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Intermittent Lactobacilli-containing Vaginal Probiotic or Metronidazole Use to Prevent Bacterial Vaginosis Recurrence: A Pilot Study Incorporating Microscopy and Sequencing

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Bacterial vaginosis (BV) is associated with HIV acquisition and adverse pregnancy outcomes. Recurrence after metronidazole treatment is high. HIV-negative, non-pregnant Rwandan BV patients were randomized to four groups (n = 17/group) after seven-day oral metronidazole treatment: behavioral counseling only (control), or counseling plus intermittent use of oral metronidazole, Ecologic Femi+ vaginal capsule (containing multiple *Lactobacillus* and one *Bifidobacterium* species), or Gynophilus LP vaginal tablet (*L. rhamnosus* 35) for two months. Vaginal microbiota assessments at all visits included Gram stain Nugent scoring and 16S rRNA gene qPCR and HiSeq sequencing. All interventions were safe. BV (Nugent 7–10) incidence was 10.18 per person-year at risk in the control group, and lower in the metronidazole (1.41/person-year; p = 0.004), Ecologic Femi+ (3.58/person-year; p = 0.043), and Gynophilus LP groups (5.36/person-year; p = 0.220). In mixed effects models adjusted for hormonal contraception/pregnancy, sexual risk-taking, and age, metronidazole and Ecologic Femi+ users, each compared to controls, had higher *Lactobacillus* and lower BV-anaerobes estimated concentrations and/or relative abundances, and were less likely to have a dysbiotic vaginal microbiota type by sequencing. Inter-individual variability was high and effects disappeared soon after intervention cessation. Lactobacilli-based vaginal probiotics warrant further evaluation because, in contrast to antibiotics, they are not expected to negatively affect gut microbiota or cause antimicrobial resistance.

Most women have a vaginal microbiota (VMB) that consists predominantly of lactobacilli[1]. The most common type of vaginal dysbiosis is bacterial vaginosis (BV), characterized by a decrease in lactobacilli and increase in fastidious anaerobes[2]. Other types of bacterial dysbiosis, vulvovaginal candidiasis, and *Trichomonas vaginalis* (TV) are also common. These conditions are associated with vaginal inflammation, thereby increasing the risk of HIV acquisition[3]. BV is also associated with pelvic inflammatory disease, infertility, and adverse pregnancy outcomes[2].

The majority of women seeking care for vaginal symptoms receive antibiotic or antifungal treatment empirically or syndromically without any diagnostic testing[4]. In some specialized clinics, women might be offered

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limited diagnostic testing, such as vaginal pH determination and/or wet mount microscopy. In research settings, BV is typically diagnosed by the Amsel criteria or Nugent scoring[5,6], with the latter currently being considered the gold standard: Gram-stained vaginal smears are scored based on microscopic visualization of three bacterial morphotypes with a score of 0–3 considered normal, 4–6 intermediate, and 7–10 BV regardless of symptoms. In the last 15 years, molecular methods have become more widely available, and have been applied to the VMB, although mostly in descriptive studies to date[1].

Evidence is mounting that 'microbiological-BV' (defined here as the absence of lactobacilli-domination by Nugent scoring or by molecular methods regardless of symptoms) can cause long-term adverse outcomes in the presence or absence of vaginal symptoms[2]. BV is notoriously difficult to treat[7–9]. About 60–80% of patients are cured after a course of oral or vaginal metronidazole or clindamycin, but recurrence rates are high[7]. Therapies to restore and maintain an optimal lactobacilli-dominated VMB after antibiotic treatment are not standard practice, but some clinicians in Europe and North America recommend twice weekly 0.75% metronidazole vaginal gel for 4–6 months to lower the risk of BV recurrence[7]. This recommendation was tested in a randomized controlled trial in the USA, which showed a statistically significant reduction in BV recurrence (34% by Nugent scoring)[10]. In addition, two African trials evaluating oral (2 g monthly) and vaginal metronidazole (five nights every three months) for BV prevention also showed significant incidence reductions (45% and 10%, respectively)[11,12].

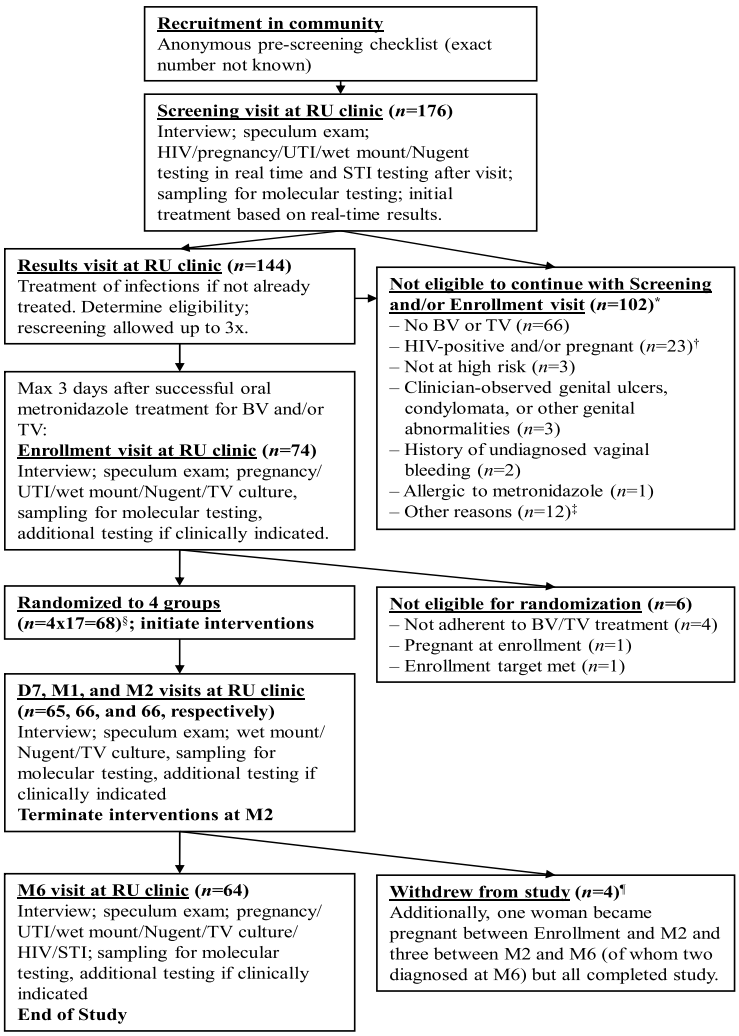
Probiotic lactobacilli may be able to restore and maintain a lactobacilli-dominated VMB, may be better able to prevent or disrupt BV-associated biofilms than antibiotics, and can likely be used safely for long periods without the risk of causing antimicrobial resistance[13]. Lactobacilli-containing vaginal probiotic clinical trials to date have shown mixed results, but eight of the 12 trials showed sufficiently promising efficacy for BV prevention to warrant further investigation[13–24]. Some trials used commercially available probiotic strains (mostly derived from the gut or fermented foods) and others vaginal strains isolated from healthy women[25], but efficacy signals were similar for the two probiotic strain categories[13–24]. None of the trials reported major safety concerns or colonization beyond the dosing period. Because 'natural' strains do not seem to outperform commercially available strains, and for pragmatic reasons, we chose to evaluate two vaginal probiotics that are currently on the market. Our aim was to assess their safety and impact on the VMB in much more detail than previous trials had done, and to develop data analytic methods to enable use of high-dimensional 16S rRNA gene sequencing data for this purpose. The two probiotics that we evaluated were Ecologic Femi+ vaginal capsule (EF+; Winclove Probiotics, Amsterdam, The Netherlands) and Gynophilus LP vaginal tablet (GynLP; Biose, Arpajon-sur-Cère, France). EF+ contains multiple lyophilized bacteria (*Bifidobacterium bifidum* W28, *Lactobacillus acidophilus* W70, *L. helveticus* W74, *L. brevis* W63, *L. plantarum* W21, and *L. salivarius* W24) in a total dose of 1.5×10^9 colony forming units (CFU). GynLP contains 1.6×10^9 CFU of Lcr Regenerans, a culture of the *L. rhamnosus* 35 (Lcr35) strain. The tablet disintegrates in the vagina after forming a gel to release Lcr35. Gynophilus (the same active ingredient as GynLP but a different formulation) had shown promise in preventing BV recurrence in a previous trial, but EF+ had not previously been studied for this indication[15].

Methods

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

Eligibility. Women were eligible for screening if they were aged 18–45, in good overall physical and mental health, and at high urogenital infection risk defined as having had more than one sex partner in the last 12 months or having been treated for a sexually transmitted infection and/or BV in the last 12 months. They were eligible for enrollment if they were confirmed HIV-negative and non-pregnant, were diagnosed with BV (Nugent score 7–10 and/or by modified Amsel criteria, defined as two of the following three criteria positive: vaginal pH > 4.5, positive whiff test, or ≥20% clue cells) and/or TV (by wet mount and/or culture), and were cured after seven days of oral metronidazole treatment (500 mg twice per day)[5]. Cure was defined as no BV by modified Amsel criteria and no TV by wet mount[6]. We did not use Nugent scores and TV culture as tests-of-cure to allow for same day enrollment but results became available after enrollment. At enrollment, 51/68 women were BV-negative by modified Amsel and Nugent criteria (score 0–6), 17/68 women were BV-negative by modified Amsel but BV-positive by Nugent criteria (score 7–10), and all women were TV-negative by both wet mount and culture and free of symptomatic vulvo-vaginal candidiasis, urinary tract infection, syphilis, and clinician-observed genital abnormalities or vaginal discharge. We did not exclude women with gonorrhea and/or chlamydia because the local testing turn-around time was slow. Positive herpes simplex type 2 serology was not a reason for exclusion. Additional exclusion criteria applied but these were rare (Fig. 1, Supplement 1).

Randomization groups and visit schedule. Women were randomized to four groups (17 women per group) within three days of completing oral metronidazole treatment: (1) behavioral counseling only (control group); (2) counseling plus 500 mg oral metronidazole twice weekly for two months; (3) counseling plus EF+ vaginal capsule once per day for the first five days followed by thrice weekly for a total of two months; and 4) counseling plus GynLP once every four days for two months. Participants applied the first dose of their intervention under direct observation at the enrollment visit, and returned to the clinic after seven days (D7), one month (M1), two months (M2; cessation of product use), and six months (M6). They were allowed to cease vaginal product use temporarily during menstruation. Product adherence was assessed by review of diary cards, used and unused products, and by a self-rating adherence scale. Symptomatic BV, vulvovaginal candidiasis, and



	Women	Swabs collected (two per woman per time point)	DNA extacted (one swab per woman per time point)	Valid sequencing results available after bioinformatics	Valid sequencing plus valid 16S qPCR results available
Screening‖	176	340	170	162	66 qPCR done for randomized women only
Enrollment‖	68	136	68	67	63
Follow-up‖	68	540	270	269	250
Self-sampled‖	12	258	131	131	0 qPCR not done
Total		1,274	639	629	379

Figure 1. Flow diagram of numbers of women seen, study procedures, and samples collected. Abbreviations: BV, bacterial vaginosis; D7, day 7 visit; DNA, desoxyribonucleic acid; M1/2/6, month 1/2/6 visit; qPCR, quantitative polymerase chain reaction; RU, Rinda Ubuzima; STI, sexually transmitted infection; TV, *Trichomonas vaginalis*; UTI, urinary tract infection. *Totals to 110 reasons among 102 women because there could be more than one reason per woman. †No speculum exam performed; molecular testing of self-sampled vaginal swabs. ‡Reasons: outside of metronidazole treatment window (n = 5), enrollment target already met (n = 4), has a mental disorder (n = 1), did not complete screening procedures and was subsequently lost to follow-up (n = 1), withdrew consent during the screening visit because she thought the reimbursement was too low (n = 1). §Three women in each randomization group were selected for self-sampling every other day during the first month of follow-up. ¶Reasons: moved away from Kigali (n = 2), lost interest because symptoms resolved (n = 1), was verbally harassed by partner and sister about study participation (n = 1). ‖These were all physician-collected. See Supplement 1 for information about samples that should have been but were not collected or were lost during processing.

urinary tract infections, and laboratory-confirmed sexually transmitted infections, diagnosed during follow-up were treated using standard oral therapies, and women were urged to continue their interventions during treatment. Visit procedures are summarized in Fig. 1 and described in Supplement 1.

Diagnostic testing. Women were tested for BV, TV, and vulvovaginal candidiasis at each visit. BV was diagnosed by Gram stain Nugent scoring and modified Amsel criteria; the vaginal pH was measured by pressing a pH paper strip (pH range 3.6–6.1 with 0.3 increments; Machery-Nagel, Düren, Germany) against the vaginal wall. TV was diagnosed when motile trichomonads were observed on wet mount and by InPouch culture (Biomed Diagnostics, White City, OR, USA). Vulvovaginal candidiasis was diagnosed when budding yeasts and/or (pseudo)hyphae were observed on wet mount. All other diagnostic tests (Supplement 1) were only done at screening, M6, and when judged clinically necessary by the physician, with the exception of pregnancy and urinalysis tests, which were repeated at enrollment prior to randomization.

16S rRNA gene sequencing and qPCR. We collected vaginal swabs in duplicate but DNA was extracted from one swab per woman only at each time point (639 samples in total): screening (176 screened women and 170 samples), enrollment and four follow-up visits (68 randomized women and 338 samples), and self-sampled swabs from 12 women (three per randomization group) who had been asked to self-sample at home every Monday, Wednesday, and Friday during the first month (12 women and 131 samples) (Fig. 1). Briefly, DNA was extracted using a combination of lysozyme lysis, Qiagen DNeasy Blood and Tissue kit (Qiagen, Manchester, UK), and bead-beating procedures (Supplement 1). Purified DNA underwent two PCR rounds: amplification of the 16S rRNA gene V3-V4 region and dual-index barcoding allowing multiplexing of up to 384 samples. Samples were sequenced on an Illumina HiSeq2500 instrument (Illumina, San Diego, CA, USA), run in rapid mode, 2 × 300 bp using a 250PE and 50PE kit. All samples collected from the same participant were included in the same run. Negative and positive controls (ZymoBiomics Microbial Community DNA standard; Zymo Research Corp, Irvine, USA) were incorporated throughout. The panbacterial 16S rRNA gene copy concentrations of physician-collected samples of enrolled women with valid sequencing results (N = 393) were determined by BactQuant qPCR assay as previously described[26].

Molecular data processing. Processing of sequencing reads, taxonomic assignments, and rarefaction are described in Supplement 1. This yielded 401 unique amplicon sequence variants (ASVs) in 629 samples, mapping to species (n = 255; 63.6%), genus (n = 116; 28.9%), or higher taxonomic level (n = 30; 7.5%). Concentrations in cells/μl per ASV per sample were estimated by multiplying the ASV-specific copy-normalized relative abundance by the sample-specific 16S rRNA gene copies concentration (explained in more detail in Supplement 1)[27,28]. Heatmaps of the 20 most common ASVs by sample are shown in Fig. S1A for relative abundances and Fig. S1B for estimated concentrations. VMB data reduction was required for molecular efficacy analyses, and was done in three different ways. First, the richness (number of ASVs) and Simpson diversity index (1-D) were calculated for each sample. Second, each ASV was assigned to one of four 'bacterial groups' based on the published literature (Supplement 2): (1) lactobacilli (all species combined, but with subcategorization into EF+ strains, the GynLP strain, and 'natural lactobacilli' in some analyses); (2) BV-anaerobes (fastidious anaerobes that have consistently been associated with BV); (3) pathobionts (bacteria that are considered more pathogenic than BV-anaerobes and that often co-occur with lactobacilli instead of BV-anaerobes, such as streptococci, staphylococci, enterococci, and *Enterobacteriaceae*)[29]; and (4) 'other bacteria' (a rest group, consisting mostly of skin bacteria and *Bifidobacterium* species). Within each sample, read counts of ASVs belonging to the same bacterial group were summed. This resulted in four relative abundances (one for each bacterial group) per sample, which sum to 1.0 for each sample. Third, we classified samples into eight mutually exclusive VMB types (each sample was assigned to only one VMB type): (1) *L. iners*-dominated (>75% lactobacilli of which *L. iners* was the most common; n = 247 samples); (2) *L. crispatus*-dominated (>75% lactobacilli of which *L. crispatus* was the most common; n = 17); (3) other lactobacilli-dominated (>75% lactobacilli of which *L. jensenii* and *L. gasseri* were the most common; n = 28); (4) lactobacilli plus BV-anaerobes (25–75% lactobacilli; n = 86); (5) polybacterial with ≥10% *Gardnerella vaginalis* (<25% lactobacilli and 10–50% *G. vaginalis*; n = 138), (6) polybacterial with <10% *G. vaginalis* (<25% lactobacilli and <10% *G. vaginalis*; n = 23), (7) *G. vaginalis*-dominated (<25% lactobacilli and >50% *G. vaginalis*; n = 41); and (8) ≥20% pathobionts (n = 49). The samples assigned to VMB types 1–7 had a maximum of 0–18.2% pathobionts per VMB type. To improve statistical power and to facilitate visualizations, the eight VMB types were condensed further into four 'pooled VMB types' as follows: (1) lactobacilli-dominated (original VMB types 1–3 combined; n = 292), (2) lactobacilli plus BV-anaerobes (original VMB type 4; n = 86); (3) BV-like (original VMB types 5–7 combined; n = 202); and ≥20% pathobionts (original VMB type 8; n = 49).

Downstream statistical analysis. Data were analyzed using STATA v13.0 (StataCorp, College Station, TX, USA). The trial safety endpoints were self-reported solicited and unsolicited (serious) adverse events and social harms, and clinician-observed speculum exam findings. Adverse events were coded using the Medical Dictionary for Regulatory Activities (medDRA v19.1, McLean, VA, USA). The primary preliminary efficacy endpoints were incident BV by Nugent score 7–10 or by modified Amsel criteria (binary variables). The secondary preliminary efficacy endpoints were the VMB composition variables derived from the sequencing data: the four bacterial group relative abundances and estimated concentrations (continuous variables) and VMB type or pooled VMB type (categorical variables). For cross-sectional analyses, we used Fisher's exact test for binary and categorical variables, and Kruskal-Wallis and Mann Whitney U tests for continuous variables. For longitudinal analyses, we used incidence rates and incidence rate ratios (which only included samples collected at study visits; Fig. 1), and mixed effects models (which included all samples, including those that were self-collected in between study visits, but Nugent scores and estimated concentrations were not available for self-collected samples; Fig. 1). Most analyses were conducted on the intent-to-treat (ITT) population (N = 68), but the BV by Nugent score and modified Amsel criteria incidence analyses were conducted on the modified ITT population (which was limited to women who were BV-negative by both modified Amsel and Nugent criteria at the time of randomization; n = 51). We compared each intervention group to the control group for one VMB endpoint at a time at screening,

enrollment, and longitudinally over time. All mixed effects models included one VMB endpoint as the outcome, participant identification number as the random effect, and randomization group (an indicator variable with the control group as the reference group) as the main fixed effect, and adjusted for covariates that were associated with at least one VMB endpoint in mixed effects models at $p < 0.05$ (Supplement 1).

Ethical statement. All participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki, sponsored by the University of Liverpool, approved by the Rwanda National Ethics Committee and the University of Liverpool Research Ethics Subcommittee for Physical Interventions, and first posted on ClinicalTrials.gov (NCT02459665) on 2 June 2015.

Results

Participant disposition. Of the 68 randomized women, only four did not complete the trial (Fig. 1), resulting in 29.93 person-years of data. The median age was 31 (interquartile range (IQR) 27–35) (Tables 1 and S1). The majority of women (92.6%) had exchanged sex for money or goods, and had had a median of five (IQR 2–18) sex partners, in the past month. All but three women used condoms, but mostly inconsistently. Two-thirds of the women (61.8%) were using hormonal contraception, and four women became pregnant during the trial. Short course metronidazole/tinidazole use for other indications during the intervention period was evenly distributed among randomization groups (Table S2: n = 1–3 per group; p = 0.688), as was short course use of other oral antibiotics (Table S2: n = 2–4 per group; p = 0.781). Furthermore, these other antibiotics did not impact lactobacilli and BV-anaerobes estimated concentrations significantly (Table S2, Fig. S2). No antifungals were used. Most women adhered with their study product >90% of the time, but this percentage was non-significantly lower in the GynLP group (68.8%) than in the metronidazole (82.4%; Fisher's exact p = 0.438) and EF+ groups (88.2%, Fisher's exact p = 0.225; Table S3).

VMB compositions at baseline. By design, all 68 randomized women at screening had BV (by Nugent and/or modified Amsel criteria) and/or TV (by wet mount and/or culture): 56 women (82.4%) had BV alone, 10 women (14.7%) had BV and TV, and two women (2.9%) had TV alone. Therefore, as expected, most women had dysbiotic VMB types by 16S rRNA gene sequencing at screening: polybacterial with ≥10% *G. vaginalis* (28/67; 41.8%), lactobacilli plus BV-anaerobes (12/67; 17.9%), polybacterial with <10% *G. vaginalis* (8/67; 11.9%), *G. vaginalis*-dominated (8/67; 11.9%), and ≥20% pathobionts (1/67; 1.5%). However, 10/67 women (14.9%) had a *L. iners*-dominated VMB type, of whom six were TV-negative, and these women might not have needed metronidazole treatment. Also by design, all randomized women were BV-negative (by modified Amsel criteria) and TV-negative (by wet mount and culture) at the time of randomization. However, almost half of the women (30/67; 44.8%) were not lactobacilli-dominated by sequencing at the time of randomization, and none of them were *L. crispatus*-dominated (which is considered the most optimal VMB state): their VMB types were *L. iners*-dominated (35/67; 52.2%), dominated by other lactobacilli (2/67; 3.0%), lactobacilli plus BV-anaerobes (18/67; 26.9%), ≥20% pathobionts (6/67; 9.0%), *G. vaginalis*-dominated (6/67; 6.0%), and polybacterial with ≥10% *G. vaginalis* (2/67; 3.0%) (Table 1).

At randomization, the estimated mean total bacterial concentration ranged from 5.54–6.34 $\log_{10}$ cells/μl in the four randomization groups, and these ranges were 5.22–5.97 for lactobacilli, 3.36–5.62 for BV-anaerobes, 1.34–2.36 for pathobionts, and 0.57–2.20 for 'other bacteria' (Table S4; relative abundance data in Table S5). The mean richness ranged from 7.5–17.8, and the mean Simpson diversity from 0.15–0.38. Randomization did not completely balance the baseline VMB compositions of the four groups: the estimated concentrations of total bacteria and BV-anaerobes were higher in the GynLP group than the control group (Mann Whitney U test $p < 0.05$), and the estimated BV-anaerobes concentration ($p < 0.01$) and Simpson diversity ($p < 0.05$) were lower in the EF+ group than the control group (Table 1).

Variables that were associated with at least one VMB endpoint in unadjusted mixed effects models (Table S6) included currently using hormonal contraception or being pregnant (associated with a higher estimated pathobionts concentration), above-average sexual risk taking based on reported numbers of partners and condom use (higher estimated pathobionts concentration), aged 30 years or older (lower estimated BV-anaerobes and pathobionts concentrations), and managing menses with a sanitary pad compared to other methods (higher Nugent score). Reporting current urogenital symptoms was also associated with VMB composition but this likely represents reverse causality. We could not exclude women with ongoing chlamydia and/or gonorrhea infections at randomization because the turn-around time of diagnostic testing was slow, but the VMB compositions after metronidazole treatment of women with and without ongoing chlamydia and/or gonorrhea infection were similar (Fig. S2).

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

During the intervention period, urogenital symptoms were reported by 13.4% of participants (genital itching, burning, and pain during sex but no vaginal discharge) with no differences between groups, and only two speculum exam and no bimanual exam findings were reported by the physician (Table 2). After product cessation, urogenital symptom reporting was similar compared to the intervention period (10.8%), but the number of speculum exam findings increased (32.8%), probably reflecting the high urogenital infection incidence in this cohort.

Sociodemographic characteristics at Scr/Enr	Screened (n = 175)*	Enrolled (n = 68)	Controls (n = 17)	Metro (n = 17)	EF+ (n = 17)	GynLP (n = 17)	p†
Age in years, median (IQR)	30 (27 – 34)	31 (27 – 35)	29 (24 – 36)	30 (27 – 34)	33 (28 – 35)	30 (27 – 35)	0.563
Sex partners last mo, median (IQR)	5 (2 – 16)	5 (2 – 18)	5 (3 – 20)	5 (2 – 10)	3 (2 – 15)	3 (2 – 20)	0.624
Condom during last sex act, n (%)*	95 (54.6)	36 (52.9)	11 (64.7)	8 (47.1)	6 (35.3)	11 (64.7)	0.279
Currently using contraception, n (%)							0.750
None	69 (40.2)	24 (35.3)	6 (35.3)	6 (35.3)	4 (23.5)	8 (47.1)	
Hormonal contraception	99 (57.6)	42‡ (61.8)	10 (58.8)	11 (64.7)	12 (70.6)	9 (52.9)	
Copper IUD	4 (2.3)	2 (2.9)	1 (5.9)	0	1 (5.9)	0	
Laboratory results at Scr	**Screened (n = 173)**	**Enrolled (n = 68)**	**Controls (n = 17)**	**Metro (n = 17)**	**EF+ (n = 17)**	**GynLP (n = 17)**	**p†**
Nugent score, mean (95% CI)*	4.7 (4.1 – 5.3)	7.4 (6.8 – 8.0)	7.6 (6.5 – 8.6)	6.8 (5.3 – 8.3)	7.1 (5.5 – 8.6)	8.2 (7.4 – 9.0)	0.333
Nugent categories, n (%)*							0.075
0–3	55 (38.2)§	5 (7.5)	1 (5.9)	2 (11.8)	2 (12.5)	0	
4–6	20 (13.9)	6 (9.0)	0	4 (23.5)	0	2 (11.8)	
7–10	69 (47.9)	56 (83.5)	16 (94.1)	11 (64.7)	14 (87.5)	15 (88.2)	
BV by modified Amsel criteria, n (%)	30 (20.6)	20 (29.4)	4 (23.5)	6 (35.5)	3 (17.7)	7 (41.2)	0.486
Candida on wet mount, n (%)	14 (9.6)	6 (8.8)	1 (5.9)	2 (11.8)	3 (17.7)	0	0.493
TV on wet mount, n (%)	9 (6.2)	6 (8.8)	3 (17.7)	1 (5.9)	1 (5.9)	1 (5.9)	0.707
TV by InPouch culture, n (%)	17 (11.8)§	11 (16.4)	3 (18.8)	1 (5.9)	5 (29.4)	2 (11.8)	0.282
UTI by dipstick, n (%)	33 (19.1)	17 (25.0)	4 (23.5)	6 (35.3)	4 (23.5)	3 (17.7)	0.760
CT by PCR, n (%)	30 (20.8)§	20 (29.4)	5 (29.4)	7 (41.2)	3 (17.7)	5 (29.4)	0.560
NG by PCR, n (%)	18 (12.5)§	13 (19.1)	5 (29.4)	4 (23.5)	2 (11.8)	2 (11.8)	0.555
HIV serology positive, n (%)	17 (9.8)	0	0	0	0	0	NA
HSV2 serology positive, n (%)	117 (67.6)	44 (64.7)	9 (52.9)	12 (70.6)	11 (64.7)	12 (70.6)	0.780
Syphilis serology positive, n (%)	13 (7.5)	4 (5.9)	0	1 (5.9)	0	3 (17.7)	0.182
Pregnancy test positive, n (%)	7 (4.1)	0	0	0	0	0	NA
Laboratory results at Enr**	**Screened**	**Enrolled (*n* = 68)**	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 17)**	**p†**
Nugent score, mean (95% CI)*	NA	3.1 (2.2 – 3.9)	3 (1.2 – 4.8)	3.3 (1.4 – 5.2)	1.7 (0.1 – 3.3)	4.3 (2.6 – 6.0)	0.187
Nugent categories, n (%)*							
0–3	NA	36 (54.6)	8 (53.3)	9 (52.9)	13 (76.5)	6 (35.3)	0.149
4–6		13 (19.7)	5 (33.3)	2 (11.8)	1 (5.9)	5 (29.4)	
7–10		17 (25.8)	2 (13.3)	6 (35.3)	3 (17.7)	6 (35.3)	
BV by modified Amsel criteria, n (%)	NA	0	0	0	0	0	NA
Candida on wet mount, n (%)	NA	4 (5.9)	2 (11.8)	1 (5.9)	0	1 (5.9)	0.897
TV by wet mount/culture, n (%)	NA	0	0	0	0	0	NA
VMB outcomes at Enr	**Screened**	**Enrolled (*n* = 67)***	**Controls (*n* = 17)**	**Metro (*n* = 17)**	**EF+ (*n* = 17)**	**GynLP (*n* = 16)***	**p†**
Richness, mean (95% CI)	NA	12.8 (10.7 – 14.8)	17.8 (12.1 – 23.5)	12.1 (8.7 – 15.5)	7.5‡‡ (4.9 – 10.2)	13.7 (10.3 – 17.1)	0.003
Simpson diversity index, mean (95% CI)	NA	0.31 (0.25 – 0.38)	0.38 (0.21 – 0.54)	0.35 (0.20 – 0.50)	0.15§§ (0.05 – 0.25)	0.38 (0.25 – 0.50)	0.022
Total bacteria estimated conc in log_{10}/μL, mean (95% CI)††	NA	5.85 (5.66 – 6.04)	5.75 (5.30 – 6.20)	5.79 (5.39 – 6.19)	5.54 (5.28 – 5.80)	6.34‡‡ (5.95 – 6.73)	0.019
Total *Lactobacillus* estimated conc in log_{10}/μL, mean (95% CI)††	NA	5.56 (5.34 – 5.78)	5.22 (4.63 – 5.82)	5.59 (5.20 – 5.97)	5.46 (5.19 – 5.73)	5.97 (5.44 – 6.49)	0.092
Total BV-anaerobes estimated conc in log_{10}/μL, mean (95% CI)††	NA	4.55 (4.15 – 4.95)	4.78 (4.17 – 5.39)	4.50 (3.77 – 5.24)	3.36‡‡ (2.43 – 4.29)	5.62‡‡ (4.99 – 6.25)	0.002
Total pathobionts estimated conc in log_{10}/μL, mean (95% CI)††	NA	2.01 (1.48 – 2.54)	2.36 (1.28 – 3.44)	2.08 (1.00 – 3.17)	1.34 (0.29 – 2.39)	2.30 (0.98 – 3.62)	0.447
Total other bacteria estimated conc in log_{10}/μL, mean (95% CI)††	NA	1.46 (1.01 – 1.92)	1.80 (0.84 – 2.76)	1.30 (0.36 – 2.24)	0.57 (-0.10 – 1.24)	2.20 (1.07 – 3.34)	0.066
VMB types, n (%):							
L. iners-dominated	NA	35 (52.2)	7 (41.2)	9 (52.9)	14 (82.4)	5 (31.3)	0.048
L. crispatus-dominated		0	0	0	0	0	
Other lactobacilli-dominated		2 (3.0)	1 (5.9)	1 (5.9)	0	0	
Lactobacilli plus BV-anaerobes		18 (26.9)	3 (17.7)	5 (29.4)	2 (11.8)	8 (50.0)	
Polybacterial with ≥10% *G. vag*		2 (3.0)	1 (5.9)	0	0	1 (5.9)	
Polybacterial with <10% *G. vag*		0	0	0	0	0	
G. vaginalis-dominated		4 (6.0)	2 (11.8)	0	0	2 (12.5)	
≥20% pathobionts		6 (9.0)	3 (17.7)	2 (11.8)	1 (5.9)	0	

Table 1. Baseline characteristics. Abbreviations: CI, confidence interval; conc, concentration; CT, *Chlamydia trachomatis*; EF+, Ecologic Femi+; Enr, enrollment visit; *G. vag, G. vaginalis*; GynLP, Gynophilus LP; HSV2, herpes simplex virus type 2; IQR, inter-quartile range; Metro, metronidazole; Mo, month; NA, not applicable; NG, *Neisseria gonorrhoeae*; Scr, screening visit; TV, *Trichomonas vaginalis*; UTI, urinary tract infection; VMB, vaginal microbiota. *Total numbers are slightly lower due to 1–4 missing values. 176 women were screened. 68 women were randomized but one woman did not have sequencing data at screening and enrollment. [†]Fisher's exact test for binary/categorical outcomes and Kruskall Wallis test for continuous outcomes, between randomization groups. [‡‡]Seven women reported using oral contraception, 18 hormonal injections, and 17 hormonal implants. [§]n = 144; women at the screening visit that were HIV-positive and/or pregnant, or were otherwise ineligible, did not undergo testing for this urogenital disease. [‖]n = 146; women at the screening visit who were HIV-positive and/or pregnant, or were otherwise ineligible, did not undergo testing for this urogenital disease. **All enrolled participants were negative for HIV and pregnancy, and were treated for UTI and syphilis if positive at screening. Twenty-three enrolled participants (six controls, seven in the metronidazole group, four in the EF+ group, and six in the GynLP group) were positive for CT and/or NG at screening and had not received treatment by the enrollment visit. [††]Total numbers are slightly lower due to invalid qPCR results: overall enrollment population is n = 63, control group n = 16, and metronidazole group n = 14. [‡‡]$p < 0.05$ by Mann Whitney U test, compared to control group. [§§]$p < 0.01$ by Mann Whitney U test, compared to control group.

A total of 41 adverse events were spontaneously reported between enrollment and M6. All of them were judged definitely not or unlikely to be related to trial participation, and they were evenly distributed among groups.

Preliminary efficacy: microscopy endpoints. In modified ITT analyses, the BV incidence rate by Nugent score during the intervention period was 10.18 per person-year at risk (PY) in the control group, and lower in the metronidazole (1.41/PY; p = 0.004), EF+ (3.58/PY; p = 0.043), and GynLP groups (5.36/PY; p = 0.220) (Table 3). Mean Nugent scores during the intervention period were highest in the control group, lowest in the metronidazole group, and in-between in the two vaginal probiotics groups (Fig. 2A). By the end of the intervention period, many women had developed microbiological-BV without symptoms. In line with standard practice, they were not treated, but they were also no longer included in the 'person-years at risk' denominator because they had already developed the endpoint. BV incidence rates were therefore much lower (1.26/PY overall) after cessation of the intervention, and similar between groups. The results for BV incidence by modified Amsel criteria were similar to those for Nugent scores (Table 3), and no vulvovaginal candidiasis was diagnosed after randomization.

Preliminary efficacy: sequencing endpoints. We took the baseline VMB composition imbalances into account by not only showing bacterial group means at each visit but also mean differences with enrollment (Fig. 2 and Table S4 for estimated concentrations, and Fig. S3 and Table S5 for relative abundances), and by using mixed effects models including participant identification number as the random effect to determine if any changes were statistically significant (Table 4). Immediately after BV treatment completion, the VMBs of most women gradually worsened (lactobacilli declined and BV-anaerobes expanded), probably due to the high-risk nature of the cohort. The mean estimated lactobacilli concentration declined to a low of 3.86 $\log_{10}$ cells/μl at M2 in the control group, but less so in the intervention groups (ranging from 4.60–5.58 $\log_{10}$ cells/μl at follow-up visits). In unadjusted mixed effects models using data from the intervention period only, metronidazole users had a higher estimated lactobacilli concentration (p = 0.043) and relative abundance (p = 0.006) than controls, EF+ users had a higher relative abundance (p = 0.014) but not estimated concentration than controls, and GynLP users had trends in the same directions that were not statistically significant. The expansion of BV-anaerobes was significantly lower in oral metronidazole users (relative abundance; p = 0.023), and in EF+ users (estimated concentration; p = 0.041), compared to controls. Mean estimated pathobionts concentrations were low in all groups throughout, ranging from 1.61–3.35 $\log_{10}$ cells/μl at follow-up visits. Mixed effects models did not identify any significant associations between randomization groups and estimated pathobionts concentrations or relative abundances, but showed trends ($0.05 < p < 0.1$) towards lower pathobionts relative abundances in the two vaginal probiotics groups compared to controls (Table 4). Mean estimated concentrations of 'other bacteria' were also low throughout, but highest in the EF+ group at the D7 and M1 visits (EF+ contains a *Bifidobacterium* strain). This was significant in unadjusted mixed effects models for relative abundances (p = 0.023) but not estimated concentrations.

The proportions of women in each randomization group at each visit having a particular VMB type corresponded with the estimated concentration and relative abundance data, and additionally showed that – in lactobacilli-dominated women – the *L. iners*-dominated VMB type was far more common than the *L. crispatus*-dominated and other lactobacilli-dominated VMB types throughout (Fig. S4). Among women with dysbiosis, the lactobacilli plus BV-anaerobes and polybacterial with ≥10% *G. vaginalis* VMB types continued to be the most common dysbiosis types during follow-up. In unadjusted mixed effects models using intervention period data only, metronidazole users and EF+ users, each compared to controls, were significantly less likely to have dysbiotic VMB types (p = 0.012 and p = 0.029, respectively) (Table 4).

The associations in unadjusted mixed effects models persisted after adjustment for hormonal contraception use/pregnancy, sexual risk taking, and age, except for the association with estimated *Lactobacillus* concentration among metronidazole users. Metronidazole users compared to controls had a significantly higher *Lactobacillus* relative abundance (p = 0.014), a significantly lower BV-anaerobes relative abundance (p = 0.049), and were significantly less likely to have BV by Nugent scoring (p = 0.031) or by VMB types (the two polybacterial and *G. vaginalis*-dominated VMB-types combined; p = 0.017) (Table 4). EF+ users compared to controls had a

Patient-reported symptoms and clinician-observed signs at Screening and Enrollment	Total ($n = 68$)	Controls ($n = 17$)	Metro ($n = 17$)	EF+ ($n = 17$)	GynLP ($n = 17$)	p*
Any (current or in last 2 weeks) urogenital symptoms at Scr, n (%)	49 (72.1)†	11 (64.7)	13 (76.5)	13 (76.5)	12 (70.6)	0.933
Any (current) urogenital symptoms at Enr, n (%)	0	0	0	0	0	NA
Any abnormal speculum exam findings at Scr, n (%)‡	3 (4.4)§	1 (5.9)	1 (5.9)	0	1 (5.9)	1
Any abnormal speculum exam findings at Enr, n (%)‡	1 (1.5)¶	0	0	0	1 (5.9)	1
AEs – Structurally assessed between Enr and M2 (during product use)						
Any current urogenital symptoms, n (%)‖	9 (13.4)**	2 (11.8)	2 (11.8)	2 (11.8)	3 (18.8)	0.894
Any abnormal speculum exam findings, n (%)‖	2 (3.0)††	0	0	0	2 (12.5)	0.054
Any abnormal bimanual exam findings n (%)‖	0	0	0	0	0	NA
AEs – Structurally assessed between M2 and M6 (after product cessation)						
Any current urogenital symptoms, n (%)‖	7 (10.8)‡‡	3 (17.7)	1 (5.9)	1 (6.3)	2 (13.3)	0.7
Any abnormal speculum exam findings, n (%)‖	21 (32.8)§§	6 (35.3)	5 (31.3)	6 (37.5)	4 (26.7)	0.951
Any abnormal bimanual exam findings n (%)‖	1 (1.6)¶¶	0	0	0	1 (6.7)	0.234
AEs – Not structurally assessed	**Total (N)**	**Controls (N)**	**Metro (N)**	**EF+ (N)**	**GynLP (N)**	**p***
Number of women with reported AEs‖‖	27	8	4	8	7	0.439
Total number reported AEs between Enr-M6	41	13	6	9	13	0.324
Total number reported AEs between Enr-M2	32	12	4	5	11	NA
Total number reported AEs between M2-M6***	9	1	2	4	2	NA
AEs – Not structurally assessed	**Total AEs ($n = 41$)**	**Controls ($n = 13$)**	**Metro ($n = 6$)**	**EF+ ($n = 9$)**	**GynLP ($n = 13$)**	**p***
Severity of reported AEs, n (%):						
Mild	2 (4.9)	0	0	1 (11.1)	1 (7.7)	
Moderate	36 (87.8)	13 (100)	5 (83.3)	6 (66.7)	12 (92.3)	NA
Severe	3 (7.3)	0	1 (16.7)	2 (22.2)	0	
Life-threatening	0	0	0	0	0	
Deemed related to study by physician, n (%):						
Definitely not related	10 (24.4)	3 (23.1)	2 (33.3)	3 (33.3)	2 (15.4)	NA
Unlikely	31 (75.6)	10 (76.9)	4 (66.7)	6 (66.7)	11 (84.6)	
Possible/probable/definitely related	0	0	0	0	0	
Outcome of reported AE, n (%):						
Fully recovered	40 (97.6)	12 (92.3)	6 (100)	9 (100)	13 (100)	
Not fully recovered, deteriorated, permanent damage, or death	0	0	0	0	0	NA
Ongoing	1 (2.4)	1 (7.7)†††	0	0	0	
Action taken by physician, n (%):						
None	6 (14.6)	1 (7.7)	0	2 (22.2)	3 (23.1)	
Medication given	35 (85.4)	12 (92.3)	6 (100)	7 (77.8)	10 (76.9)	NA
Study discontinuation	0	0	0	0	0	
Hospitalization‡‡‡	0	0	0	0	0	

Table 2. Safety endpoints. Abbreviations: AE, adverse event; EF+, Ecologic Femi+; Enr, enrollment visit; GynLP, Gynophilus LP; M2/6, month 2/6 visit; Metro, metronidazole; NA, not applicable; Scr, screening visit. *Fisher's exact test for binary outcomes and Kruskall Wallis test for continuous outcomes, between groups. †Most common symptom (89.8%) was genital itching. ‡No abnormal findings observed during bimanual exams. §Includes vaginal discharge (n = 2) and cervical polyps (n = 1). ¶Unusual cervical discharge. ‖Total numbers are slightly lower due to loss to follow-up (Fig. 1). No missing values. **Includes genital itching (n = 8) and burning (n = 3), pain during sex (n = 4), and burning when urinating, lower abdominal pain, unusual vaginal discharge, and genital/anal sores (all n = 1). ††One had ulcers/blisters in the vagina, one had lesions on the perineum and labia majora. ‡‡Includes genital itching (n = 5) and burning (n = 3), lower abdominal pain (n = 4), pain during sex, burning when urinating, urinary frequency/urgency (all n = 2), and unusual vaginal discharge (n = 1). §§Includes unusual vaginal (n = 4) and cervical (n = 13) discharge, and cervicitis (n = 10). ¶¶Cervical motion tenderness. ‖‖Most common AEs according to MedDRA coding were "Infections and infestations" (n = 14), followed by "Reproductive system and breast disorders" (n = 9) and "Gastrointestinal disorders" (n = 7; two in the no-intervention group, none in the metronidazole group, two in the EF+ group, and three in the GynLP group). No headaches were reported. Sexually transmitted infections and vulvovaginal candidiasis were not considered AEs but secondary outcomes. ***No AEs were reported after the M6 visit. †††Case of dental caries. ‡‡‡Both serious AEs involved hospitalizations that were not initiated by the study physician (see manuscript text).

BV IR Enr–M2	Controls		Metronidazole		EF+		GynLP	
	n/N*	IR (95% CI)†	n/N*	IR (95% CI)†	n/N*	IR (95% CI)†	n/N*	IR (95% CI)†
Nugent 7–10	9/11	10.18 (5.48–18.92)	2/10	1.41 (0.35–5.62)	5/12	3.58 (1.61–7.96)	6/10	5.36 (2.41–11.93)
Modified Amsel¶	11/15	7.53 (4.28–13.26)	3/11	2.04 (0.66–6.31)	6/13	3.36 (1.51–7.48)	4/9	3.35 (1.26–8.92)
BV IR M2–M6								
Nugent 7–10	4/11	0.91 (0.34–2.41)	5/8	1.86 (0.78–4.48)	6/9	1.58 (0.71–3.52)	2/7	0.76 (0.19–3.03)
Modified Amsel¶	3/12	2.96 (0.33–3.15)	5/10	2.25 (0.94–5.40)	6/8	3.84 (1.72–8.54)	3/8	1.83 (0.59–5.67)

BV IRR Enr–M2	Controls	Metronidazole vs. Controls	EF+ vs. Controls	GynLP vs. Controls
		IRR (95% CI)‡	IRR (95% CI)‡	IRR (95% CI)‡
Nugent 7–10	NA	0.14 (0.01–0.65)	0.35 (0.10–1.07)	0.53 (0.16–1.60)
Modified Amsel¶	NA	0.27 (0.05–1.00)	0.45 (0.14–1.28)	0.44 (0.10–1.47)
BV IRR M2–M6				
Nugent 7–10	NA	2.06 (0.44–10.37)	1.74 (0.41–8.41)	0.84 (0.08–5.85)
Modified Amsel¶	NA	2.22 (0.43–14.27)	3.78 (0.81–23.35)	1.80 (0.24–13.46)

Table 3. Preliminary efficacy – microscopy endpoints. Abbreviations: BV, bacterial vaginosis; CI, confidence interval; EF+, Ecologic Femi+; Enr, enrollment visit; GynLP, Gynophilus LP; IR, incidence rate; IRR, incidence rate ratio; ITT, intent to treat; M2/6, month 2/6 visit; NA, not applicable. There were no cases of vulvovaginal candidiasis during follow-up. The table presents modified ITT results: Participants who were BV-negative by modified Amsel but BV-positive by Nugent score 7–10 at the enrollment visit (n = 17) were omitted from modified ITT analyses. *Number of women (n) who developed at least one incident infection during the specified time period as a proportion of the women who had at least one follow-up visit in that time period (N). †Incident infections divided by person-years at risk. Three participants in the Enr–M2 BV by modified Amsel model, seven in the Enr – M2 BV by Nugent model, 10 in the M2–M6 BV by modified Amsel model, and 11 in the M2–M6 BV by Nugent model were omitted due to having been at risk for less than 10 person-days. ¶BV by modified Amsel criteria is defined as at least two of the following three laboratory criteria are positive: vaginal pH > 4.5, whiff test positive, and/or presence of >20% clue cells on wet mount. ‡Compared to the control group.

significantly higher *Lactobacillus* relative abundance (p = 0.025), a significantly lower estimated BV-anaerobes concentration (p = 0.046), a significantly higher relative abundance of 'other bacteria' (p = 0.009), and were significantly less likely to have BV-like VMB types (p = 0.027).

Detection of probiotic strains. During the intervention period, relevant probiotic strains were detected in 39% of samples from EF+ users and 20% of samples from GynLP users (all swabs combined, including self-sampled swabs). The detection percentages were 58% and 31%, respectively, in sensitivity analyses using non-rarefied data. Some of the EF+ strains cannot be differentiated from naturally occurring strains, and EF+-like strains were therefore detected (at low levels) in all groups at most time points (Fig. 3, Table S4). However, the mean estimated concentrations were highest in the EF+ group during the intervention period (mean estimated concentrations 0.48–1.92 $\log_{10}$ cells/μl per visit for all women combined, and 3.62–4.28 $\log_{10}$ cells/μl per visit for women who did have EF+ strains detected using rarefied data). The GynLP strain was only detected in the GynLP group during the intervention period (mean estimated concentrations 0.25–1.05 $\log_{10}$ cells/μl per visit for all women combined, and 3.72–4.55 $\log_{10}$ cells/μl per visit for women who did have GynLP detected using rarefied data). Inter- and intra-individual differences between participants were high: the highest estimated vaginal probiotic concentration detected in an individual EF+ user was 5.51 $\log_{10}$ cells/μl, and in an individual GynLP user was 6.17 $\log_{10}$ cells/μl. During the intervention period, the mean relative abundances of the probiotic strains were 0.03 in both EF+ and GynLP users, and 0.08 and 0.15, respectively, if only samples in which any strains were detected were included.

VMB transitions. The stacked graph and alluvial diagrams (Fig. S4) show that transitions from one VMB type to another were common. As expected, most transitions during the intervention period were from *Lactobacillus-dominated* states to dysbiotic states, but the reverse also occurred. Transitions between VMB types were common in all randomization groups. The percentage of actual transitions divided by potential transitions between D7 and M6 were 29/66 (43.9%) in the control group, 32/64 (50.0%) in the metronidazole group, 28/67 (41.8%) in the EF+ group and 24/56 (42.9%) in the GynLP group (Fisher's exact p = 0.792).

Incidence of sexually transmitted and urinary tract infections. As expected, the incidences of HIV (n = 2), herpes simplex type 2 (n = 1), syphilis (n = 1), gonorrhea (n = 5), chlamydia (n = 6), TV (n = 5) and urinary tract infection (n = 7) were too low to determine differences between randomization groups (Table S7).

Discussion

This trial showed that all three interventions were safe. Our preliminary efficacy results confirm that intermittent use of metronidazole reduces BV recurrence[10–12], and suggest that intermittent use of lactobacilli-containing vaginal probiotics may also reduce BV recurrence. These findings are important because some women and clinicians may prefer safe and efficacious vaginal probiotics to metronidazole since they are not expected to negatively affect other body niche microbiomes or cause antimicrobial resistance.

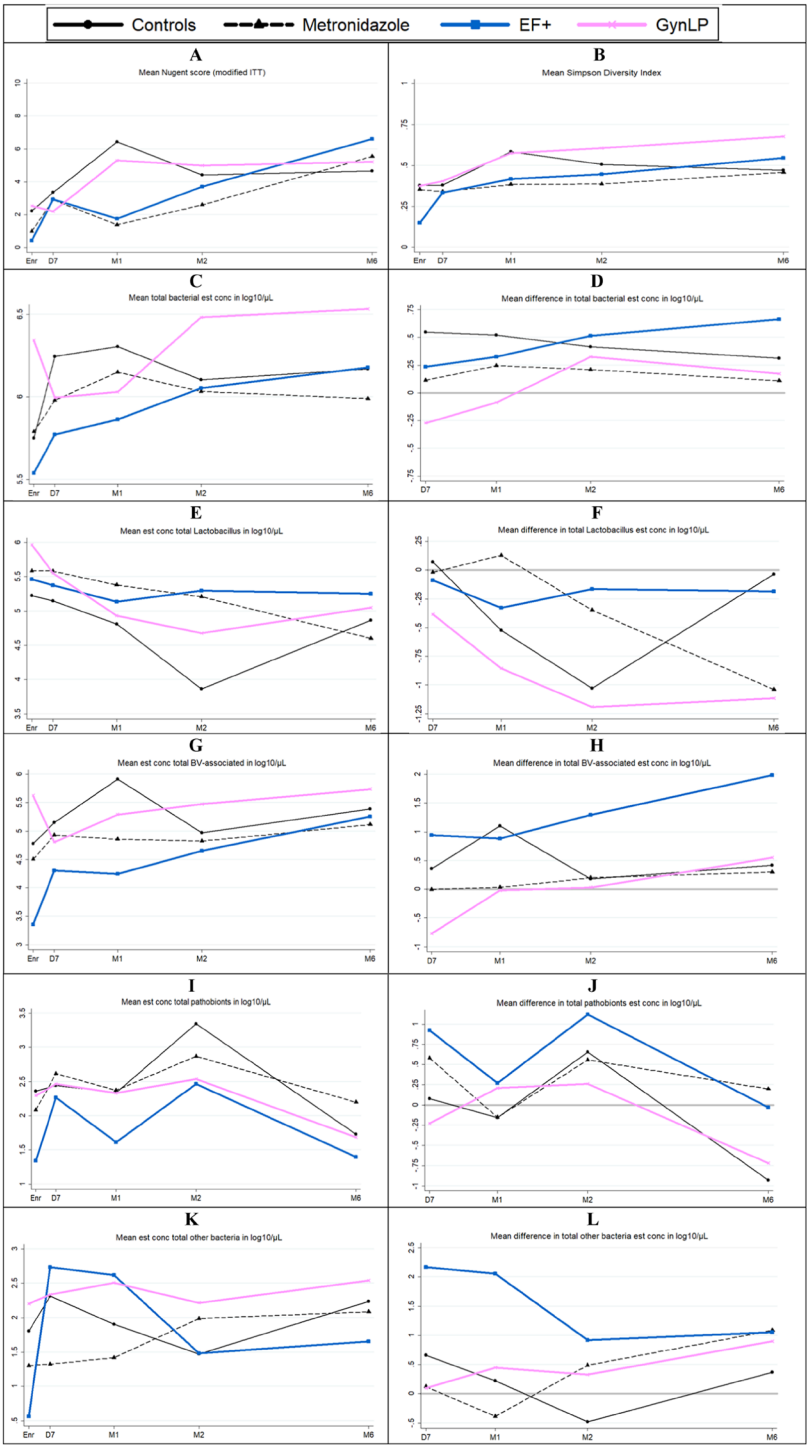


Figure 2. Preliminary efficacy by Nugent score, alpha diversity, and bacterial group estimated concentration. Abbreviations: D7, Day 7 visit; EF+, Ecologic Femi+; Enr, enrollment visit; est conc, estimated concentration; GynLP, Gynophilus LP; ITT, intention to treat; M1/2/6, month 1/2/6 visit; Scr, screening visit; VMB, vaginal microbiota. Changes in VMB outcomes over time per randomization group. See Supplement Table S4 for 95% confidence intervals. (**A**) Mean Nugent scores over time, only including women (n = 51) who were BV-negative by modified Amsel and Nugent criteria (modified ITT analysis). (**B**) Mean alpha diversity over time. (**C**) Mean estimated bacterial cell concentration over time. (**D**) Difference in mean estimated bacterial cell concentration with enrollment, over time. (**E**) Mean estimated lactobacilli concentration over time. (**F**) Difference in mean estimated lactobacilli concentration with enrollment, over time. (**G**) Mean estimated BV-anaerobes concentration over time. (**H**) Difference in mean estimated BV-anaerobes concentration with enrollment, over time. (**I**) Mean estimated pathobionts concentration over time. (**J**) Difference in mean estimated pathobionts concentration with enrollment, over time. (**K**) Mean estimated other bacteria concentration over time. (**L**) Difference in mean estimated other bacteria concentration with enrollment, over time.

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

Table 4. Preliminary efficacy – mixed effects models. Abbreviations: BV, bacterial vaginosis; CI, confidence interval; EF+, Ecologic Femi+; Enr, enrollment visit; est conc, estimated concentration; GynLP, Gynophilus LP; LA, lactobacilli plus BV-anaerobes VMB type; LD, *Lactobacillus-dominated* VMB type; Metro, metronidazole group; OR, odds ratio; PB, pathobionts VMB type; VMB, vaginal microbiota. *Compared to the control group. †Including all valid samples during product use (D7, M1, and M2 visits). Self-sampled samples were also taken during product use, but were not Gram stained nor tested by 16S rRNA gene qPCR (see Methods). ‡Estimated concentrations in log10 copies/μl, entered into the models as continuous variables. §Including all valid samples during product use (D7, M1, and M2 visits, and self-sampled samples). ¶Relative abundances, entered into the models as continuous variables constrained between 0 and 1. **Model adjusted for hormonal/pregnancy status (longitudinal assessments), sexual risk taking (longitudinal assessments; a composite variable including condom use consistency and number of sexual partners: women were considered low risk when they reported fewer than five sexual partners in the past month plus consistent condom use), and age (see Methods).

Our trial was funded as a pilot study and therefore had a modest sample size. Despite this, many of the preliminary efficacy associations for intermittent metronidazole and EF+ use reached statistical significance. While this was not the case for intermittent GynLP use, all of the trends in this group were in the same directions. We believe that the GynLP group suffered a few disadvantages compared to the other randomization groups, which may

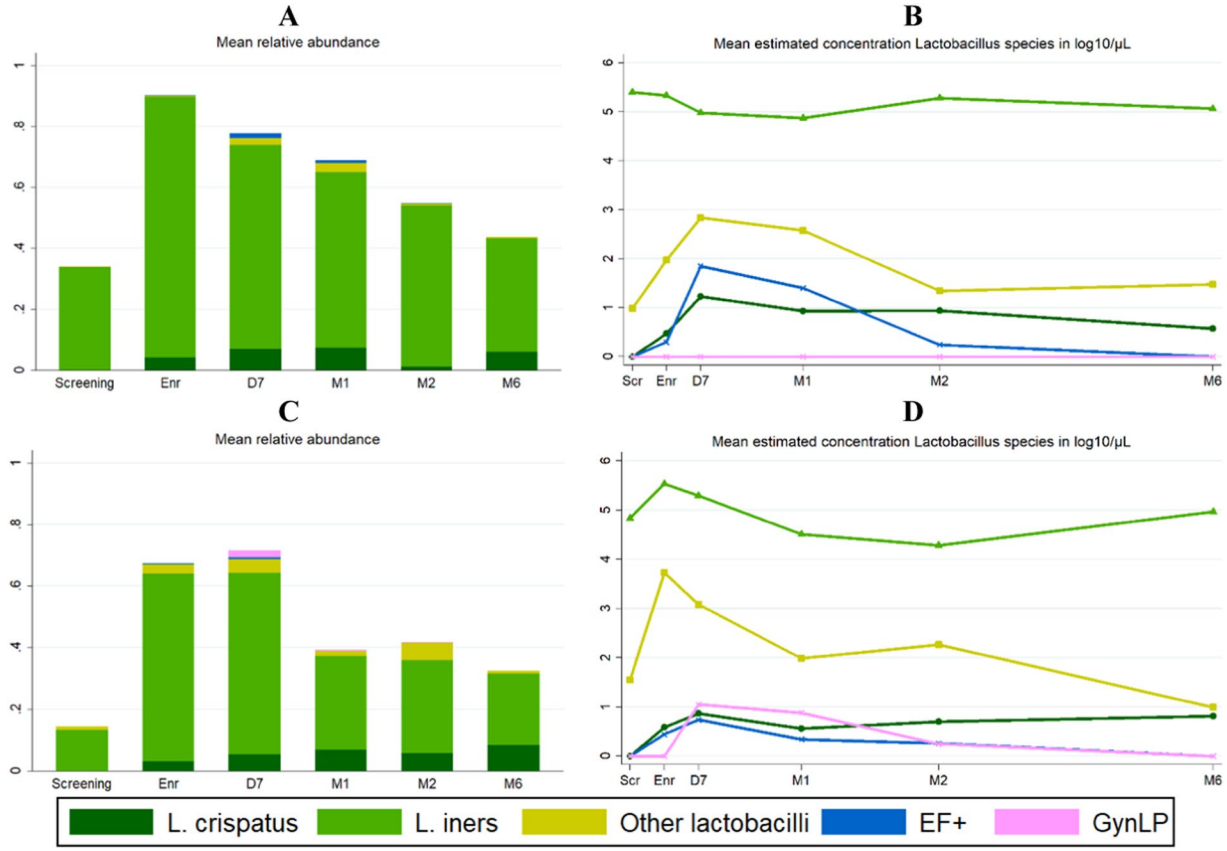


Figure 3. Detection of probiotic strains during the trial. Abbreviations: D7, Day 7 visit; EF+, Ecologic Femi+; Enr, enrollment visit; GynLP, Gynophilus LP; M1/2/6, month 1/2/6 visit; Scr, screening visit. Mean relative abundance (**A**) and mean estimated concentration (**B**) of *Lactobacillus* species over time in the EF+ group; mean relative abundance (**C**) and mean estimated concentration (**D**) of *Lactobacillus* species over time in the GynLP group. The length of bars in (**A,C**) depicts total relative abundance of all *Lactobacillus* species combined.

explain the lack of statistical significance. Randomization imbalances commonly occur in small trials[30], and in our trial, this led to women in the GynLP group being more dysbiotic at baseline than controls and EF+ users. We ameliorated this disadvantage in our analysis strategy, but we may not have been able to eradicate it. In addition, GynLP users were less adherent on average than metronidazole and EF+ users. Differences in adherence using triangulated data did not reach statistical significance, but probiotic strain detection rates (58% for EF+ samples and 31% for GynLP samples using non-rarefied data) support this claim. Biose has since simplified the dosing regimen of next generation Gynophilus products to two fixed days a week to boost adherence. Finally, GynLP dosing (once every four days) was similar to metronidazole dosing (twice weekly) but less frequent than EF+ dosing (once per day for the first five days followed by thrice weekly for the remainder of the intervention period).

Probiotic detection rates were 58% for EF+ samples and 31% for GynLP samples, and inter- and intra-individual variabilities were high (with estimated probiotic concentrations ranging from zero to 6.17 $\log_{10}$ cells/µl). This detection variability is consistent with most other vaginal probiotic studies that used sampling at non-daily intervals and molecular assessment methods[21,31–33]. A major drawback of all of those studies, including ours, is that product use was not directly observed but self-reported, and precise information about the time period between last product insertion and sample collection was lacking. The average total bacterial load of a healthy vagina is currently not known[34], but we estimate it to be in the order of 2×10^{10} bacteria (Supplement 1). The vaginal probiotic strain(s) in our trial were both applied at about 1.5×10^{9} CFU per dose, which would be about 7.5% of the total vaginal bacterial load after application if all probiotic bacteria were to remain in the vagina. We detected mean relative abundances of 7.7% for EF+ strains and 15.1% for GynLP when only samples with any relevant probiotic strains detected during product use were included (thereby eliminating any potential non-adherence). A recently published study of Gynophilus Slow Release tablet (which is almost identical to GynLP) in which women self-sampled every day showed that mean estimated vaginal concentrations of Lcr35 by qPCR were between 10^4 and 10^6 CFU/µl in women who used the tablet once every four or five days[35]. Using our estimated concentration data, we detected a similar concentration range in samples with any GynLP detected. This consistency is reassuring, but the question remains whether probiotic concentrations of this order of magnitude optimally prevent BV recurrence in the long-term. Furthermore, all studies referenced in this paragraph, including ours, have shown that probiotic strains do not persist in the vagina after dosing has ceased. The second question then is whether the colonization capacity of probiotic bacteria should be improved. Our data suggest that probiotic lactobacilli may boost 'natural' lactobacilli indirectly, which may be sufficient. Indirect effects may

include increased localized lactic acid production, modulation of cervicovaginal mucosal immune responses, and/or inhibition of biofilm formation, by probiotic bacteria[3,36,37].

Additional limitations of our study include lack of proof that the probiotic contents (strains and doses) were in agreement with the product labels, the high urogenital infection risk of this cohort (which makes prevention more challenging), and our inability to fully control for potential confounders. However, the mixed effects models were controlled for some of the best known VMB determinants (hormonal contraception, pregnancy, sexual risk taking, and age)[1,38–40]. We were not able to exclude women with gonorrhea and/or chlamydia infection at the time of randomization due to the slow laboratory turn-around time, but the VMB compositions of women with and without infection were similar, and we therefore think that this did not negatively affect our results.

With the development of better genomic and culturing methods, we are now on the cusp of a new era in vaginal probiotic research. Past vaginal probiotic studies have shown mixed results[13–24], but almost all of these studies used imprecise VMB assessments based on clinical symptoms and microscopy. The addition of sequencing methods showed that many more women than previously thought are not lactobacilli-dominated after standard antibiotic BV treatment, that host responses to antibiotic and probiotic treatment are highly variable, and that it is possible to differentiate between probiotic strains and 'natural' lactobacilli. Furthermore, others have shown that quantifying relative abundance data in the same manner as we have done in this study correlates well with species-specific quantitative PCRs of non-minority species[27,28]. This then allows for microbiota data reduction into quantitative variables that can be analyzed in mixed effects models that adjust for repeated measures and confounding.

We conclude that lactobacilli-based vaginal probiotics warrant further development to improve long-term beneficial effects. We recommend that future trials incorporate quantitative molecular methods, optimize dosing and timing of product insertion versus sample collection, and enroll women with various urogenital risk profiles. Ideally, these trials would also evaluate the effects of interventions on vaginal biofilm formation, and – eventually – the impact on pregnancy complications, HIV epidemics, and other adverse outcomes.

Data availability

Participants were not explicitly asked for consent related to use of their data by external parties or use that does not address the research questions described in the approved study protocol (publicly available at https://datacat.liverpool.ac.uk/). Data were therefore deposited in a controlled access repository at the University of Liverpool. Data can be requested by emailing the Research Data Management team (rdm@liverpool.ac.uk) and the data steward (Professor Janneke van de Wijgert; j.vandewijgert@liverpool.ac.uk). Requests will be submitted to the University of Liverpool ethics committee (ethics@liverpool.ac.uk) for approval.

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Author contributions

J.v.d.W. obtained the research funding and wrote the study protocol and data collection documents. J.v.d.W., A.N., E.L., and J.R. were members of the Trial Steering Committee. S.A., L.M., V.M., M.U., and J.v.d.W. collected the primary data. L.M. performed the diagnostic testing. M.V., C.B., A.D., and J.R. conducted or oversaw the 16S rRNA gene sequencing and qPCRs. J.v.d.W. and M.V. developed the analytical approach and performed the statistical analyses. J.v.d.W. and M.V. wrote the manuscript. All authors commented on and approved the final manuscript. JvdW had full access to the data and had final responsibility for the decision to submit for publication.

Competing interests

AN is employed by Biose (owner of trial product GynLP) and EL by Winclove Probiotics BV (owner of trial product EF+). AN and JR have financial and/or intellectual investments in competing products. The other authors report no competing interests.

Additional information

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