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SUPPLEMENT ARTICLE

Connecting the Dots: Translating the Vaginal Microbiome Into a Drug

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A *Lactobacillus*-dominated vaginal microbiota (VMB) has been associated with health and considered an important host defense mechanism against urogenital infections. Conversely, depletion of lactobacilli and increased microbial diversity, amplifies the risk of adverse gynecologic and obstetric outcomes. A common clinical condition that exemplifies dysbiosis is bacterial vaginosis (BV). BV is currently treated with antibiotics, but frequently recurs, due in part to persistent dysbiosis and failure of lactobacilli to repopulate the vagina. New treatment options are needed to address BV. The VMB is relatively simple and optimally dominated by one or several species of *Lactobacillus*. *Lactobacillus crispatus* is strongly associated with vaginal health and depleted in dysbiosis. Replenishing the dysbiotic VMB with protective *L. crispatus* CTV-05 is a promising approach to prevent recurrent infections and improve women's health. Here we discuss confirmation of this approach with the microbiome-based biologic drug, LACTIN-V (*L. crispatus* CTV-05), focusing on prevention of BV recurrence.

Keywords. LACTIN-V; *Lactobacillus crispatus* CTV-05; bacterial vaginosis (BV); vaginal microbiota (VMB); live biotherapeutic product (LBP); women's health.

THE VAGINAL MICROBIOTA AND WOMEN'S HEALTH

The role of the vaginal microbiota (VMB) in the female reproductive tract health is well established [1–3]. *Lactobacillus acidophilus* was once considered the major vaginal species until the 1980s when molecular identification methods showed it to be a complex of multiple species. Subsequently, *Lactobacillus crispatus, Lactobacillus gasseri*, and *Lactobacillus jensenii* were identified as major species of the VMB [4, 5], and more recently *Lactobacillus iners* emerged as another prevalent species [6, 7], although its role in vaginal health is still under debate [8]. Newer culture-independent techniques, using DNA sequencing techniques, revealed the same 4 *Lactobacillus* species dominating separate bacterial community state types (CSTs), as well as a heterogeneous CST that is not dominated by *Lactobacillus* [6, 7].

Although every woman harbors a unique bacterial community, the VMB is optimally dominated by H_2O_2-producing lactobacilli, which create a low-pH [9], noninflammatory environment [3]. Lactic acid produced by lactobacilli acidifies the vagina and suppresses the growth of many opportunistic pathogens [10, 11]. *L. crispatus* is the most prevalent H_2O_2-producing *Lactobacillus* species of the female reproductive tract and *L. crispatus*-dominated bacterial communities exhibit the lowest vaginal pH, lowest proinflammatory cytokine levels, and lowest risk of gynecologic and obstetric complications [10–14].

VAGINAL DYSBIOSIS

Bacterial vaginosis (BV) is a common ecological disorder of the VMB characterized by increased microbial diversity with expansion of mainly anaerobic bacteria and loss of H_2O_2-producing lactobacilli [15, 16]. BV affects 15%–50% of reproductive-aged women globally and can recur in 20%–75% within 3 months following standard antibiotic treatment [17, 18]. Dysbiosis can be associated with increased levels of proinflammatory cytokines [19] and increased numbers of activated $CD4^+$ T lymphocytes [20]. Cervicovaginal bacterial communities are major modulators of the host inflammatory response [11]. Several negative sequelae accompany proinflammatory dysbiosis, such as increased risk of sexually transmitted infections (STIs) [21, 22], including human immunodeficiency virus (HIV) [23], pelvic inflammatory disease [24], preterm birth [25], and enhanced progression of cervical cancer human papillomavirus (HPV) [26].

BV is currently treated with antibiotics (ie, metronidazole) [27]. Metronidazole kills the BV-associated anaerobic bacteria while sparing vaginal lactobacilli, which are intrinsically resistant to nitroimidazoles. However, metronidazole treatment alone does not restore a *Lactobacillus*-dominated microbiome and dysbiosis can persist.

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FROM PROBIOTICS TO LIVE BIOTHERAPEUTIC PRODUCTS

Probiotics have rapidly grown into a multibillion-dollar industry that is lightly regulated as food or dietary supplements in the United States [28]. This has led to a call for stricter requirements for scientific substantiation of putative health benefits conferred by microorganisms [29]. Although most probiotics are for gastrointestinal use, several are marketed for vaginal health. However, many of these products contain species that are not naturally present in the VMB. It is not clear whether these products can sustainably colonize or benefit the vaginal ecosystem because vaginal strains differ from those found in food or the gastrointestinal tract. A number of recent meta-analyses have been published on probiotics to treat/prevent BV [30–32], and while the products were generally safe, there was no clear or consistent indication that commercially available probiotics improve outcomes related to women's health. Because probiotic products are not regulated as drugs in the United States, they cannot make specific health claims.

Fueled by the Human Microbiome Project [33], the roles of the microbiome in health have become better appreciated and spurred the development of microbiome-based products intended to treat or prevent disease. This activity prompted the Center for Biologics Evaluation and Research at the Food and Drug Administration (FDA), to respond with a draft guidance, document in 2012, addressing the early development of live biotherapeutic products (LBPs), thus establishing a new class of biologic drugs [34]. LBP was defined as a biological product that: (1) contains live microorganisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine.

LACTIN-V: THE FIRST VMB-BASED LBP

L. crispatus has long been associated with reproductive health and has strong inverse relationships with vaginal dysbiosis and its clinical sequelae [10, 11]. For example, depletion of vaginal *Lactobacillus*, particularly H_2O_2-producing strains such as *L. crispatus*, has been associated with both BV and recurrent urinary tract infections (rUTI) [35, 36]. For these reasons, a strain of *L. crispatus* was carefully selected as the active ingredient of LACTIN-V, which became the first VMB-based LBP. LACTIN-V is being developed under an Investigational New Drug application with the FDA as an adjuvant therapy to prevent recurrence of BV and rUTI following antimicrobial treatment.

LACTIN-V contains *L. crispatus* CTV-05 (CTV-05), a specific strain isolated from the vagina of a healthy woman [5, 37]. *L. crispatus* is found naturally in the vagina of many healthy women and has also been detected in the rectum [38–40]. CTV-05 is a homofermenter of glucose to lactic acid (both D and L isomers), and an H_2O_2 producer. Unlike most commercially available probiotic *Lactobacillus* strains, which are not vaginal strains, CTV-05 adheres to vaginal epithelial cells and is capable of colonizing the vagina [37, 41, 42]. CTV-05 has an antibiotic-susceptibility profile similar to other *L. crispatus* strains and is intrinsically resistant to metronidazole. In addition, CTV-05 antagonizes a number of urogenital pathogens in vitro (Table 1). The strain has an excellent preclinical and clinical safety record. There have been no reports of *L. crispatus* causing bacteremia or endocarditis, as noted with some probiotic lactobacilli [45].

Table 1. Inhibition of Vaginal and Urinary Pathogens by CTV-05

Microorganism	Strain No.	Results: Zone of Inhibition, mm[a]
Vaginal pathogens		
N. gonorrhoeae	F6[b]	61 (S)
N. gonorrhoeae	SPD 600[b]	62.5 (S)
N. gonorrhoeae	7603389[b]	55 (S)
N. gonorrhoeae	87016589[b]	55 (S)
N. gonorrhoeae	85044571[b]	65 (S)
B. fragilis	25285[c]	32.5 (S)
B. fragilis	43860[c]	42.5 (S)
B. fragilis	43858[c]	70 (S)
S. agalactiae group B	13813[c]	57.5 (S)
S. pyogenes	Clinical isolate[d]	Complete inhibition
G. vaginalis	ATCC 14018	Complete inhibition
G. vaginalis	Clinical isolate 9[d]	Complete inhibition
G. vaginalis	Clinical isolate 10[d]	Complete inhibition
G. vaginalis	Clinical isolate 11[d]	Complete inhibition
Dialister sp.	Clinical isolate[d]	Partial inhibition
Urinary tract pathogens		
E. coli	3052-961	Complete inhibition
E. coli	3100-961	63 (S)
E. coli	3171-961	70 (S)
E. coli	3196-961	Complete inhibition
E. coli	3265-961	63 (S)
E. coli	3301-971	Complete inhibition
E. coli	3077-971	63 (S)
E. coli	3058-981	67.5 (S)
E. coli	3163-981	60 (S)
E. coli	3201-981	67.5 (S)
E. coli	49161[c]	68 (S)
E. coli	11775[c]	71.5 (S)
E. coli	29194[c]	62.5 (S)
E. coli	25922[c]	60 (S)
Staph. aureus	25923[c]	68 (S)

Abbreviations: *B., Bacillus; E., Escherichia; G., Gardnerella; N., Neisseria; S., Streptococcus; Staph., Staphylococcus;* S, sensitive.

[a]Zone of inhibition method used an agar bilayer technique to detect inhibition by *Lactobacillus crispatus* CTV-05 against the test organisms [43, 44]. For organisms requiring special nutrient agars, the process was modified to use commercially prepared agar, which was aseptically removed from a petri dish and placed directly over an MRS agar surface creating a bilayer of equal thickness. All MRS agar plates were inoculated by streaking 0.01 mL of an overnight culture of CTV-05 across the diameter of the plate. All plates were incubated under optimum conditions for 24, 48, or 72 hours prior to overlay.

[b]Strains provided by the Centers for Disease Control and Prevention.

[c]Strain obtained from the American Type Culture Collection.

[d]Clinical vaginal isolates obtained from women under IRB 06157-01 with Planned Parenthood Mar Monte.

LACTIN-V DEVELOPMENT

LACTIN-V was originally formulated as a vaginally administered gelatin capsule with a potency of 5×10^8 colony-forming units (CFU)/capsule. The capsule was tested in phase 1 and phase 2 clinical trials of healthy female volunteers and women with rUTI or BV. Following a successful phase 1 safety study in healthy women with a history of rUTI [46], a phase 2 rUTI trial (NCT00305227) was conducted in 100 women who received standard antibiotic treatment for uncomplicated cystitis, followed by LACTIN-V or placebo capsules daily for 5 days, then once weekly for 10 weeks [47]. Although not statistically significant, the rUTI incidence in the LACTIN-V arm (15%) was about half of that in the placebo arm (27%), similar to prophylactic antibiotic treatment. However, high-level vaginal colonization with *L. crispatus* (>10^6 16S rRNA gene copies/mL by quantitative polymerase chain reaction [qPCR]) in the LACTIN-V arm was associated with a significant rUTI reduction (risk ratio [RR] = 0.07; 95% confidence interval [CI], .02–.3). Interestingly, women receiving placebo who achieved comparably high levels of endogenous *L. crispatus* did not appear to have equivalent protection against rUTI (RR = 1.1), suggesting that CTV-05 was superior to the endogenous strains.

LACTIN-V capsules were tested in a phase 2 multisite randomized placebo-controlled trial of 149 women treated for BV with topical metronidazole or clindamycin (unpublished). The product was administered for 5 consecutive days, then once weekly for 10 weeks, with follow-up clinic visits at 4, 10, and 16 weeks. LACTIN-V administration appeared to modestly decrease the rate and incidence of recurrent BV compared to placebo. The time to first BV recurrence was longer in the LACTIN-V arm (118.7 days) versus the placebo arm (98.7 days). Subjects in the LACTIN-V arm who were colonized with CTV-05 were less likely to experience another BV episode than those in the placebo arm. CTV-05 colonization was determined by culture and repetitive element PCR (repPCR) [37]. In the per protocol cohort, the incidence of BV recurrence in CTV-05–colonized subjects was 12.5% compared to 30.3% in placebo, and 16% for the modified intention to treat cohort compared to 33.8% in placebo. Colonization in the most compliant per protocol cohort was 59% compared to 42% in the modified intention to treat cohort. Although not statistically significant, these results suggested for the first time that CTV-05 colonization may be a surrogate marker for efficacy, and that improving efficacy would require achieving higher colonization rates. Because the capsule formulation dissolved poorly in the vagina, likely hindering CTV-05 colonization, a specially designed vaginal applicator was developed to deliver LACTIN-V powder directly to the vaginal mucosa.

LACTIN-V VAGINAL APPLICATOR

The new dosage form was tested in a phase 1 escalating dose trial to assess safety, tolerability, and acceptability of the LACTIN-V applicator at 3 doses ranging from 5×10^8 to 2×10^9 CFU/applicator (NCT00537576) [48]. Twelve healthy women received the study product for 5 consecutive days and returned for follow-up visits on days 7 and 14. The adverse events (AEs) were mild or moderate and predominantly local (genitourinary) and evenly distributed across dose levels and treatment arms. LACTIN-V delivered at doses up to 2×10^9 CFU/applicator appeared to be safe and well tolerated.

A small phase 2a trial followed to assess colonization, safety, tolerability, and acceptability of applicator-delivered LACTIN-V (NCT00635622) [42]. All participants were treated for BV with 0.75% metronidazole vaginal gel (MetroGel), followed by LACTIN-V (2×10^9 CFU/applicator) or matching placebo applicator daily for 5 days, then once weekly for 2 weeks. The participants returned for follow-up on days 10 and 28. Overall, 61% in the LACTIN- V group were colonized with CTV-05 as determined by culture and repPCR. Among LACTIN-V users with complete adherence to the protocol, 78% were colonized [42]. The AEs were mild or moderate in severity and evenly distributed between the LACTIN-V and placebo arms. The applicator product appeared to be safe, well tolerated, and accepted by the participants.

The effects of endogenous vaginal bacteria on *L. crispatus* colonization during the phase 2a study were examined by qPCR for 7 BV-associated bacteria, (*Leptotrichia*/*Sneathia* sp., *Gardnerella vaginalis,* BVAB-1, BVAB-2, BVAB-3, *Megasphera* sp., and *Atopobium vaginae*), *L. iners,* and *L. crispatus* [49]. The concentrations of the 7 BV species declined between the screening and enrollment visits with successful Metrogel treatment, and *L. crispatus* levels generally increased upon application of LACTIN-V (Figure 1). Overall, this study provided additional evidence that vaginal colonization with *L. crispatus* following LACTIN-V treatment was associated with reduced levels of BV-associated anaerobes, potentially reducing the risk of BV recurrence. A preliminary microbiome analysis of a subset of samples from the phase 2a study showed that women colonized with CTV-05 underwent a shift from a diverse VMB at enrollment, to a *L. crispatus*-dominated bacterial community at day 28 (Figure 2A). Results from 2 women not colonized with CTV-05 are also shown (Figure 2A). Figure 2B shows the concomitant decrease in the Shannon diversity index for the 2 women colonized with CTV-05. Figure 2C shows a principal component analysis of the 4 women day 1 and day 28 post CTV-05 treatment. For women not colonized, the Shannon diversity index remained high and *L. iners* was the main *Lactobacillus* species present at enrollment and day 28.

PHASE 2 PROOF OF CONCEPT

Recently, a larger phase 2b multisite randomized, placebo-controlled trial of LACTIN-V to prevent BV recurrence was completed (NCT02766023) [51]. In this study, 228 women with BV were treated with Metrogel for 5 days, then randomized 2:1

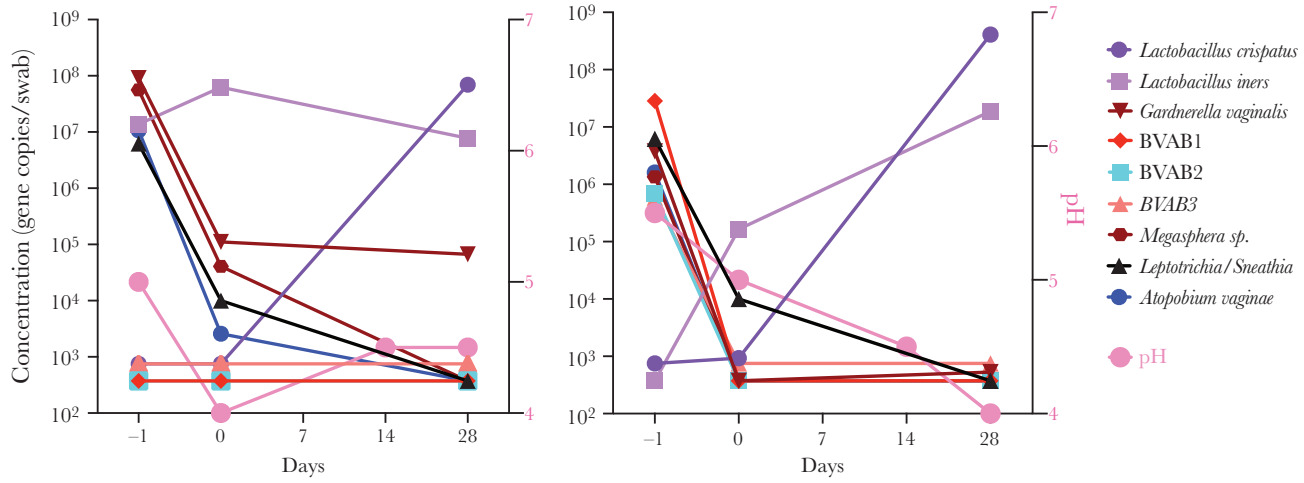

Figure 1. Changes in the concentrations of vaginal bacteria species of 2 women successfully colonized with *Lactobacillus crispatus*-CTV-05 in the LACTIN-V arm of the phase 2a trial. Vaginal swabs were taken at screening (before metronidazole treatment), at enrollment (after metronidazole and before LACTIN-V treatment), and at day 28 (after LACTIN-V treatment). DNA was extracted from vaginal swabs and bacterium-specific qPCR was performed for targeted vaginal bacteria. Bacteria concentrations are expressed as mean $\log_{10}$ 16S rRNA gene copies per swab. Vaginal pH was measured at screening (day –1), enrollment (day 0), and days 14 and 28. Abbreviations: BVAB, bacterial vaginosis-associated bacteria; qPCR, quantitative polymerase chain reaction.

to receive LACTIN-V (2×10^9 CFU/applicator) or a matching placebo applicator for 5 consecutive days, followed by twice-weekly doses for 10 additional weeks. Follow-up clinic visits occurred at weeks 4, 8, 12, and 24. The primary end points were the proportion of women with recurrent BV by week 12 and safety by week 24. Secondary end points included detectable CTV-05 colonization at each study visit and the proportion of women with recurrent BV by week 24.

In the intention-to-treat analysis, BV recurrence through the 12-week visit was significantly less common in the LACTIN-V arm than in the placebo arm (30% vs 45%, respectively; RR = 0.66; 95% CI, .44–.87; P = .01). In addition, BV recurrence remained significantly less common through the 24-week visit, 13 weeks after the last dose of LACTIN-V (39% vs 54%, respectively; RR = 0.73; 95% CI, .54–.92). The local solicited genitourinary AEs were mostly mild or moderate in severity, and their frequency and severity were similar between arms (Table 2). Additional information of solicited, unsolicited, and systemic AEs can be found in Cohen et al supplemental material [51].

CONNECTING THE DOTS

Overall, CTV-05 was detected by qPCR in 79% of women in the LACTIN-V arm at the 12-week visit and 48% at 24 weeks (Figure 3). Detectable colonization was defined as CTV-05 levels above the lower limit of detection at the 95% detection threshold (6.6×10^2 CFU/mL). Among subjects with detectable CTV-05, the median concentration (expressed as CFU/mL) in the LACTIN-V intervention arm ranged from 1.7×10^6 to 6.2×10^6 at different clinic visits during the dosing regimen through week 12, and 5.6×10^6 at week 24, approximately 13 weeks after the last dose of LACTIN-V. Although the proportion of participants with detectable colonization decreased after the last dose of LACTIN-V, the smaller number of women who remained colonized at week 24 still had median levels of CTV-05 comparable to those on treatment up to week 12 (Figure 3). This finding suggested that some women remained durably colonized for at least 13 weeks following the last dose of LACTIN-V. When the concentrations of CTV-05 at 12 and 24 weeks were further analyzed in participants with or without BV recurrence, it was evident that women who did not experience BV recurrence had significantly higher CTV-05 concentrations than those with BV recurrence (Figure 4). Women harboring $\geq 10^6$ CFU/mL CTV-05 appeared to be protected from recurrent BV.

Furthermore, the colonization of *L. crispatus* was considerably higher in the LACTIN-V arm than in the placebo arm at both week 12 (82% vs 35%, respectively) and week 24 (64% vs 22%, respectively). Thus, without LACTIN-V treatment, only about one-third of women in the study population would spontaneously recolonize with an endogenous *L. crispatus* following Metrogel treatment.

When CTV-05 dominates the VMB, it generally tracks with qPCR measurements of *L. crispatus* sp., *Lactobacillus* spp., total bacteria, and low vaginal pH. Figure 5A shows results from 2 women who were successfully colonized throughout the duration of the study to week 24, while Figure 5B shows results from 2 women who were successfully colonized during dosing through week 12, but then lost CTV-05 after the last dose of LACTIN-V. When CTV-05 levels declined in Figure 5B there was a corresponding increase in vaginal pH. An important mechanism for protection from BV recurrence is vaginal acidity, which is largely a function of lactic acid production by lactobacilli. In some instances, *L. crispatus* sp. levels were below

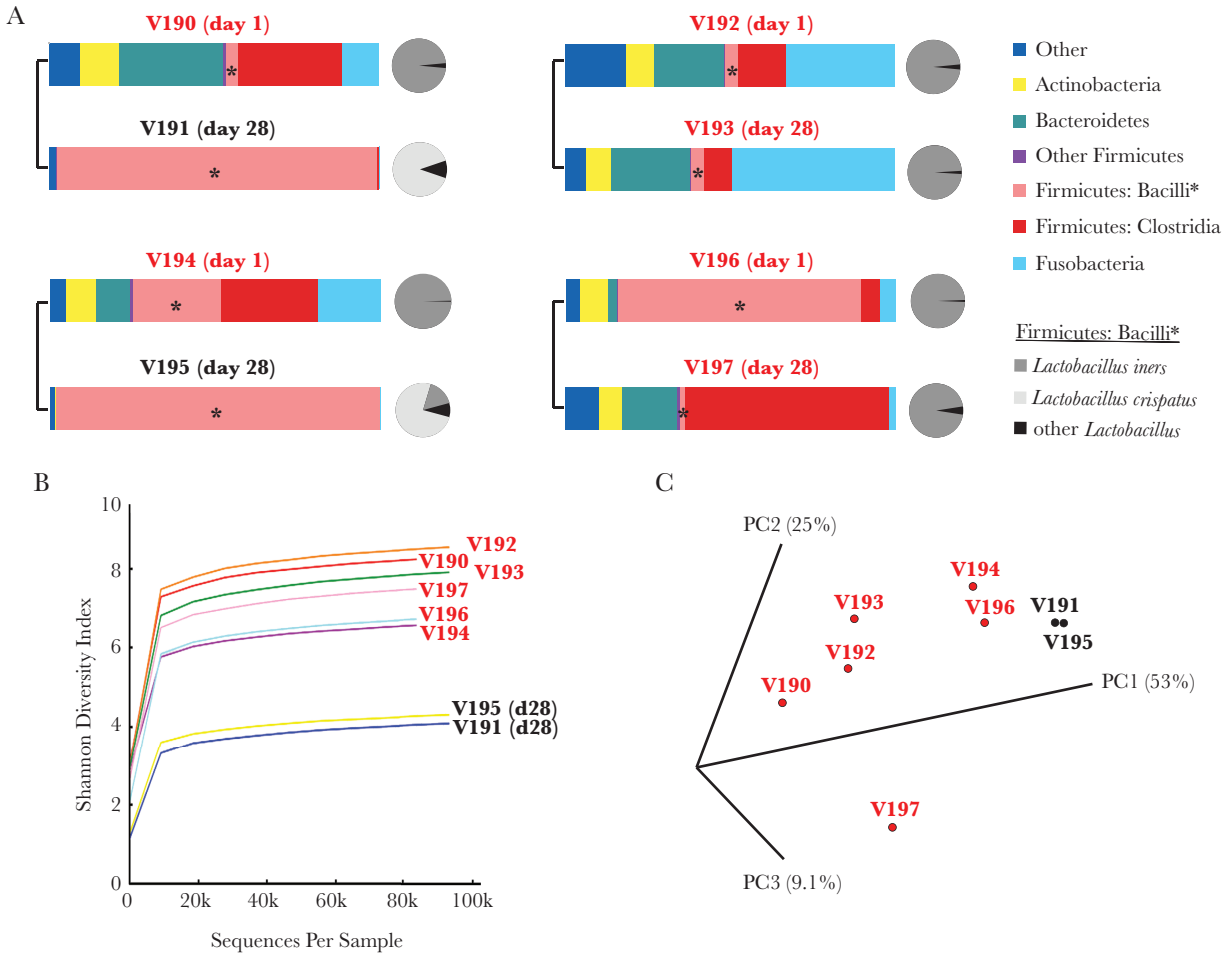

Figure 2. Changes in the microbiota of 4 women during the phase 2a LACTIN-V trial. *A*, The bacterial communities of 4 women before (day 1) and after (day 28) dosing with *Lactobacillus crispatus*-CTV-05 in the LACTIN-V arm of the phase 2a trial. Left, 2 women who were successfully colonized; right, 2 women who were not. Note that the bacterial species in the phylum Firmicutes, class Bacilli became *L. crispatus*-dominant (light grey circle) in the women who were colonized. *Lactobacillus iners* (medium grey) was the predominant lactobacillus species in women who were not colonize with *L. crispatus*. *B*, Shannon diversity indices were calculated for the 2 time points of each woman. Note that the day-28 time points for the women in whom *L. crispatus* became dominant (V191 and V195) had the lowest Shannon diversity. *C*, A principal component analysis (PCA) was performed to visualize the differences in microbiota among the 4 women at pre- and posttreatment time points. Note that the posttreatment time points for the 2 women who were successfully colonized with *L. crispatus* (in response to LACTIN-V treatment) and thus had the highest proportion of lactobacilli, V191 and V195, cluster together by PCA. Microbiome metrics (relative abundance and Shannon diversity indices) were calculated and PCA graphs were plotted using QIIME software (version 1) [50]. Individual vaginal swab samples are denoted with a "V###" abbreviation, while each of the 3 principal component axes are denoted with a "PC#" abbreviation.

detection while total *Lactobacillus* spp. levels were high, and vaginal pH was more variable (eg, subject 594). *L. iners* was suspected as the probable species in many of these cases, as observed in the phase 2a study. Metagenomic next-generation sequencing is being conducted to further identify the bacterial species present and their potential role in BV recurrence.

Table 2. Frequency and Severity of Local Genitourinary Adverse Events

	LACTIN-V		Placebo	
Symptoms	% Mild	% Moderate	% Mild	% Moderate
Abnormal vaginal discharge	41.8	25.5	34.8	34.8
Abnormal vaginal odor	35.4	21.2	21.1	30.8
External genital irritation	24.1	19.8	18.1	12.1
External genital swelling	10.6	10.6	7.5	7.5
Genital burning	18.4	15.6	10.6	18.1
Genital itching	29.7	31.9	28.7	22.7
Genital rash	9.2	5.6	6.0	4.5
Vaginal bleeding	23.4	3.5	28.7	4.5

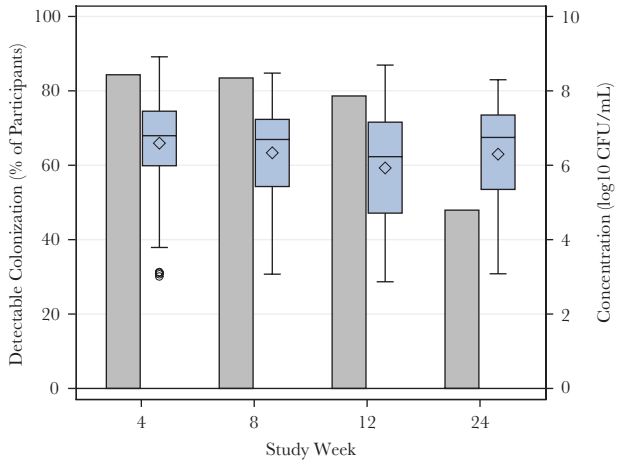

Figure 3. Detectable colonization and median concentrations of *Lactobacillus crispatus* CTV-05 in the LACTIN-V arm of the phase 2b trial at weeks 4, 8, 12, and 24. The grey bars indicate the overall percentage of women colonized with *L. crispatus* CTV-05 above the lower limit of detection in the LACTIN-V arm. The box and whiskers plots indicate maximum and minimum values of *L. crispatus* CTV-05, the horizontal lines inside the box and whiskers indicate median values and the diamonds indicate mean values. The circles below the box and whiskers bar at week 4 represent outliers. The size of each box and whiskers represents the interquartile range. Abbreviation: CFU, colony-forming units. Reproduced with permission from the New England Journal of Medicine [52].

CURRENT STATUS, NEXT STEPS, AND FUTURE PROSPECTS

In the phase 2b trial, LACTIN-V significantly reduced BV recurrence by one-third compared to placebo. This result was particularly significant in the context of the study population because most of the women were at high risk of BV recurrence. About half the participants had experienced ≥5 prior BV episodes, and nearly 70% had ≥3 BV episodes. In addition, over half the women self-identified as African American or Hispanic/Latina, 2 populations where BV is particularly prevalent [52].

CTV-05 colonization was closely correlated with prevention of BV recurrence. It appeared that colonization levels $\geq 10^6$ CFU/mL may be protective, possibly because sufficient lactic acid can be produced to drive vaginal pH down and antagonize the growth of BV-associated bacteria. Nonetheless, some women who were not well colonized with CTV-05 experienced BV recurrence. An important ongoing therapeutic goal is to determine the factors that contribute to persistent colonization of CTV-05 so that BV recurrence can be prevented in a greater proportion of women over longer time periods.

Recent studies have identified multiple factors and temporal dynamics that impact the composition of the VMB and its ability to maintain a *Lactobacillus*-dominant state and prevent BV. Host genetic factors may lead to a higher risk for disturbances of the VMB in women of African, African American, and Latina ethnicity [7, 53–56]. Genetic associations in Kenyan women suggest a role for the innate immune system and cell signaling in vaginal microbiome composition and susceptibility to nonoptimal vaginal microbiome [57]. In addition, hormonal fluctuations impact the VMB [58–62]; estrogen and vaginal glycogen levels are lowest during menses, and the presence of menstrual blood may increase pH and BV risk [63–66]. However, in the phase 2b trial neither menses nor unprotected sex decreased CTV-05 colonization, possibly due to the twice-weekly dosing regimen. In addition, evidence points toward certain hormonal contraceptives as being potentially protective against BV [61, 67–72].

Sexual behavior affects BV risk. While condoms are protective [73, 74], early sexual debut [75, 76], oral sex [77], untreated sexual partners [67, 78–83], multiple partners [76], and exposure to semen [73, 74, 78, 84] all increase BV risk. BV

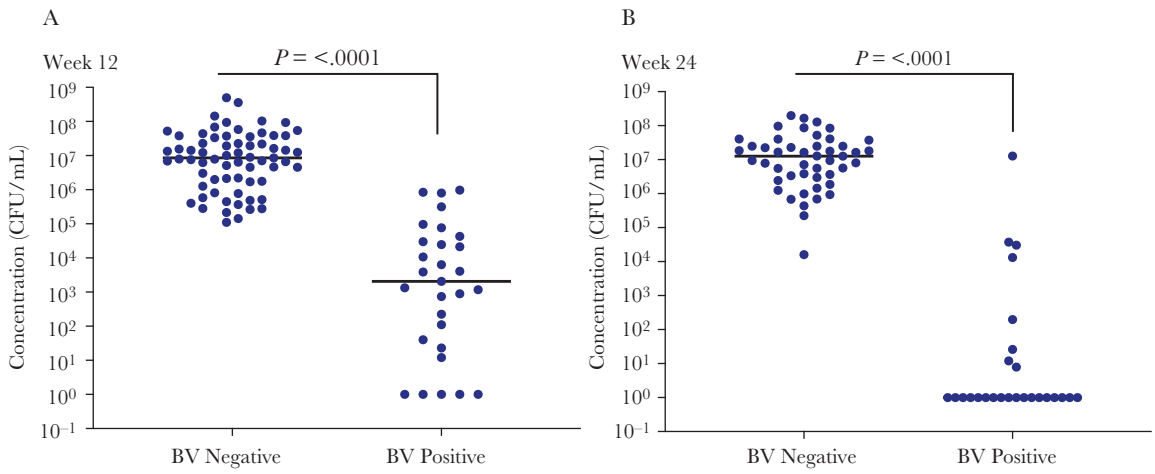

Figure 4. Concentrations of *Lactobacillus crispatus* CTV-05 in participants diagnosed with or without bacterial vaginosis (BV) at (*A*) week 12 and (*B*) week 24 of the phase 2b trial. QPCR values were plotted separately for women with and without a positive BV diagnosis at weeks 12 and 24. A positive BV diagnosis was defined as both a positive Amsel test (≥3 Amsel criteria) and Nugent score >3. Values above 10^6 colony-forming units (CFU)/mL appeared to protect women from BV recurrence. Significance of difference between groups was determined by the Mann-Whitney 2-tailed test in Prism Version 8.4.2.

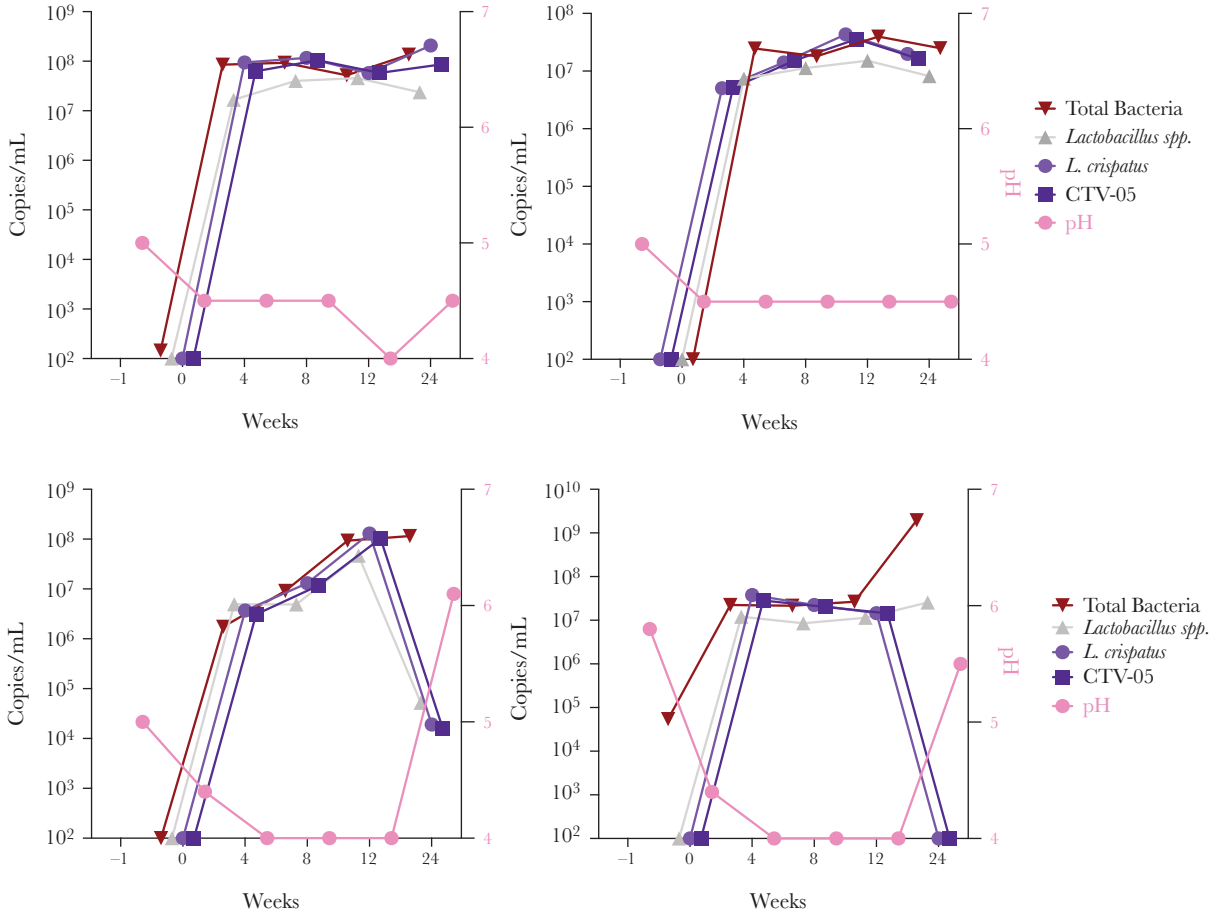

Figure 5. Examples of 2 patterns of *Lactobacillus crispatus* CTV-05 colonization following LACTIN-V administration in the phase 2b trial. *A*, Two women successfully colonized with *L. crispatus* CTV-05 throughout the 24-week duration of the study and (*B*) 2 women successfully colonized with *L. crispatus* CTV-05 only during LACTIN-V dosing through week 12. Vaginal pH was measured and vaginal swabs were collected at screening, prior to metronidazole treatment (day –1), at enrollment, after metronidazole and before LACTIN-V treatment (day 0), and then at weeks 4, 8, 12 (during treatment) and at 24 (13 weeks post treatment). DNA was extracted from vaginal swabs. Primers were designed for quantitative polymerase chain reaction to amplify pan 16S rRNA for total bacteria, and specific primers for all *Lactobacillus* species, *L. crispatus* species, and *L. crispatus* CTV-05 strain.

recurrence may be caused by reinfection from a partner [67]. A polymicrobial biofilm has been identified on desquamated epithelial cells in male urine and semen samples, suggesting a potential reinfection mechanism [85]. Concurrent partner treatment is a promising option [86]. Vaginal cleaning practices, douching [87–91], and smoking [92] are also linked to BV risk. Many of these factors influence vaginal pH, which may alter the growth of lactobacilli and BV-associated bacteria. A pH of >5 is permissive to the growth of many nonacidophilic bacteria. One hypothesis is that the production of biogenic amines by certain taxa may allow them to competitively colonize the vagina by mitigating the protective effects of low pH, thus increasing the risk of BV development [93]. Similarly, the failure of metronidazole to suppress BV bacteria (possibly due to biofilms or resistant strains) following treatment appears to have a negative impact on CTV-05 colonization.

Although transitions between *L. crispatus* and *L. iners*-dominated communities occur, temporal studies of the VMB have shown that they tend to be mutually exclusive, suggesting competition for the same niche [94]. The *L. iners*-dominated community is relatively unstable and more likely to transition to BV. *L. iners* may suppress CTV-05 colonization in some women or possibly facilitate BV-associated bacteria. In these cases, selective inhibition of *L. iners* may potentially improve *L. crispatus* colonization and clinical benefit [95].

As part of our ongoing effort to understand factors that influence BV recurrence, DNA and RNA sequencing are being conducted to analyze microbial content and gene expression profiles are investigating changes in the VMB during LACTIN-V treatment. Ultimately, these data will be combined with vaginal cytokine and chemokine data and clinical metadata to understand why some women are colonized well with CTV-05, while others are not. This multiomic approach is expected to provide insights into biomarkers to potentially customize the dosing regimen or inform on patient stratification. These approaches may identify molecular signatures of strains

of *L. crispatus* (or other species) that might complement CTV-05 and be incorporated into a second-generation product. New insights into the VMB indicate that thousands of *L. crispatus* strains may exist, and it is possible that some may colonize better or have additional beneficial features [96].

While most women are readily colonized with CTV-05 after successful metronidazole treatment and respond favorably to intermittent LACTIN-V administration, others may need more aggressive BV treatment, or a change in the frequency or duration of LACTIN-V dosing. In the absence of an approved LBP to optimize the VMB and prevent recurrent BV, clinicians may potentially consider recommending hormonal contraception, boric acid, condom-protected sex, partner treatment, and abstaining from vaginal douches and smoking to reduce risk of recurrence.

LACTIN-V represents the first microbiome-based LBP to exhibit significant efficacy in preventing BV recurrence in a rigorous FDA-regulated clinical trial. BV is associated with a number of important clinical sequelae, preterm birth, low birth weight in newborns, STI and HIV susceptibility, and oncogenic HPV progression. Successful prevention of BV and optimization of the VMB is expected to have a positive impact on these and other indications, and to usher in a new approach to improve women's health.

Notes

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