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Changes in vaginal microbiota following antimicrobial and probiotic therapy

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Background: The composition of the vaginal microbiota is known to be important for health. When infections occur, antimicrobial therapy is often poorly efficacious.

Objective and design: We used 16S rRNA gene sequencing to characterize changes in the bacterial microbiota following oral antimicrobial and probiotic interventions.

Results: While the bacterial vaginal profiles of women with vulvovaginal candidiasis were dominated by lactobacilli as in healthy women, and unchanged by therapy, *Gardnerella vaginalis*, *Prevotella*, *Atopobium*, *Sneathia*, and *Megasphaera* dominated the vagina of women with bacterial vaginosis (BV), and treatment with tinidazole plus *Lactobacillus reuteri* RC-14 + *L. rhamnosus* GR-1 resulted in an increased relative abundance of indigenous *L. iners* or *L. crispatus*.

Conclusions: The ability to restore homeostasis provides a rationale for conjoint use of probiotics with antibiotic treatment of BV.

Keywords: Lactobacillus; *bacterial vaginosis*; *vulvovaginal candidiasis*; *probiotics*

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The vaginal microbiome plays an important role in health and disease. As documented by numerous studies, including our own of over 196 women (1), *Lactobacillus* is the dominant genus not only in healthy women but also even in women with vulvovaginal candidiasis (VVC), whereas it is depleted in bacterial vaginosis (BV). Therapy for VVC and BV has changed little in over 40 years, and recurrences of both conditions are extremely common, reducing quality of life for many women (2–5). The antimicrobial failures and recurrences common in BV (6) and for some strain-specific VVC are attributed to drug resistance (7), failure of the agents to penetrate and eradicate biofilms, and inability to restore bacterial community homeostasis (8, 9). The aim of the present study was to perform a more in-depth analysis of the microbiota from the 196 women and to determine the impact of adjunctive oral administration of 2 g single dose of tinidazole plus probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 for 28 days (10), and single dose of fluconazole (150 mg) plus the probiotic (11).

Materials and methods

Clinical samples and study design

The vaginal swabs obtained as described in depth previously (1) were stored at −80°C until DNA extraction for amplification and sequencing. Samples were taken from three groups. The first group was a set of 63 healthy women, and a single vaginal swab was taken to define the 'healthy microbiome' in this population. The second

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group was a set of 62 women classified as having BV as was assessed by Amsel (12) and Nugent (13) criteria. These women were split into two equal treatment arms as outlined previously (10), with both arms being treated with a single 2 g dose of tinidazole with 31 women in one arm receiving two oral capsules of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 with 31 women in the other arm receiving two placebo cellulose and magnesium stearate gelatin capsules taken daily in the morning. Patients received either the probiotic or placebo for 28 days starting on the first day of tinidazole use as outlined previously (10). The third group was a set of 55 women diagnosed with VVC which was defined as having no vaginal discharge, itching and/or burning vaginal feeling, dyspareunia and/or dysuria, and *Candida* recovered by culturing. Twenty-six women chosen at random received a single 150 mg dose of fluconazole plus either placebo capsules as above, and 29 women received two oral capsules of *L. rhamnosus* GR-1 and *L. reuteri* RC-14. In both cases patients took the capsules in the morning for 28 days starting on the first day of antibiotic use (11). Swabs for bacterial microbiota profiling were collected before treatment and at the end of the 28-day period.

Each subject signed an informed consent statement (Ethics Review Board of the Centro de Saúde Escola da Faculdade de Medicina de Ribeirão Preto—Universidade de São Paulo; CSE-FMRP-USP protocol 0146). This study was registered online at "Comissão Nacional de Ética em Pesquisa" (CONEP document 070202), Brazil. Exclusion criteria included immunosuppression, pregnancy, and current use of antibiotics or antifungals. The samples were transferred to Canada for microbiota analysis as previously noted (1). No patient identifiers are disclosed.

V6-targeted 16S rRNA gene sequencing

Swabs were vigorously shaken in 1 mL PBS (pH 7.5) to dislodge cell material. DNA extraction was performed using the InstaGene Matrix (Bio-rad) according to manufacturer instructions. Amplification of the V6 region of the bacterial 16S rRNA gene was performed as described previously (14), using unique barcode sequences embedded in the V6 primers for each sample. The V6 region was chosen specifically due to length restrictions on the Illumina GAIIx, which was the platform available to us at the time of sequencing, and for the ability to easily distinguish *Lactobacillus* and *Gardnerella* species (14, 15). The amplified products were pooled for Illumina GAIIx 100 bp paired-end sequencing at the Next Generation Sequencing Facility at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto, Canada.

To support the use of the V6 sequence to separate *Lactobacillus* species, the minimum Levenshtein edit distances between the most common vaginal species were compared based on sequences pulled from the RDP database (Table 1). The edit distance is large enough to separate all expected species except *L. rhamnosus* with *L. casei* and *L. paracasei*. Additionally, we used full 16S rRNA gene sequence from the sequenced probiotic organisms (*L. reuteri* RC-14 and *L. rhamnosus* GR-1) to BLAST against a database of the representative V6 sequences for detection of these organisms.

Sequence processing

Paired reads were overlapped using Xorro (www.sourceforge.net/projects/xorro-overlap/) (16) to give full-length V6 sequence. Sequences with at least 97% identity were clustered into operational taxonomic units (OTUs) using UClust v. 3.0.617 (17). OTUs that did not reach 1% abundance in any one sample were removed from the total dataset. The most abundant sequence in each OTU cluster was used for taxonomic assignment by RDP SeqMatch (18). From the top 20 matches to the RDP named isolates database, the full taxonomy was retained for matches with the highest S_ab score. For multiple top matches with equal scores, the lowest common ancestor in the taxonomy was retained (e.g. genus level if multiple species matched equally well). Taxonomy assignment was verified by BLAST (19) to a custom V6 vaginal organism database. Up to one mismatch was accepted as a hit.

Data analysis

Weighted UniFrac distances (20) between samples were calculated in QIIME (21) by using a phylogenetic tree of OTU sequences built with FastTree (22). The phylogeny was based on an OTU sequence alignment with MUSCLE (23), since alignment to a consensus model was not possible with the short hypervariable V6 sequences. Shannon's diversity was calculated for each sample in QIIME. Figures were generated and statistics were performed in R (24). To compare alpha and beta diversity, the Wilcoxon rank-sum test was used with a Bonferroni multiple test correction and a cutoff of <0.01 was considered significant.

Differential relative abundances between ribotypes (OTUs and OTUs summed into lowest common taxonomy) were compared using the ALDEx2 tool (www.bioconductor.org/packages/ALDEx2/) (25). This tool estimates the sampling error by taking Monte-Carlo samples from a

Table 1. Minimum Levenshtein distance between species of *Lactobacillus* using sequences from the RDP database

Species	*L. iners*	*L. crispatus*	*L. reuteri*	*L. rhamnosus*	*L. jenensii*
L. iners	0	13	18	18	9
L. crispatus		0	14	17	17
L. reuteri			0	13	10
L. rhamnosus				0	10

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Dirichlet distribution, transforming the resulting proportions for each Monte-Carlo instance using the centered log-ratio (clr) transformation. The resultant clr abundance value per taxa represents its abundance relative to the mean abundance of all taxa in the sample (on a $\log_2$ scale). Significant differences between taxa between conditions were tested using Welch's *t*-test followed by a Benjamini–Hochberg false discovery rate (FDR) correction. The *p* and FDR values for each instance were tabulated and the expected value for 128 instances was reported in the paper. This approach removes from consideration those ribotypes where the significance is due to random sampling error, while permitting ribotypes that have relative abundances that are robust to sampling error to be identified as significantly different between the defined conditions. Taxa were considered differentially abundant using ALDEx2 with an FDR cutoff of <0.05 applied to a Welch's *t*-test.

Supplementary Table 1 contains the table of OTU read counts and associated taxonomy information, and Supplementary Table 2 contains the sample metadata and associated keys to the metadata.

Results

The healthy, asymptomatic vaginal samples set the baseline for the expected bacterial profiles in the population (Fig. 1). Most of 63 samples showed a dominance of *Lactobacillus* spp. with 44/63 samples (69.9%) having at least 60% of the sequences belonging to the *Lactobacillus* genus. *Lactobacillus iners* and *L. crispatus* were the predominant species and there were two distinguishable clusters of samples dominated by either *L. iners* or *L. crispatus*. As expected, this population had a low Shannon diversity (Fig. 2) because the microbiota composition comprised only a few abundant organisms. We observed that *Gardnerella* spp. was the second most abundant genus, and along with genera representing *Prevotella* and *Megasphaera* sequences, these genera were found to be abundant in samples containing $<60\%$ *Lactobacillus*.

Hierarchical cluster analysis using weighted UniFrac showed that samples from women with VVC were largely indistinguishable from those from healthy women (Fig. 1). These were generally dominated by *Lactobacillus* ($40/55 = 72.7\%$ with $\geq 60\%$ *Lactobacillus* spp.). In contrast, a large fraction of the women with BV formed a distinct cluster of profiles with a low proportion of *Lactobacillus*, and greater proportion of *Gardnerella vaginalis*, *Prevotella*, *Megasphaera*, *Atopobium*, *Sneathia*, and the BV-Associated Bacteria (BVAB) 1 and 2. In general, *Lactobacillus*-dominated profiles were rare in the BV group compared to the healthy and VVC groups with only $6/62 = 9.7\%$ women having $\geq 60\%$ *Lactobacillus* sequences. These initial BV microbiota profiles were similar to those of other women with BV as previously reported using the 16S rRNA V6 region (26, 27).

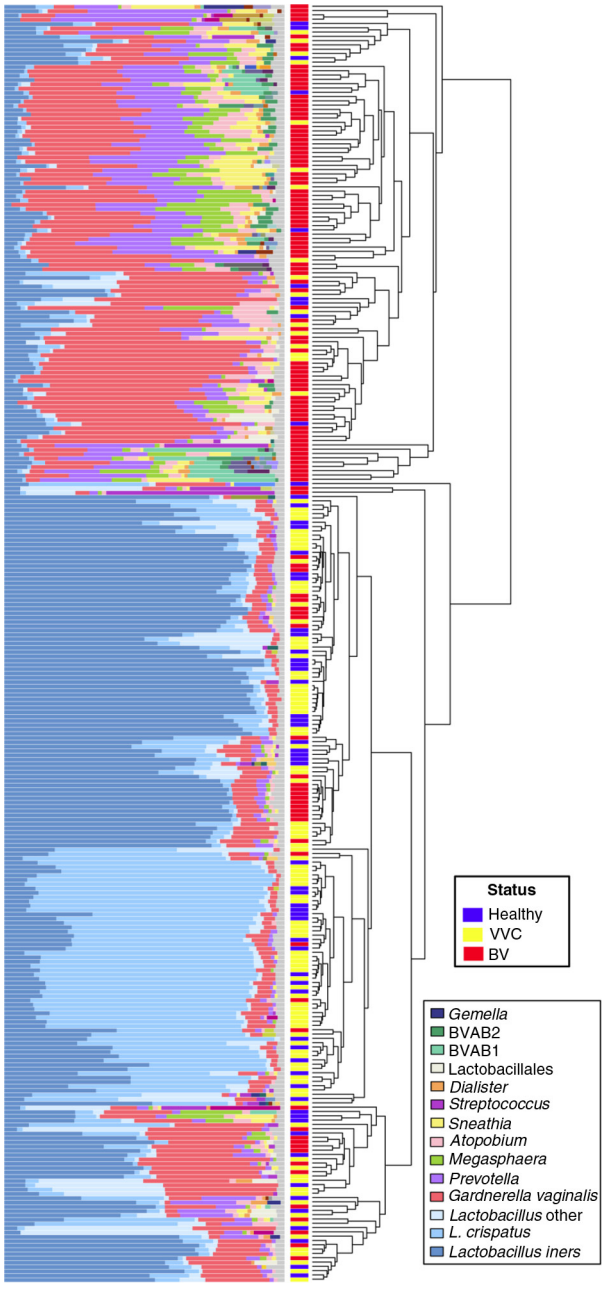

Fig. 1. Relative abundance taxa bar plot of vaginal samples from healthy asymptomatic women, and those diagnosed with BV or VVC before treatment. Taxa (groups of V6 rRNA gene sequences) are represented at the genus level where possible, with the exception of *L. iners* and *L. crispatus*, and the BV-Associated Bacteria (BVAB). The dendrogram above the bar plot represents the hierarchical average-linkage clustering of the microbiota profiles based on the computed weighted UniFrac distances.

As shown in Fig. 2 and Table 2, the vaginal microbiota profile of women with BV had a higher alpha diversity compared to women with VVC or asymptomatic women. Significant differences in the alpha diversity distributions were detected between BV and healthy microbiota, BV

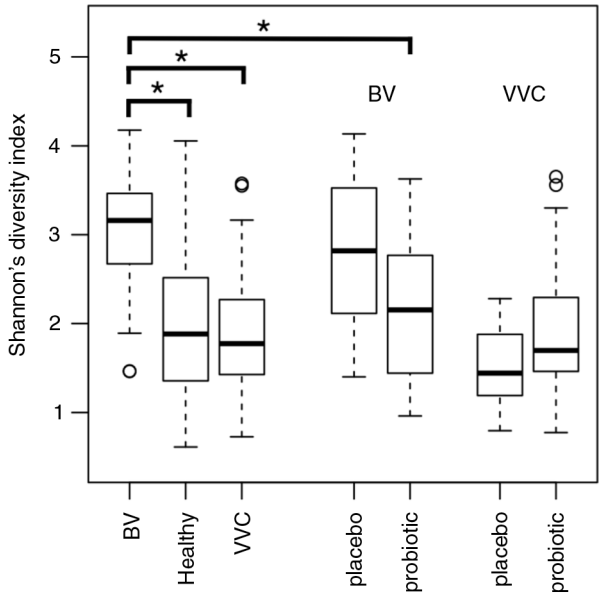

Fig. 2. Distribution of alpha diversity within groups as measured by Shannon index. The first three box plots represent the calculated Shannon index for microbiota samples at time 0 (before treatment) in the BV, healthy, and VVC groups. After treatment (28 days), the diversities were calculated within each treatment group (probiotic and placebo) per condition (BV and VVC). The difference in diversity was compared between all pairwise groups using the Wilcoxon rank-sum test with an adjusted *p*-value cutoff of <0.01. Significant group differences are marked with an asterisk.

and VVC, and BV pretreatment compared to BV post-treatment with tinidazole plus probiotic *L. rhamnosus* GR-1 + *L. reuteri* RC-14. No significant difference was found between the VVC and healthy groups, nor the VVC pretreatment and either of the posttreatment groups.

Principal coordinate analysis (PCoA) of the UniFrac distances showed that most of the variation (67.4%) could be explained by the first principal component (Supplementary Fig. 1), with the second component only contributing an additional 4.8% of the total variation. The largest split on the first component was likely due to the relative abundance of *Lactobacillus* species. The majority of the healthy asymptomatic samples clustered together, with a split between *L. iners*-dominant and *L. crispatus*-dominant samples (also seen in Fig. 1). After treatment, the majority of the antibiotic + probiotic-treated women in the BV cohort shifted on the PCoA towards a more *Lactobacillus*-dominant profile. There was no such visible shift for antibiotic + placebo in the BV cohort, nor for either VVC cohort.

The weighted UniFrac phylogenetic distance metric was used to measure the distance between the microbiota profile of each woman before/after treatment in the placebo and control groups. A lower UniFrac score indicates more similarity between the compared microbiota with a score of 0 representing microbiota profiles exactly the same in bacterial presence and abundance, while a score of 1 represents profiles with no overlapping bacterial sequences (20). Figure 3 shows box plots of the paired UniFrac distance measures for the placebo and probiotic treatment groups.

In the case of VVC, Table 2 and Fig. 3a show that the paired UniFrac distances before and after treatment for the probiotic group compared to the placebo group were not significantly different (probiotic group median = 0.08, placebo group median = 0.17, $p = 0.065$). This result suggests there was high similarity in the bacterial profiles before and after treatment. In the case of BV, Table 2 and Fig. 3 show that tinidazole with placebo treatment had a very small effect on the microbiota composition relative to the initial composition, with a median UniFrac distance of 0.22 with an interquartile range (IQR) of 0.18. In contrast, the tinidazole with probiotic treatment resulted in a large shift in UniFrac distance with a mean distance of 0.57 with an IQR of 0.52. These represent a significant difference between the probiotic-treated group compared to the placebo-treated group within the BV cohort (Bonferroni-corrected $p = 0.007$, Table 2). The overall larger UniFrac distances in the probiotic cohort indicate a larger change in the overall microbiota after treatment when compared to the placebo cohort and therefore a large effect of the probiotic treatment.

In addition to the pre- versus post-treatment distances, we examined the UniFrac distances between all pairwise samples before treatment. In the VVC cohort, the median distance of 0.29 between samples of different individuals was significantly greater than the distance between the same individual before and after treatment regardless of treatment group (probiotic = 0.08, placebo = 0.17 – See Table 2 and Supplementary Fig. 2). This indicates there was less intrapersonal variation in the bacterial profiles before and after treatment than the general interpersonal variation in the population. The same observation of relatively higher inter- versus intrapersonal variation in microbiota was present in the BV cohort (median pre-treatment UniFrac distance = 0.35, Supplementary Fig. 2), with the exception of the probiotic-treated cohort having a significantly larger change in microbiota following treatment.

The differences in relative abundance of the bacterial taxa as components of the entire microbiota before and after treatment were evaluated with ALDEx2 (25). There were no significant taxa changes for before and after treatment samples in any of the cohorts except for the BV cohort treated with tinidazole plus *L. rhamnosus* GR-1 + *L. reuteri* RC-14. Figure 4 shows the top 13 most abundant taxa are plotted by their difference in clr abundances (ALDEx2 values) and the difference in raw proportions (fractions as part of the total read set) for the samples before and after treatment. Setting a family wide

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Table 2. Results of statistical tests using Wilcoxon rank sum. Time 0 indicates before treatment samples, while time 1 is post-treatment

Data	Comparison	*p*-value	Corrected *p*-value[a]
Shannon's diversity (Fig. 2)	BV time 0 vs. healthy	7.74e-11	4.64e-10*
	BV time 0 vs. BV time 1 placebo	0.491	1
	BV time 0 vs. BV time 1 probiotic	5.32e-06	2.66e-05*
	BV time 0 vs. VVC time 0	6.30e-12	4.41e-11*
	VVC time 0 vs. healthy	0.738	1
	VVC time 0 vs. VVC time 1 placebo	0.012	0.049
	VVC time 0 vs. VVC time 1 probiotic	0.560	1
Principal component 1 coordinate position (Fig. 3b)	BV time 0 vs. BV time 1 placebo	0.139	0.417
	BV time 0 vs. BV time 1 probiotic	3.29e-07	1.65e-06*
	VVC time 0 vs. VVC time 1 placebo	0.010	0.041
	VVC time 0 vs. VVC time 1 probiotic	0.480	0.961
	Healthy vs. BV pretreatment	7.88e-16	4.73e-15*
	Healthy vs. VVC pretreatment	0.593	0.961
Weighted UniFrac distance between pre- and post-treatment samples (Fig. 3a and Supplementary Fig. 2)	BV placebo vs. probiotic group	0.003	0.007*
	VVC placebo vs. probiotic group	0.065	0.065
	VVC time 0 vs. Placebo	0.025	4.96e-2*
	VVC time 0 vs. Probiotic	8.73e-7	3.49e-6*
	BV time 0 vs. Placebo	0.0002	6.16e-4*
	BV time 0 vs. probiotic	0.044	4.96e-2*

[a]Using Bonferroni multiple test correction.
*$p < 0.01$.

FDR of <0.05 revealed that *L. iners*, *L. crispatus*, and all other *Lactobacillus* increased in abundance relative to the mean abundance of all taxa after treatment, while *Parvimonas*, BVAB2, *Sneathia*, *Atopobium*, *Dialister*, *Megasphaera*, *Prevotella* decreased in abundance compared to the mean. Examination of the taxonomic bar plots for the pre- and post-treatment BV cohort (Supplementary Fig. 3) supported that the major reason for the

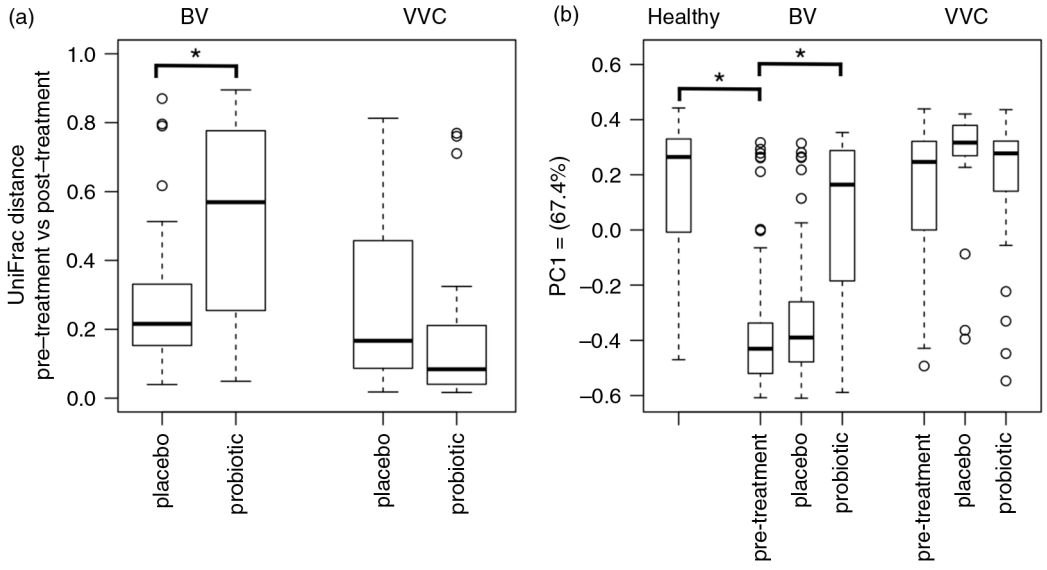

Fig. 3. Weighted UniFrac distance between pretreatment versus posttreatment samples (a) and change in principal component 1 (PC1) coordinate position (b). A weighted UniFrac distance was calculated for microbiota samples from the same individuals pre- and post-treatment (28 days) and plotted by condition and treatment group (a). In (b), the coordinate position for the first component (PC1, accounting for 67.4% of the variation) is plotted for the healthy cohort, and for the individuals in the treatment groups before and following treatment. Significantly different groups are marked with a bar and asterisk for a Bonferroni-corrected *p*-value <0.01 from a Wilcoxon rank-sum test.

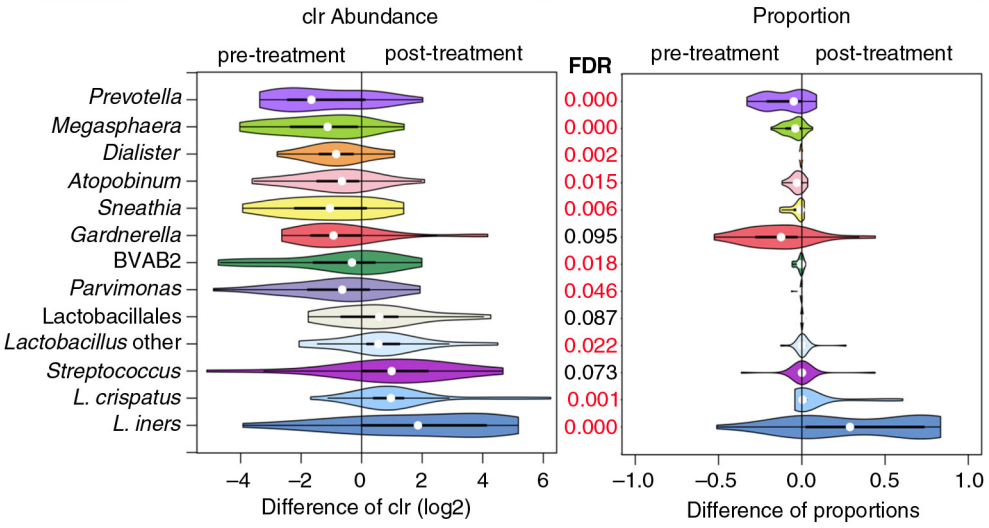

Fig. 4. Violin plots showing change in bacterial abundance before and after treatment for BV with oral probiotic *L. rhamnosus* GR-1 + *L. reuteri* RC-14 and tinidazole. The top 13 most abundant taxa across all BV samples are shown. In a violin plot, the white dot represents the median value, the black bar is the interquartile range, and the vertical width of the plot shows the density of the data along the X-axis. The left plot shows the difference in relative abundance as computed by ALDEx2. The right plot shows the difference in raw proportions for the same taxa. The reported false discovery rate (FDR) value is the result of a Benjamini–Hochberg corrected *p*-value from a Welch's *t*-test calculated within ALDEx2. Taxa with an FDR <0.05 were considered significantly different between the time points and those values are colored red. To interpret these data using *L. iners* as an example: the median change in proportions is approximately 25% more abundant after treatment, while the median change in clr abundance is approximately $2^2 =$ fourfold increase relative to the mean abundance of all taxa: this can also be described as *L. iners* becoming fourfold more abundant relative to the geometric mean abundance of all taxa following probiotic + antibiotic treatment.

large shift in the microbiota was the increased relative abundance of lactobacilli in many members of the probiotic cohort, and specifically *L. iners*. In contrast, there is very little change in these organisms in the placebo group. Correspondingly, after treatment, the median Shannon's diversity was lower in the probiotic group (median = 2.15) compared to the placebo group (median = 2.78).

Clinical signs of BV had been measured before and after the intervention by Amsel criteria (12) and Nugent scoring (13). The latter consists of a Gram-stained vaginal smear, where bacterial morphotypes presumably *Lactobacillus*, *Garnerella*, and *Mobiluncus* are enumerated. The scores are weighted heavily on the number of lactobacilli and not surprisingly a low or 'normal' Nugent score corresponded with recovery of *Lactobacillus* relative abundance after treatment (Supplementary Fig. 4, bottom). Amsel criteria indicate BV if three of four criteria are present: pH greater than 4.5; presence of thin, white, homogenous discharge; a positive 'whiff test' – the release of fishy odor after addition of potassium hydroxide; and the presence of 'clue cells' (epithelial cells covered in bacteria) by microscopy. Amsel criteria generally corresponded with *Lactobacillus*-depleted biota (Supplementary Fig. 3, below colored taxonomic bar plots).

Of note, the administered probiotics *L. rhamnosus* and *L. reuteri* were not detected at the relative abundance cutoff of 1% after one month's treatment of 61 women.

Discussion

The vaginal bacteria profiles as determined by 16S rRNA gene V6 region sequencing for the VVC study further emphasized that this condition is not associated with a disruption of the bacterial microbiota and a depletion of lactobacilli (28). Members of *Lactobacillus* genus were present in high proportions both during VVC and after antifungal therapy (Supplementary Fig. 4). Although we did not target sequencing for the yeast, the clinical study reported elsewhere showed a significant reduction in yeast following therapy (11). The bacterial profiles show a low bacterial diversity more representative of the healthy microbiotas (Figs. 2 and 3, and Supplementary Fig. 1). It has been suggested that amines produced by BV organisms can inhibit *Candida* and prevent concomitant yeast infection (29), but the reverse has not been investigated where *Candida* might inhibit BV organisms. *Candida* are known to produce a number of metabolites that might be inhibitory to bacteria (30), but none would explain why the Gram-negative bacteria appeared inhibited but not the Gram-positive lactobacilli. As BV was an enrollment exclusion, the outcome of VVC therapy on concomitant BV could not be evaluated.

If the bacterial microbiota *per se* did not influence the *Candida* and yet the cure rate was improved by probiotic use compared to antifungal alone (11), we postulate possible mechanism of action. *Candida albicans* has been

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shown to lose metabolic activity and be killed in the presence of *L. rhamnosus* GR-1 (31). A genome-wide transcriptional analyses showed significantly lower expression of the *CDR1* gene encoding an important drug efflux pump involved in fluconazole resistance, suggesting the *Candida* may not regard the lactobacilli or lactic acid as a threat to viability, and this might explain why conjoint probiotic therapy increased eradication of the fungi. The lactobacilli repressed the *PHR1* and *ALS12* genes involved in biofilm formation, and increased expression of stress-related genes. In addition, oral probiotic therapy with the two strains used here has been shown to decrease transfer of yeast from the rectum, suggesting another means of stopping the reseeding process (32).

In relation to the BV component of the study, it is known that oral administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can restore health to patients with BV (32–35), and the present study showed that this is due to recovery of indigenous lactobacilli. This supports a study (36) showing restoration of *Lactobacillus*-dominated profiles after treatment for BV with intravaginal metronidazole and probiotic *L. delbrueckii* subsp. *lactis* DM8909. In our study, the significant 10-fold increase in subjects with dominant lactobacilli compared with only twofold change with antibiotic and placebo reiterates that the current gold standard of antibiotics to treat BV is not sufficient to restore bacterial homeostasis in the vagina.

Examination of the Nugent scores after treatment showed that 11/31 patients (35.5%) of the placebo group and 23/31 patients (74.2%) of the probiotic group had a normal *Lactobacillus*-dominated Gram stain after treatment. Examination of these *Lactobacillus*-dominated samples by sequencing showed that *Lactobacillus* was not the predominant organism in every case. Only 7/11 = 63.6% (placebo) and 21/23 = 91.3% (probiotic) of those previously reported as cured by Nugent had at least 50% relative abundance of lactobacilli sequences (Supplementary Fig. 3). This discrepancy from the clinical report (10) may be caused by the different protocols: Nugent scoring counts absolute numbers of cell morphotypes visible on select microscopic fields, while amplicon sequencing determines the relative abundance of lactobacilli compared to the rest of the sampled sequences and not absolute numbers.

These findings also show that even without colonizing the vagina in high numbers, orally administered lactobacilli can still influence the vaginal ecosystem. This may be through lowering pathogen ascension from the vagina (32), increasing lactobacilli transfer from rectum to vagina (37), transiently disrupting the BV biofilms (8), promoting recovery of the indigenous lactobacilli via immune modulating effects (38) or by affecting the mRNA expression of the community (39). The cause of BV has not been established, but as with other microbial ecosystems a disturbance could lead to ecosystem degradation allowing a number of species to grow and outcompete the typically healthy lactobacilli. By administering probiotics, the system is again disturbed but in a way that displaces the BV-associated organisms and allows the indigenous species to re-establish, ideally creating a stable microbiota (40).

In summary, in addition to being the first high-throughput 16S rRNA gene sequence study of the vaginal microbiota in relation to VVC, we have shown that conjoint administration of probiotic *Lactobacillus reuteri* RC-14 + *L. rhamnosus* GR-1 induces an increase in the relative abundance of indigenous vaginal lactobacilli in women with BV. Given that current recommended treatment is not preventing the recurrence of urogenital infections (6), understanding how probiotics restore homeostasis and interfere with pathogenesis is worthy of further study.

Authors' contributions

GR helped plan the study; JMM, JCC, GBG, and RK performed the data analysis. JMM, GBG, RK, and GR wrote the paper.

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Conflict of interest and funding

None of the authors have declared a conflict of interest. JM received funding from the VoGue CIHR Team grant. This work was supported in part by the Howard Hughes Medical Institute.

Data and materials availability

Sequences have been deposited in QIIME-DB under the study ID 1888.

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