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Title

Sex is associated with the persistence of non-optimal vaginal microbiota following treatment for bacterial vaginosis: a prospective cohort study

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Running Header:

Sex drives a non-optimal vaginal microbiota

ABSTRACT

Objective

Determine the associations between factors and sexual practices and the composition of the vaginal microbiome (VM) of women treated for bacterial vaginosis (BV).

Design

Prospective cohort study

Setting

The Melbourne Sexual Health Centre, Melbourne, Australia

Population

Seventy-five reproductive-age women diagnosed with clinical BV, treated with first-line antibiotics and followed for up to 6-months.

Methods

Women self-collected vaginal swabs and completed questionnaires at enrolment, the day following antibiotics, and monthly for up to 6 months until BV recurrence, or no BV recurrence (N=430 specimens). Bacterial composition was determined using 16S rRNA gene amplicon sequencing. The effects of ongoing factors on VM composition (utilising 291 monthly specimens) were assessed using generalised estimating equations population-averaged models, which accounted for repeated measures within individuals.

Main Outcome Measures

The relative abundance of vaginal bacterial taxa

Results

Women who reported ongoing sex with a regular sexual partner (RSP) had a VM comprised of increased relative abundance of non-optimal BV-associated bacteria (Adjusted co-efficient=11.91 95%CI:3.39, 20.43, p=0.006) and a decreased relative abundance of optimal, *Lactobacillus* species. (Adjusted co-eff=-12.76, 95%CI:-23.03, -2.49, p=0.015). A history of BV was also associated with decreased relative abundance of *Lactobacillus* spp. (Adjusted co-eff=-12.35, 95%CI:-22.68, -2.01, p=0.019). The relative abundance of *Gardnerella, Atopobium* and *Sneathia* spp. increased following sex with an RSP.

Conclusions

Sex with an untreated RSP after BV treatment was associated with a VM comprised of non-optimal BV-associated bacteria. BV treatment approaches may need to include partner treatment if they are to achieve a sustained optimal VM associated with improved health outcomes.

Funding

This original trial was supported by a grant from Monash University (Near Miss) awarded to CSB and an Early Career Researcher grant from The University of Melbourne (Australia) to LAV. This work was also supported by an Australian National Health and Medical Research Council (NHMRC) Program Grant (1071269) awarded to CKF and SMG. CSB and CKF are supported by an NHMRC Leadership Investigator Grants (1173361, 1172900, respectively), GT by NHMRC Senior Research Fellowship (1117748) and EPFC by an NHMRC Emerging Leadership Investigator Grant (1172873).

Keywords

Vaginal microbiome; bacterial vaginosis; sexual practices; regular sexual partner; *Gardnerella vaginalis*; combined oral contraceptive pill; patient involvement

Tweetable abstract (110 chars)

Sex drives a return to a 'non-optimal' vaginal microbiota after antibiotics for bacterial vaginosis

Introduction

Underpinning a woman's sexual and reproductive health is an optimal vaginal microbiota (VM), which acidifies the vagina and produces antimicrobial molecules to protect against pathogens.[1,2] Bacterial vaginosis (BV) is the most common vaginal condition, affecting ~30% of women globally[3], and is associated with increased risk of HIV, sexually transmitted infections (STIs), miscarriage, preterm delivery, low birth weight and pelvic inflammatory disease.[4-7] While an optimal VM is characterised by *Lactobacillus* spp., the most common non-optimal VM is associated with the clinical syndrome of BV and is characterised by diverse facultative and strict anaerobes including *Gardnerella vaginalis, Prevotella, Atopobium vaginae,* and *Sneathia.*[8-11] Despite our increased understanding of the composition of the VM in women with BV, the pathogenesis of BV-recurrence is poorly understood. Strategies to improve sustained cure are urgently needed, with >50% of women with BV experiencing recurrence within 6 months of antibiotic treatment.[12-14]

Epidemiologically, BV-recurrence is more common in women with a regular sexual partner (RSP) and in those whose male partners inconsistently use condoms, indicating sexual exchange of BV-associated bacteria may contribute to recurrence.[15,16] Conversely, hormonal contraceptive use is associated with a 30% reduced risk of recurrent BV by meta-analysis (pooled effect size=0.69, 95%CI:0.59–0.91).[17] We previously conducted the *Strategies to Prevent BV (STOPBV)* study, a pilot randomised controlled trial (RCT) investigating the use of the combined (oestrogen and progesterone) oral contraceptive pill (COCP) to reduce BV-recurrence following antibiotic treatment.

While COCP-exposure did not improve BV-cure in the pilot RCT, we found that sex with an ongoing RSP and reporting a history of BV were both significantly associated with BV-recurrence.[16] If there is a beneficial effect of hormonal contraceptives on the VM in sexually-active women, hormonal contraceptives would be a readily available and acceptable intervention for women globally. However, it is challenging to disentangle the contribution of a specific sexual practice in women, due to the complex interplay and interaction between contraception and sexual activity. In the current study, we conducted a cohort analysis of women enrolled in *STOPBV* to investigate factors, including sexual practices, associated with the composition of the VM among women following antibiotic treatment for BV.

Methods

Participants and specimens

This study used prospectively collected specimens derived from the *STOPBV* study, an open-label pilot RCT of COCP-use following first-line antibiotics to determine the impact on BV-recurrence.[16] There was no patient engagement in the study development. Briefly, women attending the Melbourne Sexual Health Centre, Australia, diagnosed with symptomatic BV (≥3 Amsel criteria and Nugent score [NS]=4-10) were prescribed 7d oral metronidazole 400mg bd or 7d 2% intra-vaginal clindamycin cream *nocte*. Women were offered participation in the trial if they were aged 18-46 and still menstruating, not pregnant, were not currently using hormonal contraception and had no COCP contraindications. Ninety-five women were enrolled and randomly allocated 1:1 (block size 6) to intervention (21d COCP comprising 30mcg ethinyl oestradiol/150mcg levonorgestrel and 7d inactive pill, to commence the day after antibiotics) or control (continue with current non-hormonal contraceptives). Participants provided a self-collected high-vaginal swab and vaginal smear for NS assessment at enrolment (pre-antibiotics), the day following antibiotics (day 8) and monthly either in-clinic (months 3,6) or at home (months 1,2,4,5) for up to 6 months, or until BV-recurrence. Demographics, a prior history of BV and sexual practices were captured in the enrolment questionnaire. The post-antibiotic questionnaire only captured antibiotic adherence and side-effects. Ongoing contraceptive use and sexual practices were captured in monthly questionnaires. Throughout follow-up, women who returned a home smear with NS=7-10 were recalled for in-clinic assessment of clinical-BV, diagnosed consistent with enrolment (≥3 Amsel criteria and NS=4-10). Swabs were agitated in 1ml RNAlater (Thermo Fisher Scientific, Waltham, USA) and stored at -80C for microbial analysis.

DNA extraction

Ninety-five women were recruited to the original trial.[16] To be eligible for the microbiota analysis, women needed to provide at least 2 post-treatment swabs; 19 women were excluded as a consequence. DNA was extracted from 472 specimens (N=76 participants) using 200µl on a MagNA Pure 96 System (Roche Diagnostics, Mannheim, Germany) in accordance to the manufacturer's small-volume universal pathogen protocol. Two samples of RNAlater were extracted in parallel as negative controls.

Library preparation

One-step library preparation polymerase chain reaction (PCR) was performed using primers targeting the V3V4 regions of the 16S rRNA gene that comprised unique barcodes, heterogeneity spacers and an Illumina adapter sequence[18,19]. Specimens were amplified in duplicate reactions on a T100 Thermal Cycler (Bio-Rad Laboratories, California, USA).[20] For specimens which amplified successfully, reactions were pooled, purified (AMPure Kit; Agencourt Bioscience, Massachusetts, USA) and quantified (Qubit dsDNA HS Assay; Thermo Fisher Scientific). A normalised pooled library was sequenced by Micromon Genomics (Monash University, Victoria, Australia) on a MiSeq instrument using v3 600-cycle kit (Illumina, San Diego, CA, USA). Extraction and PCR negative controls were included.

Sequence analysis

Sequence data were processed as previously described.[21] Briefly, DADA2[22] was used to filter sequence reads, infer amplicon sequence variants (ASVs) and assign taxonomy. For filtering, reads were trimmed according to quality profiles and sequences with ambiguous bases or that exceeded the number of expected errors were discarded. Chimeric and short sequences (<400bp) were removed. Taxonomy was assigned using DADA2 and the SILVA reference database (v128)[23]. Species level assignment of *Lactobacillus* ASVs was confirmed by BLAST search against a database of *Lactobacillus* type strains. ASVs matching BVAB1, BVAB2 and BVAB3 were identified as previously described.[21]

We removed ASVs that were present in only one specimen, had a relative abundance of <0.001%, were detected in negative controls, or matched previously identified contaminants[24] (Table S1). Specimens with <1000 filtered reads were omitted (n=19); as a consequence, one participant had no remaining post-treatment specimens so was excluded. Six enrolment specimens were omitted after filtering, however, these women all provided at least two post-treatment specimens so remained eligible for microbiota analysis. In the event women were recalled for in-clinic Amsel assessment

following a home-smear with NS=7-10, the clinic-collected specimen was included as the BV-recurrence specimen and home-collected specimen excluded (n=15). ASVs assigned identical taxonomy were merged and a heatmap was drawn using the ComplexHeatmap package (R v3.5.0).[25] Compositional change between paired longitudinal specimens from each participant was measured using the Bray-Curtis dissimilarity score.

Statistical analysis

As the aim of the current study was to evaluate factors associated with VM composition in a treated cohort of women, only post-treatment specimens from month 1 onwards were included in statistical analyses. There was no association between COCP-exposure and BV-recurrence in the original RCT, therefore specimens from all women were considered together for analysis. Composition was first assessed using the relative abundance of either BV-associated bacteria (Table S2) or combined *Lactobacillus* spp. so that we could determine factors associated with a 'non-optimal' or 'optimal' VM, respectively. Statistical models that accounted for repeated measures within individuals were fitted using generalised estimating equations (GEE) with population-averaged models to investigate the impact of factors (selected *a priori* based on the original trial) on VM composition. The characteristics selected included COCP-exposure, sex with an ongoing RSP, self-reported BV-history, sex with a new sexual partner (NSP), and condom use. All analyses were adjusted for the number of days since antibiotic treatment. Post-hoc analyses of the influence of additional factors selected *a priori* on the VM composition were conducted: these comprised smoking, menstrual phase, douching, specific sexual practices, and sex work. Characteristics deemed significant in univariate analyses ($p<0.05$) were included in multivariable analyses.

Box plots were used to visualise the relative abundance of non-optimal BV-associated bacteria and *Lactobacillus* spp. in women by sampling stage (i.e. months post-treatment) and stratified by sex with an ongoing RSP or COCP-exposure, given the original RCT aimed to determine the effect of the pill on BV-recurrence. For each stratified group, two sample Wilcoxon rank-sum tests were conducted to determine differences in the relative abundance of either BV-associated bacteria or total *Lactobacillus* spp. between women with the exposure *vs* no exposure at each month.

We next investigated the impact of factors/practices on the relative abundance of individual taxa. We assessed taxa that had a mean relative abundance of ≥0.1% and were present in ≥10% of the 291 monthly specimens included (i.e. ≥29 had a non-zero read count) due to the limited power for very rare taxa. Relative abundances underwent centre log ratio (CLR) transformation to normalise taxon

read counts (mixOmics package).[26] Statistical models were fitted using GEE as above, to investigate the impact of key factors on the transformed abundance data as the outcome.

Finally, GEE was used to investigate the impact of factors on compositional change (Bray-Curtis dissimilarity) of the VM post-treatment. Statistical analyses were performed using STATA/IC v14.2 (StataCorp LP, College Station, USA).

Results

Participant characteristics

Baseline and longitudinal (reported monthly) factors and sexual practices of the 75 women who provided at least two post-treatment specimens are summarised in Table 1. The median age was 27 years (range=20-46) and the majority (83%) had a tertiary education and a prior history of BV (68%). Forty (53%) reported an RSP at enrolment. At baseline, all women had clinical-BV (≥3 Amsel criteria), with 66 women with NS=7-10 and nine with NS=4-6.

Throughout monthly follow-up, COCP-exposure was reported by 37/75 women, 46 women reported smoking, and 11 douched. Thirty-one women reported sex with an RSP; 28 had a male RSP and 3 had a female RSP. Sex with an NSP was reported by 40 women. Inconsistent condom use for penile-vaginal sex was reported by 51 women. Thirty-seven women experienced BV-recurrence post-treatment within 6 months; 23 with NS=4-10 and ≥3 Amsel criteria, and 14 with NS=7-10 only (i.e. did not attend for in-clinic Amsel assessment).

A total of 430 specimens were evaluated representing 69 baseline specimens, 70 were immediate post-antibiotic specimens (day 8), and 291 monthly specimens, which included 254 specimens from women without BV and 37 specimens from women with BV-recurrence. Following quality filtering, 5,109,676 reads representing 489 ASVs remained. The median number of reads per specimen was 8,398 (IQR=6,213-12,343).

Characteristics influencing vaginal microbial composition

The VM composition of each individual specimen is illustrated in Figure 1. Enrolment specimens were abundant in *Gardnerella* (n=47/69, 68%) or comprised mixed diverse taxa (n=13/69, 19%). Specimens immediately following antibiotic completion were abundant in lactobacilli (n=54/70, 77%, mostly *L. iners* (n=37/70, 53%). The majority of monthly specimens from women who did not experience BV recurrence were abundant in lactobacilli (n=235/254, 93%), and most BV-recurrence

specimens were abundant in *Gardnerella* (n=28/37, 76%) or comprised mixed diverse taxa (n=6/37, 16%).

We first investigated the factors associated with relative abundance of BV-associated bacteria (defined in Table S2) and relative abundance of *Lactobacillus* spp. in monthly specimens. Women who reported a past history of BV had a significantly increased relative abundance of BV-associated bacteria in monthly specimens (co-eff=9.33, 95%CI:1.21,17.46, p=0.024; Table 2) and, conversely, a decreased relative abundance of *Lactobacillus* spp. (co-eff=-13.75, 95%CI:-23.56,-3.94, p=0.006). Similarly, women reporting sex with an ongoing RSP had a significantly higher relative abundance of BV-associated bacteria (co-eff=13.23, 95%CI:4.54,21.92, p=0.003) and lower abundance of *Lactobacillus* spp. (co-eff=-14.88, 95%CI:-25.54,-4.22, p=0.006) compared to women reporting no RSP. Women reporting penile-vaginal sex with any partner also had an increased relative abundance of BV-associated bacteria (co-eff=9.03, 95%CI:1.63,16.43, p=0.017) compared to women reporting no sex. Specimens from women reporting inconsistent condom use for penile-vaginal sex, sex with an NSP, COCP-exposure or other investigated factors did not significantly differ in the relative abundance of these two composition groups (Table 2).

In multivariate analyses, sex with an ongoing RSP was associated with an increased relative abundance of BV-associated bacteria (Adjusted-co-eff=11.91, 95%CI:3.39,20.43, p=0.006) and a decreased relative abundance of *Lactobacillus* spp. (Adjusted-co-eff=-12.76, 95%CI:-23.03,-2.49, p=0.015)(Table 2). History of BV was only associated with a decreased relative abundance of *Lactobacillus* spp. (Adjusted-co-eff=-12.35, 95%CI:-22.68 -2.01, p=0.019).

Box plots were used to visualise the abundance of BV-associated bacteria and *Lactobacillus* spp. stratified by sex with an ongoing RSP or COCP-exposure (Figure S1). At month 1, the relative abundance of BV-associated bacteria was significantly higher in women who reported sex with an RSP *vs* those with no RSP/no sex (Z=-1.96, p=0.0498). There were no other statistically significant differences found at other time points or in women with COCP-exposure *vs* no exposure.

We next examined how factors or sexual practices influenced the abundance of individual taxa (Table 3). There were 15 taxa/species prevalent in at least 10% of specimens at a relative abundance of ≥0.1%. Women with a BV-history had a significantly increased relative abundance of *Gardnerella* (co-eff=1.11, 95%CI:0.1,2.20, p=0.048) and *Atopobium* (co-eff=0.82, 95%CI:0.15,1.49, p=0.017), and decreased relative abundance of *L. Jensenii* (co-eff=-0.91, 95%CI:-1.76,-0.07, p=0.035). Women

reporting sex with an RSP had a significantly higher relative abundance of *Gardnerella* (co-eff=1.10, 95%CI:0.06,2.15, p=0.038), *Atopobium* (co-eff=1.01, 95%CI:0.36,1.66, p=0.002) and *Sneathia* (co-eff=0.75, 95%CI:0.27,1.23, p=0.002) compared to women without an RSP. Conversely, the relative abundance of *L. antri* (co-eff=-0.75, 95%CI:-1.37,-0.13, p=0.019) and *L. fornicalis* (co-eff=-0.74, 95%CI:-1.44,-0.03, p=0.040) was lower in specimens from women reporting sex with an RSP. Women reporting inconsistent condom use for penile-vaginal sex had a higher abundance of *L. crispatus* (co-eff=0.94, 95%CI:0.01,1.87, p=0.049) compared with women using condoms for penile-vaginal sex or reporting no penile-vaginal sex. *L. gasseri* (co-eff=0.69, 95%CI:0.24,1.13, p=0.002) and *L. antri* (co-eff=0.69, 95%CI:0.21,1.18, p=0.005) were significantly higher in women reporting sex with an NSP compared with women with no NSP. As sex with an RSP was moderately correlated with both inconsistent condom use and inversely with sex with an NSP (ρ>0.3), we conducted sensitivity analyses excluding intervals of RSP-exposure, which yielded similar results (Table S3). No taxa had a significantly different relative abundance in women with COCP-exposure *vs* no COCP-exposure (Table S3).

Factors influencing compositional change of the vaginal microbiota

Compositional change was measured using the Bray-Curtis dissimilarity score calculated between consecutive longitudinal specimens. Women reporting a prior BV-history had a significantly higher Bray-Curtis score indicating increased compositional change, which remained significant after adjusting for sex with an RSP and COCP-exposure (Adjusted-co-eff=0.15,95%CI:0.04,0.25, p=0.0012)(Table S4). Neither COCP-exposure nor specific sexual practices were associated with compositional change.

Discussion

Main findings

We investigated characteristics associated with the composition of the VM in a cohort of women following BV treatment. Women reporting sex with an RSP post-antibiotic treatment were more likely to have a non-optimal VM[2] comprised of BV-associated bacteria. Additionally, the relative abundance of specific BV-associated bacteria (*Gardnerella, Atopobium, Sneathia*) was higher in women reporting an RSP compared to those without an RSP post-treatment. The increased relative abundance of BV-associated bacteria coincided with a significant decrease in the relative abundance of *Lactobacillus* spp. in these women. We measured relative rather than absolute taxa abundance, therefore changes in relative abundance may reflect an increase in concentration of BV-associated bacteria, a decrease in lactobacilli concentration, or both occurring simultaneously. Women with a

prior BV-history had a significant decrease in the relative abundance of *Lactobacillus* spp., and increase in specific BV-associated bacteria post-treatment. These data compliment the original trial findings that showed post-treatment sex with an RSP and BV-history increase the risk of BV-recurrence.[16] Any modest protective effect that the combined oestrogen-containing pill may impart in sexually-active women is unlikely to support a sustained optimal VM if BV-associated bacteria are persisting, re-emerging or re-introduced during sex.

Strengths and Limitations

There are limitations related to the original RCT from which the specimens originated.[16] To mitigate the challenges with randomisation, attrition and cross-over of allocation groups, samples were analysed as a cohort and monthly-intervals of self-reported COCP-exposure used to assess exposure rather than arm-allocation. Sensitivity analyses confirmed no effect of randomisation group on VM composition. We also found no association with menstrual phase, smoking, oral sex, anal sex or douching. Either these factors had no effect, they were overwhelmed by sexual exposure or persistent BV-associated bacteria/biofilm, their effect size was smaller than the study was powered to detect, or composition was influenced by unmeasured confounders. Other less-abundant or less-prevalent BV-associated bacteria were not significantly associated with the included factors, which may be true or due to limited statistical power or limitations associated with 16S rRNA gene sequencing. As the outcome is measured as relative abundance, the changes observed may be real or as a result of the compositional nature of the data. Although we utilised a standard methodological pipeline, biases can be introduced by DNA extraction[24], primer selection[27] and bioinformatics software utilised.[22,28] Finally, high attrition and reduced generalisability due to the single recruitment site, highlights the need for well-powered trials to further interrogate the suitability of the COCP as an adjunctive therapy to sustain an optimal VM.

Interpretation

Women with a BV-history were more likely to experience compositional change and have a VM composition characterised by a lower relative abundance of *Lactobacillus* spp. Reduced lactobacilli abundance could be explained by several different mechanisms occurring post-antibiotics including 1) failure of the VM to recolonise with lactobacilli, 2) persistence of BV-associated bacteria or biofilm or 3) re-emergence of BV-associated bacteria.[29,30] A reduction in the relative abundance of specific *Lactobacillus* spp. results in a decrease in lactic acid and rise in vaginal pH.[1,31] This creates an unfavourable environment for lactobacilli to recolonise and may reinforce growth of BV-associated bacteria. Both *Gardnerella* and *Atopobium* had a higher relative abundance in women with a BV-

history. *Gardnerella*, the most prevalent BV-associated bacteria, is hypothesised to be the key driver of BV pathogenesis.[30] BV-associated biofilms, consisting of *G. vaginalis* and other BV-associated bacteria including *A. vaginalis* and *Prevotella*, can shield bacteria from lactic acid[32], protect bacterial growth through gene expression regulation[33], and act as a reservoir of antibiotic resistant BV-associated bacteria.[34-36] In addition, specific *G. vaginalis* clades are intrinsically resistant to metronidazole.[37] Persistence may therefore result from presence of biofilm and/or antibiotic resistant strain types. Finally, BV-history may also be a marker of repeat exposure to an RSP who is re-introducing BV-associated bacteria following each treatment episode.

We found that return to a non-optimal VM following antibiotics was strongly associated with sex with an RSP, suggesting sexual transmission is integral to BV pathogenesis[29,30]. Sexual contact with an RSP after a woman has completed treatment may re-expose her to BV-associated bacteria present in her partner, resulting in increased relative abundance of BV-associated bacteria, and a decrease in lactobacilli. Our data suggest that re-exposure to BV-associated bacteria in partners is undermining the efficacy of treatment strategies in sexually-active women. Microbiological evidence supports this concept. In men, the coronal sulcus of the glans penis and the distal urethra are colonised with BV-associated bacteria, including the species we identified.[38-40] Male circumcision reduces carriage of BV-associated bacteria, which explains why female partners of uncircumcised men are more likely to have BV.[40-42] A biofilm dominated by BV-organisms has been detected in men, especially if they have a partner with BV.[43] Heterosexual couples have concordant community state types[40,44], and female couples share *Lactobacillus* spp. and have concordant Nugent scores[45,46], highlighting the exchange of both optimal and potentially pathogenic bacteria between sexually-active partners. Men also harbour lactobacilli[20,38,44], which may explain why inconsistent condom use and sex with an NSP were associated with increased abundance of specific *Lactobacillus* spp. Sensitivity analyses excluding specimens from women reporting RSP sex yielded similar results. Together, these data suggest that sex with a partner with an optimal genital microbiota may support an optimal microbiota for both partners. As sex is essential for reproduction this is entirely plausible, as one would expect unprotected sex to promote vaginal health. Conversely, if a partner is carrying BV-associated bacteria, this may result in a non-optimal genital microbiota for both partners. Ongoing vaginal microbiota transplant studies will establish if transfer of vaginal secretions from women with an optimal VM elicit a sustained optimal VM in the recipient (ClinicalTrials.gov:NCT04046900, NCT03769688).

Clearly, all bacteria may be sexually transmitted but research into the specific strain(s) transmitted and their functional contribution to a sustained non-optimal VM is warranted. *Gardnerella*[30,34,47], *Atopobium*[30,48] and *Sneathia*[49] all have virulence potential, but the aetiology of BV is unclear. Substantial genetic diversity exists within the *Gardnerella* genus. Some variants are likely to be commensal with low virulence potential, whereas others with high virulence potential may drive pathogenesis.[50] Consequently it appears that treatments that do not involve the sexual partners of women with BV have a low likelihood of supporting a sustained optimal VM. An individualised approach to BV treatment in combination with first-line antibiotics may include i) concurrent antibiotic treatment of partners, ii) adjunctive treatment with biofilm disrupting agents or bioactive agents such as lactic acid, and iii) post-antibiotic support of *Lactobacillus* spp., such as adjunctive hormonal contraceptives, probiotics or prebiotics.[29,51,52]

Conclusions

We found that women reporting sex with an RSP were more likely to experience post-treatment recurrence of a non-optimal VM dominated by BV-associated bacteria, particularly key BV- and biofilm-associated organisms including *Gardnerella*, *Atopobium* and *Sneathia* spp. This coincided with a decrease in the relative abundance of *Lactobacillus* spp. Even if COCP-exposure supports an optimal VM in some women[53], sexual practices, which also influence contraceptive practices, appear to overwhelm any beneficial effect of exogenous oestrogen on the VM. It is unlikely that any treatment strategy solely directed to sexually-active women with BV will universally achieve high levels of sustained cure and an optimal VM. The short-term follow-up post-treatment in many BV trials prevents researchers and clinicians evaluating the impact of sex and partners on the durability of the intervention.

Data Availability

The raw sequencing data are publicly available in the NCBI Sequence Read Archive (SRA) under the Bioproject number PRJNA592384. Accompanying meta-data analysed during the study are not publicly available due to the highly sensitive nature of the questions answered by study participants, which provide extensive detail on participants' sexual behaviours. The data is required to be securely stored in keeping with Alfred Hospital Ethics requirements.

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Disclosure of 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. GM reports grants from Metrodora, and the Department of Health and Human Services, State Government of Victoria, Australia, outside the submitted work; SMG reports grants, personal fees and other from Merck, outside the submitted work. The remaining authors declare that they do not have any relevant interests to disclose. Completed disclosure of interest forms are available to view online as supporting information.

Contribution to Authorship

LAV and CSB conceived and designed the original trial. LAV, CSB, CKF, GT and JSH all contributed to study design and implementation of the original trial from which the specimens were derived. LKR performed laboratory work, with supervision from GLM, JD and SMG. LKR and ELP conducted bioinformatics. LAV and LKR performed statistical analyses with statistical support from JSH and EPFC. CKF, SMG, CSB and LAV all acquired funding. LAV, LKR, ELP and CSB wrote the original draft. All authors critically revised the manuscript. All authors approved the final version of the manuscript.

Details of Ethics Approval

This study received ethical approval from the Alfred Hospital Human Research Ethics Committee (404/13; 8/Oct/2013) and registered with the University of Melbourne Human Research Ethics Committee (1340852). Informed written consent was obtained from all participants prior to any study procedures taking place.

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Table/Figure Caption List

Table 1. Baseline and longitudinal (monthly) characteristics reported by women contributing specimens to the microbiota analyses (N=75)

Table 2. Factors associated with VM composition grouped as BV-associated bacteria or total *Lactobacillus* spp.

Table 3. Centre log ratio-transformed relative abundance of individual taxa by characteristic exposure

Figure 1. Examples of participant follow-up and heatmap demonstrating vaginal microbiota (VM) composition of included specimens across time points

A) Examples of participant follow up. Participants provided a specimen and completed a questionnaire at enrolment, immediately post-antibiotics, and monthly for 6 months or until BV recurrence (indicated by arrows). B) Heatmap displays the relative abundance of the top 20 most abundant taxa detected in all specimens (N=430) provided by 75 study participants. Ward linkage clustering was applied to determine the order of specimens within each study stage (enrolment, post-antibiotic or any monthly specimen). Metadata is displayed above the heatmap: sex with an ongoing regular sexual partner (RSP) post-treatment is indicated in the top panel (dark purple= sex with an RSP, light purple = no sex/sex with a new sexual partner) and study stage is indicated in the bottom panel (enrolment=green, post-antibiotic [day 8]=orange, monthly non-BV specimen=yellow and BV-recurrence specimens=red).

Key: TRT=antibiotic treatment, M=month, BV=bacterial vaginosis, RSP=regular sexual partner

Supporting Material

Table S1. Contaminants table

Table S2. Genera contributing to the BV-associated bacteria group

Table S3. Centre log ratio-transformed relative abundance of individual taxa in women with COCP-exposure *vs* no COCP-exposure and in sensitivity analyses

Table S4. Characteristics and sexual practices associated with instability of the vaginal microbiota as measured by Bray-Curtis dissimilarity between consecutive specimens

Figure S1. Relative abundance of BV-associated bacteria and *Lactobacillus* spp. in women stratified by sex with an RSP and COCP-exposure

Table 1. Baseline and longitudinal (monthly) characteristics reported by women contributing specimens to the microbiota analyses (N=75)

Baseline Characteristics (reported at enrolment)	n women reporting exposure (%)	***Longitudinal characteristics (reported on monthly questionnaires)*** (N=75 women, 291 months)	n women reporting exposure[d], (n months exposure reported)
Age		Average number of cigarettes smoked per week	
Median, Range	27, 20-46	none	48 (150)
≤27 y	41 (55%)	1-34 per week	25 (75)
>27 y	34 (45%)	35+ per week	21 (66)
Country of birth		Any douching	
Australia/New Zealand	33 (44%)	No	74 (275)
Other[a]	42 (56%)	Yes	11 (15)
Education level		Menstrual phase	
Secondary School	13 (17%)	Menses/peri menses	56 (134)

Tertiary/masters or PhD	62 (83%)	Non-menstrual	65 (156)
Self-reported history of BV		Current use of combined oral contraceptive pill	
No	24 (32%)	No	45 (155)
Yes	51 (68%)	Yes	37 (136)
Hormonal contraception use in previous 6 mo[b]		Any penile-vaginal sex	
No	56 (75%)	No	42 (115)
Yes	19 (25%)	Yes	60 (175)
Douching (Ever)		Any receptive oral sex	
No	49 (65%)	No	56 (164)
Yes	26 (35%)	Yes	54 (126)
Current RSP		Any receptive anal sex	
No	35 (47%)	No	73 (270)
Yes	40 (53%)	Yes	16 (20)
RSP gender (N=40)		Condom use for any penile-vaginal sex	
Male	36 (90%)	Always/not practiced	53 (160)
Female	4 (10%)	Not always	51 (131)
Current sex work		Any NSP	
No	70 (93%)	No	67 (199)
Yes	5 (7%)	Yes	40 (92)
Nugent Score at enrolment		Sex with ongoing RSP	
4-6[c]	9 (12%)	No	48 (201)
7-10	66 (88%)	Yes[e]	31 (90)

Abbreviations: BV, bacterial vaginosis; mo, months; RSP, regular sexual partner; NSP, new sexual partner

Continuous variables dichotomised at median value

[a] Other country of birth comprised predominately of individuals from Britain and Ireland (31%), China, Taiwan and South East Asia (20%), and Eastern and Western Europe (16%); [b] Reflects any method of hormonal contraceptive use in the 6 mo prior to enrolment. Women reported prior implant (n = 2, removed > 2 mo ago), ring (n = 2, removed > 4 mo ago), injection (n = 2, > 3 mo ago), Mirena® (n = 1, removed > 4 mo ago) or COCP-use (n = 13, stopped > 2 mo ago) [c] All women with a Nugent score of 4-6 had at least 3 Amsel criteria; one did not have Clue cells present, two did not have a noticeable fishy odour and one did not have pH measured and the remaining five had all four criteria reported; [d] As women provided specimens and questionnaires for ≥1 month, they could contribute to more than one outcome (i.e. no smoking in month 1 but smoking 1-34 times per week in month 2); [e] Three women reported oral sex with an ongoing female RSP, the remaining reported penile-vaginal sex with an ongoing male RSP

Table 2. Factors associated with VM composition grouped as BV-associated bacteria or total *Lactobacillus* spp.

	BV-associated bacteria[a]				*Lactobacillus spp.*[b]			
	co-efficient (95%CI)	p-value[c]	Adjusted co-efficient (95%CI)	p-value[d]	co-efficient (95%CI)	p-value[c]	Adjusted co-efficient (95%CI)	p-value[d]
Time (days since antibiotic treatment)[e]	0.01 (-0.05, 0.07)	0.789	0.02 (-0.04, 0.08)	0.543	-0.05 (-0.12, 0.02)	0.184	-0.06 (-0.13, 0.01)	0.091
Past history of BV[f]	9.33 (1.21, 17.46)	**0.024**	8.00 (-0.45, 16.44)	0.063	-13.75 (-23.56, -3.94)	**0.006**	-12.35 (-22.68, -2.01)	**0.019**
COCP-exposure[g]	0.45 (-7.50, 8.40)	0.912			1.92 (-7.63, 11.46)	0.694		
Sex with RSP[h]	13.23 (4.54, 21.92)	**0.003**	11.91 (3.39, 20.43)	**0.006**	-14.88 (-25.54, -4.22)	**0.006**	-12.76 (-23.03, -2.49)	**0.015**
Inconsistent condom use[i]	5.53 (-1.79, 12.85)	0.138			-3.67 (-12.27, 4.93)	0.403		
Sex with NSP[j]	-0.88 (-8.61, 6.85)	0.824			-0.36 (-8.62, 9.33)	0.938		
Any oral sex	6.11 (-1.04, 13.25)	0.094			-2.99 (-11.34, 5.35)	0.482		
Any penile-vaginal sex[k]	9.03 (1.63, 16.43)	**0.017**			-8.18 (-16.94, 0.57)	0.067		
Any anal sex	-4.07 (-17.81, 9.67)	0.562			7.29 (-8.45, 23.04)	0.364		

Abbreviations: COCP, combined oral contraceptive pill; RSP regular (ongoing) sex partner; NSP, new (post-treatment) sex partner. Bolded text indicates significant associations at the level of $p<0.05$. With the exception of past history of BV, each variable is comprised of practices occurring in the last month as reported by participants at each study interval.

[a] The total relative abundance of ASVs assigned to the genera designated BV-associated bacteria (Supplementary Table 2); [b] The total relative abundance of ASVs assigned to the genera designated as *Lactobacillus* spp.; [c] Univariate GEE linear regression clustered for multiple specimens from each participant (75 clusters). The regression coefficient represents the mean difference of relative abundance of each VM composition group between reference (i.e. no exposure) and comparison group (i.e. exposure) for each characteristic/practice investigated. All analyses were adjusted for days since antibiotic treatment (with the exception of the Time variable); [d] Multivariate GEE linear regression clustered for multiple specimens from each participant (75 clusters). The

adjusted regression coefficient represents the mean difference of relative abundance of each VM composition group between reference (i.e. no exposure) and comparison group (i.e. exposure) for each characteristic/practice investigated adjusted for other factors included in the model; [e] Time is a continuous variable of days since antibiotic completion (i.e. after day 8); [f] Past history of BV is self-reported history of BV, compared with no past BV; [g] COCP-exposure compared with no COCP-exposure; [h] Sex with an ongoing RSP defined as post-treatment sex with the same pre-treatment RSP, with sex with female RSP defined as having received oral sex and sex with male RSP defined as penile-vaginal sex, compared with no sex or sex with a post-treatment NSP; [i] Inconsistent condom use for penile-vaginal sex compared with consistent condom use/no penile-vaginal sex; [j] Sex with a new partner within the prior follow-up interval compared with no sex/sex with an ongoing partner from a prior interval; [k] Both any penile-vaginal sex and sex with an RSP were moderately correlated so only RSP-exposure was retained so as to not overfit the model.

NB. There were no significant associations between relative abundance of BV-associated bacteria or Lactobacillus spp. and smoking, menstrual stage, sex work, douching, or when analysed by randomisation group as allocated in the original trial

Table 3. Centre log ratio-transformed relative abundance of individual taxa by characteristic exposure

Taxa	prevalence N=291	Median (IQR)	Past history of BV[a] co-efficient (95%CI)	p value[e]	Sex with RSP[b] co-efficient (95%CI)	p value[e]	Inconsistent condom use[c] co-efficient (95%CI)	p value[e]	Sex with NSP[d] co-efficient (95%CI)	p value[e]
Prevotella	80	-0.2 (-0.3, 1.7)	0.53 (-0.06, 1.13)	0.078	0.49 (-0.15, 1.13)	0.132	0.26 (-0.29, 0.82)	0.355	0.14 (-0.45, 0.73)	0.639
Gardnerella	151	1.4 (-0.2, 5.6)	1.11 (0.01, 2.20)	**0.048**	1.10 (0.06, 2.15)	**0.038**	0.43 (-0.32, 1.18)	0.265	0.06 (-0.41, 0.83)	0.870
Atopobium	66	-0.2 (-0.3, -0.1)	0.82 (0.15, 1.49)	**0.017**	1.01 (0.36, 1.66)	**0.002**	0.36 (-0.16, 0.87)	0.175	-0.28 (-0.80, 0.25)	0.305
Dialister	85	-0.2 (-0.3, 2.1)	0.29 (-0.18, 0.75)	0.226	0.42 (-0.05, 0.90)	0.082	0.11 (-0.31, 0.52)	0.616	-0.08 (-0.52, 0.36)	0.710
Sneathia	33	-0.3 (-0.4, -0.1)	0.35 (-0.10, 0.81)	0.130	0.75 (0.27, 1.23)	**0.002**	0.11 (-0.33, 0.55)	0.613	-0.23 (-0.69, 0.24)	0.337
Megasphaera	30	-0.3 (-0.4, -0.1)	0.33 (-0.06, 0.71)	0.100	0.39 (-0.03, 0.81)	0.067	0.10 (-0.27, 0.48)	0.593	-0.15 (-0.55, 0.25)	0.465
Streptococcus	64	-0.2 (-0.4, -0.1)	0.38 (-0.22, 0.98)	0.213	-0.36 (-0.95, 0.24)	0.238	-0.17 (-0.65, 0.32)	0.502	0.32 (-0.18, 0.82)	0.210
L. iners	252	7.7 (4.6, 8.8)	-0.57 (-1.68, 0.55)	0.320	-0.65 (-1.75, 0.44)	0.243	-0.60 (-1.40, 0.21)	0.148	-0.14 (-0.97, 0.70)	0.747

L. crispatus	145	-0.1 (-0.3, 7.9)	-0.52 (-2.05, 1.02)	0.509	-1.03 (-2.39, 0.34)	0.141	0.94 (0.01, 1.87)	**0.049**	0.88 (-0.06, 1.82)	0.067
L. fornicalis	123	-0.1 (-0.3, 3.4)	0.10 (-0.65, 0.85)	0.797	-0.74 (-1.44, -0.03)	**0.040**	0.40 (-0.14, 0.95)	0.147	0.48 (-0.08, 1.03)	0.093
L. jensenii	77	-0.2 (-0.4, 2.8)	-0.91 (-1.76,-0.07)	**0.035**	-0.73 (-1.52, 0.05)	0.067	0.26 (-0.31, 0.82)	0.370	0.28 (-0.30, 0.86)	0.329
L. gasseri	72	-0.2 (-0.4, -0.1)	-0.50 (-1.07, 0.07)	0.083	-0.46 (-1.02, 0.09)	0.101	0.32 (-0.13, 0.76)	0.161	0.69 (0.24, 1.13)	**0.002**
L. antri	101	-0.1 (-0.4, 3.0)	-0.31 (-0.98, 0.36)	0.366	-0.75 (-1.37, -0.13)	**0.019**	0.30 (-0.18, 0.78)	0.220	0.69 (0.21, 1.18)	**0.005**
L. coleohominis	85	-0.2 (-0.4, 1.4)	0.19 (-0.40, 0.77)	0.532	-0.26 (-0.80, 0.28)	0.340	-0.19 (-0.56, 0.18)	0.319	-0.20 (-0.58,0.17)	0.294
L. acidophilus	53	-0.2 (-0.4, -0.1)	0.01 (-0.54, 0.57)	0.962	-0.34 (-0.86, 0.18)	0.200	0.21 (-0.18, 0.60)	0.283	0.10 (-0.30, 0.49)	0.638

Abbreviations: COCP, combined oral contraceptive pill; RSP regular sexual partner; NSP, new sexual partner; IQR, interquartile range. Bolded text indicates significant associations at the level p<0.05. With the exception of past history of BV, each variable is comprised of practices occurring in the last month as reported by participants at each study interval. A negative centre log ratio (CLR)-transformed value indicates a lower abundance relative to the mean abundance of all taxa, 0 indicates abundance equal to the mean abundance of all taxa, and a positive value indicates a higher abundance relative to the mean abundance of all taxa. For example, a value of 1 indicates a 2-fold (2^1) increase relative to the mean, and a value of -1 indicates a 2-fold decrease relative to the mean. [a] self-reported past history of BV (prior to trial enrolment) relative to no past BV; [b] Sex with an RSP defined as post-treatment sex with the same pre-treatment RSP, with sex with female RSP defined as having received oral sex and sex with male RSP defined as penile-vaginal sex, compared with no sex or sex with a post-treatment NSP; [c] Inconsistent condom use for penile-vaginal sex compared with consistent condom use/no penile-vaginal sex; [d] Sex with a new partner with whom first sexual contact was within the prior follow-up interval compared with no sex/sex with an ongoing partner from a prior interval; [e] Univariate GEE linear regression clustered for multiple specimens from each participant (75 clusters). The regression coefficient represents the mean difference of CLR-transformed relative abundance of each taxa between reference (i.e. no exposure) and comparison group (i.e. exposure) for each characteristic/practice investigated. NB. There were no significant associations between relative abundance of BV-associated bacteria or *Lactobacillus* spp. and COCP-exposure vs no COCP-exposure (see Table S3)