

effects and complications may range from gastrointestinal effects to sepsis and transferring of antibiotic resistance genes to host bacteria.

Probiotic VMB therapy has mostly focused on BV and less commonly on candidiasis and other forms of dysbiosis. The studies have commonly reported on a small study sample with huge differences in terms of: (1) studied population (symptomatic versus asymptomatic; acute or recurrent episodes, diagnostic criteria), (2) clinical entity evaluated (BV, abnormal microbiome, candidiasis, any “vaginitis”), (3) type and number of strains of lactobacilli administered, (4) routes of administration (oral and/or vaginal), (6) dosage, (7) duration of use, (8) antibiotic cotreatments, (9) primary outcome measures (clinical cure, microscopic cure, pH, self-perception), and (10) evaluation time points.

Given these variations, there are several challenges in study interpretation. First, different probiotic products contain a wide variety of *Lactobacillus* and non-*Lactobacillus* species. Second, there is little evidence to date that administration of probiotics results in vaginal colonization by these strains, and most studies did not distinguish endogenous lactobacilli from probiotic strains. Third, there is a lack of quality control in manufacturing probiotic products, which means that the purity and potency of the products may not be consistent.⁵

In a systematic review of 34 eligible studies evaluating vaginal probiotics (of 2949 articles), van de Wijgert and Verwijs² reported that many clinical trials evaluating probiotics had suboptimal designs, many had considerable bias, and most were too small to have adequate statistical power to detect differences between study arms. Their results suggest that vaginally applied lactobacilli-containing probiotics may hold promise for BV cure and prevention, but much less so for cure and prevention of candidiasis. Given that molecular studies have shown that *Candida* species often coexist with lactobacilli (see part III) and that epidemiological studies have shown negative associations between BV and vulvovaginal candidiasis, these findings are not surprising. It was also found that prolonged antibiotic use was more efficient in reducing BV recurrence than probiotic use.^{6–8} In addition, there was consistent evidence that vaginal detection of probiotic strains does not last long beyond the treatment period, suggesting that none of the evaluated probiotic strains colonized the vagina.²

Lactobacillus crispatus—CTV-05 LACTIN-V

A *L. crispatus* dominated vaginal environment exhibits the lowest vaginal pH, lowest proinflammatory cytokine levels, and lowest risk of gynecologic and obstetric complications and is therefore considered the predominant species associated with vaginal health. Most studies of probiotics did not evaluate *L. crispatus* strains and used strains obtained from the gut or from traditional fermented foods.

The *L. crispatus* CTV-05 was originally isolated more than 26 years ago from the vagina of a healthy woman.^{9,10} The probiotic product was preserved in a gelatin capsule, and a series of studies were conducted to evaluate its colonization potential,⁹ to develop a fingerprinting method to distinguish the probiotic strain from endogenous lactobacilli¹⁰ and evaluate its role in the prevention of recurrent BV. These studies demonstrated that CTV-05 did not effectively colonize women who already had *L. crispatus*,⁹ that unprotected sex decreased the likelihood of successful colonization,⁹ and that there was no overall benefit in preventing recurrent BV (unpublished data, Sharon Hillier, personal communication). The product (LACTIN-V) continues under development, as an Investigational New Drug application with the Food and Drug Administration (FDA), aimed as adjuvant therapy to prevent recurrent BV and recurrent urinary tract infections (rUTIs), after antimicrobial treatment. Because the capsule formulation dissolved poorly in the vagina, possibly hampering CTV-05 colonization, a spe-

cially designed vaginal applicator was developed to deliver a powder formulation to the vagina.¹¹

A phase II rUTI trial was conducted in 100 women who received antibiotic treatment for uncomplicated cystitis, followed by LACTIN-V or placebo capsules daily for 5 days, then once weekly for 10 weeks.¹² The rUTI incidence in the LACTIN-V arm (15%) was approximately half of that in the placebo arm (27%), a result which was not statistically significant.

A recently published randomized, double-blind, placebo-controlled, phase II-b trial evaluated the ability of LACTIN-V to prevent the recurrence of BV after a course of vaginal metronidazole.¹³ The presence of symptoms was not required for recruitment, nor was it reported whether treatment affected the symptoms in those who had them. After 11 weeks of treatment, follow-up occurred through week 24. Recurrence of BV by week 12 occurred in 30% in the LACTIN-V group and in 45% in the placebo group (risk ratio = 0.66, 95% CI = 0.44–0.87, $p = .01$). The risk ratio for recurrence by follow-up week 24 was 0.73 (95% CI = 0.54–0.92). *Lactobacillus crispatus* CTV-05 was detected in 79% of participants in the LACTIN-V group at the treatment termination 12-week visit and in only 48% of participants at follow-up week 24.

Overall, these studies demonstrate that although this intervention has a substantial effect, its attenuation at follow-up week 24 suggests that continued administration may be needed to maintain this effect, and the lack of colonization among women having unprotected sex⁹ may be a serious limitation of this probiotic use.

Use of Probiotics in Various Conditions

The theoretical potential of probiotics being able to favorably modulate the dysbiotic milieu of the genital tract has been tested as a possibility to reduce the risk of preterm labor. A meta-analysis found no evidence that taking probiotics during pregnancy either increases or decreases the risk of preterm birth or other infant and maternal adverse pregnancy outcomes.¹⁴

Two studies have addressed the role of probiotics in the regression of human papillomavirus infection at 6 months: one found no differences between the groups (29.2% vs 19.2%, $p = .41$), whereas the second showed higher rate of regression in the treatment group (31.2% vs 11.6%, $p = .044$).^{15,16}

Although there is a strong consumer demand for probiotics to improve vaginal health, most probiotics are sold without making specific medical claims, resulting from significantly low level and quality of evidence supporting patient benefit. In recent years, the regulatory situation for vaginal probiotics has changed considerably, and since 2016, the US FDA and Europe's EMEA (European Medicines Agency) has required human drug approval when health claims are made.² This means that a significantly higher level and quality of evidence supporting usage benefit will be required for a biologic product containing *Lactobacillus* species to be approved as a therapeutic product.⁵ The LACTIN-V is an example of probiotic being developed as a drug for human use; it shows only a modest effect and has not been commercialized yet.

Prebiotics

In terms of microbiome, “prebiotics” refers to nutraceutical compounds that induce the bacterial growth or activity of probiotics or beneficial endogenous microorganisms.

Because prebiotic consumption has been a highly effective approach in improving intestinal health, it was evaluated whether these compounds could stimulate vaginal lactobacilli.¹⁷ Monocultures of *L. crispatus*, *L. vaginalis*, *L. gasseri*, *L. johnsonii*, *L. jensenii*, and *L. iners*, BV-associated bacteria, and *Candida albicans* were tested in vitro for their capability to utilize prebiotics consisting of lactitol, lactulose, raffinose, and oligofructose. The disaccharide lactulose was found to most broadly and specifically stimulate

vaginal lactobacilli, including *L. crispatus*, while not stimulating BV-associated bacteria or *C. albicans*.¹⁷ In a randomized clinical trial ($N = 39$), placebo was compared with galacto-oligosaccharides and *Trifolium pratense* (red clover) extract, following standard treatment with metronidazole for BV. In the treatment group, the Nugent score was consistently 3 or less, 16 days after treatment; in the placebo group, it was greater than 3 in 24% ($p = .016$). Recurrence at day 84 was similar in both arms (11% and 19%, respectively). No adverse effects were reported.¹⁸

Lactoferrin, an iron-binding glycoprotein, has been used as a possible treatment for BV and for preterm labor reduction.¹⁹ Its proposed mechanisms of action include anti-inflammatory and anti-infectious effects, as well as sequestration of iron, thus making it unavailable for bacterial metabolism.²⁰ It was found that with vaginal dysbiosis, the levels of lactoferrin increase markedly; this can represent a defensive response, by reducing the available iron.²⁰ The problem with using lactoferrin in dysbiosis is that most lactobacilli species are also dependent on iron for their metabolism. In a case series of 6 women with refractory BV, lactoferrin significantly improved the VMB after 1-month therapy, with gradual increase in lactobacilli dominance.²¹ Lactoferrin has also been successfully combined with lactobacilli (see hereinafter: Symbiotics).²²

The route of delivery of prebiotics is still a matter of debate; vaginal use, including pessaries, creams, and douches have been the most frequent. Prebiotics are usually considered safe; their undesirable effects (diarrhea, bloating, flatulence) are attributed to an intestinal osmotic effect.²³

Symbiotics

One of the limitations of prebiotics use is its dependence on the presence of lactobacilli, which are absent or nearly absent in situations of dysbiosis. Symbiotics are combinations of prebiotics and probiotics, based on the concept that the former nutraceutical may improve the bacterial growth and function of the latter.²⁴ In a randomized controlled trial involving 48 women with recurrent BV, it was shown that the association of a probiotic (*Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001) and bovine lactoferrin as an adjuvant to metronidazole, led to an improvement in terms of recurrences of BV.²²

pH Modifying Agents

Lactic acid, a metabolite of lactobacilli activity, is believed to participate in the regulation of bacterial growth. Given that lactobacilli thrive in a low pH milieu, while the growth of potentially pathogenic bacteria is limited in this environment, acidifying agents have been suggested to restore a normal VMB in women with BV. A recent systematic review described the effect of intravaginal lactic acid-containing products on BV cure and their effect on VMB composition.²⁵ Of 1,883 articles, 57 were eligible for review; the authors identified 7 different lactic acid-containing products that were evaluated in these studies, which differed regarding excipients, lactic acid concentration, and pH. Most studies had medium or high risk of bias. Three trials compared the efficacy of a lactic acid-comprising product to metronidazole for BV cure. One study found lactic acid to be equivalent to metronidazole, whereas 2 studies found it significantly inferior. Two different studies included a control group receiving a placebo or no treatment. One reported lactic acid to be superior to no treatment and the other reported lactic acid to be equivalent to placebo. Lactic acid-containing products did not significantly impact the vaginal microbiota composition. The authors concluded that there is a lack of high-quality evidence to support the use of lactic acid-containing products for BV cure or VMB modulation.

Activated Charcoal

Tominaga et al.²⁶ compared the use of activated charcoal to chloramphenicol for BV. Both groups showed improvement in malodor and diminished discharge. Activated charcoal usage significantly reduced pH with minimal reduction of lactobacilli. The use of activated charcoal was based on the concept that this porous substance has lower capacity to bind lactobacilli than other bacteria.

Phage Therapy

More than a century ago, and almost a decade before the discovery of penicillin, the practice of bacteriophage (phage) therapy was being developed as a treatment for bacterial infections, such as *Shigella dysenteriae*.²⁷ Rise of the antibiotic period led the suppression of phage therapy; however, the emergence of antibiotic resistance increased the attention on phages' therapeutic potential for bacterial infections.

Phages are bacteria-specific viruses, dependent on a bacterial host for survival, and play a crucial role in regulating bacterial populations. They typically bind to specific receptors on the bacterial cell surface and insert their genetic material into the host cell. The phage mechanistically operates through 1 of 2 paths. "Temperate" phages integrate genetic material into the bacterial genome and reproduce vertically from mother-to-daughter cell. "Lytic" phages takeover the bacterial replication machinery to produce the next generation of phage progeny and in so doing, lyse the cell. Upon reaching a critical mass of phage progeny, the lytic proteins become active and hydrolyze the peptidoglycan cell wall, releasing phages to restart the lytic cycle.²⁸ Most phages are virulent only to the bacteria that carry their matching receptor, which, in turn, determines phage-host range.²⁸ As a therapeutic modality, phages have several major advantages over antibiotics such as host specificity, self-amplification, biofilm degradation, and low toxicity to humans.^{29,30} The genome of the phages can be manipulated by using biotechnological approaches for a better delivery and treatment of bacterial infections. Two strategies of phage therapy are used: natural phage therapy and engineered or synthetic phage therapy.

The vaginal phagome (the community of bacteriophages) may participate in dysbiotic conditions, although little is known about such a relationship. A recent study found that the vaginal virome was clearly linked with bacterial community structure and BV status.³¹ One of the theories regarding BV etiology suggests involvement of sexually transmitted phages that specifically target lactobacilli and allow the proliferation of anaerobic bacteria.³² Data indicate that lytic phages can target lactobacilli,^{33,34} thereby contributing to dysbiosis in BV.

The same concept can be used in the future to treat dysbiosis, by producing specific phages targeting the microorganisms that are causing imbalance. To date, several investigators are designing phage-based therapy to treat BV by selectively targeting *Gardnerella vaginalis*.³⁵

Biofilm Disruptive Agents

Other promising therapies against BV or VMB dysbiosis include a novel boric acid-based vaginal anti-infective enhanced with ethylenediaminetetraacetic acid, with antibiofilm activity (TOL-463).³⁶ A phase II clinical trial ($n = 106$) demonstrated that TOL-463 treatment is safe and well tolerated and led to a 59% and 50% clinical cure rate of BV for insert and gel, respectively, at days 9–12. Boric acid has been shown to offer additional benefit in recurrent BV when given after conventional oral metronidazole,^{37,38} and it was postulated to result from biofilm disruption, although the exact mechanism was not explored. Ethylenediaminetetraacetic acid has been shown to enhance the antimicrobial

activity of boric acid and provide increased antibiofilm potency against *G. vaginalis* and *Candida* while sparing lactobacilli.³⁶

The fact that these products affect both the biofilms associated with BV and candidiasis,³⁶ may be an advantage, as the proper diagnosis is often not made.

Vaginal Microbiome Transplantation

The success of fecal microbiota transplant in treating *Clostridium difficile* infection has led to interest in the potential of using transplanted human material as a treatment for a wide range of conditions related to microbial dysbiosis or infection. A 2019 pilot study ($n = 5$) demonstrated the feasibility of using vaginal microbiome transplantation (VMT) from healthy donors as treatment for symptomatic, intractable, recurrent BV.³⁹ Four of the 5 women who received VMT had long-term remission, defined as marked improvement of symptoms, Amsel criteria, microscopic vaginal fluid appearance, and reconstitution of a *Lactobacillus*-dominated VMB. Notably, remission in 3 patients necessitated repeated VMT, including a change of donor in 1 patient, to elicit a long-standing clinical response. The cure lasted up to 5–21 months of follow-up, and patients' condition did not require additional treatment or repeated VMTs to preserve a normal VMB, and no adverse effects were noted.

The main risks in VMT are transfer of pathogens from the donor to the recipient, including transfer of antimicrobial-resistant microorganisms and pathogens that are undetectable by current screening methods. In addition, inadvertent transfer of sperm in vaginal fluid can result in unintended pregnancy. Long-term consequences of VMT remain unknown. Thus, stringent inclusion/exclusion criteria and extensive testing of donor samples are imperative to minimize risks. Such screening approach for universal VMT donors has been described.⁴⁰ Future studies with larger cohorts and randomized, placebo-controlled design are required to determine the efficacy and durability of VMT. Given the risk of human-to-human pathogen transfer, future trials will expectantly define specific bacterial strain mixtures that confer health benefits with the goal of eventually producing “purified” versions of these microbial cocktails for clinical use. Another theoretical treatment option in microbiome transplantation may include manipulation and bioengineering of microorganisms to confer them specific characteristics that allow health benefits, including specific drug production capability.⁴¹

Summary

Probiotics are widely used as an alternative or as an adjuvant to antibiotics and antifungals, despite the lack of good quality evidence sustaining it. The concept that lactobacilli cannot thrive in an already unfavorable milieu led to the idea of using prebiotics and symbiotics, in an attempt to favor the colonization by lactobacilli. However, even this approach seems to be insufficient, and for that reason, more complex approaches may be needed—being vaginal microbiome transplantation one of the most promising fields of research in the area.

In the future, existing antimicrobials, vaginal probiotics, live bacterial therapeutics, novel antimicrobials, biofilm disruptors, and VMT have the potential to be used alone or in combination to modify the VMB. Given the paucity of solid data, it is not clear what the best treatment strategies would be: as a stand-alone treatment, as an adjunct treatment to antibiotics, as a treatment for episodic BV or maintenance therapy for recurrent BV. In addition, dosing should be defined, as well as frequency and treatment duration.

THE RESEARCH OF VAGINAL MICROBIOME IN THE FUTURE

Much of the research done on vaginitis and vaginal dysbiosis was performed before the introduction of molecular technologies,

relying on microscopy, culture, and clinical features. Apparently, the clinical significance of the VMB in various gynecological states, health, and illness, has just begun to be discovered. Recent studies suggest that the traditional model of vaginal health does not apply to all women, and *Lactobacillus* species do not predominate in a substantial proportion of asymptomatic women, suggesting an existence of multiple “vagitypes.”⁴² These findings should be validated in large-cohort longitudinal studies, to understand their potential clinical use. More studies are essential to determine the extent to which genital bacteria are causal factors, bystanders, or consequence of disease. Large, cross-sectional studies may allow for the identification of bacteria or groups of bacteria that distinguish VMB profiles of healthy, asymptomatic women who have lactobacilli-deficient microbiome from women with clinically relevant dysbiosis. Studies that will characterize specific contributions of genetic and environmental effects on the formation and preservation of VMB may lead to better diagnostics and treatments for a variety of conditions affecting women's health. Such studies must include women of different races, ethnicities, and socioeconomic backgrounds, as substantial differences have been reported in VMB compositions among women of different ethnic origins. Future studies should include genetic, environmental, behavioral, and socioeconomic data, which may further allow identification of possible associations and confounders between VMB and medical conditions. Better understanding of the VMB may not only provide insights into microorganism-mediated mechanisms contributing to various disorders development and/or progression but may also further assist in classification, diagnosis, and treatment of female reproductive tract conditions.

Classification

Current classification of vaginal disorders evolved during the past decades and is based on symptoms, physical findings, microscopy, and cultures. In many cases, experienced clinicians do not find a defined entity based on these tools, resulting in either a diagnosis of “nonspecific vaginitis,” “vulvodynia,” or “normal” diagnoses. Another obstacle is the definition of disorders based on a cluster of findings, such as BV or desquamative inflammatory vaginitis/aerobic vaginitis, leaving a gray zone of undefined vaginal conditions, which do not completely meet currently available diagnostic criteria.

Microbiome research includes not only identification of microbial communities and particular bacterial species but also use of “omic” technologies to identify the functional products of the microbes, such as metaproteomics for proteins, metatranscriptomics (the research of genes that are transcribed under certain environmental conditions, as measured by the abundance of mRNA transcripts) for gene expression, and metabolomics for small molecules. Current advances in microbiome research may allow investigation of the clinical significance of relevant bacteria and their activity and be used to better define, in association with patient's symptoms, physical findings, and health outcomes, various conditions that are currently undefined.

In syndromes that are currently classified based on physical and microscopic criteria, such as BV, the growing data showing different microbial composition and activity may allow subclassification by combining symptoms, clinical presentation, and specific bacterial consortia. This may further allow targeting specific treatments to these different subgroups, according to bacteria identity and its metabolic features.

Diagnosis

Previous studies showed that the mere presence or absence of specific bacterial species, such as *G. vaginalis*, is not indicative of the disease state, but more importantly, it is the genetic and metabolic potential of specific bacterial species that defines a

commensal from a pathogenic strain. Thus, metagenomics and computational biology approaches providing the biological, technological, and reference genome resources may enable significant advances in the understanding of health and disease, rather than using bacterial strains as indicators or mediators of pathology.⁴³

A thorough knowledge of the relationship between the microbiome composition and biological processes within the host will hopefully allow using the microbiome as a robust, low-cost diagnostic tool to quickly detect dysfunction in biological processes and to remediate problems earlier than we otherwise could. Identification of microbial and host signatures, such as bacterial communities and/or species, immune mediators, proteins, and metabolites, may be used to exploit as biomarkers for various gynecologic conditions, and their potential progression to clinically relevant conditions, that is, obstetric complications or malignancy.

A genomic catalog of the VMB, termed VIRGO, has been released as a freely available resource that can be used in microbiome studies (<https://virgo.igs.umaryland.edu/>).⁴⁴ VIRGO presents a central reference database and analytical framework that enables an efficient and precise characterization of the microbial gene content of the VMB. VIRGO provides an accessible tool to comprehensively characterize the structure and function of vaginal metagenome and metatranscriptome data sets.

Treatment

The implication that therapeutic modulation of VMB may modify mucosal inflammation suggests that future ability to manipulate VMB may represent emerging targets to modulate obstacles of fertility, pregnancy, infectious (such as HIV acquisition), and malignancy.

The Center for Biologics Evaluation and Research at the FDA, drafted a guidance document in 2012, addressing the early development of live biotherapeutic products (LBPs), thus establishing a new class of biologic drugs.⁴⁵ Live biotherapeutic product was defined as a biological product that contains live microorganisms, such as bacteria, is applicable to the prevention, treatment or cure of a disease or condition of human beings, and is not a vaccine.

The VMB represents an interesting case study for the advancement of microbiome-based therapeutics. Compared with the gut, the VMB is relatively simple and optimally dominated by 1 or several species of bacteria. Nevertheless, studies evaluating use of single bacteria to remodulate VMB were disappointing, as was discussed previously. The preliminary encouraging results of VMT may suggest that the whole microbiome, rather than a single bacterial species, may be necessary for an effective modulation of the VMB, as was previously shown in the opposite direction by Gardner and Dukes. Designing effective interventions to revise the VMB requires improved understanding of how the VMB communities are established and maintained across the individual's lifetime and within different populations. Advancing microbiome-based therapeutics will also necessitate considering differences in the baseline composition and function of the microbiome across populations with different ancestry and socioeconomic nature.

Summary

Microbiome research has grown dramatically in recent years, motivated by innovations in technology and significant decrease in the cost of analysis. This research has revealed a large quantity of data, which has yielded immense insight into the nature of microbial communities, their interactions, and effects with their hosts and other microbes.

Further understanding of the bacterial, fungal, phage, and viral microbiomes in combination with host genetics, immunologic status, and environmental factors is needed to better understand and provide personalized medical diagnostics and interventions to improve women's health.

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