/month or less	1			
Several times/month	0.98 (0.54, 1.77)	0.948		
Several times/week	1.59 (0.84, 3.11)	0.152		
Sex with NP[e]				
No	1		1	
Yes	2.56 (1.37, 4.81)	**0.003**	2.65 (1.34, 5.22)	**0.005**
Sexual practices with FSP[f]				
Any receptive oral vaginal sex				
No[g]	1			
Yes	1.59 (0.96, 2.66)	0.074		
Sharing of sex toys				
No toys/washed/condoms used[g]	1			
Unwashed	1.50 (0.83, 2.72)	0.182		
Sexual Practices with an MSP[h]				
Any vaginal sex				
No[i]	1			
Yes	0.72 (0.32, 1.63)	0.435		
Self-reported symptoms and microbiota characteristics				
Self-reported abnormal vaginal discharge and/or odour				
No	1			
Yes	1.04 (0.49, 2.22)	0.917		
Shannon diversity	1.53 (1.02, 2.28)	**0.038**		
Community state type of index specimen[j]				
CSTI- *L. crispatus*	1		1	
CST2-*Lactobacillus* mixed	5.71 (2.47, 13.15)	**<0.001**	6.65 (2.81, 15.76)	**<0.001**
CST3-*L. iners*	2.81 (1.54, 5.12)	**0.001**	3.13 (1.67, 5.87)	**<0.001**
CST4-*G. vaginalis* and diverse[k]	1.57 (0.50, 4.95)	0.440	1.57 (0.48, 5.17)	0.457
CST5- anaerobic and diverse	14.22 (3.13,	**<0.001**	13.18 (2.83,	**<0.001**

	64.69)	61.31)

Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to female or male partner); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner

Missing data for variables included in this analysis occurred in <0.5% of intervals.

[a] Univariate GEE logistic regression clustered for multiple specimens from each participant

[b] Multivariable GEE logistic regression clustered for multiple specimens from each participant

[c] Longitudinal characteristics were measured as any exposure over the prior study interval (~90 days). No significant associations were identified between change of CST and either hormonal contraceptive use or recent menses.

[d] Douching omitted from table due to collinearity – all intervals of douching (n=5) were accompanied by a change of CST

[e] Sex with a new partner with who first sexual contact was within 90 days

[f] The following sexual practices/characteristics with an FSP were left out of the table for simplicity: digital-vaginal sex, receptive oral anal sex, digital anal sex, and current partner with BV symptoms. No significant associations between change of CST and these sexual practices were identified.

[g] Or did not have a FSP

[h] The following sexual practices/characteristics with an MSP were left out of the table for simplicity: condoms use for vaginal sex, anal sex, vaginal sex after anal sex, oral vaginal sex and digital-vaginal sex. No significant associations between change of CST and these sexual practices were identified.

[i] Or did not have a MSP

[j] Index specimen refers to the first specimen of each consecutive pair

[k] Majority of CST4 specimens are endpoint specimens which do not have accompanying change of CST information

By multinomial regression, women reporting sex with a new partner were more likely than women without a new partner to change from a *Lactobacillus* CST (i.e. CST1/2/3) to a non-*Lactobacillus* dominated CST relative to not changing CST (relative risk ratio [RRR] 4.18, 95% CI 2.06, 8.50, p<0.001). Smokers were more likely than non-smokers to change between *Lactobacillus* CSTs (RRR 2.21, 95% CI 1.15, 4.23, p=0.017) or change from a *Lactobacillus* CST to a non-*Lactobacillus* dominated CST (i.e. CST4/5; RRR 2.04, 95% CI 1.11, 3.75, p=0.021) relative to not changing CST. Figure 6 summarises changes of CST in each participant longitudinally and indicates when sex with a new partner was reported.

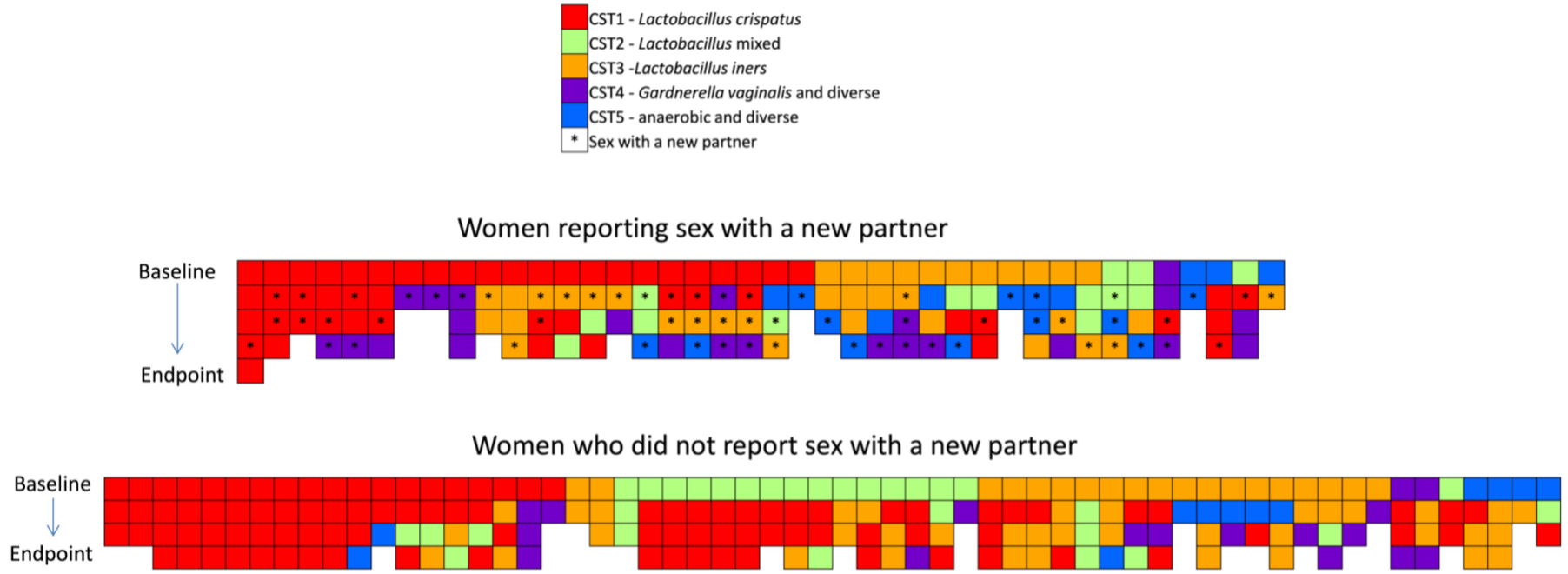


Figure 6. Longitudinal changes of community state type in women reporting sex with a new partner

*Each column represents a participant and sequential longitudinal specimens are ordered from baseline to endpoint. Boxes are coloured according to CST. * indicates a report of sex with a new partner. Most women changed CST at least once during the study. Change of CST occurred in 36/40 (90%) women who reported exposure to a new partner and 47/60 (78%) women who did not report sex with a new partner.*

Practices significantly associated with instability of the vaginal microbiota (i.e. increased Bray-Curtis scores between consecutive samples) by univariate analysis were included in a multivariable model that also included CST of the index specimen (Table 8). Sex with a new partner (adj. coefficient 0.21, 95% CI 0.11, 0.31, p<0.001) and smoking (adj. coefficient 0.09, 95% CI 0.01, 0.18, p=0.036) were associated with increased instability of the microbiota, adjusted for index specimen CST. Additionally, having a vaginal microbiota in the index specimen of CST3-*L. iners* (adj. coefficient 0.25, 95% CI 0.15, 0.34, p<0.001), CST4-*G. vaginalis* and diverse (adj. coefficient 0.24, 95% CI 0.05, 0.43, p=0.013) or CST5-anaerobic and diverse (adj. coefficient 0.44, 95% CI 0.30, 0.60, p<0.001) was associated with increased instability of the vaginal microbiota longitudinally compared to a *L. crispatus* (CST1) vaginal microbiota.

Table 8 - Characteristics and sexual practices associated with instability of the vaginal microbiota as measured by Bray-Curtis dissimilarity between consecutive specimens

Characteristic	Coeff. (95% CI) [a]	*P* value[a]	Adj Coeff. (95% CI)[b]	*P* value[b]
Self-reported past history of BV				
No	ref	-		
Yes	0.10 (-0.04, 0.23)	0.159		
Longitudinal practices[c]				
Any smoking				
No	ref	-	ref	-
Yes	0.15 (0.05, 0.25)	**0.003**	0.09 (0.01, 0.18)	**0.036**
No. of cigarettes smoked				
0/non-smoker	ref	-		
1-7	0.07 (-0.09, 0.22)	0.388		
8+	0.15 (0.01, 0.30)	**0.035**		
Any douching				
No	ref	-		
Yes	0.33 (-0.01, 0.67)	0.056		
Number of SP				
0	ref			
1	-0.12 (-0.28, 0.03)	0.119		
≥2	0.13 (-0.06, 0.32)	0.182		
Frequency of sex				
Once/month or less	ref	-		

Several times/month	-0.01 (-0.12, 0.10)	0.865		
Several times/week	0.06 (-0.07, 0.18)	0.366		
Sex with NP[d]				
No	ref	-	ref	-
Yes	0.23 (0.12, 0.33)	**<0.001**	0.21 (0.11, 0.31)	**<0.001**
Sexual practices with FSP[e]				
Any receptive oral vaginal sex				
No[f]	ref	-		
Yes	0.06 (-0.04, 0.15)	0.258		
Sharing of sex toys				
No toys/washed/condoms used[f]	ref			
Unwashed	0.04 (-0.07, 0.15)	0.478		
Sexual practices with MSP[g]				
Any vaginal sex				
No[h]	ref	-		
Yes	0.09 (-0.07, 0.26)	0.279		
Self-reported symptoms and microbiota characteristics				
Self-reported abnormal vaginal discharge and/or odour				
No	ref	-		
Yes	0.07 (-0.07, 0.22)	0.305		
Shannon diversity	0.07 (-0.00, 0.14)	0.053		
Community state type of index specimen[i]				
CSTI-*L. crispatus*	ref	-	ref	-
CST2-*Lactobacillus* mixed	0.08 (-0.05, 0.20)	0.241	0.09 (-0.03, 0.22)	0.134
CST3-*L. iners*	0.23 (0.12, 0.33)	**<0.001**	0.25 (0.15, 0.34)	**<0.001**
CST4-*G. vaginalis* and diverse[j]	0.23 (0.03, 0.43)	**0.027**	0.24 (0.05, 0.43)	**0.013**
CST5- anaerobic and diverse	0.47 (0.30, 0.64)	**<0.001**	0.44 (0.30, 0.60)	**<0.001**

Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to FSP or MSP); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner
Missing data for variables included in this analysis occurred in <0.5% of intervals.
[a] Univariate GEE linear regression clustered for multiple specimens from each participant. The regression coefficient represents the mean difference of Bray-Curtis Dissimilarity between the reference and comparison group/s for each characteristic/practice investigated
[b] Multivariable GEE linear regression clustered for multiple specimens from each participant.
[c] Longitudinal characteristics were measured as any exposure over the prior follow-up interval (~90 days). No significant associations were identified between beta diversity and either hormonal contraceptive use or recent menses.

[d] Sex with a new partner with who first sexual contact was within 90 days. Partner gender was defined by the participant
[e] The following sexual practices/characteristics with an FSP were left out of the table for simplicity: digital-vaginal sex, receptive oral anal sex, digital anal sex and current partner with BV symptoms. No significant associations between beta diversity and these sexual practices were identified.
[f] Or did not have a FSP
[g] The following sexual practices/characteristics with an MSP were left out of the table for simplicity: condoms use for vaginal sex, anal sex, vaginal sex after anal sex, oral vaginal sex and digital-vaginal sex. No significant associations between beta diversity and these sexual practices were identified.
[h] Or did not have a MSP
[i] Index specimen refers to the first specimen of each consecutive pair
[j] Majority of CST4 specimens are endpoint specimens which do not have accompanying beta diversity information

2.5.5. Practices impacting the vaginal microbiota composition

After considering factors that influence stability of the microbiota, we looked at specific characteristics and sexual practices that influenced the vaginal microbiota composition by multinomial logistic regression. In univariate analyses (Supplementary Table A2), we found women who reported sex with a new partner in the previous 90 days were more likely than women reporting no sex or sex in an ongoing relationship to have a vaginal microbiota of CST4-*G. vaginalis* abundant and diverse (RRR 4.09, 95% CI 1.69, 9.92, p=0.002) or CST5-anaerobic and diverse (RRR 5.37, 95% CI 2.18, 13.20, p<0.001) than one of CST1. Women who reported smoking were more likely than non-smokers to have anaerobic microbiota (CST5) relative to CST1 (RRR 3.01, 95% CI 1.31, 6.92, p=0.009). Women who reported receptive oral vaginal sex from an FSP or sharing of unwashed sex toys with an FSP were more likely to have a CST4 microbiota, and women who douched or had a past history of BV were more likely to have a CST5 microbiota (Supplementary Table A2). Women reporting recent menses (defined as onset of menses within 7 days of specimen collection) were more likely than women not reporting recent menses (> 7 days from specimen collection) to have a CST2-*Lactobacillus*-mixed or CST3-*L. iners* microbiota composition relative to CST1 microbiota, but were not more likely to have a *G. vaginalis* (CST4) or anaerobic microbiota (CST5).

We included past history of BV, receptive oral sex from a FSP, sex with a new partner, sharing of unwashed sex toys with an FSP smoking and recent menses in a multivariable

multinomial regression model (Table 9). Women reporting sex with a new partner were more likely than women reporting no sex or sex in an ongoing relationship to have a CST4-*G. vaginalis* and diverse (aRRR 3.45, 95% CI 1.42, 8.41, p=0.006) or CST5-anerobic and diverse vaginal microbiota (aRRR 3.62, 95% CI 1.43, 9.14, p=0.007) relative to a CST1 vaginal microbiota. Women reporting that they shared unwashed sex toys with an FSP were more likely than women not reporting this practice to have a CST4 vaginal microbiota (aRRR 2.49, 95% CI 1.05, 5.91, p=0.038). Women reporting smoking were more likely than non-smokers to have a CST5-anaerobic and diverse vaginal microbiota relative to a CST1 vaginal microbiota (aRRR 2.94, 95% CI 1.16, 7.43, p=0.023). Women with a past-history of BV were more likely to have a CST5 vaginal microbiota (aRRR 3.18, 95% CI 1.13, 8.91, p=0.028), and women reporting recent menses were more likely to have a CST2 (aRRR 3.89, 95% CI 1.58, 9.50, p=0.003) or CST3 (aRRR 2.37, 95% CI 1.14, 4.90, p=0.020) vaginal microbiota.

Table 9 - Characteristics and practices associated with vaginal microbiota composition by multivariable multinomial logistic regression

Outcome by CST	RRR (95% CI)	*P* value[a]	aRRR (95% CI)	*P* value[b]
***Lactobacillus* mixed (CST2) vs CST1**				
Self-reported past history of BV[c]	0.87 (0.35, 2.17)	0.762	0.83 (0.33, 2.09)	0.696
Smoker[d]	1.59 (0.77, 3.29)	0.214	1.75 (0.84, 3.65)	0.138
Sex with a NP[e]	0.62 (0.18, 2.19)	0.460	0.57 (0.16, 2.00)	0.384
Receptive oral vaginal sex from FSP[f]	1.09 (0.56, 2.15)	0.799	1.02 (0.51, 2.05)	0.951
Sharing of unwashed sex toys with FSP[g]	0.92 (0.36, 2.32)	0.859	0.75 (0.26, 2.18)	0.600
Onset of last menses ≤ 7 days ago[h]	3.59 (1.49, 8.68)	**0.004**	3.89 (1.58, 9.50)	**0.003**
***L. iners* (CST3) vs CST1**				
Self-reported past history of BV[c]	0.69 (0.26, 1.87)	0.467	0.66 (0.23, 1.93)	0.450
Smoker[d]	1.38 (0.71, 2.65)	0.341	1.46 (0.72, 2.94)	0.291
Sex with a NP[e]	1.77 (0.87, 3.60)	0.117	1.61 (0.77, 3.38)	0.208
Receptive oral vaginal sex from FSP[f]	1.09 (0.57, 2.12)	0.790	1.01 (0.51, 2.01)	0.977
Sharing of unwashed sex toys with FSP[g]	0.98 (0.50, 1.92)	0.952	1.02 (0.48, 2.14)	0.965
Onset of last menses ≤ 7 days ago[h]	2.19 (1.08, 4.46)	**0.030**	2.37 (1.14, 4.90)	**0.020**
***G. vaginalis* and diverse (CST4) vs CST1**				
Self-reported past history of BV[c]	1.13 (0.39, 3.27)	0.817	1.24 (0.43, 3.57)	0.686
Smoker[d]	1.72 (0.74, 4.01)	0.207	1.69 (0.70, 4.08)	0.240
Sex with a NP[e]	4.09 (1.69, 9.92)	**0.002**	3.45 (1.42, 8.41)	**0.006**
Receptive oral vaginal sex from FSP[f]	2.60 (1.09, 6.20)	**0.031**	1.94 (0.77, 4.84)	0.158
Sharing of unwashed sex toys with FSP[g]	2.38 (1.04, 5.45)	**0.039**	2.49 (1.05, 5.91)	**0.038**
Onset of last menses ≤ 7 days ago[h]	1.64 (0.55, 4.91)	0.378	1.37 (0.41, 4.64)	0.611
Anaerobic and diverse (CST5) vs CST1				
Self-reported past history of BV[c]	2.82 (1.09, 2.27)	**0.032**	3.18 1.13, 8.91)	**0.028**

Smoker[d]	3.01 (1.31, 6.92)	**0.009**	2.94 (1.16, 7.43)	**0.023**
Sex with a NP[e]	5.37 (2.18, 13.20)	**<0.001**	3.62 (1.43, 9.14)	**0.007**
Receptive oral vaginal sex from FSP[f]	2.17 (0.86, 5.46)	0.099	1.67 (0.62, 4.49)	0.308
Sharing of unwashed sex toys with FSP[g]	1.46 (0.62, 3.46)	0.387	2.07 (0.79, 5.43)	0.141
Onset of last menses ≤ 7 days ago[h]	1.03 (0.27, 4.00)	0.964	0.78 (0.01, 0.12)	0.734

Abbreviations: CST, community state type; NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; RRR, relative risk ratio; aRRR, adjusted relative risk ratio

Missing data for variables included in this analysis occurred in <0.5% of intervals.

[a] Multinomial logistic regression with CST1-*L. crispatus* as the baseline comparison group. Analysis clustered for multiple specimens from participants (100 clusters).

[b] Multinomial logistic regression as described in [a] adjusted for all other characteristics in the table.

[c] Self-reported past history of BV relative to no self-report of past history of BV.

[d] Smoker relative to non-smoker

[e] Sex with a new partner with who first sexual contact was within 90 days relative to no sex/sex with a partner with who first sexual contact was >90 days.

[f] Receptive oral vaginal sex from FSP relative to no receptive oral sex from FSP (or no FSP).

[g] Sharing of unwashed sex toys with FSP relative to no toy use/changed condoms on the sex toys/always washed the sex toys between sharing with a partner.

[h] Onset of last menses ≤ 7 days ago relative to onset of last menses > 7 days ago

2.6. Discussion

In this longitudinal cohort study of women who have sex with women, specific sexual practices influenced the bacterial diversity, stability and composition of the vaginal microbiota. Sex with a new partner (primarily representing new FSPs) was associated with an increase in bacterial diversity and an increase in compositional change (or instability) of the vaginal microbiota, both in terms of change of CST and increased Bray-Curtis dissimilarity. Furthermore, women who reported sex with a new partner were more likely than women reporting no sex/sex in an ongoing relationship to have a vaginal microbiota characterised by BV-associated anaerobic bacteria or *G. vaginalis*, relative to an optimal microbiota characterised by *L. crispatus*. This study highlights the influence of practices on the development of a non-optimal vaginal microbiota and provides microbiological support for the sexual exchange of bacteria between women. These microbiological findings complement the previously reported epidemiological data from the original cohort (Bradshaw *et al.*, 2014; Vodstrcil *et al.*, 2015) which showed sex with a new partner was associated with a 2.5-fold increased risk of BV acquisition.

There is increasing evidence to support the sexual transmission of vaginal bacteria between WSW. Longitudinal studies in this population have shown that one of the greatest risk factors for BV is having a sexual partner with a history of BV, BV symptoms or microbiologically confirmed BV (Marrazzo *et al.*, 2010b; Vodstrcil *et al.*, 2015). A recent study demonstrated that incident BV occurred at a median of 4 days post sexual activity in 93% of WSW, indicating a similar incubation period to that of other STIs (Muzny *et al.*, 2019b). An early study looking at the transmission dynamics of BV demonstrated that transfer of vaginal secretions between women resulted in BV in 11 of 15 women (Gardner & Dukes, 1955). Furthermore, high concordance of Nugent score categories between FSP (Berger *et al.*, 1995; Bradshaw *et al.*, 2014; Evans *et al.*, 2007; Marrazzo *et al.*, 2002; Vodstrcil *et al.*, 2015) and evidence that women in monogamous relationships share *Lactobacillus* strains (Marrazzo *et al.*, 2009) in their vaginas supports exchange of bacteria between women during sex. In our study, women who shared unwashed sex toys and/or received oral sex from an FSP were more likely than women not reporting these practices to have an anaerobic or *G. vaginalis* abundant vaginal microbiota than a microbiota dominated

by *L. crispatus*. Sexual practices are frequently highly correlated, so it is difficult to determine whether one activity has a greater impact on the vaginal microbiota than others. However, both oral sex with an FSP and sex toy use involve exchange of bodily fluids to varying degrees and therefore promote exchange of bacteria between women. Additionally, both practices have been reported as a risk factor for BV (Fethers *et al.*, 2008; Marrazzo *et al.*, 2010a; Marrazzo *et al.*, 2010b; Vodstrcil *et al.*, 2015). Collectively, these data suggest that female partner treatment of women with BV may be an effective strategy to improve BV cure and warrants further investigation.

Change of CST was common in our study, in accordance with previous reports that show the vaginal microbiota can be highly dynamic (Gajer *et al.*, 2012; Schwebke *et al.*, 1999; Srinivasan *et al.*, 2010). Compositional change (or instability) of the vaginal microbiota between consecutive specimens was primarily influenced by the bacteria present in the index specimen. Collectively, women with a low diversity *L. crispatus* dominated vaginal microbiota were more likely to have a stable microbiota longitudinally and were less likely to experience change of CST than women with a diverse microbiota or a microbiota abundant in *L. iners* or *G. vaginalis*. Our findings are consistent with one study (Gajer *et al.*, 2012) that analysed the vaginal microbiota of 32 women sampled twice-weekly for 16-weeks. Gajer *et al.* (2012) reported that *L. crispatus* and *L. gasseri* dominated microbiota appeared to be stable, and that sexual activity negatively impacted stability. Interestingly, practices and microbiological characteristics associated with change of CST were highly consistent with those associated with increasing instability of the microbiota (measured by Bray-Curtis), suggesting change of CST may be a useful measure of microbiota instability (Brooks *et al.*, 2017a).

Smoking had a broad ranging effect on the diversity, stability and composition of the vaginal microbiota, and past studies have shown an association between smoking and BV and/or vaginal microbiota composition that was dose dependent (Bradshaw *et al.*, 2014; Brotman *et al.*, 2014a; Hellberg *et al.*, 2000; Mehta *et al.*, 2015). There are a number of possible explanations for this association. Smokers have been shown to have reduced oestradiol levels compared non-smokers (Westhoff *et al.*, 1996), and reduced oestrogen has been associated with non-optimal *Lactobacillus*-deficient vaginal microbiota (Miller *et al.*, 2016). Furthermore, it is well established that nicotine has detrimental effects on the immune

system, including reduced production of inflammatory cytokines and decreased functionality of neutrophils and macrophages (Murphy & Mitchell, 2016), and nicotine and its derivatives have been detected in the vaginal metabolome (Nelson *et al.*, 2018). It is possible that modulation of immune responses may result in reduced clearance of *G. vaginalis* and other BV-associated bacteria (similar to what has been observed for human papillomavirus (Giuliano *et al.*, 2002)) or prevent maintenance of an optimal *Lactobacillus* vaginal microbiota. The association between smoking and vaginal microbiota instability seen in our study is interesting and may be because the microbiota composition that is found more commonly in smokers (i.e. anaerobic and diverse microbiota) is inherently more unstable than others, such as those dominated by *L. crispatus*. It is also possible that observed associations between smoking and adverse microbiota composition and instability are due to unmeasured confounding; however, the fact that this association has been shown to be dose dependent in some studies and persists in adjusted analysis provides evidence for a biological association.

A number of other factors were associated with vaginal microbiota composition, stability and/or diversity including past history of BV, menses and douching. The finding that past history of BV was associated with both increased bacterial diversity and an anaerobic microbiota may represent persistence or re-emergence of a polymicrobial BV-biofilm (Machado & Cerca, 2015; Swidsinski *et al.*, 2005), or alternatively the influence of other factors such as host genetics or immune function (Murphy & Mitchell, 2016), diet (Tuddenham *et al.*, 2019) or contraceptive practices (Joesoef *et al.*, 2001). Both douching and menses have been shown in a number of studies to adversely alter vaginal microbiota composition and stability (Brotman *et al.*, 2008a; Gajer *et al.*, 2012; van der Veer *et al.*, 2019), and consistent with this, we found that douching was associated with anaerobic and diverse vaginal microbiota and had a borderline adverse effect on microbiota stability in univariate analyses. While recent menses did not have an effect on microbiota diversity or stability in our study, it did influence microbiota composition. Women were more likely to have a vaginal microbiota abundant in *L. iners* (i.e. CST2 or CST3) if their specimen was collected within seven days of onset of menses, consistent with data that shows *L. iners* grows best on media containing blood (Falsen *et al.*, 1999; Srinivasan *et al.*, 2016).

Hormonal contraception may have a beneficial impact on the vaginal microbiota (Brooks *et al.*, 2017b). However, we found no association between hormonal contraception and microbiota diversity, stability or composition, which may be because only a small number of women reported hormonal contraceptive use in the parent cohort.

There are a number of limitations to this study. The study population comprised highly educated women who were predominately Australian born and reported Australian or English ethnicity, which may limit the generalizability of our findings. Specimens were collected every three months which limited our ability to assess immediate effect of sexual practices behaviours on the vaginal microbiota and any short-term fluctuations in microbiota composition. Specimens included in the analysis were not selected randomly or from specified study time points which may have biased results. We did not include negative controls alongside specimens during sequencing, however we removed contaminants previously identified using the same extraction methodology, primer set up and sequencing instrument (Plummer *et al.*, 2018a) and the microbiota profiles are consistent with those previously published (Chaban *et al.*, 2014; Ravel *et al.*, 2011; Vodstrcil *et al.*, 2017). Finally, this study did not assess practices or the vaginal microbiota of the sexual partner/s of participants so we cannot definitively prove sexual transmission of BV-associated bacteria is occurring between women. Nevertheless, the microbiota data presented here is consistent with epidemiological data that supports sexual transmission of BV in WSW (Marrazzo *et al.*, 2010b; Vodstrcil *et al.*, 2015).

This study shows that sex with a new partner is associated with changes in the vaginal microbiota of WSW, including increased diversity and increased abundance of bacteria commonly associated with a non-optimal vaginal microbiota. These findings suggest that sexual exchange of bacteria, including BV-associated bacteria, occurs between female sexual partners, and highlight the influence of specific practices on the development of a non-optimal vaginal microbiota. These data are important for informing strategies to promote a vaginal microbiota that is associated with optimal reproductive health, as well as new approaches to improve BV cure such as female partner treatment.

Chapter 3. The association of *Gardnerella vaginalis* clade distribution with behavioural practices and Nugent score in women who have sex with women

3.1. Overview

As discussed in Section 1.6.1 of this literature review, *G. vaginalis* is thought to play a key role in the pathogenesis of BV. However, a role for *G. vaginalis* in BV pathogenesis is complicated by the fact that it is detected in women with BV and women without BV. There is evidence of substantial genetic variation in the *Gardnerella* genus (Ahmed *et al.*, 2012; Cornejo *et al.*, 2018; Paramel Jayaprakash *et al.*, 2012), and identification of at least four genetically distinct *G. vaginalis* clades has led to the hypothesis that pathogenic and commensal *G. vaginalis* clades exist. Although there have been a number of studies investigating the distribution of *G. vaginalis* clades in women with and without BV, there have been no studies of this kind in WSW. Additionally, there have been no studies that have investigated the association between *G. vaginalis* clade distribution and sexual and behavioural practices, or between *G. vaginalis* clade distribution and the composition of the vaginal microbiota.

Chapter 3 aims to address this gap in the literature and presents a detailed analysis of the relationship between *G. vaginalis* clade distribution and 1) sexual and behavioural practices, 2) Nugent score and 3) vaginal microbiota composition in WSW. This chapter utilises specimens and data from the same cohort of women presented in **Chapter 2**: the WOW Health Cohort Study (Vodstrcil *et al.*, 2015).

Chapter 3 is presented as the accepted version of the following peer reviewed manuscript. No alterations to the text have been made, except that abbreviations, and figure and table numbers have been adjusted to generate a consistent presentation within this thesis.

Publication:

Plummer EL, Vodstrcil LA, Murray GL, Fairley CK, Danielewski JA, Garland SM, Chow EPF, Bulach DM, Fethers KA, Hocking JS, Bradshaw CS (2020). *Gardnerella vaginalis* clade distribution is associated with behavioral practices and Nugent score in women who have sex with women. *J Infect Dis* 221 (3):454-463. doi:10.1093/infdis/jiz474

Findings from this manuscript have also been presented at the following national and international conferences:

- STI & HIV 2019 World Congress, Vancouver Canada, 14 – 17 Jul 2019. [Poster] **First Prize poster award –young investigator category**
- Central Clinical School Postgraduate Symposium, Melbourne, Australia, 7 Oct 2019. [Poster] **Third place poster presentation award**

All supplementary files referred to in this chapter have been appended to this thesis in Appendix B.

A PDF of the published manuscript is in Appendix E.

3.2. Abstract

Background*: Gardnerella vaginalis* is detected in women with and without BV. Identification of four *G. vaginalis* clades raised the possibility that pathogenic and commensal clades exist. We investigated the association of behavioural practices and Nugent score with *G. vaginalis* clade distribution in WSW.

Methods: Longitudinal self-collected vaginal specimens were analysed using established *G. vaginalis* species-specific and clade-typing PCR assays. Logistic regression assessed factors associated with detection of *G. vaginalis* clades and multinomial regression assessed factors associated with number of clades.

Results: Clades 1, 2 and 3, and multi-clade communities (>2 clades) were associated with Nugent-BV. Clade 1 (odds ratio [OR]:3.36; 95% CI 1.65, 6.84) and multi-clade communities (relative-risk-ratio [RRR]:9.51; 95% CI 4.36, 20.73) were also associated with *Lactobacillus*-deficient vaginal microbiota. Clade 4 was neither associated with Nugent-BV nor *Lactobacillus*-deficient microbiota (OR:1.49; 95% CI 0.67, 3.33). Specific clades were

associated with differing behavioural practices. Clade 1 was associated with increasing number of recent sexual partners and smoking, whereas clade 2 was associated with penile-vaginal sex and sharing of sex toys with female partners.

Conclusions: Our results suggest that *G. vaginalis* clades have varying levels of pathogenicity in WSW, with acquisition occurring through sexual activity. These findings suggest that partner treatment may be an appropriate strategy to improve BV cure.

3.3. Introduction

Bacterial vaginosis is the commonest vaginal condition of reproductive aged women (Unemo *et al.*, 2017). Current BV treatments are sub-optimal with over 50% of women experiencing recurrence within 6-12 months following treatment (Bradshaw *et al.*, 2006a; Sobel *et al.*, 1993). Improving BV treatment has been impeded by its complex aetiology and pathogenesis. *G. vaginalis* is thought to play a key role in BV pathogenesis, potentially as a founder organism (Coleman & Gaydos, 2018; Muzny & Schwebke, 2013; Schwebke *et al.*, 2014b). *G. vaginalis* is almost always present in the vagina of women who have BV (Srinivasan *et al.*, 2012) and it possesses characteristics important for pathogenesis, including production of sialidase, an enzyme that degrades cervicovaginal mucus (Lewis *et al.*, 2013), and vaginolysin, a cytolysin that induces vaginal epithelial cell lysis (Gelber *et al.*, 2008). Both factors may assist *G. vaginalis* adherence to host epithelial cells and form biofilms (Swidsinski *et al.*, 2005). *G. vaginalis* is also detected in the vagina of women without BV, albeit at lower prevalence and abundance (Fethers *et al.*, 2012; Ravel *et al.*, 2011; Schwebke *et al.*, 2014a; Shipitsyna *et al.*, 2013; Vodstrcil *et al.*, 2017). Substantial genetic diversity exists within *G. vaginalis* (Ahmed *et al.*, 2012; Cornejo *et al.*, 2018; Paramel Jayaprakash *et al.*, 2012) and different genetic types/clades may have different virulence potential (Cornejo *et al.*, 2018; Janulaitiene *et al.*, 2018; Schellenberg *et al.*, 2016; Schuyler *et al.*, 2015; Shipitsyna *et al.*, 2019). Collectively, these findings suggest that commensal and pathogenic *G. vaginalis* variants exist. Different methods have been used to define *G. vaginalis* clades including *cpn60* gene analysis (Paramel Jayaprakash *et al.*, 2012), whole genome sequence analysis (Ahmed *et al.*, 2012), detection of clade-specific genes (Balashov *et al.*, 2014) and ecotyping (Cornejo *et al.*, 2018). Comparison of methods has been discussed previously (Cornejo *et al.*, 2018; Schellenberg *et al.*, 2016).

Previous studies have explored the association of specific *G. vaginalis* clades and number of *G. vaginalis* clades with BV, and while results regarding specific clades have been mixed, detection of multiple *G. vaginalis* clades is consistently associated with BV (Balashov *et al.*, 2014; Hilbert *et al.*, 2017; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019; Vodstrcil *et al.*, 2017). BV prevalence is high amongst WSW, with estimates ranging from 25-52% (Bailey *et al.*, 2004; Bradshaw *et al.*, 2014; Evans *et al.*, 2007; McCaffrey *et al.*, 1999). Despite this, there are no published data about *G. vaginalis* clade distribution in WSW. Additionally, there is limited information concerning associations between *G. vaginalis* clades and BV risk factors. Defining the role of *G. vaginalis* clades in BV pathogenesis and understanding how they are acquired may lead to the development of more effective treatment strategies. Using a multiplex real-time PCR *G. vaginalis* clade-typing assay (Balashov *et al.*, 2014) we aimed to investigate the association of *G. vaginalis* clade distribution with sexual and behavioural practices and Nugent score in a cohort of Australian WSW.

3.4. Methods

3.4.1. Participants and specimens

Participants and specimens were selected from the WOW Health cohort, which examined factors associated with incident BV (Bradshaw *et al.*, 2014; Vodstrcil *et al.*, 2015). Briefly, nonpregnant premenopausal women reporting a FSP in the 18 months prior to enrolment and who had a Nugent score <7 (Nugent *et al.*, 1991) on three consecutive vaginal smears were eligible for enrolment (Bradshaw *et al.*, 2014; Vodstrcil *et al.*, 2015). Participants completed a questionnaire and self-collected a vaginal swab and smear three-monthly until study endpoint (incident Nugent-BV [Nugent score =7-10] or 24 months without Nugent-BV [Nugent score =0-6]). Women were asked to collect specimens avoiding the time during menses. Smears were scored using the Nugent score method (Nugent *et al.*, 1991), with a score of 0-3 defined as non-BV, 4-6 as intermediate-BV and 7-10 as Nugent-BV (McKinnon *et al.*, 2019).

Within WOW, we conducted a nested cohort study that included women who developed incident Nugent-BV and women who reached the study endpoint without Nugent-BV (Vodstrcil *et al.*, 2015) (selected using a random sort command in Stata/IC (Version 14.2,

StataCorp LP, College Station, USA)). As there were seven women who developed incident Nugent-BV who co-enrolled in WOW with their ongoing FSP (Vodstrcil *et al.*, 2015), controls were frequency matched on coenrolment and age, to ensure a similar distribution of both variables. A baseline specimen and 1-4 longitudinal specimens (typically the last three specimens collected over the 24-month study period) were included from 101 women. As a result, 101 women contributed a total of 372 specimens to analyses; comprising 101 enrolment samples and 271 longitudinal samples, 48 of which were collected at incident Nugent-BV. Ethical approval was obtained from the Human Research Ethics Committees of Alfred Hospital, Melbourne (251/09) and the University of Melbourne (0932804). Participants provided informed written consent.

3.4.2. Laboratory methods

Swabs were agitated in 1ml RNAlater (Thermo Fisher Scientific, Waltham, USA) and stored at -80°C until analysed. DNA was extracted as outlined in Fethers *et al.* (2012), using the MagNA Pure 96 instrument and the DNA and Viral NA small volume kit (Roche Diagnostics, Mannheim, Germany). *G. vaginalis* was detected using a species-specific real-time PCR assay previously described (Fethers *et al.*, 2012). Presence of the four *G. vaginalis* clades (hereafter referred to as clades 1, 2, 3 and 4) was determined using a validated multiplex real-time PCR assay targeting sequences unique to each clade (Balashov *et al.*, 2014) as previously described (Vodstrcil *et al.*, 2017); the limit of detection for this assay is 10 copies per reaction (Balashov *et al.*, 2014). Specificity and sensitivity of the primer set was assessed by BLAST (Altschul *et al.*, 1990) analysis; additional details provided in Supplementary Methods B.

Identification of *G. vaginalis* clades using clade-specific PCR provides biological information on top of what can be inferred from microbiota studies utilising 16S rRNA gene sequencing. Vaginal microbiota composition was previously determined for 360 specimens using 16S rRNA gene sequencing of the V3-V4 hypervariable regions (Plummer *et al.*, 2018b). Vaginal microbiota composition was categorised into three groups based on McKinnon *et al.* (2019): optimal non-*iners Lactobacillus* microbiota (dominated by non-*iners Lactobacillus* spp.), *Lactobacillus iners* microbiota (*L. iners* was the most abundant taxon) and non-optimal

microbiota (deficient in *Lactobacillus* spp.); additional details provided in Supplementary Methods B.

3.4.3. Statistical Analysis

Univariable and multivariable logistic regression models fitted with generalised estimating equations were used to explore associations between covariates (including history of BV, self-reported symptoms (vaginal discharge and/or odour), behavioural and sexual practices, Nugent score and vaginal microbiota composition) and detection of *G. vaginalis* (present *vs* absent). Logistic regression models explored associations between covariates and detection of each *G. vaginalis* clade *vs* detection of any other clade/s. Univariable and multivariable multinomial logistic regression examined associations between covariates and number of *G. vaginalis* clades detected. This analysis determined the relative risk of having one clade or multiple clades in a specimen compared to having no *G. vaginalis* (i.e. *G. vaginalis* was not detected), generating relative risk ratios (RRR) and 95% CI. Sexual and behavioural practices deemed significant in univariable analyses ($p<0.05$) were included in multivariable analyses. Self-reported symptoms, behavioural and sexual practices were recorded for the 3-month interval preceding each specimen collection. Self-reported symptoms, Nugent score and vaginal microbiota composition were not included in multivariable analyses as they are correlated with *G. vaginalis* presence. Regression models accounted for repeated measures from individuals. We assumed an exchangeable correlation structure and used a cluster-based variance estimate for standard error. Statistical analyses were performed using Stata/IC (Version 14.2, StataCorp LP, College Station, USA).

3.5. Results

3.5.1. Specificity and sensitivity of clade-specific primers

No *G. vaginalis* isolate had more than one clade-specific amplicon detected and detection of clade-specific amplicons corresponded with clade phylogeny (Supplementary Figure B1), demonstrating high specificity of the primers. Isolates JCP8481A, JCP8481B and PSS_7772B did not have a clade-specific amplicon detected and appeared genetically unrelated to the four clades. These isolates may represent an additional clade, as previously suggested (Schellenberg *et al.*, 2009). B482 and GED7275B clustered with clade 2 isolates but the clade

2 amplicon was not detected in these isolates. Thus the primers have reduced sensitivity for clade 2.

3.5.2. Participant demographics

One hundred and one women, contributing 372 specimens, were included in analyses. Participant median age was 28 (interquartile range 23-36yrs) and most women were Australian born (n=87, 86%). At enrolment, 72 (71%) women had a FSP (median duration of relationship 2 years (interquartile range: 0.3-4 years), 44 (44%) reported smoking and 22 (22%) had a past history of BV.

3.5.3. Factors associated with *G. vaginalis* detection

Seventy-seven women (76%; 95% CI 67, 84%) had at least one specimen in which *G. vaginalis* was detected. *G. vaginalis* was detected in 184/372 specimens (49%; 95% CI 44, 55%); 130/306 specimens with Nugent score=0-3 (42%; 95% CI 67, 48%), 15/18 specimens with Nugent score=4-6 (83%; 95% CI 59, 96%) and 39/48 specimens with Nugent-BV (81%; 95% CI 67, 91%). In univariable analyses, *G. vaginalis* detection was associated with self-reported symptoms, Nugent score=4-6 and Nugent-BV (Table 10). *G. vaginalis* was also associated with having a non-optimal (*Lactobacillus* deficient) microbiota (Odds ratio [OR] 4.35; 95% CI 2.47, 7.66; p<0.001).

G. vaginalis detection was associated with smoking, history of penile-vaginal sex and frequent sex (several times per week) in univariable analyses (Table 10). Multivariable analysis of behavioural practices found that *G. vaginalis* detection was associated with smoking in the previous three months (adjusted OR [AOR] 1.76; 95% CI 1.06, 2.93; p=0.028; Table 10) and lifetime history of penile-vaginal sex (AOR 2.03; 95% CI 1.01, 4.10; p=0.047). There was a borderline association between *G. vaginalis* and frequent sex (AOR 1.61; 95% CI 0.99, 2.61; p=0.052).

Table 10 - Factors associated with detection of *G. vaginalis*

Risk factor	*G. vaginalis* absent n (%)	*G. vaginalis* present n (%) (N=184)	OR 95% CI	P value[a]	AOR 95% CI	P value[b]

	(N=188)					
Age[c]						
<28	92 (49)	84 (46)	1			
≥28	96 (51)	100 (54)	1.17 (0.67, 2.05)	0.577		
Self-reported history of BV						
No	154 (82)	142 (77)	1			
Yes	34 (18)	42 (23)	1.43 (0.68, 2.99)	0.346		
Baseline sexual practices						
No. of lifetime FSPs[c]						
<5	96 (51)	70 (38)	1			
≥5	92 (49)	114 (62)	1.69 (0.93, 3.09)	0.088		
No. of FSPs in previous 12 months[c]						
≤1	129 (69)	101 (55)	1			
>1	59 (31)	83 (45)	1.80 (0.97, 3.34)	0.062		
Lifetime history of penile-vaginal sex with a man						
No	66 (35)	36 (20)	1		1	
Yes	122 (65)	148 (80)	2.27 (1.14, 4.54)	**0.020**	2.03 (1.01, 4.10)	**0.047**
Interval characteristics in prior 3 months[d]						
Any smoking						
No	128 (68)	94 (51)	1		1	
Yes	59 (32)	90 (49)	1.88 (1.15, 3.09)	**0.012**	1.76 (1.06, 2.93)	**0.028**
Any douching						
No	183 (98)	179 (97)	1			
Yes	4 (2)	5 (3)	0.58 (0.15, 2.30)	0.441		
Any hormonal contraceptive use						
No	157 (84)	166 (90)	1			
Yes	31 (16)	18 (10)	0.62 (0.30, 1.27)	0.191		
Last menstrual						

period						
≤7 days	31 (17)	23 (13)	1			
>7 days	149 (83)	155 (87)	1.05 (0.62, 1.78)	0.858		
Number of SP						
0	25 (13)	18 (10)	1			
1	147 (79)	129 (70)	1.01 (0.52, 1.95)	0.979		
≥2	15 (8)	37 (20)	2.11 (0.92, 4.85)	0.079		
Frequency of sex						
Several times/month or less	143 (76)	115 (63)	1		1	
Several times/week	45 (24)	69 (37)	1.70 (1.07, 2.71)	**0.025**	1.61 (0.99, 2.61)	0.052
Sex with new partner[e]						
No	165 (88)	147 (80)	1			
Yes	23 (12)	37 (20)	1.39 (0.84, 2.30)	0.195		
Sexual practices with FSP in prior 3 months[f]						
Number of FSPs[c]						
0	37 (20)	32 (17)	1			
≥1	151 (80)	152 (83)	1.16 (0.68, 1.99)	0.581		
Receptive oral vaginal sex						
No[g]	77 (41)	69 (38)	1			
Yes	110 (59)	115 (63)	1.03 (0.68, 1.56)	0.874		
Sharing sex toys						
No toys/washed/ condoms used[g]	142 (76)	141 (77)	1			
Unwashed	45 (24)	43 (23)	0.87 (0.53, 1.42)	0.575		
Current partner with BV symptoms						
No/don't know[g]	187 (100)	177 (97)				
Yes	0	7 (4)	…			

Sexual practices with an MSP in prior 3 months[h]				
Number of MSPs[c]				
0	173 (92)	156 (85)	1	
≥1	15 (8)	28 (15)	1.94 (0.89, 4.23)	0.097
Any penile-vaginal sex				
No[i]	173 (93)	158 (86)	1	
Yes	14 (7)	26 (14)	1.86 (0.81, 4.24)	0.143
Self-reported symptoms and microbiota measures				
Abnormal vaginal discharge and/or odour				
No	175 (93)	152 (83)	1	
Yes	13 (7)	32 (17)	2.09 (1.13, 3.87)	**0.019**
Nugent score				
0-3	176 (93)	130 (71)	1	
4-6	3 (2)	15 (8)	3.96 (1.44, 10.93)	**0.008**
7-10	9 (5)	39 (21)	3.48 (1.95, 6.22)	**<0.001**
Vaginal microbiota type[j]				
Optimal non-*iners* *Lactobacillus* microbiota	118 (66)	79 (44)	1	
L. iners microbiota	49 (27)	45 (25)	1.10 (0.69, 1.75)	0.692
Non-optimal microbiota	13 (7)	56 (31)	4.35 (2.47, 7.66)	**<0.001**

Includes 372 specimens from 101 participants

Abbreviations: OR, odds ratio; AOR, adjusted odds ratio; CI, confidence interval; BV, bacterial vaginosis; FSP, female sexual partner; MSP, male sexual partner; SP, sexual partner (refers to total number of sexual partners in a study interval, female and male);

[a] Univariate logistic regression fitted with generalised estimating equations (GEE) clustered for multiple specimens from each participant.

[b] Multivariable logistic regression fitted with GEE, clustered for multiple specimens from each participant.

[c] Variables were dichotomized at median value

[d] Interval characteristics were measured as any exposure over the prior follow-up interval

(~90 days)
[e] Sex with a new partner with whom first sexual contact was within 90 days. May represent a new FSP or new MSP.
[f] The following characteristics /sexual practices with an FSP were left out of the table for simplicity: digital-vaginal sex, receptive oral anal sex, digital anal sex. No significant associations between *G. vaginalis* and these sexual practices were identified.
[g] Or did not have a FSP
[h] The following sexual practices with an MSP were left out of the table for simplicity: receptive oral sex, digital-vaginal sex and penile-anal sex. No significant associations between *G. vaginalis* and these sexual practices were identified.
[i] Or did not have a FSP
[j] Vaginal microbiota type available for 360 specimens from 100 women. Optimal non-*iners Lactobacillus* microbiota includes specimens predominately consisting of non-*iners Lactobacillus* spp., *Lactobacillus iners* microbiota includes specimens predominately consisting of *L. iners* and non-optimal microbiota includes specimens predominately consisting of non-*Lactobacillus* spp.

3.5.4. Factors associated with number of G. vaginalis clades

Three *G. vaginalis* positive specimens did not belong to any clade detectable by clade-specific PCR. These specimens were excluded and consequently, 369 specimens from 101 women contributed to the following analyses.

Clade 4 was the most prevalent clade (n=136/369; 37%; 95% CI 32, 42%), followed by clade 1 (n=116/369; 31%; 95% CI 27, 36%), clade 2 (n=76/369; 21%; 95% CI 17, 25%) and clade 3 (n=17/369; 5%; 95% CI 3, 7%).Clade 4 was detected in 62 women (n=62/101; 61%), clade 1 in 55 women (n=55/101; 54%), clade 2 in 44 women (n=44/101; 44%) and clade 3 in 14 women (n=14/101; 14%). Of the 181 specimens positive for a *G. vaginalis* clade, 63 had one clade detected (35%, 95% CI 28, 42%) and 118 had multiple (i.e. two or more) clades (65%, 95% CI 58, 72%). Five specimens contained all four clades. Clade 3 was only detected in multi-clade communities (Figure 7).

In univariable analyses, women reporting symptoms were more likely than asymptomatic women to have multiple *G. vaginalis* clades relative to no *G. vaginalis* (relative-risk-ratio[RRR] 4.19; 95% CI 1.85, 9.49; p=0.001, Supplementary Table B1). Women with intermediate-BV (RRR 8.72; 95% CI 2.32, 32.76; p =0.001) or Nugent-BV (RRR 8.72; 95% CI 4.05, 18.78; p <0.001) were more likely than women with Nugent score =0-3 to have multiple clades relative to no *G. vaginalis*. Having a single clade was not associated with Nugent score. Women with a non-optimal microbiota were more likely than women with an

optimal microbiota to have a single clade (RRR 3.03; 95% CI 1.20, 7.61; p =0.019) or multiple clades (RRR 9.51; 95% CI 4.36, 20.73; p<0.001) relative to no *G. vaginalis* (Supplementary Table B1).

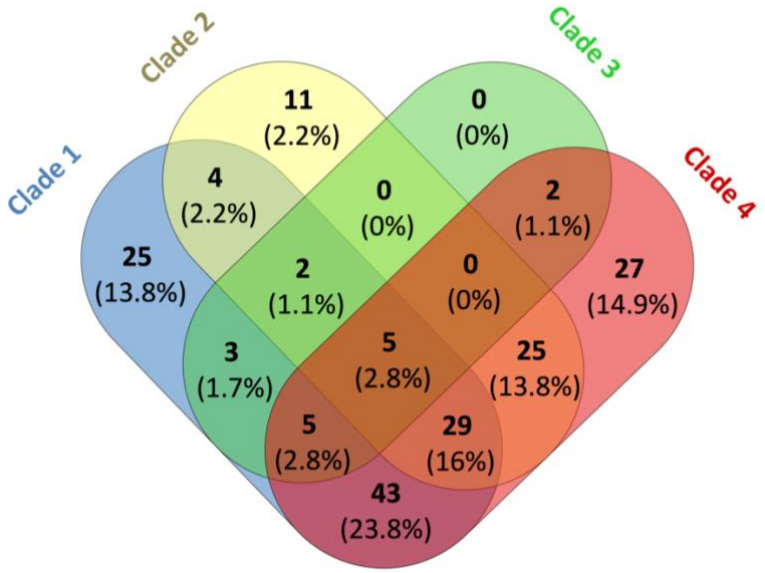


Figure 7. Distribution of *G. vaginalis* clades

Venn diagram showing the distribution and co-occurrence of G. vaginalis clades in vaginal specimens. Total number of specimens: 181 from 77 women.

In univariable analyses, having multiple *G. vaginalis* clades was associated with smoking in the previous three months and sexual practices including increased number of lifetime FSPs, history of penile-vaginal sex, increased frequency of sex, current sexual practices with a male and sex with a new partner (predominantly representing new FSPs; Supplementary Table B1). No significant associations were observed between practices and single clade communities. Smoking, lifetime number of FSPs, frequency of sex and sex with a new partner were included in multivariable analyses (Table 11); sexual practices with a male partner were rare and were omitted to prevent overfitting the model due to their correlation with sex with a new partner. Women reporting smoking in the previous three months were more likely than non-smokers to have multiple *G. vaginalis* clades relative to no *G. vaginalis* (aRRR 2.38; 95% CI 1.19, 4.74; p=0.014). No other variable was significant in adjusted analyses.

Table 11 - Multinomial adjusted logistic regression investigating behavioural practices associated with number of *G. vaginalis* clades detected

	Single clade (n=63) vs *G. vaginalis* not detected		**Multiple clades (n=118) vs *G. vaginalis* not detected**	
	aRRR (95% CI)	***P* value[a]**	**aRRR (95% CI)**	***P* value[a]**
Baseline characteristics				
No. of lifetime FSPs[b]				
<5	1		1	
≥5	0.94 (0.46, 1.91)	0.859	2.04 (0.94, 4.43)	0.072
Interval practices in prior 3 months[c]				
Smoking				
No	1		1	
Yes	1.15 (0.59, 2.25)	0.674	2.38 (1.19, 4.74)	**0.014**
Sex with a new partner[d]				
No	1		1	
Yes	0.86 (0.31, 2.40)	0.768	1.79 (0.91, 3.52)	0.091
Frequency of sex				
Several times/month or less	1		1	
Sex several times per week	1.46 (0.68, 3.14)	0.331	1.77 (0.93, 3.37)	0.082

Abbreviations: aRRR, adjusted relative risk ratio; CI, confidence interval; FSP, female sexual partner

[a] Multinomial logistic regression with no *G. vaginalis* (i.e. *G. vaginalis* not detected) as the referent group. Analysis clustered for multiple specimens from participants (101 clusters). Variables included in the adjusted analysis were lifetime number of FSPs, smoking in prior 3 months, sex with a new partner in prior 3 months and frequency of sex.

[b] Variable was dichotomized at median value

[c] Interval practices were measured as any exposure over the prior study interval (~90 days)

[d] Sex with a new partner with who first sexual contact was within 90 days. May represent a new FSP or new MSP.

3.5.5. Factors associated with detection of each *G. vaginalis* clade

Seventy-two women changed clade at least once over the study period, accounting for 120 instances of change. Acquisition of new clade/s was the most frequent change observed (n=71/120, 59%), followed by loss of clade/s (n=38/120, 32%), and a combination of loss and

acquisition of clade/s (n=11/120, 9%). Five women had stable clade distribution over time.

Four univariable analyses were conducted to assess factors associated with detection of each specific clade *vs* detection of any other clade/s (Supplementary Table B2). As multi-clade specimens were common and we wanted to examine factors associated with detection of each individual clade rather than detection of *G. vaginalis,* we excluded specimens where no *G. vaginalis* clade was detected. Seventy-seven women contributed 181 specimens to each of the four analyses.

Clade 1 detection was associated with Nugent-BV (OR 3.55; 95% CI 1.76, 7.18; p<0.001) and non-optimal vaginal microbiota (OR 3.36; 95% CI 1.65, 6.84; p=0.001). Clades 2 and 3 were both associated with intermediate-BV (Clade 2 OR 3.49; 95% CI 1.17, 10.36; p=0.025 and Clade 3 OR 4.90; 95% CI 1.11, 21.57; p=0.035) and Nugent-BV (Clade 2 OR 1.90; 95% CI 1.02, 3.55; p=0.043 and Clade 3 OR 3.67; 95% CI 1.22, 11.04; p=0.020), but were not associated with vaginal microbiota composition. Clade 4 was not associated with Nugent score or vaginal microbiota composition (Supplementary Table B2).

For each clade, behavioural practices significantly associated by univariable analysis were included in a clade-specific multivariable analysis. Detection of clades 1, 2 and 3 were associated with interval practices (i.e. those performed in the three months prior to specimen collection). Having clade 1 vs any other clade/s was associated with smoking (AOR 2.42; 95% CI 1.12, 5.25; p=0.025; Table 12) and ≥1 sexual partner of any gender (AOR 4.03; 95% CI 1.16, 14.01; p=0.028), after adjusting for sex frequency. Having clade 2 vs any other clade/s was associated with sharing of unwashed sex toys with an FSP (AOR 2.59; 95% CI 1.22, 5.51; p=0.013) and recent penile-vaginal sex (AOR 5.67; 95% CI 1.74, 18.51; p=0.004). Having clade 3 vs any other clade/s was associated with sex with a new partner (AOR 5.02; 95% CI 1.25, 20.18; p=0.023) and having a current FSP with BV symptoms (AOR 24.73 95% CI 3.37, 181.27; p=0.002). Having clade 4 vs any other clade/s was associated with having ≥5 lifetime FSPs (AOR 3.35; 95% CI 1.45, 7.75; p=0.005).

Table 12 - Practices associated with detection of specific *G. vaginalis* clades by logistic regression

***G. vaginalis* clade 1** [a]	**OR (95% CI)**	***P* value**	**AOR (95% CI)**	***P* value**
Any smoking in prior 3 months				
No	1		1	
Yes	2.29 (1.40, 6.10)	**0.004**	2.42 (1.12, 5.25)	**0.025**
Number of SP in prior 3 months				
0	1		1	
1	5.09 (1.55, 16.66)	**0.007**	4.03 (1.16, 14.01)	**0.028**
≥2	8.19 (2.05, 32.75)	**0.003**	5.25 (1.22, 22.50)	**0.026**
Frequency of sex with any SP				
Several times/month or less	1		1	
Several times/week	2.03 (1.07, 3.88)	**0.031**	1.60 (0.79, 3.25)	0.190
***G. vaginalis* clade 2** [b]	**OR (95% CI)**	***P value***	**AOR (95% CI)**	***P value***
Any sharing of sex toys in prior 3 months				
No toys/washed/condoms used	1		1	
Unwashed	2.21 (1.08, 4.52)	**0.030**	2.59 (1.22, 5.51)	**0.013**
Any penile-vaginal sex in prior 3 months				
No	1		1	
Yes	4.80 (1.52, 15.17)	**0.007**	5.67 (1.74, 18.51)	**0.004**
***G. vaginalis* clade 3** [c]	**OR (95% CI)**	***P* value**	**AOR (95% CI)**	***P* value**
Sex with a new partner in prior 3 months [d]				
No	1		1	
Yes	3.09 (1.10, 8.69)	**0.032**	2.90 (0.95, 8.92)	0.063
Current partner with BV symptoms				
No/don't know	1		1	
Yes	17.40	**0.001**	16.95	**0.001**

	(3.36, 90.11)		(3.11, 92.23)	
***G. vaginalis* clade 4** [e]	**OR (95% CI)**	***P value***	**AOR (95% CI)**	***P value***
No. of lifetime FSPs				
<5	1		1	
≥5	3.93 (1.74, 8.87)	**0.001**	3.35 (1.45, 7.75)	**0.005**
Lifetime history of vaginal sex with a man [f]				
No	1		1	
Yes	2.88 (1.13, 7.32)	**0.026**	2.09 (0.82, 5.33)	0.125

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; FSP, female sexual partner; SP, sexual partner (may refer to female or male partner)
Includes specimens positive for one or more *G. vaginalis* clade (181 specimens from 77 women).
[a] Logistic regression fitted with generalized estimating equations (GEE) with absence of clade 1 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)
[b] Logistic regression fitted with GEE with absence of clade 2 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)
[c] Logistic regression fitted with GEE with absence of clade 3 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)
[de] Sex with a new partner with who first sexual contact was within 90 days. May represent a new FSP or new MSP.
[e] Logistic regression fitted with GEE with absence of clade 4 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)
[f] Variable was dichotomized at median value

3.6. Discussion

We explored the distribution of *G. vaginalis* clades in women-who-have-sex-with-women (WSW) and found that clade 1 was the only clade associated with both Nugent-BV and a non-optimal (*Lactobacillus*-deficient) vaginal microbiota. Notably, clade 4 was the most prevalent clade but was not associated with these two vaginal states. Factors associated with detection of *G. vaginalis* included smoking, frequent sex and a history of penile-vaginal sex and clades were associated with a range of differing sexual practices and behaviours in adjusted analysis. Clades 1, 2 and 3 were associated with recent behavioural and sexual practices and clade 4 was associated with sexual practices prior to enrolment. These findings support the sexual exchange of *G. vaginalis*, and suggest that different *G. vaginalis*

clades may have varying levels of pathogenicity, differ in mode of acquisition and duration of infection, and may circulate in different populations or sexual networks.

Previous studies investigating *G. vaginalis* clade distribution that use the same clade typing applied in our study have inconsistently associated individual clades with BV (Balashov *et al.*, 2014; Hilbert *et al.*, 2017; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019; Vodstrcil *et al.*, 2017). However, most studies agree that clade 1 is associated with BV (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019). Our finding that clade 1 was associated with both Nugent-BV and non-optimal vaginal microbiota suggests that it may have increased pathogenicity compared to other clades. A recent comparative genomic and ecotyping analysis of 35 *G. vaginalis* strains highlighted key differences between ecotype 1 (corresponds to clade 1) and other *G. vaginalis* ecotypes that may contribute to its pathogenicity (Cornejo *et al.*, 2018). Ecotype 1 uniquely encodes glycosidases that may aid cervicovaginal mucus degradation, and has enriched galactose and pentose sugar metabolism pathways, which may provide ecotype 1 with an advantage when co-colonising the vagina with lactic acid producing bacteria (Cornejo *et al.*, 2018).

Our finding that clade 4 was not associated with Nugent-BV or non-optimal vaginal microbiota is supported by two studies reporting no association of clade 4 with BV (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017). Clade 4 strains lack sialidase activity (Janulaitiene *et al.*, 2018; Schellenberg *et al.*, 2016), which is associated with mucin degradation in BV, further supporting reduced pathogenicity of clade 4. However, Vodstrcil *et al.* (2017) found clade 4 was associated with Nugent-BV in a cohort of 52 young sexually inexperienced women and two additional studies noted an association between Nugent-BV and increasing prevalence and/or load of clade 4 (Hilbert *et al.*, 2017; Shipitsyna *et al.*, 2019). Inconsistencies between studies may be due to population differences including sexual practices and networks, behavioural practices such as smoking, past history of BV and ethnicity. Previous associations of clade 4 and BV may also be a result of high load infections (Hilbert *et al.*, 2017; Shipitsyna *et al.*, 2019). Another consideration is that differences between studies are a result of spurious findings due to unmeasured confounding. *G. vaginalis* clades do not often occur in isolation and associations between specific clades and BV may be due to high abundance and/or presence of other BV-associated bacteria, or due to depleted levels of optimal *Lactobacillus* spp. in the vagina. Given the polymicrobial nature

of BV, future studies should consider interactions between *G. vaginalis* clades and other inhabitants of the vaginal microbiome that may play an integral role in BV-pathogenesis.

Multi-clade *G. vaginalis* communities are common and are associated with BV (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017; Shipitsyna *et al.*, 2019; Vodstrcil *et al.*, 2017). In our study, detection of multiple clades was associated with intermediate-BV, Nugent-BV and non-optimal vaginal microbiota. Multiple clades may act synergistically to supress *Lactobacillus* spp. or form biofilms. Alternatively, there may be one *G. vaginalis* clade driving disease as well as passenger clades; in specimens where clades co-occur it is difficult to identify which specific clade/s is driving disease as other clades may rapidly cohabit following an initiation event. To investigate whether there were independent associations between individual *G. vaginalis* clades and BV, we conducted an additional analysis that included all four clades with Nugent-BV as the outcome (Supplementary Table B3). The results of the adjusted analysis were consistent with our univariable findings, with clade 1 having the strongest association with Nugent-BV (Supplementary Table B2). However, this analysis was limited by the correlation between clades so was not investigated further. The high prevalence of multi-clade communities may explain contradictory associations of specific clades with BV in previous studies.

Clades 1, 2 and 3 were significantly associated with current sexual practices and we observed a non-significant trend between detection of multiple *G. vaginalis* clades and sexual practices including increased sex frequency and sex with a new partner. Several studies document exchange of *G. vaginalis* between sexual partners. Heterosexual couples share identical *G. vaginalis* strains (Eren *et al.*, 2011) and demonstrate high concordance for *G. vaginalis* biofilm (Swidsinski *et al.*, 2010). A study using culture methods reported increased *G. vaginalis* colonisation with increasing frequency of digital-vaginal sex and sex-toy use in WSW (Mitchell *et al.*, 2011). Using PCR, *G. vaginalis* prevalence has been shown to increase following sexual debut and with increasing numbers of sexual partners (Fethers *et al.*, 2012). Differing associations seen with individual clades does not necessarily suggest that specific clades are only transmitted by specific sexual practices, but rather provides support for the sexual exchange of *G. vaginalis* clades across a range of different practices.

Smoking was associated with clade 1 detection and women who reported smoking were more likely than non-smokers to have multiple *G. vaginalis* clades detected. This is consistent with a previous report of increased *G. vaginalis* detection by culture amongst smokers compared to non-smokers (Hellberg *et al.*, 2000), and is supported by the frequent association of smoking with BV (Bradshaw *et al.*, 2014; Brotman *et al.*, 2014a; Evans *et al.*, 2007). The mechanism by which smoking may increase *G. vaginalis* in the vagina is unknown, however smokers have an altered vaginal metabolome (including high concentrations of nicotine and its derivatives) (Nelson *et al.*, 2018) and reduced oestradiol levels (Westhoff *et al.*, 1996), which may impact vaginal microbiota composition. Additionally, smoking negatively impacts immune function (Bagaitkar *et al.*, 2008), which may result in reduced clearance of *G. vaginalis*, similar to what has been observed for human papillomavirus (Giuliano *et al.*, 2002). It is also possible that our observed association between smoking and both clade 1 and multi-clade communities is a result of unmeasured confounding.

Three specimens that tested positive for *G. vaginalis* by species specific PCR did not have any clade detected by clade-specific PCR. This raises the possibility that more than four *G. vaginalis* clades exist or that genetic subgroups may exist within the four clades (Cornejo *et al.*, 2018; Schellenberg *et al.*, 2016). In early 2019, Vaneechoutte *et al.* (2019) proposed the existence of 13 different species within the *Gardnerella* genus and additional clades/subgroups are likely to be revealed as we analyse larger numbers of *G. vaginalis* isolates.

Our study has limitations. Nugent score was used to facilitate self-collection of vaginal specimens at home. Participants were not examined by a clinician, which limited our ability to assess *G. vaginalis* clade distribution in Amsel-BV. Longitudinal specimens included in the analysis typically represented the last three specimens collected from a participant and were not selected randomly or from specified study time points which may have biased results. Specimens were collected at three-monthly intervals so the immediate impact of practices on *G. vaginalis* clade distribution is unclear. Frequent sampling would address this and clarify whether *G. vaginalis* clades persist or are transient. As we did not use the PCR assay to quantitate clades, clade associations are based on detection rather than quantity and we were unable to assess associations between covariates and quantity of each clade.

Finally, the study population comprised highly educated women who were predominately Australian born and our findings may not be generalizable to other populations including sexually inexperienced women or women who exclusively have sex with men.

We report that multi-clade *G. vaginalis* communities and clades 1, 2 and 3 were associated with Nugent-BV in Australian WSW. That multi-clade communities and clade 1 were both also associated with non-optimal (*Lactobacillus* deficient) vaginal microbiota indicates increased pathogenicity. The finding that clade 4 was not associated with either BV or non-optimal vaginal microbiota strongly suggests it may be a commensal clade. Detailed comparative analyses of commensal and pathogenic *G. vaginalis* genetic types may help to identify potential mechanisms of *G. vaginalis* pathogenesis. Individual *G. vaginalis* clades were associated with differing sexual practices, adding to the growing evidence supporting the sexual exchange of *G. vaginalis*. These data have implications for BV treatment and suggest that partner treatment may be an effective strategy to improve BV cure.

Chapter 4. A prospective, open-label pilot study of concurrent male partner treatment for bacterial vaginosis

4.1. Overview

Chapters 2 and **3** of this thesis provide microbiological evidence that sexual exchange of bacteria (including BV-associated bacteria such as *Gardnerella* spp.) occurs between female sexual partners and contributes to the development of non-optimal vaginal microbiota. These data add to the growing body of evidence that sexual transmission contributes significantly to the pathogenesis of BV (Section 1.6.2). Importantly, these data highlight that preventative and treatment strategies that address sexual transmission and re-infection from an untreated sexual partner may be effective in improving long term cure of BV.

Chapter 4 presents the findings from a prospective open-label pilot study of concurrent male partner treatment for BV, that utilised the novel approach of treating male sexual partners with both oral and topical antimicrobials.

As discussed in Section 1.6.3.1 of the literature review, past partner treatment trials have failed to consistently reduce BV recurrence. However, earlier RCTs of partner treatment were poor quality and methodologically flawed, and their findings should not be considered evidence that BV is not/never sexually transmitted. A review of past RCTs concluded that high quality studies that address limitations of past studies and that incorporate accompanying genital microbiota data are needed to evaluate the efficacy of partner treatment (Mehta, 2012). Therefore, the motivation behind the study presented in **Chapter 4** was to pilot recruitment methods, the use of combined oral and topical antibiotic treatment for men, specimen collection, laboratory methods, and bioinformatic analysis methods in order to inform the design of a large RCT of partner treatment. Specific aims of **Chapter 4** were to: 1) investigate the impact of concurrent male partner treatment on the genital microbiota over a 12-week period, 2) determine the adherence, acceptability and tolerability of male partner treatment utilising combined oral and topical antibiotics, and 3) generate preliminary estimates of the efficacy of the intervention.

Chapter 4 is presented as a manuscript that was submitted to the peer reviewed journal *mBio* in June 2021 and we have been invited to submit a revision. No alterations to the text of the submitted manuscript have been made, except that abbreviations, and figure and table numbers have been adjusted to generate a consistent presentation within this thesis. Additionally, the methods section has been moved to appear before the results to be consistent with other chapters within this thesis.

Publication:

> **Plummer EL**, Vodstrcil LA, Doyle M, Danielewski JA, Murray GL, Fehler G, Fairley CK, Bulach DM, Garland SM, Chow EPF, Hocking JS, Bradshaw CS. A prospective, open-label pilot study of concurrent male partner treatment for bacterial vaginosis. Submitted to *mBio* in June 2021, revision in preparation.

Findings from this manuscript have also been presented at the following national and international conferences:

- Virtual STI & HIV 2021 World Congress, 14 – 17 Jul 2021. [Oral] - **American STD Association – Best oral presentation (Young Investigator) award**
- 2020 Virtual Impact AMR Research Colloquium, 25 Nov 2020. [Oral]
- 2020 Virtual Joint HIV&AIDS and Sexual Health Conferences, 16 – 20 Nov 2020. [Oral]
- Royal Women's Hospital Research Week, Melbourne, Australia, 18 – 19 Nov 2019. [Poster]
- STI & HIV 2019 World Congress, Vancouver Canada, 14 – 17 Jul 2019. [Poster]

All supplementary files referred to in this chapter have been appended to this thesis in Appendix C.

4.2. Abstract

Up to 50% of women receiving first-line antibiotics for bacterial vaginosis (BV) experience recurrence within 12-weeks. Evidence suggests that reinfection from an untreated regular sexual partner contributes to recurrence. We conducted a pilot study of 34 heterosexual couples to describe the impact of concurrent partner treatment on the composition of the genital microbiota over a 12-week period. We also determined the acceptability and

tolerability of concurrent partner treatment, and obtained preliminary estimates of the efficacy of the intervention to inform a randomised controlled trial (RCT). Women received first-line antibiotic treatment for BV (i.e. oral metronidazole or intravaginal clindamycin), and their male partner received oral metronidazole 400mg and 2% clindamycin cream applied topically to penile skin, both twice-daily for 7-days. The genital microbiota was characterised at three anatomical sites (women: vaginal; men: cutaneous penile, first-pass urine [representing the urethra]) using 16S rRNA gene sequencing. Immediately post-treatment, concurrent partner treatment significantly reduced the abundance of BV-associated bacteria (false discovery rate (FDR) corrected p-value<0.05) and altered the overall microbiota composition of all three anatomical sites (p=0.001). Suppression of BV-associated bacteria was sustained in the majority of women over the 12-week period (FDR p-value<0.05), despite BV-associated bacteria re-emerging at both genital sites in men. In this cohort of women at high risk for recurrence, five recurred within 12-weeks of treatment (17%; 95%CI:6-34%). Importantly, men tolerated and adhered to combination therapy. Our findings provide support for a RCT of combined oral and topical male partner treatment for BV.

4.3. Introduction

Bacterial vaginosis is a highly prevalent vaginal condition that is associated with obstetric and gynaecological sequelae, and has significant implications for healthcare expenditure (Peebles *et al.*, 2019). The vaginal microbiota in women with BV is characterised by a reduction in *Lactobacillus* spp. and an increase in obligate and facultative anaerobes collectively termed BV-associated bacteria (including *Gardnerella vaginalis, Atopobium vaginae, Prevotella* spp., *Sneathia* spp. among others) (Fredricks *et al.*, 2005). Recurrence is frustratingly common and treatments that achieve long term cure are lacking. Up to 50% of women experience recurrence within the 12-weeks following first-line antibiotic treatment (Bradshaw *et al.*, 2006a; Sobel *et al.*, 1993), and epidemiological studies have shown that women who have the same regular sexual partner pre- and post-treatment for BV are at 2-3-fold increased risk of recurrence (Bradshaw *et al.*, 2006a; Bradshaw *et al.*, 2013a; Vodstrcil *et al.*, 2019). Despite a growing body of evidence that sexual transmission plays an important role in the pathogenesis of BV acquisition and recurrence, current treatments for BV only target the affected woman (Workowski & Bolan, 2015).

Our group is conducting a programme of research investigating the acceptability, tolerability and efficacy of concurrent partner treatment for improving BV cure. We previously published an exploratory study of 22 heterosexual couples receiving concurrent antimicrobial therapy for BV (Plummer *et al.*, 2018a). Women received first-line BV-treatment (oral metronidazole or intravaginal clindamycin) and males received combined topical and oral antimicrobial therapy (oral metronidazole and topical clindamycin applied to the penile skin). As BV-associated bacteria have been detected on the coronal sulcus/glans penis and in the distal urethra, as well as in urine and semen samples (Mandar *et al.*, 2015; Mehta *et al.*, 2020b; Nelson *et al.*, 2012; Zozaya *et al.*, 2016), both oral and topical antibiotics were used in males to address multi-site carriage. In this exploratory study, we followed couples for 3-weeks post-treatment and found that suppression of BV-associated bacteria was sustained in the majority of women. These data are encouraging as women in this cohort were at high risk of recurrence, all were having unprotected sex with their regular male partner, who were predominantly uncircumcised, and the majority had a history of BV (Plummer *et al.*, 2018a). Although this earlier exploratory study provided support for continued investigation into concurrent partner treatment for BV, it was limited by a short duration of follow-up and suboptimal sampling of the urethral site in males. This affected our ability to assess the durability and long-term impact of partner treatment on the genital microbiota, and particularly at the male urethral site. This is relevant because a recent study found that both the urethral and cutaneous penile microbiota were accurate predictors of incident BV in female sexual partners, with the composition of the urethral site having slightly higher prediction accuracy than the cutaneous site (Mehta *et al.*, 2020b).

The objectives of the present study were to: 1) assess the impact of concurrent partner treatment on three anatomical sites (the vagina, cutaneous penile site and male urethra) over a 12-week period, 2) determine the adherence to, and acceptability and tolerability of concurrent male partner treatment, and 3) provide preliminary estimates of the efficacy of the intervention in order to inform an RCT.

4.4. Methods

This study was conducted and reported in accordance with the Transparent Reporting of Evaluations with Nonrandomised Designs statement (Des Jarlais *et al.*, 2004), and was

prospectively registered with the Australian New Zealand Clinical Trials Registry (ACTRN12618000219280).

4.4.1. Study design, participants and recruitment

This was a prospective, open-label pilot study of concurrent partner treatment for BV conducted at the Melbourne Sexual Health Centre, Victoria, Australia between March 2018 and March 2019. This study was originally designed as a two-arm, non-randomised trial, where participants could choose to either be in an intervention group (concurrent partner treatment) or a standard of care group (female-only treatment). While the study objectives were to obtain acceptability, tolerability and microbiota data, as well as preliminary efficacy estimates for the intervention, the design enabled couples to enrol if the male declined treatment. However, all recruited couples elected to receive concurrent partner treatment (i.e. no males who agreed to participate in the study declined treatment), so the study is reported as a single-arm study.

Women attending Melbourne Sexual Health Centre with vaginal symptoms were tested for BV using both Amsel criteria (Amsel *et al.*, 1983) and the Nugent method (Nugent *et al.*, 1991). In keeping with our standard clinical practice, women were diagnosed with BV defined by ≥3 Amsel criteria and Nugent score=4-10. Women with BV were referred to the research team for eligibility screening. Women were eligible if they were prescribed a first-line treatment for BV (oral metronidazole 400mg *BID* for seven days, or an alternative first-line treatment (i.e. 2% vaginal clindamycin cream as one applicator vaginally for seven nights if oral metronidazole was contraindicated), were aged 18-55 years, were willing and able to comply with study requirements, and had a regular male partner of ≥2 months who was willing to participate. Women were ineligible if they were pregnant or breastfeeding, HIV positive, diagnosed with current pelvic inflammatory disease, had an allergy to both metronidazole and clindamycin, diagnosed with *C. trachomatis, N. gonorrhoeae* or *T. vaginalis* at baseline, had other current sexual partners or were engaging in current sex work.

Male partners of women who agreed to participate were screened for eligibility and recruited either in clinic or via a telephone consultation. Males were eligible if they were aged ≥18 years and were willing and able to comply with study requirements. Males were

ineligible if they were HIV positive, had an allergy to metronidazole or clindamycin, had other current sexual partners or were engaging in current sex work.

4.4.2. Intervention

All males received oral metronidazole 400mg *BID* and 2% clindamycin cream which was applied topically to the head of the penis and upper shaft (under the foreskin if uncircumcised) *BID* for seven days. Couples were instructed to abstain from sexual activity until both partners had completed all treatment doses.

4.4.3. Study procedures

Prior to commencing antibiotics, participants completed a questionnaire concerning demographics, clinical and behavioural information, and self-collected genital specimens for microbiota analysis. Women self-collected a high-vaginal swab (Copan flocked swab, Italy) and males self-collected a cutaneous penile swab and a first pass urine (FPU) sample. FPU has been shown to provide an accurate representation of the urethral microbiota (Dong *et al.*, 2011) and was chosen as it was thought to be more acceptable to men than a self-collected urethral swab. The cutaneous penile swab was collected using a Copan flocked swab pre-moistened with sterile water. Males were instructed to firmly rub the swab three times around the coronal sulcus and then over the glans of the penis. Males obtained the FPU sample by urinating the first 15ml of urine into a pot and then used a sterile single-use plastic pipette to transfer the urine to a 15ml tube with 830µl of AssayAssure® Genelock (SierraMolecular, USA). Males were instructed to retract the foreskin if uncircumcised before collecting both specimens.

Couples completed questionnaires and self-collected genital specimens at day-8 (the day after finishing antibiotics), and weeks 4, 8 and 12. All follow-up was completed at home and packs returned by post, with the exception that women were required to return to the clinic for BV-assessment at week-12. At each time point, women self-collected a vaginal swab and a smear for Nugent scoring, and men self-collected a cutaneous penile swab and FPU sample. Women who experienced BV symptoms during follow-up were recalled to the clinic for BV assessment. Couples were censored from the study if the woman experienced BV recurrence (≥3 Amsel criteria and a Nugent score of 4-10). Where the woman had a Nugent

score=7-10 during follow-up, she was encouraged to attend clinic for BV assessment using combined Amsel and Nugent criteria. Women who did not report symptoms and could not attend clinic, were not treated and continued to provide specimens until study endpoint (BV recurrence or week-12 without recurrence).

4.4.4. Outcomes

The following outcomes were measured:

1) The effect of concurrent partner treatment on the composition of the vaginal and male genital (cutaneous penile and urethral) microbiota over a 12-week period. Couples contributed to this outcome if both partners returned a minimum of day-0, day-8 and endpoint specimens.

2) Adherence to, and acceptability and tolerability of combined oral and topical antibiotic treatment in male partners of women with BV. Adherence was self-reported at day-8, acceptability was assessed as the proportion of males who agreed to participate, and tolerability was assessed by self-report of adverse events at day-8. Couples contributed to this outcome if both partners completed the day-8 questionnaire.

3) Preliminary estimates of BV recurrence (defined as ≥3 Amsel criteria and Nugent score=4-10) over the 12-week study period. Couples contributed to this outcome if both partners completed the day-8 questionnaire and provided questionnaire data to study endpoint.

4.4.5. Microbiota characterisation

On receipt, vaginal and cutaneous penile swabs were agitated in 600µl of PBS and stored at -80^{o}C until DNA extraction. DNA was extracted from swab samples using a pre-lysis bead beating protocol followed by automated extraction on the MagNA Pure 96 System (Roche Diagnostics, Germany). Samples were prepared for pre-lysis bead beating as follows: 200µl of sample, 300µl of MagNA Pure 96 Bacterial Lysis Buffer (Roche Diagnostics) and 50µl of Proteinase K Solution (Recombinant, PCR Grade 18 mg/mL; Roche Diagnostics) were combined and then incubated at 65^{o}C for 10 min. Following incubation, lysate was transferred to a bead tube (bead tubes for PureLink™ Microbiome DNA Purification Kit, Invitrogen) for bead beating on the Tissue Lyser (Qiagen, Germany) at 50Hz for 5 min, after

which 500µl of lysate was transferred for extraction using the MagNA Pure 96 DNA and Viral NA large-volume kit (Roche Diagnostics) and the manufacturer's Pathogen Universal 500 3.1 protocol with an elution volume of 100µl for vaginal swabs and 50µl for penile swabs. Reagent only negative controls were extracted in parallel for the pre-lysis using PBS, and Ultrapure water (Sigma-Aldrich) for the MagNA Pure 96 extraction.

On receipt, urine samples were immediately transferred to Royal Women's Hospital, Melbourne, Australia, where they were centrifuged using a refrigerated Heraeus Megafuge 16R (ThermoFisher Scientific, USA) at 5,580 relative centrifugal force for 30 min at 4°C. Supernatant was removed to within 2mL of the pellet. The pellet was resuspended in the remaining supernatant and stored at -80°C until extraction. DNA was extracted from 1mL of urine concentrate using the *saliva and urine sample* protocol for the PureLink™ Microbiome DNA Purification Kit (Invitrogen) with an elution volume of 50µl. A sample comprising the stabilisation medium AssayAssure® Genelock (SierraMolecular, USA) and PBS was extracted for each kit as the reagent only negative controls.

PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene was performed by Micromon Genomics (Monash University, Victoria, Australia) using primers 341F/805R and dual indexing based on the 16S Metagenomics protocol (Illumina, San Diego, CA, USA). Amplicons were sequenced on the Illumina MiSeq platform using v3 chemistry (600-cycle kit; Illumina, San Diego, CA, USA) at Micromon Genomics. Reagent and PCR negative controls and positive controls (mock microbial community standards) were processed and sequenced alongside samples (Supplementary Table C1).

Adapter removal and demultiplexing was performed by Micromon Genomics. The 16S rRNA gene amplifying primer sequences were removed from sequencing reads using Cutadapt v2.4 (Martin, 2011). DADA2 v1.16.0 (Callahan *et al.*, 2016) was used to quality filter and trim sequencing reads, infer amplicon sequence variants (ASVs), merge paired reads and remove chimeras. Taxonomic classification of ASVs was also performed using DADA2 and the DADA2-formatted SILVA database v138 (Quast *et al.*, 2013). Species-level classification of *Lactobacillus* ASVs and classification of ASVs matching *Candidatus Lachnocurva vaginae* (formerly BVAB-1), BVAB-2 and *Mageeibacillus indolicus* (formerly BVAB-3) was performed as previously described (Plummer *et al.*, 2019a).

ASVs identified as likely contaminants were removed. Contaminants included ASVs that were present 1) only in negative control specimens, or 2) in higher prevalence and/or abundance in negative control specimens compared to biological specimens, and not expected in the biological context (Supplementary Table C2). We additionally removed ASVs that were of non-bacterial origin, had a total abundance of <0.001%, or were present in only one specimen.

4.4.6. Sequence analysis

RStudio v.1.3.959 (RStudio Team, 2020) running R v4.0.3 (R Core Team, 2020) was employed for all analysis and generating figures, unless stated otherwise.

Nonmetric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) were used to visualise and test for global differences in the microbiota composition following partner treatment. We performed two analyses for each genital site comparing: 1) day-0 *vs* day-8 specimens, and 2) day-0 *vs* endpoint specimens. NMDS and ANOSIM were performed with vegan (Oksanen *et al.*, 2020) using the Bray-Curtis dissimilarity index and ASV data. NMDS plots were drawn using ggplot2 (Wickham, 2016).

ASVs with identical taxonomy were merged for subsequent analyses. Stacked bar plots were drawn using ggplot2 (Wickham, 2016).

ANOVA-Like Differential Expression tool (ALDEX2) (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014) was used to identify taxa that were differentially abundant between pre-treatment and post-treatment samples, using the sample comparisons as above for each genital site: 1) day-0 *vs* day-8, and 2) day-0 *vs* endpoint. We generated 128 centre log-ratio transformed Dirichlet Monte Carlo instances, and tested for differentially abundant taxa using the Wilcoxon test, followed by a Benjamini–Hochberg FDR correction. Taxa with an FDR<0.05 were considered significant. Boxplots were drawn using ggplot2 (Wickham, 2016).

Analysis of composition of microbiomes (ANCOM) (Mandal *et al.*, 2015) v2.1 was used to identify differences longitudinally in the abundance of taxa between couples who recurred and couples who were cured. Specimens collected at day-0 and day-8 were excluded from ANCOM analyses. The ANCOM framework accounts for the compositional nature of microbiota data by applying a pseudocount of 1 to all taxa, and comparing the log-

transformed ratio of the abundance of each pair of taxa between study groups. Specifically, we regressed the log-transformed ratio of the abundance of each pair of taxa against recurrence status, with participant as a random effect. A cut-off value of 0.7 was applied to identify taxa that were differentially abundant, meaning that the null hypothesis was rejected in ≥70% of comparisons, using FDR<0.05. Structural zeros were identified (Kaul *et al.*, 2017), and taxa that were present in ≤10% of samples were excluded.

The FastSpar (Watts *et al.*, 2019) implementation of SparrCC (Friedman & Alm, 2012) was used to examine the correlation between taxa in the female and male genital microbiota of sexual partners at day-0, day-8 and longitudinally, taking into account the compositional nature of microbiota data. Taxa with an absolute correlation coefficient >0.3 and P<0.05 were considered significant.

Raw sequence data are available in the NCBI Sequence Read Archive (BioProject identifier PRJNA735440).

4.4.7. Study approval

Ethical approval was obtained from the Human Research and Ethics Committee of the Alfred Hospital, Melbourne, Australia (Project number 264/15). Written informed consent was obtained from all participants.

4.5. Results

4.5.1. Participant flow and recruitment

From March 2018 to March 2019, 115 women attending the Melbourne Sexual Health Centre who were diagnosed with BV were referred to the research team and screened for eligibility (Figure 8). Forty-three women were ineligible and 23 declined. Of note, 11 women were excluded because they felt their partner would not be interested in participating or they did not want to discuss the study with their partner. Of the 49 women who consented, 43 male partners (88%) agreed to participate. Of the 43 couples who received the intervention, seven were protocol violations (male did not return any study packs) and two completed baseline procedures only. Thirty-four couples (79%) provided both baseline (day-0) and day-8 data and contributed to adherence, acceptability and tolerability analyses.

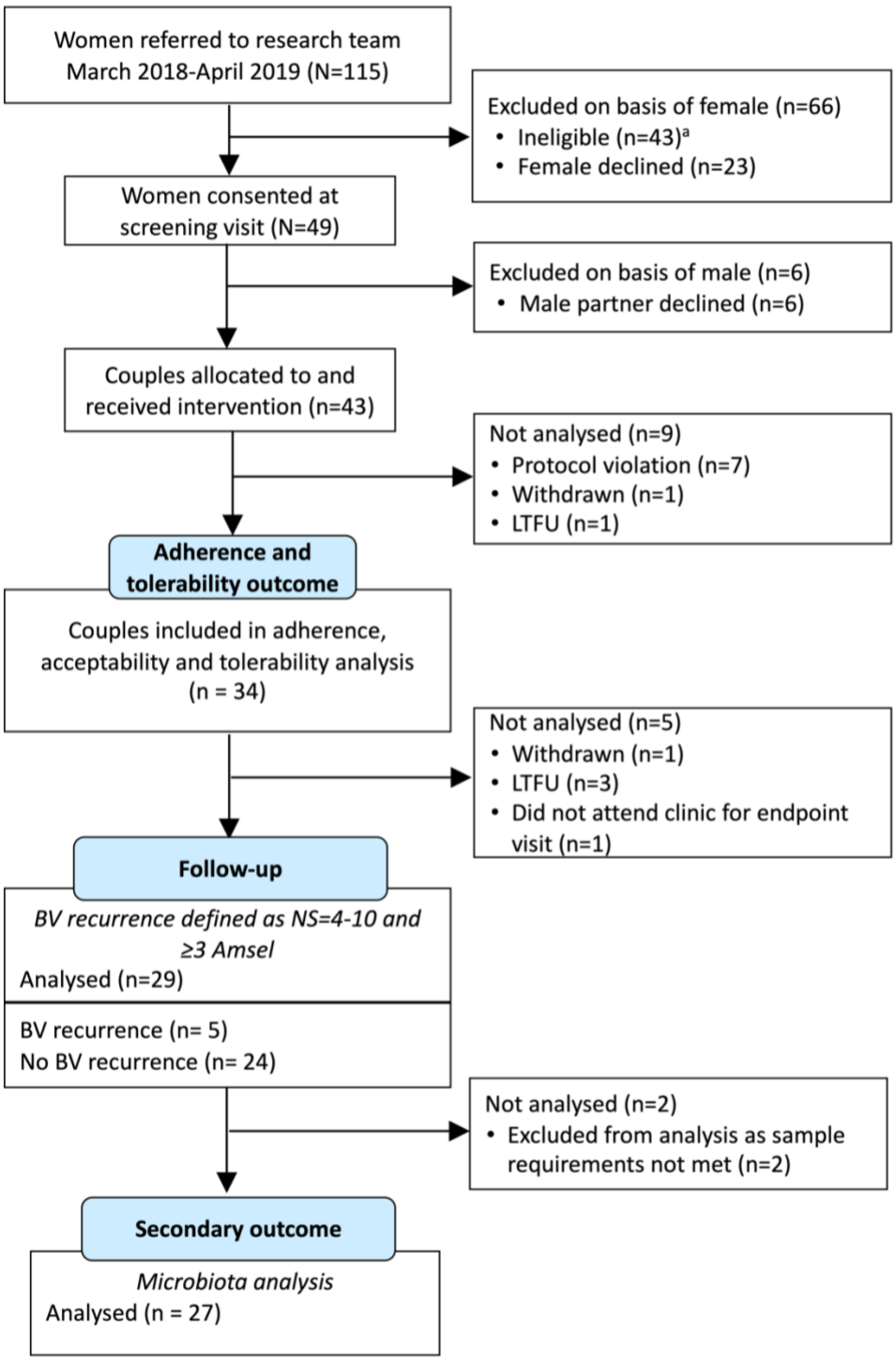


Figure 8. CONSORT Diagram of participant flow through the study

Abbreviations: LTFU, loss-to-follow-up

[a] the most common reasons for ineligibility were no BV by the study criteria (n=12), the couple was unable to comply with study procedures (n=11), or one or both partners reported other sexual partners (n=6)

[b] Endpoint specimens for microbiota analysis were missing from one female (who experienced BV recurrence) and from one male (whose female partner was cured).

4.5.2. Baseline data

Baseline demographic and behavioural characteristics for the 34 couples is provided in Table 13. Most women reported a past history of BV (n=27, 79%), 18 (53%) reported current hormonal contraceptive use, and 12 (35%) had an intrauterine device (IUD) *in situ* (six reported using an levonorgestrel IUD and six reported using a copper IUD). Most men were uncircumcised (n=29, 85%). The median duration of their relationship was 18 months (IQR=8-36 months), and all couples reported condomless penile-vaginal sex in the month prior to enrolment.

Table 13 - Demographic and behavioural characteristics of couples at baseline

	Female (n=34)	**Male (n=34)**
Age in years, median (IQR)	30 (27-34)	31 (27-37)
Country of Birth		
Australia	18 (53)	23 (70)
Other	16 (47)[a]	10 (30)[b]
Current smoker		
No	26 (79)	20 (61)
Yes	7 (21)	13 (39)
History of BV		
No	7 (21)	-
Yes	27 (79)	-
Months since last BV, median (IQR)	3 (2-12)	-
Current use of hormonal contraception		
No	16 (47)	-
Yes	18 (53)[c]	-
Current use of an intrauterine device		
No	22 (65)	-
Yes	12 (35)[d]	-
Current douching		
No	31 (91)	-
Yes	3 (9)	-
Circumcised		
No	-	29 (85)
Yes	-	5 (15)

Number of sexual partners in last 3 months[e]		
1	25 (74)	22 (71)
≥2	9 (26)	9 (29)
Number of lifetime sexual partners		
1-7	11 (34.4)	6 (19)
8-20	10 (31.2)	10 (32)
≥21	11 (34.4)	15 (48)
Duration of current partnership in months, median (IQR)[f]	18 (8-36)	21 (9-36)
Any condomless vaginal sex in last month		
No	0 (0)	0 (0)
Yes	34 (100)	32 (100)
Any condomless anal sex in last month		
No	25 (74)	24 (75)
Yes	9 (26)	8 (25)
Any oral sex received in the last month		
No	7 (21)	3 (10)
Yes	27 (79)	28 (90)
Antibiotics taken in last month		
No	28 (82)	29 (88)
Yes	6 (18)[g]	4 (12)[h]
Vaginal treatments used in last month		
No	32 (94)	-
Yes	2 (6)[i]	-
Treatments on penis used in last month		
No	-	30 (94)
Yes	-	2 (6)[j]

Data presented as n(%) unless otherwise specified; Abbreviations: SD, standard deviation; Data missing from up to 2 women and 3 men for some questions

[a] Country of birth for females not born in Australia: WHO European region (n=8), WHO Western Pacific region (n=3), WHO Americas region (n=3), WHO South-East Asian region (n=1), WHO Eastern Mediterranean region (n=1).

[b] Country of birth for males not born in Australia: WHO European region (n=8), WHO Region of the Americas (n=1), WHO Western Pacific region (n=1)

[c] Eight women reported current oral contraceptive use, six reported using a levonorgestrel IUD, three reported using a contraceptive implant, one reported using a hormonal injection (Depo Provera)

[d] Six women reported using a levonorgestrel IUD and six women reported using a copper IUD.

[e] Includes the partner they enrolled with

[f] Discrepancies are a result of independent reporting by female and male partners

[g] Metronidazole (n=3), tinidazole (n=1), doxycycline (n=1), azithromycin (n=1)

[h] Doxycycline (n=1), amoxicillin (n=1), amoxicillin/clavulanic acid (n=1), azithromycin (n=1)

[i] Clotrimazole (n=2)
[j] Clotrimazole (n=1), Daivobet 50/500 gel (contains calcipotriol; betamethasone dipropionate, n=1)

Clinical and laboratory characteristics are in Table 14. Thirty-three (97%) women had ≥3 Amsel criteria and a Nugent score of 4-10 at enrolment. The final woman had BV by Nugent criteria (Nugent score=9), as well as presence of Clue cells and vaginal pH>4.5. However, the clinician was unable to accurately assess vaginal discharge and vaginal malodour on examination as the woman had undertaken intravaginal cleaning immediately prior to examination. Self-reported penile symptoms were reported by 2 males (6%).

Table 14 - Clinical and laboratory characteristics at baseline

	Females (n=34)
Self-reported vaginal discharge	
No	6 (19)
Yes	26 (81)
Self-reported vaginal malodour	
No	6 (18)
Yes	27 (82)
Nugent score	
4-6	6 (18)
7-10	28 (82)
Amsel criteria	
2	1 (3)[a]
≥3	33 (97)
Days since LNMP ended, median (IQR)[b]	14 (6-17)
	Males (n=34)
Self-reported penile discharge	
No	31 (97)
Yes	1 (3)
Self-reported penile malodour	
No	30 (94)
Yes	2 (6)

Data presented as n(%) unless otherwise specified; Abbreviations: LNMP, Last known menstrual period;
[a] This woman had BV by Nugent criteria (Nugent score=9), as well as presence of Clue cells and a vaginal pH>4.5. However, clinician recorded vaginal discharge and vaginal malodour (i.e. amine test) were not able to be accurately reported as the woman had undertaken intravaginal cleaning immediately prior to clinical examination.
[b] LNMP missing for n=3 women, n=5 women report not menstruating due to hormonal

contraception, n=3 women were menstruating at time of BV diagnosis

4.5.3. Adherence and tolerability

All 34 women provided adherence and tolerability data, 32 were prescribed oral metronidazole (400mg *BID* for seven days) and two received 2% intravaginal clindamycin cream (one applicator vaginally for seven nights; Table 15). All males received both oral metronidazole (400mg *BID* for seven days) and 2% clindamycin cream (applied topically to the penis *BID* for seven days). Self-reported adherence to metronidazole was high; 29 women (91%) and 30 men (88%) took all tablets. Both women who received intravaginal clindamycin reported applying all doses, and 24 males (71%) reported applying all clindamycin doses. Eighteen women and 11 men reported at least one adverse event in the day-8 questionnaire, with nausea (n=13) and metallic taste (n=11) being the most common. Three men reported penile irritation and/or redness, which was mild and not treatment limiting.

Table 15 - Treatment adherence and adverse effects

	Female (n=34)	**Male (n=34)**
Prescribed Metronidazole (oral)[a]	32 (94)	34 (100)
Self-reported adherence to metronidazole		
Took all tablets	29 (91)	30 (88)
Missed 1-4	1 (3)	3 (9)
Missed >4	2 (6)	1 (3)
Prescribed Clindamycin (topical)[a]	2 (6)	34 (100)
Self-reported adherence to clindamycin		
Applied all doses	2 (100)	24 (71)
Missed 1-4	0	8 (23)
Missed >4	0	2 (6)
Self-reported adverse effect[b]		
Nausea	9 (26)	4 (12)
Vomiting	0 (0)	0 (0)
Metallic taste	7 (21)	4 (12)
Headache	6 (18)	2 (6)
Vaginal irritation	2 (6)	-
Irritation of penile skin	-	2 (6)
Redness of penile skin	-	2 (6)
Other	8 (23)[c]	6 (18)[d]

Data presented as n(%) unless otherwise specified
[a] Oral metronidazole was standard treatment for females. One woman received 28 days of metronidazole. Oral metronidazole was contraindicated in two women who subsequently received vaginal clindamycin.
[b] No adverse effects reported by women who used clindamycin.
[c] Other side effects: thrush (n=2), drowsiness or fatigue (n=2), feeling weak (n=1), vaginal dryness (n=1), gastrointestinal upset (n=1), tension behind eyes and feeling ill (n=1).
[d] Other side effects: fatigue (n=2), dizziness (n=1), tingling sensation in hands (n=1), increased appetite (n=1) and thrush (n=1, this participant also reported irritation and redness of penile skin).

4.5.4. BV recurrence over the study period

Of the 29 women to provide follow-up data to study endpoint (BV recurrence defined as Nugent score 4-10 with ≥3 Amsel criteria or week-12 without recurrence), five (17%; 95%CI: 6-34%) experienced recurrence. Table 16 presents participant practices over the study period stratified by BV recurrence. Women who recurred all had a past history of BV, all had an uncircumcised partner, all reported condomless vaginal sex after the 7-day treatment period, and three of the five had an IUD *in situ* (two had a copper IUD *in situ* and one had a levonorgestrel IUD *in situ*). One woman who recurred also reported a new sexual partner during follow-up. All couples that recurred reported 100% treatment adherence, and all reported no sexual contact, or using condoms for sex, during the 7-day treatment period.

Table 16 - Baseline and longitudinal characteristics of women stratified by BV recurrence status

	Cured (n=24)	**BV recurrence (n=5)**
Baseline characteristics		
History of BV		
No	5 (21)	0 (0)
Yes	19 (79)	5 (100)
Circumcised partner		
No	19 (79)	5 (100)
Yes	5 (21)	0 (0)
Intrauterine device *in situ*		
No	16 (67)	2 (40)
Yes	8 (33)[a]	3 (60)[b]
Current hormonal contraception use		
No	11 (46)	3 (60)
Yes	13 (54)	2 (40)

Treatment period characteristics (days 1-7)		
Treatment adherence (female)		
100%	21 (88)	5 (100)
<100%	3 (13)	0 (0)
Treatment adherence (male)		
100%	14 (58)	5 (100)
<100%	10 (42)	0 (0)
Condomless vaginal sex during treatment period		
No sex/ protected sex only	21 (88)	5 (100)
Yes	3 (13)	0 (0)
Any oral sex received		
No	22 (92)	5 (100)
Yes	2 (8)	0 (0)
Longitudinal post-treatment characteristics (day 8 to endpoint)		
Any condomless vaginal sex		
No sex/ protected sex only	1 (4)	0 (0)
Yes	23 (96)	5 (100)
Any condomless anal sex		
No sex/ protected sex only	16 (67)	3 (60)
Yes	8 (33)	2 (40)
Any oral sex received		
No	4 (17)	1 (20)
Yes	20 (83)	4 (80)
Any new sexual partner in relationship[c]		
No	21 (92)	4 (80)
Yes	3 (8)	1 (20)

[a] Four women reported using a copper IUD, four reported using a levonorgestrel IUD
[b] Two women who recurred reported using a copper IUD, one reported using a levonorgestrel IUD
[c] Three women and one man reported a new sexual partner during the follow-up period

4.5.5. Vaginal, cutaneous penile and urethral microbiota composition at baseline

A total of 27 couples (including four couples where the female partner experienced recurrence and 23 couples where the female partner was cured) were included in microbiota analyses (Figure 8), providing 131 vaginal specimens, 122 cutaneous penile specimens and 122 urethral specimens. After quality filtering, the median number of reads

per specimen was 24,284 (IQR=18,988-28,719) for vaginal specimens, 22,695 (IQR=17,698-27,046) for cutaneous penile and 22,497 (IQR=17,621-26,018) for urethral specimens.

Prior to commencing treatment (i.e. at day-0), all women had low relative abundance of *Lactobacillus* spp. and high prevalence and relative abundance BV-associated bacteria (Figure 9 and Supplementary Figure C1). Male specimens were heterogeneous in composition at day-0 (Figure 9 and Supplementary Figure C2*)*. Overall, the most abundant organisms in the cutaneous penile microbiota were *Corynebacterium*, *Finegoldia, Staphylococcus, Peptoniphilus* (grouped under BVAB others in Figure 9) and *Prevotella*. The most abundant organisms in the urethral microbiota were *Streptococcus*, *Lactobacillus iners, Gardnerella, Sneathia* and *Staphylococcus*. *Gardnerella* was also prevalent in cutaneous penile samples but at a lower average relative abundance compared to the urethra (1.4% *vs* 7.5%).

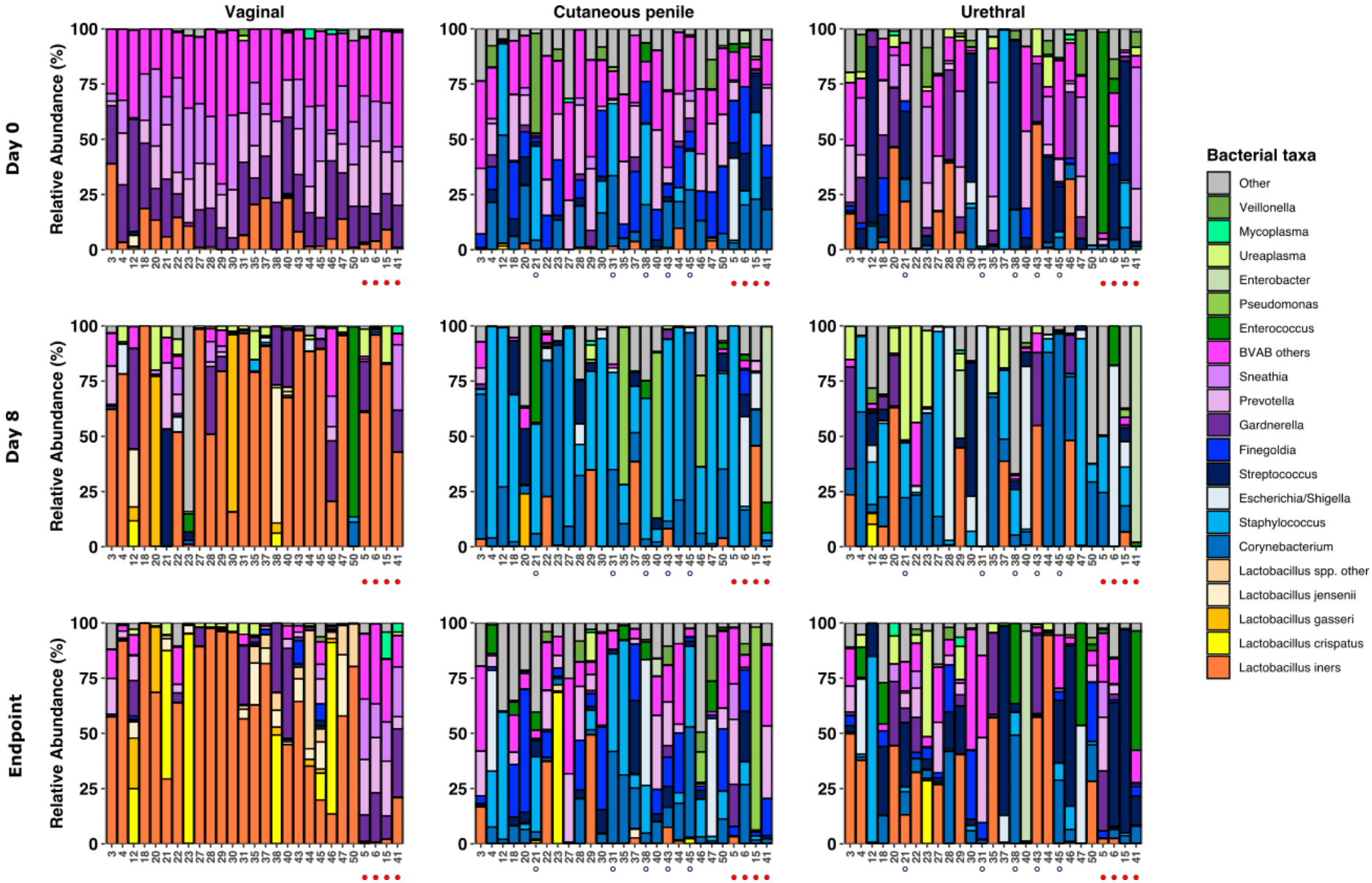

Vaginal
Cutaneous penile
Urethral
Day 0
Day 8
Endpoint
Relative Abundance (%)
0
25
50
75
100
Bacterial taxa
Other
Veillonella
Mycoplasma
Ureaplasma
Enterobacter
Pseudomonas
Enterococcus
BVAB others
Sneathia
Prevotella
Gardnerella
Finegoldia
Streptococcus
Escherichia/Shigella
Staphylococcus
Corynebacterium
Lactobacillus spp. other
Lactobacillus jensenii
Lactobacillus gasseri
Lactobacillus crispatus
Lactobacillus iners

Figure 9. Stacked bar graphs of the vaginal, cutaneous penile and male urethral microbiota

Stacked bar graphs show the relative abundance of key bacterial taxa in the vaginal, cutaneous penile and urethral microbiota of sexual partners at day-0, day-8 and endpoint. Specimens are ordered by couple number. Circumcised males are indicated by the open circle under the bar graphs, and couples where the woman experienced BV recurrence are indicated by solid red circle. Lactobacillus spp. other includes L. antri, L. casei, L. coleohominis, L. fermentum*, and* L. pontis*, and Lactobacillus species that were unable to be classified to a species level). BVAB others includes less abundant species and genera that have previously been associated with BV (*Aerococcus, Anaerococcus, Atopobium, BVAB TM7, Candidatus Lachnocurva vaginae *(previously BVAB-1), BVAB-2,* Mageeibacillus indolicus *(previously BVAB-3),* Dialister*, DNF00809,* Fusobacterium, Gemella, Megasphaera, Mobiluncus, Parvimonas, Peptoniphilus, Peptostreptococcus, Porphyromonas). *Remaining taxa are grouped in the 'Other' category.*

4.5.6. Impact of concurrent partner treatment on the composition of the female and male genital microbiota

NMDS analyses revealed that vaginal specimens collected at day-0 clustered separately from those collected immediately following the 7-day treatment period i.e. at day-8 (ANOSIM *R*-statistic=0.5101, *P*<0.001; Figure 10A) and those collected at study endpoint i.e. week-12 or BV recurrence (ANOSIM *R*-statistic=0.5352, *P*<0.001; Figure 10B). Male genital specimens collected at day-0 clustered separately from those collected at day-8 (cutaneous penile ANOSIM *R*-statistic=0.5132, *P*<0.001; Figure 10C, and urethral ANOSIM *R*-statistic=0.238, *P*<0.001; Figure 10E). However, male specimens collected at endpoint did not cluster separately from those collected at day-0, and the ANOSIM R-statistics suggest little difference between the overall composition of the male genital microbiota at day-0 compared to endpoint (cutaneous penile ANOSIM *R*-statistic=0.0475, *P*=0.05; Figure 10D and urethral ANOSIM *R*-statistic=0.0382, *P*=0.060; Figure 10F).

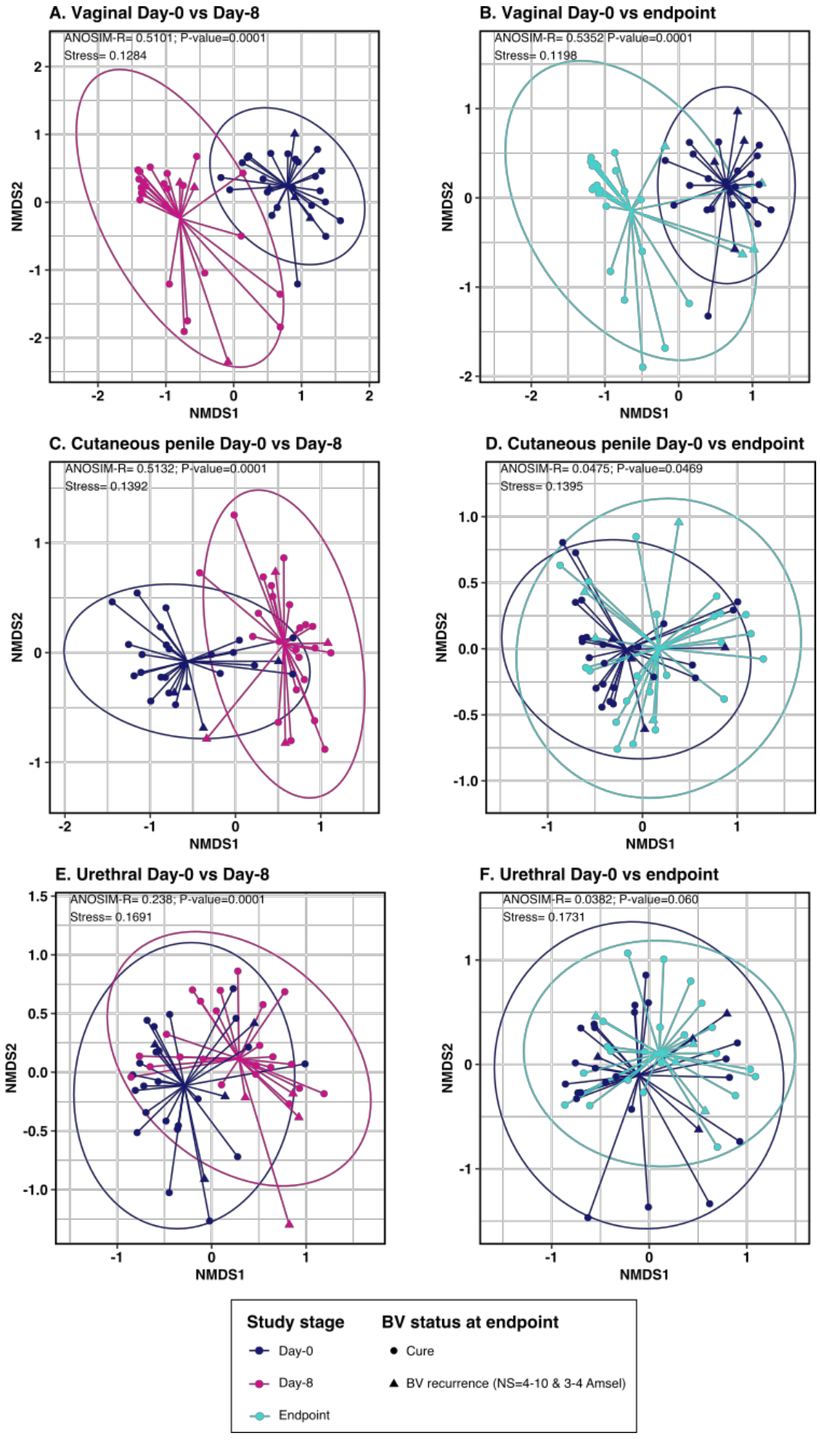

A. Vaginal Day-0 vs Day-8
ANOSIM-R= 0.5101; P-value=0.0001
Stress= 0.1284
B. Vaginal Day-0 vs endpoint
ANOSIM-R= 0.5352 P-value=0.0001
Stress= 0.1198
C. Cutaneous penile Day-0 vs Day-8
ANOSIM-R= 0.5132; P-value=0.0001
Stress= 0.1392
D. Cutaneous penile Day-0 vs endpoint
ANOSIM-R= 0.0475; P-value=0.0469
Stress= 0.1395
E. Urethral Day-0 vs Day-8
ANOSIM-R= 0.238; P-value=0.0001
Stress= 0.1691
F. Urethral Day-0 vs endpoint
ANOSIM-R= 0.0382; P-value=0.060
Stress= 0.1731
NMDS1
NMDS2
Study stage
Day-0
Day-8
Endpoint
BV status at endpoint
Cure
BV recurrence (NS=4-10 & 3-4 Amsel)

Figure 10. Nonmetric multidimensional scaling (NMDS) plots showing the global differences in microbiota composition following concurrent partner treatment

Concurrent partner treatment had a significant immediate (Figure 10A) and sustained (Figure 10B) effect on the vaginal microbiota composition. Conversely, concurrent partner treatment had a significant immediate effect on the cutaneous penile (Figure 10C) and urethral microbiota (Figure 10D), but this was not sustained at endpoint at either male site (Figures 10E and 10F). Analysis of similarity (ANOSIM) test statistics are shown in the top left corner of each plot, and a P<0.05 indicates dissimilarity in the composition of specimens collected pre-treatment vs post-treatment. NMDS and ANOSIM analyses were conducted using Bray–Curtis dissimilarities and ASV level data.

ALDEX2 (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014) was used to identify taxa that were differentially abundant between pre- and post-treatment specimens, using centre log-ratio transformed data (Supplementary Table C3 and Supplementary Table C4). The relative abundance of 18 taxa was significantly decreased in day-8 vaginal specimens compared to day-0, including *Atopobium vaginae*, *Megasphaera*, Coriobacteriales bacterium DNF00809, *Prevotella* spp. (*P. timonensis, P. disiens*), *Sneathia* spp. (*S. amnii, S. sanguinegens*) and *Gardnerella* (FDR<0.05; Figure 11A). Additionally, the relative abundance of nine taxa significantly increased in the vaginal microbiota after 7-days of treatment, including *L. iners*, *Staphylococcus, Ureaplasma* and *Corynebacterium*. At endpoint, the relative abundance of 16 taxa significantly decreased in vaginal specimens compared to day-0, including *A. vaginae, Megasphaera*, *Prevotella* spp., *Sneathia* spp., Coriobacteriales bacterium DNF00809, and *Gardnerella* (FDR<0.05; Supplementary Figure C3A). *Lactobacillus* spp. (including *L. iners* and *L. jensenii*), *Corynebacterium*, *Finegoldia*, *Ureaplasma* and *Staphylococcus* were significantly increased in vaginal specimens at endpoint compared to day-0.

The relative abundance of 11 taxa, including *Anaerococcus, Finegoldia, Peptoniphilus, Prevotella* spp. and *Dialister*, was significantly decreased in day-8 cutaneous penile specimens compared to day-0 (FDR<0.05; Figure 11B). The relative abundance of these five taxa was also significantly decreased in the urethral microbiota after 7-days of treatment (FDR<0.05; Figures 11C). Additionally, the relative abundance of *Staphylococcus* significantly

increased at day-8 at both male sites, and *Escherichia* increased at the urethra. The relative abundance of several BV-associated bacteria remained decreased at the cutaneous penile and urethral sites at endpoint; however, following FDR correction the difference was not significant ($P<0.05$ but FDR>0.05; Supplementary Figures C3B and C3C).

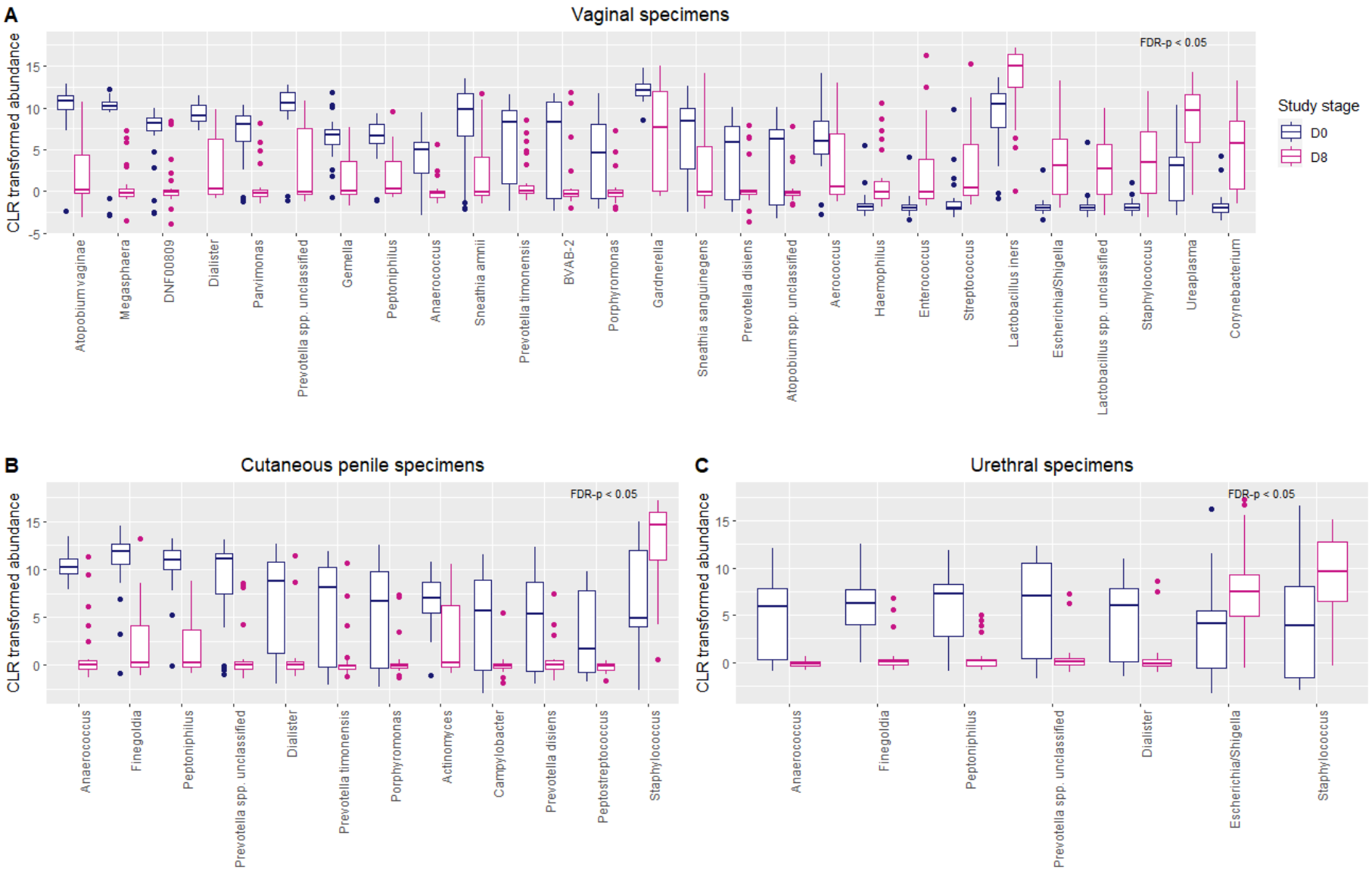

A
Vaginal specimens
CLR transformed abundance
FDR-p < 0.05
Study stage
D0
D8
Atopobium vaginae
Megasphaera
DNF00809
Dialister
Parvimonas
Prevotella spp. unclassified
Gemella
Peptoniphilus
Anaerococcus
Sneathia amnii
Prevotella timonensis
BVAB-2
Porphyromonas
Gardnerella
Sneathia sanguinegens
Prevotella disiens
Atopobium spp. unclassified
Aerococcus
Haemophilus
Enterococcus
Streptococcus
Lactobacillus iners
Escherichia/Shigella
Lactobacillus spp. unclassified
Staphylococcus
Ureaplasma
Corynebacterium
B
Cutaneous penile specimens
CLR transformed abundance
FDR-p < 0.05
Anaerococcus
Finegoldia
Peptoniphilus
Prevotella spp. unclassified
Dialister
Prevotella timonensis
Porphyromonas
Actinomyces
Campylobacter
Prevotella disiens
Peptostreptococcus
Staphylococcus
C
Urethral specimens
CLR transformed abundance
FDR-p < 0.05
Anaerococcus
Finegoldia
Peptoniphilus
Prevotella spp. unclassified
Dialister
Escherichia/Shigella
Staphylococcus

Figure 11. Differences in the relative abundance of taxa between samples collected pre-treatment and following seven days of concurrent partner treatment.

Boxplots show the centred-log ratio (CLR) transformed relative abundance of bacteria that were differentially abundant by ALDEX2 (FDR<0.05) between day-0 and day-8 specimens in the vaginal (Figure 11A), cutaneous penile (Figure 11B) and urethral microbiota (Figure 11C).

4.5.7. The composition of the female and male genital microbiota by recurrence status

ANCOM (Mandal *et al.*, 2015) was used to investigate longitudinal differences in microbiota composition between couples in whom the female partner experienced BV recurrence and those who did not. This analysis had limited power to detect differences due to the small number of women who recurred. However, eleven taxa, including *S. sanguinegens, Dialister, Gemella* and *S. amnii*, were found in significantly higher abundance in the vaginal microbiota of women who experienced BV recurrence (Supplementary Table C5). Conversely, *L. crispatus* and *L. gasseri* were present in cured women, but were not detected in women who recurred. No taxa were significantly differentially abundant between male partners of women who recurred and male partners of cured women.

4.5.8. Correlations between bacterial taxa in the female and male genital microbiota of sexual partners

We investigated the correlation between vaginal taxa and both cutaneous penile and urethral taxa using FastSpar (Watts *et al.*, 2019). Seven taxa were positively correlated between sexual partners at day-0 (Table 17), one of which (*S. sanguinegens*) showed moderate positive correlation between the vaginal microbiota and both the cutaneous penile and urethral microbiota (correlation coefficient=0.37 and 0.43, respectively). *P. disiens* and *Candidatus Lachnocurva vaginae* (BVAB-1) were both moderately positively correlated between the vagina and cutaneous penile microbiota (correlation coefficient=0.47 and 0.63, respectively). Additionally, *L. crispatus, L. gasserii* and *L. jensenii* were moderately positively correlated between the vagina and cutaneous penile microbiota (correlation coefficient=0.57, 0.48 and 0.35, respectively). *Dialister* was negatively correlated between the vaginal microbiota and both the cutaneous penile and urethral

microbiota. At day-8, four taxa were moderately positively correlated between the vaginal and cutaneous penile microbiota of sexual partners (*Anaerococcus, L. gasseri, Finegoldia* and *Corynebacterium*), and two were moderately positively correlated between the vaginal and urethral microbiota (*L. crispatus* and *L. gasseri*).

We also observed a significant positive correlation of taxa between sexual partners longitudinally. When we stratified data by BV recurrence (Supplementary Table C6), two taxa were significant correlated between the vaginal and urethral microbiota of sexual partners who recurred: *P. timonensis* was strongly positively correlated (correlation coefficient=0.97) and *L. iners* was strongly negatively correlated (correlation coefficient=-0.96). *S. amnii* also demonstrated a strong positive correlation between the vaginal and urethra microbiota of sexual partners that recurred, but the correlation was not significant (correlation coefficient=0.85, *P*=0.06). Conversely, *Lactobacillus* spp. (including *L. crispatus*, *L. iners, L. gasseri* and *L. jensenii*), *Corynebacterium*, and *Gardnerella* were moderately positively correlated between the genital microbiota of cured partners. Correlation of taxa predominately occurred between the vaginal and cutaneous penile sites; however, *L. crispatus*, *L. iners* were also correlated between the vagina and male urethra. BV-associated bacteria *P. timonensis* and *S. amnii* were also moderately positively correlated between the genital microbiota of cured partners (correlation coefficient=0.38 and 0.34, respectively), but the strength of correlation between cured couples was lower compared to couples that recurred. As above, these analyses had limited power to detect differences due to the small number of women who recurred.

Table 17 - Correlation of specific bacterial taxa between the genital microbiota of sexual couples at day-0, day-8 and longitudinally

	Day-0[a]				Day-8[b]				Longitudinal samples[c]			
	Vaginal/Penile[d]		Vaginal / Urethral		Vaginal/Penile[d]		Vaginal / Urethral		Vaginal/Penile[d]		Vaginal / Urethral	
Taxon	**Corr**	**P-value**	**Corr**	**P-value**	**Corr**	**P-value**	**Corr**	**P-value**	**Corr**	**P-value**	**Corr**	**P-value**
Aerococcus[#]	0.1636	0.3916	0.0737	0.7283	0.1109	0.5085	0.3201	0.0549	**0.4118**	**0.001**	0.2678	0.017
Anaerococcus	0.3021	0.1009	0.1247	0.5095	**0.4041**	**0.005**	-	-	-	-	-	-
Atopobium vaginae	0.0732	0.7293	0.0293	0.8931	-0.1365	0.3596	0.1617	0.2368	0.048	0.4795	0.1367	0.0989
Candidatus Lachnocurva vaginae (BVAB-1)[#]	**0.6268**	**0.001**	0.2533	0.0619	-	-	-	-	-	-	-	-
BVAB-2[#]	0.2931	0.0889	**0.3586**	**0.048**	0.0215	0.8641	-	-	-0.0145	0.8342	0.1691	0.046
Corynebacterium	0.1236	0.3716	0.1833	0.1908	**0.3943**	**0.029**	0.1698	0.3976	**0.3786**	**0.001**	-0.167	0.1489
Dialister[#]	**-0.4451**	**0.017**	**-0.5567**	**0.004**	0.2804	0.0759	0.0238	0.8721	0.2419	0.036	0.1691	0.1259
Enterobacter	0.1032	0.3696	0.1894	0.1032	-	-	-	-	-	-	-	-
Enterococcus	0.0912	0.4396	0.047	0.6773	0.0294	0.8731	-0.0732	0.6733	**0.339**	**0.001**	0.2269	0.015
Escherichia/Shigella	0.201	0.0909	0.1704	0.1768	0.2311	0.2168	0.0596	0.7293	0.2444	0.017	0.0725	0.5175
Finegoldia	-0.2017	0.2707	-0.0037	0.984	**0.4306**	**0.004**	0.0416	0.7812	-0.0385	0.7433	-0.1229	0.2947
Gardnerella	0.0522	0.7862	0.0522	0.7862	-0.0434	0.8212	-0.0025	0.991	**0.3473**	**0.004**	**0.3109**	**0.012**
L. crispatus	**0.5724**	**0.001**	**-**	**-**			**0.4517**	**0.008**	**0.4627**	**0.001**	**0.3291**	**0.002**
L. gasseri	**0.4813**	**0.001**	-	-	**0.4229**	**0.01**	**0.3835**	**0.013**	**0.4362**	**0.001**	0.2223	0.014
L. iners	0.1843	0.3407	0.2438	0.2168	0.0901	0.6523	0.1727	0.3726	**0.3654**	**0.003**	0.2876	0.011
L. jensenii	**0.3522**	**0.032**	-	-	-	-	0.2595	0.044	**0.4082**	**0.001**	0.1531	0.037
Megasphaera[#]	0.1845	0.3387	0.0627	0.6314	-	-	-	-	-0.0105	0.8771	-	-
Peptoniphilus	0.1153	0.5764	-0.3671	0.0559	0.2428	0.1499	-0.1205	0.3976	0.0869	0.4615	-0.1136	0.3257
Prevotella unclassified spp.	0.2522	0.1738	0.1586	0.4585	0.2649	0.1039	-0.0937	0.5445	0.2082	0.0869	0.1823	0.1189

P. bivia	0.0381	0.8382	0.0042	0.974	0.0324	0.8122	0.0308	0.7902	0.1859	0.0969	0.0682	0.5984
P. disiens[#]	**0.4698**	**0.01**	0.1844	0.3437	-0.1425	0.2667	-	-	**0.3583**	**0.001**	0.2828	0.011
P. timonensis[#]	0.3343	0.0759	0.1234	0.4975	0.1364	0.3636	-0.1007	0.3676	**0.4585**	**0.001**	**0.3742**	**0.002**
Pseudomonas	0.1844	0.0972	-0.0169	0.8824	-	-	-	-	0.0782	0.2148	0.0642	0.3666
Sneathia unclassified spp.[#]	0.2488	0.1558	**0.3806**	**0.026**	-	-	-	-	0.0154	0.8202	0.159	0.0689
S. amnii	0.3132	0.0729	0.3036	0.1209	0.1869	0.2068	0.1051	0.4505	**0.3932**	**0.001**	**0.4796**	**0.001**
S. sanguinegens	**0.3741**	**0.038**	**0.4302**	**0.019**	0.1865	0.1928	-	-	**0.365**	**0.005**	**0.3234**	**0.005**
Staphylococcus	0.2183	0.0619	0.1325	0.2577	0.1502	0.4525	0.1026	0.5864	**0.3088**	**0.011**	0.02	0.8691
Streptococcus	0.1469	0.3836	0.1593	0.3417	0.0903	0.6154	0.0874	0.6174	0.1882	0.0999	0.0575	0.6384
Ureaplasma	-0.0355	0.8182	-0.0416	0.8272	-0.1423	0.4446	0.1696	0.4006	0.1087	0.3397	0.1945	0.0849

Corr, SparCC correlation coefficient; - indicates the taxa was not detected in one or more specimen type for that study time point
The ten most abundant bacteria at each site and eight bacteria previously associated with BV (indicated by #) are presented in this table.
Correlations with an absolute correlation coefficient >0.3 and $P<0.05$ were considered significant and have been bolded in this table
[a] Includes 27 vaginal, 27 cutaneous penile and 27 urethral specimens from 27 couples
[b] Includes 27 vaginal, 27 cutaneous penile and 27 urethral specimens from 27 couples
[c] Includes 68 vaginal, 68 cutaneous penile and 68 urethral specimens from 27 couples
[d] Penile refers to cutaneous penile specimens

4.6. Discussion

In this pilot study of combined oral and topical antibiotic treatment of male partners of women being treated for BV we assessed 1) the impact of concurrent partner treatment on the composition of the vaginal, cutaneous penile and male urethral microbiota over a 12-week period, and 2) the adherence to and acceptability and tolerability of concurrent partner treatment and 3) generated preliminary estimates of the efficacy of the intervention over a 12-week period. We found that concurrent partner treatment significantly altered the overall composition of the genital microbiota of both partners immediately following seven days of treatment. Specifically, we observed a decrease in the abundance of anaerobic BV-associated bacteria at all three urogenital sites. Several bacterial taxa, including *Lactobacillus* spp., *Corynebacterium* and *Ureaplasma*, increased in abundance in the vaginal microbiota, and the abundance of *Staphylococcus* increased in both the cutaneous penile and urethral microbiota. This immediate effect of concurrent partner treatment on the male genital microbiota is consistent with findings from our previous exploratory study that followed couples for only 3-weeks post-treatment and provided only cutaneous penile data (Plummer *et al.*, 2018a). However, by 12-weeks, the overall composition of the male genital microbiota was not significantly different to baseline, with BV-associated bacteria re-emerging at both male sites. Despite this, the majority of women experienced suppression of BV-associated bacteria, a sustained increase in *Lactobacillus* spp. (most commonly *L. iners*), and BV cure to 12-weeks. This study was not powered to assess the effect of partner treatment on BV recurrence; however, we observed fewer than the expected number of cases of BV recurrence in a group of women with a high prevalence of risk factors for recurrence. Additionally, we found that concurrent partner treatment was well tolerated and adhered to by men. Close to 90% of approached male partners agreed to participate in the study and receive the intervention, most reported 100% adherence to treatment all treatment doses, and few side effects were reported. These findings extend the data from our previous exploratory study and provide the support and evidence to proceed to an RCT of combined oral and topical male partner treatment for BV (Vodstrcil *et al.*, 2020) .

Recurrence following recommended BV treatments is unacceptably high. Prior studies have shown that approximately 20-25% of women recur within 1-month of female-only treatment, and up 50% recur within 12-weeks (Bradshaw *et al.*, 2006a; Sobel *et al.*, 1993). Notably, women with a regular sexual partner have a 2-3 fold increased risk of recurrence (Bradshaw *et al.*, 2006a; Bradshaw *et al.*, 2013a; Vodstrcil *et al.*, 2019), and a recent placebo controlled RCT of partner treatment for BV conducted by Schwebke *et al.* (2021) reported recurrence to be as high as 80% in women (all of whom had a regular sexual partner), in the 16-weeks following standard treatment. All women in our study also had a regular sexual partner, but other known risk factors for recurrence were very common: 80% of women had a history of BV, 80% of male partners were uncircumcised, and all but one couple reported condomless sex during the follow-up period. Additionally, 35% of women had an IUD *in situ*, and IUD use, particularly copper IUD use, has been associated with increased risk of BV (Achilles *et al.*, 2018; Avonts *et al.*, 1990; Joesoef *et al.*, 2001; Madden *et al.*, 2012; Peebles *et al.*, 2020). Given the risk profile of our population, we would expect recurrence rates in our cohort to be similar to that reported by Schwebke *et al.* (2021). Encouragingly, only five women (17%) experienced recurrence within 12-weeks following concurrent partner treatment, and 24 (83%) were cured at 12-weeks post-treatment. Additionally, although our first exploratory study assessed BV using Nugent score only (Plummer *et al.*, 2018a), pooled data from our two studies provides an insight into 1-month recurrence rates following this intervention. Collectively, of 50 couples undergoing partner treatment who provided data to 4-weeks, only three women (8%; 95%CI: 2-19%) experienced recurrence within 1-month of treatment. Despite the encouraging results from our two studies, past randomised trials of male partner treatment have failed to consistently improve BV-cure (Colli *et al.*, 1997; Mengel *et al.*, 1989; Moi *et al.*, 1989; Schwebke *et al.*, 2021; Swedberg *et al.*, 1985; Vejtorp *et al.*, 1988; Vutyavanich *et al.*, 1993). Methodological limitations have been highlighted as a potential reason that early trials failed (Amaya-Guio *et al.*, 2016; Mehta, 2012); however, the recent study by Schwebke *et al.* (2021), a well-designed placebo-controlled RCT of male partner treatment with 7-days of oral metronidazole, also failed to improve BV cure. A common theme among completed trials is that they have all used only oral therapy for men. This contrasts to our study which used combined oral and topical antibiotics for males, specifically to address multi-site carriage of BV-associated bacteria in men (Mandar *et al.*, 2015; Mehta *et al.*, 2020b; Nelson *et al.*, 2012; Zozaya *et al.*, 2016). Utilising an intervention

that targets both male genital sites may have contributed to the reduction in recurrence observed in our study.

Overall, there is a lack of information on how antibiotics modify the male genital microbiota composition, with our two studies providing the only data. A currently recruiting trial (NCT03412071) is investigating the impact of four antimicrobial agents (oral tinidazole, topical metronidazole, topical clindamycin, and topical hydrogen peroxide) compared to circumcision on the composition of the cutaneous penile microbiota (Galiwango *et al.*, 2019). The study does not follow participants longitudinally following completion of antimicrobials and the treatment regimens used are non-standard regimens for BV. Nonetheless, it will add to our understanding of how different antimicrobials modify the composition of the penile microbiota. Investigating alternative oral antimicrobials may be of particular importance because consistent with our first exploratory study (Plummer *et al.*, 2018a), we observed lower male adherence to clindamycin compared to metronidazole, potentially indicating a preference for oral over topical therapy. Although the five women who recurred in our study all self-reported 100% treatment adherence, as did their male partners, non-adherence was a predictor of recurrence in the study by Schwebke *et al.* (2021). These data highlight the importance of education and emphasis on strategies to optimise adherence for males in these trials and also investigating the acceptability of alternative antimicrobials for partner treatment.

In both of our partner treatment studies completed to date we observed re-emergence of BV-associated bacteria in men that was not associated with BV recurrence in female partners. These data raise important questions. Firstly, why is the immediate effect of treatment on the male genital microbiota not sustained? Although the immediate effect of treatment on the cutaneous penile microbiota was in the same order of magnitude as the vagina (ANOSIM *R*-statistics= 0.5132 and 0.5101, respectively), the immediate effect of treatment on the urethral microbiota was not as large (ANOSIM *R*-statistic= 0.238). These data may suggest reduced treatment efficacy at the urethral site. Intrinsic resistance to metronidazole is well documented for BV-associated bacteria including *Gardnerella* and *A. vaginae* (De Backer *et al.*, 2006; Ferris *et al.*, 2004; Kharsany *et al.*, 1993; Knupp de Souza *et al.*, 2016; Nagaraja, 2008; Schuyler *et al.*, 2015; Simoes *et al.*, 2001), and studies have shown that *Gardnerella* is less resistant to clindamycin than metronidazole (Li *et al.*, 2020;

Nagaraja, 2008). Additionally, BV-associated biofilms have been detected in male urine (Swidsinski *et al.*, 2010), and bacterial biofilms comprised predominately of *Gardnerella* and *Atopobium* have been shown to re-emerge within 3-weeks of treatment with oral metronidazole (Swidsinski *et al.*, 2008). Together these data may suggest that metronidazole resistant BV-associated bacteria persist at low levels post-treatment in the urethra, potentially in the setting of an established biofilm, only to re-emergence later at both male sites. A recent and alternative hypothesis is that sequestration of metronidazole by non-target organisms such as *Lactobacillus* spp. may impact metronidazole efficacy (Lee *et al.*, 2020). Lee *et al.* (2020)found that in co-cultures, metronidazole efficacy decreased as the ratio of *Gardnerella* to *L. iners* decreased. Additionally, women with BV were more likely to fail metronidazole if they had a low pre-treatment ratio of BV-associated bacteria to *Lactobacillus* spp. (Lee *et al.*, 2020), suggesting the pre-treatment vaginal microbiota composition may impact treatment efficacy. It is unclear what implications this has for metronidazole efficacy in men, and if organisms that are present in high abundance in the male genital microbiota pre-treatment may influence treatment outcomes. However, the re-emergence of BV-associated bacteria in men may suggest that alternative treatment/s, or potentially a longer duration of treatment, could be needed to achieve sustained clearance of BV-associated bacteria from men. Finally, it is possible that BV-associated bacteria are re-introduced into the male genital microbiota from extra-penile sources (i.e. the prostate (Mandar *et al.*, 2015) or the gastrointestinal tract) via autoinoculation, or during anal sex.

Little is known about what constitutes an optimal or 'normal' genital microbiota in men. A study of 18 adolescent males reported detection of BV-associated bacteria including *Atopobium*, *Megasphaera*, *Mobiluncus*, *Prevotella* and *Gardnerella* in the coronal sulcus of sexually experienced and sexually inexperienced individuals (Nelson *et al.*, 2012). Although BV-associated bacteria are commonly detected in the genital microbiota of male partners of women with BV, their detection has also been reported in male partners of women without BV, albeit at lower abundance and prevalence (Liu *et al.*, 2015; Zozaya *et al.*, 2016). Additionally, In line with the evidence that specific *Gardnerella* spp. or clades associate with BV and others do not (Balashov *et al.*, 2014; Hill *et al.*, 2019; Janulaitiene *et al.*, 2017; Plummer *et al.*, 2019b; Shipitsyna *et al.*, 2019; Vodstrcil *et al.*, 2017), it is possible that the organisms that re-emerged in men are non-virulent species or strains that constitute a

'normal' male genital microbiota, or do not pose a BV risk to their female partner. Interestingly, a recent study reported that six bacterial species present in the penile foreskin microbiota (belonging to the *Prevotella*, *Dialister* and *Peptostreptococcus* genera), were associated with increased risk of HIV acquisition in men (Prodger *et al.*, 2021). Some of the identified species (i.e. *P. bivia, P. disiens*) have previously been associated with BV and were also identified in our study. Further investigation is needed to better understand the composition of the genital microbiota in males, and factors that influence the microbiota composition. While a low diversity *Lactobacillus* dominant vaginal microbiota has generally been accepted as associated with optimal outcomes in women, we have no such data in males, or knowledge of if there is an optimal genital microbiota in men. Further research is clearly needed to determine the contribution of BV-associated bacteria to health outcomes in men and their female partners.

The second question our data raise is: why does the return of BV-associated bacteria in men not correspond to a return of BV-associated bacteria and subsequent recurrence in all women? One hypothesis is that the organisms we see re-emerge in men are not driving BV-pathogenesis. Alternatively, although longitudinal data suggests that the majority of BV recurrence occurs within the 12-weeks following treatment (Bradshaw *et al.*, 2006a; Bradshaw *et al.*, 2013a; Schwebke *et al.*, 2021; Sobel *et al.*, 1993; Vodstrcil *et al.*, 2019), it is possible that treating male partners delays recurrence in women and we were unable to capture this within three months of follow-up. Although realistically, one would expect the effect on women, of a one week intervention in males, to be captured and most relevant to the first 1-2 months of follow-up. Another explanation is that the immediate reduction of BV-associated bacteria in both partners may have been sufficient for *Lactobacillus* spp., which are resistant to metronidazole (Charteris *et al.*, 1998; Melkumyan *et al.*, 2015), to recolonise the vagina and provide a barrier to re-infection. Additionally, we found that *Lactobacillus* spp. (including *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii*) were moderately correlated between the genital microbiota of sexual partners who were cured. In contrast, *L. iners* was negatively correlated between partners who recurred, and in general, *Lactobacillus* spp. were depleted amongst couples that recurred. Although our correlation and ANCOM analyses had limited power due to the small number of recurrences, our findings indicate that couples who share *Lactobacillus* spp. may be more likely to maintain

an optimal vaginal microbiota and less likely to experience recurrence. In support of this, increased frequency of penile-vaginal sex has been shown to increase the concentration of hydrogen peroxide–producing lactobacilli (Mitchell *et al.*, 2011). This is also supported by evidence from studies of female partnerships showing that female sexual partners share *Lactobacillus* strains (Marrazzo *et al.*, 2009). Furthermore, female sexual partners are highly concordant for Nugent score category (Bradshaw *et al.*, 2014; Evans *et al.*, 2007; Marrazzo *et al.*, 2002; Vodstrcil *et al.*, 2015), and a key determinant of persistently stable low Nugent scores (representing *Lactobacillus* dominated microbiota) over a two year period was having a BV-negative female sexual partner (i.e. a partner with a low Nugent score) (Vodstrcil *et al.*, 2015).

If concurrent partner treatment is shown to be effective for reducing BV recurrence, its success depends on its acceptability to couples. Eleven of the women we approached declined to participate because they did not want to discuss BV or the study with their partner, or felt that he would not be interested in participating. Excluding these couples biases our findings towards a higher proportion of male partners accepting treatment, however we don't know for sure if these men would have declined. Importantly, among couples that discussed the study, acceptance of partner treatment was high. A recent qualitative study of men participating in partner treatment trials for BV highlighted that open communication in the relationship was a key element to men accepting treatment (Wigan *et al.*, 2020). Furthermore, participants hypothesised that a primary reason men may decline partner treatment is that they feel BV has "nothing to do with them", and this is compounded by a lack of corresponding symptoms and no diagnostic test for men (Wigan *et al.*, 2020). This research identifies key barriers to couples accepting partner treatment, and highlights that more support is needed to help women discuss BV with their partners, and more education about BV and male carriage of BV-associated bacteria is needed to effectively engage men.

Limitations of this study include the small size and recruiting from a single sexual health clinic, both of which limit the generalisability of our findings. Additionally, we did not have a control group where men were not treated. Thus, we were unable to compare the effect of concurrent partner treatment to standard treatment (i.e. female-only treatment), or determine if treating a woman with antibiotics influences the genital microbiota of her

untreated male partner. Our currently recruiting multisite trial (Vodstrcil *et al.*, 2020) (ACTRN12619000196145) will provide the first randomised data on the efficacy of combined oral and topical partner treatment for BV compared to female-only treatment. Additionally, there are inherent limitations with 16S rRNA gene sequencing, including reduced resolution beyond the genus level. Although the aetiological agent of BV remains unknown, specific species of *Gardnerella* have been hypothesised to be integral to BV pathogenesis (Muzny *et al.*, 2019c). As *Gardnerella* species cannot be distinguished using the 16S rRNA gene sequence (Vaneechoutte *et al.*, 2019), we could not achieve this level of resolution in this study. Studies are needed to determine the effect of concurrent partner treatment at the strain-level, as well as to identify the organisms that represent an optimal genital microbiota composition in men, and if there are organisms in men that promote optimal outcomes in female partners. Furthermore, extending the follow-up duration may provide more insight into the durability of male partner treatment for BV, although onset of new partnerships becomes more likely the longer individuals are followed. Finally, there are limitations with self-reported data including recall bias and social desirability bias (Althubaiti, 2016).

In summary, our findings demonstrate that combined oral and topical antibiotic treatment has a significant and immediate effect on the composition of the female and male genital microbiota. Although our study was not powered to measure BV recurrence, we observed a lower than expected BV recurrence within the 12-weeks following concurrent partner treatment in a group of women who were at high risk of recurrence. We would expect >50% of these women to experience BV recurrence within 12-weeks of standard first-line antibiotics, and only 17% of women recurred following concurrent partner treatment. Our findings demonstrate that concurrent partner treatment is well adhered to and tolerated by those who agree to receive it. Critically, our study highlights that strategies to educate couples in a way that encourages open communication about BV are needed to ensure partner treatment is well accepted. If shown to be effective in a randomised setting, concurrent partner treatment, utilising combined oral and topical antibiotic treatment for male partners, represents a readily implementable intervention that ultimately, may be a strategy to achieve sustained BV cure and improve reproductive and sexual health outcomes for women globally.

Chapter 5. Are *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* associated with specific genital symptoms and clinical signs in nonpregnant women?

5.1. Overview

The common genital Mollicutes (also commonly referred to as the common genital mycoplasmas) were introduced in Section 1.2.4 of the literature review. With increasing use of multiplex PCR assays, laboratories commonly report on detection of the common genital Mollicutes: *M. hominis*, *U. urealyticum* and *U. parvum,* which leads clinicians to believe treatment of these organisms may be required. However, there is limited evidence supporting a role for *M. hominis*, *U. urealyticum* and *U. parvum*in causing disease in nonpregnant women, and routine reporting of their detection creates confusion amongst clinicians around the need to treat. Furthermore, previous studies that have investigated the association between *M. hominis* and the ureaplasmas and symptoms in nonpregnant women have failed to adjust for important confounders such as concurrent STI infection and BV (Horner *et al.*, 2018).

Therefore, the study presented in **Chapter 5** aimed to determine if *M. hominis*, *U. urealyticum* and *U. parvum* were associated with genital symptoms and/or clinical signs in nonpregnant women, after adjusting for important confounders, in order inform testing indications for these bacteria in a sexual health clinic setting.

Chapter 5 is presented as the accepted version of the following peer reviewed manuscript. No alterations to the text have been made, except that abbreviations, and figure and table numbers have been adjusted to generate a consistent presentation within this thesis.

Publication:

> **Plummer EL,** Vodstrcil LA, Bodiyabadu K, Murray GL, Doyle M, Latimer RL, Fairley CK, Payne M, Chow EPF, Garland SM, Bradshaw CS (2021). Are *Mycoplasma hominis, Ureaplasma urealyticum* and *Ureaplasma parvum* associated with

specific genital symptoms and clinical signs in non-pregnant women? *Clin Infect Dis.* ciab061. doi:10.1093/cid/ciab061

This manuscript received an editorial commentary:

Jensen JS (2021). To test or not to test for *Mycoplasma hominis* and the Ureaplasmas: That's (not) the question. *Clin Infect Dis*. ciab065. doi:10.1093/cid/ciab065

Findings from this manuscript have also been presented at the following national conferences:

- 2020 Virtual Joint HIV&AIDS and Sexual Health Conferences, 16 – 20 Nov 2020. [Oral]
- Central Clinical School Postgraduate Symposium, Melbourne, Australia, 4-5 Nov 2020. [Oral]

A PDF of the published manuscript is in Appendix E.

5.2. Abstract

Background: There is limited evidence supporting an association between *Mycoplasma hominis, Ureaplasma urealyticum* and *Ureaplasma parvum* and symptoms or disease in nonpregnant women. However, testing and reporting of these organisms frequently occurs, in-part due to their inclusion in multiplex-PCR assays for STI detection. We investigated if *M. hominis, U. urealyticum* and *U. parvum* were associated with symptoms and/or signs in nonpregnant women attending a sexual health service.

Methods: Eligible women attending Melbourne Sexual Health Centre completed a questionnaire regarding sexual practices and symptoms. Symptomatic women underwent examination. Women were assessed for BV and vulvovaginal candidiasis (VVC), and tested for *M. hominis, U. urealyticum* and *U. parvum*, and four non-viral STIs using a commercial multiplex-PCR.

Results: 1, 272 women were analysed. After adjusting for STIs and VVC, *M. hominis* was associated with abnormal vaginal discharge (AOR 2.70, 95% CI 1.92, 3.79), vaginal malodour (AOR 4.27, 95% CI 3.08, 5.91), vaginal pH>4.5 (AOR 4.27, 95% CI 3.22, 5.66) and presence of

clue cells (AOR 8.08, 95% CI 5.68, 11.48). *Ureaplasma* spp. were not associated with symptoms/signs. BV was strongly associated with *M. hominis* (AOR 8.01, 95% CI 5.99, 10.71), but was not associated with either *Ureaplasma* spp. In stratified analyses, *M. hominis* was associated with self-reported vaginal malodour and clinician-recorded vaginal discharge in women with BV, but not with symptoms/signs in women without BV.

Conclusion: Only *M. hominis* was associated with symptoms/signs, and these were manifestations of BV. Importantly, *M. hominis* was not associated with symptoms/signs in women without BV. These findings do not support routine testing for *M. hominis, U. urealyticum* and *U. parvum* in nonpregnant women.

5.3. Introduction

Mycoplasma hominis, Ureaplasma urealyticum and *Ureaplasma parvum* are commonly recovered from the urogenital tract of symptomatic and asymptomatic women and men. Molecular studies of nonpregnant women of reproductive age place prevalence estimates between 3.2-26.2% for *M. hominis*, 7.6-28.4% for *U. urealyticum* and 22.4-67.3% for *U. parvum* (Cox *et al.*, 2016; Foschi *et al.*, 2018; Kim *et al.*, 2014; Leli *et al.*, 2018; Lobao *et al.*, 2017; Ouzounova-Raykova *et al.*, 2011; Silva *et al.*, 2018). There is limited evidence supporting a role for *M. hominis*, *U. urealyticum* and *U. parvum* in causing symptoms and/or disease in nonpregnant women (Horner *et al.*, 2018). However, vaginal detection of *Ureaplasma* spp. (particularly *U. parvum*) has been associated with obstetric complications including preterm birth and low birth weight (Kataoka *et al.*, 2006; Payne *et al.*, 2016; Rittenschober-Bohm *et al.*, 2019; Rittenschober-Bohm *et al.*, 2018). Additionally, *Ureaplasma* spp. have been associated with significant neonatal morbidities including necrotising enterocolitis, bronchopulmonary dysplasia, intraventricular haemorrhage and meningitis (Garland & Bowman, 1996; Garland & Murton, 1987; Glaser & Speer, 2015; Viscardi, 2014).

Testing for *M. hominis*, *U. urealyticum* and *U. parvum* has become increasingly common, often due to their inclusion in commercial multiplex-PCR assays used for STI screening. The use of culture for identification of these organisms has also been practiced (even though culture cannot differentiate between *Ureaplasma* spp.), and reports of detection are often accompanied by antibiotic susceptibility findings, which can lead clinicians to believe that

antibiotic treatment is indicated. The routine testing and reporting of *M. hominis*, *U. urealyticum* and *U. parvum*, along with the high prevalence of asymptomatic colonisation and limited evidence of a causative role in disease in nonpregnant women, creates confusion for clinicians around the need to treat positive cases.

In 2018, the European STI Guidelines Editorial Board released a 'Position Statement' regarding testing for *M. hominis*, *U. urealyticum* and *U. parvum* (Horner *et al.*, 2018). Following a review of published literature concerning the aetiological role of these bacteria in STI syndromes and complications, the statement concluded that there is currently no evidence of benefit from routinely testing and treating for *M. hominis*, *U. urealyticum* and *U. parvum* in adult men and nonpregnant women (Horner *et al.*, 2018).

Importantly, the statement noted limitations with a number of the reviewed studies, including a failure to control for important confounders such as sexual practices and concurrent genital infections (Horner *et al.*, 2018). In light of this, we conducted a study to determine if *M. hominis*, *U. urealyticum* and *U. parvum* were associated with specific symptoms and/or clinical signs in nonpregnant women, after adjusting for important confounders, in order to inform testing indications for these bacteria in a sexual health clinic setting.

5.4. Methods

5.4.1. Study population

Participants and specimens used for this study were collected as part of a cross-sectional study (OhMG) that had the principal aim of informing clinical indications for testing for *Mycoplasma genitalium* and additional aims of determining the contribution of *M. hominis, U. urealyticum* and *U. parvum* to symptoms and/or clinical signs in nonpregnant women (Latimer *et al.*, 2019b). Women attending Melbourne Sexual Health Centre between April 2017-April 2019 were eligible to participate if they were sexually active, aged ≥18 years and were presenting with common genitourinary symptoms or presenting for routine STI screening. Women were ineligible if they were unable to consent for reasons of language or mental state, were sex workers, were sexual contacts of *M. genitalium* or were attending

for a *M. genitalium* test-of-cure. Alfred Hospital Ethics Committee (project 100/17) approved this project and all participants provided written informed consent.

5.4.2. Clinical and laboratory procedures

Women attending Melbourne Sexual Health Centre were assessed for eligibility and recruited by either a sexual health physician or a research nurse. All recruited women completed a questionnaire concerning sexual practices and genital symptoms experienced in the week prior to presentation. Symptomatic women (women reporting at least one symptom in the questionnaire) underwent a genital examination by a clinician and the presence or absence of the following clinical signs was recorded: abnormal vaginal discharge, vaginal malodour, vulval redness or vulvitis, cervicitis (defined as mucopurulent cervicitis and/or cervical friability), cervical contact bleeding, and cervical or adnexal motion tenderness. In keeping with standard practice at Melbourne Sexual Health Centre, asymptomatic women (women reporting none of the symptoms in the questionnaire) were not examined.

In addition to their routine clinical specimens for STI testing (results of routine STI testing are reported elsewhere (Latimer *et al.*, 2019b)), all women provided a high vaginal swab specimen (self- or clinician-collected) for study related procedures. Swabs were swirled in 600μL 1x PBS, and 200μL extracted by MagNA Pure 96 (Roche Diagnostics, Mannheim, Germany) using the DNA and Viral NA Small Volume Kit. Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae, M. genitalium*, *Trichomonas vaginalis*, *M. hominis, U. urealyticum* and *U. parvum* was performed with the AnyplexTM II STI-7e PCR assay (Seegene Inc. Republic of Korea), according to manufacturer instructions.

For all women, vaginal pH was recorded (MColorpHast^{TM} pH indicator strips; Merck KGaA, Darmstadt, Germany), and a Gram stain (and wet preparation for symptomatic women) of a vaginal smear was prepared to assess for BV, VVC and vaginal polymorphonuclear leukocyte (PMNL) cell counts. BV was defined as the presence of ≥3 Amsel criteria (Amsel *et al.*, 1983) and a Nugent score=4-10 (Nugent *et al.*, 1991). In asymptomatic women (and symptomatic women who could not be assessed by Amsel criteria due to menses/blood), BV was defined as a Nugent score=4-10 and presence of clue cells on microscopy or wet preparation. VVC was diagnosed based on the presence of typical clinical features on examination (thick white

or curdy discharge and/or vulvovaginal erythema) and/or presence of visible pseudohyphae and/or budding yeasts on microscopy.

5.4.3. Statistical methods

Proportions of *M. hominis*, *U. urealyticum* and *U. parvum* were calculated with 95% binomial confidence intervals. Logistic regression was used to identify demographic, sexual and microbiological factors associated with the detection of *M. hominis*, *U. urealyticum* and *U. parvum*. Factors with a significance level of $P<0.10$ in univariable analyses were included in multivariable models.

A logistic regression analysis that adjusted for *M. hominis*, *U. urealyticum, U. parvum*, *C. trachomatis*, *N. gonorrhoea, M. genitalium, T. vaginalis* and VVC was performed for each sign and symptom to investigate if *M. hominis*, *U. urealyticum* and *U. parvum* were independently associated with any clinical characteristic. Analyses of clinical signs were restricted to symptomatic women who were assessed for all clinical signs (n=406). As associations with individual Amsel criteria (Amsel *et al.*, 1983) were examined, we did not adjust for BV to prevent over-fitting the models. For analyses of signs/symptoms, p-values were corrected for false discovery rate (Yekutieli & Benjamini, 1999) using the Benjamini-Hochberg method and we used a significance value of $q<0.05$ (calculated in R v4.0.2 using p.adjust).

Odds ratios were presented with 95% CI and analyses were performed using Stata/IC v16(StataCorp LP, College Station, TX, USA), unless stated otherwise.

5.5. Results

5.5.1. Description of study population

During the study period, 1, 355 women were recruited (Latimer *et al.*, 2019b). Thirty-seven women were excluded post-recruitment: 25 were sex workers and 12 were inadvertently recruited twice. Thirty-seven swabs had insufficient DNA remaining for testing. Nine specimens returned an invalid test result and were excluded. A total of 1, 272 women were included in analyses; 1, 081 women reported at least one symptom and 191 reported none of the symptoms in the questionnaire. The median age of participants was 26 years (IQR: 23-

29 years, Table 18). The most frequently reported symptoms were abnormal vaginal discharge (34%), vaginal malodour (24%) and vaginal itch (21%).

5.5.2. Detection of *M. hominis*, *U. urealyticum* and *U. parvum*

M. hominis was detected in 375 women (29%, 95% CI 27, 32), *U. urealyticum* was detected in 403 women (32%, 95% CI 29, 34) and *U. parvum* was detected in 910 women (72%, 95% CI 69, 74). There was no difference in the detection of these organisms between women with one or more symptoms (symptomatic) or no symptoms (asymptomatic; Table 18). The proportion of women with other genital infections has been reported (Latimer *et al.*, 2019b): 6% (95% CI 5, 8) had *M. genitalium,* 8% (95% CI 6, 9) had *C. trachomatis*, 1% (95% CI 0, 2) had *N. gonorrhoeae*, 30% (95% CI 28, 33) had BV, 24% (95% CI 22, 27) had VVC and 1% (95% CI 0, 2) had *T. vaginalis*.

Table 18 - Univariable analysis of demographic and sexual factors associated with *M. hominis, U. urealyticum* and *U. parvum* detection

Characteristic	*M. hominis*				*U. urealyticum*				*U. parvum*			
	Negative n=897	**Positive n=375**	**OR (95% CI)**	***P* value**	**Negative n=869**	**Positive n=403**	**OR (95% CI)**	***P* value**	**Negative n=362**	**Positive n=910**	**OR (95% CI)**	***P* value**
Cohort												
Asymptomatic	142 (16)	49 (13)	1		138 (16)	53 (13)	1		55(15)	136 (15)	1	
Symptomatic	755 (84)	326 (87)	1.25 (0.88, 1.78)	0.209	731 (84)	350 (87)	1.25 (0.89, 1.75)	0.206	307 (85)	774 (85)	1.02 (0.73, 1.43)	0.911
Age in years (median, IQR)	27 (6)	26 (5)	0.97 (0.94, 0.99)	**0.002**	27 (6)	26 (6)	0.99 (0.97, 1.01)	0.186	27 (6)	27 (6)	1.00 (0.98, 1.02)	0.972
No. male partners in past 12mo (median, IQR)	4 (5)	6 (6)	1.06 (1.03, 1.09)	**<0.001**	4 (3)	6 (7)	1.07 (1.04, 1.10)	**<0.001**	4 (5)	5 (5)	1.05 (1.01, 1.09)	**0.005**
No. female partners in past 12mo (median, IQR)	0 (1)	0 (1)	1.01 (0.89, 1.15)	0.897	0 (1)	0 (1)	0.93 (0.81, 1.07)	0.319	0 (1)	0 (1)	1.35 (1.09, 1.67)	**0.007**
Condom use with male partners in past 12mo[a]												
Always	69 (8)	18 (5)	1		68 (8)	19 (5)	1		34 (10)	53 (6)	1	
Never/not always	814 (92)	353 (95)	1.66 (0.97, 2.83)	0.062	786 (92)	381 (95)	1.73 (1.03, 2.93)	**0.039**	323 (90)	844 (94)	1.68 (1.07, 2.63)	**0.024**
STI in past 6mo												
No	779 (89)	300 (82)	1		742 (87)	337 (86)	1		308 (87)	771 (87)	1	
Yes	100 (11)	66 (18)	1.71 (1.22, 2.40)	**0.002**	111 (13)	55 (14)	1.09 (0.77, 1.54)	0.624	46 (13)	120 (13)	1.04 (0.72, 1.50)	0.824

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation; mo, months; STI, sexually transmitted infection
Data are presented as n (%), unless otherwise specified.
Data missing for up to 2% of participants for some variables.
P-values < 0.05 are bolded to indicate statistically significant associations.

5.5.3. Demographic, sexual and microbiological factors associated with *M. hominis*, *U. urealyticum* and *U. parvum*

In univariable analysis, *M. hominis*, *U. urealyticum* and *U. parvum* were each significantly associated with increased numbers of male sexual partners (MSPs) in the past 12-months (Table 18). Additionally, *M. hominis* was associated with younger age (OR 0.97, 95% CI 0.94, 0.99) and self-report of STI in the previous 6-months (OR 1.71, 95% CI 1.22, 2.40), *U. parvum* was associated with increased number of female sexual partners (FSPs; OR 1.35, 95% CI 1.09, 1.67), and both *U. urealyticum* and *U. parvum* were associated with inconsistent condom use (OR 1.73, 95% CI 1.03, 2.93 and OR 1.68, 95% CI 1.07, 2.63, respectively).

M. hominis was associated with *C. trachomatis* (OR 1.76, 95% CI 1.17, 2.64), *N. gonorrhoeae* (OR 3.48, 95% CI 1.32, 9.22), and concurrent BV (OR 8.40, 95% CI 6.36, 11.10), and negatively associated with VVC (OR 0.71, 95% CI 0.53, 0.95; Table 19). Both *U. urealyticum* and *U. parvum* were associated with BV (OR 1.55, 95% CI 1.20, 2.00 and OR 1.45, 95% CI 1.09, 1.92, respectively), but neither were associated with concurrent STIs.

M. hominis was positively associated with detection of *U. urealyticum* (OR 2.65, 95% CI 2.06, 3.42) and detection of *U. parvum* (OR 1.92, 95% CI 1.44, 2.57), but *U. urealyticum* and *U. parvum* were negatively associated with each other (OR 0.48, 95% CI 0.37, 0.61).

In multivariable analyses, *M. hominis* was associated with younger age (adjusted-OR [AOR] 0.95, 95% CI 0.93, 0.98), self-report of STI in the previous 6-months (AOR 1.72, 95% CI 1.12, 2.62), co-detection of *U. urealyticum* (AOR 2.95, 95% CI 2.14, 4.05), co-detection of *U. parvum* (AOR 2.15, 95% CI 1.51, 3.07), and BV (AOR 9.26, 95% CI 6.77, 12.67; Table 20). *M. hominis* was not associated with concurrent STIs in adjusted analyses.

Table 19 - Univariable analysis of microbiological factors associated with *M. hominis, U. urealyticum* and *U. parvum* detection

	M. hominis				***U. urealyticum***				***U. parvum***			
	Negative n=897	**Positive n=375**	**OR (95% CI)**	***P* value**	**Negative n=869**	**Positive n=403**	**OR (95% CI)**	***P* value**	**Negative n=362**	**Positive n=910**	**OR (95% CI)**	***P* value**
C. trachomatis												
Negative	834 (93)	331 (88)	1		795 (91)	370 (92)	1		337 (93)	828 (91)	1	
Positive	63 (7)	44 (12)	1.76 (1.17, 2.64)	**0.006**	74 (9)	33 (8)	0.96 (0.62, 1.47)	0.845	25 (7)	82 (9)	1.33 (0.84, 2.13)	0.224
N. gonorrhoeae												
Negative	890 (99)	365 (97)	1		861 (99)	394 (98)	1		360 (99)	895 (98)	1	
Positive	7 (1)	10 (3)	3.48 (1.32, 9.22)	**0.012**	8 (1)	9 (2)	2.46 (0.94, 6.42)	0.066	2 (1)	15 (2)	3.02 (0.69, 13.26)	0.144
M. genitalium												
Negative	843 (94)	341 (91)	1		813 (94)	371 (92)	1		340 (94)	844 (93)	1	
Positive	54 (6)	34 (9)	1.56 (0.99, 2.43)	0.052	56 (6)	32 (8)	1.25 (0.80, 1.97)	0.329	22 (6)	66 (7)	1.21 (0.73, 1.99)	0.457
T. vaginalis												
Negative	892 (99)	373 (99)	1		864 (99)	401 (100)	1		359 (99)	906 (100)	1	
Positive	5 (1)	2 (1)	0.96 (0.18, 4.95)	0.958	5 (1)	2 (0)	0.86 (0.17, 4.46)	0.859	3 (1)	4 (0)	0.53 (0.12, 2.37)	0.405
Nugent score												
0-3	662 (78)	120 (33)	1		564 (68)	218 (57)	1		245 (71)	537 (62)	1	
4-6	78 (9)	48 (13)	3.39 (2.26, 5.11)	**<0.001**	79 (10)	47 (12)	1.54 (1.04, 2.28)	**0.032**	29 (8)	97 (11)	1.53 (0.98, 2.37)	0.061
7-10	106 (13)	200 (54)	10.41 (7.67, 14.13)	**<0.001**	187 (23)	119 (31)	1.65 (1.25, 2.17)	**<0.001**	73 (21)	233 (27)	1.46 (1.07, 1.97)	**0.015**
Not assessed	51	7			39	19			15	43		
Bacterial vaginosis[a]												
Negative	706 (83)	138 (38)	1		602 (73)	242 (63)	1		260 (75)	584 (67)	1	

Positive	140 (17)	230 (63)	8.40 (6.36, 11.10)	**<0.001**	228 (27)	142 (37)	1.55 (1.20, 2.00)	**0.001**	87 (25)	283 (33)	1.45 (1.09, 1.92)	**0.010**
Not assessed	51	7			39	19			15	43		
Vulvovaginal candidiasis[b]												
Negative	651 (74)	294 (80)	1		651 (76)	294 (75)	1		277 (77)	668 (75)	1	
Positive	231 (26)	74 (20)	0.71 (0.53, 0.95)	**0.023**	205 (24)	100 (25)	1.08 (0.82, 1.42)	0.584	82 (23)	223 (25)	1.13 (0.84, 1.51)	0.416
Not assessed	15	7			13	9			3	19		
M. hominis												
Negative	-	-	-	-	671 (77)	226 (56)	1		288 (80)	609 (67)	1	
Positive	-	-	-	-	198 (23)	177 (44)	2.65 (2.06, 3.42)	**<0.001**	74 (20)	301 (33)	1.92 (1.44, 2.57)	**<0.001**
U. urealyticum												
Negative	671 (75)	198 (53)	1		-	-	-	-	204 (56)	665 (73)	1	
Positive	226 (25)	177 (47)	2.65 (2.06, 3.42)	**<0.001**	-	-	-	-	158 (47)	245 (27)	0.48 (0.37, 0.61)	**<0.001**
U. parvum												
Negative	288 (32)	74 (20)	1		204 (23)	158 (39)	1		-	-	-	-
Positive	609 (68)	301 (80)	1.92 (1.44, 2.57)	**<0.001**	665 (77)	245 (61)	0.48 (0.37, 0.61)	**<0.001**	-	-	-	-

Abbreviations: n, number; OR, odds ratio; CI, confidence interval

Data are presented as n (%), unless otherwise specified.

P-values < 0.05 are bolded to indicate statistically significant associations.

[a] Bacterial vaginosis diagnosis was defined as Nugent score=4-10 and ≥3 Amsel criteria for symptomatic women and as Nugent score=4-10 and presence of clue cells for asymptomatic women

[b] Vulvovaginal candidiasis diagnosis was based on the presence of typical clinical features on examination and/or presence of visible pseudohyphae and/or budding yeasts on microscopy

Table 20 - Multivariable analysis of factors associated with detection of *M. hominis, U. urealyticum* and *U. parvum* in women

Characteristic	*M. hominis* N=1155[a]		*U. urealyticum* N=1194[a]		*U. parvum* N=1194[a]	
	AOR (95% CI)[b]	***P* value**	**AOR (95% CI)[bc]**	***P* value**	**AOR (95% CI)[bc]**	***P* value**
Age	0.95 (0.93, 0.98)	**0.001**				
No. male partners in past 12mo	1.02 (0.99, 1.04)	0.270	1.07 (1.04, 1.11)	**<0.001**	1.06 (1.02, 1.09)	**0.003**
No. female partners in past 12mo					1.31 (1.04, 1.64)	**0.023**
Inconsistent condom use	0.94 (0.50, 1.77)	0.844	1.90 (1.08, 3.34)	**0.025**	2.06 (1.28, 3.32)	**0.003**
STI in past 6mo	1.72 (1.12, 2.62)	**0.012**				
C. trachomatis detected	1.56 (0.93, 2.61)	0.091				
N. gonorrhoeae detected	1.54 (0.43, 5.52)	0.510	1.30 (0.40, 4.20)	0.666		
M. genitalium detected	1.37 (0.80, 2.34)	0.258				
U. urealyticum detected	2.95 (2.14, 4.05)	**<0.001**			0.41 (0.31, 0.53)	**<0.001**
U. parvum detected	2.15 (1.51, 3.07)	**<0.001**	0.40 (0.31, 0.53)	**<0.001**		
Concurrent BV	9.19 (6.66, 12.70)	**<0.001**	1.49 (1.13, 1.95)	**0.004**	1.41 (1.05, 1.89)	**0.023**
Concurrent VVC	1.30 (0.90, 1.87)	0.165				

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; mo, months; STI, sexually transmitted infection; BV, bacterial vaginosis; VVC, vulvovaginal candidiasis

P-values < 0.05 are bolded to indicate statistically significant associations.

[a] Numbers differ from whole study population due to missing data for some variables, and as not all women were assessed for BV or VVC.

[b] A separate multivariable logistic regression was conducted for each common genital mycoplasma (i.e. *M. hominis, U. urealyticum* and *U. parvum*), adjusting for characteristics with *P*<0.10 in univariable analyses. Nugent score was left out of all adjusted models so as to not over-fit the models as it was strongly correlated with BV (ρ=0.92).

[c] *M. hominis* was moderately correlated with BV (ρ=0.46). As a result it was left out of adjusted analyses for *U. urealyticum* and *U. parvum* to not over-fit the models. BV was included as it is a known clinically relevant syndrome.

U. urealyticum was associated with increased numbers of MSPs in the previous 12-months (AOR 1.07, 95% CI 1.04, 1.11), inconsistent condom use (AOR 1.90, 95% CI 1.08, 3.34), and BV (AOR 1.49, 95% CI 1.13, 1.95). Detection of *U. urealyticum* was negatively associated with co-detection of *U. parvum* (AOR 0.40, 95% CI 0.31, 0.53).

U. parvum was associated with higher numbers of MSPs (AOR 1.06, 95% CI 1.02, 1.09) and FSPs in the previous 12-months (AOR 1.31, 95% CI 1.04, 1.64), inconsistent condom use (AOR 2.06, 95% CI 1.28, 3.32), and BV (AOR 1.41, 95% CI 1.05, 1.89).

Due to the correlation between *M. hominis* and BV (ρ=0.46), we did not adjust analyses in Table 20 for *M. hominis*. To investigate if *M. hominis*, *U. urealyticum* and/or *U. parvum* were independently associated with BV, we performed a logistic regression that included *M. hominis*, *U. urealyticum* and *U. parvum* with BV as the outcome, adjusted for number of MSPs. In this analysis, BV was strongly associated with *M. hominis* (AOR 8.01, 95% CI 5.99, 10.71), but was not associated with either *U. urealyticum* or *U. parvum* (Figure 12).

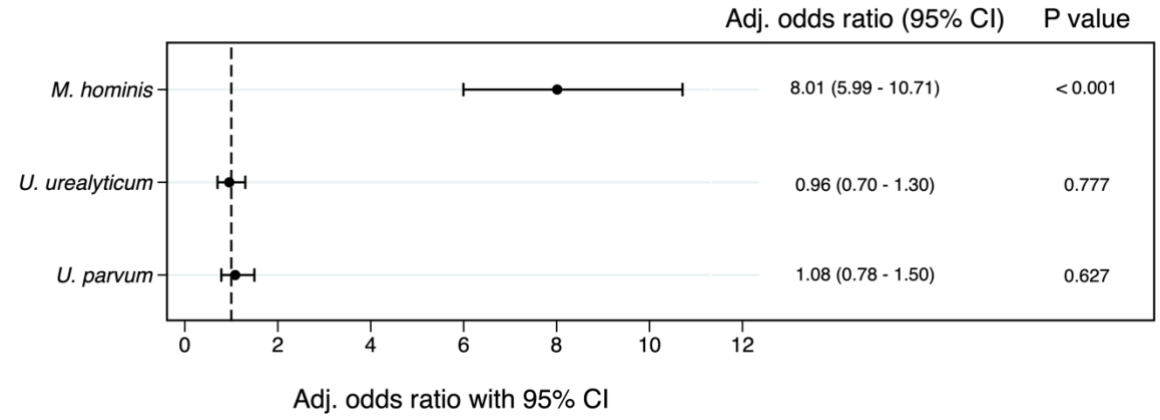


Figure 12. Association of *M. hominis*, *U. urealyticum* and *U. parvum* with bacterial vaginosis

The adjusted odds ratio reflects the association between the three common genital mycoplasmas (i.e. *M. hominis, U. urealyticum* and *U. parvum*) and bacterial vaginosis, adjusted for each other and number of male sexual partners. Adjusted odds ratios are presented with 95% confidence intervals.

5.5.4. Self-reported symptoms and clinical signs associated with *M. hominis, U. urealyticum* and *U. parvum*

The association of *M. hominis, U. urealyticum* and *U. parvum* with self-reported symptoms and clinical signs is presented in Table 21. After adjusting for genital co-infections and following FDR correction, *M. hominis* increased the odds of BV symptoms and each Amsel criterion, including abnormal vaginal discharge (self-report AOR 1.66, 95% CI 1.26, 2.18 and clinician-recorded AOR 2.70, 95% CI 1.92, 3.79), vaginal malodour (self-report AOR 3.19, 95% CI 2.38, 4.26 and clinician-recorded AOR 4.27, 95% CI 3.08, 5.91), elevated vaginal pH>4.5 (AOR 4.27, 95% CI 3.22, 5.66) and presence of clue cells (AOR 8.08, 95% CI 5.68, 11.48). However, *U. urealyticum* and *U. parvum* were not associated with any symptom or sign after adjusting for genital co-infections and following FDR correction.

Table 21 - Self-reported symptoms and clinical signs associated with detection of *M. hominis*, *U. urealyticum* and *U. parvum*

	M. hominis					*U. urealyticum*					*U. parvum*				
	Negative n=897	**Positive n=375**	**AOR (95% CI)[a]**	***P* value**	***q* value**	**Negative n=869**	**Positive n=403**	**AOR (95% CI)[a]**	***P* value**	***q* value**	**Negative n=362**	**Positive n=910**	**AOR (95% CI)[a]**	***P* value**	***q* value**
Abdominal Pain															
No	762 (85)	321 (86)	1			739 (86)	344 (86)	1			299 (83)	784 (87)	1		
Yes	130 (15)	52 (14)	0.97 (0.67, 1.41)	0.892	0.911	125 (14)	57 (14)	0.91 (0.63, 1.31)	0.621	0.775	61 (17)	121 (13)	0.76 (0.53, 1.07)	0.120	0.380
Missing	5	2				5	2				2	5			
Dyspareunia															
No	798 (90)	335 (90)	1			775 (90)	358 (90)	1			318 (89)	815 (91)	1		
Yes	86 (10)	38 (10)	1.03 (0.67, 1.58)	0.886	0.911	82 (10)	42 (11)	1.13 (0.75, 1.70)	0.571	0.775	39 (11)	85 (9)	0.87 (0.57, 1.31)	0.500	0.723
Missing	13	2				12	3				5	10			
Abnormal vaginal discharge															
No	611 (69)	221 (59)	1			572 (66)	260 (65)	1			230 (64)	602 (67)	1		
Yes	277 (31)	151 (41)	1.66 (1.26, 2.18)	**<0.001**	**<0.001**	289 (34)	139 (35)	0.92 (0.70, 1.20)	0.526	0.775	129 (36)	299 (33)	0.82 (0.63, 1.08)	0.126	0.440
Missing	9	3				8	4				3	9			
Vaginal malodour															
No	732 (82)	227 (61)	1			657 (76)	302 (75)	1			278 (77)	681 (76)	1		
Yes	158 (18)	144 (39)	3.19 (2.38, 4.26)	**<0.001**	**<0.001**	202 (24)	100 (25)	0.81 (0.60, 1.09)	0.166	0.775	83 (23)	219 (24)	0.90 (0.66, 1.22)	0.499	0.723
Missing	7	4				10	1				1	10			
Vaginal itch															
No	695 (78)	300 (80)	1			685 (79)	310 (77)	1			284 (79)	711 (78)	1		
Yes	196 (22)	73 (20)	0.93	0.676	0.911	177 (21)	92 (23)	1.15	0.390	0.775	74 (21)	195 (22)	1.07	0.685	0.723

			(0.66, 1.30)					(0.83, 1.59)					(0.77, 1.49)		
Missing	6	2				7	1				4	4			
Post, coital bleeding															
No	812 (92)	337 (91)	1			779 (91)	370 (92)	1			331 (92)	818 (91)	1		
Yes	74 (8)	34 (9)	1.09 (0.70, 1.71)	0.705	0.911	77 (9)	31 (8)	0.86 (0.55, 1.36)	0.522	0.775	28 (8)	80 (9)	1.12 (0.70, 1.77)	0.645	0.723
Missing	11	4				13	2				3	12			
Intermenstrual bleeding															
No	803 (90)	332 (89)	1			773 (90)	362 (91)	1			312 (87)	823 (91)	1		
Yes	85 (10)	40 (11)	1.28 (0.84, 1.94)	0.250	0.594	87 (10)	38 (10)	0.80 (0.52, 1.22)	0.302	0.775	46 (13)	79 (9)	0.63 (0.42, 0.95)	0.026	0.222
Missing	9	3				9	3				4	8			
Dysuria															
No	769 (86)	332 (89)	1			754 (87)	347 (86)	1			312 (87)	789 (87)	1		
Yes	122 (14)	41 (11)	0.75 (0.50, 1.12)	0.157	0.426	108 (13)	55 (14)	1.18 (0.82, 1.71)	0.378	0.775	45 (13)	118 (13)	1.10 (0.75, 1.61)	0.616	0.723
Missing	6	2				7	1				5	3			
Urinary frequency															
No	743 (83)	301 (81)	1			717 (83)	327 (82)	1			291 (81)	753 (83)	1		
Yes	150 (17)	69 (19)	1.13 (0.81, 1.58)	0.468	0.885	145 (17)	74 (18)	1.06 (0.76, 1.47)	0.734	0.775	67 (19)	152 (17)	0.86 (0.62, 1.20)	0.375	0.713
Missing	4	5				7	2				4	5			
Fever															
No	859 (97)	358 (96)	1			830 (97)	387 (96)	1			350 (97)	867 (96)	1		
Yes	29 (3)	14 (4)	1.09 (0.55, 2.16)	0.816	0.911	28 (3)	15 (4)	1.13 (0.57, 2.25)	0.719	0.775	9 (3)	34 (4)	1.56 (0.72, 3.38)	0.260	0.618
Missing	9	3				11	1				3	9			
Clinical signs associated with detection of *M. hominis*, *U. urealyticum* and *U. parvum*[b]															

	M. hominis					*U. urealyticum*					*U. parvum*				
	Negative n=250	**Positive n=156**	**AOR (95% CI)[a]**	***P* value**	***q* value**	**Negative n=282**	**Positive n=124**	**AOR (95% CI)[a]**	***P* value**	***q* value**	**Negative n=117**	**Positive n=289**	**AOR (95% CI)[a]**	***P* value**	***q* value**
Abnormal vaginal discharge															
No	88 (35)	21 (13)	1			84 (30)	25 (20)	1			39 (33)	70 (24)	1		
Yes	162 (65)	135 (87)	3.58 (2.04, 6.28)	**<0.001**	**<0.001**	198 (70)	99 (80)	1.45 (0.82, 2.56)	0.196	0.775	78 (67)	219 (76)	1.73 (1.04, 2.89)	0.035	0.222
Vaginal malodour															
No	201 (80)	78 (50)	1			202 (72)	77 (62)	1			91 (78)	188 (65)	1		
Yes	49 (20)	78 (50)	3.78 (2.35, 6.08)	**<0.001**	**<0.001**	80 (28)	47 (38)	1.18 (0.71, 1.97)	0.523	0.775	26 (22)	101 (35)	1.95 (1.14, 3.35)	0.015	0.222
Vulval redness															
No	200 (80)	123 (79)	1			231 (82)	92 (74)	1			93 (79)	230 (80)	1		
Yes	50 (20)	33 (21)	1.27 (0.70, 2.30)	0.437	0.885	51 (18)	32 (26)	1.64 (0.89, 3.00)	0.110	0.775	24 (21)	59 (20)	1.13 (0.62, 2.08)	0.685	0.723
Mucopurulent cervicitis[c]															
No	217 (87)	132 (85)	1			242 (86)	107 (86)	1			98 (84)	251 (87)	1		
Yes	33 (13)	24 (15)	1.11 (0.60, 2.05)	0.732	0.911	40 (14)	17 (14)	0.99 (0.51, 1.91)	0.975	0.975	19 (16)	38 (13)	0.75 (0.40, 1.38)	0.354	0.713
Cervical or adnexal motion tenderness															
No	198 (79)	124 (79)	1			222 (79)	100 (81)	1			92 (79)	230 (80)	1		
Yes	52 (21)	32 (21)	1.03 (0.61, 1.74)	0.911	0.911	60 (21)	24 (19)	0.88 (0.50, 1.54)	0.647	0.775	25 (21)	59 (20)	0.88 (0.51, 1.51)	0.647	0.723
Cervical contact bleeding															
No	207 (83)	131 (84)	1			235 (83)	103 (83)	1			95 (81)	243 (84)	1		
Yes	43 (17)	25 (16)	0.83	0.525	0.885	47 (17)	21 (17)	1.11	0.728	0.775	22 (19)	46 (16)	0.80	0.451	0.723

			(0.47, 1.48)					(0.61, 2.04)					(0.45, 1.43)		
Vaginal pH															
≤4.5	160 (64)	40 (26)	1			150 (53)	50 (40)	1			65 (56)	135 (47)	1		
>4.5	90 (36)	116 (74)	4.80 (3.00, 7.69)	**<0.001**	**<0.001**	132 (47)	74 (60)	1.17 (0.71, 1.92)	0.546	0.775	52 (44)	154 (53)	1.47 (0.91, 2.37)	0.120	0.380
Clue cells															
Absent	184 (74)	39 (25)	1			173 (61)	50 (40)	1			72 (62)	151 (52)	1		
Present	66 (26)	117 (75)	8.76 (5.19, 14.78)	**<0.001**	**<0.001**	109 (39)	74 (60)	1.77 (1.02, 3.09)	0.043	0.775	45 (38)	138 (48)	1.73 (1.00, 2.98)	0.051	0.242
Vaginal PMNL cell count															
<5 PMNL/hpf	150 (60)	88 (56)	1			169 (60)	69 (56)	1			67 (57)	171 (59)	1		
≥5 PMNL/hpf	100 (40)	68 (44)	1.14 (0.73, 1.80)	0.559	0.885	113 (40)	55 (44)	1.27 (0.79, 2.03)	0.325	0.775	50 (43)	118 (41)	0.96 (0.60, 1.52)	0.852	0.852

Abbreviations: n, number; AOR, adjusted odds ratio; CI, confidence interval; PMNL/hpf, polymorphonuclear leukocyte/high power field

Data are presented as n (%), unless otherwise specified

Reported q-values are p-values adjusted for the false discovery rate and q-values <0.05 are bolded to indicate statistically significant associations.

[a] Multivariable logistic regression adjusted for *C. trachomatis*, *N. gonorrhoea, M. genitalium, T. vaginalis,* VVC and common genital mycoplasmas (i.e. *M. hominis, U. urealyticum* and *U. parvum*). As we examined associations with individual Amsel criteria, we did not adjust for BV in order to prevent over-fitting the model.

[b] Analysis of clinical signs was restricted to symptomatic women who were assessed for all signs (n=406)

[c] Mucopurulent cervicitis was defined as the presence of a visible mucopurulent discharge on the cervix or extruding from the cervical os.

To account for potential confounding by BV on the relationship between *M. hominis* and symptoms/signs, data were stratified by BV status, and the association between *M. hominis* and clinical characteristics was investigated within each strata using the chi-squared or Fisher exact test. In women without BV, *M. hominis* was not associated with symptoms or signs. In women with BV, *M. hominis* was detected more frequently in women with self-reported vaginal malodour compared to women not reporting malodour (118/230 [52%] vs 53/140 [38%]; *P*=0.012), and in women with clinician-recorded vaginal discharge compared to women without discharge on examination (111/123 [90%] vs 52/65 [80%]; *P*=0.049).

5.6. Discussion

In our study of 1,272 nonpregnant women attending a large public sexual health service in Melbourne, Australia, *M. hominis* was detected in 29% (95% CI 27-32) of women, *U. urealyticum* was detected in 32% (95% CI 29-34) and *U. parvum* was detected in 72% (95% CI 69-74). In adjusted analyses, *M. hominis, U. urealyticum* and *U. parvum* were associated with classic epidemiological markers of sexual risk such as increased numbers of sexual partners, inconsistent condom use and recent STI diagnosis. *M. hominis* was strongly associated with the syndrome of BV (AOR 8.08, 95% CI 5.68-11.48), after adjusting for *U. urealyticum, U. parvum* and number of MSPs, whereas *U. urealyticum* and *U. parvum* were not associated with BV. Only *M. hominis* was associated with specific clinical characteristics, and these were manifestations of BV. In stratified analyses, *M. hominis* was only associated with vaginal malodour and abnormal vaginal discharge in women with BV and was not associated with symptoms or signs in women without BV. These findings support the hypothesis that *M. hominis* is part of the spectrum of organisms considered to be BV-associated bacteria. The high prevalence of *U. urealyticum* and *U. parvum* and the finding that these bacteria were not associated with any symptom or sign indicates that asymptomatic carriage of these bacteria is common. In agreement with the European Position Statement (Horner *et al.*, 2018), our findings do not support the routine screening or testing for *M. hominis*, *U. urealyticum* and *U. parvum* in nonpregnant women in a sexual health setting.

We found that *M. hominis* independently increased the odds of BV by 8-fold, whereas *U. urealyticum* and *U. parvum* were not associated with increased odds of BV. Previous studies

have reported associations between *M. hominis* and BV (Ceccarani *et al.*, 2019; Cox *et al.*, 2016; Foschi *et al.*, 2018; Keane *et al.*, 2000; Rumyantseva *et al.*, 2019; Vitali *et al.*, 2015; Zozaya-Hinchliffe *et al.*, 2010), including increased detection and increased load of *M. hominis* in women with BV compared to women without BV (Cox *et al.*, 2016; Keane *et al.*, 2000; Rumyantseva *et al.*, 2019). However, not all women with BV have detectable *M. hominis*, and whether *M. hominis* contributes to the initiation of BV or how it contributes to BV pathophysiology is unknown. Interestingly, *M. hominis* metabolises arginine to produce ammonia (Pollack, 1992), and the production of ammonia by other organisms (e.g. *Prevotella bivia*) has been shown to enhance growth of *Gardnerella vaginalis* (Pybus & Onderdonk, 1997), a prime suspect in the pathogenesis of BV (Muzny *et al.*, 2019c). Positive correlation in organism load of *M. hominis* and *G. vaginalis* in women co-infected with both organisms has been reported, which may suggest synergy between the two bacteria (Cox *et al.*, 2016). Although *M. hominis* displays resistance to metronidazole *in vitro,* BV-treatment with metronidazole decreases the load of *M. hominis* (Koutsky *et al.*, 1983). Conversely, doxycycline, which is active against *M. hominis,* is ineffective for treating BV (Pheifer *et al.*, 1978). Together, these data suggest that *M. hominis* may enhance the growth of more virulent BV-associated bacteria, but is unlikely to be integral to BV pathogenesis. Furthermore, the association of *M. hominis*, *U. urealyticum* and *U. parvum* with BV in univariable analysis highlights that these organisms exist in a complex bacterial milieu. As has been noted previously (Donders *et al.*, 2017b; Horner *et al.*, 2018), testing or screening for *M. hominis*, *U. urealyticum* and *U.parvum* without concurrent evaluation of the vaginal microbiome has limited value. Importantly, our data suggests that in a sexual health service, there may be more clinical value in appropriately diagnosing the syndrome of BV, rather than testing for *M. hominis*, *U. urealyticum* or *U. parvum* on their own.

We found that *U. urealyticum* and *U. parvum* were not associated with any symptom or sign, but these bacteria have been associated with negative outcomes in previous studies. This raises important considerations. For example, the pathogenicity of ureaplasmas may be serovar-specific. However, to date, studies have failed to consistently associate individual serovars with symptoms or specific clinical outcomes such as preterm birth (De Francesco *et al.*, 2009; Payne *et al.*, 2020; Rittenschober-Bohm *et al.*, 2019; Xiao *et al.*, 2011). Furthermore, there is some controversy surrounding the concept of *Ureaplasma* serovars.

Xiao *et al.* (2011) serotyped 1, 061 clinical *Ureaplasma* isolates and found that 39% contained markers for multiple serovars, 6% had no serovar detected and 4% had discordant species and serovar level results. Analysis of 271 isolates with multiple serovar markers identified 28% as being pure isolates that were hybrids of ≥2 serovars, which suggests that serotyping may have limited utility for determining pathogenicity (Xiao *et al.*, 2011). Further investigation of pathogenicity at the serovar level is needed. Other factors, such as bacterial load, may be important for pathogenicity. High-load of vaginal *Ureaplasma* spp.($>10^5$ cfu/ml) has been shown to be an independent risk factor for both chorioamnionitis and preterm delivery (Abele-Horn *et al.*, 2000). Additionally, it has been reported that *U. urealyticum* load, but not prevalence, is associated with postpartum endometritis ($>10^5$ cfu/ml; 39% *vs* 17%, *P*=0.03) (Chaim *et al.*, 2003). Assessment of bacterial load, and the subsequent host immune response (Kasper *et al.*, 2010), may be important for understanding the clinical role of *M. hominis*, *U. urealyticum* and *U. parvum*.

There are limitations to this study. Firstly, our prevalence estimates are higher than previous estimates, which is likely a result of recruiting from a STI clinic population. Women in our study had a higher prevalence of *C. trachomatis* compared to a previous study of women attending primary-care centres in Australia (8% vs 4%) (Yeung *et al.*, 2014), which reflects the higher risk nature of the population, and combined with the single recruitment site, limits the generalisability of our findings. Furthermore, 30% (95% CI 28-33) of our study population had BV (Latimer *et al.*, 2019b), which is more than double the most recent prevalence estimates for BV among Australian women attending primary-health care and sexual health clinics (12%; 95% CI 9-14) (Bradshaw *et al.*, 2013b). As a result, we may have overestimated the relationship between *M. hominis* and BV. Additionally, associations are based on organism detection rather than organism load, and serovar specific associations were not examined. As discussed above, organism load may provide further insight into the contribution of *M. hominis*, *U. urealyticum* and *U. parvum* to clinical characteristics in nonpregnant women.

To summarise, we found that *M. hominis, U. urealyticum* and *U. parvum* were common and associated with markers of sexual exposure in a population of nonpregnant women attending a sexual health service. *M. hominis* was strongly associated with concurrent BV and was associated with clinical characteristics in women with BV, however it was not

associated with symptoms or signs in women without BV. *U. urealyticum* and *U. parvum* were not associated with any symptom or sign, or independently associated with BV. These data, that have been adjusted for important confounders, provide evidence against the routine testing and screening of nonpregnant women for *M. hominis*, *U. urealyticum* and *U. parvum* in a sexual health setting, and highlight the importance of accurately diagnosing the syndrome of BV.

Chapter 6. Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: a systematic review

6.1. Overview

As discussed in Section 1.5 of the literature review, current recommended therapies for BV involve antibiotic treatment with metronidazole or clindamycin, and these therapies have unacceptably high recurrence rates. There is consensus in the BV and vaginal microbiota field that we need to investigate and develop alternative therapies to improve long term BV cure and reduce associated sequelae. A large proportion of this thesis has been dedicated to the role of sexual transmission in the pathogenesis of incident and recurrent BV, and although partner treatment is likely to represent a key strategy for improving long-term BV cure for many women (see **Chapter 4**), it is clear that partner treatment will not be the only strategy required. As discussed throughout this thesis, the optimal vaginal microbiota of reproductive aged women is typically dominated by lactic acid producing lactobacilli and women with BV have reduced abundance of these lactobacilli. Lactic acid has properties that may make it effective for treating BV (Section 1.2.5.1) and for supporting an optimal *Lactobacillus* dominated vaginal microbiota following antibiotic treatment for BV (Tachedjian *et al.*, 2017). Indeed, there are several over the counter lactic acid-containing products available that are marketed to treat BV and/or to restore and support an optimal vaginal microbiota composition. However, these products are not recommended in any treatment guidelines (Australian Sexual Health Alliance, 2018; Hay *et al.*, 2012; Sherrard *et al.*, 2018; Workowski & Bolan, 2015), nor has their use been systematically reviewed.

In light of this, **Chapter 6** presents a systematic review that describes the effect of intravaginal lactic acid-containing products on BV cure, as well as the impact of intravaginal lactic acid-containing products on the composition of the vaginal microbiota.

Chapter 6 is presented as the accepted version of the following peer reviewed manuscript that was published in *PLoS One*. No alterations to the text have been made, except that

abbreviations, and figure and table numbers have been adjusted to generate a consistent presentation within this thesis.

Publication:

> **Plummer EL**, Bradshaw CS, Doyle M, Fairley CK, Murray GL, Bateson D, Masson L, Slifirski J, Tachedjian G, Vodstrcil LA (2021). Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: A systematic review. ***PLoS One*** 16 (2):e0246953. doi:10.1371/journal.pone.0246953

Findings from this manuscript have also been presented at the following national conference:

- 2020 Virtual Joint HIV&AIDS and Sexual Health Conferences, 16 – 20 Nov 2020. [Poster]

Supplementary files referred to in this chapter have been appended in Appendix D.

A PDF of the published manuscript is in Appendix E.

6.2. Abstract

Objective: The vaginal microbiota in BV typically has low abundance of lactic acid producing lactobacilli. Lactic acid has properties that may make it effective for treating BV and/or restoring an optimal lactobacillus-dominated vaginal microbiota. We conducted a systematic review to describe the effect of intravaginal lactic acid-containing products on BV cure, and their impact on vaginal microbiota composition (PROSPERO registration: CRD42018115982).

Methods: PubMed, Embase and OVID were searched from inception to November 2019 to identify eligible studies. Included studies evaluated an intravaginal lactic acid-containing product and reported BV cure using established diagnostic methods, and/or vaginal microbiota composition using molecular methods. Studies were independently screened and assessed, and the proportion of women cured post-treatment was calculated. Study results were described in a qualitative manner.

Results: We identified 1883 articles and assessed 57 full-texts for eligibility. Seven different lactic acid-containing products were evaluated and differed with respect to excipients, lactic

acid concentration and pH. Most studies had medium or high risk of bias. Three trials compared the efficacy of a lactic acid-containing product to metronidazole for BV cure. One study found lactic acid to be equivalent to metronidazole and two studies found lactic acid to be significantly inferior to metronidazole. Two studies included a control group receiving a placebo or no treatment. One reported lactic acid to be superior than no treatment and the other reported lactic acid to be equivalent to placebo. Lactic acid-containing products did not significantly impact the vaginal microbiota composition.

Conclusion: There is a lack of high-quality evidence to support the use of lactic acid-containing products for BV cure or vaginal microbiota modulation. However, adequately powered and rigorous randomised trials with accompanying vaginal microbiota data are needed to evaluate the efficacy of lactic acid as a BV treatment strategy.

6.3. Introduction

Bacterial vaginosis is the commonest vaginal condition in reproductive aged women. BV is associated with serious sequelae including miscarriage, preterm birth and pelvic inflammatory disease, and acquisition of sexually transmitted infections including HIV (Brotman *et al.*, 2010; Cohen *et al.*, 2012; Koumans *et al.*, 1999; Low *et al.*, 2011; Myer *et al.*, 2005). Recommended first-line treatments for BV are oral or intravaginal metronidazole and intravaginal clindamycin (Workowski & Bolan, 2015). First-line treatments have equivalent four-week cure rates of ~70-85% (Oduyebo *et al.*, 2009), but BV recurrence is common (Bradshaw *et al.*, 2006a; Sobel *et al.*, 1993). Recurrences negatively impact a woman's quality of life (Bilardi *et al.*, 2013) and result in repeated clinical presentations and antibiotic use. Given the significant sequelae, treatments that improve BV cure are needed.

The optimal vaginal microbiota of reproductive aged women is typically characterised by dominance of lactic acid producing *Lactobacillus* species including *Lactobacillus crispatus, Lactobacillus gasseri* and *Lactobacillus jensenii* (Boskey *et al.*, 2001; Boskey *et al.*, 1999; Fredricks *et al.*, 2005; McKinnon *et al.*, 2019; Ravel *et al.*, 2011; Tachedjian *et al.*, 2018). Women with BV have reduced abundance of these lactobacilli and increased prevalence and abundance of anaerobic and facultative-anaerobic bacteria (Fredricks *et al.*, 2005; Ravel *et al.*, 2011). *In vitro* studies have shown that lactic acid inactivates BV-associated bacteria (O'Hanlon *et al.*, 2011) and pathogens including *C. trachomatis, N. gonorrhoeae* and HIV via

mechanisms independent of acidity alone (Aldunate *et al.*, 2013; Gong *et al.*, 2014; Graver & Wade, 2011; Tyssen *et al.*, 2018). Lactic acid has also been shown to modulate cervicovaginal epithelial cell functions to prevent *C. trachomatis* infection (Edwards *et al.*, 2019). Lactic acid also has immunomodulatory effects (Tachedjian *et al.*, 2017), and can elicit an anti-inflammatory response and reduce production of inflammatory cytokines and chemokines from cervicovaginal epithelial cells *in vitro* (Hearps *et al.*, 2017).

The antimicrobial and immunomodulatory properties of lactic acid may make it effective for the treatment of BV and/or to restore an optimal microbiota following antibiotic treatment (Tachedjian *et al.*, 2017). Lactic acid-containing products have been evaluated for BV treatment in clinical trials, and several over-the-counter lactic acid-containing products are marketed to treat BV or support optimal vaginal microbiota. However, the use of these products is not recommended by any treatment guidelines (Workowski & Bolan, 2015).

We conducted a systematic review with two objectives: 1) to describe the effect of intravaginal lactic acid-containing products for BV cure (assessed using an established diagnostic method), and 2) to describe the impact of intravaginal lactic acid-containing products on the vaginal microbiota (assessed using molecular methods).

6.4. Materials and Methods

We conducted and reported this systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis statement (Moher *et al.*, 2015)(Supplementary File D1), and registered the protocol prospectively with PROSPERO (CRD42018115982).

6.4.1. Search strategy, eligibility criteria

We searched electronic databases (PubMed, Embase, OVID Medline) from inception until 4th November 2019 using keywords: “bacterial vaginosis”, “vaginal microbiota” and “lactic acid” (search strings in Supplementary Table D1). Reference lists and conference abstracts were searched for additional studies. Conference abstracts were included if they reported adequate information. Studies were uploaded to Covidence (Veritas Health Innovation, Melbourne, Australia, www.covidence.org) and were independently reviewed for eligibility by three authors (EP, JS, MD). Disagreements were resolved with a fourth author (LV).

Studies were eligible for objective 1 (BV cure outcome) if they assessed an intravaginal lactic acid-containing product as the main or adjuvant therapy for BV cure in women diagnosed with BV. BV had to be diagnosed using an established method (e.g. Amsel criteria or modified Amsel criteria (Amsel *et al.*, 1983), Nugent score (Nugent *et al.*, 1991) or Ison-Hay method (Ison & Hay, 2002)). Studies were eligible if they were RCTs where an intravaginal lactic acid-containing product was assessed in comparison to either no treatment, a placebo or a recommended antibiotic treatment for BV. No restrictions were placed on number of participants enrolled. Studies of pregnant women and post-menopausal women were excluded.

Studies were eligible for objective 2 (vaginal microbiota outcome) if they reported use of an intravaginal lactic acid-containing product in women with or without BV, and assessed the vaginal microbiota using a molecular method such as qPCR or high throughput sequencing. In order to capture all published literature evaluating the impact of lactic acid-containing product on the vaginal microbiota composition, no restrictions were placed on study design, number of participants enrolled, age, menopause or pregnancy status.

For both objectives, we excluded studies if they were performed on animals or the data was not stratified by lactic acid-containing product use. Only English language studies were included.

6.4.2. Interventions assessed

Assessed interventions included any intravaginal lactic acid-containing product. Interventions were excluded if they contained lactic acid producing bacteria or were not used intravaginally.

6.4.3. Outcome measures

Outcome measures were: 1) BV cure defined as ≤2 Amsel criteria and/or Nugent score<4, or Ison-Hay grade 1 measured ≥7 days after the start of treatment, 2) vaginal microbiota composition assessed by molecular methods, and 3) occurrence of adverse events.

6.4.4. Data extraction

Three authors (EP, JS, MD) independently extracted the following information for each study: author details, publication year, study design, population studied, intervention details, comparator details, follow-up duration, BV diagnostic method, BV cure definition, microbiota characterisation methodology, adverse events and study findings. Disagreements in extracted data were resolved by discussion between authors. Two corresponding authors were contacted for additional details, one responded (Gottschick *et al.*, 2017a).

6.4.5. Data analysis

For objective 1 (BV cure outcome), we calculated the proportion of women cured post-treatment per treatment group with 95% confidence intervals, and described results in a narrative manner. For objective 2 (vaginal microbiota outcome), the impact of lactic acid-containing products on the vaginal microbiota was described narratively. Where an article reported ≥2 lactic acid-containing products or treatment regimens, each product/regimen was presented separately in tables.

6.4.6. Assessment of bias

Two authors (EP, MD) independently assessed the risk of bias of each study using a modified version of the RoB 2.0 (Higgins JPT *et al.*, 2016) and ROBINS-I tools (Sterne *et al.*, 2016) (Supplementary Table D2). The level of overall risk was summarised across six domains: selection bias, performance bias, measurement bias, response bias, reporting bias and other sources of bias (i.e. adjustment for confounders and insufficient description of product details). Studies were not excluded based on bias assessment.

6.5. Results

6.5.1. Study selection

Our initial search identified 1882 articles. One additional article was identified from reference lists. Following duplicate removal, 1591 articles were screened on title and abstract. We excluded 1534 articles and assessed 57 full-text articles. Fifty articles were excluded; seven of which evaluated a lactic acid-containing product for BV treatment but

were excluded because they were non-randomised (n=5), did not use standard criteria to assess BV cure (n=1) or assessed BV recurrence only (n=1; Supplementary Table D3). Seven articles were included in the review (Figure 13).

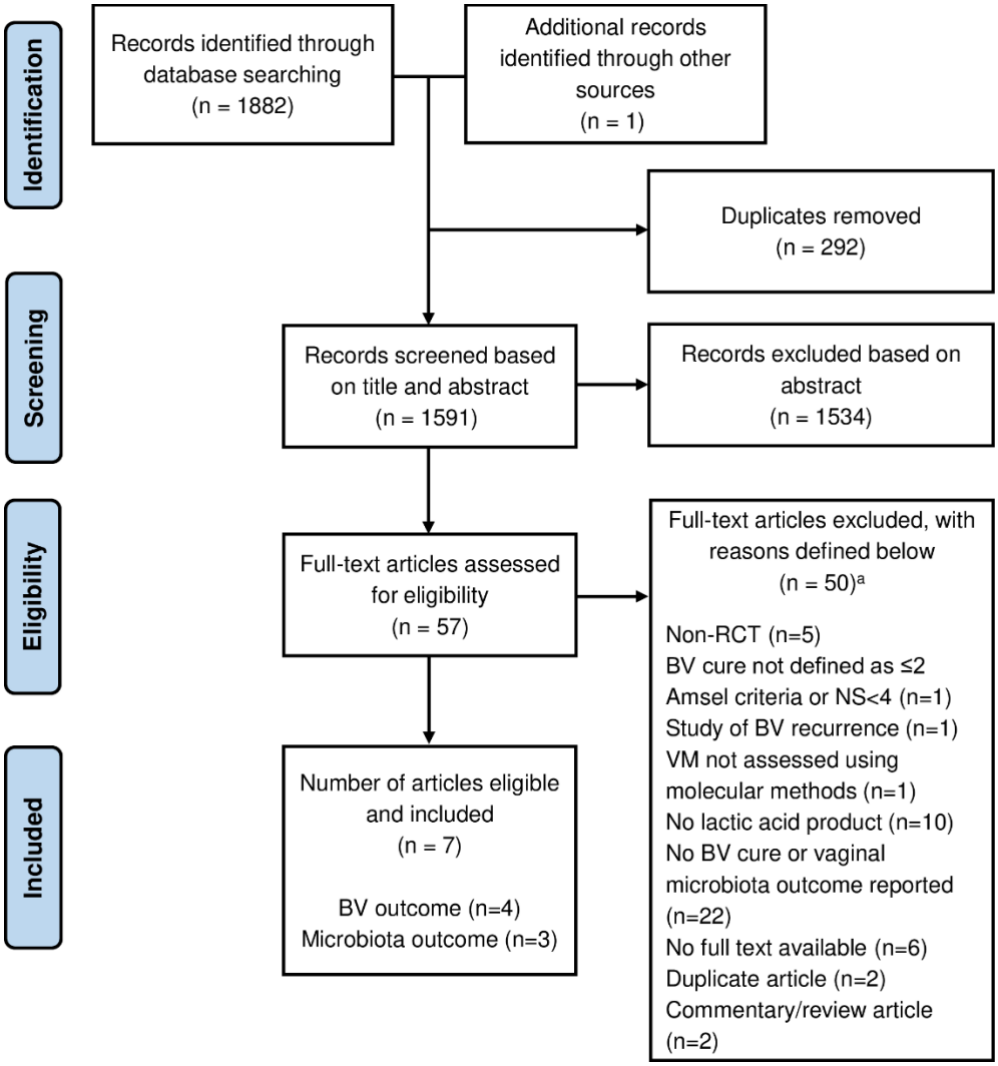


Figure 13. PRISMA flow diagram describing the literature search and article selection process

[a] *Detailed reasons for exclusion are provided in Supplementary Table D3*

6.5.2. Lactic acid-containing products evaluated

Seven different lactic acid-containing products were evaluated and differed with respect to lactic acid concentration, pH and included ingredients/excipients (Table 22). Two intravaginal gels were evaluated in three studies: Acidform was evaluated in two studies (Keller *et al.*, 2012; Simoes *et al.*, 2006) and Lactal was evaluated in one study (Andersch *et al.*, 1986).

Four different vaginal pessaries were evaluated in three studies (Boeke *et al.*, 1993; Fredstorp *et al.*, 2015; Gottschick *et al.*, 2017a) and a vaginal douche (Etos®) was evaluated in one study (van der Veer *et al.*, 2019). Excipients were not reported in one study (Fredstorp *et al.*, 2015). Lactic acid isomer details were only located for Acidform, which comprises L-lactic acid (Nelson, 2018).

Table 22 - Lactic acid-containing product details

Product name, formulation, reference	Lactic acid details	pH	Other ingredients and excipients including preservatives
Acidform[a] intravaginal gel (Keller *et al.*, 2012; Simoes *et al.*, 2006)	88mg (1.76%) per dose L-lactic acid isomer	3.55	50 mg (1%) citric acid, 20 mg (0.4%) potassium bitartrate, benzoic acid, alginic acid, xanthan gum, glycerin, sodium hydroxide and water in a 5mg dose (Nelson, 2018)
Lactal intravaginal gel (Andersch *et al.*, 1986)[b]	NR	3.5	Growth substrates for lactobacilli
Lactic acid pessary (Boeke *et al.*, 1993)	100mg lactic acid per pessary	3.3	2.4g of polyethylene glycol 1540
Vagisan®, vaginal pessary (Gottschick *et al.*, 2017a)	40mg lactic acid per pessary	~4.5	Macrogol 1500, macrogol 6000 and sodium lactate
WO3191, vaginal pessary (Gottschick *et al.*, 2017a)	Total lactic acid conc. of ~3.9% total weight	~4.5	Cocoamphopropionate (amphoteric tenside), sodium lactate
Sustained release oligomeric lactic acid (OMLA) pessary (Fredstorp *et al.*, 2015)	700mg lactic acid per pessary[c]	pH 3.5	NR
Etos® vaginal douche (van der Veer *et al.*, 2019)	Neat lactic acid conc. 0.45%, diluted conc. 0.06% (1 in 7 dilution)	Neat pH 3.42, diluted pH 3.50 (1 in 7 dilution)	Aqua, butylene glycol, caprylyl glycol, sodium pyroglutamic acid, Zea mays kernel extract, hydrolyzed milk protein, niacinamide, and adenosine triphosphate

NR, not reported;
[a] Also known as Amphora
[b] Reference Andersch *et al.* (1990) states that Lactal gel is the lactic acid-containing product in Andersch *et al.* (1986)
[c] Designed to release lactic acid over a 72hr period

6.5.3. Intravaginal lactic acid-containing products for BV cure

Four RCTs assessed the efficacy of an intravaginal lactic acid-containing product for BV cure (Table 23) (Andersch *et al.*, 1986; Boeke *et al.*, 1993; Fredstorp *et al.*, 2015; Simoes *et al.*, 2006).

Andersch *et al.* (1986) randomised women to receive once nightly Lactal gel (lactic acid concentration not specified) for seven days or twice daily oral metronidazole for seven days. No details of allocation concealment, implementation of randomisation or blinding of participants and/or Amsel outcome assessors were provided (Figure 14). One week after the start of treatment (i.e. immediately post-treatment), all women in both groups had ≤2 of 3 Amsel criteria (Table 23); 77% (n=24/31) of women receiving Lactal and 76% (n=13/17) of women receiving metronidazole were negative for all criteria assessed (positive amine test, clue cells, pH≥5.0). No adverse events were reported (Supplementary Table D4).

Table 23 - Key findings of included studies

Objective 1: Studies assessing BV cure

Reference	Study design	Intervention	Comparator	No. women randomised	Outcome measure	Duration of follow-up	BV cure results in intervention vs comparator
Andersch *et al.* (1986)	RCT[a]	Lactal gel 5ml PV/night x 7 nights	Oral MTZ 500 mg *BID* x 7 days	Lactal= 32 MTZ= 22	≤2 of 3 Amsel criteria[b]	1 week after start of treatment	31/31 (100%, 95% CI 89, 100) *vs* 17/17 (100%, 95% CI 90, 100)
Boeke *et al.* (1993)	RCT[a]	Oral placebo *BID* x 7 days and lactic acid vaginal suppository/night x 7 days	Two comparator groups: 1) Oral MTZ 500 mg *BID* x 7 days and placebo vaginal suppository/night x 7 days 2) Oral placebo *BID* x 7 days and placebo vaginal suppository/night x 7 days	Lactic acid= 41[c] MTZ= 44 Placebo= 40	≤2 of 4 Amsel criteria	2 weeks after start of treatment	18/37 (49%, 95% CI 32, 66) *vs* 35/42 (83%, 95% CI 69, 93) 16/34 (47%, 95% CI 30, 65)
						4 weeks after start of treatment	11/33 (33%, 95% CI 18, 52) *vs* 27/38 (71%, 95% CI 54, 85) 12/35 (34%, 95% CI 19, 52)
						3 months after start of treatment	12/32 (38%, 95% CI 21, 56) *vs* 29/37 (78%, 95% CI 62, 90) 11/32 (34%, 95% CI 19, 53)
Simoes *et al.* (2006)	Double-blind RCT	Acidform gel 5g PV/day x 5 days	10% MTZ gel PV/day x 5 days	Acidform= 13 MTZ= 17	≤2 of 4 Amsel criteria	12-17 days after start of treatment	3/13 (23%, 95% CI 5, 54) *vs* 15/17 (88%, 95% CI 64, 99)
						33-40 days after start of treatment	1/13 (8%, 95% CI 0, 36) *vs* 9/17 (53%, 95% CI 28, 77)

Fredstorp *et al.* (2015)	Open-label RCT	Two intervention groups: 1) OMLA pessary applied once/week for 1 week 2) OMLA pessary applied twice/week for 1 week[d]	Untreated control group	Once/week= 37 Twice/week= 35 Control= 33	≤2 of 4 Amsel criteria	1 week after start of treatment	24/34 (71%, 95% CI 53, 85) 28/35 (80%, 95% CI 63, 92) *vs* 3/30 (10%, 95% CI 2, 27)

Objective 2: Studies assessing vaginal microbiota composition

Reference	**Study design**	**Intervention**	**Comparator**	**No. women randomised**	**Outcome measure**	**Reported results**
Keller *et al.* (2012)	Single-blind RCT	Acidform gel 5g PV *BID* x 14 days	HEC placebo gel PV *BID* x 14 days	Acidform= 18 Placebo= 18	qPCR assays: *L. crispatus* *L. jensenii* *G. vaginalis* *Megasphaera* (type 1 & type 2) BVAB2	In 35[e] women without BV, no significant changes were observed in the prevalence or concentration of *L. crispatus, L. jensenii, Megasphaera* (type 1 & type 2) or BVAB2 following 14 days of gel use in either the Acidform or placebo group (compared to baseline values). There was a non-significant trend towards a decrease in *G. vaginalis* concentration in the Acidform group following 14 days of gel use compared to baseline (median of $1.36x10^6$ to $3.66x10^4$ DNA copies/swab, p = 0.083), but not in the placebo group (median of $9.8x10^5$ to

						$4.4x10^6$ DNA copies/swab, p-value not reported).
Gottschick *et al.* (2017a)[f]	Double-blind RCT	Oral MTZ 2g single dose. After 7-28 days, WO3191 pessary applied twice-weekly x 3 weeks	Oral MTZ 2g single dose. After 7-28 days, Vagisan® pessary applied twice-weekly x 3 weeks	WO3191= 18 Vagisan®= 26	16S rRNA gene sequencing of V1-V2 regions	In 36[g] women initially treated for BV with oral metronidazole, no significant changes in vaginal microbiota composition were reported during or following use of either WO3191 or Vagisan®. The cumulative relative abundance of *Lactobacillus* spp. (*L. crispatus*, *L. iners* and *L. gasseri*) was 73% in the WO3191 group prior to starting pessary use, 77% after 3 weeks of pessary use, and had decreased to 59% 12-14 weeks after last pessary use. The cumulative relative abundance of *Lactobacillus* spp. was 75% in the Vagisan® group prior to starting pessary use, 69% after 3 weeks of pessary use, and 73% 12-14 weeks after last pessary use. There was a non-significant increase in the relative abundance of *L. crispatus* in Vagisan® group from 18% prior to starting pessary use to 33% 12-14 weeks after last pessary use. There was no difference in microbiota diversity (as measured by Shannon diversity index)

						between women randomised to WO3191 and women randomised to Vagisan®.
van der Veer *et al.* (2019)	Single arm prospective cohort	Participants were followed for 3 menstrual cycles. Etos® douche was applied 3/per week for duration of cycle 2 starting on day 1 of menses.	NA	29	16S rRNA gene sequencing of V3-V4 regions	In 25[h] women without BV there was a non-statistically significant increased odds of having a diverse anaerobic vaginal microbiota relative to an *L. crispatus* microbiota during (odds ratio: 1.4; 95% CI 0.9, 2.1) and after douching with Etos® (odds ratio 1.7, 95% CI 0.9, 3.1), compared to before douching, following adjustment for menses. Douching with Etos® had no effect on microbiota diversity as measured by Shannon diversity index.

No., number; BV, bacterial vaginosis; RCT, randomised controlled trial; PV, intravaginal; MTZ, metronidazole; BID, twice a day; OMLA, oligometric lactic acid; CI, confidence interval; qPCR, quantitative PCR; NA, not applicable

[a] Details of blinding not provided.

[b] The three criteria evaluated were: positive amine test, clue cells, pH≥5.0

[c] 168 women randomised, but post-randomisation, 43 women were found to be ineligible and excluded, thus randomisation numbers presented reflect the 125 eligible women included in analyses.

[d] OMLA pessary is designed to release lactic acid over a 72hr period

[e] One woman allocated to Acidform did not receive the intervention

[f] Both the intervention (WO3191) and the comparator (Vagisan®) contain lactic acid.

[g] 36 women were included in microbiota analyses, n=13 receiving WO3191 and n=23 receiving Vagisan®

[h] Twenty-nine women were recruited, four were excluded and 25 women completed the study.

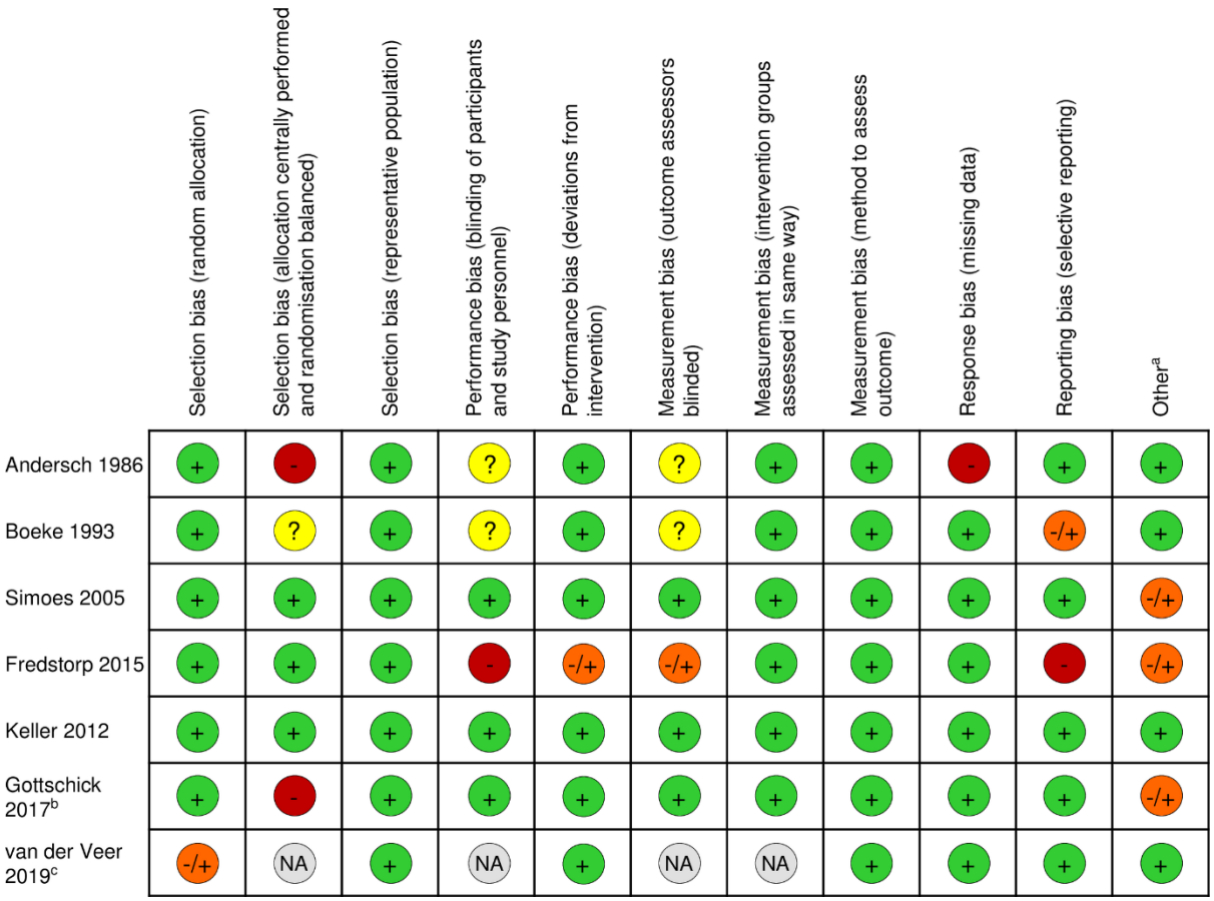


Figure 14. Risk of bias assessment

+ indicates a low risk of bias, -/+ indicates moderate risk of bias, - indicates high risk of bias, ? indicates unknown risk, NA indicates bias is not applicable to the study

[a] Other sources of bias include whether confounders were appropriately accounted for and whether lactic acid-containing product details were sufficiently described in the manuscript

[b] One study disclosed receipt of funding from the lactic acid-containing product manufacturer.

[c] Single arm prospective cohort study

In a multicentre RCT, Boeke *et al.* (1993) randomised women to receive either nightly lactic acid pessary (100mg lactic acid/pessary) plus oral placebo *BID* for seven days, oral metronidazole *BID* plus nightly placebo pessary for seven days, or oral placebo *BID* plus nightly placebo pessary for seven days. No details of allocation concealment or blinding were provided (Figure 14). Cure was assessed at three time points (2-weeks, 4-weeks and 3-months after the start of treatment) using ≤2 of 4 Amsel criteria and an additional author definition called 'strict' cure (absence of: positive amine test, clue cells, pH>4.5). Two weeks

after the start of treatment, 49% (n=18/37) of women receiving lactic acid, 83% (n=35/42) of women receiving metronidazole and 47% (n=16/34) of women receiving dual placebo had ≤2 Amsel criteria (Table 23). When assessed according to the authors definition of strict cure, none of the women receiving lactic acid pessary, 10% of women receiving metronidazole and 3% of women receiving dual placebo were cured. Similar findings were reported 4 weeks and 3-months after start of treatment (Table 23). There was no difference in adverse events reported between the three randomisation groups. Of 33 women receiving lactic acid, four reported gastrointestinal symptoms, three reported genital irritation and one reported headache/vertigo (Supplementary Table D4).

In a double-blind pilot RCT, Simoes *et al.* (2006) randomised women to receive either once daily Acidform gel (an acid buffering contraceptive gel, 88mg lactic acid/5g) for five days (n=13) or once daily 10% metronidazole intravaginal gel for five days (n=17). Randomisation was performed by the product manufacturer and researchers were provided with product tubes labelled with participant numbers so that the randomisation group was double-blinded to the researchers and participants (Figure 14). At 7-12 days post-treatment, 23% (n=3/13) of women receiving Acidform and 88% (n=15/17) women receiving metronidazole were cured (≤2 Amsel criteria). At 28-35 days post-treatment, the percent of women cured decreased to 8% (n=1/13) in the Acidform group and 53% (n=9/17) in the metronidazole group (Table 23). Four women receiving Acidform and one woman receiving metronidazole reported genital irritation (Supplementary Table D4).

Fredstorp *et al.* (2015) evaluated a sustained release oligomeric lactic acid (OMLA) pessary in a two part multicentre study. Part A of the study is not included in this review as there was no control group. In Part B, women were randomised to receive either OMLA pessary applied once per week for one week, OMLA pessary applied twice per week for a week or no treatment. Block randomisation was performed according to a computer-generated randomisation list, with block size blinded to the investigators. Sites were provided with coded envelopes, and the study was open-label (Figure 14). After one week of pessary use, 71% (n=24/34) of women receiving once-weekly pessary, 80% (n=28/35) of women receiving twice-weekly pessary and 10% (n=3/30) of women receiving no treatment had ≤2 of 3 Amsel criteria (Table 23). Vaginal itching was the most common adverse event and was reported by 11 women receiving OMLA pessary, by five applying the pessary once/week and

by six applying the pessary twice/week. Two women receiving OMLA pessary had a yeast infection, and vaginal irritation and genital burning sensation were both reported by >1 woman (exact numbers and group not provided; Supplementary Table D4). Adverse events were not recorded from control participants.

6.5.4. Impact of intravaginal lactic acid-containing products on the vaginal microbiota composition

Three studies reported a measure of vaginal microbiota composition (Table 23) (Gottschick *et al.*, 2017a; Keller *et al.*, 2012; van der Veer *et al.*, 2019).

Keller *et al.* (2012) evaluated the safety of Acidform gel *BID* (88mg lactic acid/5g) compared to HEC placebo gel *BID* in 35 sexually abstinent nonpregnant women without BV. Women were randomised 1:1 by a pharmacist. Though the treatments were not identical in appearance, participants were not informed of their allocation and laboratory personnel assessing the outcome were blinded (Fig 2). The change in prevalence and concentration of five bacteria after 14 days of gel use was assessed by qPCR. There were no significant changes in vaginal microbiota composition following 14 days of either Acidform or placebo (Table 23). A non-significant trend towards decreased *Gardnerella vaginalis* concentration following Acidform use was reported. Five women receiving Acidform reported vulvar itching, four reported vaginal or vulvar burning and three reported abdominal cramping (Supplementary Table D4). Two women receiving placebo reported vaginal or vulvar itching.

Gottschick *et al.* (2017a) evaluated the safety, tolerability and efficacy of a biofilm disrupting agent (cocoamphopropionate) administered as a pessary (WO3191, which contains lactic acid at 3.9% of total pessary weight) in a double-blind RCT. Forty-four nonpregnant women were randomised to receive either WO3191 or Vagisan® (40mg lactic acid/pessary) 7-28 days after treatment for BV with 2g single dose oral metronidazole (Table 23). No details of randomisation or allocation concealment were provided (Figure 14). Microbiota results (assessed by 16S rRNA gene sequencing) were reported for 36 women (WO3191 n=13 and Vagisan® n=23). No significant changes in vaginal microbiota composition were observed during or following use of either pessary. No safety concerns were identified for either pessary (Supplementary Table D4).

In an open-label non-comparative pilot study, van der Veer *et al.* (2019) evaluated the impact of a lactic acid-containing douche (Etos®, 0.06% lactic acid when diluted for use) on the vaginal microbiota composition of 25 nonpregnant reproductive aged women without BV (Table 23). Etos® did not significantly impact the vaginal microbiota composition (assessed by 16S rRNA gene sequencing). The study reported non-significant increased odds for having a diverse anaerobic vaginal microbiota during and after douching with Etos®, following adjustment for menses. Five women reported dryness and 2 reported an increase in vaginal symptoms post douching (Supplementary Table D4).

6.5.5. Adverse events

No major safety concerns were reported (Supplementary Table D4). Vaginal or vulvar irritation, itching, burning, redness and/or dryness were recorded in women receiving a lactic acid-containing product in five of the seven studies. Minimal differences in adverse events between lactic acid-containing product and control randomisation groups were reported.

6.5.6. Risk of bias of included studies

Risk of bias assessment is in Figure 14. Only one RCT evaluating a lactic acid-containing product for BV cure was double-blinded (Simoes *et al.*, 2006) and only one study had low bias across all six domains (Keller *et al.*, 2012).

Two studies assessing BV cure reported sample size calculations (Boeke *et al.*, 1993; Fredstorp *et al.*, 2015) and one reached the required sample size (Fredstorp *et al.*, 2015). Four studies measured treatment adherence; one study reported these results (Keller *et al.*, 2012). An additional study reported comparable treatment adherence across intervention groups, but did not provide raw data (Boeke *et al.*, 1993).

6.6. Discussion

The efficacy of lactic acid-containing products for BV cure and their impact on the vaginal microbiota composition has not been extensively evaluated. We identified four RCTs that investigated the use of intravaginal lactic acid-containing products for BV cure and three studies that investigated the impact of lactic acid-containing products on the vaginal

microbiota. Most studies were small and underpowered, had medium-high risk of bias, and the time-point at which cure was measured differed between studies. Three studies compared a lactic acid-containing product to a first-line BV treatment: one reported lactic acid to have equivalent efficacy to metronidazole and two reported lactic acid to be inferior to metronidazole. Two studies included a placebo or no treatment control group: one reported lactic acid to be superior to no treatment and the other reported lactic acid to be equivalent to placebo. Minimal effects of lactic acid-containing products on the vaginal microbiota were reported. New treatments are needed to improve BV cure and the use of lactic acid is supported by *in vitro* evidence (O'Hanlon *et al.*, 2011; Tachedjian *et al.*, 2017). However, there is limited high-quality *in vivo* evidence that supports the use of lactic acid for BV cure or modulating the vaginal microbiota. Large rigorous trials of well evaluated lactic acid-containing products with long-term follow-up and accompanying microbiota data are needed.

The lactic acid-containing products assessed varied with respect to lactic acid concentration, pH, formulation (i.e. gel, pessary, douche) and excipients. Women with lactobacillus-dominated vaginal microbiota (defined as Nugent score=0-3) have an average vaginal lactic acid concentration of approximately 0.79-1% and pH of 3.45-4.12 (O'Hanlon *et al.*, 2019; O'Hanlon *et al.*, 2013). Some products had a lactic acid concentration or pH outside of these ranges, and no study reported the concentration of lactic acid released into the vagina. Thus, it is not clear if biologically active levels of lactic acid were achieved, which may have impacted on treatment efficacy. Functional effects of lactic acid *in vitro* are usually observed within concentration ranges of 0.30-1% and pH of 3.45-4.12 (Aldunate *et al.*, 2013; O'Hanlon *et al.*, 2019; O'Hanlon *et al.*, 2013), and are mediated by the uncharged protonated form of lactic acid which predominates at pH≤3.86 (Aldunate *et al.*, 2013; Hearps *et al.*, 2017; O'Hanlon *et al.*, 2011). Accordingly, biological effects diminish as lactic acid levels decrease and pH increases. For example, at <0.3% lactic acid and pH≥4.2, the HIV virucidal activity (Aldunate *et al.*, 2013) and immunomodulatory effects (Hearps *et al.*, 2017) of lactic acid decrease. Additionally, while 1% lactic acid at pH 4.5 reduces the viability of BV-associated bacteria approximately 10^6-fold, a negligible reduction is observed with 0.1% lactic acid (O'Hanlon *et al.*, 2011). The lactic acid concentration and vaginal pH maintained after dosing are likely to be critical for achieving biological effects *in vivo*.

Other important characteristics of lactic acid-containing products need consideration, including lactic acid isomer and product osmolality. Lactic acid exists in two isomers: D- and L-lactic acid, and *Lactobacillus* spp. differ in their ability to produce each isomer. For example, *in vitro*, *L. crispatus* and *L. gasseri* produce both isomers, *L. jensenii* produces only D-lactic acid and *L. iners* produces only L-lactic acid (Witkin *et al.*, 2013). The protective effects of *L. crispatus* compared to *L. iners* are partly attributed to the ability of *L. crispatus* to produce D-lactic acid (Tachedjian *et al.*, 2017). It is hypothesised that D-lactic acid affords more protection than L-lactic acid against upper genital tract infections (Witkin *et al.*, 2013); however, this has not been studied in the context of BV. Isomer information was available for one product included in this review. In order to understand the relative contribution of each isomer to the inactivation of BV-associated bacteria, future studies of products under evaluation for BV treatment or vaginal microbiota modulation should report the L-/D-isomer ratio. Additionally, no study reported product osmolality. This is relevant because hyperosmolal products are likely to damage vaginal epithelium (Ayehunie *et al.*, 2018; Wilkinson *et al.*, 2019). Vaginal and vulvar irritation were commonly reported adverse events in women using lactic acid-containing products, and may be related to product osmolality and/or excipients or other ingredients (e.g. citric acid). Adverse events should be monitored following intravaginal lactic acid use.

Minimal changes in vaginal microbiota composition following lactic acid-containing product use were reported. Two of the three studies evaluating microbiota composition recruited women without BV and the third study assessed women recently treated with oral metronidazole. Thus, one might expect the impact of lactic acid on the vaginal microbiota composition of these women to be minimal. The non-significant association of Etos® douche with non-optimal vaginal microbiota composition (van der Veer *et al.*, 2017) may be a result of the douching action rather than an adverse impact of lactic acid, highlighting the importance of product formulation. Douching has been associated with increased risk of BV-associated bacteria detection (Sabo *et al.*, 2019), as well as increased risk of intermediate-BV and Nugent-BV by meta-analysis (Low *et al.*, 2011). However, whether douching introduces BV-associated bacteria, depletes optimal lactobacilli, or modifies the vaginal environment such that BV-associated bacteria growth is favoured is unknown.

This review has limitations. The 2019 Food and Drug Administration (FDA) guidelines for developing BV treatments recommends that clinical cure be defined as the absence of 3 Amsel criteria, specifically resolution of vaginal discharge, a negative whiff test and clue cells <20% per high-power field on wet mount (Food Drug Administration Center for Drugs Evaluation Research, 2019). In clinical practice, BV is typically diagnosed as the presence of ≥3 Amsel criteria (Workowski & Bolan, 2015), recurrence is defined as the presence of ≥3 criteria (Sobel *et al.*, 2019) and cure is reported as ≤2 criteria. Based on international clinical practice and published studies, we defined BV cure as the presence of ≤2 Amsel criteria (and/or Nugent score<4, although no included study reported cure using Nugent score) and not by the 2019 FDA guidelines. Additionally, only two studies assessed cure at a timepoint recommended by FDA guidelines (Boeke *et al.*, 1993; Simoes *et al.*, 2006). The FDA guidelines recommend cure be assessed 7-14 days post-randomisation for topical drugs administered for a short period of time (i.e. 1-2 days) or 21-30 days post-randomisation for topical drugs that that are administered for a longer period of time (i.e. 1 week) (Food Drug Administration Center for Drugs Evaluation Research, 2019). Follow-up was limited to immediately post-treatment in two studies (Andersch *et al.*, 1986; Fredstorp *et al.*, 2015), which is not only likely to be too soon after treatment cessation to adequately assess cure, it also prevented our assessment of the long-term efficacy and safety of lactic acid-containing products. If lactic acid is effective it is likely to be most effective when used as adjunctive therapy with antibiotics (Tachedjian *et al.*, 2017) and/or when used as sustained release or as periodic presumptive therapy, as has been shown with biweekly suppressive use of 0.75% metronidazole gel (Sobel *et al.*, 2006). Finally, our search was restricted to English-language records which excluded at least one study (Andreeva *et al.*, 2002).

Other lactic acid-containing products are available over-the-counter but were either not identified through our systematic search of published literature or were ineligible for inclusion in our review. An RCT of 1, 900 women comparing the clinical and cost effectiveness of intravaginal lactic acid gel to oral metronidazole for BV is currently ongoing (Armstrong-Buisseret *et al.*, 2019)(ISRCTN14161293). The primary outcome is patient reported resolution of BV symptoms 14-days post-randomisation. Initial qualitative data from ISRCTN14161293 indicates women prefer lactic acid gel to antibiotics for mild BV

episodes despite lower perceived efficacy (Anstey Watkins *et al.*, 2019), supporting the need to further investigate lactic acid-containing products for BV.

6.7. Conclusions

New treatments are needed to improve BV cure, reduce associated sequelae and improve antibiotic stewardship. *In vitro* data suggest that lactic acid may be effective for BV treatment; however, high-quality evidence supporting the use of lactic acid-containing products for BV and modification of the vaginal microbiota is lacking. Large, rigorous randomised trials of lactic acid-containing products that have been carefully evaluated with respect to pH, lactic acid concentration, L-/D-isomer ratio and osmolality are needed. Future studies should include standardised clinical endpoints, standardised timing of endpoint measurement, assessment of adverse events, long-term follow-up of participants and accompanying high-resolution vaginal microbiota data.

Chapter 7. Integrated discussion, implications for clinical practice and areas for future research

Bacterial vaginosis is the commonest vaginal condition among reproductive aged women. Recommended treatment for BV involves a multidose course of either metronidazole or clindamycin. Although both antibiotics are effective in the short term, recurrence following treatment is unacceptably high. Not only is BV associated with serious and costly obstetric and gynaecological sequelae, but post-treatment recurrences place a heavy burden on the healthcare system as they result in multiple presentations to clinical services and repeated antibiotic use. Recurrences are also highly distressing for the affected women and have detrimental effects on self-esteem, sexual relationships, and overall quality of life. We clearly need better treatments for BV, but there has been little progress made in improving the long-term cure of this common condition. There are significant challenges to improving BV cure; the aetiology of BV is unknown and the event/s that initiate BV and drive treatment failure are not well understood and are likely to be multifactorial. This thesis aimed to investigate the pathogenesis of BV with a focus on the role of sexual transmission, as well as investigate how different factors influence and modify the vaginal microbiota composition. This final chapter will summarise the aims and findings from this thesis, as well as outline implications for clinical practice and areas for future research.

Chapter 2 aimed to evaluate factors associated with the composition, diversity, and stability of the vaginal microbiota in a cohort of Australian WSW. Factors evaluated included history of BV, behavioural practices such as smoking and douching, as well as specific sexual practices. The key findings from this study were that in adjusted analyses, sex with a new partner was associated with significant increases in the bacterial diversity and instability of the vaginal microbiota. Furthermore, women who reported a new sexual partner were 3.5-fold more likely to have a non-optimal vaginal microbiota composition (i.e., one dominated by *G. vaginalis* or anaerobic and diverse bacteria) than an optimal vaginal microbiota dominated by *L. crispatus*, compared to women who were either not having sex or having sex in an established relationship.

These data provide microbiological evidence for the sexual transmission of BV-associated bacteria between women and support a role of sexual transmission in the acquisition of BV.

We did not assess the vaginal microbiota of sexual partners; therefore the evidence relating to sexual transmission is indirect. However, the microbiological data presented in **Chapter 2** is consistent with epidemiological findings from the WOW Health Cohort study (Vodstrcil *et al.*, 2015) and another longitudinal study of women with female partners (Marrazzo *et al.*, 2010b) that showed that having a sexual partner with a history of BV, BV symptoms or microbiologically confirmed BV significantly increased the risk of BV acquisition.

Chapter 3, which was closely linked to **Chapter 2** and examined the same cohort of women, aimed to describe the distribution of *G. vaginalis* clades in a cohort of WSW and investigate factors associated with *G. vaginalis*-clade distribution. Factors evaluated included history of BV, behavioural and sexual practices, Nugent score and vaginal microbiota composition. The key findings from this study were that 1) clade 1 and multi-clade communities were associated with both Nugent-BV and non-optimal (i.e. *Lactobacillus*-deficient) vaginal microbiota, 2) clade 4 was neither associated with Nugent-BV or non-optimal vaginal microbiota composition, 3) individual clades were associated with differing sexual practices.

As discussed in Section 1.6.1.1 of the literature review, our understanding of the genetic diversity of the *Gardnerella* genus continues to grow. At the time this PhD commenced in 2018, *G. vaginalis* was the only species in the *Gardnerella* genus. Between 2019 and 2021, an emended description of *G. vaginalis* was proposed and an additional 13 species have been identified: three have been named (*G. leopoldii, G. piotii* and *G. swidsinskii*) and ten currently remain undefined (Putonti *et al.*, 2021; Vaneechoutte *et al.*, 2019). Although the four-clade nomenclature does not distinguish all 14 *Gardnerella* species (see Table 3 in Section 1.6.1.1), the findings from **Chapter 3** are highly relevant. The findings presented add to our understanding of the role of *Gardnerella* spp. in BV and how *Gardnerella* spp. are acquired. Our findings suggest that *Gardnerella* spp. are acquired sexually via a range of different practices and that different *Gardnerella* spp. may have varying levels of pathogenicity.

Together, **Chapters 2** and **3** provide strong microbiological evidence to support the sexual exchange of BV-associated bacteria between women and suggest that sexual transmission of BV-associated bacteria plays an important role in the development of non-optimal vaginal microbiota and the pathogenesis of BV. These findings have implications for clinical practice as they indicate that preventative and treatment approaches that address sexual

transmission (and re-infection from a sexual partner) should be investigated as potential strategies for preventing BV acquisition and improving long term BV cure. One such approach is concurrent partner treatment.

Partner treatment trials have failed to consistently improve long-term BV cure and there are clear challenges with conducting partner treatment trials. In **Chapter 4** we presented a pilot study of concurrent male partner treatment for BV that had the primary purpose of informing the design of a male partner RCT. Several key findings arose from this study. Firstly, we found that concurrent male partner treatment (utilising combined oral and topical antibiotic treatment for men) significantly altered the composition of the vaginal, cutaneous penile and male urethral microbiota. Specifically, we observed a reduction in the abundance of BV-associated bacteria immediately post-treatment at all three genital sites. The reduction of BV-associated bacteria was sustained in most women during the 12-week follow-up period, and there were few cases of BV-recurrence during follow-up (n=5/29; 17%; 95%CI: 6-34%). Although our study was exploratory and not powered for an efficacy outcome, our finding of 17% recurrence is particularly encouraging because women had multiple risk factors for BV-recurrence. As discussed in **Chapter 4**, we would expect recurrence rates that exceed 50% in this population of women who were at high risk for recurrence (Bradshaw *et al.*, 2006a; Bradshaw *et al.*, 2013a; Schwebke *et al.*, 2021; Vodstrcil *et al.*, 2019). Another key finding from this chapter was that most of the men we approached agreed to receive partner treatment. Among these men, adherence was high and both oral and topical therapies were well tolerated. Together these data provided us with evidence to proceed to a large RCT of combined oral and topical male partner treatment for BV (ANZCTR: ACTRN12619000196145 (Vodstrcil *et al.*, 2020)). Our research group commenced recruitment to this RCT in 2019.

Importantly, partner treatment trials are needed in other populations, including WSW. Concordance of BV is high between female partners, and this represents a key challenge of conducting a gold standard RCT of female partner treatment. BV is common among WSW (Berger *et al.*, 1995; Bradshaw *et al.*, 2014; Evans *et al.*, 2007; Marrazzo *et al.*, 2002; Vodstrcil *et al.*, 2015), and data presented in **Chapters 2** and **3** strongly support the sexual exchange of BV-associated bacteria between women. Therefore, pragmatic studies that evaluate female partner treatment for BV should be conducted.

Importantly, **Chapter 4** identified key factors that are likely to influence the 'real world' success of partner treatment if it is shown to be effective for reducing BV recurrence in a randomised setting. A number of women we approached declined to participate because they felt that their partner would not be interested in taking part, or because they did not want to discuss BV or the study with him. A recent qualitative study of men participating in partner treatment trials for BV found that most men had very little knowledge about BV (Wigan *et al.*, 2020). Additionally, most men reported that beyond their concern for their partner, BV did not directly impact them due in part to the absence of symptoms (Wigan *et al.*, 2020). This highlights the need for clinicians to support and help women to discuss BV and partner treatment with their sexual partners. It also highlights that more education around male carriage of BV-associated bacteria is needed to effectively engage couples in partner treatment. Although adherence to treatment was high in our study, male adherence to topical treatment was lower than male adherence to oral treatment. As discussed in **Chapter 4**, this may indicate a preference among men for oral therapy over topical therapy. This further highlights that strategies to engage male partners and optimise adherence to partner treatment are also needed, and this may be particularly challenging in men as they rarely experience symptoms. Therefore, the acceptability and efficacy of alternative antimicrobials for partner treatment should continue to be investigated.

Chapter 5 aimed to determine the prevalence of *M. hominis, U. urealyticum* and *U. parvum* in 1,272 nonpregnant women attending the Melbourne Sexual Health Centre and investigated their association with specific symptoms and clinical signs. *M. hominis* was detected in 29% (95% CI 27-32) of women, *U. urealyticum* was detected in 32% (95% CI 29-34) and *U. parvum* was detected in 72% (95% CI 69-74). We found that in adjusted analyses, the detection of *M. hominis* and *Ureaplasma* spp. was associated with markers of sexual exposure, which supports previous research that the acquisition of these bacteria in adults occurs primarily via sexual activity. After adjusting for concurrent STIs and VVC, we found that *M. hominis* was associated with the presence of specific clinical signs and symptoms including abnormal vaginal discharge, vaginal malodour, vaginal pH>4.5 and presence of clue cells. Conversely, the *Ureaplasma* spp. were not associated with any symptom or sign in adjusted analysis. Additionally, *M. hominis* independently increased the odds of BV by 8-fold, whereas the ureaplamas were not associated with an increased odds of BV. Due to the

relationship between *M. hominis* and BV we conducted a stratified analysis and found that *M. hominis* was associated with vaginal malodour and vaginal discharge in women with BV, but was not associated with symptoms or signs in women without BV. This suggests that while *M. hominis* is one of the spectrum of organisms that is associated with BV, it may not independently contribute to genital symptoms and clinical signs in non-pregnant women.

These findings have significant implications for clinical practice. They highlight that routine screening and testing for *M. hominis*, *U. urealyticum* and *U. parvum* in nonpregnant women is not indicated in a sexual health setting. Further, our findings suggest that in a sexual health setting there may be more value in directing resources towards the diagnosis of BV, rather than testing for *M. hominis* and the ureaplasmas on their own. Our findings also have implications for antimicrobial stewardship. The routine reporting of *M. hominis*, *U. urealyticum* and *U. parvum* during STI screening and testing creates confusion around the need for treatment, particularly in primary care settings. As outlined in a position statement from the European STI guidelines Editorial Board, there are no international evidence-based guidelines for the management of *M. hominis* and *Ureaplasma* spp. (Horner *et al.*, 2018). Furthermore, there is a lack of evidence for effective treatment regimens for these organisms (Horner *et al.*, 2018), antimicrobial treatment may lead to selection of antimicrobial resistance among STIs, and other members of the microbiota including BV-associated bacteria. Discouraging the routine screening and testing of *M. hominis* and *Ureaplasma* spp. may prevent widespread antibiotic use for these organisms and lead to improvements in antimicrobial stewardship.

A novel aspect and strength of the study presented in **Chapter 3** is that it investigated the association between *Gardnerella* clades and vaginal microbiota composition (characterised using 16S rRNA gene sequencing). This is important because bacteria do not exist in isolation in the vagina, and associations between individual organisms and clinical outcomes (for example BV) may be confounded by the presence or absence of other inhabitants in the vagina. This was further highlighted in **Chapter 5** by the relationship between *M. hominis* and BV. Future studies that evaluate the role of individual organisms in BV pathogenesis (or their role in causing symptoms/clinical signs) should consider their interaction with other inhabitants of the vaginal microbiota. Studies are currently underway to explore the relationship between the vaginal microbiota composition, *M. hominis* and *Ureaplasma* spp.

and symptoms/clinical signs. Additionally, studies that quantitate the load of *M. hominis* and *Ureaplasma* spp. following treatment for BV may provide further insight into the relationship between BV and the common genital Mollicutes.

In addition to the key findings related to sexual practices, both **Chapters 2** and **3** identified additional factors that were associated with features of the vaginal microbiota and/or *G. vaginalis* clade distribution. In adjusted analyses in **Chapter 2**, smoking was associated with characteristics of a non-optimal vaginal microbiota, including increased bacterial diversity, increased vaginal microbiota instability, and having a vaginal microbiota composition dominated by *G. vaginalis* or anaerobic and diverse bacteria. In **Chapter 3**, smoking was associated with detection of multiple clades of *G. vaginalis* and detection of *G. vaginalis* clade 1, both of which were also associated with non-optimal vaginal microbiota composition and Nugent-BV. In **Chapter 2** we also reported that a history of BV was associated with increased bacterial diversity and non-optimal vaginal microbiota composition. Additionally, we found that *L. crispatus* dominated vaginal microbiota was more stable longitudinally compared to vaginal microbiota compositions that were dominated by *L. iners* or non-optimal bacteria. Collectively, these findings highlight that a range of different factors and behavioural practicess can influence the composition and stability of the vaginal microbiota. This reinforces that the pathogenesis of incident and recurrent BV is complex and likely to be multifactorial. This is important in the context of identifying, developing and evaluating preventative and treatment strategies for BV as it highlights that a one-size fits all approach is unlikely to be successful for all women. Indeed, five of the 29 women who participated in our partner treatment pilot study (**Chapter 4**) experienced BV recurrence, and although self-report is subject to bias, all five women reported 100% adherence to treatment, as did their male partners. Investigations of alternative treatment approaches that address persistence of BV-associated bacteria (i.e. alternative antibiotics, biofilm disrupters) and failure to recolonise with beneficial *Lactobacillus* spp. (i.e. probiotics, acidifying agents) are needed (Unemo *et al.*, 2017).

In **Chapter 6** we systematically reviewed the use of intravaginal lactic acid-containing products for BV cure and modifying the vaginal microbiota composition. Our systematic review evaluated seven different lactic acid-containing products across seven studies; four studies evaluated the effect of lactic acid-containing products on BV cure and three

evaluated their impact on the vaginal microbiota. The products evaluated varied with respect to formulation, included excipients, duration of use, lactic acid concentration and pH. The systematic review concluded that there is a lack of high-quality *in vivo* evidence to support the use of lactic acid for BV treatment and modification of the vaginal microbiota. Key recommendations from **Chapter 6** were that future trials that evaluate lactic acid as BV treatment strategy need to be adequately powered, evaluate products that have been carefully evaluated *in vitro*, use standardised endpoints, and include long-term follow-up, monitoring of adverse events, and high-resolution vaginal microbiota data. These recommendations should be applied when evaluating any new intervention for BV. Accompanying microbiota data may be particularly useful when evaluating the efficacy of new treatments, as a recent research indicates that the pre-treatment composition of the vaginal microbiota may influence treatment outcomes (Lee *et al.*, 2020; Verwijs *et al.*, 2019). An additional recommendation that wasn't discussed in **Chapter 6** is that where possible, future studies that evaluate BV treatments should control for (or conduct secondary analyses of) confounding factors that may influence BV cure or microbiota composition, including sexual practices, contraceptive practices, and past BV.

The studies presented in **Chapters 2, 3, 4** and **5** each complemented molecular data with a thorough epidemiological analysis that assessed meaningful outcomes and controlled for confounding factors where the sample size allowed. A key strength of this thesis is that it highlights the value of integrating microbiota data with comprehensive clinical and behavioural data. Through the work conducted during this thesis, our research group now has a standardised protocol that is used for processing 16S rRNA gene sequencing data.

Microbiota studies utilising 16S rRNA gene amplicon sequencing provide a cost-effective way to survey the microbiota composition of many samples. However, there are well known limitations with 16S rRNA gene sequencing. The limitations that are perhaps most relevant to this thesis are 1) the data generated are compositional (Gloor *et al.*, 2017) and provide a measure of relative abundance not organism load (Tettamanti Boshier *et al.*, 2020), and 2) limited resolution beyond the genus level. Studies that precisely quantitate the load of key BV-associated bacteria in women and their sexual partners are needed to understand if concurrent partner treatment eradicates these organisms from both partners. Additionally, studies that incorporate rapid sampling of couples following partner treatment are needed

to provide further insight into when these organisms come back in men (and women), and how the genital microbiota is shared between couples.

Chapter 4 highlighted several additional areas for future research. Similar to the evidence that pathogenic and commensal *Gardnerella* species may exist, it is possible that this is also true for other BV-associated bacteria, including those that we observed re-emerged in men post-treatment. Therefore, studies at the strain level are needed to better understand the concordance of the genital microbiota between sexual partners and the contribution of re-infection to BV recurrence. Ultimately, we don't know what represents an optimal genital microbiota in men, what factors influence the male genital microbiota, or what organisms in men may promote optimal outcomes in female partners. Future studies are needed to address these knowledge gaps.

7.1. Concluding remarks

Overall, this body of research adds to our understanding of what factors and behaviours influence the composition of the vaginal microbiota and modify the vaginal microbiota to a non-optimal state. The findings presented in this thesis provide evidence that sexual transmission has a key role in the pathogenesis of BV acquisition and recurrence, and that preventative and treatment approaches that address sexual transmission should continue to be explored. Importantly, this thesis highlights that several different factors can influence the vaginal microbiota composition, and that treatment approaches, in addition to those that address sexual transmission, will likely be needed to achieve sustained BV cure and promote ongoing maintenance of an optimal vaginal microbiota composition.

References

Abele-Horn, M., Scholz, M., Wolff, C., & Kolben, M. (2000). High-density vaginal *Ureaplasma urealyticum* colonization as a risk factor for chorioamnionitis and preterm delivery. *Acta Obstetricia et Gynecologica Scandinavica, 79*(11), 973-978.

Achilles, S. L., Austin, M. N., Meyn, L. A., Mhlanga, F., Chirenje, Z. M., & Hillier, S. L. (2018). Impact of contraceptive initiation on vaginal microbiota. *American Journal of Obstetrics and Gynecology, 218*(6), 622 e621-622 e610. doi:10.1016/j.ajog.2018.02.017

Adam, A., Pal, Z., Terhes, G., Szucs, M., Gabay, I. D., & Urban, E. (2018). Culture- and PCR-based detection of BV associated microbiological profile of the removed IUDs and correlation with the time period of IUD in place and the presence of the symptoms of genital tract infection. *Annals of Clinical Microbiology and Antimicrobials, 17*(1), 40. doi:10.1186/s12941-018-0293-6

Ahmed, A., Earl, J., Retchless, A., Hillier, S. L., Rabe, L. K., Cherpes, T. L., Powell, E., Janto, B., Eutsey, R., Hiller, N. L., Boissy, R., Dahlgren, M. E., Hall, B. G., Costerton, J. W., Post, J. C., Hu, F. Z., & Ehrlich, G. D. (2012). Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *Journal of Bacteriology, 194*(15), 3922-3937. doi:10.1128/JB.00056-12

Albert, A. Y., Chaban, B., Wagner, E. C., Schellenberg, J. J., Links, M. G., van Schalkwyk, J., Reid, G., Hemmingsen, S. M., Hill, J. E., Money, D., & Group, V. R. (2015). A study of the vaginal microbiome in healthy Canadian women utilizing cpn60-based molecular profiling reveals distinct *Gardnerella* subgroup community state types. *PloS One, 10*(8), e0135620. doi:10.1371/journal.pone.0135620

Aldunate, M., Srbinovski, D., Hearps, A. C., Latham, C. F., Ramsland, P. A., Gugasyan, R., Cone, R. A., & Tachedjian, G. (2015). Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. *Frontiers in Physiology, 6*, 164. doi:10.3389/fphys.2015.00164

Aldunate, M., Tyssen, D., Johnson, A., Zakir, T., Sonza, S., Moench, T., Cone, R., & Tachedjian, G. (2013). Vaginal concentrations of lactic acid potently inactivate HIV. *Journal of Antimicrobial Chemotherapy, 68*(9), 2015-2025. doi:10.1093/jac/dkt156

Althubaiti, A. (2016). Information bias in health research: definition, pitfalls, and adjustment methods. *J Multidiscip Healthc, 9*, 211-217. doi:10.2147/JMDH.S104807

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403-410. doi:10.1016/S0022-2836(05)80360-2

Alvarez-Sieiro, P., Montalban-Lopez, M., Mu, D., & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Applied Microbiology and Biotechnology, 100*(7), 2939-2951. doi:10.1007/s00253-016-7343-9

Alves, P., Castro, J., Sousa, C., Cereija, T. B., & Cerca, N. (2014). *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an in vitro biofilm formation model. *Journal of Infectious Diseases, 210*(4), 593-596. doi:10.1093/infdis/jiu131

Amabebe, E., & Anumba, D. O. C. (2018). The vaginal microenvironment: the physiologic role of lactobacilli. *Front Med (Lausanne), 5*, 181. doi:10.3389/fmed.2018.00181

Amaya-Guio, J., Viveros-Carreno, D. A., Sierra-Barrios, E. M., Martinez-Velasquez, M. Y., & Grillo-Ardila, C. F. (2016). Antibiotic treatment for the sexual partners of women with bacterial vaginosis. *Cochrane Database of Systematic Reviews, 10*, CD011701. doi:10.1002/14651858.CD011701.pub2

Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C., Eschenbach, D., & Holmes, K. K. (1983). Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *American Journal of Medicine, 74*(1), 14-22.

Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., Padavattan, N., Ismail, N., Moodley, A., Sabatini, M. E., Ghebremichael, M. S., Nusbaum, C., Huttenhower, C., Virgin, H. W., Ndung'u, T., Dong, K. L., Walker, B. D., Fichorova, R. N., & Kwon, D. S. (2015). Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity, 42*(5), 965-976. doi:10.1016/j.immuni.2015.04.019

Andersch, B., Brandberg, A., & Holst, E. (1990). [Treatment of bacterial vaginosis--an acid gel as an alternative to antibiotic treatment]. *Lakartidningen, 87*(7), 465-468.

Andersch, B., Forssman, L., Lincoln, K., & Torstensson, P. (1986). Treatment of bacterial vaginosis with an acid cream: a comparison between the effect of lactate-gel and metronidazole. *Gynecologic and Obstetric Investigation, 21*(1), 19-25. doi:10.1159/000298923

Andreeva, P., Slavchev, B., Kovachev, S., Nacheva, A., & Vacheva, R. (2002). [Treatment of bacterial vaginosis with high dosage metronidazole and lactic acid]. *Akusherstvo i Ginekologiia, 41*(5), 36-39.

Anstey Watkins, J., Ross, J. D. C., Thandi, S., Brittain, C., Kai, J., & Griffiths, F. (2019). Acceptability of and treatment preferences for recurrent bacterial vaginosis-Topical lactic acid gel or oral metronidazole antibiotic: Qualitative findings from the VITA trial. *PloS One, 14*(11), e0224964. doi:10.1371/journal.pone.0224964

Armstrong-Buisseret, L., Brittain, C., David, M., Dean, G., Griffiths, F., Hepburn, T., Jackson, L., Kai, J., Montgomery, A., Roberts, T., Thandi, S., & Ross, J. D. C. (2019). Metronidazole versus lactic acid for treating bacterial vaginosis (VITA): protocol for a randomised controlled trial to assess the clinical and cost effectiveness of topical lactic acid gel for treating second and subsequent episodes of bacterial vaginosis. *Trials, 20*(1), 648. doi:10.1186/s13063-019-3731-7

Atashili, J., Poole, C., Ndumbe, P. M., Adimora, A. A., & Smith, J. S. (2008). Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *AIDS, 22*(12), 1493-1501. doi:10.1097/QAD.0b013e3283021a37

Austin, M. N., Beigi, R. H., Meyn, L. A., & Hillier, S. L. (2005). Microbiologic response to treatment of bacterial vaginosis with topical clindamycin or metronidazole. *Journal of Clinical Microbiology, 43*(9), 4492-4497. doi:10.1128/JCM.43.9.4492-4497.2005

Australian Sexual Health Alliance. (2018, 28 March 2018). Bacterial Vaginosis. *Australian STI management guidelines for use in primary care.* Retrieved from http://sti.guidelines.org.au/sexually-transmissible-infections/infections-associated-with-sex/bacterial-vaginosis

Avonts, D., Sercu, M., Heyerick, P., Vandermeeren, I., Meheus, A., & Piot, P. (1990). Incidence of uncomplicated genital infections in women using oral contraception or an intrauterine device: a prospective study. *Sexually Transmitted Diseases, 17*(1), 23-29.

Ayehunie, S., Wang, Y. Y., Landry, T., Bogojevic, S., & Cone, R. A. (2018). Hyperosmolal vaginal lubricants markedly reduce epithelial barrier properties in a three-dimensional vaginal epithelium model. *Toxicol Rep, 5*, 134-140. doi:10.1016/j.toxrep.2017.12.011

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

Bailey, J. V., Farquhar, C., & Owen, C. (2004). Bacterial vaginosis in lesbians and bisexual women. *Sexually Transmitted Diseases, 31*(11), 691-694.

Balashov, S. V., Mordechai, E., Adelson, M. E., & Gygax, S. E. (2014). Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *Journal of Medical Microbiology, 63*(Pt 2), 162-175. doi:10.1099/jmm.0.066407-0

Balkus, J. E., Fredricks, D., Srinivasan, S., Dinwiddie, D., Holm, J., van Der Pol, W., Hughes, J., & Ravel, J. (2019a). *Pre-Congress Symposium 3 - Understanding variability and improving the reproducibility of genital microbiome research.* Paper presented at the STI & HIV 2019 World Congress, Vancouver, Canada.

Balkus, J. E., Proll, S., Holm, J., Srinivasan, S., Dinwiddie, D., Van Der Pol, W., Hoffman, N., Lefkowitz, E., Hughes, J., Van Der Pol, B., Wheeler, C. A., Wald, A., Marrazzo, J. M., Ravel, J., & Fredricks, D. N. (2019b). *A multi-site comparative study to understand sources of variability in studies of the vaginal microbiota*. Paper presented at the STI & HIV 2019 World Congress joint meeting of the 23rd ISSTDR & 20th IUSTI, Vancouver, Canada.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology, 19*(5), 455-477. doi:10.1089/cmb.2012.0021

Bassis, C. M., Allsworth, J. E., Wahl, H. N., Sack, D. E., Young, V. B., & Bell, J. D. (2017). Effects of intrauterine contraception on the vaginal microbiota. *Contraception, 96*(3), 189-195. doi:10.1016/j.contraception.2017.05.017

Bateson, D., Harvey, C., Trinh, L., Stewart, M., & Black, K. I. (2016). User characteristics, experiences and continuation rates of copper intrauterine device use in a cohort of Australian women. *Australian and New Zealand Journal of Obstetrics and Gynaecology, 56*(6), 655-661. doi:10.1111/ajo.12534

Baumann, L., Cina, M., Egli-Gany, D., Goutaki, M., Halbeisen, F. S., Lohrer, G. R., Ali, H., Scott, P., & Low, N. (2018). Prevalence of Mycoplasma genitalium in different population groups: systematic review andmeta-analysis. *Sexually Transmitted Infections, 94*(4), 255-262. doi:10.1136/sextrans-2017-053384

Bautista, C. T., Wurapa, E., Sateren, W. B., Morris, S., Hollingsworth, B., & Sanchez, J. L. (2016). Bacterial vaginosis: a synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. *Mil Med Res, 3*, 4. doi:10.1186/s40779-016-0074-5

Beigi, R. H., Austin, M. N., Meyn, L. A., Krohn, M. A., & Hillier, S. L. (2004). Antimicrobial resistance associated with the treatment of bacterial vaginosis. *American Journal of Obstetrics and Gynecology, 191*(4), 1124-1129. doi:10.1016/j.ajog.2004.05.033

Benito, R., Vazquez, J. A., Berron, S., Fenoll, A., & Saez-Neito, J. A. (1986). A modified scheme for biotyping *Gardnerella vaginalis*. *Journal of Medical Microbiology, 21*(4), 357-359. doi:10.1099/00222615-21-4-357

Berger, B. J., Kolton, S., Zenilman, J. M., Cummings, M. C., Feldman, J., & McCormack, W. M. (1995). Bacterial vaginosis in lesbians: a sexually transmitted disease. *Clinical Infectious Diseases, 21*(6), 1402-1405.

Biagi, E., Vitali, B., Pugliese, C., Candela, M., Donders, G. G., & Brigidi, P. (2009). Quantitative variations in the vaginal bacterial population associated with asymptomatic infections: a real-time polymerase chain reaction study. *European Journal of Clinical Microbiology and Infectious Diseases, 28*(3), 281-285. doi:10.1007/s10096-008-0617-0

Bilardi, J. E., Walker, S., Temple-Smith, M., McNair, R., Mooney-Somers, J., Bellhouse, C., Fairley, C. K., Chen, M. Y., & Bradshaw, C. (2013). The burden of bacterial vaginosis: women's experience of the physical, emotional, sexual and social impact of living with recurrent bacterial vaginosis. *PloS One, 8*(9), e74378. doi:10.1371/journal.pone.0074378

Boeke, A. J., Dekker, J. H., van Eijk, J. T., Kostense, P. J., & Bezemer, P. D. (1993). Effect of lactic acid suppositories compared with oral metronidazole and placebo in bacterial vaginosis: a randomised clinical trial. *Genitourinary Medicine, 69*(5), 388-392.

Bohr, L. L., Mortimer, T. D., & Pepperell, C. S. (2020). Lateral Gene Transfer Shapes Diversity of Gardnerella spp. *Front Cell Infect Microbiol, 10*, 293. doi:10.3389/fcimb.2020.00293

Borgdorff, H., Tsivtsivadze, E., Verhelst, R., Marzorati, M., Jurriaans, S., Ndayisaba, G. F., Schuren, F. H., & van de Wijgert, J. H. (2014). *Lactobacillus*-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *ISME J, 8*(9), 1781-1793. doi:10.1038/ismej.2014.26

Borgdorff, H., van der Veer, C., van Houdt, R., Alberts, C. J., de Vries, H. J., Bruisten, S. M., Snijder, M. B., Prins, M., Geerlings, S. E., Schim van der Loeff, M. F., & van de Wijgert, J. (2017). The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. *PloS One, 12*(7), e0181135. doi:10.1371/journal.pone.0181135

Boris, S., Suarez, J. E., Vazquez, F., & Barbes, C. (1998). Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infection and Immunity, 66*(5), 1985-1989.

Boskey, E. R., Cone, R. A., Whaley, K. J., & Moench, T. R. (2001). Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Human Reproduction, 16*(9), 1809-1813.

Boskey, E. R., Telsch, K. M., Whaley, K. J., Moench, T. R., & Cone, R. A. (1999). Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. *Infection and Immunity, 67*(10), 5170-5175.

Bradshaw, C. S., & Brotman, R. M. (2015). Making inroads into improving treatment of bacterial vaginosis - striving for long-term cure. *BMC Infectious Diseases, 15*, 292. doi:10.1186/s12879-015-1027-4

Bradshaw, C. S., Morton, A. N., Hocking, J., Garland, S. M., Morris, M. B., Moss, L. M., Horvath, L. B., Kuzevska, I., & Fairley, C. K. (2006a). High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *Journal of Infectious Diseases, 193*(11), 1478-1486. doi:10.1086/503780

Bradshaw, C. S., & Sobel, J. D. (2016). Current treatment of bacterial vaginosis-limitations and need for innovation. *Journal of Infectious Diseases, 214 Suppl 1*, S14-20. doi:10.1093/infdis/jiw159

Bradshaw, C. S., Tabrizi, S. N., Fairley, C. K., Morton, A. N., Rudland, E., & Garland, S. M. (2006b). The association of *Atopobium vaginae* and *Gardnerella vaginalis* with bacterial vaginosis and recurrence after oral metronidazole therapy. *Journal of Infectious Diseases, 194*(6), 828-836. doi:10.1086/506621

Bradshaw, C. S., Vodstrcil, L. A., Hocking, J. S., Law, M., Pirotta, M., Garland, S. M., De Guingand, D., Morton, A. N., & Fairley, C. K. (2013a). Recurrence of bacterial vaginosis is significantly associated with posttreatment sexual activities and hormonal contraceptive use. *Clinical Infectious Diseases, 56*(6), 777-786. doi:10.1093/cid/cis1030

Bradshaw, C. S., Walker, J., Fairley, C. K., Chen, M. Y., Tabrizi, S. N., Donovan, B., Kaldor, J. M., McNamee, K., Urban, E., Walker, S., Currie, M., Birden, H., Bowden, F., Garland, S., Pirotta, M., Gurrin, L., & Hocking, J. S. (2013b). Prevalent and incident bacterial vaginosis are associated with sexual and contraceptive behaviours in young Australian women. *PloS One, 8*(3), e57688. doi:10.1371/journal.pone.0057688

Bradshaw, C. S., Walker, S. M., Vodstrcil, L. A., Bilardi, J. E., Law, M., Hocking, J. S., Fethers, K. A., Fehler, G., Petersen, S., Tabrizi, S. N., Chen, M. Y., Garland, S. M., & Fairley, C. K. (2014). The influence of behaviors and relationships on the vaginal microbiota of women and their female partners: the WOW Health Study. *Journal of Infectious Diseases, 209*(10), 1562-1572. doi:10.1093/infdis/jit664

Breshears, L. M., Edwards, V. L., Ravel, J., & Peterson, M. L. (2015). *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiology, 15*, 276. doi:10.1186/s12866-015-0608-0

Briselden, A. M., Moncla, B. J., Stevens, C. E., & Hillier, S. L. (1992). Sialidases (neuraminidases) in bacterial vaginosis and bacterial vaginosis-associated microflora. *Journal of Clinical Microbiology, 30*(3), 663-666.

Brooks, J. P., Buck, G. A., Chen, G., Diao, L., Edwards, D. J., Fettweis, J. M., Huzurbazar, S., Rakitin, A., Satten, G. A., Smirnova, E., Waks, Z., Wright, M. L., Yanover, C., & Zhou, Y. H. (2017a). Changes in vaginal community state types reflect major shifts in the microbiome. *Microbial Ecology in Health and Disease, 28*(1), 1303265. doi:10.1080/16512235.2017.1303265

Brooks, J. P., Edwards, D. J., Blithe, D. L., Fettweis, J. M., Serrano, M. G., Sheth, N. U., Strauss, J. F., 3rd, Buck, G. A., & Jefferson, K. K. (2017b). Effects of combined oral contraceptives, depot medroxyprogesterone acetate and the levonorgestrel-releasing intrauterine system on the vaginal microbiome. *Contraception, 95*(4), 405-413. doi:10.1016/j.contraception.2016.11.006

Brotman, R. M., Bradford, L. L., Conrad, M., Gajer, P., Ault, K., Peralta, L., Forney, L. J., Carlton, J. M., Abdo, Z., & Ravel, J. (2012). Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women. *Sexually Transmitted Diseases, 39*(10), 807-812. doi:10.1097/OLQ.0b013e3182631c79

Brotman, R. M., Ghanem, K. G., Klebanoff, M. A., Taha, T. E., Scharfstein, D. O., & Zenilman, J. M. (2008a). The effect of vaginal douching cessation on bacterial vaginosis: a pilot study. *American Journal of Obstetrics and Gynecology, 198*(6), 628 e621-627. doi:10.1016/j.ajog.2007.11.043

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

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

Brotman, R. M., Klebanoff, M. A., Nansel, T. R., Yu, K. F., Andrews, W. W., Zhang, J., & Schwebke, J. R. (2010). Bacterial vaginosis assessed by Gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. *Journal of Infectious Diseases, 202*(12), 1907-1915. doi:10.1086/657320

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

Brunham, R. C., Gottlieb, S. L., & Paavonen, J. (2015). Pelvic inflammatory disease. *New England Journal of Medicine, 372*(21), 2039-2048. doi:10.1056/NEJMra1411426

Bukusi, E., Thomas, K. K., Nguti, R., Cohen, C. R., Weiss, N., Coombs, R. W., & Holmes, K. K. (2011). Topical penile microbicide use by men to prevent recurrent bacterial vaginosis in sex partners: a randomized clinical trial. *Sexually Transmitted Diseases, 38*(6), 483-489.

Bump, R. C., & Buesching, W. J., 3rd. (1988). Bacterial vaginosis in virginal and sexually active adolescent females: evidence against exclusive sexual transmission. *American Journal of Obstetrics and Gynecology, 158*(4), 935-939. doi:10.1016/0002-9378(88)90097-x

Burton, J. P., & Reid, G. (2002). Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. *Journal of Infectious Diseases, 186*(12), 1770-1780. doi:10.1086/345761

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods, 13*(7), 581-583. doi:10.1038/nmeth.3869

Calzolari, E., Masciangelo, R., Milite, V., & Verteramo, R. (2000). Bacterial vaginosis and contraceptive methods. *International Journal of Gynaecology and Obstetrics, 70*(3), 341-346. doi:10.1016/s0020-7292(00)00217-4

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods, 7*(5), 335-336. doi:10.1038/nmeth.f.303

Carda-Dieguez, M., Cardenas, N., Aparicio, M., Beltran, D., Rodriguez, J. M., & Mira, A. (2019). Variations in vaginal, penile, and oral microbiota after sexual intercourse: a case report. *Front Med (Lausanne), 6*, 178. doi:10.3389/fmed.2019.00178

Castro, J., Henriques, A., Machado, A., Henriques, M., Jefferson, K. K., & Cerca, N. (2013). Reciprocal interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on initial adherence to epithelial cells. *International Journal of Medical Sciences, 10*(9), 1193-1198. doi:10.7150/ijms.6304

Catlin, B. W. (1992). Gardnerella vaginalis: characteristics, clinical considerations, and controversies. *Clinical Microbiology Reviews, 5*(3), 213-237. doi:10.1128/CMR.5.3.213

Ceccarani, C., Foschi, C., Parolin, C., D'Antuono, A., Gaspari, V., Consolandi, C., Laghi, L., Camboni, T., Vitali, B., Severgnini, M., & Marangoni, A. (2019). Diversity of vaginal microbiome and metabolome during genital infections. *Scientific Reports, 9*(1), 14095. doi:10.1038/s41598-019-50410-x

Chaban, B., Links, M. G., Jayaprakash, T. P., Wagner, E. C., Bourque, D. K., Lohn, Z., Albert, A. Y., van Schalkwyk, J., Reid, G., Hemmingsen, S. M., Hill, J. E., & Money, D. M. (2014). Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. *Microbiome, 2*, 23. doi:10.1186/2049-2618-2-23

Chaim, W., Horowitz, S., David, J. B., Ingel, F., Evinson, B., & Mazor, M. (2003). *Ureaplasma urealyticum* in the development of postpartum endometritis. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 109*(2), 145-148. doi:10.1016/s0301-2115(03)00007-1

Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods, 69*(2), 330-339. doi:10.1016/j.mimet.2007.02.005

Chan, R. C., Reid, G., Irvin, R. T., Bruce, A. W., & Costerton, J. W. (1985). Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. *Infection and Immunity, 47*(1), 84-89.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *Journal of Food Protection, 61*(12), 1636-1643. doi:10.4315/0362-028x-61.12.1636

Chen, K. C., Forsyth, P. S., Buchanan, T. M., & Holmes, K. K. (1979). Amine content of vaginal fluid from untreated and treated patients with nonspecific vaginitis. *Journal of Clinical Investigation, 63*(5), 828-835. doi:10.1172/JCI109382

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

Cherpes, T. L., Meyn, L. A., Krohn, M. A., Lurie, J. G., & Hillier, S. L. (2003). Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clinical Infectious Diseases, 37*(3), 319-325. doi:10.1086/375819

Chojnacki, S., Cowley, A., Lee, J., Foix, A., & Lopez, R. (2017). Programmatic access to bioinformatics tools from EMBL-EBI update: 2017. *Nucleic Acids Research, 45*(W1), W550-W553. doi:10.1093/nar/gkx273

Cohen, C. R., Lingappa, J. R., Baeten, J. M., Ngayo, M. O., Spiegel, C. A., Hong, T., Donnell, D., Celum, C., Kapiga, S., Delany, S., & Bukusi, E. A. (2012). Bacterial vaginosis associated with increased

risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS Medicine, 9*(6), e1001251. doi:10.1371/journal.pmed.1001251

Coleman, J. S., & Gaydos, C. A. (2018). Molecular Diagnosis of Bacterial Vaginosis: an Update. *Journal of Clinical Microbiology, 56*(9). doi:10.1128/JCM.00342-18

Colli, E., Landoni, M., & Parazzini, F. (1997). Treatment of male partners and recurrence of bacterial vaginosis: a randomised trial. *Genitourinary Medicine, 73*(4), 267-270.

Cornejo, O. E., Hickey, R. J., Suzuki, H., & Forney, L. J. (2018). Focusing the diversity of *Gardnerella vaginalis* through the lens of ecotypes. *Evol Appl, 11*(3), 312-324. doi:10.1111/eva.12555

Cox, C., Watt, A. P., McKenna, J. P., & Coyle, P. V. (2016). *Mycoplasma hominis* and *Gardnerella vaginalis* display a significant synergistic relationship in bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases, 35*(3), 481-487. doi:10.1007/s10096-015-2564-x

Criswell, B. S., Ladwig, C. L., Gardner, H. L., & Dukes, C. D. (1969). *Haemophilus vaginalis*: vaginitis by inoculation from culture. *Obstetrics and Gynecology, 33*(2), 195-199.

Cu-Uvin, S., Hogan, J. W., Caliendo, A. M., Harwell, J., Mayer, K. H., Carpenter, C. C., & Study, H. I. V. E. R. (2001). Association between bacterial vaginosis and expression of human immunodeficiency virus type 1 RNA in the female genital tract. *Clinical Infectious Diseases, 33*(6), 894-896. doi:10.1086/322613

Datcu, R., Gesink, D., Mulvad, G., Montgomery-Andersen, R., Rink, E., Koch, A., Ahrens, P., & Jensen, J. S. (2013). Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR. *BMC Infectious Diseases, 13*, 480. doi:10.1186/1471-2334-13-480

De Backer, E., Verhelst, R., Verstraelen, H., Claeys, G., Verschraegen, G., Temmerman, M., & Vaneechoutte, M. (2006). Antibiotic susceptibility of *Atopobium vaginae*. *BMC Infectious Diseases, 6*, 51. doi:10.1186/1471-2334-6-51

De Francesco, M. A., Negrini, R., Pinsi, G., Peroni, L., & Manca, N. (2009). Detection of *Ureaplasma* biovars and polymerase chain reaction-based subtyping of *Ureaplasma parvum* in women with or without symptoms of genital infections. *European Journal of Clinical Microbiology and Infectious Diseases, 28*(6), 641-646. doi:10.1007/s10096-008-0687-z

Delgado-Diaz, D. J., Tyssen, D., Hayward, J. A., Gugasyan, R., Hearps, A. C., & Tachedjian, G. (2019). Distinct Immune Responses Elicited From Cervicovaginal Epithelial Cells by Lactic Acid and Short Chain Fatty Acids Associated With Optimal and Non-optimal Vaginal Microbiota. *Front Cell Infect Microbiol, 9*, 446. doi:10.3389/fcimb.2019.00446

Des Jarlais, D. C., Lyles, C., Crepaz, N., & Group, T. (2004). Improving the reporting quality of nonrandomized evaluations of behavioral and public health interventions: the TREND statement. *American Journal of Public Health, 94*(3), 361-366.

Devault, A. M., Mortimer, T. D., Kitchen, A., Kiesewetter, H., Enk, J. M., Golding, G. B., Southon, J., Kuch, M., Duggan, A. T., Aylward, W., Gardner, S. N., Allen, J. E., King, A. M., Wright, G., Kuroda, M., Kato, K., Briggs, D. E., Fornaciari, G., Holmes, E. C., Poinar, H. N., & Pepperell, C. S. (2017). A molecular portrait of maternal sepsis from Byzantine Troy. *Elife, 6*. doi:10.7554/eLife.20983

Dols, J. A., Smit, P. W., Kort, R., Reid, G., Schuren, F. H., Tempelman, H., Bontekoe, T. R., Korporaal, H., & Boon, M. E. (2011). Microarray-based identification of clinically relevant vaginal bacteria in relation to bacterial vaginosis. *American Journal of Obstetrics and Gynecology, 204*(4), 305 e301-307. doi:10.1016/j.ajog.2010.11.012

Donders, G., Bellen, G., Janssens, D., Van Bulck, B., Hinoul, P., & Verguts, J. (2017a). Influence of contraceptive choice on vaginal bacterial and fungal microflora. *European Journal of Clinical Microbiology and Infectious Diseases, 36*(1), 43-48. doi:10.1007/s10096-016-2768-8

Donders, G. G. (2007). Definition and classification of abnormal vaginal flora. *Best Practice & Research: Clinical Obstetrics & Gynaecology, 21*(3), 355-373. doi:10.1016/j.bpobgyn.2007.01.002

Donders, G. G., Berger, J., Heuninckx, H., Bellen, G., & Cornelis, A. (2011). Vaginal flora changes on Pap smears after insertion of levonorgestrel-releasing intrauterine device. *Contraception, 83*(4), 352-356. doi:10.1016/j.contraception.2010.08.007

Donders, G. G., Van Calsteren, K., Bellen, G., Reybrouck, R., Van den Bosch, T., Riphagen, I., & Van Lierde, S. (2009). Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. *BJOG: An International Journal of Obstetrics and Gynaecology, 116*(10), 1315-1324. doi:10.1111/j.1471-0528.2009.02237.x

Donders, G. G. G., Ruban, K., Bellen, G., & Petricevic, L. (2017b). *Mycoplasma/Ureaplasma* infection in pregnancy: to screen or not to screen. *Journal of Perinatal Medicine, 45*(5), 505-515. doi:10.1515/jpm-2016-0111

Dong, Q., Nelson, D. E., Toh, E., Diao, L., Gao, X., Fortenberry, J. D., & Van der Pol, B. (2011). The microbial communities in male first catch urine are highly similar to those in paired urethral swab specimens. *PloS One, 6*(5), e19709. doi:10.1371/journal.pone.0019709

Donlan, R. M. (2001). Biofilms and device-associated infections. *Emerging Infectious Diseases, 7*(2), 277-281. doi:10.3201/eid0702.010226

Dumonceaux, T. J., Schellenberg, J., Goleski, V., Hill, J. E., Jaoko, W., Kimani, J., Money, D., Ball, T. B., Plummer, F. A., & Severini, A. (2009). Multiplex detection of bacteria associated with normal microbiota and with bacterial vaginosis in vaginal swabs by use of oligonucleotide-coupled fluorescent microspheres. *Journal of Clinical Microbiology, 47*(12), 4067-4077. doi:10.1128/JCM.00112-09

Edwards, D. I. (1993). Nitroimidazole drugs--action and resistance mechanisms. I. Mechanisms of action. *Journal of Antimicrobial Chemotherapy, 31*(1), 9-20. doi:10.1093/jac/31.1.9

Edwards, V. L., Smith, S. B., McComb, E. J., Tamarelle, J., Ma, B., Humphrys, M. S., Gajer, P., Gwilliam, K., Schaefer, A. M., Lai, S. K., Terplan, M., Mark, K. S., Brotman, R. M., Forney, L. J., Bavoil, P. M., & Ravel, J. (2019). The cervicovaginal microbiota-host interaction modulates *Chlamydia trachomatis* infection. *mBio, 10*(4). doi:10.1128/mBio.01548-19

Eren, A. M., Zozaya, M., Taylor, C. M., Dowd, S. E., Martin, D. H., & Ferris, M. J. (2011). Exploring the diversity of *Gardnerella vaginalis* in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation. *PloS One, 6*(10), e26732. doi:10.1371/journal.pone.0026732

Eschenbach, D. A. (2007). Bacterial vaginosis: resistance, recurrence, and/or reinfection? *Clinical Infectious Diseases, 44*(2), 220-221. doi:10.1086/509584

Eschenbach, D. A., Thwin, S. S., Patton, D. L., Hooton, T. M., Stapleton, A. E., Agnew, K., Winter, C., Meier, A., & Stamm, W. E. (2000). Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. *Clinical Infectious Diseases, 30*(6), 901-907. doi:10.1086/313818

Evans, A. L., Scally, A. J., Wellard, S. J., & Wilson, J. D. (2007). Prevalence of bacterial vaginosis in lesbians and heterosexual women in a community setting. *Sexually Transmitted Infections, 83*(6), 470-475. doi:10.1136/sti.2006.022277

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., & Ravel, J. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome, 2*(1), 6. doi:10.1186/2049-2618-2-6

Falsen, E., Pascual, C., Sjoden, B., Ohlen, M., & Collins, M. D. (1999). Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov. *International Journal of Systematic Bacteriology, 49 Pt 1*, 217-221. doi:10.1099/00207713-49-1-217

Fernandes, A. D., Macklaim, J. M., Linn, T. G., Reid, G., & Gloor, G. B. (2013). ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-Seq. *PloS One, 8*(7), e67019. doi:10.1371/journal.pone.0067019

Fernandes, A. D., Reid, J. N., Macklaim, J. M., McMurrough, T. A., Edgell, D. R., & Gloor, G. B. (2014). Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S

rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome, 2*, 15. doi:10.1186/2049-2618-2-15

Ferris, M. J., Masztal, A., Aldridge, K. E., Fortenberry, J. D., Fidel, P. L., Jr., & Martin, D. H. (2004). Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infectious Diseases, 4*, 5. doi:10.1186/1471-2334-4-5

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

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

Fethers, K. A., Fairley, C. K., Morton, A., Hocking, J. S., Fehler, G., Kennedy, L. J., & Bradshaw, C. S. (2011). Low incidence of bacterial vaginosis in cohort of young Australian women. *Sexually Transmitted Diseases, 38*(2), 124-126. doi:10.1097/OLQ.0b013e3181f0bc37

Fethers, K. A., Fairley, C. K., Morton, A., Hocking, J. S., Hopkins, C., Kennedy, L. J., Fehler, G., & Bradshaw, C. S. (2009). Early sexual experiences and risk factors for bacterial vaginosis. *Journal of Infectious Diseases, 200*(11), 1662-1670. doi:10.1086/648092

Fethers, K. A., Twin, J., Fairley, C. K., Fowkes, F. J., Garland, S. M., Fehler, G., Morton, A. M., Hocking, J. S., Tabrizi, S. N., & Bradshaw, C. S. (2012). Bacterial vaginosis (BV) candidate bacteria: associations with BV and behavioural practices in sexually-experienced and inexperienced women. *PloS One, 7*(2), e30633. doi:10.1371/journal.pone.0030633

Fettweis, J. M., Brooks, J. P., Serrano, M. G., Sheth, N. U., Girerd, P. H., Edwards, D. J., Strauss, J. F., 3rd, Jefferson, K. K., & Buck, G. A. (2014). Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology, 160*(Pt 10), 2272-2282. doi:10.1099/mic.0.081034-0

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., Huang, B., Arodz, T. J., Edupuganti, L., Glascock, A. L., Xu, J., Jimenez, N. R., Vivadelli, S. C., Fong, S. S., Sheth, N. U., Jean, S., Lee, V., Bokhari, Y. A., Lara, A. M., Mistry, S. D., Duckworth, R. A., 3rd, Bradley, S. P., Koparde, V. N., Orenda, X. V., Milton, S. H., Rozycki, S. K., Matveyev, A. V., Wright, M. L., Huzurbazar, S. V., Jackson, E. M., Smirnova, E., Korlach, J., Tsai, Y. C., Dickinson, M. R., Brooks, J. L., Drake, J. I., Chaffin, D. O., Sexton, A. L., Gravett, M. G., Rubens, C. E., Wijesooriya, N. R., Hendricks-Munoz, K. D., Jefferson, K. K., Strauss, J. F., 3rd, & Buck, G. A. (2019). The vaginal microbiome and preterm birth. *Nature Medicine, 25*(6), 1012-1021. doi:10.1038/s41591-019-0450-2

Food Drug Administration Center for Drugs Evaluation Research. (2019). Bacterial Vaginosis: Developing Drugs for Treatment. Guidance for Industry. Retrieved from https://www.fda.gov/media/129530/download

Foschi, C., Salvo, M., D'Antuono, A., Gaspari, V., Banzola, N., Cevenini, R., & Marangoni, A. (2018). Distribution of genital Mollicutes in the vaginal ecosystem of women with different clinical conditions. *New Microbiologica, 41*(3), 225-229.

France, M. T., Ma, B., Gajer, P., Brown, S., Humphrys, M. S., Holm, J. B., Waetjen, L. E., Brotman, R. M., & Ravel, J. (2020). VALENCIA: a nearest centroid classification method for vaginal microbial communities based on composition. *Microbiome, 8*(1), 166. doi:10.1186/s40168-020-00934-6

France, M. T., Mendes-Soares, H., & Forney, L. J. (2016). Genomic comparisons of *Lactobacillus crispatus* and *Lactobacillus iners* reveal potential ecological drivers of community composition in the vagina. *Applied and Environmental Microbiology, 82*(24), 7063-7073. doi:10.1128/AEM.02385-16

Francis, S. C., Crucitti, T., Smekens, T., Hansen, C. H., Andreasen, A., Jespers, V., Hardy, L., Irani, J., Changalucha, J., Baisley, K., Hayes, R., Watson-Jones, D., & Buve, A. (2020). The Vaginal

Microbiota Among Adolescent Girls in Tanzania Around the Time of Sexual Debut. *Front Cell Infect Microbiol, 10*, 305. doi:10.3389/fcimb.2020.00305

Fredricks, D. N., Fiedler, T. L., & Marrazzo, J. M. (2005). Molecular identification of bacteria associated with bacterial vaginosis. *New England Journal of Medicine, 353*(18), 1899-1911. doi:10.1056/NEJMoa043802

Fredstorp, M., Jonasson, A. F., Barth, A., & Robertsson, J. (2015). A new effective, user-friendly bacterial vaginosis treatment: a randomized multicenter open-label parallel-group two-part study with a novel sustained-release pessary containing oligomeric lactic acid. *HSOA Journal of Infectious and Non Infectious Diseases, 1*(1), 100006. doi:10.24966/INID-8654/100006

Freeman, C. D., Klutman, N. E., & Lamp, K. C. (1997). Metronidazole. A therapeutic review and update. *Drugs, 54*(5), 679-708. doi:10.2165/00003495-199754050-00003

Freire-de-Lima, L., Fonseca, L. M., Oeltmann, T., Mendonca-Previato, L., & Previato, J. O. (2015). The trans-sialidase, the major *Trypanosoma cruzi* virulence factor: Three decades of studies. *Glycobiology, 25*(11), 1142-1149. doi:10.1093/glycob/cwv057

Friedman, J., & Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLoS Computational Biology, 8*(9), e1002687. doi:10.1371/journal.pcbi.1002687

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., Koenig, S. S., Fu, L., Ma, Z. S., Zhou, X., Abdo, Z., Forney, L. J., & Ravel, J. (2012). Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine, 4*(132), 132ra152. doi:10.1126/scitranslmed.3003605

Galiwango, R. M., Bagaya, B., Mpendo, J., Joag, V., Okech, B., Nanvubya, A., Ssetaala, A., Muwanga, M., & Kaul, R. (2019). Protocol for a randomized clinical trial exploring the effect of antimicrobial agents on the penile microbiota, immunology and HIV susceptibility of Ugandan men. *Trials, 20*(1), 443. doi:10.1186/s13063-019-3545-7

Garcia, E. M., Serrano, M. G., Edupuganti, L., Edwards, D. J., Buck, G. A., & Jefferson, K. K. (2021). Sequence comparison of vaginolysin from different *Gardnerella* species. *Pathogens, 10*(2). doi:10.3390/pathogens10020086

Gardner, H. L., & Dukes, C. D. (1955). *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *American Journal of Obstetrics and Gynecology, 69*(5), 962-976.

Garland, S. M., & Bowman, E. D. (1996). Role of *Ureaplasma urealyticum* and *Chlamydia trachomatis* in lung disease in low birth weight infants. *Pathology, 28*(3), 266-269. doi:10.1080/00313029600169134

Garland, S. M., & Murton, L. J. (1987). Neonatal meningitis caused by *Ureaplasma urealyticum*. *Pediatric Infectious Disease Journal, 6*(9), 868-870. doi:10.1097/00006454-198709000-00019

Gaspar, C., Donders, G. G., Palmeira-de-Oliveira, R., Queiroz, J. A., Tomaz, C., Martinez-de-Oliveira, J., & Palmeira-de-Oliveira, A. (2018). Bacteriocin production of the probiotic *Lactobacillus acidophilus* KS400. *AMB Express, 8*(1), 153. doi:10.1186/s13568-018-0679-z

Gelber, S. E., Aguilar, J. L., Lewis, K. L., & Ratner, A. J. (2008). Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *Journal of Bacteriology, 190*(11), 3896-3903. doi:10.1128/JB.01965-07

Giuliano, A. R., Sedjo, R. L., Roe, D. J., Harri, R., Baldwi, S., Papenfuss, M. R., Abrahamsen, M., & Inserra, P. (2002). Clearance of oncogenic human papillomavirus (HPV) infection: effect of smoking (United States). *Cancer Causes and Control, 13*(9), 839-846. doi:10.1023/a:1020668232219

Glaser, K., & Speer, C. P. (2015). Neonatal CNS infection and inflammation caused by *Ureaplasma* species: rare or relevant? *Expert Review of Anti-Infective Therapy, 13*(2), 233-248. doi:10.1586/14787210.2015.999670

Gliniewicz, K., Schneider, G. M., Ridenhour, B. J., Williams, C. J., Song, Y., Farage, M. A., Miller, K., & Forney, L. J. (2019). Comparison of the vaginal microbiomes of premenopausal and postmenopausal women. *Frontiers in Microbiology, 10*, 193. doi:10.3389/fmicb.2019.00193

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome Datasets Are Compositional: And This Is Not Optional. *Frontiers in Microbiology, 8*, 2224. doi:10.3389/fmicb.2017.02224

Godha, K., Tucker, K. M., Biehl, C., Archer, D. F., & Mirkin, S. (2018). Human vaginal pH and microbiota: an update. *Gynecological Endocrinology, 34*(6), 451-455. doi:10.1080/09513590.2017.1407753

Gong, Z., Luna, Y., Yu, P., & Fan, H. (2014). Lactobacilli inactivate *Chlamydia trachomatis* through lactic acid but not H2O2. *PloS One, 9*(9), e107758. doi:10.1371/journal.pone.0107758

Gosmann, C., Anahtar, M. N., Handley, S. A., Farcasanu, M., Abu-Ali, G., Bowman, B. A., Padavattan, N., Desai, C., Droit, L., Moodley, A., Dong, M., Chen, Y., Ismail, N., Ndung'u, T., Ghebremichael, M. S., Wesemann, D. R., Mitchell, C., Dong, K. L., Huttenhower, C., Walker, B. D., Virgin, H. W., & Kwon, D. S. (2017). *Lactobacillus*-deficient cervicovaginal bacterial ommunities are associated with increased HIV acquisition in young South African women. *Immunity, 46*(1), 29-37. doi:10.1016/j.immuni.2016.12.013

Gottschick, C., Deng, Z. L., Vital, M., Masur, C., Abels, C., Pieper, D. H., Rohde, M., Mendling, W., & Wagner-Dobler, I. (2017a). Treatment of biofilms in bacterial vaginosis by an amphoteric tenside pessary-clinical study and microbiota analysis. *Microbiome, 5*(1), 119. doi:10.1186/s40168-017-0326-y

Gottschick, C., Deng, Z. L., Vital, M., Masur, C., Abels, C., Pieper, D. H., & Wagner-Dobler, I. (2017b). The urinary microbiota of men and women and its changes in women during bacterial vaginosis and antibiotic treatment. *Microbiome, 5*(1), 99. doi:10.1186/s40168-017-0305-3

Gottschick, C., Szafranski, S. P., Kunze, B., Sztajer, H., Masur, C., Abels, C., & Wagner-Dobler, I. (2016). Screening of compounds against *Gardnerella vaginalis* biofilms. *PloS One, 11*(4), e0154086. doi:10.1371/journal.pone.0154086

Graver, M. A., & Wade, J. J. (2011). The role of acidification in the inhibition of *Neisseria gonorrhoeae* by vaginal lactobacilli during anaerobic growth. *Annals of Clinical Microbiology and Antimicrobials, 10*, 8. doi:10.1186/1476-0711-10-8

Gray, R. H., Kigozi, G., Serwadda, D., Makumbi, F., Nalugoda, F., Watya, S., Moulton, L., Chen, M. Z., Sewankambo, N. K., Kiwanuka, N., Sempijja, V., Lutalo, T., Kagayii, J., Wabwire-Mangen, F., Ridzon, R., Bacon, M., & Wawer, M. J. (2009). The effects of male circumcision on female partners' genital tract symptoms and vaginal infections in a randomized trial in Rakai, Uganda. *American Journal of Obstetrics and Gynecology, 200*(1), 42 e41-47. doi:10.1016/j.ajog.2008.07.069

Grunloh, D. S., Casner, T., Secura, G. M., Peipert, J. F., & Madden, T. (2013). Characteristics associated with discontinuation of long-acting reversible contraception within the first 6 months of use. *Obstetrics and Gynecology, 122*(6), 1214-1221. doi:10.1097/01.AOG.0000435452.86108.59

Gu, Z., Eils, R., & Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics, 32*(18), 2847-2849. doi:10.1093/bioinformatics/btw313

Gupta, K., Stapleton, A. E., Hooton, T. M., Roberts, P. L., Fennell, C. L., & Stamm, W. E. (1998). Inverse association of H2O2-producing lactobacilli and vaginal *Escherichia coli* colonization in women with recurrent urinary tract infections. *Journal of Infectious Diseases, 178*(2), 446-450.

Haggerty, C. L., Hillier, S. L., Bass, D. C., & Ness, R. B. (2004). Bacterial vaginosis and anaerobic bacteria are associated with endometritis. *Clinical Infectious Diseases, 39*(7), 990-995. doi:10.1086/423963

Haggerty, C. L., Totten, P. A., Ferris, M., Martin, D. H., Hoferka, S., Astete, S. G., Ondondo, R., Norori, J., & Ness, R. B. (2009). Clinical characteristics of bacterial vaginosis among women testing positive for fastidious bacteria. *Sexually Transmitted Infections, 85*(4), 242-248. doi:10.1136/sti.2008.032821

Haggerty, C. L., Totten, P. A., Tang, G., Astete, S. G., Ferris, M. J., Norori, J., Bass, D. C., Martin, D. H., Taylor, B. D., & Ness, R. B. (2016). Identification of novel microbes associated with pelvic inflammatory disease and infertility. *Sexually Transmitted Infections, 92*(6), 441-446. doi:10.1136/sextrans-2015-052285

Hardy, L., Jespers, V., Dahchour, N., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M., & Crucitti, T. (2015). Unravelling the bacterial vaginosis-associated biofilm: a multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence in situ hybridization assay using peptide nucleic acid probes. *PloS One, 10*(8), e0136658. doi:10.1371/journal.pone.0136658

Hardy, L., Jespers, V., Van den Bulck, M., Buyze, J., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M., & Crucitti, T. (2017). The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PloS One, 12*(2), e0172522. doi:10.1371/journal.pone.0172522

Harwich, M. D., Jr., Alves, J. M., Buck, G. A., Strauss, J. F., 3rd, Patterson, J. L., Oki, A. T., Girerd, P. H., & Jefferson, K. K. (2010). Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics, 11*, 375. doi:10.1186/1471-2164-11-375

Hawes, S. E., Hillier, S. L., Benedetti, J., Stevens, C. E., Koutsky, L. A., Wolner-Hanssen, P., & Holmes, K. K. (1996). Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. *Journal of Infectious Diseases, 174*(5), 1058-1063. doi:10.1093/infdis/174.5.1058

Hay, P., Patel, S., & Daniels, D. (2012). UK National Guideline for the management of Bacterial Vaginosis 2012. Retrieved from https://www.bashhguidelines.org/media/1041/bv-2012.pdf

Hay, P. E., Lamont, R. F., Taylor-Robinson, D., Morgan, D. J., Ison, C., & Pearson, J. (1994). Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. *BMJ, 308*(6924), 295-298. doi:10.1136/bmj.308.6924.295

Hearps, A. C., Tyssen, D., Srbinovski, D., Bayigga, L., Diaz, D. J. D., Aldunate, M., Cone, R. A., Gugasyan, R., Anderson, D. J., & Tachedjian, G. (2017). Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. *Mucosal Immunology, 10*(6), 1480-1490. doi:10.1038/mi.2017.27

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

Hellberg, D., Nilsson, S., & Mardh, P. A. (2000). Bacterial vaginosis and smoking. *International Journal of STD and AIDS, 11*(9), 603-606. doi:10.1258/0956462001916461

Hickey, R. J., Zhou, X., Settles, M. L., Erb, J., Malone, K., Hansmann, M. A., Shew, M. L., Van Der Pol, B., Dennis Fortenberry, J., & Forney, L. J. (2015). Vaginal microbiota of adolescent girls prior to the onset of menarche resemble those of reproductive-age women. *mBio, 6*(2). doi:10.1128/mBio.00097-15

Higgins JPT, Sterne JAC, Savović J, Page MJ, Hróbjartsson A, Boutron I, Reeves B, & Eldridge SA. (2016). A revised tool for assessing risk of bias in randomized trials. *Cochrane Database of Systematic Reviews 10*(Suppl 1), 29-31. doi:dx.doi.org/10.1002/14651858.CD201601.

Hilbert, D. W., Schuyler, J. A., Adelson, M. E., Mordechai, E., Sobel, J. D., & Gygax, S. E. (2017). *Gardnerella vaginalis* population dynamics in bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases, 36*(7), 1269-1278. doi:10.1007/s10096-017-2933-8

Hill, G. B., St Claire, K. K., & Gutman, L. T. (1995). Anaerobes predominate among the vaginal microflora of prepubertal girls. *Clinical Infectious Diseases, 20 Suppl 2*, S269-270. doi:10.1093/clinids/20.supplement_2.s269

Hill, J. E., Albert, A. Y. K., & Group, V. R. (2019). Resolution and cooccurrence patterns of *Gardnerella leopoldii, G. swidsinskii, G. piotii,* and *G. vaginalis* within the vaginal microbiome. *Infection and Immunity, 87*(12). doi:10.1128/IAI.00532-19

Hillier, S. L. (1993). Diagnostic microbiology of bacterial vaginosis. *American Journal of Obstetrics and Gynecology, 169*(2 Pt 2), 455-459.

Hillier, S. L., Krohn, M. A., Klebanoff, S. J., & Eschenbach, D. A. (1992). The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. *Obstetrics and Gynecology, 79*(3), 369-373.

Hillier, S. L., & Lau, R. J. (1997). Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. *Clinical Infectious Diseases, 25 Suppl 2*, S123-126. doi:10.1086/516221

Holst, E. (1990). Reservoir of four organisms associated with bacterial vaginosis suggests lack of sexual transmission. *Journal of Clinical Microbiology, 28*(9), 2035-2039.

Holzman, C., Leventhal, J. M., Qiu, H., Jones, N. M., Wang, J., & Group, B. V. S. (2001). Factors linked to bacterial vaginosis in nonpregnant women. *American Journal of Public Health, 91*(10), 1664-1670. doi:10.2105/ajph.91.10.1664

Honest, H., Bachmann, L. M., Knox, E. M., Gupta, J. K., Kleijnen, J., & Khan, K. S. (2004). The accuracy of various tests for bacterial vaginosis in predicting preterm birth: a systematic review. *BJOG: An International Journal of Obstetrics and Gynaecology, 111*(5), 409-422. doi:10.1111/j.1471-0528.2004.00124.x

Horner, P., Donders, G., Cusini, M., Gomberg, M., Jensen, J. S., & Unemo, M. (2018). Should we be testing for urogenital *Mycoplasma hominis, Ureaplasma parvum* and *Ureaplasma urealyticum* in men and women? - a position statement from the European STI Guidelines Editorial Board. *Journal of the European Academy of Dermatology and Venereology, 32*(11), 1845-1851. doi:10.1111/jdv.15146

Huang, W., Li, L., Myers, J. R., & Marth, G. T. (2012). ART: a next-generation sequencing read simulator. *Bioinformatics, 28*(4), 593-594. doi:10.1093/bioinformatics/btr708

Hugerth, L. W., Pereira, M., Zha, Y., Seifert, M., Kaldhusdal, V., Boulund, F., Krog, M. C., Bashir, Z., Hamsten, M., Fransson, E., Svarre-Nielsen, H., Schuppe-Koistinen, I., & Engstrand, L. (2020). Assessment of in vitro and in silico protocols for sequence-based characterization of the human vaginal microbiome. *mSphere, 5*(6). doi:10.1128/mSphere.00448-20

Hummelen, R., Fernandes, A. D., Macklaim, J. M., Dickson, R. J., Changalucha, J., Gloor, G. B., & Reid, G. (2010). Deep sequencing of the vaginal microbiota of women with HIV. *PloS One, 5*(8), e12078. doi:10.1371/journal.pone.0012078

Hutchinson, K. B., Kip, K. E., & Ness, R. B. (2007a). Condom use and its association with bacterial vaginosis and bacterial vaginosis-associated vaginal microflora. *Epidemiology, 18*(6), 702-708. doi:10.1097/EDE.0b013e3181567eaa

Hutchinson, K. B., Kip, K. E., Ness, R. B., & Gynecologic Infection Follow-Through, I. (2007b). Vaginal douching and development of bacterial vaginosis among women with normal and abnormal vaginal microflora. *Sexually Transmitted Diseases, 34*(9), 671-675. doi:10.1097/01.olq.0000258435.34879.da

Ingianni, A., Petruzzelli, S., Morandotti, G., & Pompei, R. (1997). Genotypic differentiation of *Gardnerella vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunology and Medical Microbiology, 18*(1), 61-66. doi:10.1111/j.1574-695X.1997.tb01028.x

Isik, G., Demirezen, S., Donmez, H. G., & Beksac, M. S. (2016). Bacterial vaginosis in association with spontaneous abortion and recurrent pregnancy losses. *Journal of Cytology, 33*(3), 135-140. doi:10.4103/0970-9371.188050

Ison, C. A., & Hay, P. E. (2002). Validation of a simplified grading of Gram stained vaginal smears for use in genitourinary medicine clinics. *Sexually Transmitted Infections, 78*(6), 413-415. doi:10.1136/sti.78.6.413

Jacobson, J. C., Turok, D. K., Dermish, A. I., Nygaard, I. E., & Settles, M. L. (2014). Vaginal microbiome changes with levonorgestrel intrauterine system placement. *Contraception, 90*(2), 130-135. doi:10.1016/j.contraception.2014.04.006

Janulaitiene, M., Gegzna, V., Baranauskiene, L., Bulavaite, A., Simanavicius, M., & Pleckaityte, M. (2018). Phenotypic characterization of *Gardnerella vaginalis* subgroups suggests differences in their virulence potential. *PloS One, 13*(7), e0200625. doi:10.1371/journal.pone.0200625

Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A., & Pleckaityte, M. (2017). Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infectious Diseases, 17*(1), 394. doi:10.1186/s12879-017-2501-y

Jervis-Bardy, J., Leong, L. E., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B., & Marsh, R. L. (2015). Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome, 3*, 19. doi:10.1186/s40168-015-0083-8

Jespers, V., Hardy, L., Buyze, J., Loos, J., Buve, A., & Crucitti, T. (2016). Association of sexual debut in adolescents with microbiota and inflammatory markers. *Obstetrics and Gynecology, 128*(1), 22-31. doi:10.1097/AOG.0000000000001468

Jespers, V., Kyongo, J., Joseph, S., Hardy, L., Cools, P., Crucitti, T., Mwaura, M., Ndayisaba, G., Delany-Moretlwe, S., Buyze, J., Vanham, G., & van de Wijgert, J. (2017). A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa. *Scientific Reports, 7*(1), 11974. doi:10.1038/s41598-017-12198-6

Jespers, V., Menten, J., Smet, H., Poradosu, S., Abdellati, S., Verhelst, R., Hardy, L., Buve, A., & Crucitti, T. (2012). Quantification of bacterial species of the vaginal microbiome in different groups of women, using nucleic acid amplification tests. *BMC Microbiology, 12*, 83. doi:10.1186/1471-2180-12-83

Joesoef, M. R., Karundeng, A., Runtupalit, C., Moran, J. S., Lewis, J. S., & Ryan, C. A. (2001). High rate of bacterial vaginosis among women with intrauterine devices in Manado, Indonesia. *Contraception, 64*(3), 169-172.

Juarez Tomas, M. S., Ocana, V. S., Wiese, B., & Nader-Macias, M. E. (2003). Growth and lactic acid production by vaginal *Lactobacillus acidophilus* CRL 1259, and inhibition of uropathogenic *Escherichia coli*. *Journal of Medical Microbiology, 52*(Pt 12), 1117-1124. doi:10.1099/jmm.0.05155-0

Kasper, D. C., Mechtler, T. P., Reischer, G. H., Witt, A., Langgartner, M., Pollak, A., Herkner, K. R., & Berger, A. (2010). The bacterial load of *Ureaplasma parvum* in amniotic fluid is correlated with an increased intrauterine inflammatory response. *Diagnostic Microbiology and Infectious Disease, 67*(2), 117-121. doi:10.1016/j.diagmicrobio.2009.12.023

Kataoka, S., Yamada, T., Chou, K., Nishida, R., Morikawa, M., Minami, M., Yamada, H., Sakuragi, N., & Minakami, H. (2006). Association between preterm birth and vaginal colonization by mycoplasmas in early pregnancy. *Journal of Clinical Microbiology, 44*(1), 51-55. doi:10.1128/JCM.44.1.51-55.2006

Kaul, A., Mandal, S., Davidov, O., & Peddada, S. D. (2017). Analysis of Microbiome Data in the Presence of Excess Zeros. *Frontiers in Microbiology, 8*, 2114. doi:10.3389/fmicb.2017.02114

Kaul, R., Nagelkerke, N. J., Kimani, J., Ngugi, E., Bwayo, J. J., Macdonald, K. S., Rebbaprgada, A., Fonck, K., Temmerman, M., Ronald, A. R., Moses, S., & Kibera, H. I. V. S. G. (2007). Prevalent herpes simplex virus type 2 infection is associated with altered vaginal flora and an increased susceptibility to multiple sexually transmitted infections. *Journal of Infectious Diseases, 196*(11), 1692-1697. doi:10.1086/522006

Kaur, B., Balgir, P. P., Mittu, B., Kumar, B., & Garg, N. (2013). Biomedical applications of fermenticin HV6b isolated from *Lactobacillus fermentum* HV6b MTCC10770. *Biomed Res Int, 2013*, 168438. doi:10.1155/2013/168438

Keane, F. E., Ison, C. A., & Taylor-Robinson, D. (1997). A longitudinal study of the vaginal flora over a menstrual cycle. *International Journal of STD and AIDS, 8*(8), 489-494. doi:10.1258/0956462971920631

Keane, F. E., Thomas, B. J., Gilroy, C. B., Renton, A., & Taylor-Robinson, D. (2000). The association of *Mycoplasma hominis, Ureaplasma urealyticum* and *Mycoplasma genitalium* with bacterial vaginosis: observations on heterosexual women and their male partners. *International Journal of STD and AIDS, 11*(6), 356-360. doi:10.1258/0956462001916056

Keller, M. J., Carpenter, C. A., Lo, Y., Einstein, M. H., Liu, C., Fredricks, D. N., & Herold, B. C. (2012). Phase I randomized safety study of twice daily dosing of acidform vaginal gel: candidate antimicrobial contraceptive. *PloS One, 7*(10), e46901. doi:10.1371/journal.pone.0046901

Kenyon, C., & Osbak, K. (2015). Sexual networks, HIV, race and bacterial vaginosis. *AIDS, 29*(5), 641-642. doi:10.1097/QAD.0000000000000566

Kenyon, C. R., Delva, W., & Brotman, R. M. (2019). Differential sexual network connectivity offers a parsimonious explanation for population-level variations in the prevalence of bacterial vaginosis: a data-driven, model-supported hypothesis. *BMC Women's Health, 19*(1), 8. doi:10.1186/s12905-018-0703-0

Kharsany, A. B., Hoosen, A. A., & Van den Ende, J. (1993). Antimicrobial susceptibilities of *Gardnerella vaginalis*. *Antimicrobial Agents and Chemotherapy, 37*(12), 2733-2735. doi:10.1128/aac.37.12.2733

Kim, Y., Kim, J., & Lee, K. A. (2014). Prevalence of sexually transmitted infections among healthy Korean women: implications of multiplex PCR pathogen detection on antibiotic therapy. *J Infect Chemother, 20*(1), 74-76. doi:10.1016/j.jiac.2013.08.005

Klebanoff, M. A., Schwebke, J. R., Zhang, J., Nansel, T. R., Yu, K. F., & Andrews, W. W. (2004). Vulvovaginal symptoms in women with bacterial vaginosis. *Obstetrics and Gynecology, 104*(2), 267-272. doi:10.1097/01.AOG.0000134783.98382.b0

Klebanoff, S. J., Hillier, S. L., Eschenbach, D. A., & Waltersdorph, A. M. (1991). Control of the microbial flora of the vagina by H2O2-generating lactobacilli. *Journal of Infectious Diseases, 164*(1), 94-100. doi:10.1093/infdis/164.1.94

Knupp de Souza, D. M., Diniz, C. G., Filho, D. S., Andrade de Oliveira, L. M., Coelho, D. M., Talha, L. S., Nascimento, T. C., Ferreira-Machado, A. B., & Silva, V. L. (2016). Antimicrobial susceptibility and vaginolysin in *Gardnerella vaginalis* from healthy and bacterial vaginosis diagnosed women. *J Infect Dev Ctries, 10*(9), 913-919. doi:10.3855/jidc.7161

Koumans, E. H., Markowitz, L. E., Berman, S. M., & St Louis, M. E. (1999). A public health approach to adverse outcomes of pregnancy associated with bacterial vaginosis. *International Journal of Gynaecology and Obstetrics, 67 Suppl 1*, S29-33.

Koumans, E. H., Markowitz, L. E., Hogan, V., & Group, C. B. W. (2002). Indications for therapy and treatment recommendations for bacterial vaginosis in nonpregnant and pregnant women: a synthesis of data. *Clinical Infectious Diseases, 35*(Suppl 2), S152-172. doi:10.1086/342103

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

Koutsky, L. A., Stamm, W. E., Brunham, R. C., Stevens, C. E., Cole, B., Hale, J., Davick, P., & Holmes, K. K. (1983). Persistence of *Mycoplasma hominis* after therapy: importance of tetracycline resistance and of coexisting vaginal flora. *Sexually Transmitted Diseases, 10*(4 Suppl), 374-381.

Kwak, W., Han, Y. H., Seol, D., Kim, H., Ahn, H., Jeong, M., Kang, J., Kim, H., & Kim, T. H. (2020). Complete genome of *Lactobacillus iners* KY using flongle provides insight into the genetic background of optimal adaption to vaginal econiche. *Frontiers in Microbiology, 11*, 1048. doi:10.3389/fmicb.2020.01048

Larsson, P. G., Platz-Christensen, J. J., & Sundstrom, E. (1991). Is bacterial vaginosis a sexually transmitted disease? *International Journal of STD and AIDS, 2*(5), 362-364. doi:10.1177/095646249100200511

Latimer, R. L., Read, T. R. H., Vodstrcil, L. A., Goller, J. L., Ong, J. J., Fairley, C. K., Hocking, J. S., & Bradshaw, C. S. (2019a). Clinical features and therapeutic response in women meeting criteria for presumptive treatment for pelvic inflammatory disease associated With *Mycoplasma genitalium*. *Sexually Transmitted Diseases, 46*(2), 73-79. doi:10.1097/OLQ.0000000000000924

Latimer, R. L., Vodstrcil, L., Read, T., Doyle, M., Fairley, C., Murray, G., Bodiyabadu, K., Kaiser, M., Danielewski, J. A., Mokany, E., Tan, L., Chow, E., Garland, S., & Bradshaw, C. (2019b). Oh MG! The symptoms of *Mycoplasma genitalium* in women. *Sexually Transmitted Infections, 95*.

Lee, C. Y., Cheu, R. K., Lemke, M. M., Gustin, A. T., France, M. T., Hampel, B., Thurman, A. R., Doncel, G. F., Ravel, J., Klatt, N. R., & Arnold, K. B. (2020). Quantitative modeling predicts mechanistic links between pre-treatment microbiome composition and metronidazole efficacy in bacterial vaginosis. *Nat Commun, 11*(1), 6147. doi:10.1038/s41467-020-19880-w

Lehtoranta, L., Hibberd, A. A., Reimari, J., Junnila, J., Yeung, N., Maukonen, J., Crawford, G., & Ouwehand, A. C. (2020). Recovery of vaginal microbiota after standard treatment for bacterial vaginosis infection: an observational study. *Microorganisms, 8*(6). doi:10.3390/microorganisms8060875

Leitich, H., Bodner-Adler, B., Brunbauer, M., Kaider, A., Egarter, C., & Husslein, P. (2003). Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *American Journal of Obstetrics and Gynecology, 189*(1), 139-147. doi:10.1067/mob.2003.339

Leitich, H., & Kiss, H. (2007). Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Practice & Research: Clinical Obstetrics & Gynaecology, 21*(3), 375-390. doi:10.1016/j.bpobgyn.2006.12.005

Leizer, J., Nasioudis, D., Forney, L. J., Schneider, G. M., Gliniewicz, K., Boester, A., & Witkin, S. S. (2018). Properties of epithelial cells and vaginal secretions in pregnant women when *Lactobacillus crispatus* or *Lactobacillus iners* dominate the vaginal microbiome. *Reproductive Sciences, 25*(6), 854-860. doi:10.1177/1933719117698583

Leli, C., Mencacci, A., Latino, M. A., Clerici, P., Rassu, M., Perito, S., Castronari, R., Pistoni, E., Luciano, E., De Maria, D., Morazzoni, C., Pascarella, M., Bozza, S., & Sensini, A. (2018). Prevalence of cervical colonization by *Ureaplasma parvum, Ureaplasma urealyticum, Mycoplasma hominis* and *Mycoplasma genitalium* in childbearing age women by a commercially available multiplex real-time PCR: An Italian observational multicentre study. *Journal of Microbiology, Immunology, and Infection. Wei Mian Yu Gan Ran Za Zhi, 51*(2), 220-225. doi:10.1016/j.jmii.2017.05.004

Lennard, K., Dabee, S., Barnabas, S. L., Havyarimana, E., Blakney, A., Jaumdally, S. Z., Botha, G., Mkhize, N. N., Bekker, L. G., Lewis, D. A., Gray, G., Mulder, N., Passmore, J. S., & Jaspan, H. B. (2018). Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in South African adolescent females. *Infection and Immunity, 86*(1). doi:10.1128/IAI.00410-17

Leopold, S. (1953). Heretofore undescribed organism isolated from the genitourinary system. *United States Armed Forces Medical Journal, 4*(2), 263-266.

Lessard, T., Simoes, J. A., Discacciati, M. G., Hidalgo, M., & Bahamondes, L. (2008). Cytological evaluation and investigation of the vaginal flora of long-term users of the levonorgestrel-releasing intrauterine system (LNG-IUS). *Contraception, 77*(1), 30-33. doi:10.1016/j.contraception.2007.09.009

Lewis, F. M., Bernstein, K. T., & Aral, S. O. (2017). Vaginal microbiome and its relationship to behavior, sexual health, and sexually transmitted diseases. *Obstetrics and Gynecology, 129*(4), 643-654. doi:10.1097/AOG.0000000000001932

Lewis, W. G., Robinson, L. S., Gilbert, N. M., Perry, J. C., & Lewis, A. L. (2013). Degradation, foraging, and depletion of mucus sialoglycans by the vagina-adapted Actinobacterium *Gardnerella vaginalis*. *Journal of Biological Chemistry, 288*(17), 12067-12079. doi:10.1074/jbc.M113.453654

Li, T., Zhang, Z., Wang, F., He, Y., Zong, X., Bai, H., & Liu, Z. (2020). Antimicrobial susceptibility testing of metronidazole and clindamycin against *Gardnerella vaginalis* in planktonic and biofilm formation. *Canadian Journal of Infectious Diseases & Medical Microbiology, 2020*, 1361825. doi:10.1155/2020/1361825

Lillis, R. A., Martin, D. H., & Nsuami, M. J. (2018). *Mycoplasma genitalium* iInfections in women attending a sexually transmitted disease clinic in New Orleans. *Clinical Infectious Diseases*. doi:10.1093/cid/ciy922

Ling, Z., Kong, J., Liu, F., Zhu, H., Chen, X., Wang, Y., Li, L., Nelson, K. E., Xia, Y., & Xiang, C. (2010). Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. *BMC Genomics, 11*, 488. doi:10.1186/1471-2164-11-488

Ling, Z., Liu, X., Luo, Y., Wu, X., Yuan, L., Tong, X., Li, L., & Xiang, C. (2013). Associations between vaginal pathogenic community and bacterial vaginosis in Chinese reproductive-age women. *PloS One, 8*(10), e76589. doi:10.1371/journal.pone.0076589

Lis, R., Rowhani-Rahbar, A., & Manhart, L. E. (2015). *Mycoplasma genitalium* infection and female reproductive tract disease: a meta-analysis. *Clinical Infectious Diseases, 61*(3), 418-426. doi:10.1093/cid/civ312

Liu, C. M., Hungate, B. A., Tobian, A. A., Ravel, J., Prodger, J. L., Serwadda, D., Kigozi, G., Galiwango, R. M., Nalugoda, F., Keim, P., Wawer, M. J., Price, L. B., & Gray, R. H. (2015). Penile microbiota and female partner bacterial vaginosis in Rakai, Uganda. *mBio, 6*(3), e00589. doi:10.1128/mBio.00589-15

Liu, C. M., Hungate, B. A., Tobian, A. A., Serwadda, D., Ravel, J., Lester, R., Kigozi, G., Aziz, M., Galiwango, R. M., Nalugoda, F., Contente-Cuomo, T. L., Wawer, M. J., Keim, P., Gray, R. H., & Price, L. B. (2013). Male circumcision significantly reduces prevalence and load of genital anaerobic bacteria. *mBio, 4*(2), e00076. doi:10.1128/mBio.00076-13

Liu, C. M., Prodger, J. L., Tobian, A. A., Serwadda, D., Galiwango, R. M., Nalugoda, F., Kighoma, N., Mwinike, J., Anyokorit, M., Price, L. B., Wawer, M. J., Kigozi, G., & Gray, R. H. (2016). Genital anaerobic bacterial overgrowth and the PrePex male circumcision device, Rakai, Uganda. *Journal of Infectious Diseases, 214*(4), 595-598. doi:10.1093/infdis/jiw182

Liu, C. M., Prodger, J. L., Tobian, A. A. R., Abraham, A. G., Kigozi, G., Hungate, B. A., Aziz, M., Nalugoda, F., Sariya, S., Serwadda, D., Kaul, R., Gray, R. H., & Price, L. B. (2017). Penile anaerobic dysbiosis as a risk factor for HIV infection. *mBio, 8*(4). doi:10.1128/mBio.00996-17

Lobao, T. N., Campos, G. B., Selis, N. N., Amorim, A. T., Souza, S. G., Mafra, S. S., Pereira, L. S., Dos Santos, D. B., Figueiredo, T. B., Marques, L. M., & Timenetsky, J. (2017). *Ureaplasma urealyticum* and *U. parvum* in sexually active women attending public health clinics in Brazil. *Epidemiology and Infection, 145*(11), 2341-2351. doi:10.1017/S0950268817001145

Lofmark, S., Edlund, C., & Nord, C. E. (2010). Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clinical Infectious Diseases, 50 Suppl 1*, S16-23. doi:10.1086/647939

Low, N., Chersich, M. F., Schmidlin, K., Egger, M., Francis, S. C., van de Wijgert, J. H., Hayes, R. J., Baeten, J. M., Brown, J., Delany-Moretlwe, S., Kaul, R., McGrath, N., Morrison, C., Myer, L., Temmerman, M., van der Straten, A., Watson-Jones, D., Zwahlen, M., & Hilber, A. M. (2011). Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS Medicine, 8*(2), e1000416. doi:10.1371/journal.pmed.1000416

Lusk, M. J., Konecny, P., Naing, Z. W., Garden, F. L., Cumming, R. G., & Rawlinson, W. D. (2011). *Mycoplasma genitalium* is associated with cervicitis and HIV infection in an urban Australian STI clinic population. *Sexually Transmitted Infections, 87*(2), 107-109. doi:10.1136/sti.2010.045138

Machado, A., & Cerca, N. (2015). Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *Journal of Infectious Diseases, 212*(12), 1856-1861. doi:10.1093/infdis/jiv338

Machado, A., Jefferson, K. K., & Cerca, N. (2013). Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm

formation. *International Journal of Molecular Sciences, 14*(6), 12004-12012. doi:10.3390/ijms140612004

Macklaim, J. M., Fernandes, A. D., Di Bella, J. M., Hammond, J. A., Reid, G., & Gloor, G. B. (2013). Comparative meta-RNA-seq of the vaginal microbiota and differential expression by *Lactobacillus iners* in health and dysbiosis. *Microbiome, 1*(1), 12. doi:10.1186/2049-2618-1-12

Madden, T., Grentzer, J. M., Secura, G. M., Allsworth, J. E., & Peipert, J. F. (2012). Risk of bacterial vaginosis in users of the intrauterine device: a longitudinal study. *Sexually Transmitted Diseases, 39*(3), 217-222. doi:10.1097/OLQ.0b013e31823e68fe

Malaguti, N., Bahls, L. D., Uchimura, N. S., Gimenes, F., & Consolaro, M. E. (2015). Sensitive Detection of Thirteen Bacterial Vaginosis-Associated Agents Using Multiplex Polymerase Chain Reaction. *Biomed Res Int, 2015*, 645853. doi:10.1155/2015/645853

Mandal, S., Van Treuren, W., White, R. A., Eggesbo, M., Knight, R., & Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial Ecology in Health and Disease, 26*, 27663. doi:10.3402/mehd.v26.27663

Mandar, R., Punab, M., Borovkova, N., Lapp, E., Kiiker, R., Korrovits, P., Metspalu, A., Krjutskov, K., Nolvak, H., Preem, J. K., Oopkaup, K., Salumets, A., & Truu, J. (2015). Complementary seminovaginal microbiome in couples. *Research in Microbiology, 166*(5), 440-447. doi:10.1016/j.resmic.2015.03.009

Manhanzva, M. T., Abrahams, A. G., Gamieldien, H., Froissart, R., Jaspan, H., Jaumdally, S. Z., Barnabas, S. L., Dabee, S., Bekker, L. G., Gray, G., Passmore, J. S., & Masson, L. (2020). Inflammatory and antimicrobial properties differ between vaginal *Lactobacillus* isolates from South African women with non-optimal versus optimal microbiota. *Scientific Reports, 10*(1), 6196. doi:10.1038/s41598-020-62184-8

Marrazzo, J. M. (2011). Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused? *Anaerobe, 17*(4), 186-190. doi:10.1016/j.anaerobe.2011.03.016

Marrazzo, J. M., Antonio, M., Agnew, K., & Hillier, S. L. (2009). Distribution of genital *Lactobacillus* strains shared by female sex partners. *Journal of Infectious Diseases, 199*(5), 680-683. doi:10.1086/596632

Marrazzo, J. M., Fiedler, T. L., Srinivasan, S., Thomas, K. K., Liu, C., Ko, D., Xie, H., Saracino, M., & Fredricks, D. N. (2012). Extravaginal reservoirs of vaginal bacteria as risk factors for incident bacterial vaginosis. *Journal of Infectious Diseases, 205*(10), 1580-1588. doi:10.1093/infdis/jis242

Marrazzo, J. M., Koutsky, L. A., Eschenbach, D. A., Agnew, K., Stine, K., & Hillier, S. L. (2002). Characterization of vaginal flora and bacterial vaginosis in women who have sex with women. *Journal of Infectious Diseases, 185*(9), 1307-1313. doi:10.1086/339884

Marrazzo, J. M., Thomas, K. K., Agnew, K., & Ringwood, K. (2010a). Prevalence and risks for bacterial vaginosis in women who have sex with women. *Sexually Transmitted Diseases, 37*(5), 335-339.

Marrazzo, J. M., Thomas, K. K., Fiedler, T. L., Ringwood, K., & Fredricks, D. N. (2010b). Risks for acquisition of bacterial vaginosis among women who report sex with women: a cohort study. *PloS One, 5*(6), e11139. doi:10.1371/journal.pone.0011139

Marrazzo, J. M., Thomas, K. K., & Ringwood, K. (2011). A behavioural intervention to reduce persistence of bacterial vaginosis among women who report sex with women: results of a randomised trial. *Sexually Transmitted Infections, 87*(5), 399-405. doi:10.1136/sti.2011.049213

Martin, D. H. (2012). The microbiota of the vagina and its influence on women's health and disease. *American Journal of the Medical Sciences, 343*(1), 2-9. doi:10.1097/MAJ.0b013e31823ea228

Martin, H. L., Richardson, B. A., Nyange, P. M., Lavreys, L., Hillier, S. L., Chohan, B., Mandaliya, K., Ndinya-Achola, J. O., Bwayo, J., & Kreiss, J. (1999). Vaginal lactobacilli, microbial flora, and

risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *Journal of Infectious Diseases, 180*(6), 1863-1868. doi:10.1086/315127

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal, 17*(1), 10-12.

Mayer, B. T., Srinivasan, S., Fiedler, T. L., Marrazzo, J. M., Fredricks, D. N., & Schiffer, J. T. (2015). Rapid and profound shifts in the vaginal microbiota following antibiotic treatment for bacterial vaginosis. *Journal of Infectious Diseases, 212*(5), 793-802. doi:10.1093/infdis/jiv079

McCaffrey, M., Varney, P., Evans, B., & Taylor-Robinson, D. (1999). Bacterial vaginosis in lesbians: evidence for lack of sexual transmission. *Int. J. STD. AIDS., 10*(5), 305-308.

McCormack, W. M., Almeida, P. C., Bailey, P. E., Grady, E. M., & Lee, Y. H. (1972). Sexual activity and vaginal colonization with genital mycoplasmas. *JAMA, 221*(12), 1375-1377.

McCormack, W. M., Lee, Y. H., & Zinner, S. H. (1973). Sexual experience and urethral colonization with genital mycoplasmas. A study in normal men. *Annals of Internal Medicine, 78*(5), 696-698. doi:10.7326/0003-4819-78-5-696

McKinnon, L. R., Achilles, S. L., Bradshaw, C. S., Burgener, A., Crucitti, T., Fredricks, D. N., Jaspan, H. B., Kaul, R., Kaushic, C., Klatt, N., Kwon, D. S., Marrazzo, J. M., Masson, L., McClelland, R. S., Ravel, J., van de Wijgert, J., Vodstrcil, L. A., & Tachedjian, G. (2019). The evolving facets of bacterial vaginosis: implications for HIV transmission. *AIDS Research and Human Retroviruses, 35*(3), 219-228. doi:10.1089/AID.2018.0304

McMillan, A., MacKlaim, J. M., Burton, J. P., & Reid, G. (2013). Adhesion of *Lactobacillus iners* AB-1 to human fibronectin: A key mediator for persistence in the vagina? *Reproductive Sciences, 20*(7), 791-796. doi:10.1177/1933719112466306

Mehta, S. D. (2012). Systematic review of randomized trials of treatment of male sexual partners for improved bacteria vaginosis outcomes in women. *Sexually Transmitted Diseases, 39*(10), 822-830. doi:10.1097/OLQ.0b013e3182631d89

Mehta, S. D., Agingu, W., Nordgren, R. K., Green, S. J., Bhaumik, D. K., Bailey, R. C., & Otieno, F. (2020a). Characteristics of women and their male sex partners predict bacterial vaginosis among a prospective cohort of Kenyan women with nonoptimal vaginal microbiota. *Sexually Transmitted Diseases, 47*(12), 840-850. doi:10.1097/OLQ.0000000000001259

Mehta, S. D., Donovan, B., Weber, K. M., Cohen, M., Ravel, J., Gajer, P., Gilbert, D., Burgad, D., & Spear, G. T. (2015). The vaginal microbiota over an 8- to 10-year period in a cohort of HIV-infected and HIV-uninfected women. *PloS One, 10*(2), e0116894. doi:10.1371/journal.pone.0116894

Mehta, S. D., Green, S. J., Maclean, I., Hu, H., Bailey, R. C., Gillevet, P. M., & Spear, G. T. (2012). Microbial diversity of genital ulcer disease in men enrolled in a randomized trial of male circumcision in Kisumu, Kenya. *PloS One, 7*(7), e38991. doi:10.1371/journal.pone.0038991

Mehta, S. D., Zhao, D., Green, S. J., Agingu, W., Otieno, F., Bhaumik, R., Bhaumik, D., & Bailey, R. C. (2020b). The microbiome composition of a man's penis predicts incident bacterial vaginosis in his female sex partner with high accuracy. *Front Cell Infect Microbiol, 10*, 433. doi:10.3389/fcimb.2020.00433

Melkumyan, A. R., Priputnevich, T. V., Ankirskaya, A. S., Murav'eva, V. V., & Lubasovskaya, L. A. (2015). Effects of antibiotic treatment on the *Lactobacillus* composition of vaginal microbiota. *Bulletin of Experimental Biology and Medicine, 158*(6), 766-768. doi:10.1007/s10517-015-2857-1

Menard, J. P., Fenollar, F., Henry, M., Bretelle, F., & Raoult, D. (2008). Molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. *Clinical Infectious Diseases, 47*(1), 33-43. doi:10.1086/588661

Mengel, M. B., Berg, A. O., Weaver, C. H., Herman, D. J., Herman, S. J., Hughes, V. L., & Koepsell, T. D. (1989). The effectiveness of single-dose metronidazole therapy for patients and their partners with bacterial vaginosis. *Journal of Family Practice, 28*(2), 163-171.

Miller, E. A., Beasley, D. E., Dunn, R. R., & Archie, E. A. (2016). Lactobacilli dominance and vaginal pH: why is the human vaginal microbiome unique? *Frontiers in Microbiology, 7*, 1936. doi:10.3389/fmicb.2016.01936

Mirmonsef, P., Hotton, A. L., Gilbert, D., Burgad, D., Landay, A., Weber, K. M., Cohen, M., Ravel, J., & Spear, G. T. (2014). Free glycogen in vaginal fluids is associated with *Lactobacillus* colonization and low vaginal pH. *PloS One, 9*(7), e102467. doi:10.1371/journal.pone.0102467

Mirmonsef, P., Hotton, A. L., Gilbert, D., Gioia, C. J., Maric, D., Hope, T. J., Landay, A. L., & Spear, G. T. (2016). Glycogen levels in undiluted genital fluid and their relationship to vaginal pH, estrogen, and progesterone. *PloS One, 11*(4), e0153553. doi:10.1371/journal.pone.0153553

Mirmonsef, P., Modur, S., Burgad, D., Gilbert, D., Golub, E. T., French, A. L., McCotter, K., Landay, A. L., & Spear, G. T. (2015). Exploratory comparison of vaginal glycogen and *Lactobacillus* levels in premenopausal and postmenopausal women. *Menopause, 22*(7), 702-709. doi:10.1097/GME.0000000000000397

Mitchell, C. M., Fredricks, D. N., Winer, R. L., & Koutsky, L. (2012). Effect of sexual debut on vaginal microbiota in a cohort of young women. *Obstetrics and Gynecology, 120*(6), 1306-1313. doi:http://10.1097/AOG.0b013e31827075ac

Mitchell, C. M., Manhart, L. E., Thomas, K. K., Agnew, K., & Marrazzo, J. M. (2011). Effect of sexual activity on vaginal colonization with hydrogen peroxide-producing lactobacilli and *Gardnerella vaginalis*. *Sexually Transmitted Diseases, 38*(12), 1137-1144. doi:10.1097/OLQ.0b013e31822e6121

Mitchell, C. M., Moreira, C., Fredricks, D., Paul, K., Caliendo, A. M., Kurpewski, J., Ingersoll, J., & Cu-Uvin, S. (2009). Detection of fastidious vaginal bacteria in women with HIV infection and bacterial vaginosis. *Infectious Diseases in Obstetrics and Gynecology, 2009*, 236919. doi:10.1155/2009/236919

Mitchell, C. M., Srinivasan, S., Zhan, X., Wu, M. C., Reed, S. D., Guthrie, K. A., LaCroix, A. Z., Fiedler, T., Munch, M., Liu, C., Hoffman, N. G., Blair, I. A., Newton, K., Freeman, E. W., Joffe, H., Cohen, L., & Fredricks, D. N. (2017). Vaginal microbiota and genitourinary menopausal symptoms: a cross-sectional analysis. *Menopause, 24*(10), 1160-1166. doi:10.1097/GME.0000000000000904

Moher, D., Shamseer, L., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., Shekelle, P., Stewart, L. A., & Group, P.-P. (2015). Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst Rev, 4*, 1. doi:10.1186/2046-4053-4-1

Moi, H. (1990). Prevalence of bacterial vaginosis and its association with genital infections, inflammation, and contraceptive methods in women attending sexually transmitted disease and primary health clinics. *International Journal of STD and AIDS, 1*(2), 86-94. doi:10.1177/095646249000100203

Moi, H., Erkkola, R., Jerve, F., Nelleman, G., Bymose, B., Alaksen, K., & Tornqvist, E. (1989). Should male consorts of women with bacterial vaginosis be treated? *Genitourinary Medicine, 65*(4), 263-268.

Murphy, K., & Mitchell, C. M. (2016). The interplay of host immunity, environment and the risk of bacterial vaginosis and associated reproductive health outcomes. *Journal of Infectious Diseases, 214 Suppl 1*, S29-35. doi:10.1093/infdis/jiw140

Muzny, C. A. (2018). Should partners of women with bacterial vaginosis be treated? *Contemporary OB/GYN, August 2018*.

Muzny, C. A., & Kardas, P. (2020). A narrative review of current challenges in the diagnosis and management of bacterial vaginosis. *Sexually Transmitted Diseases, 47*(7), 441-446. doi:10.1097/OLQ.0000000000001178

Muzny, C. A., Laniewski, P., Schwebke, J. R., & Herbst-Kralovetz, M. M. (2019a). Host-vaginal microbiota interactions in the pathogenesis of bacterial vaginosis. *Current Opinion in Infectious Diseases*. doi:10.1097/QCO.0000000000000620

Muzny, C. A., Lensing, S. Y., Aaron, K. J., & Schwebke, J. R. (2019b). Incubation period and risk factors support sexual transmission of bacterial vaginosis in women who have sex with women. *Sexually Transmitted Infections, 0*(0), 1-5. doi:10.1136/sextrans-2018-053824

Muzny, C. A., & Schwebke, J. R. (2013). *Gardnerella vaginalis:* Still a Prime Suspect in the Pathogenesis of Bacterial Vaginosis. *Current Infectious Disease Reports, 15*(2), 130-135. doi:10.1007/s11908-013-0318-4

Muzny, C. A., & Schwebke, J. R. (2015). Biofilms: an underappreciated mechanism of treatment failure and recurrence in vaginal infections. *Clinical Infectious Diseases, 61*(4), 601-606. doi:10.1093/cid/civ353

Muzny, C. A., & Schwebke, J. R. (2020). Asymptomatic Bacterial Vaginosis: To Treat or Not to Treat? *Current Infectious Disease Reports, 22*(12). doi:10.1007/s11908-020-00740-z

Muzny, C. A., Sunesara, I. R., Kumar, R., Mena, L. A., Griswold, M. E., Martin, D. H., Lefkowitz, E. J., Schwebke, J. R., & Swiatlo, E. (2013). Characterization of the vaginal microbiota among sexual risk behavior groups of women with bacterial vaginosis. *PloS One, 8*(11), e80254. doi:10.1371/journal.pone.0080254

Muzny, C. A., Taylor, C. M., Swords, W. E., Tamhane, A., Chattopadhyay, D., Cerca, N., & Schwebke, J. R. (2019c). An updated conceptual model on the pathogenesis of bacterial vaginosis. *Journal of Infectious Diseases, 220*(9), 1399-1405. doi:10.1093/infdis/jiz342

Muzny, C. A., Van Der Pol, W. J., Lefkowitz, E. J., Ghosh, A., Li, M., Redden, D. T., Cui, X., & Schwebke, J. (2018). Genital microbiomes of women with recurrent bacterial vaginosis and their regular male sexual partner. *Journal of Clinical and Translational Science, 2*, 13.

Myer, L., Kuhn, L., Stein, Z. A., Wright, T. C., Jr., & Denny, L. (2005). Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *Lancet Infectious Diseases, 5*(12), 786-794. doi:10.1016/S1473-3099(05)70298-X

Nagaraja, P. (2008). Antibiotic resistance of *Gardnerella vaginalis* in recurrent bacterial vaginosis. *Indian Journal of Medical Microbiology, 26*(2), 155-157. doi:10.4103/0255-0857.40531

Nardini, P., Nahui Palomino, R. A., Parolin, C., Laghi, L., Foschi, C., Cevenini, R., Vitali, B., & Marangoni, A. (2016). *Lactobacillus crispatus* inhibits the infectivity of *Chlamydia trachomatis* elementary bodies, in vitro study. *Scientific Reports, 6*, 29024. doi:10.1038/srep29024

Nasioudis, D., Beghini, J., Bongiovanni, A. M., Giraldo, P. C., Linhares, I. M., & Witkin, S. S. (2015). alpha-Amylase in vaginal fluid: association with conditions favorable to dominance of *Lactobacillus*. *Reproductive Sciences, 22*(11), 1393-1398. doi:10.1177/1933719115581000

Nelson, A. L. (2018). An overview of properties of Amphora (Acidform) contraceptive vaginal gel. *Expert Opinion on Drug Safety, 17*(9), 935-943. doi:10.1080/14740338.2018.1515197

Nelson, D. B., Hanlon, A., Hassan, S., Britto, J., Geifman-Holtzman, O., Haggerty, C., & Fredricks, D. N. (2009). Preterm labor and bacterial vaginosis-associated bacteria among urban women. *Journal of Perinatal Medicine, 37*(2), 130-134. doi:10.1515/JPM.2009.026

Nelson, D. E., Dong, Q., Van der Pol, B., Toh, E., Fan, B., Katz, B. P., Mi, D., Rong, R., Weinstock, G. M., Sodergren, E., & Fortenberry, J. D. (2012). Bacterial communities of the coronal sulcus and distal urethra of adolescent males. *PloS One, 7*(5), e36298. doi:10.1371/journal.pone.0036298

Nelson, D. E., Van Der Pol, B., Dong, Q., Revanna, K. V., Fan, B., Easwaran, S., Sodergren, E., Weinstock, G. M., Diao, L., & Fortenberry, J. D. (2010). Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. *PloS One, 5*(11), e14116. doi:10.1371/journal.pone.0014116

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

Ness, R. B., Hillier, S. L., Richter, H. E., Soper, D. E., Stamm, C., McGregor, J., Bass, D. C., Sweet, R. L., & Rice, P. (2002). Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina. *Obstetrics and Gynecology, 100*(4), 765.

Ness, R. B., Kip, K. E., Hillier, S. L., Soper, D. E., Stamm, C. A., Sweet, R. L., Rice, P., & Richter, H. E. (2005). A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *American Journal of Epidemiology, 162*(6), 585-590. doi:10.1093/aje/kwi243

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution, 32*(1), 268-274. doi:10.1093/molbev/msu300

Nugent, R. P., Krohn, M. A., & Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *Journal of Clinical Microbiology, 29*(2), 297-301.

O'Hanlon, D. E., Come, R. A., & Moench, T. R. (2019). Vaginal pH measured *in vivo*: lactobacilli determine pH and lactic acid concentration. *BMC Microbiology, 19*(1), 13. doi:10.1186/s12866-019-1388-8

O'Hanlon, D. E., Moench, T. R., & Cone, R. A. (2011). In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BMC Infectious Diseases, 11*, 200.

O'Hanlon, D. E., Moench, T. R., & Cone, R. A. (2013). Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PloS One, 8*(11), e80074. doi:10.1371/journal.pone.0080074

Oakley, B. B., Fiedler, T. L., Marrazzo, J. M., & Fredricks, D. N. (2008). Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Applied and Environmental Microbiology, 74*(15), 4898-4909. doi:10.1128/AEM.02884-07

Oduyebo, O. O., Anorlu, R. I., & Ogunsola, F. T. (2009). The effects of antimicrobial therapy on bacterial vaginosis in non-pregnant women. *Cochrane Database of Systematic Reviews*(3), CD006055. doi:10.1002/14651858.CD006055.pub2

Oksanen , J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2020). vegan: community ecology package (Version R package version 2.5-7). Retrieved from https://CRAN.R-project.org/package=vegan

Onderdonk, A. B., Delaney, M. L., & Fichorova, R. N. (2016). The human microbiome during bacterial vaginosis. *Clinical Microbiology Reviews, 29*(2), 223-238. doi:10.1128/CMR.00075-15

Onywera, H., Williamson, A. L., Ponomarenko, J., & Meiring, T. L. (2020). The penile microbiota in uncircumcised and circumcised men: relationships with HIV and human papillomavirus infections and cervicovaginal microbiota. *Front Med (Lausanne), 7*, 383. doi:10.3389/fmed.2020.00383

Ouzounova-Raykova, V. V., Markovska, R., Mizgova, G., & Mitov, I. G. (2011). Detection of the sexually transmissible genital mycoplasmas by polymerase chain reaction in women. *Sex Health, 8*(3), 445-446. doi:10.1071/SH11044

Paramel Jayaprakash, T., Schellenberg, J. J., & Hill, J. E. (2012). Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PloS One, 7*(8), e43009. doi:10.1371/journal.pone.0043009

Patterson, J. L., Stull-Lane, A., Girerd, P. H., & Jefferson, K. K. (2010). Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology, 156*(Pt 2), 392-399. doi:10.1099/mic.0.034280-0

Payne, M. S., Ireland, D. J., Watts, R., Nathan, E. A., Furfaro, L. L., Kemp, M. W., Keelan, J. A., & Newnham, J. P. (2016). *Ureaplasma parvum* genotype, combined vaginal colonisation with

Candida albicans, and spontaneous preterm birth in an Australian cohort of pregnant women. *BMC Pregnancy and Childbirth, 16*(1), 312. doi:10.1186/s12884-016-1110-x

Payne, M. S., Newnham, J. P., Doherty, D. A., Furfaro, L. L., Pendal, N. L., Loh, D. E., & Keelan, J. A. (2020). A specific bacterial DNA signature in the vagina of Australian women in midpregnancy predicts high risk of spontaneous preterm birth (the Predict1000 study). *American Journal of Obstetrics and Gynecology*. doi:10.1016/j.ajog.2020.08.034

Payne, S. C., Cromer, P. R., Stanek, M. K., & Palmer, A. A. (2010). Evidence of African-American women's frustrations with chronic recurrent bacterial vaginosis. *Journal of the American Academy of Nurse Practitioners, 22*(2), 101-108. doi:10.1111/j.1745-7599.2009.00474.x

Pedraza-Aviles, A. G., Inzunza-Montiel, A. E., Ortiz-Zaragoza, M. C., Morales-Espinosa, M. R., & Ponce-Rosas, E. R. (1995). [*Gardnerella vaginalis* biotypes: modification of a proposed system]. *Revista Latinoamericana de Microbiología, 37*(1), 19-26.

Peebles, K., Kiweewa, F. M., Palanee-Phillips, T., Chappell, C., Singh, D., Bunge, K. E., Naidoo, L., Makanani, B., Jeenarain, N., Reynolds, D., Hillier, S. L., Brown, E. R., Baeten, J. M., Balkus, J. E., & team, M. T. N. A. s. (2020). Elevated Risk of Bacterial Vaginosis among Users of the Copper Intrauterine Device: A Prospective Longitudinal Cohort Study. *Clinical Infectious Diseases*. doi:10.1093/cid/ciaa703

Peebles, K., Velloza, J., Balkus, J. E., McClelland, R. S., & Barnabas, R. V. (2019). High global burden and costs of bacterial vaginosis: a systematic review and meta-analysis. *Sexually Transmitted Diseases, 46*(5), 304-311. doi:10.1097/OLQ.0000000000000972

Peipert, J. F., Montagno, A. B., Cooper, A. S., & Sung, C. J. (1997). Bacterial vaginosis as a risk factor for upper genital tract infection. *American Journal of Obstetrics and Gynecology, 177*(5), 1184-1187. doi:10.1016/s0002-9378(97)70038-3

Pepin, J., Deslandes, S., Giroux, G., Sobela, F., Khonde, N., Diakite, S., Demeule, S., Labbe, A. C., Carrier, N., & Frost, E. (2011). The complex vaginal flora of West African women with bacterial vaginosis. *PloS One, 6*(9), e25082. doi:10.1371/journal.pone.0025082

Petrova, M. I., Reid, G., Vaneechoutte, M., & Lebeer, S. (2017). *Lactobacillus iners*: friend or foe? *Trends in Microbiology, 25*(3), 182-191. doi:10.1016/j.tim.2016.11.007

Pheifer, T. A., Forsyth, P. S., Durfee, M. A., Pollock, H. M., & Holmes, K. K. (1978). Nonspecific vaginitis: role of *Haemophilus vaginalis* and treatment with metronidazole. *New England Journal of Medicine, 298*(26), 1429-1434. doi:10.1056/NEJM197806292982601

Piot, P., Van Dyck, E., Peeters, M., Hale, J., Totten, P. A., & Holmes, K. K. (1984). Biotypes of *Gardnerella vaginalis*. *Journal of Clinical Microbiology, 20*(4), 677-679.

Plummer, E. L., Vodstrcil, L. A., Danielewski, J. A., Murray, G. L., Fairley, C. K., Garland, S. M., Hocking, J. S., Tabrizi, S. N., & Bradshaw, C. S. (2018a). Combined oral and topical antimicrobial therapy for male partners of women with bacterial vaginosis: Acceptability, tolerability and impact on the genital microbiota of couples - A pilot study. *PloS One, 13*(1), e0190199. doi:10.1371/journal.pone.0190199

Plummer, E. L., Vodstrcil, L. A., Fairley, C. K., Tabrizi, S. N., Garland, S. M., Law, M. G., Hocking, J. S., Fethers, K. A., Bulach, D. M., Murray, G. L., & Bradshaw, C. S. (2019a). Sexual practices have a significant impact on the vaginal microbiota of women who have sex with women. *Scientific Reports, 9*(1), 19749. doi:10.1038/s41598-019-55929-7

Plummer, E. L., Vodstrcil, L. A., Murray, G. L., Fairley, C. K., Danielewski, J. A., Garland, S. M., Chow, E. P. F., Bulach, D. M., Fethers, K. A., Hocking, J. S., & Bradshaw, C. S. (2019b). *Gardnerella vaginalis* clade distribution is associated with behavioural practices and Nugent Score in women who have sex with women. *Journal of Infectious Diseases*. doi:10.1093/infdis/jiz474

Plummer, E. L., Vodstrcil, L. A., Murray, G. L., Tabrizi, S. N., Garland, S. M., Fairley, C. K., Tan, A., Law, M. G., Hocking, J. S., Bulach, D. M., Philip, G. K., & Bradshaw, C. S. (2018b). *Sexual behaviours impact the vaginal microbiota of women who have sex with women*. Paper presented at the IUSTI Asia Pacific Sexual Health Congress, Pullman Hotel, Auckland, New Zealand.

Pohl, H. G., Groah, S. L., Perez-Losada, M., Ljungberg, I., Sprague, B. M., Chandal, N., Caldovic, L., & Hsieh, M. (2020). The Urine Microbiome of Healthy Men and Women Differs by Urine Collection Method. *International Neurourology Journal, 24*(1), 41-51. doi:10.5213/inj.1938244.122

Pollack, J. D. (1992). Carbohydrate metabolism and energy conservation. In J. Maniloff, R. N. McElhaney, L. R. Finch, & J. B. Baseman (Eds.), *Mycoplasmas: molecular biology and pathogenesis* (pp. 181–200). Washington D. C: American Society for Microbiology.

Potter, J. (1999). Should sexual partners of women with bacterial vaginosis receive treatment? *British Journal of General Practice, 49*(448), 913-918.

Price, L. B., Liu, C. M., Johnson, K. E., Aziz, M., Lau, M. K., Bowers, J., Ravel, J., Keim, P. S., Serwadda, D., Wawer, M. J., & Gray, R. H. (2010). The effects of circumcision on the penis microbiome. *PloS One, 5*(1), e8422. doi:10.1371/journal.pone.0008422

Prodger, J. L., Abraham, A. G., Tobian, A. A., Park, D. E., Aziz, M., Roach, K., Gray, R. H., Buchanan, L., Kigozi, G., Galiwango, R. M., Ssekasanvu, J., Nnamutete, J., Kagaayi, J., Kaul, R., & Liu, C. M. (2021). Penile bacteria associated with HIV seroconversion, inflammation, and immune cells. *JCI Insight, 6*(8). doi:10.1172/jci.insight.147363

Purwar, M., Ughade, S., Bhagat, B., Agarwal, V., & Kulkarni, H. (2001). Bacterial vaginosis in early pregnancy and adverse pregnancy outcome. *Journal of Obstetrics and Gynaecology Research, 27*(4), 175-181. doi:10.1111/j.1447-0756.2001.tb01248.x

Putonti, C., Thomas-White, K., Crum, E., Hilt, E. E., Price, T. K., & Wolfe, A. J. (2021). Genome Investigation of Urinary *Gardnerella* Strains and Their Relationship to Isolates of the Vaginal Microbiota. *mSphere, 6*(3). doi:10.1128/mSphere.00154-21

Pybus, V., & Onderdonk, A. B. (1997). Evidence for a commensal, symbiotic relationship between *Gardnerella vaginalis* and *Prevotella bivia* involving ammonia: potential significance for bacterial vaginosis. *Journal of Infectious Diseases, 175*(2), 406-413. doi:10.1093/infdis/175.2.406

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glockner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research, 41*(Database issue), D590-596. doi:10.1093/nar/gks1219

R Core Team. (2020). R: a language and environment for statistical computing.

Ralph, S. G., Rutherford, A. J., & Wilson, J. D. (1999). Influence of bacterial vaginosis on conception and miscarriage in the first trimester: cohort study. *BMJ, 319*(7204), 220-223. doi:10.1136/bmj.319.7204.220

Rampersaud, R., Planet, P. J., Randis, T. M., Kulkarni, R., Aguilar, J. L., Lehrer, R. I., & Ratner, A. J. (2011). Inerolysin, a cholesterol-dependent cytolysin produced by *Lactobacillus iners*. *Journal of Bacteriology, 193*(5), 1034-1041. doi:10.1128/JB.00694-10

Ratten, L. K., Plummer, E. L., Murray, G. L., Danielewski, J., Fairley, C. K., Garland, S. M., Hocking, J. S., Tachedjian, G., Chow, E., Bradshaw, C. S., & Vodstrcil, L. A. (2021). Sex is associated with the persistence of non-optimal vaginal microbiota following treatment for bacterial vaginosis: a prospective cohort study. *BJOG: An International Journal of Obstetrics and Gynaecology, 128*(4), 756-767. doi:10.1111/1471-0528.16430

Ravel, J., Brotman, R. M., Gajer, P., Ma, B., Nandy, M., Fadrosh, D. W., Sakamoto, J., Koenig, S. S., Fu, L., Zhou, X., Hickey, R. J., Schwebke, J. R., & Forney, L. J. (2013). Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. *Microbiome, 1*(1), 29. doi:10.1186/2049-2618-1-29

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L., & Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America, 108 Suppl 1*, 4680-4687. doi:10.1073/pnas.1002611107

Rezk, M., Elshamy, E., Shaheen, A. E., Shawky, M., & Marawan, H. (2019). Effects of a levonorgestrel intrauterine system versus a copper intrauterine device on menstrual changes and uterine artery Doppler. *International Journal of Gynaecology and Obstetrics, 145*(1), 18-22. doi:10.1002/ijgo.12778

Rittenschober-Bohm, J., Waldhoer, T., Schulz, S. M., Pimpel, B., Goeral, K., Kasper, D. C., Witt, A., & Berger, A. (2019). Vaginal *Ureaplasma parvum* serovars and spontaneous preterm birth. *American Journal of Obstetrics and Gynecology, 220*(6), 594 e591-594 e599. doi:10.1016/j.ajog.2019.01.237

Rittenschober-Bohm, J., Waldhoer, T., Schulz, S. M., Stihsen, B., Pimpel, B., Goeral, K., Hafner, E., Sliutz, G., Kasper, D. C., Witt, A., & Berger, A. (2018). First trimester vaginal *Ureaplasma* biovar colonization and preterm birth: results of a prospective multicenter study. *Neonatology, 113*(1), 1-6. doi:10.1159/000480065

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., Galuppi, M., Lamont, R. F., Chaemsaithong, P., Miranda, J., Chaiworapongsa, T., & Ravel, J. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome, 2*(1), 4. doi:10.1186/2049-2618-2-4

Rose, W. A., 2nd, McGowin, C. L., Spagnuolo, R. A., Eaves-Pyles, T. D., Popov, V. L., & Pyles, R. B. (2012). Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures. *PloS One, 7*(3), e32728. doi:10.1371/journal.pone.0032728

RStudio Team. (2020). RStudio: integrated development environment for R.

Rumyantseva, T., Khayrullina, G., Guschin, A., & Donders, G. (2019). Prevalence of *Ureaplasma* spp. and *Mycoplasma hominis* in healthy women and patients with flora alterations. *Diagnostic Microbiology and Infectious Disease, 93*(3), 227-231. doi:10.1016/j.diagmicrobio.2018.10.001

Sabo, M. C., Balkus, J. E., Richardson, B. A., Srinivasan, S., Kimani, J., Anzala, O., Schwebke, J., Feidler, T. L., Fredricks, D. N., & McClelland, R. S. (2019). Association between vaginal washing and vaginal bacterial concentrations. *PloS One, 14*(1), e0210825. doi:10.1371/journal.pone.0210825

Sakai, M., Ishiyama, A., Tabata, M., Sasaki, Y., Yoneda, S., Shiozaki, A., & Saito, S. (2004). Relationship between cervical mucus interleukin-8 concentrations and vaginal bacteria in pregnancy. *American Journal of Reproductive Immunology, 52*(2), 106-112. doi:10.1111/j.1600-0897.2004.00203.x

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., Turner, P., Parkhill, J., Loman, N. J., & Walker, A. W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology, 12*, 87. doi:10.1186/s12915-014-0087-z

Sanders, J. N., Adkins, D. E., Kaur, S., Storck, K., Gawron, L. M., & Turok, D. K. (2018). Bleeding, cramping, and satisfaction among new copper IUD users: A prospective study. *PloS One, 13*(11), e0199724. doi:10.1371/journal.pone.0199724

Santiago, G. L., Deschaght, P., El Aila, N., Kiama, T. N., Verstraelen, H., Jefferson, K. K., Temmerman, M., & Vaneechoutte, M. (2011). *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. *American Journal of Obstetrics and Gynecology, 204*(5), 450 e451-457. doi:10.1016/j.ajog.2010.12.061

Santiago, G. L., Tency, I., Verstraelen, H., Verhelst, R., Trog, M., Temmerman, M., Vancoillie, L., Decat, E., Cools, P., & Vaneechoutte, M. (2012). Longitudinal qPCR study of the dynamics of L. crispatus, L. iners, A. vaginae, (sialidase positive) G. vaginalis, and P. bivia in the vagina. *PloS One, 7*(9), e45281. doi:10.1371/journal.pone.0045281

Satpute, S. K., Banpurkar, A. G., Banat, I. M., Sangshetti, J. N., Patil, R. H., & Gade, W. N. (2016a). Multiple roles of biosurfactants in biofilms. *Current Pharmaceutical Design, 22*(11), 1429-1448. doi:10.2174/1381612822666160120152704

Satpute, S. K., Kulkarni, G. R., Banpurkar, A. G., Banat, I. M., Mone, N. S., Patil, R. H., & Cameotra, S. S. (2016b). Biosurfactant/s from lactobacilli species: properties, challenges and potential biomedical applications. *Journal of Basic Microbiology, 56*(11), 1140-1158. doi:10.1002/jobm.201600143

Schellenberg, J. J., Links, M. G., Hill, J. E., Dumonceaux, T. J., Peters, G. A., Tyler, S., Ball, T. B., Severini, A., & Plummer, F. A. (2009). Pyrosequencing of the chaperonin-60 universal target as a tool for determining microbial community composition. *Applied and Environmental Microbiology, 75*(9), 2889-2898. doi:10.1128/AEM.01640-08

Schellenberg, J. J., Paramel Jayaprakash, T., Withana Gamage, N., Patterson, M. H., Vaneechoutte, M., & Hill, J. E. (2016). *Gardnerella vaginalis* subgroups defined by cpn60 sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PloS One, 11*(1), e0146510. doi:10.1371/journal.pone.0146510

Schmieder, R., Lim, Y. W., Rohwer, F., & Edwards, R. (2010). TagCleaner: Identification and removal of tag sequences from genomic and metagenomic datasets. *BMC Bioinformatics, 11*, 341. doi:10.1186/1471-2105-11-341

Schuyler, J. A., Mordechai, E., Adelson, M. E., Sobel, J. D., Gygax, S. E., & Hilbert, D. W. (2015). Identification of intrinsically metronidazole-resistant clades of *Gardnerella vaginalis*. *Diagnostic Microbiology and Infectious Disease*. doi:10.1016/j.diagmicrobio.2015.10.006

Schwebke, J. R., & Desmond, R. (2005). Risk factors for bacterial vaginosis in women at high risk for sexually transmitted diseases. *Sexually Transmitted Diseases, 32*(11), 654-658.

Schwebke, J. R., Flynn, M. S., & Rivers, C. A. (2014a). Prevalence of *Gardnerella vaginalis* among women with *Lactobacillus*-predominant vaginal flora. *Sexually Transmitted Infections, 90*(1), 61-63. doi:10.1136/sextrans-2013-051232

Schwebke, J. R., Lensing, S. Y., Lee, J., Muzny, C. A., Pontius, A., Woznicki, N., Aguin, T., & Sobel, J. D. (2021). Treatment of male sexual partners of women with bacterial vaginosis (BV); a randomized, double-blind, placebo-controlled trial. *Clinical Infectious Diseases*. doi:10.1093/cid/ciaa1903

Schwebke, J. R., Morgan, S. C., & Weiss, H. L. (1997). The use of sequential self-obtained vaginal smears for detecting changes in the vaginal flora. *Sexually Transmitted Diseases, 24*(4), 236-239.

Schwebke, J. R., Muzny, C. A., & Josey, W. E. (2014b). Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: A conceptual model. *Journal of Infectious Diseases, 210*(3), 338-343. doi:10.1093/infdis/jiu089

Schwebke, J. R., Richey, C. M., & Weiss, H. L. (1999). Correlation of behaviors with microbiological changes in vaginal flora. *Journal of Infectious Diseases, 180*(5), 1632-1636. doi:10.1086/315065

Schwebke, J. R., Rivers, C., & Lee, J. (2009). Prevalence of *Gardnerella vaginalis* in male sexual partners of women with and without bacterial vaginosis. *Sexually Transmitted Diseases, 36*(2), 92-94. doi:10.1097/OLQ.0b013e3181886727

Sewankambo, N., Gray, R. H., Wawer, M. J., Paxton, L., McNaim, D., Wabwire-Mangen, F., Serwadda, D., Li, C., Kiwanuka, N., Hillier, S. L., Rabe, L., Gaydos, C. A., Quinn, T. C., & Konde-Lule, J. (1997). HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet, 350*(9077), 546-550.

Shannon, B., Gajer, P., Yi, T. J., Ma, B., Humphrys, M. S., Thomas-Pavanel, J., Chieza, L., Janakiram, P., Saunders, M., Tharao, W., Huibner, S., Shahabi, K., Ravel, J., & Kaul, R. (2017). Distinct effects of the cervicovaginal microbiota and Herpes Simplex Type 2 infection on female genital tract immunology. *Journal of Infectious Diseases, 215*(9), 1366-1375. doi:10.1093/infdis/jix088

Sherrard, J., Wilson, J., Donders, G., Mendling, W., & Jensen, J. S. (2018). 2018 European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge. *International Journal of STD and AIDS, 29*(13), 1258-1272. doi:10.1177/0956462418785451

Shipitsyna, E., Krysanova, A., Khayrullina, G., Shalepo, K., Savicheva, A., Guschin, A., & Unemo, M. (2019). Quantitation of all four *Gardnerella vaginalis* clades detects abnormal vaginal microbiota characteristic of bacterial vaginosis more accurately than putative *G. vaginalis* Sialidase A gene count. *Molecular Diagnosis & Therapy, 23*(1), 139-147. doi:10.1007/s40291-019-00382-5

Shipitsyna, E., Roos, A., Datcu, R., Hallen, A., Fredlund, H., Jensen, J. S., Engstrand, L., & Unemo, M. (2013). Composition of the vaginal microbiota in women of reproductive age - sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PloS One, 8*(4), e60670.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology, 7*, 539. doi:10.1038/msb.2011.75

Silva, J., Cerqueira, F., Teixeira, A. L., Bicho, M. C., Campainha, R., Amorim, J., & Medeiros, R. (2018). Genital mycoplasmas and ureaplasmas in cervicovaginal self-collected samples of reproductive-age women: prevalence and risk factors. *International Journal of STD and AIDS, 29*(10), 999-1006. doi:10.1177/0956462418774209

Simoes, J. A., Aroutcheva, A., Heimler, I., Shott, S., & Faro, S. (2001). Bacteriocin susceptibility of *Gardnerella vaginalis* and its relationship to biotype, genotype, and metronidazole susceptibility. *American Journal of Obstetrics and Gynecology, 185*(5), 1186-1190. doi:10.1067/mob.2001.118144

Simoes, J. A., Bahamondes, L. G., Camargo, R. P., Alves, V. M., Zaneveld, L. J., Waller, D. P., Schwartz, J., Callahan, M. M., & Mauck, C. K. (2006). A pilot clinical trial comparing an acid-buffering formulation (ACIDFORM gel) with metronidazole gel for the treatment of symptomatic bacterial vaginosis. *British Journal of Clinical Pharmacology, 61*(2), 211-217. doi:10.1111/j.1365-2125.2005.02550.x

Sinclair, L., Osman, O. A., Bertilsson, S., & Eiler, A. (2015). Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform. *PloS One, 10*(2), e0116955. doi:10.1371/journal.pone.0116955

Smart, S., Singal, A., & Mindel, A. (2004). Social and sexual risk factors for bacterial vaginosis. *Sexually Transmitted Infections, 80*(1), 58-62.

Smieja, M. (1998). Current indications for the use of clindamycin: A critical review. *Canadian Journal of Infectious Diseases, 9*(1), 22-28. doi:10.1155/1998/538090

Smith, S. B., & Ravel, J. (2017). The vaginal microbiota, host defence and reproductive physiology. *Journal of Physiology, 595*(2), 451-463. doi:10.1113/JP271694

Sobel, J. D., Ferris, D., Schwebke, J., Nyirjesy, P., Wiesenfeld, H. C., Peipert, J., Soper, D., Ohmit, S. E., & Hillier, S. L. (2006). Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. *American Journal of Obstetrics and Gynecology, 194*(5), 1283-1289. doi:10.1016/j.ajog.2005.11.041

Sobel, J. D., Kaur, N., Woznicki, N. A., Boikov, D., Aguin, T., Gill, G., & Akins, R. A. (2019). Prognostic indicators of recurrence of bacterial vaginosis. *Journal of Clinical Microbiology, 57*(5). doi:10.1128/JCM.00227-19

Sobel, J. D., Schmitt, C., & Meriwether, C. (1993). Long-term follow-up of patients with bacterial vaginosis treated with oral metronidazole and topical clindamycin. *Journal of Infectious Diseases, 167*(3), 783-784.

Sobel, R., & Sobel, J. D. (2015). Metronidazole for the treatment of vaginal infections. *Expert Opinion on Pharmacotherapy, 16*(7), 1109-1115. doi:10.1517/14656566.2015.1035255

Sodhani, P., Garg, S., Bhalla, P., Singh, M. M., Sharma, S., & Gupta, S. (2005). Prevalence of bacterial vaginosis in a community setting and role of the pap smear in its detection. *Acta Cytologica, 49*(6), 634-638. doi:10.1159/000326251

Spear, G. T., French, A. L., Gilbert, D., Zariffard, M. R., Mirmonsef, P., Sullivan, T. H., Spear, W. W., Landay, A., Micci, S., Lee, B. H., & Hamaker, B. R. (2014). Human alpha-amylase present in

lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by *Lactobacillus*. *Journal of Infectious Diseases, 210*(7), 1019-1028. doi:10.1093/infdis/jiu231

Spiegel, C. A., Amsel, R., & Holmes, K. K. (1983). Diagnosis of bacterial vaginosis by direct gram stain of vaginal fluid. *Journal of Clinical Microbiology, 18*(1), 170-177.

Srinivasan, S., & Fredricks, D. N. (2008). The human vaginal bacterial biota and bacterial vaginosis. *Interdisciplinary Perspectives on Infectious Diseases, 2008*, 750479. doi:10.1155/2008/750479

Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., Ross, F. J., McCoy, C. O., Bumgarner, R., Marrazzo, J. M., & Fredricks, D. N. (2012). Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PloS One, 7*(6), e37818. doi:10.1371/journal.pone.0037818

Srinivasan, S., Liu, C., Mitchell, C. M., Fiedler, T. L., Thomas, K. K., Agnew, K. J., Marrazzo, J. M., & Fredricks, D. N. (2010). Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PloS One, 5*(4), e10197. doi:10.1371/journal.pone.0010197

Srinivasan, S., Munch, M. M., Sizova, M. V., Fiedler, T. L., Kohler, C. M., Hoffman, N. G., Liu, C., Agnew, K. J., Marrazzo, J. M., Epstein, S. S., & Fredricks, D. N. (2016). More easily cultivated than identified: classical isolation with molecular identification of vaginal bacteria. *Journal of Infectious Diseases, 214 Suppl 1*, S21-28. doi:10.1093/infdis/jiw192

Sterne, J. A., Hernan, M. A., Reeves, B. C., Savovic, J., Berkman, N. D., Viswanathan, M., Henry, D., Altman, D. G., Ansari, M. T., Boutron, I., Carpenter, J. R., Chan, A. W., Churchill, R., Deeks, J. J., Hrobjartsson, A., Kirkham, J., Juni, P., Loke, Y. K., Pigott, T. D., Ramsay, C. R., Regidor, D., Rothstein, H. R., Sandhu, L., Santaguida, P. L., Schunemann, H. J., Shea, B., Shrier, I., Tugwell, P., Turner, L., Valentine, J. C., Waddington, H., Waters, E., Wells, G. A., Whiting, P. F., & Higgins, J. P. (2016). ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ, 355*, i4919. doi:10.1136/bmj.i4919

Stoner, K. A., Reighard, S. D., Vicetti Miguel, R. D., Landsittel, D., Cosentino, L. A., Kant, J. A., & Cherpes, T. L. (2012). Recalcitrance of bacterial vaginosis among herpes-simplex-virus-type-2-seropositive women. *Journal of Obstetrics and Gynaecology Research, 38*(1), 77-83. doi:10.1111/j.1447-0756.2011.01697.x

Swedberg, J., Steiner, J. F., Deiss, F., Steiner, S., & Driggers, D. A. (1985). Comparison of single-dose vs one-week course of metronidazole for symptomatic bacterial vaginosis. *JAMA, 254*(8), 1046-1049.

Swidsinski, A., Doerffel, Y., Loening-Baucke, V., Swidsinski, S., Verstraelen, H., Vaneechoutte, M., Lemm, V., Schilling, J., & Mendling, W. (2010). *Gardnerella* biofilm involves females and males and is transmitted sexually. *Gynecologic and Obstetric Investigation, 70*(4), 256-263. doi:10.1159/000314015

Swidsinski, A., Dorffel, Y., Loening-Baucke, V., Schilling, J., & Mendling, W. (2011). Response of *Gardnerella vaginalis* biofilm to 5 days of moxifloxacin treatment. *FEMS Immunology and Medical Microbiology, 61*(1), 41-46. doi:10.1111/j.1574-695X.2010.00743.x

Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L. P., & Lochs, H. (2005). Adherent biofilms in bacterial vaginosis. *Obstetrics and Gynecology, 106*(5 Pt 1), 1013-1023. doi:10.1097/01.AOG.0000183594.45524.d2

Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dorffel, Y., Scholze, J., Lochs, H., & Verstraelen, H. (2008). An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *American Journal of Obstetrics and Gynecology, 198*(1), 97 e91-96. doi:10.1016/j.ajog.2007.06.039

Tabatabaei, N., Eren, A. M., Barreiro, L. B., Yotova, V., Dumaine, A., Allard, C., & Fraser, W. D. (2019). Vaginal microbiome in early pregnancy and subsequent risk of spontaneous preterm birth: a case-control study. *BJOG: An International Journal of Obstetrics and Gynaecology, 126*(3), 349-358. doi:10.1111/1471-0528.15299

Tachedjian, G., Aldunate, M., Bradshaw, C. S., & Cone, R. A. (2017). The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Research in Microbiology, 168*(9-10), 782-792. doi:10.1016/j.resmic.2017.04.001

Tachedjian, G., O'Hanlon, D. E., & Ravel, J. (2018). The implausible "in vivo" role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota. *Microbiome, 6*(1), 29. doi:10.1186/s40168-018-0418-3

Taha, T. E., Hoover, D. R., Dallabetta, G. A., Kumwenda, N. I., Mtimavalye, L. A., Yang, L. P., Liomba, G. N., Broadhead, R. L., Chiphangwi, J. D., & Miotti, P. G. (1998). Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. *AIDS, 12*(13), 1699-1706.

Tamrakar, R., Yamada, T., Furuta, I., Cho, K., Morikawa, M., Yamada, H., Sakuragi, N., & Minakami, H. (2007). Association between *Lactobacillus* species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. *BMC Infectious Diseases, 7*, 128. doi:10.1186/1471-2334-7-128

Taylor, B. D., Darville, T., & Haggerty, C. L. (2013). Does bacterial vaginosis cause pelvic inflammatory disease? *Sexually Transmitted Diseases, 40*(2), 117-122. doi:10.1097/OLQ.0b013e31827c5a5b

Taylor-Robinson, D. (2017). Mollicutes in vaginal microbiology: *Mycoplasma hominis, Ureaplasma urealyticum, Ureaplasma parvum* and *Mycoplasma genitalium*. *Research in Microbiology, 168*(9-10), 875-881. doi:10.1016/j.resmic.2017.02.009

Taylor-Robinson, D., & McCormack, W. M. (1980). The genital mycoplasmas (first of two parts). *New England Journal of Medicine, 302*(18), 1003-1010. doi:10.1056/NEJM198005013021805

Tettamanti Boshier, F. A., Srinivasan, S., Lopez, A., Hoffman, N. G., Proll, S., Fredricks, D. N., & Schiffer, J. T. (2020). Complementing 16S rRNA Gene Amplicon Sequencing with Total Bacterial Load To Infer Absolute Species Concentrations in the Vaginal Microbiome. *mSystems, 5*(2). doi:10.1128/mSystems.00777-19

Thomason, J. L., Gelbart, S. M., Anderson, R. J., Walt, A. K., Osypowski, P. J., & Broekhuizen, F. F. (1990). Statistical evaluation of diagnostic criteria for bacterial vaginosis. *American Journal of Obstetrics and Gynecology, 162*(1), 155-160.

Tuddenham, S., Ghanem, K. G., Caulfield, L. E., Rovner, A. J., Robinson, C., Shivakoti, R., Miller, R., Burke, A., Murphy, C., Ravel, J., & Brotman, R. M. (2019). Associations between dietary micronutrient intake and molecular-Bacterial Vaginosis. *Reprod Health, 16*(1), 151. doi:10.1186/s12978-019-0814-6

Turovskiy, Y., Ludescher, R. D., Aroutcheva, A. A., Faro, S., & Chikindas, M. L. (2009). Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*. *Probiotics Antimicrob Proteins, 1*(1), 67-74. doi:10.1007/s12602-008-9003-6

Tweten, R. K. (2005). Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. *Infection and Immunity, 73*(10), 6199-6209. doi:10.1128/IAI.73.10.6199-6209.2005

Tyssen, D., Wang, Y. Y., Hayward, J. A., Agius, P. A., DeLong, K., Aldunate, M., Ravel, J., Moench, T. R., Cone, R. A., & Tachedjian, G. (2018). Anti-HIV-1 activity of lactic acid in human cervicovaginal fluid. *mSphere, 3*(4). doi:10.1128/mSphere.00055-18

Udayalaxmi, J., Bhat, G., Kotigadde, S., & Kotian, S. (2012). Effect of pH on the adherence, surface hydrophobicity and the biofilm formation of *Gardnerella Vaginalis*. *Journal of Clinical and Diagnostic Research, 6*(6), 967-969.

Unemo, M., Bradshaw, C. S., Hocking, J. S., de Vries, H. J. C., Francis, S. C., Mabey, D., Marrazzo, J. M., Sonder, G. J. B., Schwebke, J. R., Hoornenborg, E., Peeling, R. W., Philip, S. S., Low, N., & Fairley, C. K. (2017). Sexually transmitted infections: challenges ahead. *Lancet Infectious Diseases, 17*(8), e235-e279. doi:10.1016/S1473-3099(17)30310-9

Vaca, M., Guadalupe, I., Erazo, S., Tinizaray, K., Chico, M. E., Cooper, P. J., & Hay, P. (2010). High prevalence of bacterial vaginosis in adolescent girls in a tropical area of Ecuador. *BJOG: An*

International Journal of Obstetrics and Gynaecology, 117(2), 225-228. doi:10.1111/j.1471-0528.2009.02397.x

van de Wijgert, J. H. H. M. (2017). The vaginal microbiome and sexually transmitted infections are interlinked: Consequences for treatment and prevention. *PLoS Medicine, 14*(12), e1002478. doi:10.1371/journal.pmed.1002478

van de Wijgert, J. H. H. M., Borgdorff, H., Verhelst, R., Crucitti, T., Francis, S., Verstraelen, H., & Jespers, V. (2014). The vaginal microbiota: what have we learned after a decade of molecular characterization? *PloS One, 9*(8), e105998. doi:10.1371/journal.pone.0105998

Van Der Pol, W. J., Kumar, R., Morrow, C. D., Blanchard, E. E., Taylor, C. M., Martin, D. H., Lefkowitz, E. J., & Muzny, C. A. (2019). In Silico and Experimental Evaluation of Primer Sets for Species-Level Resolution of the Vaginal Microbiota Using 16S Ribosomal RNA Gene Sequencing. *Journal of Infectious Diseases, 219*(2), 305-314. doi:10.1093/infdis/jiy508

van der Veer, C., Bruisten, S. M., van der Helm, J. J., de Vries, H. J. C., & van Houdt, R. (2017). The cervico-vaginal microbiota in women notified for *Chlamydia trachomatis* infection: a case-control study at the STI outpatient clinic in Amsterdam, the Netherlands. *Clinical Infectious Diseases, 64*(1), 24-31.

van der Veer, C., Bruisten, S. M., van Houdt, R., Matser, A. A., Tachedjian, G., van de Wijgert, J., de Vries, H. J. C., & van der Helm, J. J. (2019). Effects of an over-the-counter lactic-acid containing intra-vaginal douching product on the vaginal microbiota. *BMC Microbiology, 19*(1), 168. doi:10.1186/s12866-019-1545-0

van Houdt, R., Ma, B., Bruisten, S. M., Speksnijder, A., Ravel, J., & de Vries, H. J. C. (2018). *Lactobacillus iners*-dominated vaginal microbiota is associated with increased susceptibility to *Chlamydia trachomatis* infection in Dutch women: a case-control study. *Sexually Transmitted Infections, 94*(2), 117-123. doi:10.1136/sextrans-2017-053133

Vaneechoutte, M., Guschin, A., Van Simaey, L., Gansemans, Y., Van Nieuwerburgh, F., & Cools, P. (2019). Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella*. *International Journal of Systematic and Evolutionary Microbiology, 69*(3), 679-687. doi:10.1099/ijsem.0.003200

Vejtorp, M., Bollerup, A. C., Vejtorp, L., Fanoe, E., Nathan, E., Reiter, A., Andersen, M. E., Stromsholt, B., & Schroder, S. S. (1988). Bacterial vaginosis: a double-blind randomized trial of the effect of treatment of the sexual partner. *British Journal of Obstetrics and Gynaecology, 95*(9), 920-926.

Verhelst, R., Verstraelen, H., Claeys, G., Verschraegen, G., Van Simaey, L., De Ganck, C., De Backer, E., Temmerman, M., & Vaneechoutte, M. (2005). Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. *BMC Microbiology, 5*, 61. doi:10.1186/1471-2180-5-61

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M., & Vaneechoutte, M. (2009). Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiology, 9*, 116. doi:10.1186/1471-2180-9-116

Verstraelen, H., Verhelst, R., Roelens, K., & Temmerman, M. (2012). Antiseptics and disinfectants for the treatment of bacterial vaginosis: a systematic review. *BMC Infectious Diseases, 12*, 148. doi:10.1186/1471-2334-12-148

Verwijs, M. C., Agaba, S. K., Darby, A. C., & van de Wijgert, J. (2019). Impact of oral metronidazole treatment on the vaginal microbiota and correlates of treatment failure. *American Journal of Obstetrics and Gynecology*. doi:10.1016/j.ajog.2019.08.008

Viscardi, R. M. (2014). *Ureaplasma* species: role in neonatal morbidities and outcomes. *Archives of Disease in Childhood: Fetal and Neonatal Edition, 99*(1), F87-92. doi:10.1136/archdischild-2012-303351

Vitali, B., Cruciani, F., Picone, G., Parolin, C., Donders, G., & Laghi, L. (2015). Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases, 34*(12), 2367-2376. doi:10.1007/s10096-015-2490-y

Vodstrcil, L. A., Hocking, J. S., Law, M., Walker, S., Tabrizi, S. N., Fairley, C. K., & Bradshaw, C. S. (2013). Hormonal contraception is associated with a reduced risk of bacterial vaginosis: a systematic review and meta-analysis. *PloS One, 8*(9), e73055. doi:10.1371/journal.pone.0073055

Vodstrcil, L. A., Plummer, E., Fairley, C. K., Tachedjian, G., Law, M. G., Hocking, J. S., Worthington, K., Grant, M., Okoko, N., & Bradshaw, C. S. (2019). Combined oral contraceptive pill-exposure alone does not reduce the risk of bacterial vaginosis recurrence in a pilot randomised controlled trial. *Scientific Reports, 9*(1), 3555. doi:10.1038/s41598-019-39879-8

Vodstrcil, L. A., Plummer, E. L., Doyle, M., Fairley, C. K., McGuiness, C., Bateson, D., Hocking, J. S., Law, M. G., Petoumenos, K., Donovan, B., Chow, E. P. F., Bradshaw, C. S., & StepUp RCT Team. (2020). Treating male partners of women with bacterial vaginosis (StepUp): a protocol for a randomised controlled trial to assess the clinical effectiveness of male partner treatment for reducing the risk of BV recurrence. *BMC Infectious Diseases, 20*(1), 834. doi:10.1186/s12879-020-05563-w

Vodstrcil, L. A., Twin, J., Garland, S. M., Fairley, C. K., Hocking, J. S., Law, M. G., Plummer, E. L., Fethers, K. A., Chow, E. P., Tabrizi, S. N., & Bradshaw, C. S. (2017). The influence of sexual activity on the vaginal microbiota and *Gardnerella vaginalis* clade diversity in young women. *PloS One, 12*(2), e0171856. doi:10.1371/journal.pone.0171856

Vodstrcil, L. A., Walker, S. M., Hocking, J. S., Law, M., Forcey, D. S., Fehler, G., Bilardi, J. E., Chen, M. Y., Fethers, K. A., Fairley, C. K., & Bradshaw, C. S. (2015). Incident bacterial vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. *Clinical Infectious Diseases, 60*(7), 1042-1053. doi:10.1093/cid/ciu1130

Vutyavanich, T., Pongsuthirak, P., Vannareumol, P., Ruangsri, R. A., & Luangsook, P. (1993). A randomized double-blind trial of tinidazole treatment of the sexual partners of females with bacterial vaginosis. *Obstetrics and Gynecology, 82*(4 Pt 1), 550-554.

Watts, S. C., Ritchie, S. C., Inouye, M., & Holt, K. E. (2019). FastSpar: rapid and scalable correlation estimation for compositional data. *Bioinformatics, 35*(6), 1064-1066. doi:10.1093/bioinformatics/bty734

Wertz, J., Isaacs-Cosgrove, N., Holzman, C., & Marsh, T. L. (2008). Temporal Shifts in Microbial Communities in Nonpregnant African-American Women with and without Bacterial Vaginosis. *Interdisciplinary Perspectives on Infectious Diseases, 2008*, 181253. doi:10.1155/2008/181253

Wessels, J. M., Felker, A. M., Dupont, H. A., & Kaushic, C. (2018). The relationship between sex hormones, the vaginal microbiome and immunity in HIV-1 susceptibility in women. *Disease Models & Mechanisms, 11*(9). doi:10.1242/dmm.035147

Wessels, J. M., Lajoie, J., Vitali, D., Omollo, K., Kimani, J., Oyugi, J., Cheruiyot, J., Kimani, M., Mungai, J. N., Akolo, M., Stearns, J. C., Surette, M. G., Fowke, K. R., & Kaushic, C. (2017). Association of high-risk sexual behaviour with diversity of the vaginal microbiota and abundance of *Lactobacillus*. *PloS One, 12*(11), e0187612. doi:10.1371/journal.pone.0187612

Westhoff, C., Gentile, G., Lee, J., Zacur, H., & Helbig, D. (1996). Predictors of ovarian steroid secretion in reproductive-age women. *American Journal of Epidemiology, 144*(4), 381-388.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Wiesenfeld, H. C., Hillier, S. L., Krohn, M. A., Amortegui, A. J., Heine, R. P., Landers, D. V., & Sweet, R. L. (2002). Lower genital tract infection and endometritis: insight into subclinical pelvic

inflammatory disease. *Obstetrics and Gynecology, 100*(3), 456-463. doi:10.1016/s0029-7844(02)02118-x

Wiesenfeld, H. C., Hillier, S. L., Krohn, M. A., Landers, D. V., & Sweet, R. L. (2003). Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clinical Infectious Diseases, 36*(5), 663-668. doi:10.1086/367658

Wigan, R., Vaughn, C., Vodstrcil, L., Doyle, M., Kaiser, M., McGuiness, C., Bradshaw, C. S., & Bilardi, J. E. (2020). "It's just an issue and you deal with it... you just deal with it, you move on and you do it together.": Men's experiences of bacterial vaginosis and the acceptability of male partner treatment. *PloS One, 15*(6), e0235286. doi:10.1371/journal.pone.0235286

Wilkinson, E. M., Laniewski, P., Herbst-Kralovetz, M. M., & Brotman, R. M. (2019). Personal and clinical vaginal lubricants: impact on local vaginal microenvironment and implications for epithelial cell host response and barrier function. *Journal of Infectious Diseases, 220*(12), 2009-2018. doi:10.1093/infdis/jiz412

Witkin, S. S. (2015). The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth. *BJOG: An International Journal of Obstetrics and Gynaecology, 122*(2), 213-218. doi:10.1111/1471-0528.13115

Witkin, S. S., & Linhares, I. M. (2017). Why do lactobacilli dominate the human vaginal microbiota? *BJOG: An International Journal of Obstetrics and Gynaecology, 124*(4), 606-611. doi:10.1111/1471-0528.14390

Witkin, S. S., Mendes-Soares, H., Linhares, I. M., Jayaram, A., Ledger, W. J., & Forney, L. J. (2013). Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: Implications for protection against upper genital tract infections. *mBio, 4*(4). doi:10.1128/mBio.00460-13

Workowski, K. A., & Bolan, G. A. (2015). Sexually transmitted diseases treatment guidelines, 2015. *MMWR: Recommendations and Reports, 64*(RR-03), 1-137.

Wu, H., Moser, C., Wang, H. Z., Hoiby, N., & Song, Z. J. (2015). Strategies for combating bacterial biofilm infections. *Int J Oral Sci, 7*(1), 1-7. doi:10.1038/ijos.2014.65

Xiao, B., Wu, C., Song, W., Niu, X., Qin, N., Liu, Z., & Xu, Q. (2019). Association analysis on recurrence of bacterial vaginosis revealed microbes and clinical variables important for treatment outcome. *Front Cell Infect Microbiol, 9*, 189. doi:10.3389/fcimb.2019.00189

Xiao, L., Paralanov, V., Glass, J. I., Duffy, L. B., Robertson, J. A., Cassell, G. H., Chen, Y., & Waites, K. B. (2011). Extensive horizontal gene transfer in ureaplasmas from humans questions the utility of serotyping for diagnostic purposes. *Journal of Clinical Microbiology, 49*(8), 2818-2826. doi:10.1128/JCM.00637-11

Yekutieli, D., & Benjamini, Y. (1999). Resampling-based false discovery rate controlling multiple test procedures for correlated test. *Journal of Statistical Planning and Inference, 82*, 171-196.

Yen, S., Shafer, M. A., Moncada, J., Campbell, C. J., Flinn, S. D., & Boyer, C. B. (2003). Bacterial vaginosis in sexually experienced and non-sexually experienced young women entering the military. *Obstetrics and Gynecology, 102*(5 Pt 1), 927-933. doi:10.1016/s0029-7844(03)00858-5

Yeoman, C. J., Yildirim, S., Thomas, S. M., Durkin, A. S., Torralba, M., Sutton, G., Buhay, C. J., Ding, Y., Dugan-Rocha, S. P., Muzny, D. M., Qin, X., Gibbs, R. A., Leigh, S. R., Stumpf, R., White, B. A., Highlander, S. K., Nelson, K. E., & Wilson, B. A. (2010). Comparative genomics of Gardnerella vaginalis strains reveals substantial differences in metabolic and virulence potential. *PloS One, 5*(8), e12411. doi:10.1371/journal.pone.0012411

Yeung, A. H., Temple-Smith, M., Fairley, C. K., Vaisey, A. M., Guy, R., Law, M. G., Low, N., Bingham, A. L., Gunn, J., Kaldor, J., Donovan, B., & Hocking, J. S. (2014). *Chlamydia* prevalence in young attenders of rural and regional primary care services in Australia: a cross-sectional survey. *Medical Journal of Australia, 200*(3), 170-175. doi:10.5694/mja13.10729

Yoshimura, K., Morotomi, N., Fukuda, K., Nakano, M., Kashimura, M., Hachisuga, T., & Taniguchi, H. (2011). Intravaginal microbial flora by the 16S rRNA gene sequencing. *American Journal of Obstetrics and Gynecology, 205*(3), 235 e231-239. doi:10.1016/j.ajog.2011.04.018

Zhao, X., Yu, Z., & Ding, T. (2020). Quorum-sensing regulation of antimicrobial resistance in bacteria. *Microorganisms, 8*(3). doi:10.3390/microorganisms8030425

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

Zhou, X., Hansmann, M. A., Davis, C. C., Suzuki, H., Brown, C. J., Schutte, U., Pierson, J. D., & Forney, L. J. (2010). The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunology and Medical Microbiology, 58*(2), 169-181. doi:10.1111/j.1574-695X.2009.00618.x

Zigler, R. E., & McNicholas, C. (2017). Unscheduled vaginal bleeding with progestin-only contraceptive use. *American Journal of Obstetrics and Gynecology, 216*(5), 443-450. doi:10.1016/j.ajog.2016.12.008

Zozaya, M., Ferris, M. J., Siren, J. D., Lillis, R., Myers, L., Nsuami, M. J., Eren, A. M., Brown, J., Taylor, C. M., & Martin, D. H. (2016). Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome, 4*, 16. doi:10.1186/s40168-016-0161-6

Zozaya-Hinchliffe, M., Lillis, R., Martin, D. H., & Ferris, M. J. (2010). Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. *Journal of Clinical Microbiology, 48*(5), 1812-1819. doi:10.1128/JCM.00851-09

Zvirbliene, A., Pleckaityte, M., Lasickiene, R., Kucinskaite-Kodze, I., & Zvirblis, G. (2010). Production and characterization of monoclonal antibodies against vaginolysin: mapping of a region critical for its cytolytic activity. *Toxicon, 56*(1), 19-28. doi:10.1016/j.toxicon.2010.03.007

Appendices

This thesis includes five appendices:

Appendix	Thesis Chapter	Article title
A	2	Sexual practices have a significant impact on the vaginal microbiota of women who have sex with women
B	3	*Gardnerella vaginalis* clade distribution is associated with behavioral practices and Nugent score in women who have sex with women
C	4	A prospective, open-label pilot study of concurrent male partner treatment for bacterial vaginosis
D	6	Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: A systematic review
E	2,3,5,6	Includes pdf version of published manuscripts

Appendix A. Supplementary files: Chapter 2

Name of file in Appendix A and as referred to in Chapter 2	Name of file in PDF version of published manuscript
Supplementary Table A1	Supplementary Table 1
Supplementary Table A2	Supplementary Table 2

Supplementary Table A1 – Effect of new sexual partner exposure on microbiota diversity, stratified by oral sex

		Diversity ceoff. (95% CI) [a]	*P* value
No oral sex (n=138)	No new partner (n=124)	ref	
	New partner (n=14)	0.263 (-0.07, 0.60)	0.127
Oral sex (n=221)	No new partner (n=175)	ref	
	New partner (n=46)	0.517 (0.27, 0.76)	**<0.001**

[a] Univariate GEE linear regression, where participant ID is panel variable. The regression coefficient represents the mean difference of Shannon diversity between the reference (i.e. no new sexual partner) and comparison group (i.e. new sexual partner).

Supplementary Table A2 – Characteristics and practices associated with vaginal microbiota composition by univariate multinomial logistic regression

Characteristic	CST2 *Lactobacillus* mixed (N=44) [a]		CST3 *L. iners* (N=93) [a]		CST4 *G. vaginalis* and diverse (N=40) [a]		CST5 Anaerobic and diverse (N=31) [a]	
	RRR (95% CI)	*P* value	RRR (95% CI)	*P* value	RRR (95% CI)	*P* value	RRR (95% CI)	*P* value
Self-reported past history of BV								
No	1		1		1		1	
Yes	0.87 (0.35, 2.17)	0.762	0.69 (0.26, 1.87)	0.467	1.13 (0.39, 3.27)	0.817	2.82 (1.09, 2.27)	**0.032**
Longitudinal practices[b]								
Any smoking[c]								
No	1		1		1		1	
Yes	1.59 (0.77, 3.29)	0.214	1.38 (0.71, 2.65)	0.341	1.72 (0.74, 4.01)	0.207	3.01 (1.31, 6.92)	**0.009**
Any douching[d]								
No	1		1				1	
Yes	1.73 (0.15, 20.04)	0.660	2.48 (0.58, 10.69)	0.222	…	…	5.14 (1.20, 21.96)	**0.027**
Onset of last menses								
> 7 days ago	1		1		1		1	
≤ 7 days ago	3.59 (1.49, 8.68)	**0.004**	2.19 (1.08, 4.46)	**0.030**	1.64 (0.55, 4.91)	0.378	1.03 (0.27, 4.00)	0.964
Number of SP								

0	1		1		1		1	
1	0.60 (0.22, 1.68)	0.334	0.68 (0.20, 2.27)	0.528	0.72 (0.18, 2.85)	0.639	0.45 (0.13, 1.61)	0.222
≥2	0.51 (0.12, 2.25)	0.374	0.83 (0.20, 3.40)	0.800	1.72 (0.36, 8.26)	0.498	1.91 (0.48, 7.65)	0.360
Frequency of sex								
Once/month or less	1		1		1		1	
Several times/month	1.15 (0.46, 2.90)	0.763	0.79 (0.39, 1.61)	0.513	0.73 (0.27, 2.00)	0.543	1.51 (0.59, 3.85)	0.386
Several times/week	1.62 (0.64, 4.07)	0.306	1.00 (0.45, 2.21)	1.000	1.49 (0.52, 4.33)	0.459	1.37 (0.47, 3.93)	0.563
Sex with NP[e]								
No	1		1		1		1	
Yes	0.62 (0.18, 2.19)	0.460	1.77 (0.87, 3.60)	0.117	4.09 (1.69, 9.92)	**0.002**	5.37 (2.18, 13.20)	**<0.001**
Sexual practices with FSP								
Any receptive oral vaginal sex								
No[f]	1		1		1		1	
Yes	1.09 (0.56, 2.15)	0.799	1.09 (0.57, 2.12)	0.790	2.60 (1.09, 6.20)	**0.031**	2.17 (0.86, 5.46)	0.099
Sharing of sex toys with FSP								
No toys/washed/condoms used[f]	1		1		1		1	
Unwashed	0.92 (0.36, 2.32)	0.859	0.98 (0.50, 1.92)	0.952	2.38 (1.04, 5.45)	**0.039**	1.46 (0.62, 3.46)	0.387
Sexual practices with MSP								

Any vaginal sex							
No[g]	1		1		1		1	
Yes	0.61 (0.11, 3.32)	0.564	2.64 (0.94, 7.44)	0.066	1.41 (0.29, 6.82)	0.666	2.45 (0.64, 9.30)	0.189
Any receptive oral vaginal sex							
No[g]	1		1		1		1	
Yes	0.98 (0.15, 6.41)	0.983	3.96 (1.05, 14.92)	**0.042**	1.67 (0.24, 11.60)	0.605	3.96 (0.76, 20.59)	0.102
Any anal sex							
No[g]	1		1		1		1	
Yes	3.49 (0.22, 54.60)	0.373	12.21 (1.25, 119.66)	**0.032**	3.85 (0.24, 60.51)	0.338	10.34 (0.91, 117.70)	0.060
Self-reported symptoms							
Self-reported abnormal vaginal discharge and/or odour							
No	1		1		1		1	
Yes	0.23 (0.03, 1.78)	0.159	1.06 (0.44, 2.56)	0.904	3.29 (1.21, 8.91)	**0.019**	4.03 (1.54, 10.59)	**0.005**

Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to FSP or MSP); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner

Missing data for variables included in this analysis occurred in <0.5% of intervals.

[a] Multinomial logistic regression with CST1-*L. crispatus* as baseline comparison group (n=152). Analysis clustered for multiple specimens from participants (100 clusters).

[b] Longitudinal characteristics were measured as any exposure over the prior follow-up interval (~90 days). No significant associations were identified between microbiota composition and hormonal contraceptive use.

[c] There was no dose-response relationship between smoking and Shannon diversity

[d] Douching was rarely practice, as such it was not included in adjusted analyses.
[e] Sex with a new partner with who first sexual contact was within 90 days
[f] Or did not have a FSP
[g] Or did not have a MSP

Appendix B. Supplementary files: Chapter 3

Name of file in Appendix B and as referred to in Chapter 3	Name of file in PDF version of published manuscript
Supplementary Methods B	Supplementary Methods
Supplementary Table B1	Supplementary Table 1
Supplementary Table B2	Supplementary Table 2
Supplementary Table B3	Supplementary Table 3
Supplementary Figure B1	Supplementary Figure 1

Supplementary Methods B

Specificity and sensitivity of clade-specific PCR primers

Primers for the clade specific PCR were designed based on a limited number of *G. vaginalis* isolates (Balashov *et al.*, 2014). We investigated the specificity and sensitivity of the primer set using 60 publicly available *G. vaginalis* genomes. We conducted a comparative analysis of *G. vaginalis* isolates using the Nullarbor pipeline (v 2.0.20181015) (https://github.com/tseemann/nullarbor) and using strain 409-05 (Accession number NC_013721) as the reference genome. Fastq files for 60 *G. vaginalis* isolates were either retrieved from the Short Read Archive (https://www.ncbi.nlm.nih.gov/sra) or generated using art_illumina (Huang *et al.*, 2012) from genome sequences available on GenBank (draft or closed) accessed from https://www.ncbi.nlm.nih.gov/genome/genomes/1967?.

Nullarbor performs a number of steps including de novo assembly of each isolate using SPAdes (v3.13.0) (Bankevich *et al.*, 2012) and SNP detection and determination of the core genome SNPs for the isolate set using Snippy (v4.4.1) (https://github.com/tseemann/snippy). Phylogeny was inferred using the core genome SNP differences and IQTree (v1.6.11) (Nguyen *et al.*, 2015).

We then performed a BLAST (Altschul *et al.*, 1990) search on the assembled contigs to identify the presence of the clade specific amplicon sequence in each isolate (Balashov *et al.*, 2014), query sequences for each clade are shown below:

Clade 1 amplicon sequence

TGGCACTGGCAAAGTTTACAACGATTTTTATATTAATCAACTTGTTGAATTGCTAACGCACTATGGTC
CTATTTTTTCCGTATGGCTTGATGGTGCTTGCGGCGAGGGTGAGAATGGTAAAACGCAAACTTATGA
CTGG

Clade 2 amplicon sequence

GCAAAGTCGCCTGAACGTATTAGAAAAGCTCAAAAAAGTTAATCCAAATATGACTTTATTTGTTTTTT

GCACTGTTATGCGAGCGCCTGCGTATAACAGCAGCGATGAGGAGCCTGATTATTAC

Clade 3 amplicon sequence

TCGTTGACTTTTGGGCAACATGGTGCGGTCCGTGCCGTTCATTTGGTCCAATTTTTGAATCTGTAAGC
AATAATCATCCAGATATTGCTTTTGTTAAAATCGACATTGACGAAAATCAGCAAATAGCAGAAGAAG
CAGAA

Clade 4 amplicon sequence

CAAGTTGCACTCTTCGAGCTGGCGCAAGTGGTGGTGTGCTTCAGCCGAGTATGTCGTCTGGAGCTTG
CGTAGG

The phylogenetic tree was updated to reflect which clade specific amplicon/s was detected in each *G. vaginalis* isolate.

Microbiota analysis

Laboratory methods

Dual index primers 341F/805R with heterogeneity spacers (Fadrosh *et al.*, 2014; Shipitsyna *et al.*, 2013; Sinclair *et al.*, 2015) were used for PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene. Libraries were sequenced by Micromon Genomics (Micromon, Monash University, Victoria, Australia) on the MiSeq platform (Illumina, San Diego, CA, USA). Sequence reads are available in the NCBI Sequence Read Archive under Bioproject PRJNA434520.

Bioinformatic analysis

Barcodes were extracted using QIIME v1.9.0 (Caporaso *et al.*, 2010) and demultiplexing was performed using idemp (https://github.com/yhwu/idemp). Primers and heterogeneity spacers were removed using TagCleaner standalone version 0.16 (Schmieder *et al.*, 2010). Reads were processed using DADA2 v1.6.0 (Callahan *et al.*, 2016). Reads were truncated based on quality profiles (at 250 bases for read 1 and 220 bases for read 2) and were discarded if they had ambiguous bases or exceeded the number of expected errors based on quality scores. Chimeras were identified and removed. Taxonomy was assigned using the default RDP Classifier implemented in DADA2 and the Silva reference database (v128). Species level assignment was performed using exact matching in the DADA2 package. Taxonomy for *Lactobacillus* spp. was confirmed by a BLAST search against a database of 16S rRNA gene sequences from 158 type strains.

BVAB1 has previously been misclassified as *Shuttleworthia* (Oakley *et al.*, 2008) and BVAB3 is named as *Fastidiosiplia* in the SILVA database (Van Der Pol *et al.*, 2019). We aligned *Shuttleworthia* and *Fastidiosiplia* ASVs against BVAB1 (NCBI GenBank AY724739.1), BVAB2 (AY724740.1) and BVAB3 (AY724741.1) using Clustal Omega (EMBL-EBI) (Chojnacki *et al.*,

2017; Sievers *et al.*, 2011). *Shuttleworthia* ASV had 100% identity to BVAB1. Two *Fastidiosiplia* ASVs had high identity to BVAB2 (99.50 and 100% identity, respectively), and a third *Fastidiosiplia* ASV had 100% identity to BVAB3. The ASVs were reclassified accordingly.

ASVs were removed if they had a total abundance of less than 0.001% or were present in only one specimen. The ASV table was screened for contaminants previously identified in negative controls (Plummer *et al.*, 2018a), as well as common sequencing contaminants (removed ASVs belonging to *Facklamia* and *Shewanella* genera and Halomonadaceae family) (Jervis-Bardy *et al.*, 2015; Salter *et al.*, 2014).

Microbiota group classification

Vaginal microbiota composition was categorised into three groups based on McKinnon *et al.* (2019): optimal non-*iners Lactobacillus* microbiota, *Lactobacillus iners* microbiota and non-optimal microbiota. Characteristics of the three vaginal microbiota groups and the number of specimens in each group are summarised below:

1. Optimal non-*iners Lactobacillus* microbiota - Dominated by non-*iners Lactobacillus* spp. (i.e. > 50% relative abundance of non-iners *Lactobacillus* spp. including *L. crispatus, L. gasseri, L. jensenii* and *L. fornicalis*). N=197 specimens. 148 specimens had >90% relative abundance of non-*iners Lactobacillus* spp., 22 had 75-90%, 27 had 50-75%.
2. *L. iners* microbiota - *L. iners* most abundant taxon present in specimen. N=94 specimens. 42 specimens had ≥90% relative abundance of *L. iners*, 27 had 75-90%, 20 had 50-75%. 5 had <50% but in each case *L. iners* was the most abundant taxon present.
3. Non-optimal microbiota - Specimens where the combined relative abundance of non-Lactobacillus species exceeds the relative abundance of non-*iners Lactobacillus* spp. N=69 specimens. 40 specimens had >90% combined relative abundance of non-*Lactobacillus* spp, 16 had 75-90%, 11 had 50-75%, 2 <50% but in both cases the combined relative abundance of non-*Lactobacillus* spp. > abundance of non-*iners Lactobacillus* spp.

Supplementary Table B1. Factors associated with number of *G. vaginalis* clades detected by multinomial regression

Risk factor	***G. Vaginalis* not detected n=188**	**Single clade n=63**			**Multiple clades (i.e. 2 or more) n=118**		
	n(%)	n(%)	RRR (95% CI)	*P* value[a]	n(%)	RRR (95% CI)	*P* value[a]
Age							
<28	92 (49)	29 (46)	1		54 (46)	1	
≥28	96 (51)	34 (54)	1.12 (0.57, 2.20)	0.734	64 (54)	1.14 (0.57, 2.26)	0.717
Self-reported past history of BV							
No	154 (82)	51 (81)	1		88 (75)	1	
Yes	34 (18)	12 (19)	1.07 (0.44, 2.60)	0.889	30 (25)	1.54 (0.66, 3.59)	0.314
Baseline sexual practices							
No. of lifetime FSPs[b]							
<5	96 (51)	33 (52)	1		35 (30)	1	
≥5	92 (49)	30 (48)	0.95 (0.48, 1.89)	0.881	83 (70)	2.47 (1.18, 5.19)	**0.017**
Number of FSP in last 12 months[b]							
≤1	129 (69)	44 (70)	1		55 (47)	1	
>1	59 (31)	19 (30)	0.94 (0.45, 1.98)	0.879	63 (53)	2.50 (1.20, 5.23)	**0.015**
Lifetime history of penile-vaginal sex with a man							
No	66 (35)	18 (29)	1		16 (14)	1	

Yes	122 (65)	45 (71)	1.35 (0.64, 2.87)	0.431	102 (86)	3.45 (1.44, 8.24)	**0.005**
Interval practices in prior 3 months[c]							
Any smoking							
No	128 (68)	41 (65)	1		50 (42)	1	
Yes	59 (32)	22 (35)	1.16 (0.62, 2.19)	0.637	68 (58)	2.95 (1.49, 5.84)	**0.002**
Any douching							
No	183 (98)	63 (100)			113 (96)	1	
Yes	4 (2)	0	…	…	5 (4)	2.02 (0.58, 7.10)	0.272
Any hormonal contraception							
No	157 (84)	57 (90)	1		106 (90)	1	
Yes	31 (16)	6 (10)	0.53 (0.22, 1.32)	0.175	12 (10)	0.57 (0.25, 1.30)	0.181
Last menstrual period							
≤7 days ago	31 (17)	10 (16)	1		13 (12)	1	
>7 days ago	149 (83)	53 (84)	1.10 (0.45, 2.70)	0.831	99 (88)	1.58 (0.75, 3.35)	0.228
Number of SP							
0	25 (13)	10 (16)	1		8 (7)	1	
1	147 (79)	45 (71)	0.77 (0.27, 2.18)	0.616	81 (69)	1.72 (0.58, 5.10)	0.326
≥2	15 (8)	8 (13)	1.33 (0.41, 4.35)	0.634	29 (25)	6.04 (1.74, 20.97)	**0.005**
Frequency of sex with any SP							

Several times/month or less	143 (76)	43 (68)	1		71 (60)	1	
Several times/week	45 (24)	20 (32)	1.48 (0.69, 3.15)	0.311	47 (40)	2.10 (1.07, 4.13)	**0.031**
Sex with a new partner[d]							
No	165 (88)	56 (89)	1		88 (75)	1	
Yes	23 (12)	7 (111)	0.90 (0.33, 2.42)	0.830	30 (25)	2.45 (1.29, 4.64)	**0.006**
Sexual practices with an FSP in prior 3 months[e]							
Number of FSPs[b]							
0	37 (20)	109 (16)	1		21 (18)	1	
≥1	151 (80)	53 (84)	1.30 (0.47, 3.63)	0.618	97 (82)	1.13 (0.50, 2.56)	0.766
Any receptive oral vaginal sex							
No[f]	77 (41)	25 (40)	1		42 (36)	1	
Yes	110 (59)	38 (60)	1.06 (0.53, 2.12)	0.860	76 (64)	1.27 (0.68, 2.35)	0.454
Any sharing of sex toys							
No toys/washed/condoms used[f]	142 (76)	53 (84)	1		85 (72)	1	
Unwashed	45 (24)	10 (16)	0.60 (0.26, 1.36)	0.218	33 (28)	1.23 (0.61, 2.44)	0.564
Current partner with BV symptoms							
No/don't know[f]	187 (100)	63 (100)			111 (94)		
Yes	0	0	…	…	7 (6)	…	…
Sexual practices with an MSP in prior 3 months[g]							
Number of MSPs[b]							
0	173 (92)	61 (97)	1		93 (79)	1	

≥1	15 (8)	2 (3)	0.38 (0.08, 1.77)	0.218	25 (21)	3.10 (1.12, 8.55)	**0.029**
Any penile-vaginal sex							
No[h]	173 (93)	61 (97)	1		95 (81)	1	
Yes	14 (7)	2 (3)	0.41 (0.09, 1.92)	0.255	23 (19)	2.99 (1.03, 8.65)	**0.043**
Any digital-vaginal sex							
No[h]	175 (94)	61 (97)	1		96 (81)	1	
Yes	12 (6)	2 (3)	0.48 (0.11, 2.16)	0.338	22 (19)	3.34 (1.23, 9.09)	**0.018**
Any penile-anal sex							
No[h]	185 (99)	62 (98)	1		109 (92)	1	
Yes	2 (1)	1 (2)	1.49 (0.13, 16.71)	0.746	9 (8)	7.64 (1.49, 39.13)	**0.015**
Self-reported symptoms and microbiota measures							
Abnormal vaginal discharge and/or odour							
No	175 (93)	60 (95)	1		90 (76)	1	
Yes	13 (7)	3 (5)	0.67 (0.18, 2.50)	0.554	28 (24)	4.19 (1.85, 9.49)	**0.001**
Nugent score							
0 to 3	176 (94)	54 (86)	1		74 (63)	1	
4 to 6	3 (2)	3 (5)	3.26 (0.49, 21.76)	0.223	11 (9)	8.72 (2.32, 32.76)	**0.001**
7 to 10	9 (5)	6 (10)	2.17 (0.72, 6.52)	0.167	33 (28)	8.72 (4.05, 18.78)	**<0.001**

Vaginal microbiota type[i]							
Optimal non-*iners Lactobacillus* microbiota	118 (66)	36 (59)	1		42 (36)	1	
L. iners microbiota	49 (27)	13 (21)	0.87 (0.37, 2.05)	0.749	31 (27)	1.78 (0.92, 3.42)	0.085
Non-optimal microbiota	13 (7)	12 (20)	3.03 (1.20, 7.61)	**0.019**	44 (38)	9.51 (4.36, 20.73)	**<0.001**

Abbreviations: RRR, relative risk ratio; CI, confidence interval; BV, bacterial vaginosis; FSP, female sexual partner; MSP, male sexual partner; SP, sexual partner (refers to total number of sexual partners in a study interval, female and male)

[a] Multinomial logistic regression with no *G. vaginalis* (i.e. *G. vaginalis* not detected) as the referent group. Analysis clustered for multiple specimens from participants (101 clusters). Includes 369 specimens from 101 participants.

[b] Variables were dichotomized at median value

[c] Interval characteristics were measured as any exposure over the prior follow-up interval (~90 days)

[d] Sex with a new partner with who first sexual contact was within 90 days. May represent a new FSP or new MSP.

[e] The following characteristics /sexual practices with an FSP were left out of the table for simplicity: digital-vaginal sex, receptive oral anal sex, digital anal sex. No significant associations between these practices and the number of *G. vaginalis* clades detected were identified.

[f] Or did not have a FSP

[g] The following sexual practices with an MSP were left out of the table for simplicity: condoms use for vaginal sex, receptive oral vaginal sex. No significant associations between these practices and the number of *G. vaginalis* clades detected were identified.

[h] Or did not have a MSP

[i] Vaginal microbiota type available for 360 specimens from 100 women

Supplementary Table B2. Practices associated with detection of specific *G. vaginalis* clades by logistic regression[a]

	Clade 1 detected vs any other clade/s n=116/181		**Clade 2 detected vs any other clade/s n=76/181**		**Clade 3 detected vs any other clade/s n=17/181**		**Clade 4 detected vs any other clade/s n=136/181**	
	OR (95% CI)	*P* value[b]	OR (95% CI)	*P* value[c]	OR (95% CI)	*P* value[d]	OR (95% CI)	*P* value[e]
Age								
<28	1		1		1		1	
≥28	0.57 (0.27, 1.21)	0.142	1.60 (0.76, 3.26)	0.217	1.65 (0.54, 5.00)	0.378	1.17 (0.53, 2.60)	0.695
Self-reported past history of BV								
No	1		1		1		1	
Yes	0.65 (0.26, 1.65)	0.368	2.26 (0.92, 5.56)	0.074	1.60 (0.49, 5.17)	0.436	1.62 (0.57, 4.63)	0.368
Baseline sexual practices								
No. of lifetime FSPs[f]								
<5	1		1		1		1	
≥5	0.98 (0.43, 2.23)	0.962	1.40 (0.64, 3.03)	0.399	1.42 (0.44, 4.63)	0.557	3.93 (1.74, 8.87)	**0.001**
Number of FSP in last 12 months[f]								
≤1	1		1		1		1	
>1	2.26 (0.96, 5.29)	0.061	1.63 (0.76, 3.48)	0.211	0.85 (0.28, 2.59)	0.774	2.73 (1.11, 6.74)	**0.029**
Ever had vaginal sex with a man								
No	1		1		1		1	
Yes	2.10	0.128	0.67	0.388	…	…	2.88	**0.026**

	(0.81, 5.45)		(0.27, 1.66)				(1.13, 7.32)	
Interval practices in prior 3 months[g]								
Any smoking								
No	1		1		1		1	
Yes	2.92	**0.004**	1.35	0.392	1.83	0.287	1.28	0.533
	(1.40, 6.10)		(0.68, 2.68)		(0.60, 5.57)		(0.59, 2.75)	
Any douching								
No	1		1		1		1	
Yes	0.52	0.484	2.89	0.295	2.34	0.487	1.20	0.874
	(0.09, 3.21)		(0.40, 21.06)		(0.21, 25.80)		(0.12, 11.57)	
Any hormonal contraception								
No	1				1		1	
Yes	0.72	0.540	0.87	0.794	0.56	0.587	2.62	0.218
	(0.25, 2.06)		(0.30, 2.54)		(0.07, 4.60)		(0.57, 12.09)	
Last menstrual period								
≤7 days ago	1		1		1		1	
>7 days ago	1.35	0.453	0.67	0.334	0.45	0.204	1.58	0.315
	(0.62, 2.93)		(0.29, 1.52)		(0.13, 1.54)		(0.65, 3.84)	
Number of SP								
0	1		1		1		1	
1	5.09	**0.007**	0.67	0.457	1.30	0.689	1.10	0.874
	(1.55, 16.66)		(0.23, 1.94)		(0.35, 4.79)		(0.34, 3.56)	
≥2	8.19	**0.003**	0.83	0.759	…	…	1.80	0.419
	(2.05, 32.75)		(0.24, 2.82)				(0.43, 7.45)	

Frequency of sex with any SP								
Several times/month or less	1		1		1		1	
Several times/week	2.03	**0.031**	1.61	0.136	1.18	0.757	1.26	0.539
	(1.07, 3.88)		(0.86, 3.02)		(0.41, 3.36)		(0.61, 2.60)	
Sex with a new partner[h]								
No	1		1		1		1	
Yes	1.58	0.210	2.07	**0.041**	3.09	**0.032**	1.67	0.260
	(0.77, 3.23)		(1.03, 4.15)		(1.10, 8.69)		(0.68, 4.06)	
Interval practices with an FSP in prior 3 months[i]								
Number of FSPs[f]								
0	1		1		1		1	
≥1	2.49	**0.027**	0.52	0.130	1.89	0.458	0.84	0.720
	(1.11, 5.61)		(0.23, 1.21)		(0.35, 10.13)		(0.32, 2.22)	
Any receptive oral vaginal sex								
No[j]	1		1		1		1	
Yes	1.57	0.142	0.95	0.877	1.21	0.729	1.56	0.207
	(0.86, 2.87)		(0.51, 1.77)		(0.41, 3.57)		(0.78, 3.11)	
Any sharing of sex toys								
No toys/washed/condoms used[j]	1		1		1		1	
Unwashed	1.90	0.098	2.21	**0.030**	1.79	0.299	1.72	0.232
	(0.89, 4.07)		(1.08, 4.52)		(0.60, 5.35)		(0.71, 4.18)	
Current partner with BV symptoms								
No/don't know[j]	1		1		1		1	
Yes	1.60	0.569	4.38	0.094	17.40	**0.001**	0.51	0.401
	(0.32, 7.98)				(3.36, 90.11)		(0.11, 2.44)	

			(0.78, 24.67)					
Interval practices with an MSP in prior 3 months[k]								
Number of MSPs[f]								
0	1		1		1		1	
≥1	2.54 (0.74, 8.77)	0.140	5.62 (1.81, 17.50)	**0.003**	0.64 (0.11, 3.69)	0.620	3.41 (0.73, 15.88)	0.118
Any penile-vaginal sex								
No[l]	1		1		1		1	
Yes	2.25 (0.64, 7.88)	0.205	4.80 (1.52, 15.17)	**0.007**	0.71 (0.12, 4.12)	0.706	3.02 (0.64, 14.17)	0.161
Any digital-vaginal sex								
No[l]	1		1		1		1	
Yes	3.37 (0.84, 13.52)	0.086	5.89 (1.76, 19.73)	**0.004**	0.75 (0.13, 4.30)	0.744	2.86 (0.62, 13.19)	0.179
Any penile-anal sex								
No[l]	1		1		1		1	
Yes	1.38 (0.38, 5.02)	0.626	4.71 (1.06, 21.00)	**0.042**	1.13 (0.13, 9.54)	0.913	0.88 (0.21, 3.63)	0.860
Self-reported symptoms and microbiota measures								
Abnormal vaginal discharge and/or odour								

No	1		1		1		1	
Yes	1.16 (0.55, 2.47)	0.692	1.87 (0.87, 4.02)	0.107	2.68 (0.88, 8.17)	0.083	1.80 (0.67, 4.86)	0.247
Nugent score								
0 to 3	1		1		1		1	
4 to 6	1.44 (0.52, 3.95)	0.480	3.49 (1.17, 10.36)	**0.025**	4.90 (1.11, 21.57)	**0.035**	0.84 (0.27, 2.63)	0.766
7 to 10	3.55 (1.76, 7.18)	**<0.001**	1.90 (1.02, 3.55)	**0.043**	3.67 (1.22, 11.04)	**0.020**	1.72 (0.78, 3.81)	0.180
Vaginal microbiota type[m]								
Optimal non-*iners Lactobacillus* microbiota	1		1		1		1	
L. iners microbiota	1.68 (0.82, 3.45)	0.160	1.24 (0.59, 2.58)	0.574	3.26 (0.83, 12.82)	0.091	0.64 (0.29, 1.42)	0.275
Non-optimal microbiota	3.36 (1.65, 6.84)	**0.001**	1.60 (0.82, 3.10)	0.165	2.98 (0.81, 11.01)	0.102	1.49 (0.67, 3.33)	0.326

Abbreviations: OR, odds ratio; CI, confidence interval; BV, bacterial vaginosis; FSP, female sexual partner; MSP, male sexual partner; SP, sexual partner (refers to total number of sexual partners in a study interval, female and male)

[a] Includes only specimens positive for one or more *G. vaginalis* clade. This includes a total of 181 specimens from 77 participants

[b] Logistic regression fitted with generalised estimating equations (GEE) with detection of clade/s 2, 3 and/or 4 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)

[c] Logistic regression fitted with GEE with detection of clade/s 1, 3 and/or 4 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)

[d] Logistic regression fitted with GEE with detection of clade/s 1, 2 and/or 4 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)

[e] Logistic regression fitted with GEE with detection of clade/s 1, 2 and/or 3 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters)

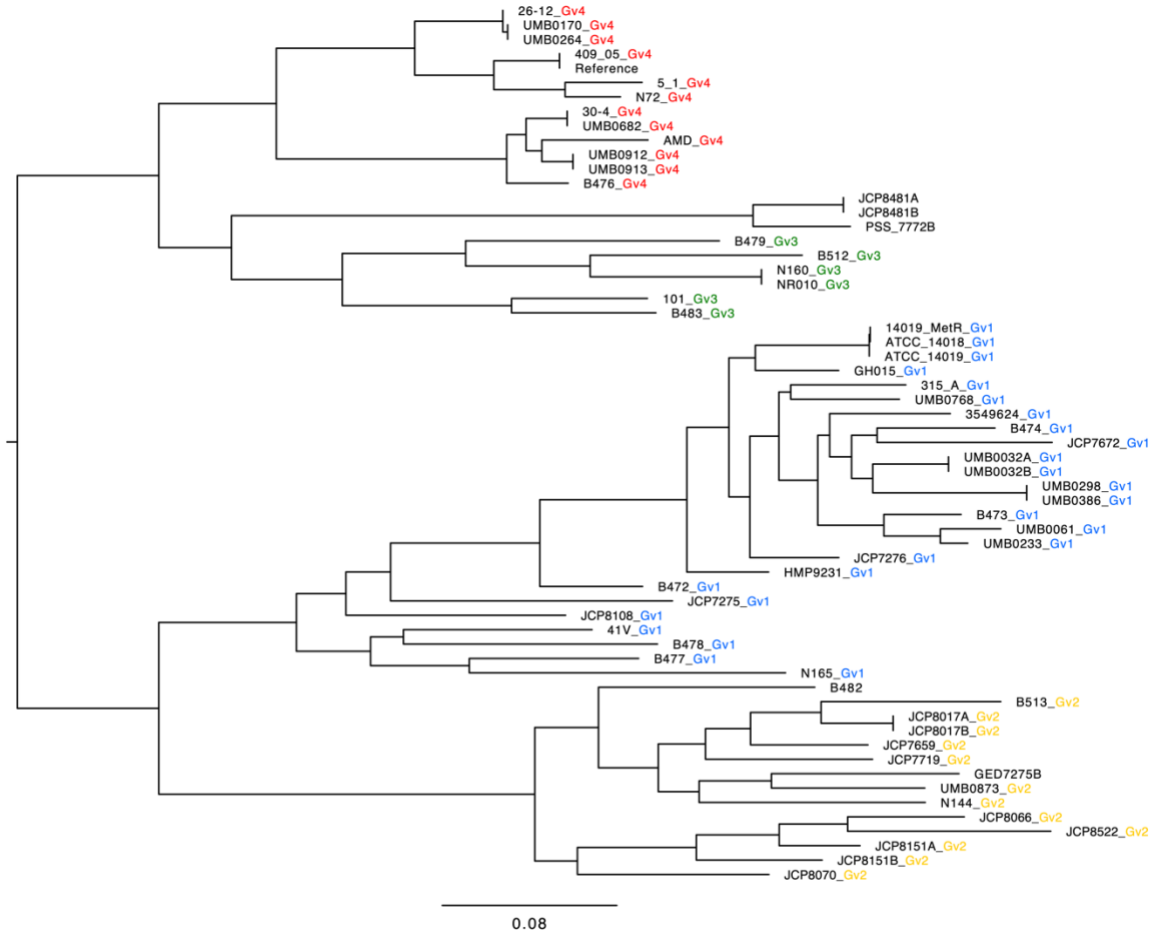
[f] Variables were dichotomized at median value
[g] Longitudinal characteristics were measured as any exposure over the prior follow-up interval (~90 days)
[h] Sex with a new partner with who first sexual contact was within 90 days. May represent a new FSP or new MSP.
[i] The following characteristics /sexual practices with an FSP were left out of the table for simplicity: digital-vaginal sex, receptive oral anal sex, digital anal sex. No significant associations between these sexual practices and *G. vaginalis* clades were identified.
[j] Or did not have a FSP
[k] The following sexual practices with an MSP were left out of the table for simplicity: condoms use for vaginal sex and vaginal sex following anal sex. No significant associations between these sexual practices and *G. vaginalis* clades were identified.
[l] Or did not have a MSP
[m] Vaginal microbiota type available for 360 specimens from 100 women. Optimal non-*iners Lactobacillus* microbiota includes specimens predominately consisting of non-*iners Lactobacillus* spp., *Lactobacillus iners* microbiota includes specimens predominately consisting of *L. iners* and non-optimal microbiota includes specimens predominately consisting of non-*Lactobacillus* spp.

Supplementary Table B3. *G. vaginalis* clades associated with Nugent score by multinomial regression

	Nugent score 4-6 (intermediate-BV) vs Nugent score 0-3 (no BV)		Nugent score 7-10 (Nugent-BV) vs Nugent score 0-3 (no BV)	
	aRRR (95% CI)	***P value*[a]**	**aRRR (95% CI)**	***P value*[a]**
Clade 1	1		1	
	1.66 (0.40, 6.81)	0.482	5.07 (1.82, 14.12)	**0.002**
Clade 2	1		1	
	5.77 (1.70, 19.64)	**0.005**	2.88 (1.35, 6.16)	**0.006**
Clade 3	1		1	
	5.12 (1.21, 21.72)	**0.027**	3.27 (1.11, 9.68)	**0.032**
Clade 4	1		1	
	0.99 (0.21, 4.64)	0.994	2.39 (0.89, 6.46)	0.085

Abbreviations: aRRR, adjusted relative risk ratio; CI, confidence interval

[a] Adjusted multinomial logistic regression with Nugent score 0-3 (no BV) as the referent group. Analysis clustered for multiple specimens from participants (77 clusters). Includes 181 specimens from 77 participants. Variables in the adjusted analysis include *G. vaginalis* clade 1, clade 2, clade 3 and clade 4.



Supplementary Figure B1 - *Phylogenetic relationship of 60 G. vaginalis isolates based on the alignment of the core genome SNPs. Strain 409-05 (Accession number NC_013721) was used as the reference genome. Presence of a clade-specific amplicon in each isolate is indicated on the tree (Gv1 indicates presence of clade 1 amplicon, Gv2 indicates presence of clade 2 amplicon, Gv3 indicates presence of clade 3 amplicon and Gv4 indicates presence of clade 4 amplicon)*

Appendix C. Supplementary files: Chapter 4

Supplementary Table C1. List of controls processed alongside biological specimens

Control type	Details
Reagent/Extraction Controls	PBS AssayAssure® Genelock (SierraMolecular, USA) Ultrapure water
PCR negative controls	
Positive control	ZymoBIOMICS™ Microbial Community Standard Catalog No. D6300 BEI resources HM-276D Genomic DNA from Microbial Mock Community B

Supplementary Table C2. ASVs identified as potential contaminants

ASV no.		Phylum	Class	Order	Family	Genus	Total prevalence (%)	Total read count	Vaginal prevalence (%)	Vaginal read count	Cutaneous penile prevalence (%)	Cutaneous penile read count	Urine prevalence (%)	Urine read count	Negative control prevalence (%)
ASV2	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia-Caballeronia-Paraburkholderia	36	737907	6	85	17	6176	87	630598	51
ASV5	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	48	446900	22	376	45	46620	68	45644	95
ASV44	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	17	47528	0	0	0	0	55	32894	12
ASV121	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	Leifsonia	13	13937	0	0	0	0	43	9901	10
ASV26	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	25	96086	2	351	25	34426	36	20065	85
ASV120	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	17	13813	0	0	13	1419	31	1783	51
ASV100	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	19	16653	0	0	16	1167	26	2187	83
ASV110	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	13	15471	1	9	15	2211	17	1130	39
ASV227	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Curvibacter	10	5564	0	0	8	436	16	262	44
ASV257	Bacteria	Bacteroidota	Bacteroidia	Cytophagales	Spirosomaceae	Flectobacillus	8	4795	0	0	7	725	14	467	22
ASV205	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	8	6467	0	0	6	278	14	708	29
ASV354	Bacteria	Proteobacteria	Alphaproteobacteria	Paracaedibacterales	Paracaedibacteraceae	Candidatus_Finniella	7	3056	1	2	2	164	14	489	29
ASV202	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	7	6841	0	0	3	173	12	380	34
ASV141	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	9	11465	0	0	10	1129	10	704	37
ASV583	Bacteria	Proteobacteria	Alphaproteobacteria	SAR11_clade	Clade_I	Clade_Ia	12	1110	16	377	8	171	9	399	24
ASV321	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	7	3548	1	6	7	400	9	364	24
ASV277	Bacteria	Firmicutes	Bacilli	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	7	4345	0	0	5	222	9	289	37
ASV926	Bacteria	Proteobacteria	Gammaproteobacteria	SAR86_clade	NA	NA	8	423	10	173	5	60	8	158	10
ASV1183	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Formosa	6	235	3	32	5	59	6	101	15
ASV143	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	6	11444	2	14	8	1047	6	744	17
ASV438	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	4	2091	0	0	5	380	5	100	15
ASV251	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	5	4967	0	0	5	3066	5	297	20
ASV1164	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Ascidiaceihabitans	5	245	6	74	3	36	5	99	12
ASV692	Bacteria	Patescibacteria	Saccharimonadia	Saccharimonadales	NA	NA	3	811	0	0	2	21	5	267	7
ASV611	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3	1016	0	0	1	61	5	210	12
ASV476	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	3	1738	1	2	0	0	5	165	17
ASV231	Bacteria	Actinobacteriota	Actinobacteria	Corynebacteriales	Nocardiaceae	Rhodococcus	6	5718	0	0	13	1006	5	1127	15
ASV1099	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Planktomarina	4	292	2	17	5	93	5	155	10
ASV522	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium-Methylorubrum	3	1360	0	0	4	122	5	115	12
ASV1452	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Flavobacteriaceae	NS4_marine_group	5	144	7	64	4	26	5	39	7
ASV168	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	5	8725	1	13	6	1137	4	881	29

Supplemer

ASV no.	Negative control read count	Notes	Sequence
ASV2	101048	*Excluded as identified as highly abundant in AssayAssure® Genelock (urine stabilisation medium)	TGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTTGGCTCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGCTAAGACCGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTGGTGACTGGCAGGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV5	354260		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV44	14634	*Excluded as identified as highly abundant in AssayAssure® Genelock (urine stabilisation medium)	TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATCGATCAGTCAGGGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTGATACTGTCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV121	4036	*Excluded as identified as highly abundant in AssayAssure® Genelock (urine stabilisation medium)	TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGTTCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACA
ASV26	41244		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV120	10611		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCTTTTACCAGGGATGATAATGACAGTACCTGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGTTACTCAAGTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGCCTTTGAAACTAGGTGACTGGAATCTTGGAGAGGCGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACTCGCTGGACAAGTATTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV100	13299		TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTTGAATAAGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV110	12121		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTTCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV227	4866		TGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGGTTTGACTTAATACGTTGGACTCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTATGTAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTAGTGACTGCATAGCTGGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV257	3603		TAGGGAATATTGGGCAATGGACGGAAGTCTGACCCAGCCATGCCGCGTGCAGGATGAAGGCGTTATGCGTTGTAAACTGCTTTTTATAGGAAGAAGGAGACCTTGCGAGGTTTGGTGACGGTACTATAAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGTTTATTAAGTCAGTGGTGAAAGACGGTCGCTCAACGATTGCAGTGCCATTGATACTGGTAGACTTGAGTTCGTATGAGGTAGCTGGAATGGATAGTGTAGCGGTGAAATGCATAGATATTATCCAGAACTCCAATTGCGTAGGCAGGTTACTAATACGATACTGACGCTGATGCACGAAAGTGTGGGGATCAAACA
ASV205	5481		TGAGGAATATTGGTCAATGGTCGCAAGACTGAACCAGCCATGCCGCGTGCAGGATGAAGCATCTATGGTGTGTAAACTGCTTTTGTACGGGAAGAAACACTCCTACGAGTAGGAGCTTGACGGTACCGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGCTTTATAAGTCAGTGGTGAAATCCGGCAGCTTAACTGTCGAATTGCCATTGATACTGTAGGGCTTGAATTGTTGTGAAGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGATATTACATGGAATACCAATTGCGAAGGCAGGTTACTAACAATACATTGACGCTGATGGACGAAAGCGTGGGTAGCGAACA
ASV354	2401		TGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGACGATAATGACGGTACCTGACGAATAAGCTCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGTCTTATAAGTTTGGTGTGAAAGCCCTGGGCTTAACCTAGGAACAGCATTGAATACTGTAAGACTTGAGACCGAGAGAGGGAAATGGAATTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCGATTTCCTGGCTCGGTACTGACGCTAAGGCGCGAAAGCGTGGGGAGCAAACA
ASV202	6288		TAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV141	9632		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV583	163		TGGGGAATCTTGCACAATGGAGGAAACTCTGATGCAGCGATGCCGCGTGAGTGAAGAAGGCCCTTGGGTTGTAAAGCTCTTTCGTCGGGGAAGAAAATGACTGTACCCGAATAAGAAGGTCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGACCTAGCGTAGTTCGGAATTACTGGGCTTAAAGAGTTCGTAGGTGGTTGAAAAAGTTAGTGGTGAAATCCCAGAGCTTAACTCTGGAACTGCCATTAAAACTTTTCAGCTAGAGTATGATAGAGGAAAGCAGAATTTCTAGTGTAGAGGTGAAATTCGTAGATATTAGAAAGAATACCAATTGCGAAGGCAGCTTTCTGGATCATTACTGACACTGAGGAACGAAAGCATGGGTAGCGAAGA
ASV321	2778		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGCAGTGTGTTAATAGCACATTGCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV277	3834		TAGGGAATTTTCGGCAATGGGGGAAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTGTTGTAAGGGAAGAACGATAGTTAGAGGGAATGCTAGCTATATGACGGTACCTTACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGAGCGCAGGCGGTTTATTAAGTTTATGGTGAAAGTTCGGGGCTCAACCCCGTGATGCCATAGAAACTGGTAGACTTGAGTACAGGAGAGGTTAGTGGAATTCCATGTGTAGCGGTAAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTAACTGGCCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAATA
ASV926	32		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTAAGCAGGGAGAAAAAGTTATAAGTTAATACCTTATAACCCTGATGTTACCTGCAGAATAAGCACCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCTGGGCTCAACCTAGGAACTGCATCCAAAACTAACTCACTAGAGTACGATAGAGGGAGGTAGAATTCATAGTGTAGCGGTGGAATGCGTAGATATTATGAAGAATACCAGTGGCGAAGGCGGCCTCCTGGATCTGTACTGACACTGAGGTGCGAAAGCGTGGGTAGCGAACA
ASV1183	43		TGAGGAATATTGGACAATGGAGGCAACTCTGATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTTTATATAGGAAGAAACCTACTTACGTGTAAGTAGCTGACGGTACTATAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGTGGATAATTAAGTCAGAGGTGAAATCTTGCAGCTCAACTGTAAAATTGCCTTTGATACTGGTTATCTTGAGTTATTATGAAGTAGTTAGAATATGTAGTGTAGCGGTGAAATGCATAGATATTACATAGAATACCAATTGCGAAGGCAGATTACTAATAATCAACTGACACTGATGGACGAAAGCGTGGGTAGCGAACA
ASV143	9639		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATACTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV438	1611		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGTGGGGAAGAAAGCCTGGTGGTTAATACCCGTCAGGAAGGACATCACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAACA
ASV251	1604		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGTAGTGTGTTAATAGCACATTGCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV1164	36		TGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTCGTAAAGCTCTTTCGCCTGTGATGATAATGACAGTAGCAGGTAAAGAAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATTGGAAAGTTGGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTCCAAAACTATCAGTCTAGAGTTCGAGAGAGGTGAGTGGAATTCCAAGTGTAGAGGTGAAATTCGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGTGCGAAAGTGTGGGGAGCAAACA
ASV692	523		TGAGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGCAGGATGAAGGCCCTCGGGTCGTAAACTGCTTTTATAAGTGAAGAATATGACGGTAACTTATGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTCATACGTAGGGTGCAAGCATTATCCGGAGTGACTGGGCGTAAAGAGTTGCGTAGGTGGTTTGTTAAGTAGGTAGTGAAATCTGGCGGCTCAACCGTACAGGCTATTACCTAAACTGGCAAACTCGAGAATGGTAGAGGTAACTGGAATTTCTTGTGTAGGAGTGAAATCCGTAGATATAAGAAGGAACACCAATGGCGTAGGCAGGTTACTGGACCATTTCTGACACTAAGGCACGAAAGCGTGGGGAGCGAACG
ASV611	745		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCGAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTAGGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGGCTCATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV476	1571		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGTGGGGAAGAAAGCCTTCCGGTTAATACCCGGGAGGAAGGACATCACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAACA
ASV231	3585		TGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTCAACTGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACA
ASV1099	27		TAGGGAATCTTAGACAATGGGGGCAACCCTGATCTAGCCATGCCGCGTGTGTGATGAAGGCCCTAGGGTCGTAAAGCACTTTCGCCAGAGATGATAATGACAGTATCTGGTAAAGAAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATTAGTAAGTTAGAGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTAATACTGCTAGTCTTGAGTTCGAGAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGCTCGATACTGACGCTGAGGTGCGAAAGTGTGGGGAGCAAACA
ASV522	1123		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAGAATGGCCTTCGATACTGGGACGCTTGAGTATGGTAGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCGGTGGCGAAGGCGGCCAACTGGACCATTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV1452	15		TAAGGAATATTGGACAATGGACGAAAGTCTGATCCAGCCATGCCGCGTGCAGGATGACAGCCCTACGGGTTGTAAACTGCTTTTATACAGGAAGAAACACTCCGACGTGTCGGAGCTTGACGGTACTGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGCAGGCTGTTTTTTAAGTCAGAGGTGAAATCCTACAGCTCAACTGTAGAACTGCCTTTGATACTGGAAAACTTGAGTTATTGTGAAGTGGTTAGAATGTGTGGTGTAGCGGTGAAATGCATAGATATCACACAGAATACCGATTGCGAAGGCAGATCACTAACAATATACTGACGCTCATGGACGAAAGCGTGGGGAGCGAACA
ASV168	6694		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA

Supplementary Table C2. ASVs identified as potential contaminants

ASV no.		Phylum	Class	Order	Family	Genus	Total prevalence (%)	Total read count	Vaginal prevalence (%)	Vaginal read count	Cutaneous penile prevalence (%)	Cutaneous penile read count	Urine prevalence (%)	Urine read count	Negative control prevalence (%)
ASV208	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	4	6530	1	2	5	4480	4	191	17
ASV332	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	Advenella	1	3290	0	0	0	0	4	349	2
ASV1434	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	3	144	2	21	5	66	3	47	5
ASV567	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	3	1187	0	0	1	16	3	90	17
ASV429	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3	2165	0	0	5	397	3	163	17
ASV242	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia/Shigella	4	5114	0	0	4	555	3	59	24
ASV665	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	NA	3	875	1	27	3	260	3	46	7
ASV1205	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Cryomorphaceae	NA	2	245	0	0	3	88	3	135	5
ASV1326	Bacteria	Proteobacteria	Alphaproteobacteria	Puniceispirillales	SAR116_clade	Candidatus_Puniceispirillum	3	174	3	42	3	44	3	66	5
ASV335	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3	3238	0	0	2	226	3	60	17
ASV556	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	2	1281	0	0	2	108	3	153	12
ASV764	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Cryomorphaceae	NA	5	667	5	70	5	96	2	29	7
ASV262	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	3	4665	0	0	4	820	2	198	17
ASV340	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	5	3183	0	0	4	106	2	39	39
ASV309	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	3	3810	0	0	3	337	2	329	17
ASV160	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	2	9717	0	0	3	7661	2	1843	10
ASV766	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	2	674	0	0	1	15	2	41	10
ASV825	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	2	575	0	0	1	58	2	81	10
ASV1031	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	1	352	0	0	1	20	2	25	2
ASV11	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia	13	214042	21	2243	15	29623	1	53	20
ASV180	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3	7865	0	0	3	1088	1	218	20
ASV378	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	2	2713	0	0	3	2308	1	50	5
ASV950	Bacteria	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus	1	416	0	0	2	174	1	48	2
ASV624	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ochrobactrum	2	973	0	0	1	81	1	21	10
ASV1502	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Cryomorphaceae	NA	2	130	3	59	1	14	1	34	5
ASV491	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Pseudochrobactrum	1	1603	0	0	1	17	1	37	2
ASV734	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Anaerobacillus	1	712	0	0	1	3	1	8	10
ASV793	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium_sensu_stricto_13	1	640	0	0	1	40	1	33	10
ASV877	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	1	503	0	0	1	27	1	17	5
ASV1415	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Morganellaceae	Proteus	1	158	0	0	1	29	1	20	2
ASV1398	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	1	162	0	0	1	137	1	13	2

Supplemer

ASV no.	Negative control read count	Notes	Sequence
ASV208	1857		TGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAAGCTTCTCCTAATA CGAGAGGCCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTATGTAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCATGGCTAGAGTACGGTAGAGGGGGATGGAATTCCGCGTGTA GCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV332	2941		TGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCAGGGAAGAAAAGGTTTCGGATAAT ACCTGGAACTGATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG TGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTCCCGAACTAGAGTATGTCAGAGGGGGGTGGAATTCCACGTGTA GCAGTGAAATGCGTAGATATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV1434	10		TGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGAAAGTTTAGTAGTTAAT ACCTGCTAGATGTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTCTGTTAAGCTAGATGTGAAAGCCCCGCGCTCAACGTGGGAGGGTCATTTAGAACTGGCAGACTAGAGTCTTGGAGAGGGGAGTGGAATTCCAGGTGT AGCGGTGAAATGCGTAGAGATCTGGAGGAACATCAATGGCGAAGGCAACTCCCTGGCCAAAGACTGACGCTCATGTGCGAAAGTGTGGGTAGCGAACA
ASV567	1081		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGTGGGGAAGAACGCCTGGTGGTTAAT ACCCGTCAGGAAGGACATCACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCG CGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGT AGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAACA
ASV429	1605		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAAT ACGTAATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV242	4500		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGATGAAGGGAGTAAAGTTAA TACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTA GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV665	542		TGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTTGTTAGGGAAGAAAAGCTAGTTTTTAAT AAAAATTAGTGATGACGGTACCTAAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTATTGGGCGTAAAGCG AGTGCAGACGGTTACTTAAGCCAGATGTGAAATCCCCAAGCTTAACTTGGGACGTGCATTTGGAACTGGGTGACTAGAGTGTGTCAGAGGGAGGTAGAATTCCACATGTA GCGGTGGAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAAACA
ASV1205	22		TGAGGAATATTGGTCAATGGAGGAAACTCTGAACCAGCCATGCCGCGTGAAGGATGACGGCCCTATGGGTTGTAAACTTCTTTTATAGAGGAAGAAACTTGTCTACGTGTA GACAACTGACGGTACTCTACGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGATTCATTGGGTTTAAAGGGTCCGTAG GCGGGTCTTTAAGTCAGTGGTGAAAGCCGACAGCTCAACTGTCGAACTGCCATTGATACTGGAGATCTTGAGTACAAATGAAGTAGGCGGAATGAGTCATGTAGCGGTGA AATGCATAGATATGACTCAGAACACCGATTGCGAAGGCAGCTTACTAACATGTAACTGACGCTGAGGGACGAAAGCGTGGGGAGCGAACA
ASV1326	22		TGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGATGAAGGCCTTAGGGTTGTAAAGCACTTTCACTGGTGAAGATGATGACGGTAACCAG AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCGAGCGTTGTTCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGTCTTTTAAGTTAG GCGTGAAAGCCCCGGGCTCAACCCGGGAACTGCGCTTAAGACTGGAAGACTAGAAAACGGAAGAGGGTAGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGG GAAGAACACCAGTGGCGAAAGCGGCTACCTGGTCCGATTTTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV335	2952		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAAT ACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV556	1020		TAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAA CTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC GCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGATAGTGGAATTCCAAGTGTA GCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV764	472		TGAGGAATATTGGTCAATGGAGGCAACTCTGAACCAGCCATGCCGCGTGAAGGATGACGGCCCTATGGGTTGTAAACTTCTTTTATAGAGGAAGAAACTTGTCTACGTGTA GACAACTGACGGTACTCTACGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGATTCATTGGGTTTAAAGGGTCCGTAG GCGGGTCTTTAAGTCAGTGGTGAAAGCCGACAGCTCAACTGTCGAACTGCCATTGATACTGGAGACCTTGAGTACAAATGAAGTAGGCGGAATGAGTCATGTAGCGGTGA AATGCATAGATATGACTCAGAACACCGATTGCGAAGGCAGCTTACTAACATGTAACTGACGCTGAGGGACGAAAGCGTGGGGAGCGAACA
ASV262	3647		TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAAAGAAGAACAAGGATGAGAGTAA CTGCTCATCCCCTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAG CGCAGGCGGTTCTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGAGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG CGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACA
ASV340	3038		TAGGGAATCATCCGCAATGGACGAAAGTCTGACGGTGCAACGCCGCGTGAGTGATGAAGGATTTCGGTTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAA TAGGTCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG CGCGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGT AGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV309	3144		TGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAA TATCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV160	213		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAAT ACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCG TGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGT AGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACA
ASV766	618		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTACCCGGGAAGATAATGACTGTACCGGG AGAATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCTTTGTAAGTCAGA GGTGAAAGCCTGGAGCTCAACTCCAGAACTGCCTTTGAGACTGCATCGCTTGAATCCAGGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGA AGAACACCAGTGGCGAAGGCGGCTCACTGGACTGGTATTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV825	436		TAGGGAATCATCCGCAATGGACGAAAGTCTGACGGTGCAACGCCGCGTGAGTGATGAAGGATTTCGGTTCGTAAAGCTCTGTTGTTAGGGAAGAAACCGTGCCGAACGAA CTAGGTCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGC GCGCGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTG TAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV1031	307		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAAT ACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGT GCGTAGGTGGTTGTTTAAGTCTGTCGTGAAAGCCCTGGGCTCAACCTGGGAATTGCGATGGAAACTGGGCGACTAGAGTGTGGCAGAGGGTAGTGGAATTCCTGGTGTA GCAGTGAAATGCGTAGAGATCAGGAGGAACATCCGTGGCGAAGGCGACTGCCTGGGCCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACA
ASV11	182123		TGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATGGCTCTGGTTAAT ACCTGGGGTCGATGACGGTACCGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG TGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTGGTGACTGCACGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCCACGTGTA GCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV180	6559		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGAGCAGTAAATTAAT ACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV378	355		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAAT ACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCG TGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTA GCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACA
ASV950	194		TAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAA CTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGC GCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTA GCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACA
ASV624	871		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGG AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGACTTTTAAGTCAGG GGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGA GGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV1502	23		TGAGGAATATTGGTCAATGGAGGAAACTCTGAACCAGCCATGCCGCGTGAAGGATGACGGCCCTATGGGTTGTAAACTTCTTTTATATGGGAAGAAACTTATCTACGTGTA GATAACTGACGGTACCATACGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGATTCATTGGGTTTAAAGGGTCCGTAG GCGGATTTTTAAGTCAGTGGTGAAAGCCGACAGCTCAACTGTCGAACTGCCATTGATACTGGAAATCTTGAGTACAAATGAAGTAGGCGGAATGAGTCATGTAGCGGTGA AATGCATAGATATGACTCAGAACACCGATTGCGAAGGCAGCTTACTAACATGTAACTGACGCTGAGGGACGAAAGCGTGGGGAGCGAACA
ASV491	1549		TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGG AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGACTTTTAAGTCAGG GGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGAAGTCTTGAGTATGGAAGAGGTAAGTGGAATTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA GGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV734	701		TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTTCAAA TAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGC GCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAATCTCGCGGCTCAACCGCGAGCGGCCATTGGAAACTGGGAGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGT AGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV793	567		TGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGAATGAAGAAGGCCTTAGGGTTGTAAAGTTCTGTTTTCTGGGACGATAATGACGGTACCAGA GGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGATGCGTAGGCGGATGTTTAAGTGAG ATGTGAAATACCCGAGCTCAACTTGGGTGCTGCATTTCAAACTGGATATCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGG AAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGCTGAGGCATGAAAGCGTGGGGAGCAAACA
ASV877	459		TGGGGAATTTTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTAGTAGCTAAT ATCTATTACTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTGTGTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGAGACTGCACGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCCACGTGTAG CAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV1415	109		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAAGTTAA TACCTTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCACGTGTA GCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV1398	12		TGGGGAATCTTGCGCAATGGGCGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCACCGGGGACGATAATGACGGTACCCGG AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGATCGTTAAGTCAG AGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTGATACTGGCGATCTTGAGTATGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGG AAGAACACCAGTGGCGAAGGCGACATACTGGCTCATTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA

Supplementary Table C2. ASVs identified as potential contaminants

ASV no.		Phylum	Class	Order	Family	Genus	Total prevalence (%)	Total read count	Vaginal prevalence (%)	Vaginal read count	Cutaneous penile prevalence (%)	Cutaneous penile read count	Urine prevalence (%)	Urine read count	Negative control prevalence (%)
ASV785	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	Nesterenkonia	1	650	0	0	0	0	1	51	2
ASV679	Bacteria	Actinobacteriota	Actinobacteria	Corynebacteriales	Dietziaceae	Dietzia	2	839	0	0	5	296	1	56	2
ASV529	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	Micrococcus	2	1400	0	0	5	767	1	204	2
ASV895	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	2	484	0	0	3	133	1	56	7
ASV466	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	1	1826	0	0	1	139	1	7	7
ASV835	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Citrobacter	1	567	0	0	1	22	1	27	7
ASV1321	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1	171	0	0	1	139	1	16	2
ASV413	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2	2337	0	0	0	0	1	6	22
ASV912	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	Cloacibacterium	0	458	0	0	0	0	1	58	2
ASV973	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Pseudochrobactrum	0	394	0	0	0	0	1	32	2
ASV992	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Anaerobacillus	0	376	0	0	0	0	1	23	2
ASV1004	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Phyllobacterium	2	366	0	0	3	310	0	0	7
ASV1168	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1	260	0	0	2	152	0	0	2
ASV1181	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	1	255	0	0	2	244	0	0	2
ASV720	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	1	736	0	0	1	52	0	0	12
ASV1176	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	1	133	0	0	1	73	0	0	5
ASV507	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	1	1470	0	0	1	3	0	0	10
ASV494	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	1	1589	0	0	1	141	0	0	7
ASV810	Bacteria	Patescibacteria	Gracilibacteria	Candidatus_Peribacteria	NA	NA	0	608	0	0	1	83	0	0	2
ASV1034	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	1	336	0	0	1	19	0	0	7
ASV1094	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia	0	300	0	0	1	31	0	0	2
ASV1276	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0	204	0	0	1	6	0	0	2
ASV1156	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	0	235	0	0	1	66	0	0	2
ASV407	Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	0	2399	0	0	0	0	0	0	5
ASV564	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	0	1218	0	0	0	0	0	0	2
ASV585	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	0	1142	0	0	0	0	0	0	2
ASV598	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0	1073	0	0	0	0	0	0	2
ASV737	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	0	708	0	0	0	0	0	0	5
ASV1201	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	NA	0	247	0	0	0	0	0	0	5
ASV1268	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Anaerobacillus	0	207	0	0	0	0	0	0	5

Supplemer ASV no.	Negative control read count	Notes	Sequence
ASV785	599		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCAGCAGGGAAGAAGCTTTTGTGACGGTA CCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGCCGCG TCTGCTGTGAAAGCCCGGGGCTTAACTCCGGGTGTGCAGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATA TCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACA
ASV679	487		TGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGTCTTCGGATTGTAAACCCCTTTCAGTAGGGACGAAGCGCAAGTGACGGT ACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC GTCGTCCGTGAAAACTCGGAGCTTAACTCCGAGCTTGCGGGCGATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATA TCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGTAGTAACTGACGCTGAGGAGCGAAAGCATGGGTAGCGAACA
ASV529	429		TGGGGAATATTGCACAATGGGCGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGT ACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC GTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGAT ATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACA
ASV895	295		TGGGGAATATTGCACAATGGGCGAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTCAGTAGCTAA TACCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV466	1680		TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAAGAAGGCGTTGTGGTTAAT AACCGCAACGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAG CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
ASV835	518		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAA TACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAATACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV973	362		TGGGGAATATTGGACAATGGGCGAAAGCCTGACCGAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAA TACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV1004	56		TGAGGAATATTGGTCAATGGGTGCAAGCCTGAACCAGCCATCCCGCGTGAAGGACGACTGCCCTATGGGTTGTAAACTTCTTTTGTATAGGGATAAACCTACCCTCGTGAGG GTAGCTGAAGGTACTATACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGAGGGTGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAG GCGGACTTATAAGTCAGTGGTGAAATCCTGTCGCTTAACGATAGAACTGCCATTGATACTGTAAGTCTTGAGTATATTTGAGGTAGCTGGAATAAGTAGTGTAGCGGTGAA ATGCATAGATATTACTTAGAACACCAATTGCGAAGGCAGGTTACCAAGATATAACTGACGCTGAGGGACGAAAGCGTGGGGAGCGAACA
ASV912	400		TGGGGAATATTGGACAATGGGCGAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTAGGGTTGTAAATCTCTTTCACCGGTGAAGATAATGACGGTAACCGG AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGACTTTTAAGTCAGG GGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGAAGTCTTGAGTATGGAAGAGGTAAGTGGAATTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA GGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV992	353		TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTTCAAA TAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGC GCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAATCTCGCGGCTCAACCGCGAGCGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGT AGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV1004	56		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAAT ACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV1168	108		TGGGGAATTTTGGACAATGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTCGGCAGGAAAGAAACGTCATGGGTTAAT ACCCCGTGAAACTGACGGTACCTGACAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTAG CAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV1181	11		TGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTTTTGTCAGGGAAGAAAAGGTTCTGGTTAAT ACCTGGGACTCATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTATGCAAGACAGAGGTGAAATCCCCGGGCTCAACCTGGGAACTGCCTTTGTGACTGCATAGCTAGAGTACGGTAGAGGGGGATGGAATTCCGCGTGTA GCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAGCCATCCCTGGACCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV720	684		TGGGGAATTTTGGACAATGGGGCAACCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTAGTAGCGAAT AACTATTACTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTGTGTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGAGACTGCAGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCCACGTGTAG CAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV507	1467		TGGGGAATTTTGGACAATGGGGCAACCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCGTAACTAATA TTAGGTGTGGATGACGGTACCGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGGGCTAACCTGGGAATTGCATTGGTGACTGCACGGCTAGAGTGTGTCAGAGGGGGTAGAATTCCACGTGTA GCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV1276	198		TGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTTTTGTCAGGGAAGAAAAGGTTCTGGCTAAT ACCTGGGACTCATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTATGCAAGACAGAGGTGAAATCCCCGGGCTCAACCTGGGAACTGCCTTTGTGACTGCATGGCTAGAGTACGGTAGAGGGGGATGGAATTCCGCGTGTA GCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV1156	169		TGGGGAATTTTGGACAATGGGGCAACCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCGGCAGGAAGAACGGTAGTAGCGAAT AACTACTACTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT GCGCAGGCGGTTGTGTAAGTCAGATGTGAAATCCCTGGGCTAACCTGGGCGGTCAGTTAAGACTGATAGACTTGAGTATGGGAGAGGGTAGTGGAATTTCCGGTGT AGCGGTGAAATGCGTAGAGATCGGAAGGAACACCAGTGGCGAAGGCGGCTACCTGGCCTGATACTGACACTGAGGAACGAAAGCGTGGGGAGCAAACA
ASV407	2399		TAGGGAATCATCCGCAATGGACGAAAGTCTGACGGTGCAACGCCGCGTGAGTGATGAAGGATTTCGGTTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAA TAGGTCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG CGCGCAGGCGGTCTTTTAAATCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTA GCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV564	1218		TAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTA ACTGCTCGACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG CGCGCAGGCGGTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTA GCAGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
ASV585	1142		TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAA TACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGACTCAACCTGGGAACTGCATTCAAAACTGACGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
ASV598	1073		TGGGGAATTTTGGACAATGGGGCAACCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGGGCTAA TATCCCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG TGCGCAGGCGGTTTGTAAGACTGTCGTGAAAGCCCCGGGCTCAACCTGGGAATGGCGATGGTGACTGCAAGGCTAGAGTTTGTCAGAGGGGGGTAGAATTCCACGTGT AGCAGTGAAATGCGTAGATATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAAACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA
ASV1201	247		TGAGGAATATTGGTCAATGGGCGCGAGCCTGAACCAGCCAAGTCGCGTGAGGGAGGACGGCCCTACGGGTTGTAAACCTCTTTTGCCGGGGAGCAACGGGCGTCACGTGT GGCGCCACTGAGAGTACCCGGAGAAAAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCG TAGGCGGATCGTTAAGTCAGTGGTCAAATTGAGGGGCTCAACCCTTCCCGCCATTGAAACTGGCGTTCTTGAGTGGAAGAGAAGTATGCGGAATGCGTGGTGTAGCGGT GAAATGCATAGATATCACGCAGAACCCGATTGGAAGGCAGCATGCCGGCTTCCGACTGACGCTGAAGCACGAAAGCGTGGGGATCGAACA
ASV1268	207		TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTTCAAA TAGGTCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG CGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGCCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGT AGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA

Supplementary Table C3. Taxa that are differentially abundant between day-0 and day-8 specimens (ALDEX2 analysis)

	Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance Day-8	Effect size[d]
Vaginal	*Atopobium vaginae*	5.32E-07	**1.18E-05**	10.81	1.25	-1.59
	Megasphaera	8.19E-06	**7.13E-05**	10.26	0.28	-1.54
	DNF00809	6.98E-06	**7.63E-05**	8.11	0.20	-1.32
	Dialister	1.74E-07	**7.45E-06**	9.14	2.03	-1.25
	Parvimonas	4.88E-05	**3.33E-04**	8.09	0.17	-1.10
	Prevotella spp. unclassified	5.43E-06	**6.28E-05**	10.57	1.31	-1.09
	Gemella	2.85E-05	**2.24E-04**	6.78	1.09	-1.01
	Peptoniphilus	6.77E-05	**4.69E-04**	6.84	1.39	-1.01
	Anaerococcus	7.37E-04	**2.84E-03**	5.01	-0.02	-0.86
	Sneathia amnii	2.07E-04	**1.08E-03**	9.75	1.02	-0.81
	Prevotella timonensis	1.59E-03	**5.80E-03**	8.20	0.76	-0.80
	BVAB-2	1.59E-03	**5.73E-03**	8.35	0.11	-0.77
	Porphyromonas	3.37E-03	**9.94E-03**	4.65	-0.02	-0.66
	Gardnerella	3.28E-04	**1.85E-03**	12.16	7.71	-0.64
	Sneathia sanguinegens	1.96E-03	**7.12E-03**	8.53	0.90	-0.62
	Prevotella disiens	4.49E-03	**1.26E-02**	5.71	0.10	-0.61
	Atopobium spp. unclassified	1.24E-02	**2.89E-02**	6.02	-0.02	-0.57
	Aerococcus	1.26E-02	**3.31E-02**	6.00	2.46	-0.45
	Haemophilus	2.41E-02	**4.94E-02**	-1.75	0.54	0.43

	Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance Day-8	Effect size[d]
	Enterococcus	1.64E-02	**3.48E-02**	-1.77	0.74	0.45
	Streptococcus	1.23E-02	**2.89E-02**	-1.39	2.05	0.55
	Lactobacillus iners	3.48E-04	**1.93E-03**	10.55	15.16	0.71
	Escherichia/Shigella	8.32E-04	**3.21E-03**	-1.98	3.10	0.75
	Lactobacillus spp. unclassified	1.48E-03	**3.99E-03**	-1.90	2.89	0.79
	Staphylococcus	3.97E-04	**1.75E-03**	-1.76	3.52	0.86
	Ureaplasma	1.33E-05	**1.23E-04**	2.96	9.70	0.92
	Corynebacterium	2.36E-05	**1.91E-04**	-1.70	5.78	1.11
	Sneathia spp. unclassified	3.86E-02	7.47E-02	6.49	0.17	-0.45
	Lactobacillus gasseri	4.86E-02	7.87E-02	-1.74	0.05	0.26
Cutaneous penile	*Anaerococcus*	1.49E-08	**2.02E-06**	10.25	0.62	-2.32
	Finegoldia	1.42E-06	**5.38E-05**	11.78	1.96	-1.66
	Peptoniphilus	4.00E-07	**1.92E-05**	11.04	1.56	-1.65
	Prevotella spp. unclassified	1.18E-05	**2.95E-04**	11.00	0.47	-1.20
	Dialister	1.87E-04	**2.09E-03**	8.75	0.15	-0.91
	Prevotella timonensis	4.13E-04	**3.96E-03**	8.00	0.25	-0.86
	Porphyromonas	4.25E-04	**4.19E-03**	6.79	0.25	-0.81
	Actinomyces	8.33E-04	**8.69E-03**	7.11	1.26	-0.77
	Campylobacter	1.11E-03	**8.27E-03**	5.68	-0.02	-0.77
	Prevotella disiens	2.85E-03	**1.67E-02**	5.53	0.26	-0.72

	Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance Day-8	Effect size[d]
	Peptostreptococcus	1.44E-02	**4.96E-02**	2.61	-0.17	-0.59
	Staphylococcus	2.19E-04	3.36E-03	5.04	14.59	0.84
	Negativicoccus	1.34E-02	5.02E-02	3.42	0.09	-0.54
	Escherichia/Shigella	1.31E-02	5.30E-02	1.44	5.45	0.49
	DNF00809	1.71E-02	5.51E-02	3.54	-0.21	-0.58
	Prevotella bivia	1.56E-02	6.12E-02	4.78	0.22	-0.56
	Howardella	3.43E-02	8.68E-02	3.34	0.06	-0.53
	Varibaculum	3.64E-02	9.33E-02	2.28	-0.18	-0.48
	Pseudomonas	3.71E-02	9.62E-02	-1.63	0.86	0.36
	Fenollaria	3.99E-02	1.02E-01	1.98	0.22	-0.38
Urethral	*Anaerococcus*	1.49E-04	**2.82E-03**	6.05	-0.14	-1.05
	Finegoldia	1.60E-04	**3.19E-03**	6.21	0.37	-1.00
	Peptoniphilus	2.41E-04	**4.36E-03**	7.17	0.42	-1.00
	Prevotella spp. unclassified	9.12E-04	**1.10E-02**	7.21	0.28	-0.79
	Dialister	1.17E-03	**1.21E-02**	6.04	0.16	-0.79
	Escherichia/Shigella	3.89E-03	**2.90E-02**	3.91	7.43	0.50
	Staphylococcus	1.64E-03	**1.81E-02**	4.17	9.58	0.65
	Prevotella timonensis	1.35E-02	5.30E-02	3.71	0.06	-0.61
	Porphyromonas	1.76E-02	6.41E-02	2.44	-0.05	-0.55
	Campylobacter	1.86E-02	6.64E-02	2.98	-0.06	-0.55

Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance Day-8	Effect size[d]
Prevotella disiens	2.15E-02	7.22E-02	2.02	-0.13	-0.51
Gardnerella	1.86E-02	7.30E-02	7.59	1.30	-0.46
Rothia	2.55E-02	7.49E-02	-1.07	1.80	0.44
Atopobium vaginae	2.28E-02	7.67E-02	4.47	0.06	-0.54
Sneathia sanguinegens	2.35E-02	7.81E-02	2.19	-0.09	-0.45
Veillonella	3.22E-02	9.91E-02	4.73	0.59	-0.46
Sneathia amnii	3.48E-02	1.02E-01	1.85	0.02	-0.41
Streptococcus	3.35E-02	1.11E-01	9.02	6.47	-0.32

[a]Only those taxa with a p-value <0.05 are shown in this table

[b]Expected P value of Wilcoxon rank test

[c]Expected Benjamini-Hochberg corrected P value of Wilcoxon test. Bold indicates statistically significant difference at FDR<0.05

[d]median difference in CLR transformed abundance between Day-0 vs Day-8 groups /median of the largest difference in CLR transformed abundance within Day-0 and Day-8 groups

Supplementary Table C4. Taxa that are differentially abundant between day-0 and endpoint specimens (ALDEX2 analysis)

	Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance endpoint	Effect size[d]
Vaginal	*Atopobium vaginae*	2.68E-07	**1.23E-05**	11.10	-0.51	-1.82
	Megasphaera	1.17E-06	**2.95E-05**	10.57	-0.68	-1.82
	DNF00809	6.26E-06	**9.69E-05**	8.45	-0.43	-1.30
	Dialister	1.56E-06	**4.18E-05**	9.43	3.87	-1.02
	BVAB-2	2.42E-04	**1.48E-03**	8.69	-0.29	-0.92
	Sneathia amnii	6.87E-05	**5.84E-04**	9.97	0.40	-0.90
	Parvimonas	2.96E-04	**1.94E-03**	8.43	-0.06	-0.89
	Sneathia sanguinegens	8.60E-04	**4.75E-03**	8.87	-0.13	-0.80
	Prevotella spp. unclassified	7.18E-05	**6.62E-04**	10.91	5.67	-0.79
	Prevotella timonensis	4.79E-04	**2.91E-03**	8.51	0.31	-0.79
	Aerococcus	1.72E-04	**1.23E-03**	6.30	1.13	-0.77
	Gemella	1.90E-04	**1.39E-03**	7.08	1.67	-0.72
	Gardnerella	3.70E-05	**4.35E-04**	12.45	8.86	-0.67
	Sneathia spp. unclassified	2.92E-03	**1.26E-02**	6.76	-0.66	-0.66
	Atopobium spp. unclassified	1.39E-02	**4.38E-02**	6.33	-0.17	-0.64
	Prevotella amnii	1.61E-02	**5.00E-02**	0.76	-1.00	-0.43
	Ureaplasma	8.25E-03	**2.84E-02**	3.31	8.50	0.54
	Lactobacillus jensenii	4.87E-03	**1.80E-02**	-1.44	1.88	0.58

	Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance endpoint	Effect size[d]
	Lactobacillus iners	2.42E-04	**1.73E-03**	10.84	14.25	0.59
	Corynebacterium	4.22E-03	**1.51E-02**	-1.46	2.79	0.68
	Staphylococcus	4.93E-04	**2.79E-03**	-1.47	3.92	0.92
	Lactobacillus spp. unclassified	9.75E-05	**7.07E-04**	-1.61	5.72	0.99
	Finegoldia	3.06E-06	**5.64E-05**	1.58	6.89	1.11
	Streptococcus	1.89E-02	5.16E-02	-1.29	2.21	0.55
	Mageeibacillus indolicus (BVAB-3)	3.56E-02	8.90E-02	0.50	-1.13	-0.46
Cutaneous penile	*Prevotella timonensis*	4.37E-03	1.07E-01	8.17	0.32	-0.64
	Atopobium vaginae	9.62E-03	1.33E-01	2.22	-1.04	-0.60
	DNF00809	1.18E-02	1.50E-01	3.54	-0.54	-0.58
	Actinomyces	1.03E-02	1.69E-01	7.24	5.33	-0.41
	Prevotella disiens	2.56E-02	2.16E-01	5.68	0.89	-0.40
	Peptoniphilus	1.60E-02	1.89E-01	11.21	9.39	-0.35
	Staphylococcus	1.27E-02	1.83E-01	5.23	10.85	0.34
	Enterococcus	4.89E-02	2.86E-01	-0.44	3.19	0.35
Urethral	*Atopobium vaginae*	2.68E-03	6.98E-02	4.38	-0.71	-0.69
	Parvimonas	3.56E-02	2.82E-01	1.85	-0.61	-0.46
	BVAB-2	3.99E-02	2.70E-01	1.13	-0.86	-0.42
	Lactobacillus iners	3.21E-02	3.06E-01	5.75	8.02	0.25
	Corynebacterium	3.25E-02	3.26E-01	8.87	10.14	0.26

Taxon[a]	p-value[b]	FDR-p[c]	Median CLR abundance Day-0	Median CLR abundance endpoint	Effect size[d]
Staphylococcus	1.76E-03	7.92E-02	4.16	7.18	0.37

[a]Only those taxa with a p-value <0.05 are shown in this table

[b]Expected P value of Wilcoxon rank test

[c]Expected Benjamini-Hochberg corrected P value of Wilcoxon test. Bold indicates statistically significant difference at FDR<0.05

[d]median difference in CLR transformed abundance between Day-0 vs Endpoint groups /median of the largest difference in CLR transformed abundance within Day-0 and Endpoint groups

Supplementary Table C5. Analysis of Composition of Microbiomes (ANCOM) of longitudinal vaginal, cutaneous penile and urethral specimens from couples that were cured vs couples who recurred[a]

	Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Vaginal microbiota	*Atopobium*	-0.14	0	0.14	Inf	TRUE	TRUE	TRUE	TRUE
	Ezakiella	-0.24	0	0.24	Inf	TRUE	TRUE	TRUE	TRUE
	Flavobacterium	-0.17	0	0.17	Inf	TRUE	TRUE	TRUE	TRUE
	Fusobacterium	0.00	0	0.00	Inf	TRUE	TRUE	TRUE	TRUE
	Murdochiella	-0.20	0	0.20	Inf	TRUE	TRUE	TRUE	TRUE
	Rothia	-0.15	0	0.15	Inf	TRUE	TRUE	TRUE	TRUE
	Lactobacillus antri	0.09	0	-0.09	Inf	TRUE	TRUE	TRUE	TRUE
	Lactobacillus coleohominis	0.07	0	-0.07	Inf	TRUE	TRUE	TRUE	TRUE
	Lactobacillus crispatus	0.76	0	-0.76	Inf	TRUE	TRUE	TRUE	TRUE
	Lactobacillus gasseri	0.45	0	-0.45	Inf	TRUE	TRUE	TRUE	TRUE
	Sneathia sanguinegens	**-0.06**	**1.02**	**1.08**	**40**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Corynebacterium	**-0.54**	**-0.23**	**0.31**	**33**	FALSE	FALSE	**TRUE**	**TRUE**
	Dialister	**-0.39**	**0.14**	**0.52**	**31**	FALSE	FALSE	**TRUE**	**TRUE**
	Gemella	**-0.19**	**0.65**	**0.84**	**31**	FALSE	FALSE	**TRUE**	**TRUE**
	Sneathia amnii	**-0.19**	**1.01**	**1.20**	**31**	FALSE	FALSE	**TRUE**	**TRUE**
	Parvimonas	0.07	0.18	0.12	30	FALSE	FALSE	FALSE	TRUE
	Peptostreptococcus	-0.21	-0.19	0.02	28	FALSE	FALSE	FALSE	TRUE

Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Atopobium vaginae	-0.06	0.23	0.29	23	FALSE	FALSE	FALSE	FALSE
Muribaculaceae	-0.20	0.03	0.23	12	FALSE	FALSE	FALSE	FALSE
Actinomyces	-0.55	-1.38	-0.84	7	FALSE	FALSE	FALSE	FALSE
Aerococcus	-0.22	0.12	0.34	2	FALSE	FALSE	FALSE	FALSE
Alloprevotella	0.06	-0.01	-0.07	6	FALSE	FALSE	FALSE	FALSE
Anaerococcus	-0.05	-0.30	-0.25	1	FALSE	FALSE	FALSE	FALSE
Bifidobacterium	-0.10	-0.82	-0.71	7	FALSE	FALSE	FALSE	FALSE
Campylobacter	-0.38	-0.71	-0.33	12	FALSE	FALSE	FALSE	FALSE
Enterococcus	-0.19	-0.66	-0.47	7	FALSE	FALSE	FALSE	FALSE
Escherichia/Shigella	-0.37	-0.33	0.04	1	FALSE	FALSE	FALSE	FALSE
Fenollaria	-0.30	-0.94	-0.64	13	FALSE	FALSE	FALSE	FALSE
Finegoldia	0.06	-0.94	-1.00	15	FALSE	FALSE	FALSE	FALSE
Granulicatella	-0.20	-0.31	-0.11	8	FALSE	FALSE	FALSE	FALSE
Haemophilus	-0.22	0.26	0.48	7	FALSE	FALSE	FALSE	FALSE
Howardella	-0.42	-0.89	-0.47	14	FALSE	FALSE	FALSE	FALSE
Lactobacillus	0.21	0.24	0.04	25	FALSE	FALSE	FALSE	FALSE
Lawsonella	-0.63	-0.42	0.21	8	FALSE	FALSE	FALSE	FALSE
Mobiluncus	-0.14	-0.21	-0.07	7	FALSE	FALSE	FALSE	FALSE
Mycoplasma	-0.12	0.40	0.52	3	FALSE	FALSE	FALSE	FALSE
Neisseria	-0.21	-0.38	-0.18	6	FALSE	FALSE	FALSE	FALSE
Peptococcus	-0.10	-0.57	-0.47	6	FALSE	FALSE	FALSE	FALSE

	Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
	Peptoniphilus	-0.37	-0.11	0.25	3	FALSE	FALSE	FALSE	FALSE
	Porphyromonas	-0.16	-0.27	-0.12	6	FALSE	FALSE	FALSE	FALSE
	Prevotella spp. unclassified	0.00	0.14	0.13	7	FALSE	FALSE	FALSE	FALSE
	Solobacterium	-0.13	-0.46	-0.32	11	FALSE	FALSE	FALSE	FALSE
	Staphylococcus	-0.71	0.06	0.78	16	FALSE	FALSE	FALSE	FALSE
	Streptococcus	-0.19	-0.35	-0.15	7	FALSE	FALSE	FALSE	FALSE
	Sutterella	-0.33	0.00	0.34	12	FALSE	FALSE	FALSE	FALSE
	Ureaplasma	1.09	-0.52	-1.61	1	FALSE	FALSE	FALSE	FALSE
	Veillonella	-0.23	0.05	0.28	8	FALSE	FALSE	FALSE	FALSE
	Gardnerella	0.92	2.65	1.73	0	FALSE	FALSE	FALSE	FALSE
	Lactobacillus iners	4.74	2.03	-2.70	1	FALSE	FALSE	FALSE	FALSE
	Lactobacillus jensenii	1.22	0.28	-0.95	4	FALSE	FALSE	FALSE	FALSE
	Prevotella bivia	-0.12	0.93	1.05	18	FALSE	FALSE	FALSE	FALSE
	Prevotella disiens	-0.39	0.29	0.68	8	FALSE	FALSE	FALSE	FALSE
	Prevotella melaninogenica	-0.16	-0.11	0.05	8	FALSE	FALSE	FALSE	FALSE
	Prevotella timonensis	-0.02	0.39	0.41	1	FALSE	FALSE	FALSE	FALSE
Cutaneou	***Alloscardovia***	**-0.09**	**0**	**0.09**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Arcanobacterium	**-0.22**	**0**	**0.22**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Ezakiella	**-0.16**	**0**	**0.16**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**

Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Fusobacterium	**-0.25**	**0**	**0.25**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
Mycoplasma	**0.17**	**0**	**-0.17**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
Pseudomonas	**0.05**	**0**	**-0.05**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
Lactobacillus gasseri	**-0.26**	**0**	**0.26**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
Streptococcus	1.16	2.38	1.22	0	FALSE	FALSE	FALSE	FALSE
Enterococcus	0.98	2.37	1.39	0	FALSE	FALSE	FALSE	FALSE
Gardnerella	0.59	1.72	1.12	0	FALSE	FALSE	FALSE	FALSE
Corynebacterium	0.91	1.10	0.19	0	FALSE	FALSE	FALSE	FALSE
Gemella	-0.26	0.96	1.22	0	FALSE	FALSE	FALSE	FALSE
Sneathia amnii	-0.14	0.93	1.07	0	FALSE	FALSE	FALSE	FALSE
Prevotella bivia	0.11	0.75	0.64	0	FALSE	FALSE	FALSE	FALSE
Prevotella timonensis	-0.10	0.66	0.76	0	FALSE	FALSE	FALSE	FALSE
Sneathia sanguinegens	-0.08	0.59	0.67	0	FALSE	FALSE	FALSE	FALSE
Lactobacillus iners	1.17	0.54	-0.63	0	FALSE	FALSE	FALSE	FALSE
Prevotella disiens	-0.02	0.49	0.51	1	FALSE	FALSE	FALSE	FALSE
Peptoniphilus	0.34	0.49	0.14	0	FALSE	FALSE	FALSE	FALSE
Anaerococcus	-0.01	0.41	0.42	0	FALSE	FALSE	FALSE	FALSE
Granulicatella	-0.29	0.41	0.70	0	FALSE	FALSE	FALSE	FALSE
Ureaplasma	0.47	0.20	-0.27	0	FALSE	FALSE	FALSE	FALSE
Prevotella spp. unclassified	0.03	0.12	0.09	0	FALSE	FALSE	FALSE	FALSE

Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Facklamia	-0.15	0.11	0.27	0	FALSE	FALSE	FALSE	FALSE
Finegoldia	0.07	0.04	-0.03	0	FALSE	FALSE	FALSE	FALSE
Aerococcus	0.29	-0.02	-0.31	0	FALSE	FALSE	FALSE	FALSE
Haemophilus	0.14	-0.07	-0.21	0	FALSE	FALSE	FALSE	FALSE
Dialister	-0.24	-0.12	0.12	0	FALSE	FALSE	FALSE	FALSE
Dermabacter	-0.28	-0.13	0.14	0	FALSE	FALSE	FALSE	FALSE
Howardella	-0.32	-0.17	0.15	0	FALSE	FALSE	FALSE	FALSE
Actinotignum	0.07	-0.17	-0.24	0	FALSE	FALSE	FALSE	FALSE
Prevotella melaninogenica	-0.14	-0.25	-0.11	0	FALSE	FALSE	FALSE	FALSE
Atopobium deltae	-0.15	-0.29	-0.14	0	FALSE	FALSE	FALSE	FALSE
Negativicoccus	-0.24	-0.30	-0.06	0	FALSE	FALSE	FALSE	FALSE
Peptococcus	-0.28	-0.32	-0.04	0	FALSE	FALSE	FALSE	FALSE
Peptostreptococcus	-0.16	-0.36	-0.19	0	FALSE	FALSE	FALSE	FALSE
Veillonella	-0.15	-0.38	-0.22	0	FALSE	FALSE	FALSE	FALSE
Rothia	-0.07	-0.41	-0.34	0	FALSE	FALSE	FALSE	FALSE
Porphyromonas	-0.01	-0.53	-0.52	0	FALSE	FALSE	FALSE	FALSE
Micrococcus	-0.17	-0.54	-0.37	0	FALSE	FALSE	FALSE	FALSE
Escherichia/Shigella	-0.03	-0.59	-0.56	0	FALSE	FALSE	FALSE	FALSE
Varibaculum	-0.13	-0.60	-0.47	1	FALSE	FALSE	FALSE	FALSE
Murdochiella	-0.15	-0.61	-0.45	0	FALSE	FALSE	FALSE	FALSE

	Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
	Actinomyces	-0.27	-0.62	-0.35	0	FALSE	FALSE	FALSE	FALSE
	Fenollaria	0.02	-0.66	-0.68	0	FALSE	FALSE	FALSE	FALSE
	Prevotellaceae	0.01	-0.68	-0.69	0	FALSE	FALSE	FALSE	FALSE
	Staphylococcus	-0.30	-0.76	-0.46	0	FALSE	FALSE	FALSE	FALSE
	Campylobacter	-0.32	-0.82	-0.50	0	FALSE	FALSE	FALSE	FALSE
	Propionimicrobium	-0.13	-0.83	-0.70	0	FALSE	FALSE	FALSE	FALSE
	Mobiluncus	-0.12	-0.89	-0.77	0	FALSE	FALSE	FALSE	FALSE
	Sutterella	-0.47	-1.07	-0.60	0	FALSE	FALSE	FALSE	FALSE
	Lawsonella	-0.44	-2.08	-1.64	0	FALSE	FALSE	FALSE	FALSE
Urethral	***Atopobium parvulum***	**-0.26**	**0**	**0.26**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Coriobacteriales Inc Se	**-0.43**	**0**	**0.43**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Acinetobacter	**0.06**	**0**	**-0.06**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Cutibacterium	**-0.31**	**0**	**0.31**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Enterobacter	**-0.02**	**0**	**0.02**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Micrococcus	**0.00**	**0**	**0.00**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Murdochiella	**-0.17**	**0**	**0.17**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Pseudoclavibacter	**-0.17**	**0**	**0.17**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Ruminococcaceae UCG014	**-0.21**	**0**	**0.21**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Lactobacillus crispatus	**-0.11**	**0**	**0.11**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
	Lactobacillus gasseri	**-0.13**	**0**	**0.13**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**

Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Lactobacillus jensenii	**-0.13**	**0**	**0.13**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
Prevotella buccalis	**-0.07**	**0**	**0.07**	**Inf**	**TRUE**	**TRUE**	**TRUE**	**TRUE**
Finegoldia	2.05	2.66	0.61	0	FALSE	FALSE	FALSE	FALSE
Prevotella bivia	0.27	1.80	1.53	0	FALSE	FALSE	FALSE	FALSE
Prevotella disiens	0.07	1.75	1.68	0	FALSE	FALSE	FALSE	FALSE
Anaerococcus	0.95	1.63	0.68	0	FALSE	FALSE	FALSE	FALSE
Peptoniphilus	1.24	1.62	0.38	0	FALSE	FALSE	FALSE	FALSE
Prevotella spp. unclassified	1.00	1.57	0.57	0	FALSE	FALSE	FALSE	FALSE
Dialister	0.09	1.33	1.25	0	FALSE	FALSE	FALSE	FALSE
Prevotella timonensis	0.39	1.28	0.89	0	FALSE	FALSE	FALSE	FALSE
Corynebacterium	2.18	1.19	-0.99	0	FALSE	FALSE	FALSE	FALSE
Pseudomonas	-0.02	1.06	1.08	0	FALSE	FALSE	FALSE	FALSE
Gemella	-0.52	0.94	1.46	0	FALSE	FALSE	FALSE	FALSE
Porphyromonas	0.16	0.69	0.53	0	FALSE	FALSE	FALSE	FALSE
Fenollaria	0.16	0.53	0.37	0	FALSE	FALSE	FALSE	FALSE
Veillonella	0.31	0.32	0.00	0	FALSE	FALSE	FALSE	FALSE
Neisseria	-0.02	0.26	0.28	0	FALSE	FALSE	FALSE	FALSE
Haemophilus	-0.15	0.15	0.30	0	FALSE	FALSE	FALSE	FALSE
Gardnerella	-0.44	0.12	0.56	0	FALSE	FALSE	FALSE	FALSE
Aerococcus	-0.10	0.12	0.21	0	FALSE	FALSE	FALSE	FALSE

Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Prevotella melaninogenica	-0.27	0.10	0.37	0	FALSE	FALSE	FALSE	FALSE
Peptostreptococcus	-0.31	-0.03	0.28	0	FALSE	FALSE	FALSE	FALSE
Actinomyces	-0.50	-0.04	0.46	0	FALSE	FALSE	FALSE	FALSE
Prevotellaceae	0.03	-0.07	-0.10	0	FALSE	FALSE	FALSE	FALSE
Rothia	-0.05	-0.09	-0.04	0	FALSE	FALSE	FALSE	FALSE
Sutterella	-0.35	-0.10	0.26	0	FALSE	FALSE	FALSE	FALSE
Campylobacter	0.04	-0.11	-0.15	0	FALSE	FALSE	FALSE	FALSE
Granulicatella	-0.28	-0.12	0.15	0	FALSE	FALSE	FALSE	FALSE
Fusobacterium	-0.39	-0.13	0.27	0	FALSE	FALSE	FALSE	FALSE
Prevotella nanceiensis	-0.25	-0.21	0.03	0	FALSE	FALSE	FALSE	FALSE
Negativicoccus	-0.32	-0.22	0.10	0	FALSE	FALSE	FALSE	FALSE
Muribaculaceae	-0.15	-0.22	-0.07	0	FALSE	FALSE	FALSE	FALSE
Staphylococcus	1.67	-0.27	-1.94	0	FALSE	FALSE	FALSE	FALSE
Prevotella corporis	-0.22	-0.27	-0.05	0	FALSE	FALSE	FALSE	FALSE
Atopobium deltae	-0.16	-0.33	-0.17	1	FALSE	FALSE	FALSE	FALSE
Mobiluncus	0.05	-0.39	-0.44	0	FALSE	FALSE	FALSE	FALSE
Streptococcus	0.70	-0.40	-1.10	0	FALSE	FALSE	FALSE	FALSE
Dermabacter	-0.45	-0.41	0.04	0	FALSE	FALSE	FALSE	FALSE
Peptococcus	-0.20	-0.42	-0.22	0	FALSE	FALSE	FALSE	FALSE
Enterococcus	0.52	-0.44	-0.96	0	FALSE	FALSE	FALSE	FALSE
Bergeyella	-0.14	-0.44	-0.30	0	FALSE	FALSE	FALSE	FALSE

Taxon	Mean CLR abundance in cure cases	Mean CLR abundance in recurrence cases	CLR-mean difference[b]	W-statistic[c]	Detected 0.9[d]	Detected 0.8[e]	Detected 0.7[f]	Detected 0.6[g]
Howardella	-0.54	-0.51	0.03	0	FALSE	FALSE	FALSE	FALSE
Alloprevotella	-0.17	-0.55	-0.38	0	FALSE	FALSE	FALSE	FALSE
Atopobium spp. unclassified	-0.26	-0.58	-0.32	0	FALSE	FALSE	FALSE	FALSE
Actinotignum	-0.13	-0.61	-0.48	0	FALSE	FALSE	FALSE	FALSE
Ezakiella	-0.09	-0.61	-0.52	0	FALSE	FALSE	FALSE	FALSE
Solobacterium	-0.36	-0.62	-0.26	0	FALSE	FALSE	FALSE	FALSE
Facklamia	-0.27	-0.62	-0.35	0	FALSE	FALSE	FALSE	FALSE
Enhydrobacter	-0.20	-0.64	-0.43	0	FALSE	FALSE	FALSE	FALSE
Alloscardovia	-0.17	-0.69	-0.52	0	FALSE	FALSE	FALSE	FALSE
Propionimicrobium	-0.64	-0.69	-0.05	0	FALSE	FALSE	FALSE	FALSE
Prevotella bergensis	-0.15	-0.86	-0.71	0	FALSE	FALSE	FALSE	FALSE
Ureaplasma	-0.20	-0.99	-0.79	0	FALSE	FALSE	FALSE	FALSE
Lawsonella	-1.16	-1.00	0.17	0	FALSE	FALSE	FALSE	FALSE
Arcanobacterium	-0.25	-1.11	-0.85	1	FALSE	FALSE	FALSE	FALSE
Lactobacillus iners	-0.04	-1.29	-1.25	0	FALSE	FALSE	FALSE	FALSE
Varibaculum	-0.32	-1.36	-1.04	0	FALSE	FALSE	FALSE	FALSE
Escherichia/Shigella	0.32	-1.68	-2.00	0	FALSE	FALSE	FALSE	FALSE

CLR, centre log-ratio transformed relative abundance; Inf = indicates a taxa that was identified as a structural zero

[a]Specimens collected at day-0 and day-8 have been excluded from this analysis. Recurrence was defined as 3-4 Amsel criteria and NS=4-10 during the follow-up period

[b]CLR-mean difference represents the difference in the mean-CLR transformed abundance of a taxon between recurrence and cure cases

[c]W statistic - the number of comparisons in which the null hypothesis as rejected fot that taxon
[d]A value of TRUE indicates that the null hypothesis was rejected in ≥90% of comparisons for that taxon
[e]A value of TRUE indicates that the null hypothesis was rejected in ≥80% of comparisons for that taxon
[f]A value of TRUE indicates that the null hypothesis was rejected in ≥70% of comparisons for that taxon
[g]A value of TRUE indicates that the null hypothesis was rejected in ≥60% of comparisons for that taxon

Supplementary Table C6. Correlation of specific bacterial taxa between the genital microbiota of sexual partners longitudinally, stratified by BV recurrence status

	Cure cases longitudinal[a]				Recurrence cases longitudinal[b]			
	Vaginal/Penile[c]		Vaginal/Urethral		Vaginal/Penile[c]		Vaginal/Urethral	
Taxon	**Corr**	**P-value**	**Corr**	**P-value**	**Corr**	**P-value**	**Corr**	**P-value**
Aerococcus	-	-	-	-	0.678	0.1102	0.6474	0.1172
Anaerococcus	0.121	0.3387	-0.2141	0.0719	-0.6097	0.3806	-0.3777	0.5534
Atopobium vaginae	0.0588	0.4216	0.1783	0.033	-	-	-	-
Candidatus Lachnocurva vaginae (BVAB-1)	-	-	-	-	-	-	-	-
BVAB-2	-0.0204	0.7812	0.2487	0.008	0.1949	0.6603	-	-
Corynebacterium	**0.339**	**0.004**	-0.1708	0.1748	-	-	-	-
Dialister	0.1966	0.0919	0.1051	0.3706	0.4554	0.4795	0.6013	0.2867
Enterobacter	-	-	-	-	-	-	-	-
Enterococcus	**0.3297**	**0.001**	0.2139	0.032	0.143	0.7596	0.0017	0.9961
Escherichia/Shigella	0.2481	0.025	0.0527	0.6344	0.173	0.7478	0.4172	0.4103
Finegoldia	-0.0087	0.9461	-0.104	0.3936	-0.4885	0.4475	-0.0069	0.9899
Gardnerella	**0.3495**	**0.003**	0.2976	0.009	-0.7743	0.5634	-0.9361	0.5374
Lactobacillus crispatus	**0.4677**	**0.001**	**0.3299**	**0.003**	-	-	-	-
Lactobacillus gasseri	**0.413**	**0.001**	0.2178	0.026	-	-	-	-
Lactobacillus iners	**0.3967**	**0.001**	**0.3152**	**0.014**	-0.3783	0.5474	**-0.9563**	**0.0399**
Lactobacillus jensenii	**0.3752**	**0.001**	0.1316	0.0809	-	-	-	-
Megasphaera	0.0076	0.9091	-	-	-	-	-	-
Peptoniphilus	0.0824	0.5035	-0.1825	0.1449	-0.4508	0.4685	-0.0386	0.946
Prevotella unclassified spp.	0.1942	0.1159	0.1075	0.3696	0.3471	0.5824	0.8418	0.0729

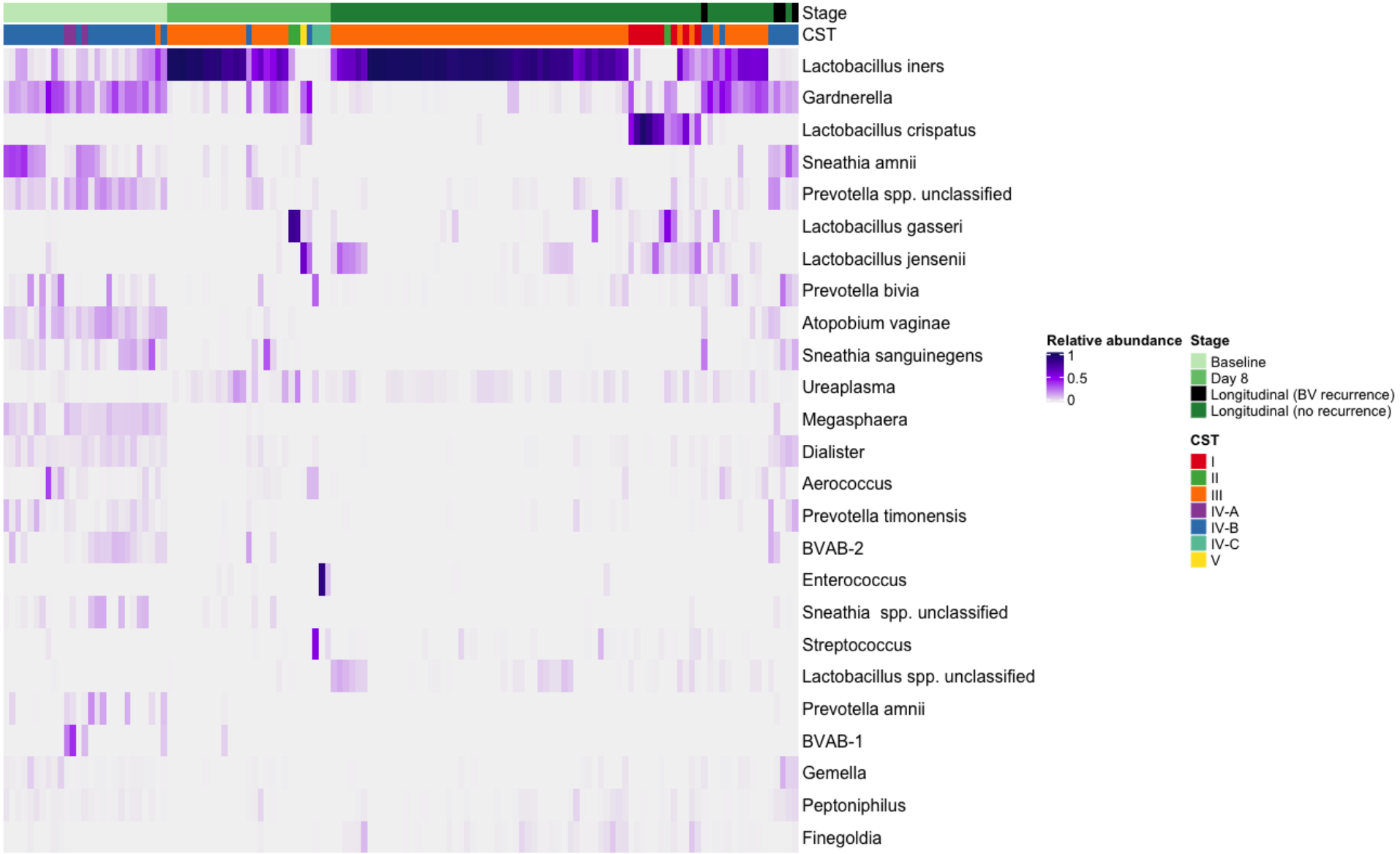
Prevotella bivia	0.1384	0.2398	-0.0051	0.976	0.6383	0.2607	0.7131	0.1778
Prevotella disiens	0.299	0.012	0.1426	0.2008	0.0182	0.9829	0.7521	0.1328
Prevotella timonensis	**0.3751**	**0.001**	0.2322	0.03	0.8648	0.0608	**0.9662**	**0.0079**
Pseudomonas	-	-	-	-	-	-	-	-
Sneathia unclassified spp.	0.018	0.7952	0.0805	0.2827	-	-	0.6504	0.1029
Sneathia amnii	0.0983	0.2318	**0.3371**	**0.005**	0.6107	0.2266	0.8521	0.0579
Sneathia sanguinegens	0.1509	0.044	0.2266	0.014	-0.0249	0.9698	-0.0793	0.9099
Staphylococcus	0.2773	0.019	-0.0039	0.969	0.6512	0.131	0.7155	0.1121
Streptococcus	0.2151	0.0649	0.0497	0.6893	-0.0853	0.8613	0.7438	0.0611
Ureaplasma	0.116	0.2957	0.1738	0.1828	0.0866	0.8809	0.1245	0.8549

Corr, SparCC correlation coefficient; - indicates the taxa was not detected in one or more specimen type for that study time point. The 10 ten most abundant bacteria at each site and 8 bacteria previously associated with BV are presented in this table. Correlations with an absolute correlation coefficient >0.3 and P<0.05 were considered significant and have been bolded in this table

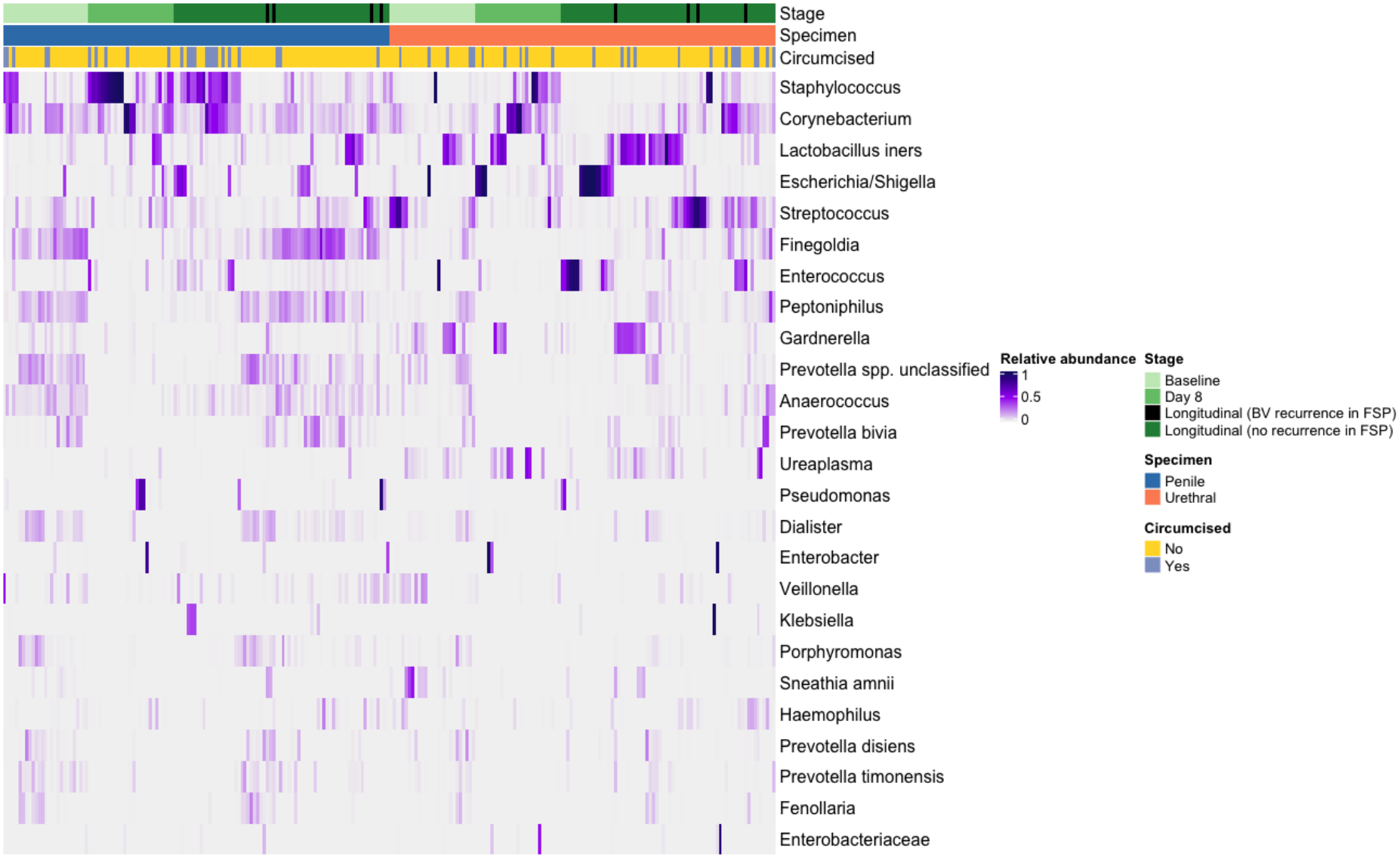
[a] Includes 63 vaginal, 63 cutaneous penile and 63 urethral specimens from 23 couples where the female was cured

[b] Includes 5 vaginal, 5 cutaneous penile and 5 urethral specimens from 4 couples where the female experienced BV recurrence during the follow-up period

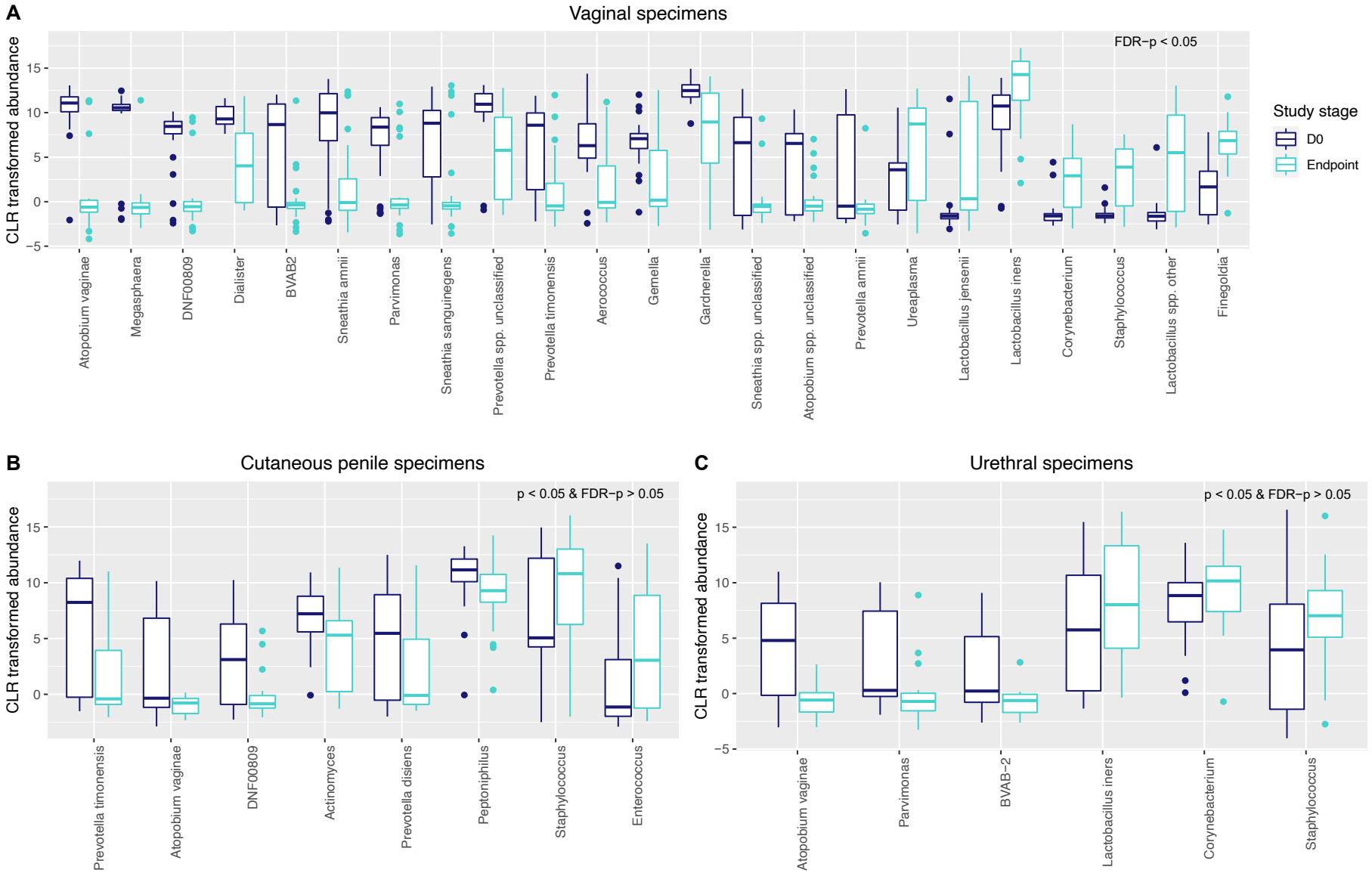
[c] Penile refers to cutaneous penile specimens


Stage
CST
Lactobacillus iners
Gardnerella
Lactobacillus crispatus
Sneathia amnii
Prevotella spp. unclassified
Lactobacillus gasseri
Lactobacillus jensenii
Prevotella bivia
Atopobium vaginae
Sneathia sanguinegens
Ureaplasma
Megasphaera
Dialister
Aerococcus
Prevotella timonensis
BVAB-2
Enterococcus
Sneathia spp. unclassified
Streptococcus
Lactobacillus spp. unclassified
Prevotella amnii
BVAB-1
Gemella
Peptoniphilus
Finegoldia
Relative abundance
1
0.5
0
Stage
Baseline
Day 8
Longitudinal (BV recurrence)
Longitudinal (no recurrence)
CST
I
II
III
IV-A
IV-B
IV-C
V

Supplementary Figure C1 - *Heatmap of relative abundance of bacterial taxa in all vaginal specimens, ordered by study stage (baseline, day-8, longitudinal (BV recurrence) and longitudinal (no recurrence)). Specimens collected at the time of BV recurrence are indicated in black in the top metadata bar. Ward linkage clustering was applied to determine the order of specimens within each study stage. VALENCIA (France et al., 2020) was used to assign community state types (CSTs) to vaginal specimens: CST I: dominated by L. crispatus, CST II: dominated by L. gasseri, CST III: dominated by L. iners, CST IV-A: low relative abundance of Lactobacillus spp. with high to moderate abundance of Candidatus Lachnocurva vaginae (BVAB-1) and Gardnerella, CST IV-B: low relative abundance of Lactobacillus spp. with high to moderate abundance of Gardnerella and A. vaginae, CST IV-C: low relative abundance of Lactobacillus spp., Candidatus Lachnocurva vaginae and Gardnerella, CST V: dominated by L. jensenii. The heatmap was drawn using ComplexHeatmap (Gu et al., 2016).*


Stage
Specimen
Circumcised
Staphylococcus
Corynebacterium
Lactobacillus iners
Escherichia/Shigella
Streptococcus
Finegoldia
Enterococcus
Peptoniphilus
Gardnerella
Prevotella spp. unclassified
Anaerococcus
Prevotella bivia
Ureaplasma
Pseudomonas
Dialister
Enterobacter
Veillonella
Klebsiella
Porphyromonas
Sneathia amnii
Haemophilus
Prevotella disiens
Prevotella timonensis
Fenollaria
Enterobacteriaceae
Relative abundance
1
0.5
0
Stage
Baseline
Day 8
Longitudinal (BV recurrence in FSP)
Longitudinal (no recurrence in FSP)
Specimen
Penile
Urethral
Circumcised
No
Yes

Supplementary Figure C2- *Heatmap of relative abundance of bacterial taxa in all male specimens, ordered by specimen type (cutaneous penile, urethral) and then by study stage (baseline, day-8, longitudinal (BV recurrence in female partner), longitudinal (no recurrence in female partner)). Specimens collected at the time of BV recurrence in the female partner are indicated in black in the top metadata bar. Circumcision status is also indicated above the heatmap. Ward linkage clustering was applied to determine the order of specimens within each study stage and the heatmap was drawn using ComplexHeatmap (Gu et al., 2016).*


A
Vaginal specimens
FDR−p < 0.05
CLR transformed abundance
15
10
5
0
−5
Atopobium vaginae
Megasphaera
DNF00809
Dialister
BVAB2
Sneathia amnii
Parvimonas
Sneathia sanguinegens
Prevotella spp. unclassified
Prevotella timonensis
Aerococcus
Gemella
Gardnerella
Sneathia spp. unclassified
Atopobium spp. unclassified
Prevotella amnii
Ureaplasma
Lactobacillus jensenii
Lactobacillus iners
Corynebacterium
Staphylococcus
Lactobacillus spp. other
Finegoldia
Study stage
D0
Endpoint
B
Cutaneous penile specimens
p < 0.05 & FDR−p > 0.05
CLR transformed abundance
15
10
5
0
Prevotella timonensis
Atopobium vaginae
DNF00809
Actinomyces
Prevotella disiens
Peptoniphilus
Staphylococcus
Enterococcus
C
Urethral specimens
p < 0.05 & FDR−p > 0.05
CLR transformed abundance
15
10
5
0
−5
Atopobium vaginae
Parvimonas
BVAB−2
Lactobacillus iners
Corynebacterium
Staphylococcus

Supplementary Figure C3 - *Boxplots showing the centred-log ratio (CLR) transformed relative abundance of bacteria that were differentially abundant by ALDEX2 between day-0 and endpoint specimens in the vaginal (Panel A; FDR<0.05), cutaneous penile (Panel B; p<0.05 but FDR>0.05) and urethral microbiota (Panel C; p<0.05 but FDR>0.05).*

Appendix D. Supplementary files: Chapter 6

Name of file in Appendix D and as referred to in Chapter 6	Name of file in PDF version of published manuscript
Supplementary File D1	S1 File
Supplementary Table D1	S1 Table
Supplementary Table D2	S2 Table
Supplementary Table D3	S3 Table
Supplementary Table D4	S4 Table

Supplementary File D1 - PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Appendix S2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Section/topic	#	Checklist item	Reported on page #

Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	NA
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	7, Fig. 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 2
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table 3
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table 2
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	16
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	16-17
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	18
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	17-19

FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	1

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Supplementary Table D1. Database search strings

Database	Search string	Date of most recent search
PubMed	((((bacterial AND vagin* OR vaginosis OR vaginitis OR (vagin* AND infect*) OR gardnerella OR (vagina* AND bacteri*) OR (vagina* AND microbio*) OR (vagina* AND flora) OR (cervicovagina* AND flora) OR (cervicovagina* AND microbio*)))) AND ((lact* AND acid OR lactic OR lactate))) AND english[Language]	4-Nov-19
OVID Medline	(((bacterial and vagin*) or vaginosis or vaginitis or (vagin* and infect*) or gardnerella or (vagina* and bacteri*) or (vagina* and microbio*) or (vagina* and flora) or (cervicovagina* and flora) or (cervicovagina* and microbio*)) and ((lact* and acid) or lactic or lactate)).af. and English.lg.	4-Nov-19
Embase	(bacterial AND vagin* OR vaginosis OR vaginitis OR (vagin* AND infect*) OR gardnerella OR (vagina* AND bacteri*) OR (vagina* AND microbio*) OR (vagina* AND flora) OR (cervicovagina* AND flora) OR (cervicovagina* AND microbio*)) AND (lact* AND acid OR lactic OR lactate) AND 'english':la	4-Nov-19

Supplementary Table D2. Bias assessment tool

First author name and year:				**Reviewer name:**
What outcome/s does study contribute to? BV, microbiota or both:				
	Bias item	**Level of risk of bias (low, moderate, high or unclear)**	**Risk judgement**	**Support for judgement**
Selection bias	Were participants randomly allocated? If not randomised, were they sequentially enrolled?	**Yes (low risk):** patients randomly allocated treatment	**+**	
		No (moderate risk): not randomised, but sequentially enrolled	**-/+**	
		No (high risk): patients self-selected their treatment or were given treatment without any listing/explanation of eligibility criteria	**-**	
		Could not be determined (unknown risk)	**?**	
	Allocation concealment. Was allocation performed centrally e.g. (phone, web, pharmacy), and was allocation concealed?	**Yes (low risk):** allocation performed centrally using predetermined random sequence. e.g. Sequentially numbered sealed envelopes, identical drug containers e	**+**	
		No (high risk): allocation not performed centrally, allocation sequence was predictable or known to staff.	**-**	
		Could not be determined (unknown risk)	**?**	
	Was the recruited population representative of the general population?	**Yes (low risk):** population was clearly representative	**+**	
		No (high risk): population was clearly not representative i.e. pregnant women, sex workers etc	**-**	
		Could not be determined (unknown risk)	**?**	
Performance bias	Were study participants and personnel blinded to what intervention a participant	**Yes (low risk):** participants and personnel blinded, blinding was effective and unlikely that blinding could have been broken	**+**	
		No (moderate risk): no blinding but outcome unlikely to be influenced	**-/+**	

	received, and was blinding effective?	**No (high risk):** no blinding or incomplete or broken blinding, outcome likely to be influenced	**-**
		Could not be determined (unknown risk)	**?**
	Were there deviations from the intended intervention beyond what would be expected in usual practice? If so, were deviations likely to affect the outcome?	**No (Low risk):** there were no deviations from the intended intervention	**+**
		Yes (moderate risk): the study deviated from the intended intervention but the deviations were unlikely to have affected the outcome	**-/+**
		Yes (high risk): the study deviated from the intended intervention and the deviations were likely to have affected the outcome	**-**
		Could not be determined (unknown risk)	**?**
Measurement bias	**BV outcome -** Were the assessors of the outcome blinded to the intervention?	**Yes (low risk):** assessors were blinded to the intervention and it is unlikely that blinding could have been broken	**+**
		No (moderate risk): no blinding but measurement of outcome unlikely to be influenced	**-/+**
		No (high risk): no blinding or incomplete or broken blinding, measurement of outcome likely to be influenced.	**-**
		Could not be determined (unknown risk)	**?**
	BV outcome - Were the intervention groups assessed in the same way?	**Yes (low risk):** the intervention groups were assessed in the same way	**+**
		No (high risk): the intervention groups were not assessed in the same way	**-**
		Could not be determined (unknown risk)	**?**
	BV outcome - Methodology used to assess outcome	**Low risk:** ≥3 Amsel's criteria and/or Nugent score: 7-10	**+**
		Moderate risk: <3 Amsel's criteria i.e. clue cells, discharge, pH or amine/odour	**-/+**
		Could not be determined (unknown risk)	**?**

	Microbiota outcome - Were the assessors of the outcome blinded to the intervention?	**Yes (low risk):** assessors of the outcome were blinded to the intervention and it is unlikely that blinding could have been broken.	**+**
		No (moderate risk): no blinding of assessors of the outcome but measurement of outcome unlikely to be influenced	**-/+**
		No (high risk): no blinding or incomplete or broken blinding of assessors of the outcome, measurement of outcome likely to be influenced.	**-**
		Could not be determined (unknown risk)	**?**
	Microbiota outcome - Were the intervention groups assessed in the same way?	**Yes (low risk):** the intervention groups were assessed in the same way	**+**
		No (high risk): the intervention groups were not assessed in the same way	**-**
		Could not be determined (unknown risk)	**?**
	Microbiota outcome - Methodology used to assess outcome	**Low risk:** 16S rRNA gene sequencing or qPCR assays	**+**
		Moderate risk: Nugent score only	**-/+**
		Could not be determined (unknown risk)	**?**
Response bias	Assessment of missing data	**Low risk:** no missing data, OR data is missing but reasons for missing are not related to outcome, missing data is balanced across groups, proportion of missing data is not enough to have significant impact on outcome	**+**
		High risk: data is missing and reasons for missing data are not provided or related to outcome or imbalanced between groups. Large enough numbers of missing data to have an impact on results.	**-**
		Could not be determined (unknown risk)	**?**
Reporting bias	Was selective reporting performed?	**No (low risk):** all pre-specified outcomes relevant to the review were reported in the pre-specified way	**+**

		Yes (high risk): outcomes are not reported as expected or as pre-specified in protocol/paper	**-**
		Could not be determined (unknown risk)	**?**
Other	Was confounding accounted for appropriately? (applicable to non RCTs)	**Yes (low risk):** confounding variables taken into account in the design and/or analysis (e.g. through matching, stratification, interaction terms, multivariate analysis, or other statistical adjustment)	**+**
		No (High risk): no adjustment or consideration of confounding variables	**-**
		Could not be determined (unknown risk)	**?**
	Are the lactic acid treatment details sufficiently described in the manuscript?	**Yes (low risk):** manuscript clearly details formulation and mode of administration of lactic acid treatment	**+**
		No (moderate risk): details of lactic acid treatment are missing from the manuscript, however drug name provided so details can be obtained from other sources	**-/+**
		No (high risk): no details of lactic acid treatment formulation in manuscript.	**-**
		Could not be determined (unknown risk)	**?**

Developed using the RoB 2.0 (*Revised* Cochrane *risk of bias tool* for *randomized trials*) and ROBINS-I (Risk Of Bias In Non-randomised Studies - of Interventions) tools. + indicates a low risk of bias, -/+ indicates moderate risk of bias, - indicates high risk of bias, ? indicates unknown risk.

Supplementary Table D3. Full text articles excluded and reasons for exclusion

Reference	Reason/s for exclusion
Rapisarda AMC, Caldaci L, Valenti G, Brescia R, Sapia F, Sarpietro G, *et al*. Efficacy of vaginal preparation containing *Lactobacillus acidophilus*, lactic acid and deodorized garlic extract in treatment and prevention of symptomatic bacterial vaginitis: result from a single-arm pilot study. Italian Journal of Gynaecology & Obstetrics. 2018;30(1):21-31.	Non-RCT Contains a lactic acid producing probiotic
Hirnle L, Malolepsza-Jarmolowska K, Kubis AA, Hirnle P. Evaluation of bacterial vaginosis therapy in pregnant women with vaginal tablets containing lactic acid complexed with eudragit® E-100 which undergo gelation at the site of application. Advances in Clinical and Experimental Medicine. 2006;15(4):645-51.	Non-RCT Pregnant women Definition of BV at baseline is unclear and difficult to determine the number of women with NS=7-10 at enrolment who were cured (NS<4) at endpoint
Holst E, Brandberg A. Treatment of bacterial vaginosis in pregnancy with a lactate gel. Scand J Infect Dis. 1990;22(5):625-6.	Non-RCT Pregnant women
Bahamondes MV, Portugal PM, Brolazo EM, Simoes JA, Bahamondes L. Use of a lactic acid plus lactoserum intimate liquid soap for external hygiene in the prevention of bacterial vaginosis recurrence after metronidazole oral treatment. Rev Assoc Med Bras (1992). 2011;57(4):415-20.	Non-RCT Study of BV recurrence Product used externally
Di Pierro F, Catacchio V, Candidi C, Zerbinati N, Alfonso R. Rhatany-based preparation in vulvovaginitis and vaginosis. Gazzetta Medica Italiana Archivio per le Scienze Mediche. 2009;168(6):339-46.	Non-RCT BV cure not defined as ≤2 Amsel criteria and/or NS<4 Contains a lactic acid producing probiotic
Decena DC, Co JT, Manalastas RM, Jr., Palaypayon EP, Padolina CS, Sison JM, *et al*. Metronidazole with Lactacyd vaginal gel in bacterial vaginosis. J Obstet Gynaecol Res. 2006;32(2):243-51.	BV cure not defined as ≤2 Amsel criteria and/or NS<4

Andersch B, Lindell D, Dahlen I, Brandberg A. Bacterial vaginosis and the effect of intermittent prophylactic treatment with an acid lactate gel. Gynecol Obstet Invest. 1990;30(2):114-9.	Study of BV recurrence
Amaral E, Perdigao A, Souza MH, Mauck C, Waller D, Zaneveld L, *et al*. Vaginal safety after use of a bioadhesive, acid-buffering, microbicidal contraceptive gel (ACIDFORM) and a 2% nonoxynol-9 product. Contraception. 2006;73(5):542-7.	Study of women without BV where vaginal microbiota is not assessed using molecular methods (Assessed only by Nugent score)
Andersch B, Lindell D, Dahlen I, Brandberg A. Bacterial vaginosis and the effect of intermittent prophylactic treatment with an acid lactate gel. Gynecol Obstet Invest. 1990;30(2):114-9.	Duplicate record
Boeke AJ, Dekker JH, van Eijk JT, Kostense PJ, Bezemer PD. Effect of lactic acid suppositories compared with oral metronidazole and placebo in bacterial vaginosis: a randomised clinical trial. Genitourinary medicine. 1993;69(5):388-92	Duplicate record
Brittingham A, Wilson WA. The antimicrobial effect of boric acid on trichomonas vaginalis. Sexually Transmitted Diseases. 2014;41(12):718-22.	No BV cure or vaginal microbiota outcome
Carati D, Zizza A, Guido M, De Donno A, Stefanizzi R, Serra R, *et al*. Safety, efficacy, and tolerability of differential treatment to prevent and treat vaginal dryness and vulvovaginitis in diabetic women. Clinical and experimental obstetrics & gynecology. 2016;43(2):198-202.	No BV cure or vaginal microbiota outcome
Clackson TE, Coombs GH. The antagonistic effects of acetate and lactate upon the trichomonacidal activity of metronidazole. Journal of Antimicrobial Chemotherapy. 1983;11(5):401-6.	No BV cure or vaginal microbiota outcome
Eusaph AZ, Nighat R, Arshad A. Lactacyd FH as an adjuvant therapy for vulvovaginal infections in Pakistani women: Fresh study, a satisfaction survey. Journal of the Pakistan Medical Association. 2016;66(5):521-7.	No BV cure or vaginal microbiota outcome
Guaraldi C, Costantino M, Costantino D. Tyndallized lactic ferments: New possible therapies in treating vaginitis. Minerva Ginecologica. 2017;69(1):112-5.	No BV cure or vaginal microbiota outcome
Jones CP, Carter B, Thomas WL. The treatment of resistant or recurrent vaginal trichomoniasis with lactic acid jelly and lactic acid douches. 1960;149(Suppl):128-38.	No BV cure or vaginal microbiota outcome

Kale V, Patil M, Khandagade A. Development of vaginal tablets containing probiotic and prebiotic. International Journal of Pharmaceutical Sciences Review and Research. 2012;15(1):31-5.	No BV cure or vaginal microbiota outcome
Lee YK, Chung HH, Kim JW, Park NH, Song YS, Kang SB. Vaginal pH-balanced gel for the control of atrophic vaginitis among breast cancer survivors: A randomized controlled trial. Obstetrics and Gynecology. 2011;117(4):922-7.	No BV cure or vaginal microbiota outcome
Malolepsza-Jarmolowska K. Studies on gynaecological hydrophilic lactic acid preparations, Part 7: use of chitosan as lactic acid carrier in intravaginal tablets (globuli vaginales). Die Pharmazie. 2006;61(9):780-2.	No BV cure or vaginal microbiota outcome
Malolepsza-Jarmolowska K. Studies on gynecological hydrophilic lactic acid preparations. Part 8: use of chitosan as lactic acid carrier in intravaginal tablets. Acta poloniae pharmaceutica. 2007;64(1):69-72.	No BV cure or vaginal microbiota outcome
Malolepsza-Jarmolowska K, Kubis AA, Hirnle L. Studies on gynaecological hydrophilic lactic acid preparations. Part 5: The use of Eudragit E-100 as lactic acid carrier in intravaginal tablets. Die Pharmazie. 2003;58(4):260-2.	No BV cure or vaginal microbiota outcome
Malolepsza-Jarmolowska K, Kubis AA, Hirnle L. Studies on gynaecological hydrophilic lactic acid preparations, part 6: use of Eudragit E-100 as lactic acid carrier in intravaginal tablets. Die Pharmazie. 2003;58(5):334-6.	No BV cure or vaginal microbiota outcome
Mauck CK, Brache V, Kimble T, Thurman A, Cochon L, Littlefield S*, et al*. A phase I randomized postcoital testing and safety study of the Caya diaphragm used with 3% Nonoxynol-9 gel, ContraGel or no gel. Contraception. 2017;96(2):124-30.	No BV cure or vaginal microbiota outcome
Melvin L, Glasier A, Elton R, Cameron ST. pH-balanced tampons: Do they effectively control vaginal pH? BJOG: An International Journal of Obstetrics and Gynaecology. 2008;115(5):639-45.	No BV cure or vaginal microbiota outcome
Passloer HJ. Problems of povidone-iodine and LEC as an alternative in vaginal disinfection. International Journal of Experimental and Clinical Chemotherapy. 1990;3(4):235-7.	No BV cure or vaginal microbiota outcome
Sanchez Carazo JL, Gimeno Carpio E, Grifol ALA. Comparison of three imidazolic regimens in the treatment of vaginal candidosis. European Journal of Sexually Transmitted Diseases. 1986;3(4):223-5.	No BV cure or vaginal microbiota outcome
Swidsinski A, Loening-Baucke V, Mendling W, Swidsinski S. Positive effects of local therapy with a vaginal lactic acid gel on dysuria and E.coli bacteriuria question our current views on recurrent cystitis. Archives of Gynecology and Obstetrics. 2012;285(6):1619-25.	No BV cure or vaginal microbiota outcome

Tansupasiri A, Puangsricharern A, Itti-arwachakul A, Asavapiriyanont S. Satisfaction and tolerability of combination of lactoserum and lactic acid on the external genitalia in Thai women. Journal of the Medical Association of Thailand. 2005;88(12):1753-7.	No BV cure or vaginal microbiota outcome
Tedeschi C, Benvenuti C. Comparison of vaginal gel isoflavones versus no topical treatment in vaginal dystrophy: results of a preliminary prospective study. Gynecol Endocrinol. 2012;28(8):652-4.	No BV cure or vaginal microbiota outcome
Verstraelen H, Vervaet C, Remon J-P. Rationale and Safety Assessment of a Novel Intravaginal Drug-Delivery System with Sustained DL-Lactic Acid Release, Intended for Long-Term Protection of the Vaginal Microbiome. PLoS ONE. 2016;11(4):e0153441.	No BV cure or vaginal microbiota outcome
Weissenbacher ER, Schulze K. Tampovagan®-C lactic acid - Investigation of the galenic preparation and clinical and microbiological efficacy in non-specific vaginitis. International Journal of Feto-Maternal Medicine. 1991;4(2):79-86.	No BV cure or vaginal microbiota outcome
Brzezinski A, Stern T, Arbel R, Rahav G, Benita S. Efficacy of a novel pH-buffering tampon in preserving the acidic vaginal pH during menstruation. International Journal of Gynecology and Obstetrics. 2004;85(3):298-300.	No BV cure or vaginal microbiota outcome
Lamotte C, Neut C, Decrocq N, Djotni H. Saforelle® and the vulvovaginal ecosystem. Fundamental and Clinical Pharmacology. 2011;25:53	No full text available
Maneksha S. Comparison of povidone iodine (betadine) vaginal pessaries and lactic acid pessaries in the treatment of vaginitis. Journal of International Medical Research. 1974;2(3):236-9.	No full text available
Tedeschi C, Benvenuti C. Use of topical+oral isoflavones in vaginal dystrophy. Climacteric. 2011;14:196-7.	No full text available
Piot P, Van Dyck E, Godts P, Vanderheyden J. A placebo-controlled, double-blind comparison of tinidazole and triple sulfonamide cream for the treatment of nonspecific vaginitis. American Journal of Obstetrics and Gynecology. 1983;147(1):85-9.	No full text available
Thomas M, Culwell K, Howard B, Dart C. Gynecologic infections and colposcopy findings from a phase 3 efficacy and safety study of a contraceptive vaginal gel compared with nonoxynol-9. Contraception. 2016;94(4):415.	No full text available

Viravaidya S, Manonai J, Sarit-Apirak S, Wattanayingcharoenchai R. Effects of topical antiseptic agent on vaginal symptoms, ph and infection in postmenopausal women using pessary for pelvic organ prolapse. International Urogynecology Journal and Pelvic Floor Dysfunction. 2012;23(2):S131-S2.	No full text available
Fleury F, Hodgson C. Single-dose treatment of vulvovaginal candidiasis with a new 500 mg clotrimazole vaginal tablet. Advances in Therapy. 1984;1(5):349-56.	No lactic acid therapy
Ahmad FJ, Alam MA, Khan ZI, Khar RK, Ali M. Development and *in vitro* evaluation of an acid buffering bioadhesive vaginal gel for mixed vaginal infections. Acta Pharmaceutica. 2008;58(4):407-19.	No lactic acid therapy
Alam MA, Ahmad FJ, Khan ZI, Khar RK, Ali M. Development and evaluation of acid-buffering bioadhesive vaginal tablet for mixed vaginal infections. AAPS PharmSciTech. 2007;8(4):E109.	No lactic acid therapy
Alioua S, Abdi A, Fhoula I, Bringel F, Boudabous A, Ouzari I. Diversity of vaginal lactic acid bacterial microbiota in 15 Algerian pregnant women with and without bacterial vaginosis by using culture independent method. Journal of Clinical and Diagnostic Research. 2016;10(9):DC23-DC7.	No lactic acid therapy
Apolikhina IA, Sukhih GT, Teterina TA, Aslanyan KO, Kuzmin SG, Vorozhtsov GN. Antiviral and antimycotic effects of PDT with ALAsense. Photodiagnosis and Photodynamic Therapy. 2011;8(2):170-1.	No lactic acid therapy
Holloway P, Bojovic T, Bojovic D, Bontekoe R, Boon T, Schuren F, *et al*. A reduction in gardnerella vaginales and bacteroides in cervical samples of women treated for bacterial vaginosis following treatment with a topical mucoadhesive gel. Cytopathology. 2011;22:79.	No lactic acid therapy
Minis EE, Moron A, Forney L, Leizer J, Bongiovanni AM, Linhares IM, *et al*. Second trimester D-lactic acid measurement: A simple assay to predict dominant vaginal bacteria and risk for short cervix and preterm birth. American Journal of Obstetrics and Gynecology. 2018;218(1):S421-S2.	No lactic acid therapy
Nyirjesy P, Robinson J, Mathew L, Lev-Sagie A, Reyes I, Culhane JF. Alternative therapies in women with chronic vaginitis. Obstetrics and Gynecology. 2011;117(4):856-61.	No lactic acid therapy
Ozmen S, Turhan NO, Seckin NC. Gardnerella-associated vaginitis: Comparison of three treatment modalities. Turkish Journal of Medical Sciences. 1998;28(2):171-3.	No lactic acid therapy
Quaranta L, Ottolina J, Parma M, Chionna R, Sileo F, Dindelli M, *et al*. An alternative approach for the treatment of vaginal atrophy. Minerva Ginecologica. 2014;66(4):377-81.	No lactic acid therapy

Davis S. Vaginal pH-balanced gel for the control of atrophic vaginitis in breast cancer survivors. Climacteric. 2011;14(4):507-8.	Commentary /Review article
Aldunate M, Srbinovski D, Hearps AC, Latham CF, Ramsland PA, Gugasyan R, *et al*. Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. Front Physiol. 2015;6:164.	Commentary /Review article

Supplementary Table D4. Adverse events reported in included studies

Study	Lactic acid-containing product	Summary of adverse events in women receiving lactic acid-containing product	Summary of adverse in control group where applicable
Andersch *et al*, 1986	Lactal	No women reported side effects	30% of women receiving MTZ reported mild side effects including nausea, metallic taste or gastrointestinal discomfort
Boeke *et al*, 1993	Lactic acid pessary	Most participants (76%) reported no side effects. 12% of women receiving lactic acid pessary reported gastrointestinal side effects, 9% reported vaginal side effects and 1 woman reported headache. No women receiving lactic acid had *Candida ablicans* detected.	Most participants receiving MTZ (80%) reported no side effects. 7% of women reported gastrointestinal side effects, 8% reported vaginal side effects, 6% reported bad taste or other adverse event and 13% had *C. albicans* detected. Most participants receiving placebo (80%) reported no side effects. 11% of women reported gastrointestinal side effects, 3% reported vaginal side effects, 3% reported headache headache/vertigo, 6% reported other adverse events and 9% had *C. albicans* detected
Simoes *et al*, 2005	Acidform	Genital irritation was reported by 4/13 women receiving Acidform. 7/13 women were Candida positive (4/13 had symptomatic Candida infection)	Genital irritation was reported by 1/17 women receiving MTZ. 7/17 women were Candida positive (3/17 had symptomatic Candida infection)

Keller *et al*, 2012	Acidform	65% of women who received Acidform had at least one local adverse event. Commonly reported side effects were vulvar itching, vaginal or vulvar burning and abdominal cramping.	Two women receiving placebo reported vaignal or vulvar itching.
Fredstorp *et al*, 2015	Sustained release oligomeric lactic acid (OMLA) pessary	The most common side-effects were vaginal itching (mentioned by <20% of participants) and/or vaginal burning. Two women developed a yeast infection.	NA - Adverse events were not reported for women in the control group.
Gottschick *et al*, 2017	WO3191 pessary and Vagisan® pessary	No safety concerns identified with either WO3191 or Vagisan®. Tolerability of the products was judged as being good/very good in most women. The most commonly reported adverse event was bacterial vaginosis, which was comparable in both groups.	NA
van der Veer *et al*, 2019	Etos® vaginal douche	Five women reported dryness and 2 reported an increase in vaginal symptoms post douching. Women were three-fold more likely to test positive for *Candida* at the end of cycle 2 (menstrual cycle when women douched with Etos), compared to cycle 1 (before douching) [OR 3.0, 95% CI 1.2–7.2; p = 0.017]	NA

NA, not applicable; MTZ, metronidazole.

Appendix E. pdf versions of published articles

(See next page)

SCIENTIFIC REPORTS
nature research

OPEN

Sexual practices have a significant impact on the vaginal microbiota of women who have sex with women

Erica L. Plummer[1,2,10*], Lenka A. Vodstrcil[1,2,10], Christopher K. Fairley[1,2], Sepehr N. Tabrizi[3,4,5], Suzanne M. Garland[3,4,5], Matthew G. Law[6], Jane S. Hocking[7], Katherine A. Fethers[2], Dieter M. Bulach[8,9], Gerald L. Murray[3,4,5,11] & Catriona S. Bradshaw[1,2,7,11]

Women-who-have-sex-with-women (WSW) are at increased risk of bacterial vaginosis (BV). We investigated the impact of practices and past BV on the vaginal microbiota within a two-year longitudinal cohort of Australian WSW. Self-collected vaginal swabs were used to characterise the vaginal microbiota using 16S-rRNA gene sequencing. Hierarchical clustering defined community state types (CSTs). Bacterial diversity was calculated using the Shannon diversity index and instability of the vaginal microbiota was assessed by change of CST and Bray-Curtis dissimilarity. Sex with a new partner increased the bacterial diversity (adjusted-coefficient = 0.41, 95%CI: 0.21,0.60, $p < 0.001$) and instability of the vaginal microbiota, in terms of both change of CST (adjusted-odds-ratio = 2.65, 95%CI: 1.34,5.22, $p = 0.005$) and increased Bray-Curtis dissimilarity (adjusted-coefficient = 0.21, 95%CI: 0.11,0.31, $p < 0.001$). Women reporting sex with a new partner were more likely than women reporting no new partner to have a vaginal microbiota characterised by *Gardnerella vaginalis* (adjusted-relative-risk-ratio[aRRR] = 3.45, 95%CI: 1.42,8.41, $p = 0.006$) or anaerobic BV-associated bacteria (aRRR = 3.62, 95%CI: 1.43,9.14, $p = 0.007$) relative to a *Lactobacillus crispatus* dominated microbiota. Sex with a new partner altered the vaginal microbiota of WSW by increasing the diversity and abundance of BV-associated bacteria. These findings highlight the influence of practices on the development of a non-optimal vaginal microbiota and provide microbiological support for the sexual exchange of bacteria between women.

The vaginal microbiota has an important role in protecting against a range of adverse obstetric and gynaecological outcomes including miscarriage, preterm birth, and transmission and acquisition of sexually transmitted diseases (STIs) and HIV[1–4]. The optimal vaginal microbiota of reproductive aged women is typically characterised by low bacterial diversity and high relative abundance of *Lactobacillus* spp., commonly *Lactobacillus crispatus*[5–7].

Bacterial vaginosis (BV) is the most common vaginal dysbiosis and is characterised by a decrease in lactobacilli and increase in the diversity and abundance of facultative and strict anaerobic bacteria including *Gardnerella vaginalis*[5,8,9]. The pathogenesis of BV is complex and mounting epidemiological and microbiological evidence suggests that sexual activity has a role in both BV incidence and recurrence. Inconsistent condom use and new or increased numbers of sexual partners have been shown by meta-analysis to increase BV risk[10]. Other sexual practices associated with increased risk of BV include penile-vaginal sex[11,12], vaginal sex after anal sex[12], receptive oral sex with a female partner[13,14], and shared use of sex toys between women[13,15,16]. BV prevalence is high

[1]Central Clinical School, Monash University, The Alfred Centre, Melbourne, Victoria, Australia. [2]Melbourne Sexual Health Centre, Alfred Health, Carlton, Victoria, Australia. [3]Women's Centre for Infectious Diseases, The Royal Women's Hospital, Parkville, Victoria, Australia. [4]Murdoch Children's Research Institute, Parkville, Victoria, Australia. [5]Department of Obstetrics and Gynaecology, The University of Melbourne, Parkville, Victoria, Australia. [6]Kirby Institute, UNSW Australia, Kensington, NSW, Australia. [7]Melbourne School of Population and Global Health, The University of Melbourne, Parkville, Victoria, Australia. [8]Microbiological Diagnostic Unit Public Health Laboratory, The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, VIC, Australia. [9]Melbourne Bioinformatics, The University of Melbourne, Carlton, Victoria, Australia. [10]These authors contributed equally: Erica L. Plummer and Lenka A. Vodstrcil. [11]These authors jointly supervised this work: Gerald L. Murray and Catriona S. Bradshaw. *email: erica.plummer@monash.edu

Characteristic	Total (N = 100)
Age[a]	
≤28	52
>28	48
Country of Birth[b]	
Australia	86
Other	14
Self-reported past history of BV	
No	78
Yes	22
Douching (ever)[c]	
No	79
Yes	20
Baseline sexual practices	
Current regular FSP	
No[d]	28
Yes	72
Number of FSPs in previous 12 months[a]	
≤1	60
>1	40
Ever had vaginal sex with a man	
No	26
Yes[e]	74
Community State Type (CST) at baseline	
CST1-*L. crispatus*	41
CST2-*Lactobacillus* mixed	19
CST3-*L. iners*	30
CST4-*G. vaginalis* and diverse	3
CST5- anaerobic and diverse[f]	7

Table 1. Characteristics of study participants at baseline. Abbreviations: BV, bacterial vaginosis; FSP, female sexual partner; [a]Continuous variables dichotomised at median value. [b]n = 81 women reported Australian or English ethnicity, n = 11 reported a European ethnicity, n = 8 reported a non-European ethnicity (other ethnicities reported were Chinese, Malaysian/Indian, Indian, Israeli, Chilean). [c]Data missing from one participant. [d]n = 6 women reported a current MSP (male sexual partner) at baseline. [e]n = 47 women reported ≥ 1 MSP in previous 12 months. [f]The top five most prevalent taxa in CST5: *Dialister* spp., *Prevotella* spp., *G. vaginalis*, *L. iners* and *Peptoniphilus* spp.

amongst women who have sex with women (WSW) with estimates ranging from 25–52%[16–21]. Whether increased prevalence of BV in WSW is due to sexual practices or other factors is not known.

A number of studies have found sexual activity is associated with disturbance of the vaginal microbiota[22–25], however there are limited data describing how specific sexual practices influence the vaginal microbiota in WSW. Mitchell *et al.*[26] used culture methods and found that sharing of sex toys with female partners was associated with reduced concentration of *Lactobacillus*, and digital vaginal sex and sex toy use was associated with increased colonization of *G. vaginalis*.

Understanding how specific sexual practices influence the composition of the vaginal microbiota and contribute to vaginal dysbiosis and BV is important in order to develop effective treatment and prevention strategies. The primary objective of our study was to describe the impact of sexual practices on the vaginal microbiota of a subset of women participating in a cohort of Australian WSW.

Results

Description of participants at baseline and longitudinally. Baseline characteristics and sexual practices of participants are summarised in Table 1. Specimens from 102 women were initially selected for inclusion in the study; however two were removed post quality control of sequencing data (as described below), leaving 100 women in the study population. The median age of participants at enrolment was 28 years (interquartile range[IQR] 24–37 years). Most women were Australian born (86%), had tertiary level education (81%) and had a female sexual partner (FSP) at enrolment (72%). Twenty-two percent of women reported a past history of BV.

Longitudinally, most women reported receiving oral sex from an FSP (85%) and use of sex toys with an FSP (72%). Fourteen women (14%) reported vaginal sex with a male during the study period. Forty women (40%) reported sex with a new partner in one or more interval (25 women reported one new sexual partner and 15 women two or more new partners over the study period). New partners were predominantly female; 28 women

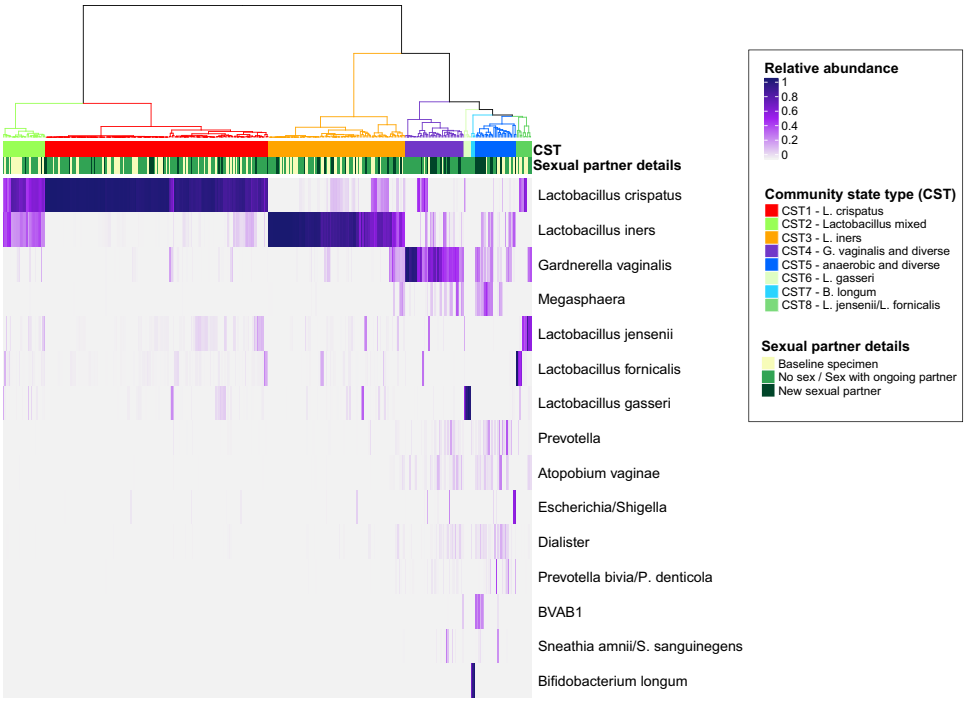


Figure 1. The vaginal microbiota of women who have sex with women. The heatmap displays the relative abundance of the 15 most abundant bacteria detected in women in this study. Hierarchical clustering of Euclidean distances with Ward linkage was used to determine eight community state types (CST): CST1-*L. crispatus*, CST2-*Lactobacillus* mixed, and CST3-*L. iners* (n = 93), CST4-*G. vaginalis* and diverse, CST5-anaerobic and diverse, CST6-*L. gasseri*, CST7-*Bifidobacterium longum*, CST8-*L. jensenii/L. fornicalis*. Exposure to a new sexual partner in the preceding 90 days is indicated above the heatmap.

reported a female new partner/s, three women reported having a male new partner/s and nine women reported both female and male new partners.

A total of 372 specimens from 102 women underwent sequencing and 5,061,171 sequence reads were generated. Following quality control, 4,942,634 reads representing 393 ASVs remained. Nine specimens had <1000 reads and were excluded; consequently two participants were excluded from analysis as one did not have an enrolment specimen and one did not have longitudinal specimens post quality control. Thus, a total of 360 specimens from 100 women were included in analyses. This included 100 enrolment specimens and 260 longitudinal specimens, 47 of which represented incident BV. The median number of reads per specimen was 12,504 (IQR 7,460–18,344).

Vaginal community state types. Hierarchical clustering identified eight community state types (CSTs), Fig. 1. Five CSTs were characterised by *Lactobacillus*: CST1-*L. crispatus* (n = 152 specimens), CST2-*Lactobacillus* mixed (comprised of *L. crispatus* and *L. iners;* n = 29), and CST3-*L. iners* (n = 93), CST6-*L. gasseri* (n = 5), CST8-*L. jensenii/L. fornicalis* (n = 10). The remaining three CSTs were: CST4-*G. vaginalis* and diverse (n = 40 specimens), CST5-anaerobic and diverse (n = 28), CST7-*Bifidobacterium longum* (n = 3). The five most prevalent taxa identified in specimens in CST5 were BV-associated bacteria *Dialister* spp., *Prevotella* spp., *G. vaginalis*, *L. iners* and *Peptoniphilus* spp. For analysis purposes, the two small *Lactobacillus* CSTs (CST6 and CST8) were combined with CST2-*Lactobacillus* mixed, and CST7-*B. longum* was combined with other anaerobic dominated specimens in CST5-anaerobic and diverse.

All women at baseline had normal (Nugent Score [NS] = 0–3, n = 92/100) or intermediate microbiota (NS = 4–6, n = 8/100) by the NS method[9]. Most women (n = 90) clustered into a *Lactobacillus* dominated CST (CST1-*L. crispatus* (n = 41), CST2-*Lactobacillus* mixed (n = 19) and CST3-*L. iners* (n = 30)).

Of the longitudinal specimens with normal (NS = 0–3, n = 204) and intermediate (NS = 4–6, n = 9) microbiota, most (89%) clustered to a *Lactobacillus* CST [CST1-*L. crispatus* (n = 111/213, 52%), CST2-*Lactobacillus* mixed (n = 24/213; 11%) and CST3-*L. iners* (n = 55/213; 26%)]. The majority of incident BV specimens (NS = 7–10, n = 47) clustered with CST4-*G. vaginalis* and diverse (n = 26/47; 55%) and CST5-anaerobic and diverse (n = 12/47; 26%).

Factors associated with vaginal microbiota diversity. In univariate analyses, sex with a new partner compared with no sex or sex in an ongoing relationship (defined as relationship for >3 months) was significantly associated with increased bacterial diversity of the vaginal microbiota (coefficient = 0.49, 95%CI: 0.30,0.68, $p < 0.001$; Table 2). Other characteristics associated with increased microbiota diversity included self-reported past history of BV, smoking, having two or more sexual partners in a study interval (i.e. the period of time

Characteristic	n women reporting exposure, n intervals exposure reported (N = 100 women and 360 intervals)	Coeff. (95% CI)[a]	*P value*[a]	Adj Coeff. (95% CI)[b]	*P value*[b]
Self-reported past history of BV					
No	78, 285	ref		ref	
Yes	22, 75	0.28 (0.06,0.50)	**0.013**	0.26 (0.04,0.48)	**0.018**
Longitudinal characteristics[c]					
Any smoking[d,e]					
No	47, 210	ref		ref	
Yes	53, 149	0.18 (0.01,0.35)	**0.036**	0.08 (−0.09,0.25)	0.352
Any douching[e]					
No	94, 351	ref			
Yes	6, 8	0.50 (−0.02,1.01)	0.060		
Number of SP					
0	3, 38[f]	ref			
1	67, 270[f]	0.03 (−0.23,0.29)	0.818		
≥2	30, 52[f]	0.36 (0.04,0.67)	**0.027**		
Frequency of sex					
Once/month or less	14, 118[g]	ref		ref	
Several times/month	36, 131[g]	0.17 (−0.02,0.36)	0.074	0.13 (−0.05,0.31)	0.164
Several times/week	50, 111[g]	0.30 (0.10,0.51)	**0.003**	0.20 (0.00,0.41)	**0.049**
Sex with NP[h]					
No	60, 300	ref		ref	
Yes	40, 60	0.49 (0.30,0.68)	**<0.001**	0.41 (0.21,0.60)	**<0.001**
Sexual practices with an FSP[i]					
Any receptive oral vaginal sex					
No[j,e]	15, 138	ref		ref	
Yes	85, 221	0.24 (0.08,0.40)	**0.003**	0.15 (−0.02,0.31)	0.076
Any digital anal sex					
No[j,e]	77, 317	ref			
Yes	23, 41	0.32 (0.08,0.57)	**0.010**		
Sharing of sex toys[e]					
No toys/washed/ condoms used[j]	58, 272	ref			
Unwashed	42, 87	0.18 (−0.00,0.37)	0.051		
Sexual practices with an MSP[k]					
Any vaginal sex					
No[l, e]	86, 321	ref			
Yes	14, 38	0.07 (−0.21,0.35)	0.637		
Self-reported symptoms					
Self-reported abnormal vaginal discharge and/or odour					
No	72, 317	ref			
Yes	28, 43	0.32 (0.09,0.55)	**0.007**		

Table 2. Characteristics and sexual practices associated with bacterial diversity of the vaginal microbiota. Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to FSP or MSP); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner Missing data for variables included in this analysis occurred in <0.5% of intervals. [a]Univariate GEE linear regression, where participant ID is panel variable. The regression coefficient represents the mean difference of Shannon diversity between the reference and comparison group/s for each characteristic/practice investigated. [b]Multivariable GEE linear regression, where participant ID is panel variable. [c]Longitudinal characteristics were measured as any exposure over the prior study interval (~90 days). No significant associations were identified between Shannon diversity and either hormonal contraceptive use or recent menses. [d]There was no dose-response relationship between smoking and Shannon diversity. [e]Missing data from a maximum of two intervals for some variables. [f]For women reporting different numbers of sexual partners in two or more intervals, the category representing the highest number of sexual partners has been used to calculate n women reporting exposure. [g]For women reporting different frequencies of sexual activity in two or more intervals, the most frequent category has been used to calculate n women reporting exposure. [h]Sex with a new partner with who first sexual contact was within 90 days. [i]No significant associations were identified between Shannon diversity and the following sexual practices with an FSP: Any digital vaginal sex from an FSP, any receptive oral anal sex from an FSP and current FSP with BV symptoms. These practices have been left out to simplify the table. [j]Or did not have a FSP. [k]No significant associations were identified between Shannon diversity and the following sexual practices with an MSP: Condoms used for vaginal sex, vaginal sex after anal sex, any receptive oral vaginal sex from an MSP, any digital vaginal sex from an MSP, any anal sex from an MSP. These practices have been left out to simplify the table. [l]Or did not have a MSP.

Characteristic	OR 95% CI	*P value*[a]	AOR 95% CI	*P value*[b]
Self-reported past history of BV				
No	1			
Yes	1.21 (0.64,2.29)	0.553		
Longitudinal practices[cd]				
Any smoking[c]				
No	1		1	
Yes	2.10 (1.25,3.54)	**0.005**	1.79 (1.03,3.11)	**0.039**
No. of cigarettes smoked				
0/non-smoker	1			
1–7	1.98 (0.87,4.49)	0.104		
8+	1.61 (0.78,3.33)	0.198		
Number of SP				
0	1			
1	0.74 (0.33,1.67)	0.464		
≥2	2.12 (0.73,6.14)	0.165		
Frequency of sex				
Once/month or less	1			
Several times/month	0.98 (0.54,1.77)	0.948		
Several times/week	1.59 (0.84,3.11)	0.152		
Sex with NP[e]				
No	1		1	
Yes	2.56 (1.37,4.81)	**0.003**	2.65 (1.34,5.22)	**0.005**
Sexual practices with FSP[f]				
Any receptive oral vaginal sex				
No[g]	1			
Yes	1.59 (0.96,2.66)	0.074		
Sharing of sex toys				
No toys/washed/condoms used[g]	1			
Unwashed	1.50 (0.83,2.72)	0.182		
Sexual Practices with an MSP[h]				
Any vaginal sex				
No[i]	1			
Yes	0.72 (0.32,1.63)	0.435		
Self-reported symptoms and microbiota characteristics				
Self-reported abnormal vaginal discharge and/or odour				
No	1			
Yes	1.04 (0.49,2.22)	0.917		
Shannon diversity	1.53 (1.02,2.28)	**0.038**		
Community state type (CST) of index specimen[j]				
CSTI- *L. crispatus*	1		1	
CST2-*Lactobacillus* mixed	5.71 (2.47,13.15)	**<0.001**	6.65 (2.81,15.76)	**<0.001**
CST3-*L. iners*	2.81 (1.54,5.12)	**0.001**	3.13 (1.67,5.87)	**<0.001**
CST4-*G. vaginalis* and diverse[k]	1.57 (0.50,4.95)	0.440	1.57 (0.48,5.17)	0.457
CST5- anaerobic and diverse	14.22 (3.13,64.69)	**<0.001**	13.18 (2.83,61.31)	**<0.001**

Table 3. Characteristics and sexual practices associated with instability of the vaginal microbiota as measured by change of CST between consecutive specimens. Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to female or male partner); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner. Missing data for variables included in this analysis occurred in <0.5% of intervals. [a]Univariate GEE logistic regression clustered for multiple specimens from each participant. [b]Multivariable GEE logistic regression clustered for multiple specimens from each participant. [c]Longitudinal characteristics were measured as any exposure over the prior study interval (~90 days). No significant associations were identified between change of CST and either hormonal contraceptive use or recent menses. [d]Douching omitted from table due to collinearity – all intervals of douching (n = 5) were accompanied by a change of CST. [e]Sex with a new partner with who first sexual contact was within 90 days. [f]The following sexual practices/characteristics with an FSP were left out of the table for simplicity: digital vaginal sex, receptive oral anal sex, digital anal sex, and current partner with BV symptoms. No significant associations between change of CST and these sexual practices were identified. [g]Or did not have a FSP. [h]The following sexual practices/characteristics with an MSP were left out of the table for simplicity: condoms use for vaginal sex, anal sex, vaginal sex after anal sex, oral

vaginal sex and digital vaginal sex. No significant associations between change of CST and these sexual practices were identified. [i]Or did not have a MSP. [j]Index specimen refers to the first specimen of each consecutive pair. [k]Majority of CST4 specimens are endpoint specimens which do not have accompanying change of CST information.

between two specimen collections), frequent sexual activity (several times/week), receptive oral sex from any FSP and self-report of BV symptoms (abnormal vaginal odour and/or vaginal discharge; Table 2). Douching and sharing of sex toys had a borderline association with increased diversity.

We included sex with a new partner, frequency of sex, smoking, oral sex and past history of BV in a multivariable model (Table 2). Digital anal sex was not included in adjusted analyses to limit over-fitting the model. Sex with a new partner (adjusted coefficient = 0.41, 95%CI: 0.21,0.60, $p < 0.001$), frequent sex (adj. coefficient = 0.20, 95%CI: 0.00,0.41, $p = 0.049$) and past history of BV (adj. coefficient = 0.26, 95%CI: 0.04,0.48, $p = 0.018$) were significantly associated with increased diversity of the vaginal microbiota. Smoking and receptive oral sex with an FSP were not associated with diversity adjusted analyses.

To explore the relationship between oral sex, exposure to a new sexual partner, and microbiota diversity, we investigated 1) the impact of new partner exposure on diversity stratified by receptive oral sex, and 2) investigated the interaction between new partner exposure and receptive oral sex. Although new partner exposure was significantly associated with microbiota diversity in women reporting oral sex and not in women who did not practice oral sex (Supplementary Table 1), the 95% confidence intervals overlapped, suggesting no statistical difference in the effect of new partner by oral sex, and furthermore there was no evidence of interaction when formally tested ($p = 0.110$).

Factors associated with instability of the vaginal microbiota. Compositional change (instability) was measured by change of CST and Bray-Curtis dissimilarity score, calculated between consecutive longitudinal specimens.

Eighty-three women (83%) experienced at least one change of CST during the study period, accounting for 138 instances of CST change. Interestingly, changing between different *Lactobacillus* CSTs (n = 66/138, 48% of all CST changes) was as common as changing from a *Lactobacillus* CST to CST4-*G. vaginalis* and diverse or CST5-anaerobic and diverse (n = 50/138, 36%).

Practices significantly associated with change of CST by univariate analysis (smoking and sex with a new partner) were included in a multivariable model with CST of index specimen (i.e. the first specimen of each consecutive pair; Table 3). In the adjusted analysis, sex with a new partner (adjusted odds ratio [AOR] 2.65, 95%CI: 1.34,5.22, $p = 0.005$) and smoking (AOR 1.79, 95%CI: 1.03,3.11, $p = 0.039$) were both associated with an increased odds of change of CST when adjusted for CST of index specimen. Additionally, women with a vaginal microbiota classified as CST2-*Lactobacillus* mixed (AOR = 6.65, 95%CI: 2.81,15.76, $p < 0.001$), CST3-*L. iners* (AOR = 3.13, 95%CI: 1.67,5.87, $p < 0.001$) or CST5-anaerobic and diverse (AOR 13.18, 95%CI: 2.83,61.31, $p < 0.001$) were more likely to change CST in the next interval compared with women with a vaginal microbiota of CST1-*L. crispatus*. Having a CST4-*G. vaginalis* and diverse microbiota was not significantly associated with change of CST, likely because the majority of CST4 samples represented an endpoint specimen i.e. incident BV (n = 26/40, 65%).

By multinomial regression, women reporting sex with a new partner were more likely than women without a new partner to change from a *Lactobacillus* CST (i.e. CST1/2/3) to a non-*Lactobacillus* dominated CST relative to not changing CST (relative risk ratio [RRR] = 4.18, 95%CI: 2.06,8.50, $p < 0.001$). Smokers were more likely than non-smokers to change between *Lactobacillus* CSTs (RRR = 2.21, 95%CI: 1.15,4.23, $p = 0.017$) or change from a *Lactobacillus* CST to a non-*Lactobacillus* dominated CST (i.e. CST4/5; RRR = 2.04, 95%CI: 1.11,3.75, $p = 0.021$) relative to not changing CST. Figure 2 summarises changes of CST in each participant longitudinally and indicates when sex with a new partner was reported.

Practices significantly associated with instability of the vaginal microbiota (i.e. increased Bray-Curtis scores between consecutive samples) by univariate analysis were included in a multivariable model that also included CST of the index specimen (Table 4). Sex with a new partner (adj. coefficient = 0.21, 95%CI: 0.11, 0.31, $p < 0.001$) and smoking (adj. coefficient = 0.09, 95%CI: 0.01, 0.18, $p = 0.036$) were associated with increased instability of the microbiota, adjusted for index specimen CST. Additionally, having a vaginal microbiota in the index specimen of CST3-*L. iners* (adj. coefficient = 0.25, 95%CI: 0.15,0.34, $p < 0.001$), CST4-*G. vaginalis* and diverse (adj. coefficient = 0.24, 95%CI: 0.05,0.43, $p = 0.013$) or CST5-anaerobic and diverse (adj. coefficient = 0.44, 95%CI: 0.30,0.60, $p < 0.001$) was associated with increased instability of the vaginal microbiota longitudinally compared to a *L. crispatus* (CST1) vaginal microbiota.

Practices impacting the vaginal microbiota composition. After considering factors that influence stability of the microbiota, we looked at specific characteristics and sexual practices that influenced the vaginal microbiota composition by multinomial logistic regression. In univariate analyses (Supplementary Table 2), we found women who reported sex with a new partner in the previous 90 days were more likely than women reporting no sex or sex in an ongoing relationship to have a vaginal microbiota of CST4-*G. vaginalis* abundant and diverse (RRR = 4.09, 95%CI: 1.69,9.92, $p = 0.002$) or CST5-anaerobic and diverse (RRR = 5.37, 95%CI: 2.18,13.20, $p < 0.001$) than one of CST1. Women who reported smoking were more likely than non-smokers to have anaerobic microbiota (CST5) relative to CST1 (RRR = 3.01,95%CI: 1.31,6.92, $p = 0.009$). Women who reported receptive oral vaginal sex from an FSP or sharing of unwashed sex toys with an FSP were more likely to have a CST4 microbiota, and women who douched or had a past history of BV were more likely to have a CST5 microbiota (Supplementary Table 2). Women reporting recent menses (defined as onset of menses within 7 days

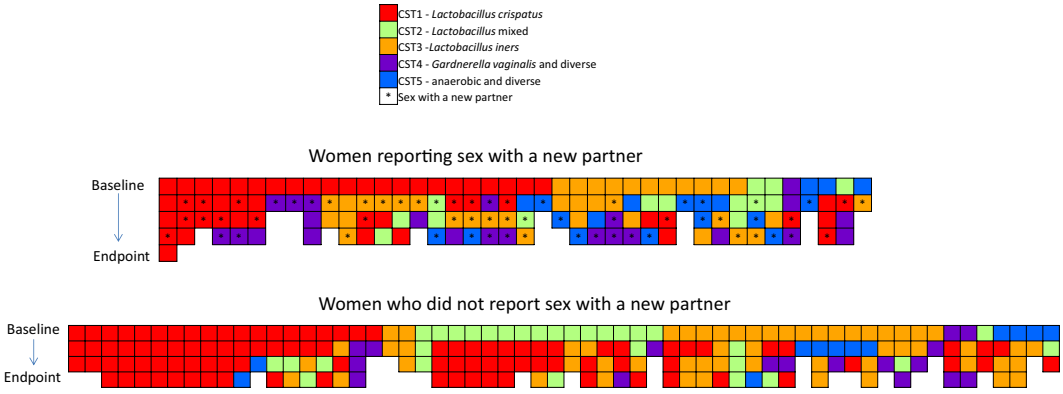


Figure 2. Longitudinal changes of community state type in women reporting sex with a new partner. Each column represents a participant and sequential longitudinal specimens are ordered from baseline to endpoint. Boxes are coloured according to community state type (CST). *Indicates a report of sex with a new partner. Most women changed CST at least once during the study. Change of CST occurred in 36/40 (90%) women who reported exposure to a new partner and 47/60 (78%) women who did not report sex with a new partner.

of specimen collection) were more likely than women not reporting recent menses (>7 days from specimen collection) to have a CST2-*Lactobacillus*-mixed or CST3-*L. iners* microbiota composition relative to CST1 microbiota, but were not more likely to have a *G. vaginalis* (CST4) or anaerobic microbiota (CST5).

We included past history of BV, receptive oral sex from a FSP, sex with a new partner, sharing of unwashed sex toys with an FSP smoking and recent menses in a multivariable multinomial regression model (Table 5). Women reporting sex with a new partner were more likely than women reporting no sex or sex in an ongoing relationship to have a CST4-*G. vaginalis* and diverse (adjusted-RRR = 3.45, 95%CI: 1.42,8.41, p = 0.006) or CST5-anerobic and diverse vaginal microbiota (adjusted-RRR = 3.62, 95%CI: 1.43,9.14, p = 0.007) relative to a CST1 vaginal microbiota. Women reporting that they shared unwashed sex toys with an FSP were more likely than women not reporting this practice to have a CST4 vaginal microbiota (adjusted-RRR = 2.49, 95%CI: 1.05,5.91, p = 0.038). Women reporting smoking were more likely than non-smokers to have a CST5-anaerobic and diverse vaginal microbiota relative to a CST1 vaginal microbiota (adjusted-RRR = 2.94, 95%CI: 1.16,7.43, p = 0.023). Women with a past-history of BV were more likely to have a CST5 vaginal microbiota (adjusted-RRR = 3.18, 95%CI: 1.13,8.91, p = 0.028), and women reporting recent menses were more likely to have a CST2 (adjusted-RRR = 3.89, 95%CI: 1.58,9.50, p = 0.003) or CST3 (adjusted-RRR = 2.37, 95%CI: 1.14,4.90, p = 0.020) vaginal microbiota.

Discussion

In this longitudinal cohort study of women who have sex with women, specific sexual practices influenced the bacterial diversity, stability and composition of the vaginal microbiota. Sex with a new partner (primarily representing new FSPs) was associated with an increase in bacterial diversity and an increase in compositional change (or instability) of the vaginal microbiota, both in terms of change of CST and increased Bray-Curtis dissimilarity. Furthermore, women who reported sex with a new partner were more likely than women reporting no sex/sex in an ongoing relationship to have a vaginal microbiota characterised by BV-associated anaerobic bacteria or *G. vaginalis*, relative to an optimal microbiota characterised by *L. crispatus*. This study highlights the influence of practices on the development of a non-optimal vaginal microbiota and provides microbiological support for the sexual exchange of bacteria between women. These microbiological findings complement the previously reported epidemiological data from the original cohort[13,18] which showed sex with a new partner was associated with a 2.5-fold increased risk of BV acquisition.

There is increasing evidence to support the sexual transmission of vaginal bacteria between WSW. Longitudinal studies in this population have shown that one of the greatest risk factors for BV is having a sexual partner with a history of BV, BV symptoms or microbiologically confirmed BV[13,14]. A recent study demonstrated that incident BV occurred at a median of 4 days post sexual activity in 93% of WSW, indicating a similar incubation period to that of other STIs[27]. An early study looking at the transmission dynamics of BV demonstrated that transfer of vaginal secretions between women resulted in BV in 11 of 15 women[28]. Furthermore, high concordance of Nugent Score categories between FSP[13,15,17–19] and evidence that women in monogamous relationships share *Lactobacillus* strains[29] in their vaginas supports exchange of bacteria between women during sex. In our study, women who shared unwashed sex toys and/or received oral sex from an FSP were more likely than women not reporting these practices to have an anaerobic or *G. vaginalis* abundant vaginal microbiota than a microbiota dominated by *L. crispatus*. Sexual practices are frequently highly correlated, so it is difficult to determine whether one activity has a greater impact on the vaginal microbiota than others. However, both oral sex with an FSP and sex toy use involve exchange of bodily fluids to varying degrees and therefore promote exchange of bacteria between women. Additionally, both practices have been reported as a risk factor for BV[10,13,14,16]. Collectively, these data suggest that female partner treatment of women with BV may be an effective strategy to improve BV cure and warrants further investigation.

Characteristic	Coeff. 95% CI[a]	*P value*[a]	Adj Coeff. 95% CI[b]	*P value*[b]
Self-reported past history of BV				
No	ref	—		
Yes	0.10 (−0.04,0.23)	0.159		
Longitudinal practices[c]				
Any smoking				
No	ref	—	ref	—
Yes	0.15 (0.05,0.25)	**0.003**	0.09 (0.01,0.18)	**0.036**
No. of cigarettes smoked				
0/non-smoker	ref	—		
1–7	0.07 (−0.09,0.22)	0.388		
8+	0.15 (0.01,0.30)	**0.035**		
Any douching				
No	ref	—		
Yes	0.33 (−0.01,0.67)	0.056		
Number of SP				
0	ref			
1	−0.12 (−0.28,0.03)	0.119		
≥2	0.13 (−0.06,0.32)	0.182		
Frequency of sex				
Once/month or less	ref	—		
Several times/month	−0.01 (−0.12,0.10)	0.865		
Several times/week	0.06 (−0.07,0.18)	0.366		
Sex with NP[d]				
No	ref	—	ref	—
Yes	0.23 (0.12,0.33)	**<0.001**	0.21 (0.11,0.31)	**<0.001**
Sexual practices with FSP[e]				
Any receptive oral vaginal sex				
No[f]	ref	—		
Yes	0.06 (−0.04,0.15)	0.258		
Sharing of sex toys				
No toys/washed/condoms used[f]	ref			
Unwashed	0.04 (−0.07,0.15)	0.478		
Sexual practices with MSP[g]				
Any vaginal sex				
No[h]	ref	—		
Yes	0.09 (−0.07,0.26)	0.279		
Self-reported symptoms and microbiota characteristics				
Self-reported abnormal vaginal discharge and/or odour				
No	ref	—		
Yes	0.07 (−0.07,0.22)	0.305		
Shannon diversity	0.07 (−0.00,0.14)	0.053		
Community state type (CST) of index specimen[i]				
CSTI-*L. crispatus*	ref	—	ref	—
CST2-*Lactobacillus* mixed	0.08 (−0.05,0.20)	0.241	0.09 (−0.03,0.22)	0.134
CST3-*L. iners*	0.23 (0.12,0.33)	**<0.001**	0.25 (0.15,0.34)	**<0.001**
CST4-*G. vaginalis* and diverse[j]	0.23 (0.03,0.43)	**0.027**	0.24 (0.05,0.43)	**0.013**
CST5- anaerobic and diverse	0.47 (0.30,0.64)	**<0.001**	0.44 (0.30,0.60)	**<0.001**

Table 4. Characteristics and sexual practices associated with instability of the vaginal microbiota as measured by Bray-Curtis dissimilarity between consecutive specimens. Abbreviations: BV, bacterial vaginosis; SP, sexual partner (may refer to FSP or MSP); NP, new partner (may refer to FSP or MSP); FSP, female sexual partner; MSP, male sexual partner. Missing data for variables included in this analysis occurred in <0.5% of intervals. [a]Univariate GEE linear regression clustered for multiple specimens from each participant. The regression coefficient represents the mean difference of Bray-Curtis Dissimilarity between the reference and comparison group/s for each characteristic/practice investigated. [b]Multivariable GEE linear regression clustered for multiple specimens from each participant. [c]Longitudinal characteristics were measured as any exposure over the prior follow-up interval (~90 days). No significant associations were identified between beta diversity and either hormonal contraceptive use or recent menses. [d]Sex with a new partner with who first sexual contact was within 90 days. Partner gender was defined by the participant. [e]The following sexual practices/characteristics with an FSP were left out of the table for simplicity: digital vaginal sex, receptive oral anal sex, digital anal sex

and current partner with BV symptoms. No significant associations between beta diversity and these sexual practices were identified. [f]Or did not have a FSP. [g]The following sexual practices/characteristics with an MSP were left out of the table for simplicity: condoms use for vaginal sex, anal sex, vaginal sex after anal sex, oral vaginal sex and digital vaginal sex. No significant associations between beta diversity and these sexual practices were identified. [h]Or did not have a MSP. [i]Index specimen refers to the first specimen of each consecutive pair. [j]Majority of CST4 specimens are endpoint specimens which do not have accompanying beta diversity information.

Change of CST was common in our study, in accordance with previous reports that show the vaginal microbiota can be highly dynamic[22,23,30]. Compositional change (or instability) of the vaginal microbiota between consecutive specimens was primarily influenced by the bacteria present in the index specimen. Collectively, women with a low diversity *L. crispatus* dominated vaginal microbiota were more likely to have a stable microbiota longitudinally and were less likely to experience change of CST than women with a diverse microbiota or a microbiota abundant in *L. iners* or *G. vaginalis*. Our findings are consistent with one study[22] that analysed the vaginal microbiota of 32 women sampled twice-weekly for 16-weeks. Gajer *et al.*[22] reported that *L. crispatus* and *L. gasseri* dominated microbiota appeared to be stable, and that sexual activity negatively impacted stability. Interestingly, practices and microbiological characteristics associated with change of CST were highly consistent with those associated with increasing instability of the microbiota (measured by Bray-Curtis), suggesting change of CST may be a useful measure of microbiota instability[31].

Smoking had a broad ranging effect on the diversity, stability and composition of the vaginal microbiota, and past studies have shown an association between smoking and BV and/or vaginal microbiota composition that was dose dependent[18,32–34]. There are a number of possible explanations for this association. Smokers have been shown to have reduced oestradiol levels compared non-smokers[35], and reduced oestrogen has been associated with non-optimal *Lactobacillus*-deficient vaginal microbiota[36]. Furthermore, it is well established that nicotine has detrimental effects on the immune system, including reduced production of inflammatory cytokines and decreased functionality of neutrophils and macrophages[37], and nicotine and its derivatives have been detected in the vaginal metabolome[38]. It is possible that modulation of immune responses may result in reduced clearance of *G. vaginalis* and other BV-associated bacteria (similar to what has been observed for human papillomavirus[39]) or prevent maintenance of an optimal *Lactobacillus* vaginal microbiota. The association between smoking and vaginal microbiota instability seen in our study is interesting and may be because the microbiota composition that is found more commonly in smokers (i.e. anaerobic and diverse microbiota) is inherently more unstable than others, such as those dominated by *L. crispatus*. It is also possible that observed associations between smoking and adverse microbiota composition and instability are due to unmeasured confounding; however, the fact that this association has been shown to be dose dependent in some studies and persists in adjusted analysis provides evidence for a biological association.

A number of other factors were associated with vaginal microbiota composition, stability and/or diversity including past history of BV, menses and douching. The finding that past history of BV was associated with both increased bacterial diversity and an anaerobic microbiota may represent persistence or re-emergence of a polymicrobial BV-biofilm[40,41], or alternatively the influence of other factors such as host genetics or immune function[37], diet[42] or contraceptive practices[43]. Both douching and menses have been shown in a number of studies to adversely alter vaginal microbiota composition and stability[22,44,45], and consistent with this, we found that douching was associated with anaerobic and diverse vaginal microbiota and had a borderline adverse effect on microbiota stability in univariate analyses. While recent menses did not have an effect on microbiota diversity or stability in our study, it did influence microbiota composition. Women were more likely to have a vaginal microbiota abundant in *L. iners* (i.e. CST2 or CST3) if their specimen was collected within seven days of onset of menses, consistent with data that shows *L. iners* grows best on media containing blood[46,47].

Hormonal contraception may have a beneficial impact on the vaginal microbiota[48]. However, we found no association between hormonal contraception and microbiota diversity, stability or composition, which may be because only a small number of women reported hormonal contraceptive use in the parent cohort.

There are a number of limitations to this study. The study population comprised highly educated women who were predominately Australian born and reported Australian or English ethnicity, which may limit the generalizability of our findings. Specimens were collected every three months which limited our ability to assess immediate effect of sexual practices behaviours on the vaginal microbiota and any short-term fluctuations in microbiota composition. Specimens included in the analysis were not selected randomly or from specified study time points which may have biased results. We did not include negative controls alongside specimens during sequencing, however we removed contaminants previously identified using the same extraction methodology, primer set up and sequencing instrument[49] and the microbiota profiles are consistent with those previously published[6,25,50]. Finally, this study did not assess practices or the vaginal microbiota of the sexual partner/s of participants so we cannot definitively prove sexual transmission of BV-associated bacteria is occurring between women. Nevertheless, the microbiota data presented here is consistent with epidemiological data that supports sexual transmission of BV in WSW[13,14].

This study shows that sex with a new partner is associated with changes in the vaginal microbiota of WSW, including increased diversity and increased abundance of bacteria commonly associated with a non-optimal vaginal microbiota. These findings suggest that sexual exchange of bacteria, including BV-associated bacteria, occurs between female sexual partners, and highlight the influence of specific practices on the development of a non-optimal vaginal microbiota. These data are important for informing strategies to promote a vaginal microbiota that is associated with optimal reproductive health, as well as new approaches to improve BV cure such as female partner treatment.

Outcome by CST	RRR (95% CI)	*P value*[a]	Adjusted RRR (95% CI)	*P value*[b]
***Lactobacillus* mixed (CST2) vs CST1**				
Self-reported past history of BV[c]	0.87 (0.35,2.17)	0.762	0.83 (0.33,2.09)	0.696
Smoker[d]	1.59 (0.77,3.29)	0.214	1.75 (0.84,3.65)	0.138
Sex with a NP[e]	0.62 (0.18,2.19)	0.460	0.57 (0.16,2.00)	0.384
Receptive oral vaginal sex from FSP[f]	1.09 (0.56,2.15)	0.799	1.02 (0.51,2.05)	0.951
Sharing of unwashed sex toys with FSP[g]	0.92 (0.36,2.32)	0.859	0.75 (0.26,2.18)	0.600
Onset of last menses ≤7 days ago[h]	3.59 (1.49,8.68)	**0.004**	3.89 (1.58,9.50)	**0.003**
***L. iners* (CST3) vs CST1**				
Self-reported past history of BV[c]	0.69 (0.26,1.87)	0.467	0.66 (0.23,1.93)	0.450
Smoker[d]	1.38 (0.71,2.65)	0.341	1.46 (0.72,2.94)	0.291
Sex with a NP[e]	1.77 (0.87,3.60)	0.117	1.61 (0.77,3.38)	0.208
Receptive oral vaginal sex from FSP[f]	1.09 (0.57,2.12)	0.790	1.01 (0.51,2.01)	0.977
Sharing of unwashed sex toys with FSP[g]	0.98 (0.50,1.92)	0.952	1.02 (0.48,2.14)	0.965
Onset of last menses ≤7 days ago[h]	2.19 (1.08,4.46)	**0.030**	2.37 (1.14,4.90)	**0.020**
***G. vaginalis* and diverse (CST4) vs CST1**				
Self-reported past history of BV[c]	1.13 (0.39,3.27)	0.817	1.24 (0.43,3.57)	0.686
Smoker[d]	1.72 (0.74, 4.01)	0.207	1.69 (0.70,4.08)	0.240
Sex with a NP[e]	4.09 (1.69,9.92)	**0.002**	3.45 (1.42,8.41)	**0.006**
Receptive oral vaginal sex from FSP[f]	2.60 (1.09,6.20)	**0.031**	1.94 (0.77,4.84)	0.158
Sharing of unwashed sex toys with FSP[g]	2.38 (1.04,5.45)	**0.039**	2.49 (1.05,5.91)	**0.038**
Onset of last menses ≤7 days ago[h]	1.64 (0.55,4.91)	0.378	1.37 (0.41,4.64)	0.611
Anaerobic and diverse (CST5) vs CST1				
Self-reported past history of BV[c]	2.82 (1.09,2.27)	**0.032**	3.18 (1.13,8.91)	**0.028**
Smoker[d]	3.01 (1.31,6.92)	**0.009**	2.94 (1.16,7.43)	**0.023**
Sex with a NP[e]	5.37 (2.18,13.20)	**<0.001**	3.62 (1.43,9.14)	**0.007**
Receptive oral vaginal sex from FSP[f]	2.17 (0.86,5.46)	0.099	1.67 (0.62,4.49)	0.308
Sharing of unwashed sex toys with FSP[g]	1.46 (0.62,3.46)	0.387	2.07 (0.79,5.43)	0.141
Onset of last menses ≤7 days ago[h]	1.03 (0.27,4.00)	0.964	0.78 (0.01,0.12)	0.734

Table 5. Characteristics and practices associated with vaginal microbiota composition by multivariable multinomial logistic regression. Abbreviations: CST, community state type; NP, new partner (may refer to FSP or MSP); FSP, female sexual partner. Missing data for variables included in this analysis occurred in <0.5% of intervals. [a]Multinomial logistic regression with CST1-*L. crispatus* as the baseline comparison group. Analysis clustered for multiple specimens from participants (100 clusters). [b]Multinomial logistic regression as described in a adjusted for all other characteristics in the table. [c]Self-reported past history of BV relative to no self-report of past history of BV. [d]Smoker relative to non-smoker. [e]Sex with a new partner with who first sexual contact was within 90 days relative to no sex/sex with a partner with who first sexual contact was >90 days. [f]Receptive oral vaginal sex from FSP relative to no receptive oral sex from FSP (or no FSP). [g]Sharing of unwashed sex toys with FSP relative to no toy use/changed condoms on the sex toys/always washed the sex toys between sharing with a partner. [h]Onset of last menses ≤7 days ago relative to onset of last menses >7 days ago.

Methods

Participant and specimen selection. Participants were selected from the Women On Women's (WOW) Health study, a two-year cohort of 298 WSW designed to examine epidemiological and microbiological factors associated with incident BV[13,18]. Women reported a FSP within 18 months prior to enrolment and were BV negative (NS < 7[9]) on three consecutive baseline vaginal smears collected one week apart. Women self-collected a vaginal swab and smear, and completed a detailed questionnaire every three months until study endpoint (diagnosis of incident BV [NS = 7–10] or 24 months without BV). Women were instructed to avoid specimen collection on the heaviest days of their menstrual cycle[13].

For the microbiota sub-study, we included all women who developed incident BV (n = 51) and an equal number of women who did not (initially controls were over-selected using a random sort command in Stata/IC (v14.2, StataCorp LP, College Station, USA)). Seven of the 51 women to develop incident BV co-enrolled in the original cohort with their FSP[13]. As such, controls were then frequency matched on co-enrolment status and age to ensure a similar distribution of both variables (for example, the last non co-enrolled control was replaced with the next randomly selected co-enrolled control). Each woman contributed a baseline specimen and an endpoint specimen (BV-specimen from women with incident BV or the 24 month specimen from women without BV). Up to three interim specimens were included for each woman (typically the last two specimens collected prior to the endpoint specimen). If a specimen could not be used/located, an earlier specimen from that participant was used.

Ethical approval was obtained from the Human Research Ethics Committees of Alfred Hospital, Melbourne, Australia and the University of Melbourne. All research was performed in accordance with the National Statement

on Ethical Conduct in Human Research. Informed written consent was obtained from all participants for the use of their specimens in the current study.

Laboratory methods. Swabs were agitated in 1 mL RNAlater (Thermo Fisher Scientific, Waltham, USA) and stored at −80 °C prior to DNA extraction using the MagNA Pure 96 instrument and the DNA and Viral NA small volume kit (Roche Diagnostics, Mannheim, Germany). Dual index primers 341 F/805 R with heterogeneity spacers[51–53] were used for PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene. Libraries were sequenced by Micromon Genomics (Micromon, Monash University, Victoria, Australia) on the MiSeq platform (Illumina, San Diego, CA, USA). Sequence reads are available in the NCBI Sequence Read Archive under Bioproject PRJNA434520.

Sequence data analysis. Barcodes were extracted using QIIME v1.9.0[54] and demultiplexing was performed using idemp (https://github.com/yhwu/idemp). Primers and heterogeneity spacers were removed using TagCleaner standalone version 0.16[55]. Reads were processed using DADA2 v1.6.0[56]. Reads were truncated based on quality profiles (at 250 bases for read 1 and 220 bases for read 2) and were discarded if they had ambiguous bases or exceeded the number of expected errors based on quality scores. Chimeras were identified and removed. Taxonomy was assigned using the default RDP Classifier implemented in DADA2 and the Silva reference database (v128)[57]. Species level assignment was performed using exact matching in the DADA2 package and taxonomy for *Lactobacillus* spp. was confirmed by a BLAST search against a database of 16S rRNA gene sequences from 158 type strains. Not all ASVs were able to be assigned to species level.

BV-associated bacteria (BVAB)−1 has previously been misclassified as *Shuttleworthia*[58] and BVAB3 is named as *Fastidiosiplia* in the Silva database[59]. We aligned *Shuttleworthia* and *Fastidiosiplia* ASVs against BVAB1 (NCBI GenBank AY724739.1), BVAB2 (AY724740.1) and BVAB3 (AY724741.1) using Clustal Omega (EMBL-EBI)[60,61]. *Shuttleworthia* ASV had 100% identity to BVAB1. Two *Fastidiosiplia* ASVs had high identity to BVAB2 (99.50 and 100% identity, respectively), and a third *Fastidiosiplia* ASV had 100% identity to BVAB3. The ASVs were reclassified accordingly.

ASVs were removed if they had a total abundance of less than 0.001% or were present in only one specimen. The ASV table was screened for contaminants previously identified in negative controls[49], as well as common sequencing contaminants (removed ASVs belonging to *Facklamia* and *Shewanella* genera and Halomonadaceae family)[62,63]. Specimens with fewer than 1000 reads were excluded from analysis. Participants were excluded if they did not have an enrolment specimen or did not have any follow-up specimens.

Diversity metrics and CST were generated using the Vegan package[64] and R Studio [V 1.1.419, Boston, USA] employing R v3.4.3. Alpha diversity was calculated using the Shannon Diversity Index using ASV data. ASVs assigned to the same taxonomy were merged and the relative abundance of each taxon was used for CST identification. Hierarchical clustering of Euclidean distances with Ward linkage was performed on the relative abundance of each taxon and a scree plot of within cluster distances was used to inform the number of CSTs. Bray-Curtis dissimilarity scores were calculated between consecutive paired specimens from each participant. The heatmap was generated using the ComplexHeatmap package[65] and the same metrics used to identify CSTs. Change of CST was defined as change or no change in CST between consecutive paired specimens.

Statistical analysis. Statistical models that accounted for repeated measures within individuals were fitted using generalised estimating equations (GEE) to investigate the impact of characteristics and practices on the diversity (Shannon-Diversity Index) and instability (change of CST or Bray-Curtis dissimilarity) of the vaginal microbiota. GEE linear regression analyses were used when the outcome was Shannon-Diversity Index or Bray-Curtis dissimilarity, with the regression coefficient representing the mean difference of each outcome between the reference and comparison group/s for each characteristic/practice investigated. GEE logistic regression was used when change of CST was the outcome. Characteristics and practices deemed significant in univariate analyses ($p < 0.05$) were included in multivariable analyses.

We also analysed the type of CST change observed. Specimens were allocated one of four change type between sequential specimens: (1) no change; (2) change from one *Lactobacillus* CST to another *Lactobacillus* CST; (3) change from one *Lactobacillus* CST to a non-*Lactobacillus* CST; or (4) change from a non-*Lactobacillus* CST to any other CST. Multinomial regression was used to investigate the relationship between practices and type of CST change relative to the risk of no change, generating relative risk ratios and 95% confidence intervals.

Multinomial regression was also used to assess associations between characteristics and microbiota composition (i.e. CST-classification of a sample). CST1-*L. crispatus* was the reference group for all analyses. This analysis calculated the risk of having a vaginal microbiota of a specific CST (details of CSTs provided in results below) compared to the risk of a vaginal microbiota of CST1, clustering for multiple samples from individual participants.

Characteristics and practices deemed significant in univariate analyses ($p < 0.05$) were included in multivariable analyses. Statistical analyses were performed using STATA v14.2, unless otherwise specified.

Data availability

The raw sequencing data are publicly available in the NCBI Sequence Read Archive (SRA) under the Bioproject number PRJNA434520.

Received: 23 May 2019; Accepted: 3 December 2019;
Published online: 24 December 2019

References

1. Cohen, C. R. *et al*. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS Med* **9**, e1001251, https://doi.org/10.1371/journal.pmed.1001251 (2012).
2. Koumans, E. H., Markowitz, L. E., Berman, S. M. & St Louis, M. E. A public health approach to adverse outcomes of pregnancy associated with bacterial vaginosis. *Int J Gynaecol Obstet* **67**(Suppl 1), S29–33 (1999).
3. Myer, L., Kuhn, L., Stein, Z. A., Wright, T. C. Jr. & Denny, L. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *Lancet Infect Dis* **5**, 786–794, https://doi.org/10.1016/S1473-3099(05)70298-X (2005).
4. Brotman, R. M. *et al*. Bacterial vaginosis assessed by Gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. *J Infect Dis* **202**, 1907–1915, https://doi.org/10.1086/657320 (2010).
5. Fredricks, D. N., Fiedler, T. L. & Marrazzo, J. M. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med* **353**, 1899–1911, https://doi.org/10.1056/NEJMoa043802 (2005).
6. Ravel, J. *et al*. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA* **108**(Suppl 1), 4680–4687, https://doi.org/10.1073/pnas.1002611107 (2011).
7. Verhelst, R. *et al*. Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. *BMC Microbiol* **5**, 61, https://doi.org/10.1186/1471-2180-5-61 (2005).
8. Srinivasan, S. *et al*. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* **7**, e37818, https://doi.org/10.1371/journal.pone.0037818 (2012).
9. Nugent, R. P., Krohn, M. A. & Hillier, S. L. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J Clin Microbiol* **29**, 297–301 (1991).
10. Fethers, K. A., Fairley, C. K., Hocking, J. S., Gurrin, L. C. & Bradshaw, C. S. Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clin Infect Dis* **47**, 1426–1435, https://doi.org/10.1086/592974 (2008).
11. Fethers, K. A. *et al*. Early sexual experiences and risk factors for bacterial vaginosis. *J Infect Dis* **200**, 1662–1670, https://doi.org/10.1086/648092 (2009).
12. Cherpes, T. L., Hillier, S. L., Meyn, L. A., Busch, J. L. & Krohn, M. A. A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sex Transm Dis* **35**, 78–83, https://doi.org/10.1097/OLQ.0b013e318156a5d0 (2008).
13. Vodstrcil, L. A. *et al*. Incident bacterial vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. *Clin Infect Dis* **60**, 1042–1053, https://doi.org/10.1093/cid/ciu1130 (2015).
14. Marrazzo, J. M., Thomas, K. K., Fiedler, T. L., Ringwood, K. & Fredricks, D. N. Risks for acquisition of bacterial vaginosis among women who report sex with women: a cohort study. *PLoS One* **5**, e11139, https://doi.org/10.1371/journal.pone.0011139 (2010).
15. Marrazzo, J. M. *et al*. Characterization of vaginal flora and bacterial vaginosis in women who have sex with women. *J Infect Dis* **185**, 1307–1313, https://doi.org/10.1086/339884 (2002).
16. Marrazzo, J. M., Thomas, K. K., Agnew, K. & Ringwood, K. Prevalence and risks for bacterial vaginosis in women who have sex with women. *Sex Transm Dis* **37**, 335–339 (2010).
17. Evans, A. L., Scally, A. J., Wellard, S. J. & Wilson, J. D. Prevalence of bacterial vaginosis in lesbians and heterosexual women in a community setting. *Sex Transm Infect* **83**, 470–475, https://doi.org/10.1136/sti.2006.022277 (2007).
18. Bradshaw, C. S. *et al*. The influence of behaviors and relationships on the vaginal microbiota of women and their female partners: the WOW Health Study. *J Infect Dis* **209**, 1562–1572, https://doi.org/10.1093/infdis/jit664 (2014).
19. Berger, B. J. *et al*. Bacterial vaginosis in lesbians: a sexually transmitted disease. *Clin Infect Dis* **21**, 1402–1405 (1995).
20. Bailey, J. V., Farquhar, C. & Owen, C. Bacterial vaginosis in lesbians and bisexual women. *Sex Transm Dis* **31**, 691–694 (2004).
21. McCaffrey, M., Varney, P., Evans, B. & Taylor-Robinson, D. Bacterial vaginosis in lesbians: evidence for lack of sexual transmission. *Int. J. STD. AIDS.* **10**, 305–308 (1999).
22. Gajer, P. *et al*. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* **4**, 132ra152, https://doi.org/10.1126/scitranslmed.3003605 (2012).
23. Schwebke, J. R., Richey, C. M. & Weiss, H. L. Correlation of behaviors with microbiological changes in vaginal flora. *J Infect Dis* **180**, 1632–1636, https://doi.org/10.1086/315065 (1999).
24. Pepin, J. *et al*. The complex vaginal flora of West African women with bacterial vaginosis. *PLoS One* **6**, e25082, https://doi.org/10.1371/journal.pone.0025082 (2011).
25. Vodstrcil, L. A. *et al*. The influence of sexual activity on the vaginal microbiota and Gardnerella vaginalis clade diversity in young women. *PLoS One* **12**, e0171856, https://doi.org/10.1371/journal.pone.0171856 (2017).
26. Mitchell, C., Manhart, L. E., Thomas, K. K., Agnew, K. & Marrazzo, J. M. Effect of sexual activity on vaginal colonization with hydrogen peroxide-producing lactobacilli and Gardnerella vaginalis. *Sex Transm Dis* **38**, 1137–1144, https://doi.org/10.1097/OLQ.0b013e31822e6121 (2011).
27. Muzny, C. A., Lensing, S. Y., Aaron, K. J. & Schwebke, J. R. Incubation period and risk factors support sexual transmission of bacterial vaginosis in women who have sex with women. *Sex Transm Infect* **0**, 1–5, https://doi.org/10.1136/sextrans-2018-053824 (2019).
28. Gardner, H. L. & Dukes, C. D. Haemophilus vaginalis vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *Am J Obstet Gynecol* **69**, 962–976 (1955).
29. Marrazzo, J. M., Antonio, M., Agnew, K. & Hillier, S. L. Distribution of genital Lactobacillus strains shared by female sex partners. *J Infect Dis* **199**, 680–683, https://doi.org/10.1086/596632 (2009).
30. Srinivasan, S. *et al*. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One* **5**, e10197, https://doi.org/10.1371/journal.pone.0010197 (2010).
31. Brooks, J. P. *et al*. Changes in vaginal community state types reflect major shifts in the microbiome. *Microb Ecol Health Dis* **28**, 1303265, https://doi.org/10.1080/16512235.2017.1303265 (2017).
32. Brotman, R. M. *et al*. Association between cigarette smoking and the vaginal microbiota: a pilot study. *BMC Infect Dis* **14**, 471, https://doi.org/10.1186/1471-2334-14-471 (2014).
33. Mehta, S. D. *et al*. The vaginal microbiota over an 8- to 10-year period in a cohort of HIV-infected and HIV-uninfected women. *PLoS One* **10**, e0116894, https://doi.org/10.1371/journal.pone.0116894 (2015).
34. Hellberg, D., Nilsson, S. & Mardh, P. A. Bacterial vaginosis and smoking. *International journal of STD & AIDS* **11**, 603–606, https://doi.org/10.1258/0956462001916461 (2000).
35. Westhoff, C., Gentile, G., Lee, J., Zacur, H. & Helbig, D. Predictors of ovarian steroid secretion in reproductive-age women. *Am. J. Epidemiol.* **144**, 381–388 (1996).
36. Miller, E. A., Beasley, D. E., Dunn, R. R. & Archie, E. A. Lactobacilli Dominance and Vaginal pH: Why Is the Human Vaginal Microbiome Unique? *Front Microbiol* **7**, 1936, https://doi.org/10.3389/fmicb.2016.01936 (2016).
37. Murphy, K. & Mitchell, C. M. The Interplay of Host Immunity, Environment and the Risk of Bacterial Vaginosis and Associated Reproductive Health Outcomes. *J Infect Dis* **214**(Suppl 1), S29–35, https://doi.org/10.1093/infdis/jiw140 (2016).
38. Nelson, T. M. *et al*. Cigarette smoking is associated with an altered vaginal tract metabolomic profile. *Sci Rep* **8**, 852, https://doi.org/10.1038/s41598-017-14943-3 (2018).

39. Giuliano, A. R. *et al*. Clearance of oncogenic human papillomavirus (HPV) infection: effect of smoking (United States). *Cancer Causes Control* **13**, 839–846, https://doi.org/10.1023/a:1020668232219 (2002).
40. Machado, A. & Cerca, N. Influence of biofilm formation by Gardnerella vaginalis and other anaerobes on bacterial vaginosis. *J Infect Dis* **212**, 1856–1861, https://doi.org/10.1093/infdis/jiv338 (2015).
41. Swidsinski, A. *et al*. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* **106**, 1013–1023, https://doi.org/10.1097/01.AOG.0000183594.45524.d2 (2005).
42. Tuddenham, S. *et al*. Associations between dietary micronutrient intake and molecular-Bacterial Vaginosis. *Reprod Health* **16**, 151, https://doi.org/10.1186/s12978-019-0814-6 (2019).
43. Joesoef, M. R. *et al*. High rate of bacterial vaginosis among women with intrauterine devices in Manado, Indonesia. *Contraception* **64**, 169–172, https://doi.org/10.1016/s0010-7824(01)00246-3 (2001).
44. Brotman, R. M. *et al*. The effect of vaginal douching cessation on bacterial vaginosis: a pilot study. *Am J Obstet Gynecol* **198**, 628 e621–627, https://doi.org/10.1016/j.ajog.2007.11.043 (2008).
45. van der Veer, C. *et al*. Effects of an over-the-counter lactic-acid containing intra-vaginal douching product on the vaginal microbiota. *BMC Microbiol* **19**, 168, https://doi.org/10.1186/s12866-019-1545-0 (2019).
46. Srinivasan, S. *et al*. More Easily Cultivated Than Identified: Classical Isolation With Molecular Identification of Vaginal Bacteria. *J Infect Dis* **214**(Suppl 1), S21–28, https://doi.org/10.1093/infdis/jiw192 (2016).
47. Falsen, E., Pascual, C., Sjoden, B., Ohlen, M. & Collins, M. D. Phenotypic and phylogenetic characterization of a novel Lactobacillus species from human sources: description of Lactobacillus iners sp. nov. *Int J Syst Bacteriol* **49**(Pt 1), 217–221, https://doi.org/10.1099/00207713-49-1-217 (1999).
48. Brooks, J. P. *et al*. Effects of combined oral contraceptives, depot medroxyprogesterone acetate and the levonorgestrel-releasing intrauterine system on the vaginal microbiome. *Contraception* **95**, 405–413, https://doi.org/10.1016/j.contraception.2016.11.006 (2017).
49. Plummer, E. L. *et al*. Combined oral and topical antimicrobial therapy for male partners of women with bacterial vaginosis: Acceptability, tolerability and impact on the genital microbiota of couples - A pilot study. *PLoS One* **13**, e0190199, https://doi.org/10.1371/journal.pone.0190199 (2018).
50. Chaban, B. *et al*. Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. *Microbiome* **2**, 23, https://doi.org/10.1186/2049-2618-2-23 (2014).
51. Fadrosh, D. W. *et al*. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* **2**, 6, https://doi.org/10.1186/2049-2618-2-6 (2014).
52. Shipitsyna, E. *et al*. Composition of the vaginal microbiota in women of reproductive age - sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS One* **8**, e60670 (2013).
53. Sinclair, L., Osman, O. A., Bertilsson, S. & Eiler, A. Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform. *PLoS One* **10**, e0116955, https://doi.org/10.1371/journal.pone.0116955 (2015).
54. Caporaso, J. G. *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336, https://doi.org/10.1038/nmeth.f.303 (2010).
55. Schmieder, R., Lim, Y. W., Rohwer, F. & Edwards, R. TagCleaner: Identification and removal of tag sequences from genomic and metagenomic datasets. *BMC Bioinformatics* **11**, 341, https://doi.org/10.1186/1471-2105-11-341 (2010).
56. Callahan, B. J. *et al*. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**, 581–583, https://doi.org/10.1038/nmeth.3869 (2016).
57. Quast, C. *et al*. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**, D590–596, https://doi.org/10.1093/nar/gks1219 (2013).
58. Oakley, B. B., Fiedler, T. L., Marrazzo, J. M. & Fredricks, D. N. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Appl Environ Microbiol* **74**, 4898–4909, https://doi.org/10.1128/AEM.02884-07 (2008).
59. Van Der Pol, W. J. *et al*. In Silico and Experimental Evaluation of Primer Sets for Species-Level Resolution of the Vaginal Microbiota Using 16S Ribosomal RNA Gene Sequencing. *J Infect Dis* **219**, 305–314, https://doi.org/10.1093/infdis/jiy508 (2019).
60. Sievers, F. *et al*. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**, 539, https://doi.org/10.1038/msb.2011.75 (2011).
61. Chojnacki, S., Cowley, A., Lee, J., Foix, A. & Lopez, R. Programmatic access to bioinformatics tools from EMBL-EBI update: 2017. *Nucleic Acids Res* **45**, W550–W553, https://doi.org/10.1093/nar/gkx273 (2017).
62. Jervis-Bardy, J. *et al*. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome* **3**, 19, https://doi.org/10.1186/s40168-015-0083-8 (2015).
63. Salter, S. J. *et al*. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol* **12**, 87, https://doi.org/10.1186/s12915-014-0087-z (2014).
64. vegan: Community Ecology Package v. R package version 2.4-1 (2016).
65. Gu, Z., Eils, R. & Schlesner, M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**, 2847–2849, https://doi.org/10.1093/bioinformatics/btw313 (2016).

Acknowledgements

This work was supported by The Australian National Health and Medical Research Council Project Grant (1020457) awarded to CSB and the Australian National Health and Medical Research Council Program (1071269) awarded to S.M.G., C.K.F. and M.G.L. E.L.P. was supported by an Australian Government Research Training Program (RTP) Scholarship. The authors thank Glenda Fehler, Susan Peterson, Dr. Marcus Chen, Dr. Sandra Walker, Dr. Jade Bilardi, and Clare Bellhouse for their contributions to the original WOW Cohort study, from which this microbiota sub-study arose. The authors thank Dr. Jimmy Twin for contributions to the laboratory work.

Author contributions

C.S.B., L.A.V., S.N.T., C.K.F. and J.S.H. conceived and designed the study. C.S.B., C.K.F., L.A.V., M.G.L., K.A.F. and J.S.H. contributed to the original cohort study from which this microbiota analysis arose. SNT oversaw the laboratory work. E.L.P. and L.A.V. analysed the data, with statistical support from M.G.L., J.S.H. and C.S.B. L.A.V., S.M.G., C.K.F., D.M.B., S.N.T., G.L.M. and C.S.B. provided additional supervision and oversight. C.S.B., S.N.T., J.S.H., C.K.F., S.M.G. and M.G.L. acquired funding. E.L.P., L.A.V., G.L.M. and C.S.B. wrote the original draft. All authors critically revised the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-55929-7.

Correspondence and requests for materials should be addressed to E.L.P.

The Journal of Infectious Diseases

MAJOR ARTICLE

Gardnerella vaginalis Clade Distribution Is Associated With Behavioral Practices and Nugent Score in Women Who Have Sex With Women

Erica L. Plummer,[1,2] Lenka A. Vodstrcil,[1,2] Gerald L. Murray,[3,4,5] Christopher K. Fairley,[1,2] Jennifer A. Danielewski,[3,4] Suzanne M. Garland,[3,4,5] Eric P. F. Chow,[1,2] Dieter M. Bulach,[6,7] Katherine A. Fethers,[2] Jane S. Hocking,[8] and Catriona S. Bradshaw[1,2]

[1]Central Clinical School, Monash University, The Alfred Centre, Melbourne, Victoria, Australia, [2]Melbourne Sexual Health Centre, Alfred Health, Carlton, Victoria, Australia, [3]Women's Centre for Infectious Diseases, The Royal Women's Hospital, Parkville, Victoria, Australia, [4]Murdoch Children's Research Institute, Parkville, Victoria, Australia, [5]Department of Obstetrics and Gynaecology, The University of Melbourne, Parkville, Victoria, Australia, [6]Microbiological Diagnostic Unit Public Health Laboratory, The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, Victoria, Australia, [7]Melbourne Bioinformatics, The University of Melbourne, Carlton, Victoria, Australia, [8]Melbourne School of Population and Global Health, The University of Melbourne, Carlton, Victoria, Australia

Background. *Gardnerella vaginalis* is detected in women with and without bacterial vaginosis (BV). Identification of 4 *G. vaginalis* clades raised the possibility that pathogenic and commensal clades exist. We investigated the association of behavioral practices and Nugent Score with *G. vaginalis* clade distribution in women who have sex with women (WSW).

Methods. Longitudinal self-collected vaginal specimens were analyzed using established *G. vaginalis* species-specific and clade-typing polymerase chain reaction assays. Logistic regression assessed factors associated with detection of *G. vaginalis* clades, and multinomial regression assessed factors associated with number of clades.

Results. Clades 1, 2, and 3 and multiclade communities (<2 clades) were associated with Nugent-BV. Clade 1 (odds ratio [OR], 3.36; 95% confidence interval [CI], 1.65–6.84) and multiclade communities (relative risk ratio [RRR], 9.51; 95% CI, 4.36–20.73) were also associated with *Lactobacillus*-deficient vaginal microbiota. Clade 4 was neither associated with Nugent-BV nor *Lactobacillus*-deficient microbiota (OR, 1.49; 95% CI, 0.67–3.33). Specific clades were associated with differing behavioral practices. Clade 1 was associated with increasing number of recent sexual partners and smoking, whereas clade 2 was associated with penile-vaginal sex and sharing of sex toys with female partners.

Conclusions. Our results suggest that *G. vaginalis* clades have varying levels of pathogenicity in WSW, with acquisition occurring through sexual activity. These findings suggest that partner treatment may be an appropriate strategy to improve BV cure.

Keywords. bacterial vaginosis; *Gardnerella vaginalis*; sexual practices; women who have sex with women.

Bacterial vaginosis (BV) is the most common vaginal condition of reproductive-aged women [1]. Current BV treatments are suboptimal with over 50% of women experiencing recurrence within 6–12 months after treatment [2, 3]. Improving BV treatment has been impeded by its complex aetiology and pathogenesis. *Gardnerella vaginalis* is thought to play a key role in BV pathogenesis, potentially as a founder organism [4–6]. *Gardnerella vaginalis* is almost always present in the vagina of women who have BV [7], and it possesses characteristics important for pathogenesis, including production of sialidase, an enzyme that degrades cervicovaginal mucus [8], and vaginolysin, a cytolysin that induces vaginal epithelial cell lysis [9]. Both factors may assist *G. vaginalis* adherence to host epithelial cells and form biofilms [10]. *Gardnerella vaginalis* is also detected in the vagina of women without BV, albeit at lower prevalence and abundance [11–15]. Substantial genetic diversity exists within *G. vaginalis* [16–18], and different genetic types/clades may have different virulence potential [18–22]. These findings, taken together, suggest that commensal and pathogenic *G. vaginalis* variants exist. Different methods have been used to define *G. vaginalis* clades including *cpn60* gene analysis [17], whole-genome sequence analysis [16], detection of clade-specific genes [23], and ecotyping [18]. Comparison of methods has been discussed previously [18, 20].

Previous studies have explored the association of specific *G. vaginalis* clades and number of *G. vaginalis* clades with BV, and although results regarding specific clades have been mixed, detection of multiple *G. vaginalis* clades is consistently associated with BV [12, 21, 23–25]. Bacterial vaginosis prevalence is high amongst women who have sex with women (WSW), with estimates ranging from 25% to 52% [26–29]. Despite this, there are no published data about *G. vaginalis* clade distribution in WSW. In addition, there is limited information concerning associations between *G. vaginalis* clades and BV risk factors.

Received 8 July 2019; editorial decision 9 September 2019; accepted 11 September 2019; published online September 23, 2019.

Correspondence: Erica L. Plummer, BSc, MSc, Melbourne Sexual Health Centre, 580 Swanston Street, Carlton, VIC, 3053, Australia (erica.plummer@monash.edu).

The Journal of Infectious Diseases® 2020;221:454–63

DOI: 10.1093/infdis/jiz474

Defining the role of *G. vaginalis* clades in BV pathogenesis and understanding how they are acquired may lead to the development of more effective treatment strategies. Using a multiplex real-time polymerase chain reaction (PCR) *G. vaginalis* clade-typing assay [23], we aimed to investigate the association of *G. vaginalis* clade distribution with sexual and behavioral practices and Nugent Score in a cohort of Australian WSW.

METHODS

Participants and Specimens

Participants and specimens were selected from the Women On Women's (WOW) Health cohort, which examined factors associated with incident BV [27, 30]. In brief, nonpregnant premenopausal women reporting a female sexual partner (FSP) in the 18 months before enrollment and who had a Nugent Score (NS) <7 [31] on 3 consecutive vaginal smears were eligible for enrollment [27, 30]. Participants completed a questionnaire and self-collected a vaginal swab and smear at 3-monthly intervals until the study endpoint (incident Nugent-BV [NS = 7–10] or 24 months without Nugent-BV [NS = 0–6]). Women were asked to collect specimens avoiding the time during menses. Smears were scored using the NS method [31]: a score of 0–3 was defined as non-BV, a score of 4–6 was defined as intermediate-BV, and a score of 7–10 was defined as Nugent-BV [32].

Within WOW, we conducted a nested cohort study that included women who developed incident Nugent-BV and women who reached the study endpoint without Nugent-BV [30] (selected using a random sort command in Stata/IC [version 14.2; StataCorp LP, College Station, TX]). Because there were 7 women who developed incident Nugent-BV who coenrolled in WOW with their ongoing FSP [30], controls were frequency matched on coenrollment and age, to ensure a similar distribution of both variables. A baseline specimen and 1–4 longitudinal specimens (typically the last 3 specimens collected over the 24-month study period) were included from 101 women. As a result, 101 women contributed a total of 372 specimens to analyses, comprising 101 enrollment samples and 271 longitudinal samples, 48 of which were collected at incident Nugent-BV. Ethical approval was obtained from the Human Research Ethics Committees of Alfred Hospital, Melbourne (251/09) and the University of Melbourne (0932804). Participants provided informed written consent.

Laboratory Methods

Swabs were agitated in 1 mL RNAlater (Thermo Fisher Scientific, Waltham, MA) and stored at −80°C until analyzed. Deoxyribonucleic acid (DNA) was extracted as outlined in Fethers et al [13], using the MagNA Pure 96 instrument and the DNA and Viral NA small volume kit (Roche Diagnostics, Mannheim, Germany). *Gardnerella vaginalis* was detected using a species-specific real-time PCR assay previously described [13]. Presence of the 4 *G. vaginalis* clades (hereafter referred to as clades 1, 2, 3, and 4) was determined using a validated multiplex real-time PCR assay targeting sequences unique to each clade [23] as previously described [12]; the limit of detection for this assay is 10 copies per reaction [23]. Specificity and sensitivity of the primer set was assessed by BLAST [33] analysis; additional details are provided in Supplementary Methods.

Identification of *G. vaginalis* clades using clade-specific PCR provides biological information on top of what can be inferred from microbiota studies utilizing 16S ribosomal ribonucleic acid (rRNA) gene sequencing. Vaginal microbiota composition was previously determined for 360 specimens using 16S rRNA gene sequencing of the V3-V4 hypervariable regions [34]. Vaginal microbiota composition was categorized into 3 groups based on McKinnon et al [32]: optimal non-*Lactobacillus iners* microbiota (dominated by non-*L. iners* spp.), *L. iners* microbiota (*L. iners* was the most abundant taxon), and nonoptimal microbiota (deficient in *Lactobacillus* spp.); additional details are provided in Supplementary Methods.

Statistical Analysis

Univariable and multivariable logistic regression models fitted with generalized estimating equations were used to explore associations between covariates (including history of BV, self-reported symptoms [vaginal discharge and/or odor], behavioral and sexual practices, NS, and vaginal microbiota composition) and detection of *G. vaginalis* (present vs absent). Logistic regression models explored associations between covariates and detection of each *G. vaginalis* clade versus detection of any other clade/s. Univariable and multivariable multinomial logistic regression examined associations between covariates and number of *G. vaginalis* clades detected. This analysis determined the relative risk of having 1 clade or multiple clades in a specimen compared with having no *G. vaginalis* (ie, *G. vaginalis* was not detected), generating relative risk ratios (RRRs) and 95% confidence intervals (CIs). Sexual and behavioral practices deemed significant in univariable analyses ($P < .05$) were included in multivariable analyses. Self-reported symptoms and behavioral and sexual practices were recorded for the 3-month interval preceding each specimen collection. Self-reported symptoms, NS, and vaginal microbiota composition were not included in multivariable analyses because they are correlated with *G. vaginalis* presence. Regression models accounted for repeated measures from individuals. We assumed an exchangeable correlation structure and used a cluster-based variance estimate for standard error. Statistical analyses were performed using Stata/IC (version 14.2; StataCorp LP).

RESULTS

Specificity and Sensitivity of Clade-Specific Primers

No *G. vaginalis* isolate had more than 1 clade-specific amplicon detected, and detection of clade-specific amplicons corresponded with clade phylogeny (Supplementary Figure

1), demonstrating high specificity of the primers. Isolates JCP8481A, JCP8481B, and PSS_7772B did not have a clade-specific amplicon detected and appeared genetically unrelated to the 4 clades. These isolates may represent an additional clade, as previously suggested [35]. B482 and GED7275B clustered with clade 2 isolates, but the clade 2 amplicon was not detected in these isolates. Thus, the primers have reduced sensitivity for clade 2.

Participant Demographics

One hundred one women, contributing 372 specimens, were included in analyses. Participant median age was 28 (interquartile range, 23–36 years) and most women were Australian born (n = 87, 86%). At enrollment, 72 (71%) women had an FSP (median duration of relationship, 2 years; interquartile range, 0.3–4 years), 44 (44%) reported smoking, and 22 (22%) had a past history of BV.

Factors Associated With *Gardnerella vaginalis* Detection

Seventy-seven women (76%; 95% CI, 67%–84%) had at least 1 specimen in which *G. vaginalis* was detected. *Gardnerella vaginalis* was detected in 184 of 372 specimens (49%; 95% CI, 44%–55%); 130 of 306 specimens with NS = 0–3 (42%; 95% CI, 67%–48%), 15 of 18 specimens with NS = 4–6 (83%; 95% CI, 59%–96%), and 39 of 48 specimens with Nugent-BV (81%; 95% CI, 67%–91%). In univariable analyses, *G. vaginalis* detection was associated with self-reported symptoms; NS = 4–6 and Nugent-BV (Table 1). *Gardnerella vaginalis* was also associated with having a nonoptimal (*Lactobacillus* deficient) microbiota (odds ratio [OR], 4.35; 95% CI, 2.47–7.66; $P < .001$).

Gardnerella vaginalis detection was associated with smoking, history of penile-vaginal sex, and frequent sex (several times per week) in univariable analyses (Table 1). Multivariable analysis of behavioral practices found that *G. vaginalis* detection was associated with smoking in the previous 3 months (adjusted OR [aOR], 1.76; 95% CI, 1.06–2.93; $P = .028$) (Table 1) and lifetime history of penile-vaginal sex (aOR, 2.03; 95% CI, 1.01–4.10; $P = .047$). There was a borderline association between *G. vaginalis* and frequent sex (aOR, 1.61; 95% CI, 0.99–2.61; $P = .052$).

Factors Associated With Number of *Gardnerell vaginalis* Clades

Three *G. vaginalis*-positive specimens did not belong to any clade detectable by clade-specific PCR. These specimens were excluded and, consequently, 369 specimens from 101 women contributed to the following analyses.

Clade 4 was the most prevalent clade (n = 136 of 369; 37%; 95% CI, 32%–42%), followed by clade 1 (n = 116 of 369; 31%; 95% CI, 27%–36%), clade 2 (n = 76 of 369; 21%; 95% CI, 17%–25%), and clade 3 (n = 17 of 369; 5%; 95% CI, 3%–7%). Clade 4 was detected in 62 women (n = 62 of 101; 61%), clade 1 was detected in 55 women (n = 55 of 101; 54%), clade 2 was detected in 44 women (n = 44 of 101; 44%), and clade 3 was detected in 14 women (n = 14 of 101; 14%). Of the 181 specimens positive for a *G. vaginalis* clade, 63 had 1 clade detected (35%; 95% CI, 28%–42%) and 118 had multiple (ie, 2 or more) clades (65%; 95% CI, 58%–72%). Five specimens contained all 4 clades. Clade 3 was only detected in multiclade communities (Figure 1).

In univariable analyses, women reporting symptoms were more likely than asymptomatic women to have multiple *G. vaginalis* clades relative to no *G. vaginalis* (RRR, 4.19; 95% CI, 1.85–9.49; $P = .001$) (Supplementary Table 1). Women with intermediate-BV (RRR, 8.72; 95% CI, 2.32–32.76; $P = .001$) or Nugent-BV (RRR, 8.72; 95% CI, 4.05–18.78; $P < .001$) were more likely than women with NS = 0–3 to have multiple clades relative to no *G. vaginalis*. Having a single clade was not associated with NS. Women with a nonoptimal microbiota were more likely than women with an optimal microbiota to have a single clade (RRR, 3.03; 95% CI, 1.20–7.61; $P = .019$) or multiple clades (RRR, 9.51; 95% CI, 4.36–20.73; $P < .001$) relative to no *G. vaginalis* (Supplementary Table 1).

In univariable analyses, having multiple *G. vaginalis* clades was associated with smoking in the previous 3 months and sexual practices including increased number of lifetime FSPs, history of penile-vaginal sex, increased frequency of sex, current sexual practices with a male, and sex with a new partner (predominantly representing new FSPs) (Supplementary Table 1). No significant associations were observed between practices and single clade communities. Smoking, lifetime number of FSPs, frequency of sex, and sex with a new partner were included in multivariable analyses (Table 2); sexual practices with a male partner were rare and were omitted to prevent overfitting the model due to their correlation with sex with a new partner. Women reporting smoking in the previous 3 months were more likely than nonsmokers to have multiple *G. vaginalis* clades relative to no *G. vaginalis* (adjusted RRR, 2.38; 95% CI, 1.19–4.74; $P = .014$). No other variable was significant in adjusted analyses.

Factors Associated With Detection of Each *Gardnerell vaginalis* Clade

Seventy-two women changed clade at least once over the study period, accounting for 120 instances of change. Acquisition of new clade/s was the most frequent change observed (n = 71 of 120, 59%), followed by loss of clade/s (n = 38 of 120, 32%), and a combination of loss and acquisition of clade/s (n = 11 of 120, 9%). Five women had stable clade distribution over time.

Four univariable analyses were conducted to assess factors associated with detection of each specific clade versus detection of any other clade/s (Supplementary Table 2). Because multiclade specimens were common and we wanted to examine factors associated with detection of each individual clade rather than detection of *G. vaginalis*, we excluded specimens in which no *G. vaginalis* clade was detected. Seventy-seven women contributed 181 specimens to each of the 4 analyses.

Clade 1 detection was associated with Nugent-BV (OR, 3.55; 95% CI, 1.76–7.18; $P < .001$) and nonoptimal vaginal microbiota

Table 1. Factors Associated With Detection of *Gardnerella vaginalis*

Risk Factor	*G. vaginalis* Absent n (%) (N = 188)	*G. vaginalis* Present n (%) (N = 184)	OR (95% CI)	*P* Value[a]	aOR (95% CI)	*P* Value[b]
Age[c]						
<28	92 (49)	84 (46)	1			
≥28	96 (51)	100 (54)	1.17 (.67–2.05)	.577		
Self-Reported History of BV						
No	154 (82)	142 (77)	1			
Yes	34 (18)	42 (23)	1.43 (.68–2.99)	.346		
Baseline Sexual Practices						
No. of lifetime FSPs[c]						
<5	96 (51)	70 (38)	1			
≥5	92 (49)	114 (62)	1.69 (.93–3.09)	.088		
No. of FSPs in Previous 12 Months[c]						
≤1	129 (69)	101 (55)	1			
>1	59 (31)	83 (45)	1.80 (.97–3.34)	.062		
Lifetime History of Penile-Vaginal Sex With a Man						
No	66 (35)	36 (20)	1		1	
Yes	122 (65)	148 (80)	2.27 (1.14–4.54)	**.020**	2.03 (1.01–4.10)	**.047**
Interval Characteristics in Prior 3 Months[d]						
Any Smoking						
No	128 (68)	94 (51)	1		1	
Yes	59 (32)	90 (49)	1.88 (1.15–3.09)	**.012**	1.76 (1.06–2.93)	**.028**
Any Douching						
No	183 (98)	179 (97)	1			
Yes	4 (2)	5 (3)	0.58 (.15–2.30)	.441		
Any Hormonal Contraceptive Use						
No	157 (84)	166 (90)	1			
Yes	31 (16)	18 (10)	0.62 (.30–1.27)	.191		
Last Menstrual Period						
≤7 days	31 (17)	23 (13)	1			
>7 days	149 (83)	155 (87)	1.05 (.62–1.78)	.858		
Number of SP						
0	25 (13)	18 (10)	1			
1	147 (79)	129 (70)	1.01 (.52–1.95)	.979		
≥2	15 (8)	37 (20)	2.11 (.92–4.85)	.079		
Frequency of Sex						
Several times/month or less	143 (76)	115 (63)	1		1	
Several times/week	45 (24)	69 (37)	1.70 (1.07–2.71)	**.025**	1.61 (.99–2.61)	.052
Sex With New Partner[e]						
No	165 (88)	147 (80)	1			
Yes	23 (12)	37 (20)	1.39 (.84–2.30)	.195		
Sexual Practices With FSP in Prior 3 Months[f]						
Number of FSPs[c]						
0	37 (20)	32 (17)	1			
≥1	151 (80)	152 (83)	1.16 (.68–1.99)	.581		
Receptive Oral Vaginal Sex						
No[g]	77 (41)	69 (38)	1			
Yes	110 (59)	115 (63)	1.03 (.68–1.56)	.874		
Sharing Sex Toys						
No toys/washed/ condoms used[g]	142 (76)	141 (77)	1			
Unwashed	45 (24)	43 (23)	0.87 (.53–1.42)	.575		
Current Partner With BV Symptoms						
No/don't know[g]	187 (100)	177 (97)				

Table 1. Continued

Risk Factor	*G. vaginalis* Absent n (%) (N = 188)	*G. vaginalis* Present n (%) (N = 184)	OR (95% CI)	*P* Value[a]	aOR (95% CI)	*P* Value[b]
Yes	0	7 (4)	…			
Sexual Practices With an MSP in Prior 3 Months[h]						
Number of MSPs[c]						
0	173 (92)	156 (85)	1			
≥1	15 (8)	28 (15)	1.94 (.89–4.23)	.097		
Any Penile-Vaginal Sex						
No[i]	173 (93)	158 (86)	1			
Yes	14 (7)	26 (14)	1.86 (.81–4.24)	.143		
Self-Reported Symptoms And Microbiota Measures						
Abnormal Vaginal Discharge and/or Odour						
No	175 (93)	152 (83)	1			
Yes	13 (7)	32 (17)	2.09 (1.13–3.87)	**.019**		
Nugent Score						
0–3	176 (93)	130 (71)	1			
4–6	3 (2)	15 (8)	3.96 (1.44–10.93)	**.008**		
7–10	9 (5)	39 (21)	3.48 (1.95–6.22)	**<.001**		
Vaginal Microbiota Type[j]						
Optimal non-*Lactobacillus iners* microbiota	118 (66)	79 (44)	1			
L. iners microbiota	49 (27)	45 (25)	1.10 (.69–1.75)	.692		
Nonoptimal microbiota	13 (7)	56 (31)	4.35 (2.47–7.66)	**<.001**		

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; BV, bacterial vaginosis; FSP, female sexual partner; OR, odds ratio; MSP, male sexual partner; SP, sexual partner (refers to total number of sexual partners in a study interval, female and male).

NOTE: Includes 372 specimens from 101 participants.

[a]Univariate logistic regression fitted with generalized estimating equations (GEE) clustered for multiple specimens from each participant.

[b]Multivariable logistic regression fitted with GEE, clustered for multiple specimens from each participant.

[c]Variables were dichotomized at median value.

[d]Interval characteristics were measured as any exposure over the prior follow-up interval (~90 days).

[e]Sex with a new partner with whom first sexual contact was within 90 days. May represent a new FSP or new MSP.

[f]The following characteristics/sexual practices with an FSP were left out of the table for simplicity: digital vaginal sex, receptive oral anal sex, digital anal sex. No significant associations between *G. vaginalis* and these sexual practices were identified.

[g]Or did not have an FSP.

[h]The following sexual practices with an MSP were left out of the table for simplicity: receptive oral sex, digital vaginal sex, and penile-anal sex. No significant associations between *G. vaginalis* and these sexual practices were identified.

[i]Or did not have an FSP.

[j]Vaginal microbiota type available for 360 specimens from 100 women. Optimal non-*L. iners* microbiota includes specimens predominately consisting of non-*L. iners* spp., *L. iners* microbiota includes specimens predominately consisting of *L. iners*, and nonoptimal microbiota includes specimens predominately consisting of non-*Lactobacillus* spp.

P-values < .05 are bolded to indicate statistically significant associations.

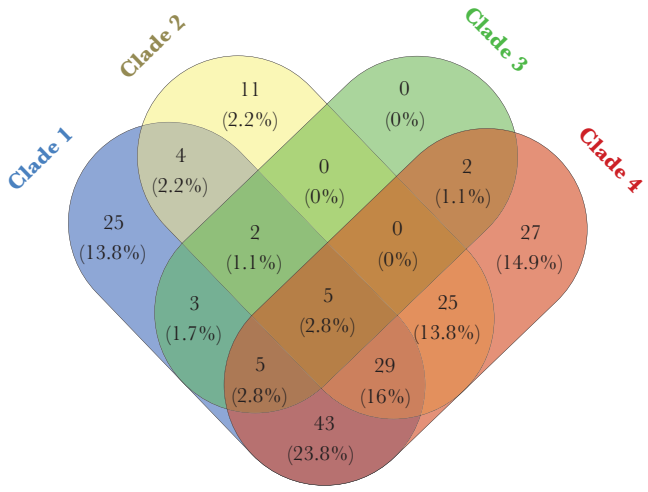


Figure 1. Distribution of *Gardnerella vaginalis* clades. Venn diagram showing the distribution and co-occurrence of *G. vaginalis* clades in vaginal specimens. Total number of specimens: 181 from 77 women.

(OR, 3.36; 95% CI, 1.65–6.84; *P* = .001). Clades 2 and 3 were both associated with intermediate-BV (clade 2 OR = 3.49, 95% CI = 1.17–10.36, *P* = .025; and clade 3 OR = 4.90, 95% CI = 1.11–21.57, *P* = .035) and Nugent-BV (clade 2 OR = 1.90, 95% CI = 1.02–3.55, *P* = .043; and clade 3 OR = 3.67, 95% CI = 1.22–11.04, *P* = .020), but they were not associated with vaginal microbiota composition. Clade 4 was not associated with NS or vaginal microbiota composition (Supplementary Table 2).

For each clade, behavioral practices significantly associated by univariable analysis were included in a clade-specific multivariable analysis. Detection of clades 1, 2, and 3 were associated with interval practices (ie, those performed in the 3 months before specimen collection). Having clade 1 versus any other clade/s was associated with smoking (aOR, 2.42; 95% CI, 1.12–5.25; *P* = .025) (Table 3) and ≥1 sexual partner of any gender (aOR, 4.03; 95% CI, 1.16–14.01; *P* = .028), after adjusting

Table 2. Multinomial Adjusted Logistic Regression Investigating Behavioral Practices Associated With Number of *Gardnerella Vaginalis* Clades Detected

	Single Clade (n = 63) vs *G. vaginalis* Not Detected		Multiple Clades (n = 118) vs *G. vaginalis* Not Detected	
Risk Factor	aRRR (95% CI)	*P* Value[a]	aRRR (95% CI)	*P* Value[a]
Baseline Characteristics				
No. of Lifetime FSPs[b]				
<5	1		1	
≥5	0.94 (.46–1.91)	.859	2.04 (.94–4.43)	.072
Interval Practices in Prior 3 Months[c]				
Smoking				
No	1		1	
Yes	1.15 (.59–2.25)	.674	2.38 (1.19–4.74)	**.014**
Sex With a New Partner[d]				
No	1		1	
Yes	0.86 (.31–2.40)	.768	1.79 (.91–3.52)	.091
Frequency of Sex				
Several times/month or less	1		1	
Sex several times per week	1.46 (.68–3.14)	.331	1.77 (.93–3.37)	.082

Abbreviations: aRRR, adjusted relative risk ratio; CI, confidence interval; FSP, female sexual partner; MSP, male sexual partner.

NOTE: Variables included in the adjusted analysis were lifetime number of FSPs, smoking in prior 3 months, sex with a new partner in prior 3 months, and frequency of sex.

[a]Multinomial logistic regression with no *G. vaginalis* (ie, *G. vaginalis* not detected) as the referent group. Analysis clustered for multiple specimens from participants (101 clusters).

[b]Variable was dichotomized at median value.

[c]Interval practices were measured as any exposure over the prior study interval (~90 days).

[d]Sex with a new partner with whom first sexual contact was within 90 days. May represent a new FSP or new MSP.

P-values < .05 are bolded to indicate statistically significant associations.

for sex frequency. Having clade 2 versus any other clade/s was associated with sharing of unwashed sex toys with an FSP (aOR, 2.59; 95% CI, 1.22–5.51; $P = .013$) and recent penile-vaginal sex (aOR, 5.67; 95% CI, 1.74–18.51; $P = .004$). Having clade 3 versus any other clade/s was associated with sex with a new partner (aOR, 5.02; 95% CI, 1.25–20.18; $P = .023$) and having a current FSP with BV symptoms (aOR, 24.73; 95% CI, 3.37–181.27; $P = .002$). Having clade 4 versus any other clade/s was associated with having ≥5 lifetime FSPs (aOR, 3.35; 95% CI, 1.45–7.75; $P = .005$).

DISCUSSION

We explored the distribution of *G. vaginalis* clades in WSW and found that clade 1 was the only clade associated with both Nugent-BV and a nonoptimal (*Lactobacillus* deficient) vaginal microbiota. Also of note, clade 4 was the most prevalent clade but was not associated with these 2 vaginal states. Factors associated with detection of *G. vaginalis* included smoking, frequent sex, and a history of penile-vaginal sex, and clades were associated with a range of differing sexual practices and behaviors in adjusted analysis. Clades 1, 2, and 3 were associated with recent behavioral and sexual practices, and clade 4 was associated with sexual practices before enrollment. These findings support the sexual exchange of *G. vaginalis*, and they suggest that different *G. vaginalis* clades may have varying levels of pathogenicity, differ in mode of acquisition and duration of infection, and may circulate in different populations or sexual networks.

Previous studies investigating *G. vaginalis* clade distribution that use the same clade typing applied in our study have inconsistently associated individual clades with BV [12, 21, 23–25]. However, most studies agree that clade 1 is associated with BV [21, 23, 25]. Our finding that clade 1 was associated with both Nugent-BV and nonoptimal vaginal microbiota suggests that it may have increased pathogenicity compared with other clades. A recent comparative genomic and ecotyping analysis of 35 *G. vaginalis* strains highlighted key differences between ecotype 1 (corresponds to clade 1) and other *G. vaginalis* ecotypes that may contribute to its pathogenicity [18]. Ecotype 1 uniquely encodes glycosidases that may aid cervicovaginal mucus degradation, and it has enriched galactose and pentose sugar metabolism pathways, which may provide ecotype 1 with an advantage when cocolonizing the vagina with lactic acid-producing bacteria [18].

Our finding that clade 4 was not associated with Nugent-BV or nonoptimal vaginal microbiota is supported by 2 studies reporting no association of clade 4 with BV [23, 25]. Clade 4 strains lack sialidase activity [19, 20], which is associated with mucin degradation in BV, further supporting reduced pathogenicity of clade 4. However, Vodstrcil et al [12] found that clade 4 was associated with Nugent-BV in a cohort of 52 young, sexually inexperienced women, and 2 additional studies noted an association between Nugent-BV and increasing prevalence and/or load of clade 4 [21, 24]. Inconsistencies between studies may be due to population differences including sexual practices and networks,

Table 3. Practices Associated With Detection of Specific *Gardnerella vaginalis* Clades by Logistic Regression[a]

G. vaginalis Clade 1[b]	OR (95% CI)	*P* Value	aOR (95% CI)	*P* Value
Any Smoking in Prior 3 Months				
No	1		1	
Yes	2.29 (1.40–6.10)	**.004**	2.42 (1.12–5.25)	**.025**
Number of SP in Prior 3 Months				
0	1		1	
1	5.09 (1.55–16.66)	**.007**	4.03 (1.16–14.01)	**.028**
≥2	8.19 (2.05–32.75)	**.003**	5.25 (1.22–22.50)	**.026**
Frequency of Sex With Any SP				
Several times/month or less	1		1	
Several times/week	2.03 (1.07–3.88)	**.031**	1.60 (.79–3.25)	.190
G. vaginalis Clade 2[c]	OR (95% CI)	*P* Value	aOR (95% CI)	*P* Value
Any Sharing of Sex Toys in Prior 3 Months				
No toys/washed/condoms used	1		1	
Unwashed	2.21 (1.08–4.52)	**.030**	2.59 (1.22–5.51)	**.013**
Any Penile-Vaginal Sex in Prior 3 Months				
No	1		1	
Yes	4.80 (1.52–15.17)	**.007**	5.67 (1.74–18.51)	**.004**
G. vaginalis Clade 3[d]	OR (95% CI)	*P* Value	aOR (95% CI)	*P* Value
Sex With a New Partner in Prior 3 Months[e]				
No	1		1	
Yes	3.09 (1.10–8.69)	**.032**	2.90 (.95–8.92)	.063
Current Partner With BV Symptoms				
No/don't know	1		1	
Yes	17.40 (3.36–90.11)	**.001**	16.95 (3.11–92.23)	**.001**
G. vaginalis Clade 4[f]	OR (95% CI)	*P* Value	aOR (95% CI)	*P* Value
No. of Lifetime FSPs				
<5	1		1	
≥5	3.93 (1.74–8.87)	**.001**	3.35 (1.45–7.75)	**.005**
Lifetime History of Vaginal Sex With a Man[g]				
No	1		1	
Yes	2.88 (1.13–7.32)	**.026**	2.09 (.82–5.33)	.125

Abbreviations: aOR, adjusted odds ratio; BV, bacterial vaginosis; CI, confidence interval; FSP, female sexual partner; MSP, male sexual partner; OR, odds ratio; SP, sexual partner (may refer to female or male partner).

[a]Includes specimens positive for 1 or more *G. vaginalis* clade (181 specimens from 77 women).

[b]Logistic regression fitted with generalized estimating equations (GEE) with absence of clade 1 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters).

[c]Logistic regression fitted with GEE with absence of clade 2 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters).

[d]Logistic regression fitted with GEE with absence of clade 3 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters).

[e]Sex with a new partner with whom first sexual contact was within 90 days. May represent a new FSP or new MSP.

[f]Logistic regression fitted with GEE with absence of clade 4 as the referent category. Analysis clustered for multiple specimens from participants (77 clusters).

[g]Variable was dichotomized at median value.

P-values < .05 are bolded to indicate statistically significant associations.

behavioral practices such as smoking, past history of BV, and ethnicity. Previous associations of clade 4 and BV may also be a result of high load infections [21, 24]. Another consideration is that differences between studies are a result of spurious findings due to unmeasured confounding. *Gardnerella vaginalis* clades do not often occur in isolation, and associations between specific clades and BV may be due to high abundance and/or presence of other BV-associated bacteria or due to depleted levels of optimal *Lactobacillus* spp. in the vagina. Given the polymicrobial nature of BV, future studies should consider interactions between *G. vaginalis* clades and other inhabitants of the vaginal microbiome that may play an integral role in BV pathogenesis.

Multiclade *G. vaginalis* communities are common and are associated with BV [12, 21, 23, 25]. In our study, detection of multiple clades was associated with intermediate-BV, Nugent-BV, and nonoptimal vaginal microbiota. Multiple clades may act synergistically to supress *Lactobacillus* spp. or form biofilms. Alternatively, there may be one *G. vaginalis* clade driving disease as well as passenger clades; in specimens in which clades co-occur, it is difficult to identify which specific clade/s is driving disease because other clades may rapidly cohabit after an initiation event. To investigate whether there were independent associations between individual *G. vaginalis* clades and BV, we conducted an additional analysis that included all 4 clades with Nugent-BV as the outcome (Supplementary Table

3). The results of the adjusted analysis were consistent with our univariable findings, with clade 1 having the strongest association with Nugent-BV (Supplementary Table 2). However, this analysis was limited by the correlation between clades so was not investigated further. The high prevalence of multiclade communities may explain contradictory associations of specific clades with BV in previous studies.

Clades 1, 2, and 3 were significantly associated with current sexual practices, and we observed a nonsignificant trend between detection of multiple *G. vaginalis* clades and sexual practices including increased sex frequency and sex with a new partner. Several studies document exchange of *G. vaginalis* between sexual partners. Heterosexual couples share identical *G. vaginalis* strains [36] and demonstrate high concordance for *G. vaginalis* biofilm [37]. A study using culture methods reported increased *G. vaginalis* colonization with increasing frequency of digital-vaginal sex and sex-toy use in WSW [38]. Using PCR, *G. vaginalis* prevalence has been shown to increase after sexual debut and with increasing numbers of sexual partners [13]. Differing associations seen with individual clades does not necessarily suggest that specific clades are only transmitted by specific sexual practices, but rather provides support for the sexual exchange of *G. vaginalis* clades across a range of different practices.

Smoking was associated with clade 1 detection, and women who reported smoking were more likely than nonsmokers to have multiple *G. vaginalis* clades detected. This is consistent with a previous report of increased *G. vaginalis* detection by culture amongst smokers compared to nonsmokers [39], and it is supported by the frequent association of smoking with BV [26, 27, 40]. The mechanism by which smoking may increase *G. vaginalis* in the vagina is unknown; however, smokers have an altered vaginal metabolome (including high concentrations of nicotine and its derivatives) [41] and reduced oestradiol levels [42], which may impact vaginal microbiota composition. In addition, smoking negatively impacts immune function [43], which may result in reduced clearance of *G. vaginalis*, similar to what has been observed for human papillomavirus [44]. It is also possible that our observed association between smoking and both clade 1 and multiclade communities is a result of unmeasured confounding.

Three specimens that tested positive for *G. vaginalis* by species-specific PCR did not have any clade detected by clade-specific PCR. This raises the possibility that more than 4 *G. vaginalis* clades exist or that genetic subgroups may exist within the 4 clades [18, 20]. In early 2019, Vaneechoutte et al [45] proposed the existence of 13 different species within the *Gardnerella* genus, and additional clades/subgroups are likely to be revealed as we analyze larger numbers of *G. vaginalis* isolates.

Our study has limitations. Nugent Score was used to facilitate self-collection of vaginal specimens at home. Participants were not examined by a clinician, which limited our ability to assess *G. vaginalis* clade distribution in Amsel-BV. Longitudinal specimens included in the analysis typically represented the last 3 specimens collected from a participant and were not selected randomly or from specified study time points, which may have biased results. Specimens were collected at 3-month intervals so the immediate impact of practices on *G. vaginalis* clade distribution is unclear. Frequent sampling would address this and clarify whether *G. vaginalis* clades persist or are transient. Because we did not use the PCR assay to quantitate clades, clade associations are based on detection rather than quantity, and we were unable to assess associations between covariates and quantity of each clade. Finally, the study population comprised highly educated women who were predominately Australian born, and our findings may not be generalizable to other populations including sexually inexperienced women or women who exclusively have sex with men.

CONCLUSIONS

We report that multiclade *G. vaginalis* communities and clades 1, 2, and 3 were associated with Nugent-BV in Australian WSW. That multiclade communities and clade 1 were both also associated with nonoptimal (*Lactobacillus* deficient) vaginal microbiota indicates increased pathogenicity. The finding that clade 4 was not associated with either BV or nonoptimal vaginal microbiota strongly suggests it may be a commensal clade. Detailed comparative analyses of commensal and pathogenic *G. vaginalis* genetic types may help to identify potential mechanisms of *G. vaginalis* pathogenesis. Individual *G. vaginalis* clades were associated with differing sexual practices, adding to the growing evidence supporting the sexual exchange of *G. vaginalis*. These data have implications for BV treatment and suggest that partner treatment may be an effective strategy to improve BV cure.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank Glenda Fehler, Susan Peterson, Matthew Law, Dr. Marcus Chen, Dr. Sandra Walker, Dr. Jade Bilardi, and Clare Bellhouse for their contributions to the original Women On Women's (WOW) Cohort study, from which this study arose. We also thank Prof Sepehr Tabrizi and Dr. Jimmy Twin for contributions to the laboratory work.

Financial support. This work funded by The Australian National Health and Medical Research Council Project Grant (1020457; to C. S. B.) and the Australian National Health and Medical Research Council Program Grant (1071269; to S. M. G. and C. K. F.). E. L. P. was supported by an Australian Government Research Training Program Scholarship.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

1. Unemo M, Bradshaw CS, Hocking JS, et al. Sexually transmitted infections: challenges ahead. Lancet Infect Dis **2017**; 17:e235–79.
2. Bradshaw CS, Morton AN, Hocking J, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. J Infect Dis **2006**; 193:1478–86.
3. Sobel JD, Schmitt C, Meriwether C. Long-term follow-up of patients with bacterial vaginosis treated with oral metronidazole and topical clindamycin. J Infect Dis **1993**; 167:783–4.
4. Muzny CA, Schwebke JR. *Gardnerella vaginalis*: still a prime suspect in the pathogenesis of bacterial vaginosis. Curr Infect Dis Rep **2013**; 15:130–5.
5. Schwebke JR, Muzny CA, Josey WE. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. J Infect Dis **2014**; 210:338–43.
6. Coleman JS, Gaydos CA. Molecular diagnosis of bacterial vaginosis: an update. J Clin Microbiol **2018**; 56:e00342-18.
7. Srinivasan S, Hoffman NG, Morgan MT, et al. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. PLoS One **2012**; 7:e37818.
8. Lewis WG, Robinson LS, Gilbert NM, Perry JC, Lewis AL. Degradation, foraging, and depletion of mucus sialoglycans by the vagina-adapted Actinobacterium *Gardnerella vaginalis*. J Biol Chem **2013**; 288:12067–79.
9. Gelber SE, Aguilar JL, Lewis KL, Ratner AJ. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J Bacteriol **2008**; 190:3896–903.
10. Swidsinski A, Mendling W, Loening-Baucke V, et al. Adherent biofilms in bacterial vaginosis. Obstet Gynecol **2005**; 106:1013–23.
11. Schwebke JR, Flynn MS, Rivers CA. Prevalence of *Gardnerella vaginalis* among women with lactobacillus-predominant vaginal flora. Sex Transm Infect **2014**; 90:61–3.
12. Vodstrcil LA, Twin J, Garland SM, et al. The influence of sexual activity on the vaginal microbiota and *Gardnerella vaginalis* clade diversity in young women. PLoS One **2017**; 12:e0171856.
13. Fethers K, Twin J, Fairley CK, et al. Bacterial vaginosis (BV) candidate bacteria: associations with BV and behavioural practices in sexually-experienced and inexperienced women. PLoS One **2012**; 7:e30633.
14. Shipitsyna E, Roos A, Datcu R, et al. Composition of the vaginal microbiota in women of reproductive age–sensitive and specific molecular diagnosis of bacterial vaginosis is possible? PLoS One **2013**; 8:e60670.
15. Ravel J, Gajer P, Abdo Z, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A **2011**; 108 (Suppl 1):4680–7.
16. Ahmed A, Earl J, Retchless A, et al. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. J Bacteriol **2012**; 194:3922–37.
17. Paramel Jayaprakash T, Schellenberg JJ, Hill JE. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. PLoS One **2012**; 7:e43009.
18. Cornejo OE, Hickey RJ, Suzuki H, Forney LJ. Focusing the diversity of *Gardnerella vaginalis* through the lens of ecotypes. Evol Appl **2018**; 11:312–24.
19. Janulaitiene M, Gegzna V, Baranauskiene L, Bulavaitė A, Simanavicius M, Pleckaityte M. Phenotypic characterization of *Gardnerella vaginalis* subgroups suggests differences in their virulence potential. PLoS One **2018**; 13:e0200625.
20. Schellenberg JJ, Paramel Jayaprakash T, Withana Gamage N, Patterson MH, Vaneechoutte M, Hill JE. *Gardnerella vaginalis* subgroups defined by cpn60 sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. PLoS One **2016**; 11:e0146510.
21. Shipitsyna E, Krysanova A, Khayrullina G, et al. Quantitation of all four *gardnerella vaginalis* clades detects abnormal vaginal microbiota characteristic of bacterial vaginosis more accurately than putative *G. vaginalis* Sialidase A gene count. Mol Diagn Ther **2019**; 23:139–47.
22. Schuyler JA, Mordechai E, Adelson ME, Sobel JD, Gygax SE, Hilbert DW. Identification of intrinsically metronidazole-resistant clades of *Gardnerella vaginalis*. Diagn Microbiol Infect Dis **2016**; 84:1–3.
23. Balashov SV, Mordechai E, Adelson ME, Gygax SE. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. J Med Microbiol **2014**; 63:162–75.
24. Hilbert DW, Schuyler JA, Adelson ME, Mordechai E, Sobel JD, Gygax SE. *Gardnerella vaginalis* population dynamics in bacterial vaginosis. Eur J Clin Microbiol Infect Dis **2017**; 36:1269–78.
25. Janulaitiene M, Paliulyte V, Grinceviciene S, et al. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. BMC Infect Dis **2017**; 17:394.
26. Evans AL, Scally AJ, Wellard SJ, Wilson JD. Prevalence of bacterial vaginosis in lesbians and heterosexual women in a community setting. Sex Transm Infect **2007**; 83:470–5.
27. Bradshaw CS, Walker SM, Vodstrcil LA, et al. The influence of behaviors and relationships on the vaginal microbiota of

women and their female partners: the WOW Health Study. J Infect Dis **2014**; 209:1562–72.
28. McCaffrey M, Varney P, Evans B, Taylor-Robinson D. Bacterial vaginosis in lesbians: evidence for lack of sexual transmission. Int J STD AIDS **1999**; 10:305–8.
29. Bailey JV, Farquhar C, Owen C. Bacterial vaginosis in lesbians and bisexual women. Sex Transm Dis **2004**; 31:691–4.
30. Vodstrcil LA, Walker SM, Hocking JS, et al. Incident bacterial vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. Clin Infect Dis **2015**; 60:1042–53.
31. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J Clin Microbiol **1991**; 29:297–301.
32. McKinnon LR, Achilles SL, Bradshaw CS, et al. The evolving facets of bacterial vaginosis: implications for HIV transmission. AIDS Res Hum Retroviruses **2019**; 35:219–28.
33. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol **1990**; 215:403–10.
34. Plummer EL, Vodstrcil LA, Murray GL, et al. Sexual behaviours impact the vaginal microbiota of women who have sex with women. IUSTI Asia Pacific Sexual Health Congress; November 1–3, **2018**; Auckland, New Zealand.
35. Schellenberg J, Links MG, Hill JE, et al. Pyrosequencing of the chaperonin-60 universal target as a tool for determining microbial community composition. Appl Environ Microbiol **2009**; 75:2889–98.
36. Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ. Exploring the diversity of *Gardnerella vaginalis* in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation. PLoS One **2011**; 6:e26732.
37. Swidsinski A, Doerffel Y, Loening-Baucke V, et al. *Gardnerella* biofilm involves females and males and is transmitted sexually. Gynecol Obstet Invest **2010**; 70:256–63.
38. Mitchell C, Manhart LE, Thomas KK, Agnew K, Marrazzo JM. Effect of sexual activity on vaginal colonization with hydrogen peroxide-producing lactobacilli and *Gardnerella vaginalis*. Sex Transm Dis **2011**; 38:1137–44.
39. Hellberg D, Nilsson S, Mårdh PA. Bacterial vaginosis and smoking. Int J STD AIDS **2000**; 11:603–6.
40. Brotman RM, He X, Gajer P, et al. Association between cigarette smoking and the vaginal microbiota: a pilot study. BMC Infect Dis **2014**; 14:471.
41. Nelson TM, Borgogna JC, Michalek RD, et al. Cigarette smoking is associated with an altered vaginal tract metabolomic profile. Sci Rep **2018**; 8:852.
42. Westhoff C, Gentile G, Lee J, Zacur H, Helbig D. Predictors of ovarian steroid secretion in reproductive-age women. Am J Epidemiol **1996**; 144:381–8.
43. Bagaitkar J, Demuth DR, Scott DA. Tobacco use increases susceptibility to bacterial infection. Tob Induc Dis **2008**; 4:12.
44. Giuliano AR, Sedjo RL, Roe DJ, et al. Clearance of oncogenic human papillomavirus (HPV) infection: effect of smoking (United States). Cancer Causes Control **2002**; 13:839–46.
45. Vaneechoutte M, Guschin A, Van Simaey L, Gansemans Y, Van Nieuwerburgh F, Cools P. Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus Gardnerella. Int J Syst Evol Microbiol **2019**; 69:679–87.

Clinical Infectious Diseases

MAJOR ARTICLE

Are *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* Associated With Specific Genital Symptoms and Clinical Signs in Nonpregnant Women?

Erica L. Plummer,[1,2] Lenka A. Vodstrcil,[1,2] Kaveesha Bodiyabadu,[3,4,5] Gerald L. Murray,[3,4,6] Michelle Doyle,[2] Rosie L. Latimer,[1,2] Christopher K. Fairley,[1,2] Matthew Payne,[7] Eric P. F. Chow,[1,2,8] Suzanne M. Garland,[3,4,6] and Catriona S. Bradshaw[1,2,8]

[1]Central Clinical School, Monash University, Melbourne, Australia; [2]Melbourne Sexual Health Centre, Alfred Health, Melbourne, Australia; [3]Murdoch Children's Research Institute, Parkville, Australia; [4]Women's Centre for Infectious Diseases, The Royal Women's Hospital, Parkville, Australia; [5]SpeeDx Pty Ltd, Sydney, Australia; [6]Department of Obstetrics and Gynaecology, The University of Melbourne, Parkville, Australia; [7]Division of Obstetrics and Gynaecology, The University of Western Australia, Perth, Australia; and [8]Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Australia

Background. There is limited evidence supporting an association between *Mycoplasma hominis, Ureaplasma urealyticum,* and *Ureaplasma parvum* with symptoms or disease in nonpregnant women. However, testing and reporting of these organisms frequently occurs, in part due to their inclusion in multiplex-PCR assays for sexually transmitted infection (STI) detection. We investigated if *M. hominis, U. urealyticum,* and *U. parvum* were associated with symptoms and/or signs in nonpregnant women attending a sexual health service.

Methods. Eligible women attending the Melbourne Sexual Health Centre completed a questionnaire regarding sexual practices and symptoms. Symptomatic women underwent examination. Women were assessed for bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC), and tested for *M. hominis, U. urealyticum,* and *U. parvum*, and 4 nonviral STIs using a commercial multiplex-PCR.

Results. 1272 women were analyzed. After adjusting for STIs and VVC, *M. hominis* was associated with abnormal vaginal discharge (aOR = 2.70, 95%CI:1.92–3.79), vaginal malodor (aOR = 4.27, 95%CI:3.08–5.91), vaginal pH > 4.5 (aOR = 4.27, 95%CI:3.22–5.66), and presence of clue cells (aOR = 8.08, 95%CI:5.68–11.48). *Ureaplasma* spp. were not associated with symptoms/signs. Bacterial vaginosis was strongly associated with *M. hominis* (aOR = 8.01, 95%CI:5.99–10.71), but was not associated with either *Ureaplasma* spp. In stratified analyses, *M. hominis* was associated with self-reported vaginal malodor and clinician-recorded vaginal discharge in women with BV, but not with symptoms/signs in women without BV.

Conclusions. Only *M. hominis* was associated with symptoms/signs, and these were manifestations of BV. Importantly, *M. hominis* was not associated with symptoms/signs in women without BV. These findings do not support routine testing for *M. hominis, U. urealyticum,* and *U. parvum* in nonpregnant women.

Keywords. *Mycoplasma hominis*; *Ureaplasma urealyticum*; *Ureaplasma parvum*; nonpregnant women; symptoms; bacterial vaginosis.

Mycoplasma hominis, Ureaplasma urealyticum, and *Ureaplasma parvum* are commonly recovered from the urogenital tract of symptomatic and asymptomatic women and men. Molecular studies of nonpregnant women of reproductive age place prevalence estimates between 3.2%–26.2% for *M. hominis*, 7.6%–28.4% for *U. urealyticum,* and 22.4%–67.3% for *U. parvum* [1–7]. There is limited evidence supporting a role for *M. hominis*, *U. urealyticum,* and *U. parvum* in causing symptoms and/or disease in nonpregnant women [8]. However, vaginal detection of *Ureaplasma* spp. (particularly *U. parvum*) has been associated with obstetric complications including preterm birth and low birth weight [9–12]. Additionally, *Ureaplasma* spp. have been associated with significant neonatal morbidities including necrotizing enterocolitis, bronchopulmonary dysplasia, intraventricular hemorrhage, and meningitis [13–16].

Testing for *M. hominis*, *U. urealyticum,* and *U. parvum* has become increasingly common, often due to their inclusion in commercial multiplex-polymerase chain reaction (PCR) assays used for sexually transmitted infection (STI) screening. The use of culture for identification of these organisms has also been practiced (even though culture cannot differentiate between *Ureaplasma* spp.), and reports of detection are often accompanied by antibiotic susceptibility findings, which can lead clinicians to believe that antibiotic treatment is indicated. The routine testing and reporting of *M. hominis*, *U. urealyticum,* and *U. parvum*, along with the high prevalence of asymptomatic

Received 10 November 2020; editorial decision 23 December 2020; published online 27 January 2021.

Correspondence: E. L. Plummer, Central Clinical School, Monash University, Melbourne Sexual Health Centre, 580 Swanston Street, Carlton, 3053, VIC, Australia (erica.plummer@monash.edu).

DOI: 10.1093/cid/ciab061

colonization and limited evidence of a causative role in disease in nonpregnant women, creates confusion for clinicians around the need to treat positive cases.

In 2018, the European STI Guidelines Editorial Board released a "Position Statement" regarding testing for *M. hominis*, *U. urealyticum,* and *U. parvum* [8]. Following a review of published literature concerning the etiological role of these bacteria in STI syndromes and complications, the statement concluded that there is currently no evidence of benefit from routinely testing and treating for *M. hominis*, *U. urealyticum,* and *U. parvum* in adult men and nonpregnant women [8].

Importantly, the statement noted limitations with a number of the reviewed studies, including a failure to control for important confounders such as sexual practices and concurrent genital infections [8]. In light of this, we conducted a study to determine if *M. hominis*, *U. urealyticum,* and *U. parvum* were associated with specific symptoms and/or clinical signs in nonpregnant women, after adjusting for important confounders, in order to inform testing indications for these bacteria in a sexual health clinic setting.

METHODS

Study Population

Participants and specimens used for this study were collected as part of a cross-sectional study (OhMG) that had the principal aim of informing clinical indications for testing for *Mycoplasma genitalium* and additional aims of determining the contribution of *M. hominis, U. urealyticum,* and *U. parvum* to symptoms and/or clinical signs in nonpregnant women [17]. Women attending the Melbourne Sexual Health Centre (MSHC) between April 2017 and April 2019 were eligible to participate if they were sexually active, aged ≥18 years, and were presenting with common genitourinary symptoms or presenting for routine STI screening. Women were ineligible if they were unable to consent for reasons of language or mental state, were sex workers, were sexual contacts of *M. genitalium,* or were attending for a *M. genitalium* test-of-cure. Alfred Hospital Ethics Committee (project 100/17) approved this project and all participants provided written informed consent.

Clinical and Laboratory Procedures

Women attending MSHC were assessed for eligibility and recruited by either a sexual health physician or a research nurse. All recruited women completed a questionnaire concerning sexual practices and genital symptoms experienced in the week prior to presentation. Symptomatic women (women reporting at least one symptom in the questionnaire) underwent a genital examination by a clinician, and the presence or absence of the following clinical signs was recorded: abnormal vaginal discharge, vaginal malodor, vulval redness or vulvitis, cervicitis (defined as mucopurulent cervicitis and/or cervical friability), cervical contact bleeding, and cervical or adnexal motion tenderness. In keeping with standard practice at MSHC, asymptomatic women (women reporting none of the symptoms in the questionnaire) were not examined.

In addition to their routine clinical specimens for STI testing (results of routine STI testing are reported elsewhere [17]), all women provided a high vaginal swab specimen (self- or clinician-collected) for study-related procedures. Swabs were swirled in 600 µL 1× PBS, and 200 µL extracted by MagNA Pure 96 (Roche Diagnostics, Mannheim, Germany) using the DNA and Viral NA Small Volume Kit. Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae, M. genitalium*, *Trichomonas vaginalis*, *M. hominis, U. urealyticum,* and *U. parvum* was performed with the AnyplexTM II STI-7e PCR assay (Seegene Inc., Republic of Korea), according to manufacturer instructions.

For all women, vaginal pH was recorded (MColorpHast™ pH indicator strips; Merck KGaA, Darmstadt, Germany), and a Gram stain (and wet preparation for symptomatic women) of a vaginal smear was prepared to assess for bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and vaginal polymorphonuclear leukocyte (PMNL) cell counts. Bacterial vaginosis (BV) was defined as the presence of ≥3 Amsel criteria [18] and a Nugent Score (NS) = 4–10 [19]. In asymptomatic women (and symptomatic women who could not be assessed by Amsel criteria due to menses/blood), BV was defined as a NS = 4–10 and presence of clue cells on microscopy or wet preparation. Vulvovaginal candidiasis (VVC) was diagnosed based on the presence of typical clinical features on examination (thick white or curdy discharge and/or vulvovaginal erythema) and/or presence of visible pseudohyphae and/or budding yeasts on microscopy.

Statistical Methods

Proportions of *M. hominis*, *U. urealyticum,* and *U. parvum* were calculated with 95% binomial confidence intervals (CIs). Logistic regression was used to identify demographic, sexual, and microbiological factors associated with the detection of *M. hominis*, *U. urealyticum,* and *U. parvum*. Factors with a significance level of $P < .10$ in univariable analyses were included in multivariable models.

A logistic regression analysis that adjusted for *M. hominis*, *U. urealyticum, U. parvum*, *C. trachomatis*, *N. gonorrhoea, M. genitalium, T. vaginalis,* and VVC was performed for each sign and symptom to investigate if *M. hominis*, *U. urealyticum,* and *U. parvum* were independently associated with any clinical characteristic. Analyses of clinical signs were restricted to symptomatic women who were assessed for all clinical signs (n = 406). As associations with individual Amsel criteria [18] were examined, we did not adjust for BV to prevent overfitting the models. For analyses of signs/symptoms, *P*-values

Table 1. Univariable Analysis of Demographic and Sexual Factors Associated With *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *U. parvum* Detection

Characteristic	*M. hominis*				*U. urealyticum*				*U. parvum*			
	Negative n = 897	Positive n = 375	OR (95% CI)	*P*	Negative n = 869	Positive n = 403	OR (95% CI)	*P*	Negative n = 362	Positive n = 910	OR (95% CI)	*P*
Cohort												
Asymptomatic	142 (16)	49 (13)	1		138 (16)	53 (13)	1		55(15)	136 (15)	1	
Symptomatic	755 (84)	326 (87)	1.25 (0.88–1.78)	.209	731 (84)	350 (87)	1.25 (0.89–1.75)	.206	307 (85)	774 (85)	1.02 (0.73–1.43)	.911
Age in years (median, IQR)	27 (6)	26 (5)	0.97 (0.94–0.99)	**.002**	27 (6)	26 (6)	0.99 (0.97–1.01)	.186	27 (6)	27 (6)	1.00 (0.98–1.02)	.972
No. male partners in past 12 mo (median, IQR)	4 (5)	6 (6)	1.06 (1.03–1.09)	**<.001**	4 (3)	6 (7)	1.07 (1.04–1.10)	**<.001**	4 (5)	5 (5)	1.05 (1.01–1.09)	**.005**
No. female partners in past 12 mo (median, IQR)	0 (1)	0 (1)	1.01 (0.89–1.15)	.897	0 (1)	0 (1)	0.93 (0.81–1.07)	.319	0 (1)	0 (1)	1.35 (1.09–1.67)	**.007**
Condom use with male partners in past 12 mo[a]												
Always	69 (8)	18 (5)	1		68 (8)	19 (5)	1		34 (10)	53 (6)	1	
Never/not always	814 (92)	353 (95)	1.66 (.97–2.83)	.062	786 (92)	381 (95)	1.73 (1.03–2.93)	**.039**	323 (90)	844 (94)	1.68 (1.07–2.63)	**.024**
STI in past 6 mo												
No	779 (89)	300 (82)	1		742 (87)	337 (86)	1		308 (87)	771 (87)	1	
Yes	100 (11)	66 (18)	1.71 (1.22–2.40)	**.002**	111 (13)	55 (14)	1.09 (.77–1.54)	.624	46 (13)	120 (13)	1.04 (.72–1.50)	.824

Data are presented as n (%), unless otherwise specified.
Data are missing for up to 2% of participants for some variables.
P-values < .05 are bolded to indicate statistically significant associations.
Abbreviations: CI, confidence interval; IQR, interquartile range; OR, odds ratio; SD, standard deviation; mo, months; STI, sexually transmitted infection; *U. parvum*, *Ureaplasma parvum*.

were corrected for false discovery rate (FDR) [20] using the Benjamini-Hochberg method, and we used a significance value of $q < 0.05$ (calculated in R v4.0.2 using p.adjust).

Odds ratios (OR) were presented with 95%CI, and analyses were performed using Stata/IC v16(StataCorp LP, College Station, TX, USA), unless stated otherwise.

RESULTS

Description of Study Population

During the study period, 1355 women were recruited [17]. Thirty-seven women were excluded postrecruitment: 25 were sex workers and 12 were inadvertently recruited twice. Thirty-seven swabs had insufficient DNA remaining for testing. Nine specimens returned an invalid test result and were excluded. A total of 1272 women were included in analyses; 1081 women reported at least one symptom, and 191 reported none of the symptoms in the questionnaire. The median age of participants was 26 years (interquartile range [IQR]: 23–29 years, Table 1). The most frequently reported symptoms were abnormal vaginal discharge (34%), vaginal malodor (24%), and vaginal itch (21%).

Detection of *M. hominis, U. urealyticum,* and *U. parvum*

Mycoplasma hominis was detected in 375 women (29%, 95%CI:27–32), *U. urealyticum* was detected in 403 women (32%, 95%CI:29–34), and *U. parvum* was detected in 910 women (72%, 95%CI:69–74). There was no difference in the detection of these organisms between women with one or more symptoms (symptomatic) or no symptoms (asymptomatic; Table 1). The proportion of women with other genital infections has been reported [17]: 6% (95%CI:5–8) had *M. genitalium,* 8% (95%CI:6–9) had *C. trachomatis*, 1% (95%CI:0–2) had *N. gonorrhoeae*, 30% (95%CI:28–33) had BV, 24% (95%CI:22–27) had VVC, and 1% (95%CI:0–2) had *T. vaginalis*.

Demographic, Sexual, and Microbiological Factors Associated with *M. hominis, U. urealyticum,* and *U. parvum*

In univariable analysis, *M. hominis*, *U. urealyticum,* and *U. parvum* were each significantly associated with increased numbers of male sexual partners (MSPs) in the past 12 months (Table 1). Additionally, *M. hominis* was associated with younger age (OR = 0.97, 95%CI:0.94–0.99) and self-report of STI in the previous 6 months (OR = 1.71, 95%CI:1.22–2.40), *U. parvum* was associated with increased number of female sexual partners (FSPs; OR = 1.35, 95%CI:1.09–1.67), and both *U. urealyticum* and *U. parvum* were associated with inconsistent condom use (OR = 1.73, 95%CI:1.03–2.93 and OR = 1.68, 95%CI:1.07–2.63, respectively).

Mycoplasma hominis was associated with *C. trachomatis* (OR = 1.76, 95%CI:1.17–2.64), *N. gonorrhoeae* (OR = 3.48, 95%CI:1.32–9.22), and concurrent BV (OR = 8.40, 95%CI:6.36–11.10), and negatively associated with VVC (OR = 0.71,

Table 2. Univariable Analysis of Microbiological Factors Associated With *Mycoplasma hominis, Ureaplasma urealyticum,* and *U. parvum* Detection

	M. hominis				*U. urealyticum*				*U. parvum*			
	Negative n = 897	Positive n = 375	OR (95% CI)	*P*	Negative n = 869	Positive n = 403	OR (95% CI)	*P*	Negative n = 362	Positive n = 910	OR (95% CI)	*P*
C. trachomatis												
Negative	834 (93)	331 (88)	1		795 (91)	370 (92)	1		337 (93)	828 (91)	1	
Positive	63 (7)	44 (12)	1.76 (1.17–2.64)	**.006**	74 (9)	33 (8)	.96 (.62–1.47)	.845	25 (7)	82 (9)	1.33 (.84–2.13)	.224
N. gonorrhoeae												
Negative	890 (99)	365 (97)	1		861 (99)	394 (98)	1		360 (99)	895 (98)	1	
Positive	7 (1)	10 (3)	3.48 (1.32–9.22)	**.012**	8 (1)	9 (2)	2.46 (.94–6.42)	.066	2 (1)	15 (2)	3.02 (.69–13.26)	.144
M. genitalium												
Negative	843 (94)	341 (91)	1		813 (94)	371 (92)	1		340 (94)	844 (93)	1	
Positive	54 (6)	34 (9)	1.56 (.99–2.43)	.052	56 (6)	32 (8)	1.25 (.80–1.97)	.329	22 (6)	66 (7)	1.21 (.73–1.99)	.457
T. vaginalis												
Negative	892 (99)	373 (99)	1		864 (99)	401 (100)	1		359 (99)	906 (100)	1	
Positive	5 (1)	2 (1)	.96 (.18–4.95)	.958	5 (1)	2 (0)	.86 (.17–4.46)	.859	3 (1)	4 (0)	.53 (.12–2.37)	.405
Nugent Score												
0–3	662 (78)	120 (33)	1		564 (68)	218 (57)	1		245 (71)	537 (62)	1	
4–6	78 (9)	48 (13)	3.39 (2.26–5.11)	**<.001**	79 (10)	47 (12)	1.54 (1.04–2.28)	**.032**	29 (8)	97 (11)	1.53 (.98–2.37)	.061
7–10	106 (13)	200 (54)	1.41 (7.67–14.13)	**<.001**	187 (23)	119 (31)	1.65 (1.25–2.17)	**<.001**	73 (21)	233 (27)	1.46 (1.07–1.97)	**.015**
Not assessed	51	7			39	19			15	43		
Bacterial vaginosis[a]												
Negative	706 (83)	138 (38)	1		602 (73)	242 (63)	1		260 (75)	584 (67)	1	
Positive	140 (17)	230 (63)	8.40 (6.36–11.10)	**<.001**	228 (27)	142 (37)	1.55 (1.20–2.00)	**.001**	87 (25)	283 (33)	1.45 (1.09–1.92)	**.010**
Not assessed	51	7			39	19			15	43		
Vulvovaginal candidiasis[b]												
Negative	651 (74)	294 (80)	1		651 (76)	294 (75)	1		277 (77)	668 (75)	1	
Positive	231 (26)	74 (20)	.71 (.53–.95)	**.023**	205 (24)	100 (25)	1.08 (.82–1.42)	.584	82 (23)	223 (25)	1.13 (.84–1.51)	.416
Not assessed	15	7			13	9			3	19		
M. hominis												
Negative	-	-	-	-	671 (77)	226 (56)	1		288 (80)	609 (67)	1	
Positive	-	-	-	-	198 (23)	177 (44)	2.65 (2.06–3.42)	**<.001**	74 (20)	301 (33)	1.92 (1.44–2.57)	**<.001**
U. urealyticum												
Negative	671 (75)	198 (53)	1		-	-	-	-	204 (56)	665 (73)	1	
Positive	226 (25)	177 (47)	2.65 (2.06–3.42)	**<.001**	-	-	-	-	158 (47)	245 (27)	.48 (.37–.61)	**<.001**
U. parvum												
Negative	288 (32)	74 (20)	1		204 (23)	158 (39)	1		-	-	-	-
Positive	609 (68)	301 (80)	1.92 (1.44–2.57)	**<.001**	665 (77)	245 (61)	.48 (.37–.61)	**<.001**	-	-	-	-

Data are presented as n (%), unless otherwise specified.

P-values < .05 are bolded to indicate statistically significant associations.

Abbreviations: *C. trachomatis, Chlamydia trachomatis*; CI, confidence interval; *M. genitalium, Mycoplasma genitalium*; *M. hominis, Mycoplasma hominis*; n, number; *N. gonorrhoeae, Neisseria gonorrhoeae*; OR, odds ratio; *T. vaginalis, Trichomonas vaginalis*; *U. parvum, Ureaplasma parvum*; *U. urealyticum, Ureaplasma urealyticum.*

[a]Bacterial vaginosis diagnosis was defined as Nugent Score = 4–10 and ≥3 Amsel criteria for symptomatic women and as Nugent Score = 4–10 and presence of clue cells for asymptomatic women.

[b]Vulvovaginal candidiasis diagnosis was based on the presence of typical clinical features on examination and/or presence of visible pseudohyphae and/or budding yeasts on microscopy.

95%CI:0.53–0.95; Table 2). Both *U. urealyticum* and *U. parvum* were associated with BV (OR = 1.55, 95%CI:1.20–2.00 and OR = 1.45, 95%CI:1.09–1.92, respectively), but neither were associated with concurrent STIs.

Mycoplasma hominis was positively associated with detection of *U. urealyticum* (OR = 2.65, 95%CI:2.06–3.42) and detection of *U. parvum* (OR = 1.92, 95%CI:1.44–2.57), but *U. urealyticum* and *U. parvum* were negatively associated with each other (OR = 0.48, 95%CI:0.37–0.61).

In multivariable analyses, *M. hominis* was associated with younger age (adjusted-OR [aOR] = 0.95, 95%CI:0.93–0.98), self-report of STI in the previous 6 months (aOR = 1.72, 95%CI:1.12–2.62), co-detection of *U. urealyticum* (aOR = 2.95, 95%CI:2.14–4.05), co-detection of *U. parvum* (aOR = 2.15, 95%CI:1.51–3.07), and BV (aOR = 9.26, 95%CI:6.77–12.67; Table 3); *M. hominis* was not associated with concurrent STIs in adjusted analyses.

Ureaplasma urealyticum was associated with increased numbers of MSPs in the previous 12 months (aOR = 1.07, 95%CI:1.04–1.11), inconsistent condom use (aOR = 1.90, 95%CI:1.08–3.34), and BV (aOR = 1.49, 95%CI:1.13–1.95). Detection of *U. urealyticum* was negatively associated with co-detection of *U. parvum* (aOR = 0.40, 95%CI:0.31–0.53).

Ureaplasma parvum was associated with higher numbers of MSPs (aOR = 1.06, 95%CI:1.02–1.09) and FSPs in the previous 12 months (aOR = 1.31, 95%CI:1.04–1.64), inconsistent condom use (aOR = 2.06, 95%CI:1.28–3.32), and BV (aOR = 1.41, 95%CI:1.05–1.89).

Due to the correlation between *M. hominis* and BV (ρ = 0.46), we did not adjust analyses in Table 3 for *M. hominis*. To investigate if *M. hominis*, *U. urealyticum,* and/or *U. parvum* were independently associated with BV, we performed a logistic regression that included *M. hominis*, *U. urealyticum,* and *U. parvum* with BV as the outcome, adjusted for number of MSPs. In this analysis, BV was strongly associated with *M. hominis* (aOR = 8.01, 95%CI:5.99–10.71), but was not associated with either *U. urealyticum* or *U. parvum* (Figure 1).

Self-Reported Symptoms and Clinical Signs Associated with *M. hominis, U. urealyticum,* and *U. parvum*

The association of *M. hominis, U. urealyticum,* and *U. parvum* with self-reported symptoms and clinical signs is presented in Table 4. After adjusting for genital co-infections and following FDR correction, *M. hominis* increased the odds of BV symptoms and each Amsel criterion, including abnormal vaginal discharge (self-report aOR = 1.66, 95%CI:1.26–2.18 and clinician-recorded aOR = 2.70, 95%CI:1.92–3.79), vaginal malodor (self-report aOR = 3.19, 95%CI:2.38–4.26 and clinician-recorded aOR = 4.27, 95%CI:3.08–5.91), elevated vaginal pH > 4.5 (aOR = 4.27, 95%CI:3.22–5.66), and presence of clue cells (aOR = 8.08, 95%CI:5.68–11.48). However, *U. urealyticum* and *U. parvum* were not associated with any symptom or sign after adjusting for genital co-infections and following FDR correction.

To account for potential confounding by BV on the relationship between *M. hominis* and symptoms/signs, data were stratified by BV status, and the association between *M. hominis* and clinical characteristics was investigated within each strata using the chi-squared or Fisher exact test. In women without BV, *M. hominis* was not associated with symptoms or signs. In women with BV, *M. hominis* was detected more frequently in women with self-reported vaginal malodor compared to

Table 3. Multivariable Analysis of Factors Associated With Detection of *Mycoplasma hominis, Ureaplasma urealyticum,* and *U. parvum* in Women

	M. hominis N = 1155[a]		*U. urealyticum* N = 1194[a]		*U. parvum* N = 1194[a]	
Characteristic	aOR (95% CI)[b]	*P*	aOR (95% CI)[bc]	*P*	aOR (95% CI)[bc]	*P*
Age	.95 (.93–.98)	**.001**				
No. male partners in past 12 mo	1.02 (.99–1.04)	.270	1.07 (1.04–1.11)	**<.001**	1.06 (1.02–1.09)	**.003**
No. female partners in past 12 mo					1.31 (1.04–1.64)	**.023**
Inconsistent condom use	.94 (.50–1.77)	.844	1.90 (1.08–3.34)	**.025**	2.06 (1.28–3.32)	**.003**
STI in past 6 mo	1.72 (1.12–2.62)	**.012**				
C. trachomatis detected	1.56 (.93–2.61)	.091				
N. gonorrhoeae detected	1.54 (.43–5.52)	.510	1.30 (.40–4.20)	.666		
M. genitalium detected	1.37 (.80–2.34)	.258				
U. urealyticum detected	2.95 (2.14–4.05)	**<.001**			.41 (.31–.53)	**<.001**
U. parvum detected	2.15 (1.51–3.07)	**<.001**	.40 (.31–.53)	**<.001**		
Concurrent BV	9.19 (6.66–12.70)	**<.001**	1.49 (1.13–1.95)	**.004**	1.41 (1.05–1.89)	**.023**
Concurrent VVC	1.30 (.90–1.87)	.165				

P-values <.05 are bolded to indicate statistically significant associations.

Abbreviations: aOR, adjusted odds ratio; BV, bacterial vaginosis; *C. trachomatis, Chlamydia trachomatis*; CI, confidence interval; mo, months; *N. gonorrhoeae, Neisseria gonorrhoea*; *M. genitalium, Mycoplasma genitalium*; STI, sexually transmitted infection; *U. parvum, Ureaplasma parvum*; VVC, vulvovaginal candidiasis.

[a]Numbers differ from whole study population due to missing data for some variables, and as not all women were assessed for BV or VVC.

[b]A separate multivariable logistic regression was conducted for each common genital mycoplasma (ie, *M. hominis, U. urealyticum,* and *U. parvum*), adjusting for characteristics with *P* < .10 in univariable analyses. Nugent Score was left out of all adjusted models so as to not overfit the models as it was strongly correlated with BV (ρ = 0.92).

[c]*M. hominis* was moderately correlated with BV (ρ = 0.46). As a result it was left out of adjusted analyses for *U. urealyticum* and *U. parvum* to not overfit the models. BV was included as it is a known clinically relevant syndrome.

women not reporting malodor (118/230 [52%] vs 53/140 [38%]; $P = .012$), and in women with clinician-recorded vaginal discharge compared to women without discharge on examination (111/123 [90%] vs 52/65 [80%]; $P = .049$).

DISCUSSION

In our study of 1272 nonpregnant women attending a large public sexual health service in Melbourne, Australia, *M. hominis* was detected in 29% (95%CI:27–32) of women, *U. urealyticum* was detected in 32% (95%CI:29–34), and *U. parvum* was detected in 72% (95%CI:69–74). In adjusted analyses, *M. hominis, U. urealyticum,* and *U. parvum* were associated with classic epidemiological markers of sexual risk such as increased numbers of sexual partners, inconsistent condom use, and recent STI diagnosis. *Mycoplasma hominis* was strongly associated with the syndrome of BV (aOR = 8.08, 95%CI:5.68–11.48), after adjusting for *U. urealyticum, U. parvum,* and number of MSPs, whereas *U. urealyticum* and *U. parvum* were not associated with BV. Only *M. hominis* was associated with specific clinical characteristics, and these were manifestations of BV. In stratified analyses, *M. hominis* was only associated with vaginal malodor and abnormal vaginal discharge in women with BV and was not associated with symptoms or signs in women without BV. These findings support the hypothesis that *M. hominis* is part of the spectrum of organisms considered to be BV-associated bacteria. The high prevalence of *U. urealyticum* and *U. parvum* and the finding that these bacteria were not associated with any symptom or sign indicates that asymptomatic carriage of these bacteria is common. In agreement with the European Position Statement [8], our findings do not support the routine screening or testing for *M. hominis*, *U. urealyticum,* and *U. parvum* in nonpregnant women in a sexual health setting.

We found that *M. hominis* independently increased the odds of BV by 8-fold, whereas *U. urealyticum* and *U. parvum* were not associated with increased odds of BV. Previous studies have reported associations between *M. hominis* and BV [1, 2, 21–25], including increased detection and increased load of *M. hominis* in women with BV compared to women without BV [1, 21, 22]. However, not all women with BV have detectable *M. hominis*, and whether *M. hominis* contributes to the initiation of BV or how it contributes to BV pathophysiology is unknown. Interestingly, *M. hominis* metabolizes arginine to produce ammonia [26], and the production of ammonia by other organisms (eg, *Prevotella bivia*) has been shown to enhance growth of *Gardnerella vaginalis* [27], a prime suspect in the pathogenesis of BV [28]. Positive correlation in organism load of *M. hominis* and *G. vaginalis* in women co-infected with both organisms has been reported, which may suggest synergy between the 2 bacteria [1]. Although *M. hominis* displays resistance to metronidazole in vitro, BV treatment with metronidazole decreases the load of *M. hominis* [29]. Conversely, doxycycline, which is active against *M. hominis,* is ineffective for treating BV [30]. Together, these data suggest that *M. hominis* may enhance the growth of more virulent BV-associated bacteria, but is unlikely to be integral to BV pathogenesis. Furthermore, the association of *M. hominis*, *U. urealyticum,* and *U. parvum* with BV in univariable analysis highlights that these organisms exist in a complex bacterial milieu. As has been noted previously [8, 31], testing or screening for *M. hominis*, *U. urealyticum,* and *U parvum* without concurrent evaluation of the vaginal microbiome has limited value. Importantly, our data suggest that in a sexual health service, there may be more clinical value in appropriately diagnosing the syndrome of BV, rather than testing for *M. hominis*, *U. urealyticum,* or *U. parvum* on their own.

We found that *U. urealyticum* and *U. parvum* were not associated with any symptom or sign, but these bacteria have been associated with negative outcomes in previous studies. This raises important considerations. For example, the pathogenicity of ureaplasmas may be serovar-specific. However, to date,

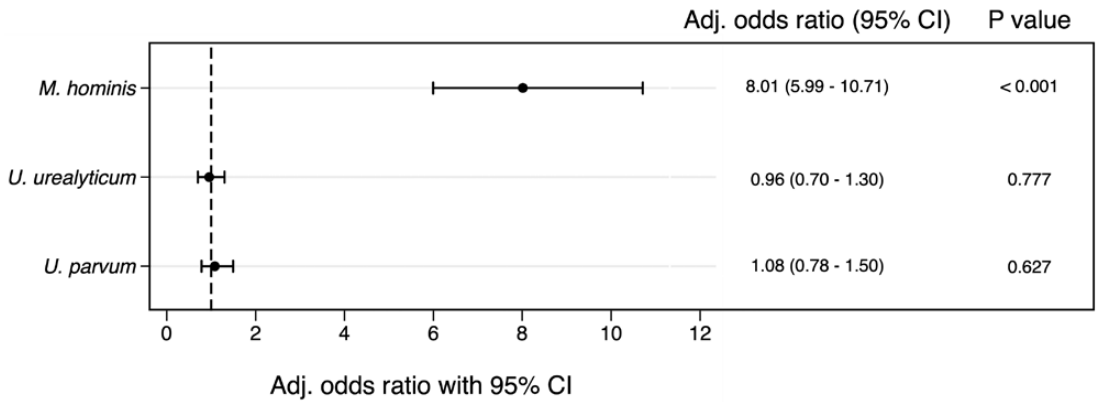


Figure 1. Association of *Mycoplasma hominis, Ureaplasma urealyticum,* and *U. parvum* with bacterial vaginosis. The adjusted odds ratio reflects the association between the 3 common genital mycoplasmas (ie, *M. hominis, U. urealyticum,* and *U. parvum*) and bacterial vaginosis, adjusted for each other and number of male sexual partners. Adjusted odds ratios are presented with 95% confidence intervals. Abbreviation: CI, confidence interval.

Table 4. Self-Reported Symptoms and Clinical Signs Associated With Detection of *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *U. parvum*[b]

	M. hominis					*U. urealyticum*					*U. parvum*				
	Negative n = 897	Positive n = 375	aOR (95% CI)[a]	*P value*	*q value*	Negative n = 869	Positive n = 403	aOR (95% CI)[a]	*P value*	*q value*	Negative n = 362	Positive n = 910	aOR (95% CI)[a]	*P value*	*q value*
Abdominal pain															
No	762 (85)	321 (86)	1			739 (86)	344 (86)	1			299 (83)	784 (87)	1		
Yes	130 (15)	52 (14)	.97 (.67–1.41)	.892	.911	125 (14)	57 (14)	.91 (.63–1.31)	.621	.775	61 (17)	121 (13)	.76 (.53–1.07)	.120	.380
Missing	5	2				5	2				2	5			
Dyspareunia															
No	798 (90)	335 (90)	1			775 (90)	358 (90)	1			318 (89)	815 (91)	1		
Yes	86 (10)	38 (10)	1.03 (.67–1.58)	.886	.911	82 (10)	42 (11)	1.13 (.75–1.70)	.571	.775	39 (11)	85 (9)	.87 (.57–1.31)	.500	.723
Missing	13	2				12	3				5	10			
Abnormal vaginal discharge															
No	611 (69)	221 (59)	1			572 (66)	260 (65)	1			230 (64)	602 (67)	1		
Yes	277 (31)	151 (41)	1.66 (1.26–2.18)	**<.001**	**<.001**	289 (34)	139 (35)	.92 (.70–1.20)	.526	.775	129 (36)	299 (33)	.82 (.63–1.08)	.126	.440
Missing	9	3				8	4				3	9			
Vaginal malodor															
No	732 (82)	227 (61)	1			657 (76)	302 (75)	1			278 (77)	681 (76)	1		
Yes	158 (18)	144 (39)	3.19 (2.38–4.26)	**<.001**	**<.001**	202 (24)	100 (25)	.81 (.60–1.09)	.166	.775	83 (23)	219 (24)	.90 (.66–1.22)	.499	.723
Missing	7	4				10	1				1	10			
Vaginal itch															
No	695 (78)	300 (80)	1			685 (79)	310 (77)	1			284 (79)	711 (78)	1		
Yes	196 (22)	73 (20)	.93 (.66–1.30)	.676	.911	177 (21)	92 (23)	1.15 (.83–1.59)	.390	.775	74 (21)	195 (22)	1.07 (.77–1.49)	.685	.723
Missing	6	2				7	1				4	4			
Postcoital bleeding															
No	812 (92)	337 (91)	1			779 (91)	370 (92)	1			331 (92)	818 (91)	1		
Yes	74 (8)	34 (9)	1.09 (.70–1.71)	.705	.911	77 (9)	31 (8)	.86 (.55–1.36)	.522	.775	28 (8)	80 (9)	1.12 (.70–1.77)	.645	.723
Missing	11	4				13	2				3	12			
Intermenstrual bleeding															
No	803 (90)	332 (89)	1			773 (90)	362 (91)	1			312 (87)	823 (91)	1		
Yes	85 (10)	40 (11)	1.28 (.84–1.94)	.250	.594	87 (10)	38 (10)	.80 (.52–1.22)	.302	.775	46 (13)	79 (9)	.63 (.42–.95)	.026	.222
Missing	9	3				9	3				4	8			
Dysuria															
No	769 (86)	332 (89)	1			754 (87)	347 (86)	1			312 (87)	789 (87)	1		
Yes	122 (14)	41 (11)	.75 (.50–1.12)	.157	.426	108 (13)	55 (14)	1.18 (.82–1.71)	.378	.775	45 (13)	118 (13)	1.10 (.75–1.61)	.616	.723
Missing	6	2				7	1				5	3			
Urinary frequency															
No	743 (83)	301 (81)	1			717 (83)	327 (82)	1			291 (81)	753 (83)	1		
Yes	150 (17)	69 (19)	1.13 (.81–1.58)	.468	.885	145 (17)	74 (18)	1.06 (.76–1.47)	.734	.775	67 (19)	152 (17)	.86 (.62–1.20)	.375	.713
Missing	4	5				7	2				4	5			
Fever															
No	859 (97)	358 (96)	1			830 (97)	387 (96)	1			350 (97)	867 (96)	1		
Yes	29 (3)	14 (4)	1.09 (.55–2.16)	.816	.911	28 (3)	15 (4)	1.13 (.57–2.25)	.719	.775	9 (3)	34 (4)	1.56 (.72–3.38)	.260	.618
Missing	9	3				11	1				3	9			

Table 4. Continued

Clinical signs associated with detection of *M. hominis, U. urealyticum,* and *U. parvum*[b]

	M. hominis					U. urealyticum					U. parvum				
	Negative n = 250	Positive n = 156	aOR (95% CI)[a]	*P value*	*q value*	Negative n = 282	Positive n = 124	aOR (95% CI)[a]	*P value*	*q value*	Negative n = 117	Positive n = 289	aOR (95% CI)[a]	*P value*	*q value*
Abnormal vaginal discharge															
No	88 (35)	21 (13)	1			84 (30)	25 (20)	1			39 (33)	70 (24)	1		
Yes	162 (65)	135 (87)	3.58 (2.04–6.28)	**<.001**	**<.001**	198 (70)	99 (80)	1.45 (.82–2.56)	.196	.775	78 (67)	219 (76)	1.73 (1.04–2.89)	.035	.222
Vaginal malodor															
No	201 (80)	78 (50)	1			202 (72)	77 (62)	1			91 (78)	188 (65)	1		
Yes	49 (20)	78 (50)	3.78 (2.35–6.08)	**<.001**	**<.001**	80 (28)	47 (38)	1.18 (.71–1.97)	.523	.775	26 (22)	101 (35)	1.95 (1.14–3.35)	.015	.222
Vulval redness															
No	200 (80)	123 (79)	1			231 (82)	92 (74)	1			93 (79)	230 (80)	1		
Yes	50 (20)	33 (21)	1.27 (.70–2.30)	.437	.885	51 (18)	32 (26)	1.64 (.89–3.00)	.110	.775	24 (21)	59 (20)	1.13 (.62–2.08)	.685	.723
Mucopurulent cervicitis[c]															
No	217 (87)	132 (85)	1			242 (86)	107 (86)	1			98 (84)	251 (87)	1		
Yes	33 (13)	24 (15)	1.11 (.60–2.05)	.732	.911	40 (14)	17 (14)	.99 (.51–1.91)	.975	.975	19 (16)	38 (13)	.75 (.40–1.38)	.354	.713
Cervical or adnexal motion tenderness															
No	198 (79)	124 (79)	1			222 (79)	100 (81)	1			92 (79)	230 (80)	1		
Yes	52 (21)	32 (21)	1.03 (.61–1.74)	.911	.911	60 (21)	24 (19)	.88 (.50–1.54)	.647	.775	25 (21)	59 (20)	.88 (.51–1.51)	.647	.723
Cervical contact bleeding															
No	207 (83)	131 (84)	1			235 (83)	103 (83)	1			95 (81)	243 (84)	1		
Yes	43 (17)	25 (16)	.83 (.47–1.48)	.525	.885	47 (17)	21 (17)	1.11 (.61–2.04)	.728	.775	22 (19)	46 (16)	.80 (.45–1.43)	.451	.723
Vaginal pH															
≤4.5	160 (64)	40 (26)	1			150 (53)	50 (40)	1			65 (56)	135 (47)	1		
>4.5	90 (36)	116 (74)	4.80 (3.00–7.69)	**<.001**	**<.001**	132 (47)	74 (60)	1.17 (.71–1.92)	.546	.775	52 (44)	154 (53)	1.47 (.91–2.37)	.120	.380
Clue cells															
Absent	184 (74)	39 (25)	1			173 (61)	50 (40)	1			72 (62)	151 (52)	1		
Present	66 (26)	117 (75)	8.76 (5.19–14.78)	**<.001**	**<.001**	109 (39)	74 (60)	1.77 (1.02–3.09)	.043	.775	45 (38)	138 (48)	1.73 (1.00–2.98)	.051	.242
Vaginal PMNL cell count															
<5 PMNL/hpf	150 (60)	88 (56)	1			169 (60)	69 (56)	1			67 (57)	171 (59)	1		
≥5 PMNL/hpf	100 (40)	68 (44)	1.14 (.73–1.80)	.559	.885	113 (40)	55 (44)	1.27 (.79–2.03)	.325	.775	50 (43)	118 (41)	.96 (.60–1.52)	.852	.852

Data are presented as n (%), unless otherwise specified.

Reported q-values are *P*-values adjusted for the false discovery rate and q-values < 0.05 are bolded to indicate statistically significant associations.

Abbreviations: aOR, adjusted odds ratio; BV, bacterial vaginosis; *C. trachomatis*, *Chlamydia trachomatis*; CI, confidence interval; *M. genitalium*, *Mycoplasma genitalium*; *M. hominis*, *Mycoplasma hominis*; n, number; *N. gonorrhoea*, *Neisseria gonorrhoeae*; PMNL/hpf, polymorphonuclear leukocyte/high power field; *T. vaginalis*, *Trichomonas vaginalis*; *U. parvum*, *Ureaplasma parvum*; *U. urealyticum*, *Ureaplasma urealyticum*; VVC, vulvovaginal candidiasis.

[a] Multivariable logistic regression adjusted for *C. trachomatis, N. gonorrhoea, M. genitalium, T. vaginalis,* VVC, and common genital mycoplasmas (ie, *M. hominis, U. urealyticum,* and *U. parvum*). As we examined associations with individual Amsel criteria, we did not adjust for BV in order to prevent overfitting the model.

[b] Analysis of clinical signs was restricted to symptomatic women who were assessed for all signs (n = 406)

[c] Mucopurulent cervicitis was defined as the presence of a visible mucopurulent discharge on the cervix or extruding from the cervical os.

studies have failed to consistently associate individual serovars with symptoms or specific clinical outcomes such as preterm birth [12, 32–34]. Furthermore, there is some controversy surrounding the concept of *Ureaplasma* serovars. Xiao et al. [34] serotyped 1061 clinical *Ureaplasma* isolates and found that 39% contained markers for multiple serovars, 6% had no serovar detected, and 4% had discordant species and serovar level results. Analysis of 271 isolates with multiple serovar markers identified 28% as being pure isolates that were hybrids of ≥2 serovars, which suggests that serotyping may have limited utility for determining pathogenicity [34]. Further investigation of pathogenicity at the serovar level is needed. Other factors, such as bacterial load, may be important for pathogenicity. High-load of vaginal *Ureaplasma* spp. ($>10^5$ cfu/mL) has been shown to be an independent risk factor for both chorioamnionitis and preterm delivery [35]. Additionally, it has been reported that *U. urealyticum* load, but not prevalence, is associated with postpartum endometritis ($>10^5$ cfu/mL; 39% vs 17%, $P = .03$) [36]. Assessment of bacterial load, and the subsequent host immune response [37], may be important for understanding the clinical role of *M. hominis*, *U. urealyticum,* and *U. parvum*.

There are limitations to this study. First, our prevalence estimates are higher than previous estimates, which is likely a result of recruiting from a STI clinic population. Women in our study had a higher prevalence of *C. trachomatis* compared to a previous study of women attending primary-care centers in Australia (8% vs 4%) [38], which reflects the higher risk nature of the population, and combined with the single recruitment site, limits the generalizability of our findings. Furthermore, 30% (95%CI:28–33) of our study population had BV [17], which is more than double the most recent prevalence estimates for BV among Australian women attending primary-health care and sexual health clinics (12%; 95%CI:9–14) [39]. As a result, we may have overestimated the relationship between *M. hominis* and BV. Additionally, associations are based on organism detection rather than organism load, and serovar specific associations were not examined. As discussed above, organism load may provide further insight into the contribution of *M. hominis*, *U. urealyticum,* and *U. parvum* to clinical characteristics in nonpregnant women.

To summarize, we found that *M. hominis, U. urealyticum,* and *U. parvum* were common and associated with markers of sexual exposure in a population of nonpregnant women attending a sexual health service. *Mycoplasma hominis* was strongly associated with concurrent BV and was associated with clinical characteristics in women with BV, however it was not associated with symptoms or signs in women without BV. *Ureaplasma urealyticum* and *U. parvum* were not associated with any symptom or sign, or independently associated with BV. These data, that have been adjusted for important confounders, provide evidence against the routine testing and screening of nonpregnant women for *M. hominis*, *U. urealyticum,* and *U. parvum* in a sexual health setting, and highlight the importance of accurately diagnosing the syndrome of BV.

Notes

Financial support. This work was supported by a Victorian Medical Research Acceleration Fund (including salary support for K. B.). Melbourne Sexual Health Centre received funding from SpeeDx Pty Ltd (Australia) in support of this research project. SpeeDx provided diagnostic kits for testing. E. L. P. and R. L. L. were supported by an Australian Government Research Training Program (RTP) Scholarship. C. S. B. and C. K. F. are supported by an Australian NHMRC Leadership Investigator Grant (GNT1173361 and GNT1172900, respectively). E. P. F. C. is supported by an Australian NHMRC Emerging Leadership Investigator Grant (GNT1172873). M. P. is funded by a NHMRC project grant (APP1144040). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Potential conflicts of interest. E. P. F. C. reports investigator grants from National Health and Medical Research Council and Gilead Sciences, outside the submitted work. All other authors declare no conflicts of interest for this project. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Cox C, Watt AP, McKenna JP, Coyle PV. *Mycoplasma hominis* and *Gardnerella vaginalis* display a significant synergistic relationship in bacterial vaginosis. Eur J Clin Microbiol Infect Dis **2016**; 35:481–7.
2. Foschi C, Salvo M, D'Antuono A, et al. Distribution of genital mollicutes in the vaginal ecosystem of women with different clinical conditions. New Microbiol **2018**; 41:225–9.
3. Kim Y, Kim J, Lee KA. Prevalence of sexually transmitted infections among healthy Korean women: implications of multiplex PCR pathogen detection on antibiotic therapy. J Infect Chemother **2014**; 20:74–6.
4. Leli C, Mencacci A, Latino MA, et al. Prevalence of cervical colonization by *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma hominis,* and *Mycoplasma genitalium* in childbearing age women by a commercially available multiplex real-time PCR: An Italian observational multicentre study. J Microbiol Immunol Infect **2018**; 51:220–5.
5. Silva J, Cerqueira F, Teixeira AL, et al. Genital mycoplasmas and ureaplasmas in cervicovaginal self-collected samples of reproductive-age women: prevalence and risk factors. Int J STD AIDS **2018**; 29:999–1006.
6. Lobão TN, Campos GB, Selis NN, et al. *Ureaplasma urealyticum* and *U. parvum* in sexually active women attending public health clinics in Brazil. Epidemiol Infect **2017**; 145:2341–51.
7. Ouzounova-Raykova VV, Markovska R, Mizgova G, Mitov IG. Detection of the sexually transmissible genital mycoplasmas by polymerase chain reaction in women. Sex Health **2011**; 8:445–6.
8. Horner P, Donders G, Cusini M, Gomberg M, Jensen JS, Unemo M. Should we be testing for urogenital *Mycoplasma hominis*, *Ureaplasma parvum,* and *Ureaplasma urealyticum* in men and women?–A position statement from the European STI Guidelines Editorial Board. J Eur Acad Dermatol Venereol **2018**; 32:1845–51.
9. Payne MS, Ireland DJ, Watts R, et al. *Ureaplasma parvum* genotype, combined vaginal colonisation with *Candida albicans*, and spontaneous preterm birth in an Australian cohort of pregnant women. BMC Pregnancy Childbirth **2016**; 16:312.
10. Kataoka S, Yamada T, Chou K, et al. Association between preterm birth and vaginal colonization by mycoplasmas in early pregnancy. J Clin Microbiol **2006**; 44:51–5.
11. Rittenschober-Böhm J, Waldhoer T, Schulz SM, et al. First trimester vaginal *Ureaplasma* biovar colonization and preterm birth: results of a prospective multicenter study. Neonatology **2018**; 113:1–6.
12. Rittenschober-Bohm J, Waldhoer T, Schulz SM, et al. Vaginal *Ureaplasma parvum* serovars and spontaneous preterm birth. Am J Obstet Gynecol **2019**; 220:594 e1–e9.
13. Viscardi RM. *Ureaplasma* species: role in neonatal morbidities and outcomes. Arch Dis Child Fetal Neonatal Ed **2014**; 99:F87–92.
14. Glaser K, Speer CP. Neonatal CNS infection and inflammation caused by *Ureaplasma* species: rare or relevant? Expert Rev Anti Infect Ther **2015**; 13:233–48.
15. Garland SM, Bowman ED. Role of *Ureaplasma urealyticum* and *Chlamydia trachomatis* in lung disease in low birth weight infants. Pathology **1996**; 28:266–9.
16. Garland SM, Murton LJ. Neonatal meningitis caused by *Ureaplasma urealyticum*. Pediatr Infect Dis J **1987**; 6:868–70.

17. Latimer RL, Vodstrcil L, Read T, et al. Oh MG! The symptoms of *Mycoplasma genitalium* in women. Sex Transm Infect **2019**; 95(Suppl 1):A1–A376.
18. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am J Med **1983**; 74:14–22.
19. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol **1991**; 29:297–301.
20. Yekutieli D, Benjamini Y. Resampling-based false discovery rate controlling multiple test procedures for correlated test. J Stat Plan Inference **1999**; 82:171–96.
21. Keane FE, Thomas BJ, Gilroy CB, Renton A, Taylor-Robinson D. The association of *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Mycoplasma genitalium* with bacterial vaginosis: observations on heterosexual women and their male partners. Int J STD AIDS **2000**; 11:356–60.
22. Rumyantseva T, Khayrullina G, Guschin A, Donders G. Prevalence of *Ureaplasma* spp. and *Mycoplasma hominis* in healthy women and patients with flora alterations. Diagn Microbiol Infect Dis **2019**; 93:227–31.
23. Zozaya-Hinchliffe M, Lillis R, Martin DH, Ferris MJ. Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. J Clin Microbiol **2010**; 48:1812–9.
24. Ceccarani C, Foschi C, Parolin C, et al. Diversity of vaginal microbiome and metabolome during genital infections. Sci Rep **2019**; 9:14095.
25. Vitali B, Cruciani F, Picone G, Parolin C, Donders G, Laghi L. Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. Eur J Clin Microbiol Infect Dis **2015**; 34:2367–76.
26. Pollack JD. Carbohydrate metabolism and energy conservation. In: Maniloff J, McElhaney RN, Finch LR, Baseman JB, eds. Mycoplasmas: molecular biology and pathogenesis. Washington D. C: American Society for Microbiology, **1992**:181–200.
27. Pybus V, Onderdonk AB. Evidence for a commensal, symbiotic relationship between *Gardnerella vaginalis* and *Prevotella bivia* involving ammonia: potential significance for bacterial vaginosis. J Infect Dis **1997**; 175:406–13.
28. Muzny CA, Taylor CM, Swords WE, et al. An updated conceptual model on the pathogenesis of bacterial vaginosis. J Infect Dis **2019**; 220:1399–405.
29. Koutsky LA, Stamm WE, Brunham RC, et al. Persistence of *Mycoplasma hominis* after therapy: importance of tetracycline resistance and of coexisting vaginal flora. Sex Transm Dis **1983**; 10:374–81.
30. Pheifer TA, Forsyth PS, Durfee MA, Pollock HM, Holmes KK. Nonspecific vaginitis: role of *Haemophilus vaginalis* and treatment with metronidazole. N Engl J Med **1978**; 298:1429–34.
31. Donders GGG, Ruban K, Bellen G, Petricevic L. *Mycoplasma/Ureaplasma* infection in pregnancy: to screen or not to screen. J Perinat Med **2017**; 45:505–15.
32. Payne MS, Newnham JP, Doherty DA, et al. A specific bacterial DNA signature in the vagina of Australian women in midpregnancy predicts high risk of spontaneous preterm birth (the Predict1000 study). Am J Obstet Gynecol **2021**; 224:206.e1–23.
33. De Francesco MA, Negrini R, Pinsi G, Peroni L, Manca N. Detection of *Ureaplasma* biovars and polymerase chain reaction-based subtyping of *Ureaplasma parvum* in women with or without symptoms of genital infections. Eur J Clin Microbiol Infect Dis **2009**; 28:641–6.
34. Xiao L, Paralanov V, Glass JI, et al. Extensive horizontal gene transfer in ureaplasmas from humans questions the utility of serotyping for diagnostic purposes. J Clin Microbiol **2011**; 49:2818–26.
35. Abele-Horn M, Scholz M, Wolff C, Kolben M. High-density vaginal *Ureaplasma urealyticum* colonization as a risk factor for chorioamnionitis and preterm delivery. Acta Obstet Gynecol Scand **2000**; 79:973–8.
36. Chaim W, Horowitz S, David JB, Ingel F, Evinson B, Mazor M. *Ureaplasma urealyticum* in the development of postpartum endometritis. Eur J Obstet Gynecol Reprod Biol **2003**; 109:145–8.
37. Kasper DC, Mechtler TP, Reischer GH, et al. The bacterial load of *Ureaplasma parvum* in amniotic fluid is correlated with an increased intrauterine inflammatory response. Diagn Microbiol Infect Dis **2010**; 67:117–21.
38. Yeung AH, Temple-Smith M, Fairley CK, et al. *Chlamydia* prevalence in young attenders of rural and regional primary care services in Australia: a cross-sectional survey. Med J Aust **2014**; 200:170–5.
39. Bradshaw CS, Walker J, Fairley CK, et al. Prevalent and incident bacterial vaginosis are associated with sexual and contraceptive behaviours in young Australian women. PLoS One **2013**; 8:e57688.

PLOS ONE

RESEARCH ARTICLE

Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: A systematic review

Erica L. Plummer[1,2]*, Catriona S. Bradshaw[1,2], Michelle Doyle[2], Christopher K. Fairley[1,2], Gerald L. Murray[3,4,5], Deborah Bateson[6,7], Lindi Masson[1,8,9,10,11], Josephine Slifirski[2], Gilda Tachedjian[8,12,13], Lenka A. Vodstrcil[1,2]

1 Central Clinical School, Monash University, Melbourne, Victoria, Australia, **2** Melbourne Sexual Health Centre, Alfred Hospital, Carlton, Victoria, Australia, **3** Women's Centre for Infectious Diseases, The Royal Women's Hospital, Parkville, Victoria, Australia, **4** Murdoch Children's Research Institute, Parkville, Victoria, Australia, **5** Department of Obstetrics and Gynaecology, The University of Melbourne, Parkville, Victoria, Australia, **6** Family Planning New South Wales, Ashfield, New South Wales, Australia, **7** Discipline of Obstetrics, Gynaecology and Neonatology, University of Sydney, Camperdown, New South Wales, Australia, **8** Burnet Institute, Melbourne, Victoria, Australia, **9** Division of Medical Virology, Department of Pathology, University of Cape Town, Cape Town, South Africa, **10** Institute of Infectious Disease and Molecular Medicine (IDM), University of Cape Town, Cape Town, South Africa, **11** Centre for the AIDS Programme of Research in South Africa, Durban, South Africa, **12** Department of Microbiology, Monash University, Clayton, Victoria, Australia, **13** Department of Microbiology and Immunology, University of Melbourne, at the Peter Doherty Institute of Infection and Immunity, Melbourne, Victoria, Australia

* erica.plummer@monash.edu

Citation: Plummer EL, Bradshaw CS, Doyle M, Fairley CK, Murray GL, Bateson D, et al. (2021) Lactic acid-containing products for bacterial vaginosis and their impact on the vaginal microbiota: A systematic review. PLoS ONE 16(2): e0246953. https://doi.org/10.1371/journal.pone.0246953

Editor: Lisa Susan Wieland, University of Maryland School of Medicine, UNITED STATES

Received: September 22, 2020

Accepted: January 28, 2021

Published: February 11, 2021

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: ELP was supported by an Australian Government Research Training Program (RTP) Scholarship. CSB and CKF are supported by an Australian NHMRC Leadership Investigator Grant (GNT1173361 and GNT1172900, respectively). GT was supported by NHMRC Senior Research Fellowship (GNT1117748) and NHMRC Project

Abstract

Objective

The vaginal microbiota in bacterial vaginosis (BV) typically has low abundance of lactic acid producing lactobacilli. Lactic acid has properties that may make it effective for treating BV and/or restoring an optimal lactobacillus-dominated vaginal microbiota. We conducted a systematic review to describe the effect of intravaginal lactic acid-containing products on BV cure, and their impact on vaginal microbiota composition (PROSPERO registration: CRD42018115982).

Methods

PubMed, Embase and OVID were searched from inception to November 2019 to identify eligible studies. Included studies evaluated an intravaginal lactic acid-containing product and reported BV cure using established diagnostic methods, and/or vaginal microbiota composition using molecular methods. Studies were independently screened and assessed, and the proportion of women cured post-treatment was calculated. Study results were described in a qualitative manner.

Results

We identified 1,883 articles and assessed 57 full-texts for eligibility. Seven different lactic acid-containing products were evaluated and differed with respect to excipients, lactic acid

Grant (GNT1164982). LAV was supported by NHMRC Project Grant (APP1138165). GT, CSB, CKF, DB are supported by Medical Research Future Fund (MRFF) Frontier in Health and Medical Research Program Stage One (MRFF75913). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: GT is a coinventor on patent application AU201501042 and United States Patent No. US 9,801,839 B2 claiming the anti-inflammatory effects of lactic acid. No other authors report a conflict of interest.

concentration and pH. Most studies had medium or high risk of bias. Three trials compared the efficacy of a lactic acid-containing product to metronidazole for BV cure. One study found lactic acid to be equivalent to metronidazole and two studies found lactic acid to be significantly inferior to metronidazole. Two studies included a control group receiving a placebo or no treatment. One reported lactic acid to be superior than no treatment and the other reported lactic acid to be equivalent to placebo. Lactic acid-containing products did not significantly impact the vaginal microbiota composition.

Conclusion

There is a lack of high-quality evidence to support the use of lactic acid-containing products for BV cure or vaginal microbiota modulation. However, adequately powered and rigorous randomised trials with accompanying vaginal microbiota data are needed to evaluate the efficacy of lactic acid as a BV treatment strategy.

Introduction

Bacterial vaginosis (BV) is the commonest vaginal condition in reproductive aged women. BV is associated with serious sequelae including miscarriage, preterm birth and pelvic inflammatory disease, and acquisition of sexually transmitted infections including HIV [1–5]. Recommended first-line treatments for BV are oral or intravaginal metronidazole and intravaginal clindamycin [6]. First-line treatments have equivalent four-week cure rates of ~70–85% [7], but BV recurrence is common [8, 9]. Recurrences negatively impact a woman's quality of life [10] and result in repeated clinical presentations and antibiotic use. Given the significant sequelae, treatments that improve BV cure are needed.

The optimal vaginal microbiota of reproductive aged women is typically characterised by dominance of lactic acid producing *Lactobacillus* species including *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii* [11–16]. Women with BV have reduced abundance of these lactobacilli and increased prevalence and abundance of anaerobic and facultative-anaerobic bacteria [13, 14]. *In vitro* studies have shown that lactic acid inactivates BV-associated bacteria [17] and pathogens including *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and HIV via mechanisms independent of acidity alone [18–21]. Lactic acid has also been shown to modulate cervicovaginal epithelial cell functions to prevent *C. trachomatis* infection [22]. Lactic acid also has immunomodulatory effects [23], and can elicit an anti-inflammatory response and reduce production of inflammatory cytokines and chemokines from cervicovaginal epithelial cells *in vitro* [24].

The antimicrobial and immunomodulatory properties of lactic acid may make it effective for the treatment of BV and/or to restore an optimal microbiota following antibiotic treatment [23]. Lactic acid-containing products have been evaluated for BV treatment in clinical trials, and several over-the-counter lactic acid-containing products are marketed to treat BV or support optimal vaginal microbiota. However, the use of these products is not recommended by any treatment guidelines [6].

We conducted a systematic review with two objectives: 1) to describe the effect of intravaginal lactic acid-containing products for BV cure (assessed using an established diagnostic method), and 2) to describe the impact of intravaginal lactic acid-containing products on the vaginal microbiota (assessed using molecular methods).

Materials and methods

We conducted and reported this systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis statement [25] (S1 File), and registered the protocol prospectively with PROSPERO (CRD42018115982).

Search strategy, eligibility criteria

We searched electronic databases (PubMed, Embase, OVID Medline) from inception until 4th November 2019 using keywords: "bacterial vaginosis", "vaginal microbiota" and "lactic acid" (search strings in S1 Table). Reference lists and conference abstracts were searched for additional studies. Conference abstracts were included if they reported adequate information. Studies were uploaded to Covidence (Veritas Health Innovation, Melbourne, Australia, www.covidence.org) and were independently reviewed for eligibility by three authors (EP, JS, MD). Disagreements were resolved with a fourth author (LV).

Studies were eligible for objective 1 (BV cure outcome) if they assessed an intravaginal lactic acid-containing product as the main or adjuvant therapy for BV cure in women diagnosed with BV. BV had to be diagnosed using an established method (e.g. Amsel criteria or modified Amsel criteria [26], Nugent Score [NS] [27] or Ison-Hay method [28]). Studies were eligible if they were randomised controlled trials (RCT) where an intravaginal lactic acid-containing product was assessed in comparison to either no treatment, a placebo or a recommended antibiotic treatment for BV. No restrictions were placed on number of participants enrolled. Studies of pregnant women and post-menopausal women were excluded.

Studies were eligible for objective 2 (vaginal microbiota outcome) if they reported use of an intravaginal lactic acid-containing product in women with or without BV, and assessed the vaginal microbiota using a molecular method such as quantitative PCR (qPCR) or high throughput sequencing. In order to capture all published literature evaluating the impact of lactic acid-containing product on the vaginal microbiota composition, no restrictions were placed on study design, number of participants enrolled, age, menopause or pregnancy status.

For both objectives, we excluded studies if they were performed on animals or the data was not stratified by lactic acid-containing product use. Only English language studies were included.

Interventions assessed

Assessed interventions included any intravaginal lactic acid-containing product. Interventions were excluded if they contained lactic acid producing bacteria or were not used intravaginally.

Outcome measures

Outcome measures were: 1) BV cure defined as $\leq$2 Amsel criteria and/or NS<4, or Ison-Hay grade 1 measured $\geq$7 days after the start of treatment, 2) vaginal microbiota composition assessed by molecular methods, and 3) occurrence of adverse events.

Data extraction

Three authors (EP, JS, MD) independently extracted the following information for each study: author details, publication year, study design, population studied, intervention details, comparator details, follow-up duration, BV diagnostic method, BV cure definition, microbiota characterisation methodology, adverse events and study findings. Disagreements in extracted data were resolved by discussion between authors. Two corresponding authors were contacted for additional details, one responded [29].

Data analysis

For objective 1 (BV cure outcome), we calculated the proportion of women cured post-treatment per treatment group with 95% confidence intervals, and described results in a narrative manner. For objective 2 (vaginal microbiota outcome), the impact of lactic acid-containing products on the vaginal microbiota was described narratively. Where an article reported ≥2 lactic acid-containing products or treatment regimens, each product/regimen was presented separately in tables.

Assessment of bias

Two authors (EP, MD) independently assessed the risk of bias of each study using a modified version of the RoB 2.0 [30] and ROBINS-I tools [31] (S2 Table). The level of overall risk was summarised across six domains: selection bias, performance bias, measurement bias, response bias, reporting bias and other sources of bias (i.e. adjustment for confounders and insufficient description of product details). Studies were not excluded based on bias assessment.

Results

Study selection

Our initial search identified 1882 articles. One additional article was identified from reference lists. Following duplicate removal, 1591 articles were screened on title and abstract. We excluded 1534 articles and assessed 57 full-text articles. Fifty articles were excluded; seven of which evaluated a lactic acid-containing product for BV treatment but were excluded because they were non-randomised (n = 5), did not use standard criteria to assess BV cure (n = 1) or assessed BV-recurrence only (n = 1; S3 Table). Seven articles were included in the review (Fig 1).

Lactic acid-containing products evaluated

Seven different lactic acid-containing products were evaluated and differed with respect to lactic acid concentration, pH and included ingredients/excipients (Table 1). Two intravaginal gels were evaluated in three studies: Acidform was evaluated in two studies [32, 33] and Lactal was evaluated in one study [34]. Four different vaginal pessaries were evaluated in three studies [29, 35, 36] and a vaginal douche (Etos®) was evaluated in one study [37]. Excipients were not reported in one study [36]. Lactic acid isomer details were only located for Acidform, which comprises L-lactic acid [38].

Intravaginal lactic acid-containing products for BV cure

Four RCTs assessed the efficacy of an intravaginal lactic acid-containing product for BV cure (Table 2) [32, 34–36].

Andersch *et al.* [34] randomised women to receive once nightly Lactal gel (lactic acid concentration not specified) for seven days or twice daily (*bid*) oral metronidazole for seven days. No details of allocation concealment, implementation of randomisation or blinding of participants and/or Amsel outcome assessors were provided (Fig 2). One week after the start of treatment (i.e. immediately post-treatment), all women in both groups had ≤2 of 3 Amsel criteria (Table 2); 77% (n = 24/31) of women receiving Lactal and 76% (n = 13/17) of women receiving metronidazole were negative for all criteria assessed (positive amine test, clue cells, pH≥5.0). No adverse events were reported (S4 Table).

In a multicentre RCT, Boeke *et al.* [35] randomised women to receive either nightly lactic acid pessary (100mg lactic acid/pessary) plus oral placebo *bid* for seven days, oral

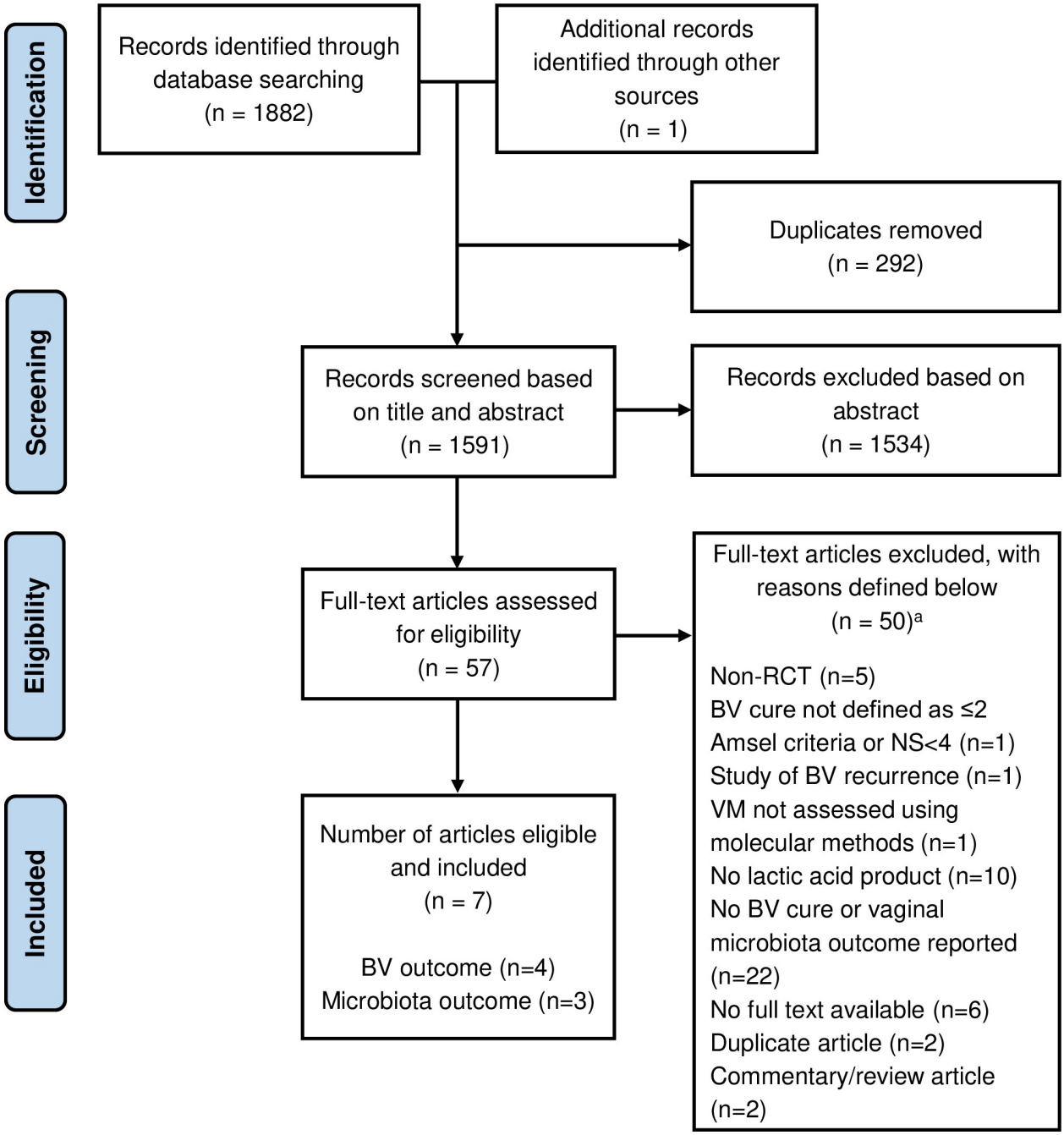


Fig 1. PRISMA flow diagram describing the literature search and article selection process. [a] Detailed reasons for exclusion are provided in S3 Table.

https://doi.org/10.1371/journal.pone.0246953.g001

metronidazole *bid* plus nightly placebo pessary for seven days, or oral placebo *bid* plus nightly placebo pessary for seven days. No details of allocation concealment or blinding were provided (Fig 2). Cure was assessed at three time points (2-weeks, 4-weeks and 3-months after the start of treatment) using ≤2 of 4 Amsel criteria and an additional author definition called 'strict' cure (absence of: positive amine test, clue cells, pH>4.5). Two weeks after the start of treatment, 49% (n = 18/37) of women receiving lactic acid, 83% (n = 35/42) of women receiving

Table 1. Lactic acid-containing product details.

Product name, formulation, reference	Lactic acid details	pH	Other ingredients and excipients including preservatives
Acidform[a] intravaginal gel [32, 33]	88mg (1.76%) per dose L-lactic acid isomer	3.55	50 mg (1%) citric acid, 20 mg (0.4%) potassium bitartrate, benzoic acid, alginic acid, xanthan gum, glycerin, sodium hydroxide and water in a 5mg dose [38]
Lactal intravaginal gel [34] [b]	NR	3.5	Growth substrates for lactobacilli
Lactic acid pessary [35]	100mg lactic acid per pessary	3.3	2.4g of polyethylene glycol 1540
Vagisan®, vaginal pessary [29]	40mg lactic acid per pessary	~4.5	Macrogol 1500, macrogol 6000 and sodium lactate
WO3191, vaginal pessary [29]	Total lactic acid conc. of ~3.9% total weight	~4.5	Cocoamphopropionate (amphoteric tenside), sodium lactate
Sustained release oligomeric lactic acid (OMLA) pessary [36]	700mg lactic acid per pessary[c]	pH 3.5	NR
Etos® vaginal douche [37]	Neat lactic acid conc. 0.45%, diluted conc. 0.06% (1 in 7 dilution)	Neat pH 3.42, diluted pH 3.50 (1 in 7 dilution)	Aqua, butylene glycol, caprylyl glycol, sodium pyroglutamic acid, Zea mays kernel extract, hydrolyzed milk protein, niacinamide, and adenosine triphosphate

NR, not reported.

[a] Also known as Amphora.

[b] Reference [39] states that Lactal gel is the lactic acid-containing product in Andersch *et al.* [34].

[c] Designed to release lactic acid over a 72hr period.

https://doi.org/10.1371/journal.pone.0246953.t001

metronidazole and 47% (n = 16/34) of women receiving dual placebo had ≤2 Amsel criteria (Table 2). When assessed according to the authors definition of strict cure, none of the women receiving lactic acid pessary, 10% of women receiving metronidazole and 3% of women receiving dual placebo were cured. Similar findings were reported 4 weeks and 3-months after start of treatment (Table 2). There was no difference in adverse events reported between the three randomisation groups. Of 33 women receiving lactic acid, four reported gastrointestinal symptoms, three reported genital irritation and one reported headache/vertigo (S4 Table).

In a double-blind pilot RCT, Simoes *et al.* [32] randomised women to receive either once daily Acidform gel (an acid buffering contraceptive gel, 88mg lactic acid/5g) for five days (n = 13) or once daily 10% metronidazole intravaginal gel for five days (n = 17). Randomisation was performed by the product manufacturer and researchers were provided with product tubes labelled with participant numbers so that the randomisation group was double-blinded to the researchers and participants (Fig 2). At 7–12 days post-treatment, 23% (n = 3/13) of women receiving Acidform and 88% (n = 15/17) women receiving metronidazole were cured (≤2 Amsel criteria). At 28–35 days post-treatment, the percent of women cured decreased to 8% (n = 1/13) in the Acidform group and 53% (n = 9/17) in the metronidazole group (Table 2). Four women receiving Acidform and one woman receiving metronidazole reported genital irritation (S4 Table).

Fredstorp *et al.* [36] evaluated a sustained release oligomeric lactic acid (OMLA) pessary in a two part multicentre study. Part A of the study is not included in this review as there was no control group. In Part B, women were randomised to receive either OMLA pessary applied once per week for one week, OMLA pessary applied twice per week for a week or no treatment. Block randomisation was performed according to a computer-generated randomisation list, with block size blinded to the investigators. Sites were provided with coded envelopes, and the study was open-label (Fig 2). After one week of pessary use, 71% (n = 24/34) of women receiving once-weekly pessary, 80% (n = 28/35) of women receiving twice-weekly pessary and 10% (n = 3/30) of women receiving no treatment had ≤2 of 3 Amsel criteria (Table 2). Vaginal

Table 2. Key findings of included studies.

Objective 1: Studies assessing BV cure

Reference	Study design	Intervention	Comparator	No. women randomised	Outcome measure	Duration of follow-up	BV cure results in intervention vs comparator
Andersch, 1986 [34]	RCT[a]	Lactal gel 5ml PV/night x 7 nights	Oral MTZ 500 mg bid x 7 days	Lactal = 32	≤2 of 3 Amsel criteria[b]	1 week after start of treatment	31/31 (100%, 95% CI 89–100) *vs*
				MTZ = 22			17/17 (100%, 95% CI 90–100)
Boeke, 1993 [35]	RCT[a]	Oral placebo bid x 7 days and lactic acid vaginal suppository/night x 7 days	Two comparator groups:	Lactic acid = 41[c]	≤2 of 4 Amsel criteria	2 weeks after start of treatment	18/37 (49%, 95%CI 32–66) *vs*
				MTZ = 44			35/42 (83%, 95%CI 69–93)
			1) Oral MTZ 500 mg bid x 7 days and placebo vaginal suppository/night x 7 days	Placebo = 40			16/34 (47%, 95%CI 30–65)
						4 weeks after start of treatment	11/33 (33%, 95%CI 18–52) *vs*
							27/38 (71%, 95%CI 54–85)
			2) Oral placebo bid x 7 days and placebo vaginal suppository/night x 7 days				12/35 (34%, 95%CI 19–52)
						3 months after start of treatment	12/32 (38%, 95%CI 21–56) *vs*
							29/37 (78%, 95%CI 62–90)
							11/32 (34%, 95%CI 19–53)
Simoes, 2006 [32]	Double-blind RCT	Acidform gel 5g PV/day x 5 days	10% MTZ gel PV/day x 5 days	Acidform = 13	≤2 of 4 Amsel criteria	12–17 days after start of treatment	3/13 (23%, 95%CI 5–54) *vs*
				MTZ = 17			15/17 (88%, 95%CI 64–99)
						33–40 days after start of treatment	1/13 (8%, 95%CI 0–36) *vs*
							9/17 (53%, 95%CI 28–77)
Fredstorp, 2015 [36]	Open-label RCT	Two intervention groups:	Untreated control group	Once/week = 37	≤2 of 4 Amsel criteria	1 week after start of treatment	24/34 (71%, 95%CI 53–85)
				Twice/ week = 35			28/35 (80%, 95%CI 63–92) *vs*
		1) OMLA pessary applied once/ week for 1 week		Control = 33			3/30 (10%, 95%CI 2–27)
		2) OMLA pessary applied twice/ week for 1 week[d]					

Objective 2: Studies assessing vaginal microbiota composition

Reference	Study design	Intervention	Comparator	No. women randomised	Outcome measure	Reported results
Keller, 2012 [33]	Single-blind RCT	Acidform gel 5g PV bid x 14 days	HEC placebo gel PV bid x 14 days	Acidform = 18	qPCR assays:	In 35[e] women without BV, no significant changes were observed in the prevalence or concentration of *L. crispatus*, *L. jensenii*, *Megasphaera* (type 1 & type 2) or BVAB2 following 14 days of gel use in either the Acidform or placebo group (compared to baseline values).
				Placebo = 18	*L. crispatus*	
					L. jensenii	
					G. vaginalis	
					Megasphaera (type 1 & type 2)	
					BVAB2	There was a non-significant trend towards a decrease in *G. vaginalis* concentration in the Acidform group following 14 days of gel use compared to baseline (median of 1.36x106 to 3.66x104 DNA copies/ swab, p = 0.083), but not in the placebo group (median of 9.8x105 to 4.4x106 DNA copies/swab, p-value not reported).

(*Continued*)

Table 2. (Continued)

Gottschick, 2017 [29] [f]	Double-blind RCT	Oral MTZ 2g single dose. After 7–28 days, WO3191 pessary applied twice-weekly x 3 weeks	Oral MTZ 2g single dose. After 7–28 days, Vagisan® pessary applied twice-weekly x 3 weeks	WO3191 = 18 Vagisan® = 26	16S rRNA gene sequencing of V1-V2 regions	In 36[g] women initially treated for BV with oral metronidazole, no significant changes in vaginal microbiota composition were reported during or following use of either WO3191 or Vagisan®.
						The cumulative relative abundance of *Lactobacillus* spp. (*L. crispatus*, *L. iners* and *L. gasseri*) was 73% in the WO3191 group prior to starting pessary use, 77% after 3 weeks of pessary use, and had decreased to 59% 12–14 weeks after last pessary use.
						The cumulative relative abundance of *Lactobacillus* spp. was 75% in the Vagisan® group prior to starting pessary use, 69% after 3 weeks of pessary use, and 73% 12–14 weeks after last pessary use. There was a non-significant increase in the relative abundance of *L. crispatus* in Vagisan® group from 18% prior to starting pessary use to 33% 12–14 weeks after last pessary use.
						There was no difference in microbiota diversity (as measured by Shannon diversity index) between women randomised to WO3191 and women randomised to Vagisan®.
van der Veer, 2019 [37]	Single arm prospective cohort	Participants were followed for 3 menstrual cycles. Etos® douche was applied 3/per week for duration of cycle 2 starting on day 1 of menses.	NA	29	16S rRNA gene sequencing of V3-V4 regions	In 25[h] women without BV there was a non-statistically significant increased odds of having a diverse anaerobic vaginal microbiota relative to an *L. crispatus* microbiota during (odds ratio: 1.4; 95% CI 0.9–2.1) and after douching with Etos® (odds ratio: 1.7; 95%CI 0.9–3.1), compared to before douching, following adjustment for menses.
						Douching with Etos® had no effect on microbiota diversity as measured by Shannon diversity index.

No., number; BV, bacterial vaginosis; RCT, randomised controlled trial; PV, intravaginal; MTZ, metronidazole; bid, twice a day; OMLA, oligometric lactic acid; CI, confidence interval; qPCR, quantitative PCR; NA, not applicable.

[a] Details of blinding not provided.

[b] The three criteria evaluated were: positive amine test, clue cells, pH≥5.0.

[c] 168 women randomised, but post-randomisation, 43 women were found to be ineligible and excluded, thus randomisation numbers presented reflect the 125 eligible women included in analyses.

[d] OMLA pessary is designed to release lactic acid over a 72hr period.

[e] One woman allocated to Acidform did not receive the intervention.

[f] Both the intervention (WO3191) and the comparator (Vagisan®) contain lactic acid.

[g] 36 women were included in microbiota analyses, n = 13 receiving WO3191 and n = 23 receiving Vagisan®.

[h] Twenty-nine women were recruited, four were excluded and 25 women completed the study.

https://doi.org/10.1371/journal.pone.0246953.t002

itching was the most common adverse event and was reported by 11 women receiving OMLA pessary, by five applying the pessary once/week and by six applying the pessary twice/week. Two women receiving OMLA pessary had a yeast infection, and vaginal irritation and genital

burning sensation were both reported by >1 woman (exact numbers and group not provided; S4 Table). Adverse events were not recorded from control participants.

Impact of intravaginal lactic acid-containing products on the vaginal microbiota composition

Three studies reported a measure of vaginal microbiota composition (Table 2) [29, 33, 37].

Keller *et al.* [33] evaluated the safety of Acidform gel *bid* (88mg lactic acid/5g) compared to HEC placebo gel *bid* in 35 sexually abstinent non-pregnant women without BV. Women were randomised 1:1 by a pharmacist. Though the treatments were not identical in appearance, participants were not informed of their allocation and laboratory personnel assessing the outcome were blinded (Fig 2). The change in prevalence and concentration of five bacteria after 14 days of gel use was assessed by qPCR. There were no significant changes in vaginal microbiota

Fig 2. Risk of bias assessment. + indicates a low risk of bias, -/+ indicates moderate risk of bias,—indicates high risk of bias,? indicates unknown risk, NA indicates bias is not applicable to the study. [a] Other sources of bias include whether confounders were appropriately accounted for and whether lactic acid-containing product details were sufficiently described in the manuscript. [b] One study disclosed receipt of funding from the lactic acid-containing product manufacturer. [c] Single arm prospective cohort study.

https://doi.org/10.1371/journal.pone.0246953.g002

composition following 14 days of either Acidform or placebo (Table 2). A non-significant trend towards decreased *Gardnerella vaginalis* concentration following Acidform use was reported. Five women receiving Acidform reported vulvar itching, four reported vaginal or vulvar burning and three reported abdominal cramping (S4 Table). Two women receiving placebo reported vaginal or vulvar itching.

Gottschick *et al.* [29] evaluated the safety, tolerability and efficacy of a biofilm disrupting agent (cocoamphopropionate) administered as a pessary (WO3191, which contains lactic acid at 3.9% of total pessary weight) in a double-blind RCT. Forty-four non-pregnant women were randomised to receive either WO3191 or Vagisan® (40mg lactic acid/pessary) 7–28 days after treatment for BV with 2g single dose oral metronidazole (Table 2). No details of randomisation or allocation concealment were provided (Fig 2). Microbiota results (assessed by 16S rRNA gene sequencing) were reported for 36 women (WO3191 n = 13 and Vagisan® n = 23). No significant changes in vaginal microbiota composition were observed during or following use of either pessary. No safety concerns were identified for either pessary (S4 Table).

In an open-label non-comparative pilot study, van de Veer *et al.* [37] evaluated the impact of a lactic acid-containing douche (Etos®, 0.06% lactic acid when diluted for use) on the vaginal microbiota composition of 25 non-pregnant reproductive aged women without BV (Table 2). Etos® did not significantly impact the vaginal microbiota composition (assessed by 16S rRNA gene sequencing). The study reported non-significant increased odds for having a diverse anaerobic vaginal microbiota during and after douching with Etos®, following adjustment for menses. Five women reported dryness and 2 reported an increase in vaginal symptoms post douching (S4 Table).

Adverse events

No major safety concerns were reported (S4 Table). Vaginal or vulvar irritation, itching, burning, redness and/or dryness were recorded in women receiving a lactic acid-containing product in five of the seven studies. Minimal differences in adverse events between lactic acid-containing product and control randomisation groups were reported.

Risk of bias of included studies

Risk of bias assessment is in Fig 2. Only one RCT evaluating a lactic acid-containing product for BV cure was double-blinded [32] and only one study had low bias across all six domains [33].

Two studies assessing BV cure reported sample size calculations [35, 36] and one reached the required sample size [36]. Four studies measured treatment adherence; one study reported these results [33]. An additional study reported comparable treatment adherence across intervention groups, but did not provide raw data [35].

Discussion

The efficacy of lactic acid-containing products for BV cure and their impact on the vaginal microbiota composition has not been extensively evaluated. We identified four RCTs that investigated the use of intravaginal lactic acid-containing products for BV cure and three studies that investigated the impact of lactic acid-containing products on the vaginal microbiota. Most studies were small and underpowered, had medium-high risk of bias, and the time-point at which cure was measured differed between studies. Three studies compared a lactic acid-containing product to a first-line BV treatment: one reported lactic acid to have equivalent efficacy to metronidazole and two reported lactic acid to be inferior to metronidazole. Two studies included a placebo or no treatment control group: one reported lactic acid to be superior to

no treatment and the other reported lactic acid to be equivalent to placebo. Minimal effects of lactic acid-containing products on the vaginal microbiota were reported. New treatments are needed to improve BV cure and the use of lactic acid is supported by *in vitro* evidence [17, 23]. However, there is limited high-quality *in vivo* evidence that supports the use of lactic acid for BV cure or modulating the vaginal microbiota. Large rigorous trials of well evaluated lactic acid-containing products with long-term follow-up and accompanying microbiota data are needed.

The lactic acid-containing products assessed varied with respect to lactic acid concentration, pH, formulation (i.e. gel, pessary, douche) and excipients. Women with lactobacillus-dominated vaginal microbiota (defined as NS = 0–3) have an average vaginal lactic acid concentration of approximately 0.79–1% and pH of 3.45–4.12 [40, 41]. Some products had a lactic acid concentration or pH outside of these ranges, and no study reported the concentration of lactic acid released into the vagina. Thus, it is not clear if biologically active levels of lactic acid were achieved, which may have impacted on treatment efficacy. Functional effects of lactic acid *in vitro* are usually observed within concentration ranges of 0.30–1% and pH of 3.45–4.12 [18, 40, 41], and are mediated by the uncharged protonated form of lactic acid which predominates at pH$\leq$3.86 [17, 18, 24]. Accordingly, biological effects diminish as lactic acid levels decrease and pH increases. For example, at <0.3% lactic acid and pH$\geq$4.2, the HIV virucidal activity [18] and immunomodulatory effects [24] of lactic acid decrease. Additionally, while 1% lactic acid at pH 4.5 reduces the viability of BV-associated bacteria approximately 10^6-fold, a negligible reduction is observed with 0.1% lactic acid [17]. The lactic acid concentration and vaginal pH maintained after dosing are likely to be critical for achieving biological effects *in vivo*.

Other important characteristics of lactic acid-containing products need consideration, including lactic acid isomer and product osmolality. Lactic acid exists in two isomers: D- and L-lactic acid, and *Lactobacillus* spp. differ in their ability to produce each isomer. For example, *in vitro*, *L. crispatus* and *L. gasseri* produce both isomers, *L. jensenii* produces only D-lactic acid and *L. iners* produces only L-lactic acid [42]. The protective effects of *L. crispatus* compared to *L. iners* are partly attributed to the ability of *L. crispatus* to produce D-lactic acid [23]. It is hypothesised that D-lactic acid affords more protection than L-lactic acid against upper genital tract infections [42]; however, this has not been studied in the context of BV. Isomer information was available for one product included in this review. In order to understand the relative contribution of each isomer to the inactivation of BV-associated bacteria, future studies of products under evaluation for BV treatment or vaginal microbiota modulation should report the L-/D-isomer ratio. Additionally, no study reported product osmolality. This is relevant because hyperosmolal products are likely to damage vaginal epithelium [43, 44]. Vaginal and vulvar irritation were commonly reported adverse events in women using lactic acid-containing products, and may be related to product osmolality and/or excipients or other ingredients (e.g. citric acid). Adverse events should be monitored following intravaginal lactic acid use.

Minimal changes in vaginal microbiota composition following lactic acid-containing product use were reported. Two of the three studies evaluating microbiota composition recruited women without BV and the third study assessed women recently treated with oral metronidazole. Thus, one might expect the impact of lactic acid on the vaginal microbiota composition of these women to be minimal. The non-significant association of Etos® douche with non-optimal vaginal microbiota composition [45] may be a result of the douching action rather than an adverse impact of lactic acid, highlighting the importance of product formulation. Douching has been associated with increased risk of BV-associated bacteria detection [46], as well as increased risk of intermediate-BV and Nugent-BV by meta-analysis [4]. However,

whether douching introduces BV-associated bacteria, depletes optimal lactobacilli, or modifies the vaginal environment such that BV-associated bacteria growth is favoured is unknown.

This review has limitations. The 2019 Food and Drug Administration (FDA) guidelines for developing BV treatments recommends that clinical cure be defined as the absence of 3 Amsel criteria, specifically resolution of vaginal discharge, a negative whiff test and clue cells <20% per high-power field on wet mount [47]. In clinical practice, BV is typically diagnosed as the presence of ≥3 Amsel criteria [6], recurrence is defined as the presence of ≥3 criteria [48] and cure is reported as ≤2 criteria. Based on international clinical practice and published studies, we defined BV cure as the presence of ≤2 Amsel criteria (and/or NS<4, although no included study reported cure using NS) and not by the 2019 FDA guidelines. Additionally, only two studies assessed cure at a timepoint recommended by FDA guidelines [32, 35]. The FDA guidelines recommend cure be assessed 7–14 days post-randomisation for topical drugs administered for a short period of time (i.e. 1–2 days) or 21–30 days post-randomisation for topical drugs that that are administered for a longer period of time (i.e. 1 week) [47]. Follow-up was limited to immediately post-treatment in two studies [34, 36], which is not only likely to be too soon after treatment cessation to adequately assess cure, it also prevented our assessment of the long-term efficacy and safety of lactic acid-containing products. If lactic acid is effective it is likely to be most effective when used as adjunctive therapy with antibiotics [23] and/or when used as sustained release or as periodic presumptive therapy, as has been shown with biweekly suppressive use of 0.75% metronidazole gel [49]. Finally, our search was restricted to English-language records which excluded at least one study [50].

Other lactic acid-containing products are available over-the-counter but were either not identified through our systematic search of published literature or were ineligible for inclusion in our review. An RCT of 1,900 women comparing the clinical and cost effectiveness of intravaginal lactic acid gel to oral metronidazole for BV is currently ongoing [51] (ISRCTN14161293). The primary outcome is patient reported resolution of BV symptoms 14-days post-randomisation. Initial qualitative data from ISRCTN14161293 indicates women prefer lactic acid gel to antibiotics for mild BV episodes despite lower perceived efficacy [52], supporting the need to further investigate lactic acid-containing products for BV.

Conclusions

New treatments are needed to improve BV cure, reduce associated sequelae and improve antibiotic stewardship. *In vitro* data suggest that lactic acid may be effective for BV treatment; however, high-quality evidence supporting the use of lactic acid-containing products for BV and modification of the vaginal microbiota is lacking. Large, rigorous randomised trials of lactic acid-containing products that have been carefully evaluated with respect to pH, lactic acid concentration, L-/D-isomer ratio and osmolality are needed. Future studies should include standardised clinical endpoints, standardised timing of endpoint measurement, assessment of adverse events, long-term follow-up of participants and accompanying high-resolution vaginal microbiota data.

Supporting information

S1 File. PRISMA checklist.
(DOC)

S1 Table. Database search strings.
(DOCX)

S2 Table. Bias assessment tool.
(DOCX)

S3 Table. Full text articles excluded and reasons for exclusion.
(DOCX)

S4 Table. Adverse events reported in included studies.
(DOCX)

Acknowledgments

The authors would like to thank Dr Anna Hearps for their reading of the manuscript.

Author Contributions

Conceptualization: Erica L. Plummer, Catriona S. Bradshaw, Lenka A. Vodstrcil.

Data curation: Erica L. Plummer.

Formal analysis: Erica L. Plummer, Michelle Doyle, Josephine Slifirski, Lenka A. Vodstrcil.

Investigation: Erica L. Plummer, Catriona S. Bradshaw, Michelle Doyle, Josephine Slifirski, Lenka A. Vodstrcil.

Methodology: Erica L. Plummer, Catriona S. Bradshaw, Gilda Tachedjian, Lenka A. Vodstrcil.

Project administration: Erica L. Plummer.

Supervision: Catriona S. Bradshaw, Christopher K. Fairley, Gerald L. Murray, Lenka A. Vodstrcil.

Visualization: Erica L. Plummer, Catriona S. Bradshaw, Christopher K. Fairley, Gerald L. Murray, Deborah Bateson, Lindi Masson, Gilda Tachedjian, Lenka A. Vodstrcil.

Writing – original draft: Erica L. Plummer, Catriona S. Bradshaw, Gilda Tachedjian, Lenka A. Vodstrcil.

Writing – review & editing: Erica L. Plummer, Catriona S. Bradshaw, Michelle Doyle, Christopher K. Fairley, Gerald L. Murray, Deborah Bateson, Lindi Masson, Josephine Slifirski, Gilda Tachedjian, Lenka A. Vodstrcil.

References

1. Brotman RM, Klebanoff MA, Nansel TR, Yu KF, Andrews WW, Zhang J, et al. Bacterial vaginosis assessed by Gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. J Infect Dis. 2010; 202(12):1907–15. https://doi.org/10.1086/657320 PMID: 21067371
2. Koumans EH, Markowitz LE, Berman SM, St Louis ME. A public health approach to adverse outcomes of pregnancy associated with bacterial vaginosis. Int J Gynaecol Obstet. 1999; 67 Suppl 1:S29–33. https://doi.org/10.1016/s0020-7292(99)00136-8 PMID: 10661734
3. Myer L, Kuhn L, Stein ZA, Wright TC Jr., Denny L. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. Lancet Infect Dis. 2005; 5(12):786–94. https://doi.org/10.1016/S1473-3099(05)70298-X PMID: 16310150
4. Low N, Chersich MF, Schmidlin K, Egger M, Francis SC, van de Wijgert JH, et al. Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. PLoS Med. 2011; 8(2):e1000416. https://doi.org/10.1371/journal.pmed.1000416 PMID: 21358808
5. Cohen CR, Lingappa JR, Baeten JM, Ngayo MO, Spiegel CA, Hong T, et al. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. PLoS Med. 2012; 9(6):e1001251. https://doi.org/10.1371/journal.pmed.1001251 PMID: 22745608

6. Workowski KA, Bolan GA, Centers for Disease C, Prevention. Sexually transmitted diseases treatment guidelines, 2015. MMWR Recomm Rep. 2015; 64(RR-03):1–137. PMID: 26042815

7. Oduyebo OO, Anorlu RI, Ogunsola FT. The effects of antimicrobial therapy on bacterial vaginosis in non-pregnant women. Cochrane Database Syst Rev. 2009(3):CD006055. https://doi.org/10.1002/14651858.CD006055.pub2 PMID: 19588379

8. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB, Moss LM, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. J Infect Dis. 2006; 193(11):1478–86. https://doi.org/10.1086/503780 PMID: 16652274

9. Sobel JD, Schmitt C, Meriwether C. Long-term follow-up of patients with bacterial vaginosis treated with oral metronidazole and topical clindamycin. J Infect Dis. 1993; 167(3):783–4. https://doi.org/10.1093/infdis/167.3.783 PMID: 8440952

10. Bilardi JE, Walker S, Temple-Smith M, McNair R, Mooney-Somers J, Bellhouse C, et al. The burden of bacterial vaginosis: women's experience of the physical, emotional, sexual and social impact of living with recurrent bacterial vaginosis. PLoS One. 2013; 8(9):e74378. https://doi.org/10.1371/journal.pone.0074378 PMID: 24040236

11. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA. Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. Infect Immun. 1999; 67(10):5170–5. https://doi.org/10.1128/IAI.67.10.5170-5175.1999 PMID: 10496892

12. Boskey ER, Cone RA, Whaley KJ, Moench TR. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. Human reproduction (Oxford, England). 2001; 16 (9):1809–13.

13. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med. 2005; 353(18):1899–911. https://doi.org/10.1056/NEJMoa043802 PMID: 16267321

14. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011; 108 Suppl 1:4680–7. https://doi.org/10.1073/pnas.1002611107 PMID: 20534435

15. Tachedjian G, O'Hanlon DE, Ravel J. The implausible "in vivo" role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota. Microbiome. 2018; 6(1):29. https://doi.org/10.1186/s40168-018-0418-3 PMID: 29409534

16. McKinnon LR, Achilles SL, Bradshaw CS, Burgener A, Crucitti T, Fredricks DN, et al. The evolving facets of bacterial vaginosis: implications for HIV transmission. AIDS research and human retroviruses. 2019; 35(3):219–28. https://doi.org/10.1089/AID.2018.0304 PMID: 30638028

17. O'Hanlon DE, Moench TR, Cone RA. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. BMC Infect Dis. 2011; 11:200. https://doi.org/10.1186/1471-2334-11-200 PMID: 21771337

18. Aldunate M, Tyssen D, Johnson A, Zakir T, Sonza S, Moench T, et al. Vaginal concentrations of lactic acid potently inactivate HIV. J Antimicrob Chemother. 2013; 68(9):2015–25. https://doi.org/10.1093/jac/dkt156 PMID: 23657804

19. Gong Z, Luna Y, Yu P, Fan H. Lactobacilli inactivate *Chlamydia trachomatis* through lactic acid but not H2O2. PLoS One. 2014; 9(9):e107758. https://doi.org/10.1371/journal.pone.0107758 PMID: 25215504

20. Graver MA, Wade JJ. The role of acidification in the inhibition of *Neisseria gonorrhoeae* by vaginal lactobacilli during anaerobic growth. Ann Clin Microbiol Antimicrob. 2011; 10:8. https://doi.org/10.1186/1476-0711-10-8 PMID: 21329492

21. Tyssen D, Wang YY, Hayward JA, Agius PA, DeLong K, Aldunate M, et al. Anti-HIV-1 activity of lactic acid in human cervicovaginal fluid. mSphere. 2018; 3(4). https://doi.org/10.1128/mSphere.00055-18 PMID: 29976641

22. Edwards VL, Smith SB, McComb EJ, Tamarelle J, Ma B, Humphrys MS, et al. The cervicovaginal microbiota-host interaction modulates *Chlamydia trachomatis* infection. mBio. 2019; 10(4). https://doi.org/10.1128/mBio.01548-19 PMID: 31409678

23. Tachedjian G, Aldunate M, Bradshaw CS, Cone RA. The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. Res Microbiol. 2017; 168(9–10):782–92. https://doi.org/10.1016/j.resmic.2017.04.001 PMID: 28435139

24. Hearps AC, Tyssen D, Srbinovski D, Bayigga L, Diaz DJD, Aldunate M, et al. Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. Mucosal Immunol. 2017; 10(6):1480–90. https://doi.org/10.1038/mi.2017.27 PMID: 28401934

25. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Syst Rev. 2015; 4:1. https://doi.org/10.1186/2046-4053-4-1 PMID: 25554246

26. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am J Med. 1983; 74(1):14–22. https://doi.org/10.1016/0002-9343(83)91112-9 PMID: 6600371

27. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J Clin Microbiol. 1991; 29(2):297–301. https://doi.org/10.1128/JCM.29.2.297-301.1991 PMID: 1706728

28. Ison CA, Hay PE. Validation of a simplified grading of Gram stained vaginal smears for use in genitourinary medicine clinics. Sex Transm Infect. 2002; 78(6):413–5. https://doi.org/10.1136/sti.78.6.413 PMID: 12473800

29. Gottschick C, Deng ZL, Vital M, Masur C, Abels C, Pieper DH, et al. Treatment of biofilms in bacterial vaginosis by an amphoteric tenside pessary-clinical study and microbiota analysis. Microbiome. 2017; 5(1):119. https://doi.org/10.1186/s40168-017-0326-y PMID: 28903767

30. Higgins JPT, Sterne JAC, Savović J, Page MJ, Hróbjartsson A, Boutron I, et al. A revised tool for assessing risk of bias in randomized trials. Cochrane Database of Systematic Reviews 2016; 10(Suppl 1):29–31.

31. Sterne JA, Hernan MA, Reeves BC, Savovic J, Berkman ND, Viswanathan M, et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. BMJ. 2016; 355:i4919. https://doi.org/10.1136/bmj.i4919 PMID: 27733354

32. Simoes JA, Bahamondes LG, Camargo RP, Alves VM, Zaneveld LJ, Waller DP, et al. A pilot clinical trial comparing an acid-buffering formulation (ACIDFORM gel) with metronidazole gel for the treatment of symptomatic bacterial vaginosis. Br J Clin Pharmacol. 2006; 61(2):211–7. https://doi.org/10.1111/j.1365-2125.2005.02550.x PMID: 16433875

33. Keller MJ, Carpenter CA, Lo Y, Einstein MH, Liu C, Fredricks DN, et al. Phase I randomized safety study of twice daily dosing of acidform vaginal gel: candidate antimicrobial contraceptive. PLoS One. 2012; 7(10):e46901. https://doi.org/10.1371/journal.pone.0046901 PMID: 23056520

34. Andersch B, Forssman L, Lincoln K, Torstensson P. Treatment of bacterial vaginosis with an acid cream: a comparison between the effect of lactate-gel and metronidazole. Gynecol Obstet Invest. 1986; 21(1):19–25. https://doi.org/10.1159/000298923 PMID: 3485071

35. Boeke AJ, Dekker JH, van Eijk JT, Kostense PJ, Bezemer PD. Effect of lactic acid suppositories compared with oral metronidazole and placebo in bacterial vaginosis: a randomised clinical trial. Genitourinary medicine. 1993; 69(5):388–92. https://doi.org/10.1136/sti.69.5.388 PMID: 8244360

36. Fredstorp M, Jonasson AF, Barth A, Robertsson J. A new effective, user-friendly bacterial vaginosis treatment: a randomized multicenter open-label parallel-group two-part study with a novel sustained-release pessary containing oligomeric lactic acid. HSOA Journal of Infectious and Non Infectious Diseases. 2015; 1(1):100006.

37. van der Veer C, Bruisten SM, van Houdt R, Matser AA, Tachedjian G, van de Wijgert J, et al. Effects of an over-the-counter lactic-acid containing intra-vaginal douching product on the vaginal microbiota. BMC Microbiol. 2019; 19(1):168. https://doi.org/10.1186/s12866-019-1545-0 PMID: 31345159

38. Nelson AL. An overview of properties of Amphora (Acidform) contraceptive vaginal gel. Expert Opin Drug Saf. 2018; 17(9):935–43. https://doi.org/10.1080/14740338.2018.1515197 PMID: 30136907

39. Andersch B, Brandberg A, Holst E. [Treatment of bacterial vaginosis—an acid gel as an alternative to antibiotic treatment]. Lakartidningen. 1990; 87(7):465–8. PMID: 2308407

40. O'Hanlon DE, Come RA, Moench TR. Vaginal pH measured *in vivo*: lactobacilli determine pH and lactic acid concentration. BMC Microbiol. 2019; 19(1):13. https://doi.org/10.1186/s12866-019-1388-8 PMID: 30642259

41. O'Hanlon DE, Moench TR, Cone RA. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. PLoS One. 2013; 8(11):e80074. https://doi.org/10.1371/journal.pone.0080074 PMID: 24223212

42. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: Implications for protection against upper genital tract infections. mBio. 2013; 4(4). https://doi.org/10.1128/mBio.00460-13 PMID: 23919998

43. Ayehunie S, Wang YY, Landry T, Bogojevic S, Cone RA. Hyperosmolal vaginal lubricants markedly reduce epithelial barrier properties in a three-dimensional vaginal epithelium model. Toxicol Rep. 2018; 5:134–40. https://doi.org/10.1016/j.toxrep.2017.12.011 PMID: 29854584

44. Wilkinson EM, Laniewski P, Herbst-Kralovetz MM, Brotman RM. Personal and clinical vaginal lubricants: impact on local vaginal microenvironment and implications for epithelial cell host response and barrier function. J Infect Dis. 2019; 220(12):2009–18. https://doi.org/10.1093/infdis/jiz412 PMID: 31539059

45. van der Veer C, Bruisten SM, van der Helm JJ, de Vries HJC, van Houdt R. The cervico-vaginal microbiota in women notified for *Chlamydia trachomatis* infection: a case-control study at the STI outpatient clinic in Amsterdam, the Netherlands. Clinical Infectious Diseases. 2017; 64(1):24–31. https://doi.org/10.1093/cid/ciw586 PMID: 27567124

46. Sabo MC, Balkus JE, Richardson BA, Srinivasan S, Kimani J, Anzala O, et al. Association between vaginal washing and vaginal bacterial concentrations. PLoS One. 2019; 14(1):e0210825. https://doi.org/10.1371/journal.pone.0210825 PMID: 30677048

47. Food Drug Administration Center for Drugs Evaluation Research. Bacterial Vaginosis: Developing Drugs for Treatment. Guidance for Industry.: U.S. Department of Health and Human Services, Food and Drug Administration; 2019 [Available from: https://www.fda.gov/media/129530/download].

48. Sobel JD, Kaur N, Woznicki NA, Boikov D, Aguin T, Gill G, et al. Prognostic indicators of recurrence of bacterial vaginosis. J Clin Microbiol. 2019; 57(5). https://doi.org/10.1128/JCM.00227-19 PMID: 30842235

49. Sobel JD, Ferris D, Schwebke J, Nyirjesy P, Wiesenfeld HC, Peipert J, et al. Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. Am J Obstet Gynecol. 2006; 194(5):1283–9. https://doi.org/10.1016/j.ajog.2005.11.041 PMID: 16647911

50. Andreeva P, Slavchev B, Kovachev S, Nacheva A, Vacheva R. [Treatment of bacterial vaginosis with high dosage metronidazole and lactic acid]. Akush Ginekol (Sofiia). 2002; 41(5):36–9. PMID: 12440337

51. Armstrong-Buisseret L, Brittain C, David M, Dean G, Griffiths F, Hepburn T, et al. Metronidazole versus lactic acid for treating bacterial vaginosis (VITA): protocol for a randomised controlled trial to assess the clinical and cost effectiveness of topical lactic acid gel for treating second and subsequent episodes of bacterial vaginosis. Trials. 2019; 20(1):648. https://doi.org/10.1186/s13063-019-3731-7 PMID: 31775859

52. Anstey Watkins J, Ross JDC, Thandi S, Brittain C, Kai J, Griffiths F. Acceptability of and treatment preferences for recurrent bacterial vaginosis-Topical lactic acid gel or oral metronidazole antibiotic: Qualitative findings from the VITA trial. PLoS One. 2019; 14(11):e0224964. https://doi.org/10.1371/journal.pone.0224964 PMID: 31730666