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Importance of Vaginal Microbes in Reproductive Health

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Abstract

Over 250 species of bacteria have been detected in the vagina using genomic sequencing. *Lactobacillus iners* and *L crispatus* dominate in most women who have a clinically healthy status. Unfortunately, the abundance profiles can change dramatically with significant increases in pathogens associated with bacterial vaginosis (BV) and aerobic vaginitis (AV). The BV microbiota have at least 4 different abundance profiles, indicating this is a complex condition, yet one that is treated with essentially 2 antimicrobial agents which were never designed for eradicting these organisms in dense biofilms. Future studies will uncover which abundance profiles are particularly associated with a risk of preterm labor, and hopefully identify the mechanisms involved in the switch from healthy to a BV or AV state. The use of probiotic lactobacilli vaginally and orally has shown great promise in helping to restore and maintain a healthy vagina, and studies have shown that certain strains have the capacity to interfere with the inflammatory pathway leading to preterm delivery. There is enormous need for new diagnostic and therapeutic modalities, especially to save the lives of millions of babies in resource-disadvantaged countries.

Keywords

vaginal microbiota, *Lactobacillus*, bacterial vaginosis, aerobic vaginitis, probiotics

Vaginal Microbes

The human vagina is colonized by a diverse community of microorganisms known as the vaginal microbiota, which can have a profound impact on the health of women, their partners, and newborn infants. Alterations in the microbial composition of the vaginal tract have been linked to several adverse health outcomes including bacterial vaginosis[1] ([BV] a condition in which the normal protective lactobacilli are replaced by high quantities of commensal anaerobes, resulting in symptomatic vaginitis in many women)[2] and aerobic vaginitis[3] ([AV] an alteration in the vaginal microbiota associated with aerobic microorganisms, mainly group B streptococci and *Escherichia coli*).[4] Bacterial vaginosis is a common infectious condition among women of reproductive age, causing symptoms and signs such as discharge, odor, and irritation. In addition, its prevalence is associated with increased risk of pelvic inflammatory disease, sexually transmitted infections, including HIV infection, and preterm delivery in pregnant women.[5-7]

For decades, diagnosis and therapy for BV and AV have been based upon the organisms detected by culture. However, in the past 10 years, the severe limitations of culturing methods have been brought to light, and DNA-based culture-independent techniques have identified a number of fastidious and strictly anaerobic organisms that play a major role in vaginal health.[8,9] Even more recent studies employing high-throughput sequencing techniques have further increased our understanding of the complexity of vaginal microbiota.[10-14]

A vaginal microbial profile determined clinically and microscopically to be "normal" or "healthy" is predominantly colonized by lactobacilli, particularly *Lactobacillus iners* and *L crispatus*.[12] The fastidious *L iners* is especially interesting as it is present in both healthy and aberrant microbiota profiles, raising the question of its role in transition between the different clinical states.[15-17] Analysis of *L iners* whole genome sequence revealed a specific adaptation mechanism to the vaginal niche apparently by acquiring genes that extract nutrients from the environment, assist it survive the fluctuating pH and other stress conditions, and by possessing cell-anchor proteins for adherence to the vaginal epithelium.[17] While its presence in BV and lack of ability to produce hydrogen peroxide (H_2O_2) has been suggested by some to mean it is not protective against

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infection, its ability to persist even after antibiotic use, could in fact suggest it has a role in helping the host recover from the infectious state. By being the primary lactobacilli present, it may create an environment that allows other *Lactobacillus* species, such as *L crispatus* or *L jensenii*, to prosper and re-create a healthy microbiota. To date, the conditions that lead to recovery of the latter organisms is unknown. On the other hand, the organism has a cell lysin[17,18] that could be associated with BV discharge and microscopy findings of lysed epithelial cells.[4] Much remains to be determined about *L iners*, but a recent transcriptomic study shows that it clearly has the ability to alter its gene expression in healthy and BV states (Macklaim et al unpublished).

The mechanisms whereby lactobacilli help prevent infection, in addition to their highly adaptive capability, likely include production of antimicrobial and antiadhesive substances that displace and inhibit pathogen colonization, competition for nutrients, enhancing the epithelial barrier function, and modulating host immunity.[19,20] These will be discussed in greater depth in a later section dealing with probiotic applications for reproductive health.

Bacterial Vaginosis-Associated Reproductive Failure

The identification of at least 4 microbiota abundance profiles associated with BV[10,21] are important for several reasons. The rapid change to a BV state is remarkable in that lightly colonized vaginal cells become engulfed with dense populations of anaerobic and facultative microbes, such as *Prevotella bivia* and *Atopobium vaginae*, in conjunction with *Gardnerella vaginalis*.[12,22] This occurs in pre- and postmenopausal women and is therefore not explained by menstruation or hormone fluctuations. There are clearly major signalling processes that occur, which stimulate pathogen growth at the expense of lactobacilli. The changes, which may relate to host factors including immune fluctuations,[23] appear to cause a functionally universal response rather than one that only promotes a specific pathogen. Thus, for example, *Prevotella* may take advantage of this situation and dominate, or likewise *Atopobium* or other pathogens, all resulting in what is referred to as a BV state.

The different profiles likely have important clinical consequences. At present, BV is diagnosed and treated as if it was a single condition. The metronidazole or clindamycin treatment options, unchanged for 40 or so years, were designed to primarily kill *Gardnerella*. Now, armed with microbiota insight, and years of seeing patients fail on these treatments or face repeated recurrences,[12,24-26] physicians have to reconsider what it is they are diagnosing and treating. Most patients who present with symptomatic BV complain of foul odor or discharge. So far, no microbial profile has been shown to coincide with discharge.[12] However, some amine-producing organisms such as *Atopobium* have been linked with odor, and therefore one would assume that treatment would target its eradication.[12] This is not the case with the current antimicrobials. The problem becomes compounded if a pregnant woman is diagnosed with BV. In this instance, it may be that one microbiota profile is particularly linked with increased risk of preterm labor. Further studies are necessary to test this hypothesis and to explain why so far antibiotic treatment of BV has failed to reduce the occurrence of preterm labor.[27-30] If for example a higher abundance of *Atopobium* and *Lachnospiriceae* and *Veillonellaceae* species is the primary cause of preterm labor, therapy could be developed to specifically target these pathogens and not impact the indigenous microbiota.

Preterm birth, which occurs before 37 weeks of gestational age, remains the leading cause of neonatal morbidity and mortality. Nowadays, the incidence of preterm birth is about 12% to 13% in the United States and 5% to 9% in the other developed countries.[31] Multiple factors, such as pregnancy history, nutritional status, and psychological condition, have been associated with an increased risk of preterm birth and among them the presence of BV has been demonstrated to raise from 1.5- to 3-fold the risk of this obstetric complication.[27,31] This has many add-on effects to the life-long health and function of the newborn, as well as adding significantly to health care costs. In developing countries where intensive care facilities are often limited or nonexistent, and where many families cannot afford the necessary medical care, babies die, often at home. A recent study reported that 3.6 million infants die annually in the first 4 weeks of life, and while progress is being made in some areas, there has been little or no effect in reducing global deaths from preterm birth and for intrapartum-related neonatal deaths.[32] The figures are likely higher, based upon an estimation in 2005 that 12.9 million births (9.6% of all births worldwide), were preterm, with approximately 11 million (85%) occurring in Africa and Asia.[33] This is an unacceptable high number of lives lost.

Another important clinical issue is asymptomatic BV. Microbial deep sequencing has so far not shown a difference in abundance profiles for women who are symptomatic versus asymptomatic for BV. This may be due to failings in the current diagnostic methods: the Nugent score which is based upon microscopical identification of gram-stained morphological bacterial types, and the Amsel criteria which consist of a clinical diagnosis based upon microscopy, pH, odor, and discharge, the latter 2 being subjectively evaluated.[34,35] Generally, asymptomatic BV is not treated, yet if the microbial profiles are indeed indicative of an infectious process that raises the risk of preterm delivery, this issue may have to be reconsidered. Microbiome sequencing is becoming less and less expensive and faster, thus in the not-so-distant future, physicians may have such data to aid in their decision making. For now, it would be worth investigating the vaginal sequencing profiles of women with a history of BV and preterm delivery, to determine whether they represent a subset of specific microbial types and abundances that have not been detected with existing diagnostic tools. Such studies should be longitudinal in women perceived to be at highest risk, in order that alterations can be detected as they could occur weeks before any obvious symptoms and signs arise. Such aberrations may induce an inflammatory cascade that leads to preterm labor.[36] By the time that BV is detected and treated, the cascade has already been

initiated and therefore antibiotic therapy is ineffective at preventing preterm labor and birth.

Another clinical problem associated with BV and reproductive health, could be with conception. Several studies of women undergoing in vitro fertilization (IVF) have shown that high rates of BV are observed in patients with infertility, and that IVF patients with abnormal vaginal microbiota and lower numbers of H_2O_2-producing lactobacilli tend to have decreased conception rates and/or increased early pregnancy loss after IVF.[37-40] The mechanism by which BV may interfere with conception is unclear, but evidence has been presented to support a positive correlation between an imbalanced vaginal microbiota and increased concentrations of proinflammatory cytokines in the uterus.[38,40,41-44] Cytokines play a direct role in the implantation process and early embryonic development, and perturbation of the delicate balance between pro- and anti-inflammatory cytokines may increase the risk of intrauterine infection and impair endometrial receptivity in IVF patients.[45-47]

Aerobic Gram-Positive Cocci and Infection

In terms of aerobic organisms, group B streptococcus (GBS), which colonizes the gastrointestinal and urogenital tracts of up to 50% of healthy adults,[48] increases the frequency of premature labor and perinatal transmission of the organism, when present in the vagina, resulting in high mortality and morbidity rates of neonates. Effective approaches to prevent neonatal GBS infections include perinatal antibiotic therapy and more recently, vaccination with conjugated GBS capsular polysaccharides or specific GBS proteins.[49]

Staphylococcus aureus is a prominent human pathogen causative in a variety of infections, ranging from mild skin lesions to life-threatening toxic shock syndrome. Traditional antibiotic therapy, although highly effective, has led to the emergence of antibiotic-resistant strains, such as methicillin-resistant *S aureus* (MSRA).[50] Community-acquired MRSA infections have occurred in both newborns and pregnant and postpartum women, and transmission of MRSA strains in these groups could be correlated with vaginal colonization of *S aureus* in pregnant women.[51-54] A large-scale surveillance study performed by Chen et al reported that the prevalence of *S aureus* in the vaginal tracts of pregnant women is substantial (17.1%) and significantly associated with GBS co-colonization, which is believed to pose a pregnancy-related threat to mother and/or neonate.[53] The antagonistic activity of human vaginal lactobacilli against these pathogenic bacteria has been widely reported,[54-56] suggesting the potential use of probiotic lactobacilli as an alternative approach to treat and prevent vaginal infections.

Probiotic Strategies to Promote Reproductive Health

It has been recognized since at least the 1970s that the vagina of healthy women is colonized predominantly by lactobacilli, while infection and adverse outcomes have been associated in many patients with the displacement of these organisms. This has led to the concept of replenishing lactobacilli to prevent and potentially even treat conditions such as BV and AV. This is termed a probiotic application, the definition of which is "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host".[57]

The rationale for selection of strains and much of the published literature on this topic has been reviewed on a number of occasions, most recently[20] in 2009. So far, the most convincing results for improving vaginal health have come from studies employing the combination of *Lactobacillus rhamnosus* GR-1 with *Lactobacillus reuteri* RC-14 (formely *L fermentum* B-54) inserted directly into the vagina and administered orally with a view to increasing the transfer of lactobacilli and decreasing pathogen ascension from the rectum to the vagina.[58-64]

In relation to reproductive health, studies have shown that *L rhamnosus* GR-1 is able to upregulate the anti-inflammatory cytokine interleukin 10 (IL-10) and colony-stimulating factor 3 (CSF3), and independently downregulate the proinflammatory cytokine tumor necrosis factor-α (TNF-α) in human placental trophoblast cells.[65-67] Tumor necrosis factor-α has been associated with infection-mediated preterm birth,[68,69] likely through several pathways, including increasing prostaglandin synthesis and matrix metalloproteinase 2 (MMP-2) and MMP-9 expression, which results in premature rupture of fetal membranes, induction of uterine contractions, and eventually preterm labor.[70-72] Both IL–10 and CSF3 are important anti-inflammatory cytokines expressed at the human maternal–fetal interface, essential for modulating inflammation response following exposure to pathogens.[73,74] Decrease in the production of TNF-α and the increase in IL-10 and CSF3 with the treatment of *L rhamnosus* GR-1 supernatant provides mechanisms by which this probiotic could protect against intrauterine infections and prevent preterm birth, but this remains to be proven in clinical trials.

The *L reuteri* RC-14 probiotic strain was selected as the species is common in the intestine and it can be found in the healthy vagina of some women. However, mostly, it was chosen for its activity against gram-positive cocci and its biosurfactant capabilities which are believed to help reduce pathogen colonization.[75] The mode of action of *L reuteri* strains was believed to be mostly due to producing a bacteriocin called reuterin,[76] however, *L reuteri* RC-14 does not produce this compound,[77] and another vaginal isolate *L reuteri* L22 appears to function primarily by the production of bacteriocin and H_2O_2.[54] The *L reuteri* RC-14 biosurfactant contains a number of collagen-binding proteins believed to compete with *S aureus* for binding to host sites, and immunomodulatory functions that can prevent wound sepsis.[78,79]

Further studies of the anti-staphylococci activity showed that *L reuteri* RC-14 is able to repress the expression of staphylococcal virulence factors SSL11 and TSST-1, the latter causing menstruation-associated toxic shock syndrome in women.[80,81] Two cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Tyr-L-Pro) have recently been isolated from the

supernatant of *L reuteri* RC-14, and shown to serve as the signaling molecules that interfere with the regulatory cascade of virulence expression in *S. aureus*.[80] These findings present potential alternative approaches to antibiotic therapy to modulate the vaginal microbiota and prevent infection. If virulence factors, and perhaps odor-causing metabolic pathways could be inhibited, it may be possible to restore and retain vaginal health and avoid the development of drug-resistant strains.[82,83] The ''disarmed'' pathogens might be easier to displace or at least keep in too low an abundance to induce adverse clinical effects.

The application of beneficial bacteria to the vagina to improve fertility rates in IVF patients has been assessed in a pilot study. A commercial formulation containing 6×10^9 living cells of *L acidophilus*, *Bifidobacterium bifidum*, and *B longum* were administered intravaginally to women immediately after oocyte retrieval. Transvaginal IVF-embryo transfer was then performed 48 to 72 hours later.[84] There was no significant difference in the prevalence of vaginal lactobacilli before and after probiotics supplementation in the study groups, and the presence of lactobacilli before oocyte retrieval or embryo transfer did not exert any effect on the pregnancy rate in both the control and study groups. Surprisingly, patients without vaginal lactobacilli during oocyte retrieval and embryo transfer appeared to have an even higher pregnancy rate than those with lactobacilli (50% and 29.1%, respectively) in the control group. However, the experimental design has several flaws,[85] with the control and study groups allocated by pseudorandomization, and patients not being blinded. In addition, no characteristics of the administered or indigenous vaginal lactobacilli were provided, suggesting the product was far from appropriate for this type of test. Clearly, additional studies are needed to determine whether probiotics can raise the chances of successful IVF.

Future Studies

The advent of deep-sequencing technology will continue to uncover the types of organisms associated with a healthy vagina. In doing so, it is possible that some women will be found to be healthy yet colonized by a microbial profile that would otherwise be regarded as aberrant.[2,86] This makes it imperative that such assessments also investigate the individual's clinical profile, and if possible vaginal metabolomic profile, to rule out presence of toxic substances such as carcinogens, and immunological profile to ensure there are no signs of impairment.

Studies are needed that sample longitudinally at regular time points to determine when the switches from normal to BV/AV or vice versa occur. Understanding the microbial communities, their by-products and effects on the host will be critical for developing new strategies to maintain and restore vaginal health in females. Such products need to include diagnostic and therapeutic improvements, whether pharmaceutical, food, or natural health products. These should include current and new probiotic and prebiotic (defined as the selective stimulation of growth and/or activity/activities of one or a limited number of microbial genus/genera/species in the gut microbiota that confer/confers health benefits to the host) products.[87] Such studies should evaluate the impact on the vaginal microbiota, with different doses, duration, and routes of probiotic administration. The concept of prebiotics has not been applied much to the vagina, but essentially aims to stimulate the growth of nonpathogenic lactobacilli and bifidobacteria to allow them to better control the emergence of pathogens. One attempt to do this using intravaginally inserted skim milk powder had some success in terms of reducing UTI recurrences,[88] but the concept has not been tested against recurrent AV or BV. Such applications should not also stimulate pathogens, and attempts to find nutrients that do this have proved difficult, especially since *Candida* can utilize many of the nutrients used by lactobacilli.[88]

In resource-disadvantaged countries, sophisticated microbiota detection and administration of clinical interventions are impossible at present. Assistance in lowering the enormous death rates can only come from 2 ways. The first is for scientists in developed countries to use the latest molecular, genetic, and other technology to better understand the causes of preterm labor, especially the triggers either produced directly by bacteria or via the host, that lead to irreversible preterm reactions. This will require isolating and growing presently difficult-to-culture organisms such as BV-associated bacteria 1 (BVA1), BVA2, and BVA3[89-91] and understanding the role of different clones of the same species, such as *Gardnerella vaginalis*.[92] It will also necessitate studies of the host response[93] and factors such as Toll-like receptor ligands and short chain fatty acids, produced by bacterial microbiota that can greatly influence immune function[94] and potentially the preterm labor cascade. Second, for diagnostic and interventional products to be applied to the developing world, including remote and rural areas, they must be simple, easy to use and understand, and preferably inexpensive. This might seem an impossible task and may require humanitarian donations or aid in the first instance, but it should be an overarching goal. Community kitchens in impoverished parts of Tanzania, Kenya, and Rwanda have been established which produce locally sourced probiotic yogurt that has shown some benefits in terms of gut health and energy levels and on lowering risk of recurrent BV.[95-97] Additional success has been achieved in a resource-disadvantaged area in Brazil.[98] This proves that simple, nutrition-based solutions can be used to translate a concept that was created through rigorous science and clinical testing in the developed world, to resource-disadvantaged communities. Other more direct use of probiotics or probiotics into the vagina[58,99,100] are also worthy of consideration, but retention of viability of the strains, accessibility to the participants in need and appropriate storage of capsules, as well as their price, will be challenges that have to be overcome.

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