Gardnerella and vaginal health: the truth is out there

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TABLE OF CONTENTS

ABSTRACT .................................................................................................................................................. 2
INTRODUCTION ........................................................................................................................................... 3
THE VAGINAL MICROBIOTA IN HEALTH ............................................................................................. 3
UNBALANCED VAGINAL MICROBIOTA IN DISEASE ............................................................................. 6
Bacterial vaginosis (BV) .......................................................................................................................... 7
\textit{Is BV a disease?} ................................................................................................................................ 7
\textit{Clinical features and diagnosis of BV} ............................................................................................ 9
\textit{Treatment of BV} ........................................................................................................................... 10
\textit{Etiology of BV} ............................................................................................................................... 12
\textit{Bacterial species involved in BV} ..................................................................................................... 13
THE EPIDEMIOLOGY OF BV IN RELATION TO SEXUAL BEHAVIOR – IS BV A STI? ............ 14
ABSTRACT

The human vagina is a dynamic ecosystem in which homeostasis depends on mutually beneficial interactions between the host and their microorganisms. However, the vaginal ecosystem can be thrown off balance by a wide variety of factors. Bacterial vaginosis (BV) is the most common vaginal infection in women of childbearing age, but its etiology is not yet fully understood, with different controversial theories being raised over the years. What is generally accepted is that BV is often characterized by a shift in the composition of the normal vaginal microbiota, from a Lactobacillus species dominated microbiota to a mixture of anaerobic and facultative anaerobic bacteria. During BV, a polymicrobial biofilm develops in the vaginal microenvironment, being mainly composed of Gardnerella species. The interactions between vaginal microorganisms are thought to play a pivotal role in the shift from health to disease and might also increase the risk of sexually transmitted infections acquisition. Here we review the current knowledge regarding the specific interactions that occur in the vaginal niche and discuss mechanisms by which these interactions might be mediated. Furthermore, we discuss the importance of novel strategies to fight chronic vaginal infections.
INTRODUCTION

The female vaginal environment is a complex and dynamic nutrient-rich milieu for microorganisms, resulting in a unique microbiome (Lloyd-Price, Abu-Ali and Huttenhower 2016). Apart from being a passage for the menstrual flow, sperm and the baby, the human vagina and its microbiota have an impact on conception, pregnancy, the mode and timing of the baby delivery and the risk of acquisition sexually transmitted infections (STIs) (Amabebe and Anumba 2018a).

The healthy vaginal mucosa of reproductive-aged women consists of a stratified squamous non-keratinized epithelium of about 28 cell layers covered by a mucosal stratum constantly lubricated by cervicovaginal fluid (Patton et al. 2000). The apical layers of the vaginal epithelium are comprised of dead cornified cells that are uninfectable, serving thus, as a shield against pathogens (Anderson, Marathe and Pudney 2014). Still, these protective layers are constantly being challenged and eventually can be disrupted, enabling the invasion of pathogens and the development of infections (Cone 2014). Most of the time, these infections are governed by diverse interactions among existing pathogens in the vaginal environment, such as the case of bacterial vaginosis (BV). In this review, we will briefly discuss some of the underlying aspects shaping the communities that have a key impact on the development of BV. The relation between this condition and other vaginal infections (or unbalances of the vaginal microbiota) will also be addressed as well as its association with sexual intercourse. Lastly, the focus will be on the limitations of the current antibiotic treatment and the importance of finding and developing novel strategies to effectively treat BV and other vaginal infections.

THE VAGINAL MICROBIOTA IN HEALTH

Since the first microbiological study of the human vagina published in 1892 by Albert Döderlein, the vaginal microbiota of healthy reproductive women has been described as principally containing Gram-positive bacilli of the genus *Lactobacillus* (Döderlein 1892). Generally, vaginal colonization with lactobacilli is believed to promote a
protective environment since these bacteria prevent other microbes from colonizing the vaginal tract, using several mechanisms (Vaneechoutte 2017; Kovachev 2018).

One of the best defense mechanisms studied is related to the production of lactic acid by the majority of Lactobacillus spp., which contributes to the maintenance of the vaginal pH below 4.5 (Tachedjian et al. 2017; Godha et al. 2018). This acidic environment represents an efficient mechanism of protection of the vaginal milieu since it makes the environment unwelcoming to many other bacteria while favoring the presence of lactobacilli (O’Hanlon, Moench and Cone 2013; Prabhurajeshwar and Chandrakanth 2017). In addition to lactic acid, Lactobacillus spp. are also known to produce broad-spectrum bacteriocins which might play an important role in fending off non-indigenous bacteria or pathogenic microorganisms (Dover et al. 2008; Stoyancheva et al. 2014) through permeabilization of their membrane (Oscáriz and Pisabarro 2001). Furthermore, lactobacilli produce hydrogen peroxide that could act as a natural microbicide within the vaginal ecosystem (Atassi and Servin 2010; Sgibnev and Kremleva 2015). However, it has been described that physiological concentrations of this metabolite produced no detectable inactivation of BV-associated bacteria when these were incubated under optimal, anaerobic growth conditions (O’Hanlon, Moench and Cone 2011). Therefore, hydrogen peroxide role in the vaginal environment is still being debated (Tachedjian, O’Hanlon and Ravel 2018). Lactobacillus spp. are also able to interfere with the adhesion of pathogenic bacteria to the vaginal epithelium, as has been shown in several in vitro studies (Castro et al. 2013, 2015; Leccese Terraf et al. 2017). This ability of lactobacilli has an important role since the pathogen adhesion and colonization on the host cells often represent the first step of the infection process (Ribet and Cossart 2015).

Besides Lactobacillus spp., the vaginal microbiota of asymptomatic women of reproductive age also harbors other distinct taxa (Drell et al. 2013). Based on the differences in the composition and abundance of vaginal bacterial species, the vaginal microbiota of childbearing-age women has been devised in five major types, known as community state types (CST). Four of these CST are dominated by Lactobacillus crispatus (CST I), Lactobacillus gasseri (CST II), Lactobacillus iners (CST III), and Lactobacillus jensenii (CST V), while the CST IV does not contain a significant number of lactobacilli, but is composed of a varied array of facultative and strictly anaerobic bacteria, including Gardnerella, Atopobium, Prevotella, Mobiluncus, Sneathia, Eggerthella, Finegoldia, Megasphaera, Peptoniphilus, Corynebacterium,
Streptococcus, and Aerococcus (Ravel et al. 2011; Drell et al. 2013). The proportion of each CST varies among the four ethnic groups (Asian, white, black, and Hispanic), as described in Figure 1. Interestingly, these variations among CST appear to be driven by a combination of genetic, behavioral, cultural, and other uncharacterized underlying factors (Ma, Forney and Ravel 2012; Borgdorff et al. 2017). However, all CST contain members that have been assigned to genera known to produce lactic acid, such as Lactobacillus, Atopobium, Megasphaera, and Streptococcus, being suggested that this ability may be conserved among communities (Ravel et al. 2011). Overall, these findings challenged the wisdom that the occurrence of high numbers of lactobacilli is synonymous with "normal and healthy" since approximately 30% of healthy women lack considerable numbers of Lactobacillus spp. (Forney, Foster and Ledger 2006; Ravel et al. 2011; Gajer et al. 2012).

In addition to the protective effects of the beneficial endogenous vaginal microbiota, the colonization of pathogenic microorganisms in the female reproductive tract is prevented by local components of the immune system (Hickey et al. 2011; Nguyen et al. 2014). The innate immune system represents the first line of response to infection and, for this reason, has a pivotal role in the host (Amjadi et al. 2014). In the female reproductive tract, the innate immune system consists of several components that provide specific protective barriers against the invasion of pathogens (Farage et al. 2011). The lining mucosa, made up of epithelial cells and mucus, acts as a physical barrier (Tjabringa et al. 2005; Hickey et al. 2011). Mucus is composed of glycoproteins, known as mucins, which trap pathogens in a thick gel phase, preventing their ascending in the upper female reproductive tract (Taherali, Varum and Basit 2018). Contrariwise, pattern recognition receptors, especially Toll-like receptors (Fazeli, Bruce and Anumba 2005; Kumar, Kawai and Akira 2011) and natural antimicrobial peptides (Yarbrough, Winkle and Herbst-Kralovetz 2015) form a chemical barrier. Toll-like receptors recognize conserved pathogen-associated molecular patterns synthesized by various microorganisms, being thought that the expression of Toll-like receptors by the epithelium plays an important role in antigen detection and initiation of the immune response (Nasu and Narahara 2010). On the other hand, antimicrobial peptides, small molecules normally with less than 50 amino acids, which are mostly represented by defensin (Yarbrough, Winkle and Herbst-Kralovetz 2015), elafin (Wira et al. 2011), cathelicidin (Doss et al. 2010), lysozyme (Wira et al. 2011), secretory leukocyte
protease inhibitor (Orfanelli et al. 2014), and lactoferrin (Valenti et al. 2018), are produced in the vaginal environment (Zhang and Gallo 2016) and have broad-spectrum antibacterial activity. Moreover, these substances play additional biological functions including cell proliferation, cytokine induction, chemotaxis, and modulation of innate and adaptive immunity (Amjadi et al. 2014). Overall, the beneficial endogenous vaginal microbiota together with the immune system provides protection in the vaginal environment whose state has a significant impact on the health of women, their partners, as well as their newborns (Li et al. 2012). Alterations in the composition of the vaginal microbiota have been linked to several adverse health outcomes, as discussed in the next section.

UNBALANCED VAGINAL MICROBIOTA IN DISEASE

The vaginal microbiota has been indicated to be a temporal dynamic ecosystem subject to changes over the menstrual cycle (Gajer et al. 2012; Nugeyre et al. 2019). Moreover, microbial communities present in the vagina may undergo different types of acute and chronic disturbances caused by endogenous and exogenous factors including phase of the menstrual cycle (Lopes et al. 2011), aging (Uchihashi et al. 2015), stress (Amabebe and Anumba 2018b), hormonal contraceptives (Fosch et al. 2018), pregnancy (Romero et al. 2014), use of antibiotics (Macklaim et al. 2015), vaginal douching (Luong et al. 2010), vaginal lubricants (Marrazzo et al. 2010a), and sexual activity (Vodstrcil et al. 2017). These alterations can cause periods of increased host susceptibility that negatively impact the ability of the vaginal community to resist pathogen colonization (Huang et al. 2014), leading thus to microbial unbalances in the urogenital tract, that can lead to infection and disease development (Donders et al. 2000). The most common vaginal infections are caused by bacteria (such as vaginal bacteriosis, commonly known as BV, or aerobic vaginitis), by fungus (vulvovaginal candidiasis) and by protozoa (trichomoniasis) as listed in Table 1 and represented in Figure 2. It is also important to note that some STIs can also influence the vaginal microbiota (van de Wiigert 2017). Table 2 briefly lists associations between BV and the most common vaginal infections or STIs.
Bacterial vaginosis (BV)

Worldwide, BV is the most common gynecological infection among women of childbearing age, affecting approximately 30% of women in the general population and 50% of African American women (Kenyon, Colebunders and Crucitti 2013). Microbiologically, BV is characterized by a dramatic shift in the vaginal microbiota from the dominant lactic acid and hydrogen peroxide-producing lactobacilli to a polymicrobial microbiota, consisting of strictly and facultative anaerobic bacteria, where Gardnerella vaginalis plays a pivotal role (Onderdonk, Delaney and Fichorova 2016). It is worth noting that an emended description of G. vaginalis was recently proposed with delineation of 13 genomic species within the genus Gardnerella (Vaneechoutte et al. 2019). As such, in this review, we will use the term Gardnerella spp. when discussing previous publications.

In the last years, BV has emerged as a global issue of concern due to its association with a wide array of adverse outcomes. It has been reported that BV significantly increases the risk of development of gynecological postoperative infections (Lin et al. 1999), pelvic inflammatory disease (Ness et al. 2005), urinary tract infections (UTIs) (Harmanli et al. 2000) and infertility (Salah et al. 2013). Moreover, BV has been also associated with adverse pregnancy outcomes such as miscarriage and recurrent pregnancy losses (Isik et al. 2016), preterm delivery and low birth weight (Svare et al. 2006) and increased neonatal morbidity (Dingens et al. 2016). Furthermore, BV facilitates the transmission of STIs agents including the human immunodeficiency virus (Haddad et al. 2018), human papillomavirus (Gillet et al. 2011), Neisseria gonorrhoeae and Chlamydia trachomatis (Wiesenfeld et al. 2003).

Is BV a disease?

Despite advances in our understanding of BV, there are still a lot of controversies. BV has been described as a disease (Eschenbach 1993), a condition (Holzman et al. 2001), a vaginal inflammation (Forsum et al. 2005), a disorder (Patterson et al. 2010), a clinical syndrome (Workowski and Bolan 2015), a dysbiosis or microbial imbalance (Muzny and Schwebke 2016), an infection (Bagnall and Rizzolo 2017), and in some women, a normal situation in which women do not present any symptoms (Gibbs 2007). It should be noted that while the term condition can be applied to an unspecific state of health,
whether well or ill, when it is conferring illness, a condition can further be classified as a disease or a disorder (Merchant et al. 2019). It has been proposed that in order to fit the definition of a disease, it is required the appearance of precise signs and symptoms (Scully 2004; Tikkinen et al. 2012). On the other hand, a disorder denotes an abnormality of regular functions in the body or part of the body and could be a result of the disease or even lead to the development of other diseases (Cooper 2004).

Conversely, the concept of infection is traditionally used to describe when a microorganism that causes disease enters the host and begins to multiply (World Health Organization Regional Office for Europe 2001). However, it should not be neglected that some infections can be asymptomatic, never leading to disease development, such as what can occur during hepatitis B (Liang 2009) or cytomegalovirus infection (Caliendo et al. 2002). Indeed, a similar situation also occurs in BV, since approximately half of the women who experience BV are asymptomatic (Eschenbach et al. 1988; Gibbs 2007; Turovskiy et al. 2011). It is, therefore, reasonable to assume that asymptomatic women colonized by *Gardnerella* might be suffering an infection, but not suffering a disease, as represented in Figure 3. The infection could occur early in women’s life and remain asymptomatic (Catlin 1992; Centers for Disease Control and Prevention 2016). In fact, a similar situation occurs with many opportunistic pathogens, such as with *Staphylococcus epidermidis* (Le, Park and Otto 2018). In cases of symptomatic BV infection, there is a presence of clinical symptoms as further discussed below, in the section *Clinical features and diagnosis of BV*. It should be noted that the recent acknowledgement of the existence of up to 13 different *Gardnerella* species (Vaneechoutte et al. 2019) might explain some of the controversial studies to date. It is not known, so far, if different species are more associated with asymptomatic colonization or more prone to develop symptomatic infection leading to disease (Hill et al. 2019; Khan, Voordouw and Hill 2019).

Another point of controversy is related to the inflammatory response associated with BV. Often BV is not inflammatory (Weissenbacher et al. 2010; Danielsson, Teigen and Moi 2011) but in some cases it has been reported an association of BV with cervicitis (Schwebke and Weiss 2002) with increased inflammatory markers (Sturm-Ramirez et al. 2000). Furthermore, recent reviews of studies on cytokines, chemokines, antimicrobial factors, and cellular immune parameters indicated that inflammation may occur in some BV patients (Mitchell and Marrazzo 2014; Borgdorff et al. 2016). It was
therefore suggested that the differences in the inflammatory response among women with BV could either be associated with microbial and host diversity or could be the result of differences in the study design (Mitchell and Marrazzo 2014).

Some authors now question that what is commonly called BV might, in fact, be different clinical conditions (Cerca et al. 2017; Reid 2018, 2019). If true, this would explain many of the controversial studies. In this regard, Reid proposed that the term of BV should be dropped, as it currently offers no adequate description of a single condition, suggesting two potential terms: vaginal dysbiosis and vaginal inflammation (Reid 2018). In any case, to avoid underdiagnosis or misdiagnosis of vaginal infections, each situation should be properly examined by evaluating the presence and abundance of specific bacteria as well as the clinical signs and symptoms (Schwiertz et al. 2006).

*Clinical features and diagnosis of BV*

In symptomatic women, BV is characterized by the presence of a profuse vaginal discharge and fishy vaginal odor (Frobenius and Bogdan 2015). The abnormal vaginal discharge results in part from the degradation of the protective vaginal mucin gel, which is performed by mucin-degrading enzymes produced by BV-associated bacteria (Olmsted et al. 2003). The fishy odor is due to the volatilization of amines produced as a result of the metabolism of anaerobic bacteria (Wolrath et al. 2001). In clinical settings, BV is commonly diagnosed using the Amsel criteria, which include the presence of at least three of the following precepts: (i) thin and homogenous discharge, (ii) vaginal pH over 4.5, (iii) positive “whiff test” (detection of fishy odor through the addition of 10% potassium hydroxide to vaginal fluid) and (iv) presence of clue cells on microscopic examination of vaginal fluid (Amsel et al. 1983). However, these clinical signs are not always present, making Amsel criteria somewhat subjective (Sha et al. 2005).

In an attempt to improve the accuracy in BV diagnosis, Nugent and colleagues proposed a Gram stain scoring system for examining vaginal smears (Nugent, Krohn and Hillier 1991). This method derived from the modification of the Gram-stained protocol proposed by Spiegel and colleagues (Spiegel, Amsel and Holmes 1983) and currently it is regarded as the gold standard for BV diagnosis (Sha et al. 2005). According to the Nugent criteria, Gram-stained smears are used for identification, classification, and
quantification of the following bacterial morphotypes: large Gram-positive bacilli (*Lactobacillus* spp.), small Gram-variable rods (*Gardnerella* spp. and *Bacteroides* spp.), and curved Gram-variable rods (*Mobiluncus* spp.), as summarized in Table 3. Each morphotype is scored in a scale from 0 to 4+, regarding the number of morphotypes observed per oil immersion field. Thus, a score of 0–3 is considered normal vaginal microbiota, 4–6 as intermediate microbiota and 7–10 as BV (Figure 4). Nevertheless, Nugent score has some disadvantages, especially related to the inter-observer variability and it requires skilled personnel to perform it. Importantly, the relationship between Gram stain score and diagnosis by the clinical criteria is imperfect. Gram stain is more sensitive, whereas the Amsel criteria can be more specific. Overall, the concordance between them is of 80% to 90% (Livengood 2009). These shortcomings of standard methods make BV diagnosis a challenging task, and, therefore, alternative methods for BV diagnosis have been investigated. The molecular methodologies, such as polymerase chain reaction (PCR) (Fredricks *et al.* 2007), quantitative PCR (qPCR) (Hilbert *et al.* 2016) or fluorescence *in situ* hybridization (Machado *et al.* 2015), have allowed the detection or even quantification of the main BV-associated bacteria. In fact, they have improved our knowledge of how microbial species interact among themselves and with the human host. However, most of these alternative methods are expensive and many of them still require validation (Africa 2013). A recent review of molecular methods for BV diagnosis discusses in detail how the field has evolved and current shortcomings. Despite the wide variety of diagnostic assays available to diagnose BV, the authors concluded that clinicians will need to consider costs, result time, and accuracy in their decision to select a particular assay to test for BV (Coleman and Gaydos 2018).

*Treatment of BV*

The Centers for Disease Control and Prevention and the International Union against Sexual Transmitted Infections recommend that all symptomatic women should be treated, since they recognize numerous benefits of therapy including the relief of the symptoms and signs of infection and reduction in the risk of STIs and BV-associated complications, mainly in pregnancy (Sherrard *et al.* 2011; Workowski and Bolan 2015). However, there is insufficient evidence to recommend routine treatment of asymptomatic women (Schwebke 2000; Gibbs 2007; Nygren *et al.* 2008).
Conventionally, BV is treated with either metronidazole, clindamycin or tinidazole (Workowski and Bolan 2015). Despite some studies reported short-term high clinical cure rates of antibiotic therapy (Paavonen et al. 2000; Thulkar, Kriplani and Agarwal 2012), high recurrence levels have been demonstrated within 3–12 months (Bradshaw et al. 2006a; Bilardi et al. 2016). Therefore, treatment of recurrent BV can be difficult and may require extended courses of antibiotic therapy to obtain a long-lasting cure (Bagnall and Rizzolo 2017).

Currently, metronidazole, the most widely known of nitroimidazole drug class, represents the first line therapy for BV and also for trichomoniasis (Sobel and Sobel 2015). However, several side effects are associated with metronidazole therapy, such as nausea, vomiting and gastrointestinal complaints (Miljkovic et al. 2014; Sobel and Sobel 2015). Clindamycin is the second recommended antimicrobial agent for the treatment of BV, with similar efficacy as metronidazole (Paavonen et al. 2000; Menard 2011). This lincosamide antibiotic has various formulations including vaginal dosage forms (ovules and cream) and oral (systemic) pills (Menard 2011). Importantly, topical clindamycin tented to cause a lower rate of adverse side effects (metallic taste in the mouth, nausea, vomiting) than oral metronidazole. Nonetheless, the administration of clindamycin seems to be a risk factor for the development of Clostridium difficile infection (Mullish and Williams 2018). Furthermore, because both clindamycin ovules and cream are oil-based, their use might interfere with the safety of latex condoms and diaphragms (Workowski and Bolan 2015). Finally, tinidazole is currently considered an alternative antimicrobial agent for BV treatment, particularly whenever metronidazole and clindamycin are not tolerated (Workowski and Bolan 2015). Being a second-generation nitroimidazole, tinidazole requires lower dosages and is administered less frequently than metronidazole due to its longer half-life (Wood and Monro 1975). The increasing evidence that BV is a recurrent infection (Wilson 2004) sparked the interest of the scientific community in exploring emerging therapeutic alternatives (Machado et al. 2016), which will be also addressed in the section Importance of novel strategies to fight chronic vaginal infections on this review.
**Etiology of BV**

BV etiology is a matter of controversy. It is still not clear if the shift from healthy to BV microbiota could occur because BV pathogens overgrowth and outcompete the resident lactobacilli or if the initial loss of lactobacilli is the trigger for subsequent BV pathogens colonization (Martin 2012; Onderdonk, Delaney and Fichorova 2016). *In vitro*, it was previously shown that BV-associated *Gardnerella* spp. is able to displace pre-adhered *L. crispatus* and initiate vaginal colonization (Castro et al. 2015). Conversely, the hypothesis of the depletion of lactobacilli as the cause of BV has not been fully supported by the fact that some women maintain a “healthy” vaginal environment without lactobacilli (Jung et al. 2017). Curiously, as also mentioned above, some strains of *Atopobium* spp., *Leptotrichia* spp., and *Megasphaera* spp. are reportedly capable of producing lactic acid. Therefore, the presence of non-lactobacilli vaginal microbiota and the lack of beneficial lactobacilli may not necessarily be sufficient to cause BV (Zhou et al. 2004; Gajer et al. 2012).

As such, the lack of basic information about etiopathogenesis of BV led to the postulation of two main hypotheses. The first is the primary pathogen hypothesis, which infers that a single pathogenic species, *Gardnerella* spp., is the etiological agent of BV, usually transmitted by sexual contact (Muzny and Schwebke 2013). In contrast, the second is the polymicrobial hypothesis, which argues that *Gardnerella* spp. acts in concert with other bacteria, principally anaerobes, to cause BV (Josey and Schwebke 2008).

Historically, in 1955, Gardner and Dukes identified what they called *Haemophilus vaginalis* (first classification attributed to *G. vaginalis*) as the etiological agent of BV, as they claimed *H. vaginalis* fulfilled all the Koch’s postulates (Koch 1876), as summarized in Table 4. However, a later study pointed out some failures in these experiments since they showed that the artificial infection with a pure culture of *H. vaginalis* did not always cause BV (Criswell et al. 1969). The assumption was then made that *H. vaginalis* was not the specific causative agent of BV, failing one of Koch’s postulates. Afterwards, it was found that several other anaerobic bacteria were presented during BV episodes (Spiegel et al. 1983; Holst et al. 1984; Hill 1993), and this led to the postulation of the polymicrobial etiology hypothesis (Josey and Schwebke 2008).
This hypothesis was supported by the demonstrations that anaerobic activity is instrumental in producing the symptoms of BV, namely the vaginal odor, as a result of the production of amines as a byproduct of anaerobic metabolism (Chen et al. 1979; Wolrath et al. 2001). However, the presence of any specific bacterium in BV has been rarely supported by microbiological functional studies, demonstrating, thus, a lack of virulence profile characterization of such species (Machado and Cerca 2015). Notwithstanding all these findings, the polymicrobial hypothesis is still incongruent with the epidemiological profile of BV since multiple studies have been revealing that BV reflects the behavior of a sexually transmitted or enhanced disease (Fethers et al. 2008; Verstraelen et al. 2010; Leppäluoto 2011).

**Bacterial species involved in BV**

Even though the current knowledge about BV etiology remains scarce, the common consensus is that BV is always associated with the overgrowth of numerous bacterial species, including *Gardnerella* spp., *Atopobium vaginae*, *Fusobacterium nucleatum*, *Mobiluncus mulieris*, *Mycoplasma hominis*, *Prevotella bivia*, and *Ureaplasma urealyticum* (Livengood 2009). With the advance in culture-independent methods, the spectrum of anaerobes detected in women with BV was greatly expanded with the addition of *Bifidobacterium*, *Dialister*, *Eggerthella*, *Leptotrichia*, *Megasphaera*, and *Slackia* organisms, as well as other bacteria related to *Arthrobacter*, *Caulobacter*, and *Butyrivibrio* organisms (Romero et al. 2014; Muzny et al. 2018). Furthermore, the Vaginal Human Microbiome Project has detected several newly described bacteria in the *Clostridiales* order, which were initially designated BV-associated bacteria (BVAB): BVAB1, BVAB2, or BVAB3 (Fredricks, Fiedler and Marruzzo 2005; Huang et al. 2014). To date, only BVAB3 has been cultured and biochemically characterized and the remaining two BVAB (BVAB1, BVAB2) have not yet been isolated by culture (Austin et al. 2015). The species name of BVAB3 was proposed as *Mageeibacillus indolicus* (Austin et al. 2015). Interestingly, differences in the BV vaginal microbiota between American women and women of European ancestry were found, with American women more likely to be colonized by *Anaerococcus tetradius*, BVAB1, BVAB3, *Coriobacteriaceae*, *Sneathia*, *Parvimonas*, *Dialister*, *Megasphaera*, *Bulleidia*, *Prevotella*, and *Atopobium* species, while women of European ancestry were more
likely to be colonized by *M. hominis*, *Dialister micraerophilus*, and *Gemella* species (Huang *et al.* 2014).

A particular species that is often found in BV is *L. iners* (Wertz *et al.* 2008; Shipitsyna *et al.* 2013), being thus evident that not all vaginal *Lactobacillus* spp. are necessarily beneficial and protective. Indeed, *L. iners* is very different from other lactobacilli, not producing D-lactic acid (Mendes-Soares *et al.* 2014; Edwards *et al.* 2019) and carrying some pathogenicity factors, such as inerolysin, a cytotoxin that was found to be up-regulated at least six-fold in women presenting BV (Macklaim *et al.* 2011, 2013). Interestingly, it has been suggested that *L. iners* is a dominant part of the vaginal microbiota in a transitional stage between BV and normal microbiota (Ferris *et al.* 2007; Jakobsson and Forsum 2007). Nevertheless, to date, the role that *L. iners* plays in the vaginal microenvironment still remains controversial and further investigations are needed to clarify this matter.

Unfortunately, despite the development of a more comprehensive picture of the vaginal microbiota during BV through the use of high-throughput 16S rRNA sequencing, the significance of these findings remains unclear, since it is not known whether these microorganisms are pathogens that cause BV or if they simply are opportunistic microorganisms that take advantage of the temporary higher pH environment and thus increase in numerical dominance (Ma, Forney and Ravel 2012).

**THE EPIDEMIOLOGY OF BV IN RELATION TO SEXUAL BEHAVIOR – IS BV A STI?**

As discussed above, there is strong evidence that BV is associated with the acquisition of other infections, including STIs. It has also been suggested that BV might be sexually transmitted (Muzny and Schwebke 2016) and thus, in this regard, several epidemiological studies have described many sexual risk factors that may enhance its acquisition. According to these studies, women are more probable to have BV if they: (i) report a high number of lifetime sexual partners (Fethers *et al.* 2008), (ii) have a new sexual partner (Schwebke and Desmond 2005), (iii) were at young age on coitarche (Verstraelen *et al.* 2010), (iv) use oral contraception instead of condom (Silva *et al.* 2013), (v) identify themselves as commercial sex workers (Schwebke 2005), or (vi) have high frequency of intercourse (Vallor *et al.* 2001). In addition, there are several studies regarding women who have sex with women that also support the sexual
transmission of BV (Bradshaw et al. 2014; Vodstrcil et al. 2015; Muzny et al. 2019a). Moreover, males as asymptomatic carriers possibly could be also considered being responsible for the transmission of BV, since their preputial space and distal urethra is suspected to act as a reservoir of BV-associated bacteria, which might be transferred to the female partners through sexual contact and where these may act as BV-inducing microorganisms (Swidsinski et al. 2010; Liu et al. 2015; Zozaya et al. 2016).

Despite the fact that BV may present a close relationship with sexual behavior, there is also some criticism and controversial studies (Morris, Rogers and Kinghorn 2001; Fethers et al. 2008). Of note, Gardnerella has also been isolated from adolescent women with no sexual activity (Bump and Buesching 1988) and recurrent BV has also been reported in a virgin adolescent (Papanikolaou et al. 2002). It is noteworthy, that in both studies the virginal status of the adolescents was carefully examined by a physician reveling an intact hymen on the vagina. Hence, an alternative infectious disease model emerged, in which BV was described as a sexually enhanced disease rather than a sexually transmitted infection, as summarized in Figure 5. Verstraelen and colleagues proposed two mechanisms that could support this alternative model (Verstraelen et al. 2010). Thus, it was thought that unprotected sexual intercourse is associated with an alteration of the physicochemical vaginal environment, affecting also the vaginal microbiota. The alkaline prostatic content of the ejaculate raises the vaginal pH and makes the environment less favorable to the survival of lactobacilli (Boskey et al. 1999), promoting at the same time the growth of BV-associated microorganisms (Hay 2005). As such, condom utilization would protect against BV development by hamper acidification of the vaginal environment and not by preventing transmission of an infectious agent. However, this has not been demonstrated yet. They also suggested that both protected and unprotected vaginal penetration could, in some way, promote the transfer of perianal, perineal, and perivulvar bacteria to the vagina, inducing BV (Verstraelen et al. 2010). Additionally, non-coital sexual behaviors, including receptive oral (Marrazzo et al. 2010b) and anal sex (Cherpes et al. 2008) and non-penetrative digito-genital contact (Fethers et al. 2009), might alter the vaginal microbiota equilibrium through the transfer of BV-associated bacteria from the rectal and perineal regions to the vulvar region and the vagina, possibly also enhancing BV development. In addition, it is noteworthy that women with BV alone or with concurrent Candida spp. infections present a high risk of coinfection with STIs, as has been shown in a recent
study developed by Van Der Pol and colleagues (Van Der Pol et al. 2019). Overall, the BV epidemiology in relation to sexual behavior still remains controversial and it is not surprising that BV has been referred to as "one of the most prevalent enigmas in the field of medicine" (Schwebke 1997; Larsson et al. 2005; Marrazzo 2011). Although BV presents high clinical importance, the exact global prevalence is unknown since it varies according to the characteristics of the studied population (Kenyon, Colebunders and Crucitti 2013).

POLYMICROBIAL NATURE OF VAGINAL BIOFILMS IN BV

A shift in the paradigm of BV research occurred in 2005 when Swidsinski and colleagues revealed the presence of a polymicrobial biofilm adhering to the vaginal epithelial cells in BV, using fluorescence in situ hybridization (Swidsinski et al. 2005). This biofilm was shown to contain high concentrations of a variety of bacterial groups, being Gardnerella spp. the most predominant member. Several other studies validated these findings and it is currently accepted that BV-related biofilms are strongly associated with Gardnerella spp. (Swidsinski et al. 2013, 2014, 2015; Hardy et al. 2015; Machado et al. 2015).

Biofilms can be defined as structured communities of bacteria embedded in a self-produced matrix of extracellular polymeric substances (Flemming et al. 2016). These complex structures often contain channels which allow circulation of nutrients. Also, they may contain genetically identical cells in separate regions of the biofilm that exhibit different patterns of gene expression (López, Vlamakis and Kolter 2010). This results in certain advantages to the biofilm community, including an enhanced tolerance and a better persistence toward adverse environmental stress conditions (Castro et al. 2017; Romero-Lastra et al. 2017; Kot, Sytykiewicz and Sprawka 2018). The formation of the biofilm is a dynamic and complex process that involves multiple interactions between single or multiple bacterial species and the host cells (Kriebel et al. 2018). Its life cycle generally includes several stages: (i) adhesion to a substrate, (ii) production of extracellular polymeric substances with the development of a mature biofilm structure and (iii) dispersal by the detachment of aggregates or by the release of single cells (Machado and Cerca 2015).
To date, the exact process of the development of a biofilm in BV remains unknown (Hardy et al. 2017; Jung et al. 2017). However, there is evidence supporting that the first stage of biofilm formation, corresponding to microbial adhesion to vaginal epithelial cells, is an essential factor to elicit BV (Swidsinski et al. 2005). This process minimizes the contact of microbes with potentially deleterious extracellular enzymes and antibodies as well as reduces their chances of being flushed away in vaginal fluid or urine (Verstraelen and Swidsinski 2013; Salo et al. 2016). Notable is the fact that the ability of \textit{Gardnerella} spp. to colonize vaginal cells was already recognized in the eighties (Johnson and Boustouller 1987; Scott, Curran and Smyth 1989). Indeed, vaginal epithelial cells covered with bacteria, the so-called clue-cells, which represent one of the Amsel criteria used to diagnose BV, are exactly what one expects to see in the case of biofilm formation. Interestingly, clue cells were recognized for decades (Amsel et al. 1983; Cook et al. 1989; Nelson and Macones 2002), but only recently they were associated to the biofilm formation process (Swidsinski et al. 2005).

More recently, Machado and colleagues demonstrated that \textit{Gardnerella} spp. was able to adhere to epithelial cells and displace pre-coated \textit{L. crispatus}, while other BV-associated species, including \textit{A. vaginae}, \textit{M. mulieris}, \textit{F. nucleatum}, and \textit{P. bivia} were outcompeted by the protective lactobacilli (Machado et al. 2013). A subsequent study confirmed that \textit{Gardnerella} spp. has a higher virulence potential and ability to adhere to epithelial cells than 29 other BV-associated bacteria (Alves et al. 2014). Still an enigma is whether \textit{Gardnerella} spp. alone is able to trigger BV or whether \textit{Gardnerella} spp. has to interact with other bacteria to cause BV. This will be discussed in the next section.

Interactions within vaginal microbes

The importance of interspecies interactions within biofilm communities has been described for bacteria present in the oral cavity (Kolenbrander et al. 2010; Kriebel et al. 2018), gastrointestinal tract (von Rosenvinge et al. 2013), lung environment (Boisvert et al. 2016), as well as in the vaginal environment (Hardy et al. 2017). Interactions among species can be either synergistic, which are able to exert their effect by modifying the environment, so it becomes appropriate for neighboring species or by producing specific metabolites which stimulate the growth of other organisms (Pybus and Onderdonk 1999), or antagonistic (Moons, Michiels and Aertsen 2009). The last can
result in competition over nutrients and growth inhibition. Regarding the interactions that occur between the microbial members within vaginal biofilms communities, our understanding is still in its infancy (Hardy et al. 2017). However, such interactions might have a significant impact on the vaginal environment, influencing the success of antimicrobial therapy. Similar to what occurs in the oral cavity, it has now been hypothesized that Gardnerella spp. is the initial colonizer that enables other BV-related bacteria to subsequently adhere and incorporate the early biofilm (Verstraelen and Swidsinski 2013; Muzny et al. 2019b), as depicted in Figure 6.

Interactions between Gardnerella spp., BV-associated pathogens and commensal bacteria

Recognizing BV as a polymicrobial condition, several studies have suggested that interactions between BV-associated species may contribute to its progression and pathogenesis, as summarized in Table 5. Accordingly, our research group has started to investigate bacterial interactions within dual-species biofilms following the hypothesis that Gardnerella spp. is the early colonizer during BV. Using an in vitro model that allows a Gardnerella spp. biofilm to develop and then introduces a second species, our group demonstrated that some of the BV-associated species had the ability to establish synergistic interactions and augment Gardnerella spp. pre-formed biofilm, while others presented antagonistic activity (Castro and Cerca 2015). By performing confocal laser scanning microscopy, we observed that the biofilm structures among bacterial consortia differentiate in at least three unique dual-species biofilm morphotypes (Castro, Machado and Cerca 2019). Interestingly, the impact of the second BV-associated species in Gardnerella spp. virulence, as assessed by the quantification of key genes, such as the genes encoding for vaginolysin or sialidase, varied significantly, suggesting that some, but not all species, could be contributing to enhanced symptoms associated with BV (Castro, Machado and Cerca 2019).

Among synergistic interactions reported in BV, a few studies have identified specific nutritional pathways involving BV-associated bacteria. An early in vitro study reported nutritional pathways to upkeep the synergistic relationship observed between Gardnerella spp. and P. bivia. Growth of P. bivia in a vaginal defined medium supplemented with amino acids or peptone resulted in ammonia production while the growth of Gardnerella spp. under the same conditions was accompanied by ammonia
utilization (Chen et al. 1979). Consequently, ammonia flow from *P. bivia* to *Gardnerella* spp. was proposed as a mechanism to support this commensal interaction (Pybus and Onderdonk 1997). Additionally, more evidence of such bacterial cooperation was supported by a study from our research group, where we demonstrated that *Gardnerella* spp. growth increased in the presence of *P. bivia*, and *P. bivia* reached higher numbers when co-cultured with *Gardnerella* spp. (Machado, Jefferson and Cerca 2013). Besides these findings, a more recent study showed, in a mice model, that the presence of *Gardnerella* spp. enhanced the invasive potential of *P. bivia*, facilitating its ascension into the uterus (Gilbert et al. 2019).

Another early study reported an enhancement of *Peptostreptococcus anaerobius* growth in the presence of *P. bivia*, but not in pure culture (Pybus and Onderdonk 1998). After analyzing *P. bivia* culture supernatants, these authors found an increased concentration of amino acids comparative to controls followed by the growth of *P. anaerobius* and amino acids utilization. Moreover, supplementation of the growth medium with amino acids in concentrations similar to those accessible after prior growth with *P. bivia* had a growth-stimulatory effect on *P. anaerobius*. Thus, increased availability of amino acids was suggested as a mechanism to support the commensal synergism of *P. bivia* with *P. anaerobius*. Another in vitro study supported the synergistic role between these two species, with *Gardnerella* spp. enhancing the growth of *P. anaerobius* when a tryptic soy agar medium supplemented with 0.5% glucose was used (Teixeira et al. 2010).

Whereas these are in vitro observations, studies performed in vivo also demonstrated the existence of potential synergies among vaginal microorganisms involved in BV. Accordingly, by investigating the composition and spatial organization of bacteria in biopsy specimens from patients with BV, Swidsinski and colleagues found that *A. vaginae* was homogeneously intermixed with *Gardnerella* spp. in an adherent biofilm specific for this condition. *Gardnerella* spp. was the predominant species in the biofilm, followed by *A. vaginae*, which composed up to 40% of the biofilm mass (Swidsinski et al. 2005). Later, Hardy and colleagues confirmed the synergy between *Gardnerella* spp. and *A. vaginae* in samples with BV-biofilms from participants from a clinical trial in Rwanda (Hardy et al. 2016). Additionally, synergistic interactions between *Gardnerella* spp. and *Mycoplasma hominis* (Cox et al. 2016) or *A. vaginae* and *Prevotella* spp. (Datcu et al. 2013) have been also demonstrated in clinical samples.
Contrary to synergistic interactions which are beneficial for the microorganisms present in the vaginal environment, antagonistic interactions result in a negative effect for at least one species (Moons, Michiels and Aertsen 2009). Antagonistic interactions among organisms within a community are unavoidable due to competition for nutrients, with effects on the viability and growth of competitors, or preference for colonization of new surfaces (Stubbendieck, Vargas-Bautista and Straight 2016). Within the vagina, these antagonistic interrelationships have been also observed, being described that production of lactic acid by lactobacilli have a detrimental effect on many BV-associated species (Amabebe and Anumba 2018a). This effect has been only discussed in a few in vivo studies, but there are many in vitro experiments that have addressed the antagonism effect between lactobacilli and bacteria involved in BV. Thus, starting with early studies (Skarin and Sylwan 1986; Nagy, Petterson and Mardh 1991) and continuing to the most recent ones (Bertuccini et al. 2017), it has been demonstrated that different Lactobacillus spp. inhibit the growth and adhesion on epithelial cells of several bacterial species cultured from the vaginal content of women with BV, as described in Table 5. Additionally, using an ex vivo porcine vaginal mucosal model, Breshears and colleagues demonstrated that L. crispatus is able to produce lactic acid and inhibits the growth of Gardnerella spp. in co-colonization experiments (Breshears et al. 2015). However, the molecular mechanisms by which Lactobacillus spp. interact with pathogenic vaginal bacteria and host cells are still largely unknown (Younes et al. 2018). A future direction of these studies could be to examine metabolic, adhesion and coaggregation processes that maintain the biofilms, as well as to determine the proteome and transcriptome of these bacterial communities.

Interactions between Gardnerella spp. and other STIs agents

As described above, BV is characterized by a polymicrobial biofilm where BV-associated species establish synergistic interactions, that include (i) co-aggregation (Rickard et al. 2003), (ii) metabolic cooperation (Castro et al. 2017), (iii) increased resistance to antibiotics (Bradshaw and Sobel 2016) or (iv) to the host immune response (Castro, Jefferson and Cerca 2018). Such bacterial interspecies cooperation could have important clinical implications, causing persistent, slowly progressing and chronic infections (Lebeaux, Ghigo and Beloin 2014; Hardy et al. 2017). Additionally, as
discussed previously, there is epidemiological data linking BV-associated microbiota to the acquisition of STIs (Gallo et al. 2012), suggesting that BV-associated bacteria and STIs agents can establish ecological interactions, as briefly described in Table 6. Together, this raises an interesting question: can STIs agents incorporate the *Gardnerella* spp. biofilm and increase the risk of reproductive health complications? In order to answer this question, Filardo and colleagues analyzed the ecological interactions between *Gardnerella* spp. and *C. trachomatis* (Filardo et al. 2019). They proposed that biofilm-related *Gardnerella* spp. genital infections may act as a reservoir of *C. trachomatis* and, thus, contribute to the transmission of the infection in the population, as well as to its dissemination into the upper genital tract, increasing the risk of developing severe reproductive sequelae (Filardo et al. 2019). The strong relationship between BV and chlamydial infections highlights the importance of normal vaginal microbiota in the defense against STIs acquisition.

It is also noteworthy that the wide panoply of BV-associated pathogens influences the epithelial homeostasis, through the reduction of the cervicovaginal fluid viscosity due to the production of mucin-degrading enzymes (Wiggins et al. 2001). These enzymes, such as sialidases, α-fucosidase, α- and β- galactosidase, N-acetyl-glucosaminidase, glycine and arginine aminopeptidases are involved in the degradation of the gel layer coating the cervical epithelium, causing micro-abrasions or alterations of epithelial cells (Olmsted et al. 2003; Moncla et al. 2015). Therefore, such enzymes may promote virulence through destroying the protective mucosa barrier and hence increase susceptibility to *C. trachomatis* and *N. gonorrhoeae* colonization (Wiesenfeld et al. 2003) and viral STIs microbes (Gillet et al. 2011; Borgdorff et al. 2016). Specifically, it was verified that such detrimental changes in the mucosal barrier could facilitate cervical HPV infection by facilitating adherence, invasion and eventually incorporation of HPV oncopgenes into the genome of cells of the transformation zone (Gillet et al. 2011). Of note, abnormal vaginal microbiota could also be implicated in the maintenance of subclinical HPV (Gillet et al. 2011). Similar to what is described for HPV, an increased acquisition of HIV has been also associated with detrimental changes caused by *Gardnerella* spp. and other vaginal pathogens to the mucosal barrier (Borgdorff et al. 2016). Also, during *T. vaginalis* colonization, it was demonstrated an enhancement of the paracellular permeability of the cervicovaginal epithelium by
disturbing the integrity of the tight junction complex caused as a result of co-colonization with *Gardnerella* spp. and other CST-IV bacteria (Hinderfeld *et al.* 2019).

Aside from these studies, most of the other investigations that focus on the interaction between *Gardnerella* spp. and STIs agents are associated with the inflammatory response. The changes in immune homeostasis could be induced through different mechanisms: production of pro-inflammatory cytokines (Kremleva and Sgibnev 2016) or recruitment of immune cells (Torcia 2019). In this sense, the preexisting mucosal immune milieu at the site of sexual STIs agents exposure is a key determinant of STIs acquisition risk (Kaul *et al.* 2015). Interestingly, there is one study that provides evidence for a cause-effect relationship between trichomoniasis and BV (Fichorova *et al.* 2013). On the one hand, *T. vaginalis*, *Gardnerella* spp., and *A. vaginae* amplified pro-inflammatory responses by inducing increased interleukin (IL)-8 production. On the other hand, co-infections with these microbes seem to influence the protective innate-immune responses by suppressing the secretory leukocyte protease inhibitor (Fichorova *et al.* 2013), an antimicrobial peptide responsible for the protection of local tissue against the detrimental consequences of inflammation.

Regarding HSV-2 infection, the biological mechanism that is responsible for its association with vaginal dysbiosis is not clear (Torcia 2019). However, there is some evidence showing that the intermittent HSV-2 reactivation leads to immune activation in the genital environment, favoring changes in microbiota composition and epithelial shedding (Cherpes *et al.* 2005; Van de Perre *et al.* 2008; Torcia 2019). Such changes in the vaginal environment might be inhospitable to healthy microbiota and therefore could be an underappreciated but important risk for incident BV (Esber *et al.* 2015).

Finally, *Gardnerella* spp. and other BV-associated bacteria seem to increase HIV acquisition risk by inducing genital inflammation (Anahtar *et al.* 2016; Gosmann *et al.* 2017). This can occur due to two possible mechanisms: (i) proinflammatory cytokines, such as IL-1α and TNF-α, are produced after stimulation of innate immune receptors on both epithelial cells and local dendritic cells (Bamias, Arseneau and Cominelli 2014; Anahtar *et al.* 2016) or (ii) genital antigen-presenting cells sense activated bacterial products, in particular LPS, produce cytokines and chemokines which increase the recruitment of activated CD4+ lymphocytes (Anahtar *et al.* 2016). Together, these
experiments highlight the importance of understanding the interactions between vaginal microbiota and STIs agents.

Taking into account that BV is associated with the increased risk of STIs acquisition, it has been suggested that interventions targeting genital microbiota, by using effective microbicides, might reduce STIs acquisition in women. However, more mechanistic studies are needed in order to leverage these interactions to improve prevention and treatment strategies.

**HOW POLYMICROBIAL INTERACTIONS INFLUENCE ANTIMICROBIAL THERAPY?**

With the knowledge that BV is associated with a polymicrobial biofilm, there was an emergent need to start focusing on investigating the effect of antibiotics on *in vivo* and *in vitro* developed BV biofilms in order to improve the treatment options. Unfortunately, available studies addressing this subject are still scarce, and to date, as far as we are aware, no studies have been reported in how polymicrobial interactions can enhance antimicrobial tolerance in BV (Hardy *et al.* 2017; Jung *et al.* 2017). Nevertheless, relevant information can be inferred from the studies concerning polymicrobial communities that have been explored antimicrobial activity in otitis media (Perez *et al.* 2014) or in cystic fibrosis (Lopes *et al.* 2012; Lee *et al.* 2014; Manavathu, Vager and Vazquez 2014).

When assessing the impact of polymicrobial interactions in cases of otitis media, Perez and colleagues demonstrated that dual-species biofilms formed by *Moraxella catarrhalis* and *Streptococcus pneumoniae* have offered both bacteria the advantage of being more resistant to β-lactam antibiotics and bacterial clearance. These authors showed that β-lactamase produced by *M. catarrhalis* provided passive protection to *S. pneumoniae* against amoxicillin killing, while *S. pneumoniae* protected *M. catarrhalis* from azithromycin killing by an unknown mechanism (Perez *et al.* 2014). Lopes and colleagues demonstrated that *Dolosigranulum pigrum* and *Inquilinus limosus*, two unusual antibiotic-sensitive species isolated from the airways of patients with cystic fibrosis, became significantly more tolerant to several antibiotics, including gentamicin, levofloxacin, and clindamycin, upon co-culture in biofilm conditions with *Pseudomonas aeruginosa* (Lopes *et al.* 2012). Likewise, mixed-species biofilms composed of *P. aeruginosa, Pseudomonas fluorescens,* and *Klebsiella pneumoniae* were more tolerant
to tobramycin and sodium dodecyl sulfate surfactant compared to mono-species biofilms, suggesting that increased tolerance stems from a cross-protection beneficial to the entire community (Lee et al. 2014).

Other studies carried out on bacterial-fungi interactions also demonstrated an increased tolerance to antibiotics. Manavathu and colleagues developed a dual-species biofilm of *P. aeruginosa* and *Aspergillus fumigatus*, both highly prevalent in the airways of cystic fibrosis patients, and revealed that *P. aeruginosa* cells associated with the dual-species biofilms had reduced susceptibility to cefepime compared to those of mono-species biofilms, while *A. fumigatus* demonstrated similar antifungal drug susceptibility in mono- and dual-species biofilms (Manavathu, Vager and Vazquez 2014). Other investigations that showed an increased antimicrobial tolerance in dual-species biofilms, compared to mono-species, are the studies between *C. albicans* and *E. coli* (De Brucker et al. 2015) or *C. albicans* and *S. aureus* (Harriott and Noverr 2009). In both cases, the biofilm matrices and extracellular polymeric substances provided cross-species protection. Accordingly, *C. albicans* exopolysaccharide, β-1,3-glucan, can bind with ofloxacin, and *E. coli* cells embedded within *C. albicans* biofilms were found to have increased tolerance to ofloxacin compared to *E. coli* mono-species biofilms (De Brucker et al. 2015). A similar situation was observed for the mixed biofilms of *C. albicans* and *S. aureus*, where *S. aureus* cells coated in the matrix secreted by *C. albicans* showed enhanced tolerance to vancomycin (Harriott and Noverr 2009).

Based on these previous studies, we hypothesize that in BV-associated biofilms, similar interactions could also occur. Such possibility is supported by *in vivo* studies. Bradshaw and colleagues followed up 139 women with BV that were treated with oral metronidazole and examined at 1, 3, 6, 12 months or until they reached a Nugent score of 7-10 and recurrence of *Gardnerella* spp. and *A. vaginae* infection was established. Their results showed that recurrence rates of BV were significantly higher in women colonized with both *Gardnerella* spp. and *A. vaginae* (83%), as compared to women infected with *Gardnerella* spp. but not *A. vaginae* (38%), suggesting that the association between these 2 bacteria enhanced the tolerance to metronidazole, with direct impact on treatment failure (Bradshaw et al. 2006b). Other *in vivo* study, in which 18 patients diagnosed with BV were treated with oral regime of metronidazole for 1 week, showed that the vaginal polymicrobial *Gardnerella* spp. biofilm was temporarily suppressed during metronidazole treatment, but quickly recovered its activity following treatment
interruption (Swidsinski et al. 2008). Importantly, Swidsinski and colleagues found that high numbers of *Gardnerella* spp. and *A. vaginae* were present on the vaginal epithelial cells during the follow-up examination, further highlighting a possible synergism between these two species, regarding antimicrobial tolerance.

Considering *in vivo* observations, antimicrobial therapy failure and high recurrence levels of BV can be also explained by some *in vitro* studies. Not surprisingly, most of the *in vitro* experiments addressing antimicrobial therapy are focused on *Gardnerella* spp. Back in 1985, it was already demonstrated that of 11 *Gardnerella* spp. isolates, 4 were resistant to metronidazole (Jones et al. 1985). More recently, another study has demonstrated that *Gardnerella* spp. presents high *in vitro* resistance rates to metronidazole with a MIC value of >128 µg mL\(^{-1}\) (Anukam and Reid 2008). Furthermore, a study conducted in our research group analyzing 14 isolates of *Gardnerella* spp. showed that all isolates tested were resistant to metronidazole, while almost 36% and 86% of the isolates were resistant to clindamycin and tinidazole, respectively (Castro et al. 2015).

Besides *Gardnerella* spp., there are also a few studies addressing other common BV-associated species, such as *A. vaginae* and *Mobiluncus* spp. Noteworthy, *in vitro* resistance of *A. vaginae* to metronidazole was demonstrated in up to 50% of the isolates tested (Ferris et al. 2004; De Backer et al. 2006, 2010). Regarding *Mobiluncus* spp., resistance to metronidazole has been found to be more prevalent in *M. curtisii* (up to 100% of the isolates tested) than in *M. mulieris* (less than 50% of the isolates tested) (Spiegel 1987; Bahar et al. 2005). Alves and colleagues also showed that many other BV-associated bacteria have *in vitro* resistance to metronidazole (Alves et al. 2014), however, that study only tested 1 strain per species and, therefore, the prevalence of this phenomena could not be assessed.

Regarding the impact of clinically approved antibiotics on BV-associated *in vitro* biofilms, only a few papers have been described so far. The first study to assess clindamycin efficiency in *Gardnerella* spp. biofilms found that 1600 µg mL\(^{-1}\) was able to reduce up to 2-log of the viable cell count in preformed biofilms (Turovskiy et al. 2012). Higher concentrations of either metronidazole (2000 µg mL\(^{-1}\)) or clindamycin (20000 µg mL\(^{-1}\)) were able to kill biofilm-associated *Gardnerella* spp. cells after 8 h of incubation (Algouri, Volski and Chikindas 2015). Afterwards, Thellin and colleagues
demonstrated that concentrations of 600 µg mL$^{-1}$ and 100 µg mL$^{-1}$ of metronidazole and clindamycin, respectively, administered on 72 h biofilms of *Gardnerella* spp. were sufficient to achieve 100% cells mortality (Thellin et al. 2016). Despite the apparent success of these *in vitro* experiments, the concentrations used in those studies were a lot higher than the peak serum concentrations (Ralph et al. 1974; Dan, Yampolsky and Poch 1997) and therefore could not be used in treatment. When using clinically achievable concentrations, Gottschick and colleagues found that metronidazole (0.001 µg mL$^{-1}$) had the ability to prevent the development of *Gardnerella* spp. biofilms, if used preemptively, but could not disrupt the existing biofilms and did not affect the viability of their cells (Gottschick et al. 2016).

Interestingly, the evidence found *in vitro* biofilms is supported by our recent study in which we have found that genes involved in antimicrobial resistance were up-regulated in *Gardnerella* spp. biofilm cells (Castro et al. 2017). Moreover, we later observed that this up-regulation of genes was further enhanced in specific dual-species BV biofilms (Castro, Machado and Cerca 2019), providing some mechanistic evidence that explains why some polymicrobial communities might have increased antimicrobial resistance and, consequently, lead to BV recurrence, which has been associated with the chronic nature of this infection. Overall, understanding the molecular basis and biological effect of these inter-bacterial processes may provide novel information necessary to define new targets and strategies for BV control.

Importance of novel strategies to fight chronic vaginal infections

Similar to what was described above for BV, increased cases of recurrence are being observed in other vaginal infections (Seña, Bachmann and Hobbs 2014; Denning et al. 2018). This is of particular concern because we are already heading toward a post-antibiotic era in which many bacterial infections will be impossible to treat (Hauser, Mecsas and Moir 2016). The same situation can be expected for fungal infections (Casadevall, Kontoyiannis and Robert 2019), whose recurrence affects millions of women worldwide, being a common cause of significant morbidity among them (Sobel 2016). Unluckily, the case of viral vaginitis is not far from the above-mentioned situations with viruses being resistant to the common antiviral drugs, and the preventive
therapies which are represented by vaccines still in development for some of them (Johnston, Gottlieb and Wald 2016; Safrit et al. 2016).

Concerning this issue, there are several attempts to use diverse compounds such as antimicrobial therapy adjuvants, in order to increase the efficacy of the common antibiotic treatment. These adjuvants, when used alone have little antimicrobial activity, but when co-administered with antibiotic, they either (i) block the main bacterial resistance mechanisms or (ii) enhance the antimicrobial action of the drug (González-Bello 2017). In this regard, several clinical studies supported the concept that lactobacilli can work as antimicrobial adjuvants since they able to increase the efficacy of metronidazole (Anukam et al. 2006; Larsson et al. 2011; Bodean et al. 2013; Heczko et al. 2015). Interestingly, the utilization of DNase in combination with metronidazole led to greater Gardnerella spp. biofilm disruption than either agent alone (Hymes et al. 2013). A similar study demonstrated that lysozyme in combination with metronidazole or clindamycin also improved the antimicrobial activity of the tested agents against Gardnerella spp. in vitro biofilms (Thellin et al. 2016). Algburi and colleagues also showed that natural antimicrobials subtilosin and lauramide arginine ethyl ester exhibited a synergistic effect with metronidazole and clindamycin when applied on biofilms of Gardnerella spp. (Algburi, Volski and Chikindas 2015). Moreover, more recently, it was shown that cationic amphiphiles displayed a positive effect either with metronidazole or clindamycin against BV-associated bacteria (Algburi et al. 2017; Weeks et al. 2019).

Besides the fact that these therapeutic strategies are promising, there are also attempts to totally replace current antibiotic treatment, as described in Table 7 and as it has been recently reviewed (Machado et al. 2016; Falconi-McCahill 2019). However, many of the alternative approaches tend to achieve a reduction of the symptoms, instead of being targeting directly the causes of BV, with little attention being put in the microbial interactions occurring during disease. As discussed before, the vaginal environment in disease is a complex niche being governed by still poorly understood relationships among the present microbial species. Therefore, it is of utmost importance to focus attention on how the microbial interactions in BV and other vaginal infections are affecting antimicrobial therapies, in order to speed up the process of finding and developing novel treatment or preventive strategies effective against recurrent vaginal infections.
GARDNERELLA SPP. BEYOND BV

Apart from vaginal infections, *Gardnerella* spp. has also been found in other types of infections. However, in some of these situations, it is neither clear what is the origin of the microorganism nor the mode of transmission and its role in the infection. Globally, when *Gardnerella* spp. is detected in infections occurred in women, often it is also reported a gynecological condition or procedure that could be the event leading to the development of infection. In cases of infection in men, a possible sexual transmission or UTIs can be the cause.

*Gardnerella* spp. association to UTIs have been described both in women and men. Some studies have demonstrated that UTIs are more common in women suffering from BV (Hillebrand *et al.* 2002; Sharami, Afrakhteh and Shakiba 2007; Sumati and Saritha 2009). However, one important limitation of these studies is the fact that it is not reported whether the development of the UTI is preceded by BV or vice-versa. The proximity of the vaginal canal with the urinary tract and the microbial alterations characteristic of BV, with an overgrowth of pathogenic bacteria and the lack of protecting microbiota, might allow the colonization with uropathogens and consequently facilitate women to develop UTIs (Lam, Birch and Fairley 1988; Harmanli *et al.* 2000; Kline and Lewis 2016). Further studies support the increased ability of women with BV to develop UTIs, showing that the artificial colonization of vagina with *Lactobacillus*, by means of probiotic treatment with *L. crispatus*, may be beneficial for women prone to recurrent UTIs (Stapleton *et al.* 2011). In the context of UTIs, *Gardnerella* spp. involvement was associated with different health problems including balanoposthitis (Kinghorn *et al.* 1982), pyelonephritis (Pritchard 2018), cystitis and prostatitis (Sturm 1989).

Other clinical situations where *Gardnerella* spp. has been detected was in bloodstream infections in women in the context of vaginal infections (Tankovic *et al.* 2017), pregnancy (Flórez *et al.* 1994), gynecological procedures that may introduce the bacterium in the bloodstream (Agostini *et al.* 2003; McCool and DeDonato 2012), or in immunocompromised patients (Saikali *et al.* 2017). Curiously, there has been one reported case of bacteremia in a newborn, where the mother was diagnosed with
endometritis and the transmission of *Gardnerella* spp. probably occurred by aspiration of maternal amniotic fluid (Amaya, Al-Dossary and Demmler 2002). Furthermore, while rare, bloodstream infections caused by *Gardnerella* spp. have been reported in men (Legrand *et al.* 1989; Lagacé-Wiens *et al.* 2008). In one report, an uncircumcised man, with a previous history of diabetes mellitus and hypertension and whose sexual partner had recurrent BV was infected with *Gardnerella* spp. having serious consequences on vital organs, with the development of infective endocarditis and emboli in the kidney and brain (Yoon *et al.* 2010). In another case, the patient was affected with the development of multiple abscess affecting the lungs and kidney, but no predisposing factors were found (Calvert, Collins and Bateman 2005).

The presence of *Gardnerella* spp. in osteoarticular infections has also been detected, such as in acute hip arthritis (Sivadon-Tardy *et al.* 2009), disk space infections (Hodge, Levy and Smith 1995), discitis and vertebral osteomyelitis (Graham *et al.* 2009), osteomyelitis and hip abscess (Shah, Nanjappa and Greene 2017), joint infections (Hoarau *et al.* 2012), reactive arthritis (El Mezouar *et al.* 2014), and spinal epidural abscesses (Stewart *et al.* 2018). *Gardnerella* spp. is also reported as the pathogen involved in some infrequent infections such as wound infection (Sturm, de Leeuw and de Pree 1983), tubo-ovarian abscess (Burgess, Daramola and Lacey 1997), meningitis (Berardi-Grassias *et al.* 1988), retinal vasculitis (Neri *et al.* 2009), cephalohematoma (Nightingale *et al.* 1986), and hydropneumothorax (Murray *et al.* 2019).

As discussed above, some antimicrobial agents are indicated for the treatment of BV. Regarding extra-vaginal infections where *Gardnerella* spp. is involved, there is no consensus in the recommendation for treatment. Overall, in the cases reviewed, the patient was initially treated with broad-spectrum antibiotics such as ampicillin. When the microorganism was identified as *Gardnerella* spp., often the treatment was changed to include metronidazole or clindamycin therapy.

**CONCLUDING REMARKS**

The vaginal microbiota plays a mutually beneficial relationship with their host and has a major impact on health and disease. Despite various studies have already addressed the importance of the vaginal microbiota and its relationship with vaginal infections and STIs, studies on the interactions among the microbial populations are lagging behind. In
a context of dysbiosis of the vaginal microbiota, *Gardnerella* spp. seems to have a special role, since this bacterium is highly detected in BV, the most prevalent vaginal infection worldwide. However, other microbes can also colonize the vaginal ecosystem, establishing ecological interactions with *Gardnerella* spp., which include the examples documented in this review.

Despite all the efforts to unveil the mechanisms involved in the interactions among vaginal microbes, the biological relevance of such interactions remains largely unknown. Because the vaginal epithelium is an important entry point for microbes, including to STIs agents, a deeper understanding of the mechanisms of adhesion and signaling involved in polymicrobial interactions will provide a new perspective on the role of known virulence determinants. Furthermore, instead of infection being thought of as a defined host-pathogen relationship, it should be envisioned as a spectrum of host-microbe pathogenic mechanisms, microbe-microbe interactions, host immunity-mediated antimicrobial defenses, and environmental factors. As such, future studies should focus on exploring mechanistic *in vitro* models as well as implementing animal model systems to study polymicrobial vaginal interactions in order to understand the complex dynamics within mixed microbial communities and their importance during interactions with the host.

The key challenges now are to unravel precise details of the unique biology of polymicrobial interactions under conditions of co-existence in the vagina. With the application of powerful RNA-sequencing, DNA microarray, proteomic, and metabolomics technologies, there are now tools available to undertake such efforts. The identification of potential targets for the inhibition of co-adhesion and biofilm development may ultimately provide the means to modify microbial vaginal colonization and thus reduce the impact of polymicrobial diseases on women health. This might form the basis for novel, ecologically-based strategies for the control of vaginal infections, other than the current use of antimicrobial agents, which are associated with high recurrence rates.
FUNDING
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Figure 1. Representation of the different ethnic groups of women within each community state type (CST) proposed by Ravel et al. 2011. The study cohort consisted of 96 Asian women, 97 white women, 104 black women, and 97 Hispanic women, showing the relationship between ethnic background and vaginal bacterial community composition.
Figure 2. Schematic representation of the healthy and unbalanced vaginal microbiota according to the most common vaginal infections.
**Figure 3.** Representation of the putative model of BV infection.
**Figure 4.** Gram-staining vaginal smears illustrate the vaginal microbiota. (a) Normal vaginal epithelial cells. (b) Intermediate vaginal microbiota. (c) BV-associated microbiota, showing a vaginal clue cell, which corresponds to vaginal squamous epithelial cells coated with *Gardnerella* spp. and other anaerobic bacteria. Original magnification: 1000 times.
Figure 5. Representation of the epidemiological profile of BV in relation to sexual behavior. This figure was created based on the information presented in the article by Verstraelen et al. 2010.
Figure 6. Conceptual multi-species model of the BV-associated biofilm development. In multi-species BV-related biofilms, secondary pathogens are able to incorporate the initially formed biofilm by *Gardnerella* spp. which is already adhered to the vaginal epithelium. Following, a synergistic relationship can be formed, allowing the biofilm to prosper.
Table 1. Main features of the normal vaginal microbiota and the most common vaginal infections.

<table>
<thead>
<tr>
<th></th>
<th>Vaginal fluid</th>
<th>Vaginal fluid pH</th>
<th>Clinical inflammation and symptoms</th>
<th>Microscopic features</th>
<th>Sexually transmitted</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>White, no or milky odor, variable viscosity along the cycle</td>
<td>3.5 – 4.5</td>
<td>No</td>
<td>Mainly normal intermediate and superficial vaginal cells, numerous lactobacilli, very scarce leukocytes</td>
<td>Not applicable</td>
<td>(Frobenius and Bogdan 2015; Palmeira-de-Oliveira, Palmeira-de-Oliveira and Martinez-de-Oliveira 2015; Sherrard et al. 2018)</td>
</tr>
<tr>
<td>Bacterial vaginosis</td>
<td>Abundant, whitish gray, rotten fish odor, low viscosity</td>
<td>&gt; 4.5</td>
<td>Odorous discharge (or no symptoms at all), absence of redness; no or slight inflammation</td>
<td>Clue cells, scarce or no lactobacilli, no leukocytes, abundant bacteria</td>
<td>Controversial</td>
<td>(Frobenius and Bogdan 2015; Palmeira-de-Oliveira, Palmeira-de-Oliveira and Martinez-de-Oliveira 2015; Sherrard et al. 2018)</td>
</tr>
<tr>
<td>Aerobic vaginitis</td>
<td>Abundant watery, yellow, no fish odor, low viscosity</td>
<td>&gt; 4.5</td>
<td>Erythema</td>
<td>Scarc or no lactobacilli, leukocytes, abundant bacteria</td>
<td>No</td>
<td>(Donders et al. 2002; Frobenius and Bogdan 2015; Palmeira-de-Oliveira, Palmeira-de-Oliveira and Martinez-de-Oliveira 2015; Sherrard et al. 2018)</td>
</tr>
<tr>
<td>Vulvovaginal candidiasis</td>
<td>White, none or ferment odor, “cottage cheese-like”, creamy or floccular, high viscosity</td>
<td>3.5 – 4.5</td>
<td>Diffuse redness, swelling and fissuring to the vulva, burning and pruritus</td>
<td>Some deeper vaginal cells present, variable number of lactobacilli and leukocytes, blastoconidia and pseudohyphae</td>
<td>No</td>
<td>(Sobel 2007; Frobenius and Bogdan 2015; Palmeira-de-Oliveira, Palmeira-de-Oliveira and Martinez-de-Oliveira 2015; Sherrard et al. 2018)</td>
</tr>
<tr>
<td>Trichomoniasis</td>
<td>Yellow/ green aqueous discharge, fishy/ putrid odor, low viscosity</td>
<td>&gt; 4.5</td>
<td>Erythema, red plaques, vulvar irritation and pruritus</td>
<td>Protozoa identification, particularly if motile numerous bacteria and leukocytes, many parabasal cells</td>
<td>Yes</td>
<td>(Palmeira-de-Oliveira, Palmeira-de-Oliveira and Martinez-de-Oliveira 2015; Edwards et al. 2016; Sherrard et al. 2018)</td>
</tr>
</tbody>
</table>
Table 2. *Association of BV with other vaginal infections.*

<table>
<thead>
<tr>
<th>Bacterial vaginosis (BV)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AEROBIC VAGINITIS (AV)</strong></td>
<td></td>
</tr>
<tr>
<td>Mixed situations (AV and BV) can be found, representing either a transient form or</td>
<td>(Vieira-Baptista et al. 2016; Donders et al. 2017)</td>
</tr>
<tr>
<td>prolonged co-infection</td>
<td></td>
</tr>
<tr>
<td><strong>VULVOVAGINAL CANDIDIASIS (VVC)</strong></td>
<td></td>
</tr>
<tr>
<td>VVC is a common side effect of BV treatment with antibiotics, indicating that the</td>
<td>(Pirotta, Gunn and Chondros 2003)</td>
</tr>
<tr>
<td>vaginal microbiota might be related to the colonization of yeast</td>
<td></td>
</tr>
<tr>
<td>Co-colonization of Candida spp., Gardnerella spp. and other BV-associated bacteria</td>
<td>(Wei et al. 2012)</td>
</tr>
<tr>
<td>on Pap smears</td>
<td></td>
</tr>
<tr>
<td><strong>TRICHOMONIASIS</strong></td>
<td></td>
</tr>
<tr>
<td>Co-occurrence of trichomoniasis and BV was found in approximately half of women</td>
<td>(Sutton et al. 2007)</td>
</tr>
<tr>
<td>infected with <em>Trichomonas vaginalis</em></td>
<td></td>
</tr>
<tr>
<td>Vaginal microbiota belonging to CST-IV was significantly associated with <em>T. vaginalis</em></td>
<td>(Brotman et al. 2012)</td>
</tr>
<tr>
<td>detection</td>
<td></td>
</tr>
<tr>
<td><em>T. vaginalis</em> vaginal colonization had a negative impact in lactobacilli but not in</td>
<td>(Fichorova et al. 2013)</td>
</tr>
<tr>
<td>BV-associated species</td>
<td></td>
</tr>
<tr>
<td>Nugent score higher than 3 was associated with a significantly increased risk of</td>
<td>(Balkus et al. 2014)</td>
</tr>
<tr>
<td>acquiring trichomoniasis</td>
<td></td>
</tr>
<tr>
<td><strong>CHLAMYDIA/ GONORRHEA</strong></td>
<td></td>
</tr>
<tr>
<td>Women with BV were 3.4 times more likely to test positive for chlamydia and 4.1 times</td>
<td>(Wiesenfeld et al. 2003)</td>
</tr>
<tr>
<td>more likely to test positive for gonorrhea compared to women without BV</td>
<td></td>
</tr>
<tr>
<td>Incident chlamydia/ gonorrhea was associated with BV severity, as measured by a high</td>
<td>(Allsworth and Peipert 2011)</td>
</tr>
<tr>
<td>Nugent score (8–10)</td>
<td></td>
</tr>
<tr>
<td>Women with a BV-associated microbiota experiencing a 2-fold increased risk for STIs</td>
<td>(Allsworth and Peipert 2011)</td>
</tr>
<tr>
<td>compared to women with normal vaginal microbiota</td>
<td></td>
</tr>
<tr>
<td>Antecedent episodes of BV are associated with an increased risk of subsequent</td>
<td>(Bautista et al. 2017)</td>
</tr>
<tr>
<td>chlamydia and gonorrhea infection</td>
<td></td>
</tr>
<tr>
<td><strong>VIRAL VAGINITIS</strong></td>
<td></td>
</tr>
<tr>
<td>Nugent scores of 4 or higher were significantly associated with a 32% increase in</td>
<td>(Allsworth, Lewis and Peipert 2008)</td>
</tr>
<tr>
<td>concurrent herpes simplex virus type 2 (HSV-2) and an 8% increase in HSV type 1 (HSV-1)</td>
<td></td>
</tr>
<tr>
<td>BV was 60% greater prevalent among HSV-2-positive women when compared with HSV-2-</td>
<td>(Esber et al. 2015).</td>
</tr>
<tr>
<td>negative women, implying HSV-2 infection is an important BV risk factor</td>
<td></td>
</tr>
<tr>
<td>An increased association of prevalent and incident human papillomavirus (HPV) was</td>
<td>Watts et al. 2005; King et al. 2011</td>
</tr>
<tr>
<td>shown in women with both intermediate and BV microbiota</td>
<td></td>
</tr>
<tr>
<td>Women who were HPV-positive had a lower proportion of protective vaginal</td>
<td>(Lee et al. 2013; Brotman et al. 2017)</td>
</tr>
</tbody>
</table>
Lactobacillus spp. when compared with HPV-negative women (al. 2014).

Vaginal dysbiosis with increased risk of acquisition and transmission of human immunodeficiency virus type 1 (HIV-1). A meta-analysis of 23 studies showed that BV was associated with a 60% increase in the risk of acquiring HIV-1 (Atashili et al. 2008; Sturm-Ramirez et al. 2000; Pyles et al. 2014; McKinnon et al. 2019).

Table 3. Scheme for grading Gram-stained vaginal contents.

<table>
<thead>
<tr>
<th>Score</th>
<th>Lactobacillus Morphotypes</th>
<th>Gardnerella and Bacteroides spp. Morphotypes</th>
<th>Curved Gram-Variable Rods</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>3+</td>
<td>1+</td>
<td>1+ or 2+</td>
</tr>
<tr>
<td>2</td>
<td>2+</td>
<td>2+</td>
<td>3+ or 4+</td>
</tr>
<tr>
<td>3</td>
<td>1+</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>4+</td>
<td></td>
</tr>
</tbody>
</table>

VAGINAL MICROBIOTA DIAGNOSIS BY NUGENT SCORE SYSTEM

<table>
<thead>
<tr>
<th>Total score</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 3</td>
<td>Normal vaginal microbiota</td>
</tr>
<tr>
<td>4 – 6</td>
<td>Intermediate vaginal microbiota</td>
</tr>
<tr>
<td>7 – 10</td>
<td>Bacterial vaginosis in vaginal microbiota</td>
</tr>
</tbody>
</table>

*Morphotypes are scored as the average number seen per oil immersion field. Quantification of each individual score: 0 for no morphotype present; 1+ for 1 morphotype present; 2+, 1 to 4 morphotypes present; 3+, 5 to 30 morphotypes present; 4+, 30 or more morphotypes present. The total score is the sum of the average classification of Lactobacillus, Gardnerella and Bacteroides, and finally Mobiluncus spp. Adapted from Nugent, Krohn and Hillier 1991.*
Table 2. Koch’s postulates and Gardner and Duke’s conclusions.

<table>
<thead>
<tr>
<th>Koch’s postulates (Koch 1876)</th>
<th>Gardner and Duke observations (Gardner and Dukes 1955)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The etiologic microbe should be found in every case of the disease</td>
<td>1. 92% of patients with a primary diagnosis of BV were found to have <em>H. vaginalis</em> infection</td>
</tr>
<tr>
<td>2. The bacterium must be isolated from a diseased organism and grown on pure culture</td>
<td>2. This was accomplished in each of the 141 cases with positive <em>H. vaginalis</em> cultures</td>
</tr>
<tr>
<td>3. The etiologic microbe should be isolated in pure culture on lifeless media and be capable of causing the characteristic disease anew upon inoculation in a susceptible host</td>
<td>3. A patient, known to be free of disease, was inoculated with <em>H. vaginalis</em>. The patient developed clinical manifestations of the disease and the organism was recovered in pure culture</td>
</tr>
<tr>
<td>4. The etiologic microbe should be re-isolated from the experimentally inoculated host</td>
<td>4. This requirement was fulfilled since pure cultures of the bacterium were successfully obtained from the patient’s culture material</td>
</tr>
</tbody>
</table>
Table 3. *Bacterial interactions occurring in the context of BV and their predictive ecological effects.*

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Interaction</th>
<th>Mechanism</th>
<th>Effect in host</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SYNERGISM WITHIN MICROBES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gardnerella</em> spp. and <em>Prevotella bivia</em></td>
<td><em>P. bivia</em> produced ammonia which was utilized by <em>Gardnerella</em> spp. which produced amino acids that were utilized by <em>P. bivia</em></td>
<td>Ammonia and amino acids cycle</td>
<td>Presence of high vaginal pH</td>
<td>(Pybus and Onderdonk 1997)</td>
</tr>
<tr>
<td><em>Peptostreptococcus anaerobius</em> and <em>P. bivia</em></td>
<td>Amino acids accumulation in <em>P. bivia</em> culture supernatants and subsequent growth of <em>P. anaerobius</em> in the conditioned supernatants</td>
<td><em>P. anaerobius</em> was able to grow in vaginal defined medium with <em>P. bivia</em>, but not in pure culture. Amino acids serve as a source for <em>P. anaerobius</em> growth</td>
<td>Increased risk for female pelvic infections, adverse pregnancy outcome, and intra-amniotic infection</td>
<td>(Pybus and Onderdonk 1998)</td>
</tr>
<tr>
<td><em>Gardnerella</em> spp. and <em>Atopobium vaginae</em></td>
<td><em>A. vaginae</em> was homogeneously intermixed with <em>Gardnerella</em> spp. in BV-associated biofilms</td>
<td>Unknown</td>
<td>Presence of clue cells</td>
<td>(Swidsinski et al. 2005)</td>
</tr>
<tr>
<td><em>Gardnerella</em> spp. and <em>P. anaerobius</em> <em>Gardnerella</em> spp. strains were able to enhance the growth of <em>P. anaerobius</em></td>
<td>Production of synergistic compounds by <em>Gardnerella</em> spp.</td>
<td>Bacterial interactions present an important role in the ecology of the vaginal microbiota</td>
<td></td>
<td>(Teixeira et al. 2010)</td>
</tr>
<tr>
<td><em>Gardnerella</em> spp. and <em>Eggerthella, Dialister</em> sp. type 2, <em>A. vaginae</em>, and <em>Aerococcus christensenii</em></td>
<td>Metabolic co-dependencies between these bacteria</td>
<td>Unknown</td>
<td>Possible contribution to increase the incidence of BV</td>
<td>(Srinivasan et al. 2012)</td>
</tr>
<tr>
<td><em>A. vaginae</em> and <em>Prevotella spp.</em></td>
<td>Both bacterial species could have metabolic co-dependencies</td>
<td>Unknown</td>
<td>A combination of <em>Prevotella</em> spp. and/or <em>A. vaginae</em> seems to diagnose BV with high accuracy</td>
<td>(Datcu et al. 2013)</td>
</tr>
<tr>
<td><strong>Gardnerella spp. and Fusobacterium nucleatum, Mobiluncus mulieris, A. vaginae or P. bivia</strong></td>
<td><em>In vitro</em> dual-species biofilms of <em>Gardnerella</em> spp. derived a growth benefit from the addition of a second species, regardless of the species. <em>Gardnerella</em> spp. biofilms enhanced the growth of <em>P. bivia</em> and to a minor extent of <em>F. nucleatum</em>.</td>
<td><em>F. nucleatum</em> was shown to be able to join an initial <em>Gardnerella</em> spp. biofilm (intermediate colonizer)</td>
<td>The symbiotic relationships established between <em>Gardnerella</em> spp. and other anaerobes in BV biofilms could contribute to the progression of BV</td>
<td>(Machado, Jefferson and Cerca 2013)</td>
</tr>
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</tr>
<tr>
<td><strong>Gardnerella spp. and Actinomyces neuii, Brevibacterium ravenspurgense, Corynebacterium amycolatum, Corynebacterium tuscaniense, Staphylococcus saprophyticus, Enterococcus faecalis, Nosocomicoccus ampullae, Staphylococcus simulans, Staphylococcus warneri, Streptococcus anginosus, Propionibacterium acnes or Escherichia coli</strong></td>
<td>These bacterial species were able to cause an increase of the biomass of a pre-established <em>Gardnerella</em> spp. biofilm</td>
<td>Unknown</td>
<td>Could be associated with a high number of clue cells</td>
<td>(Castro and Cerca 2015)</td>
</tr>
<tr>
<td><strong>Gardnerella spp. and A. vaginae</strong></td>
<td><em>Gardnerella</em> spp. and <em>A. vaginae</em> are important constituents of the vaginal biofilm</td>
<td>Unknown</td>
<td>Presence of clue cells</td>
<td>(Hardy <em>et al.</em> 2016)</td>
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<td><strong>Gardnerella spp. and E. coli or E. faecalis</strong></td>
<td><strong>E. coli</strong> and <strong>E. faecalis</strong> were able to incorporate and enhance a pre-formed <strong>Gardnerella</strong> spp. Biofilm</td>
<td>In dual-species biofilms, these bacterial species seem to be able to co-aggregate</td>
<td>Uropathogens can associate in BV biofilm</td>
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<td><strong>Gardnerella spp. and Mycoplasma hominis</strong></td>
<td>Strong association between <strong>Gardnerella</strong> spp. and <strong>M. hominis</strong> were found in women with BV</td>
<td>A potential quorum sensing-like interaction or co-response to an environmental stimulus</td>
<td>The transmission of one of these bacteria could trigger the outgrowth of the other and start a process leading to BV</td>
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<td><strong>Gardnerella spp. and P. bivia</strong></td>
<td><strong>Gardnerella</strong> spp. facilitated uterine infection by <strong>P. bivia</strong></td>
<td>The presence of <strong>Gardnerella</strong> spp. enhanced the invasive potential of <strong>P. bivia</strong>, facilitating its ascension into the uterus</td>
<td>BV bacteria may actively inhibit inflammatory responses</td>
<td></td>
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<tr>
<td><strong>Gardnerella spp. and A. vaginae, A. neuii, C. tuscaniense, M. mulieris, S. anginosus, P. bivia, C. amycolatum, N. ampullae, P. acnes, B. ravenspurgen se, E. faecalis, S. saprophyticus, S. simulans, S. hominis, S. warneri</strong></td>
<td>Despite all BV-associated species were able to increase the cell number of a pre-established <strong>Gardnerella</strong> spp. biofilm, not all bacterial species enhanced the <strong>Gardnerella</strong> spp. virulence according to transcriptomic findings</td>
<td>Increased expression of genes associated with cytotoxicity, biofilm formation, antimicrobial resistance, and evasion of immune response by <strong>Gardnerella</strong> spp. in presence of specific BV-associated bacteria in dual-species biofilms</td>
<td>Bacterial interactions between co-infecting bacteria can profoundly affect the progress of BV and its clinical outcome</td>
<td></td>
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</table>

**ANTAGONISM WITHIN MICROBES**

<table>
<thead>
<tr>
<th><strong>Lactobacillus spp. and Gardnerella spp., Mobiluncus spp., Bacteroides, and anaerobic cocci</strong></th>
<th><strong>Lactobacillus</strong> inhibited the growth of bacteria isolated from women with BV</th>
<th>The capacity of <strong>Lactobacillus</strong> to acidify the medium with a consequent decrease of pH and inhibition of growth</th>
<th><strong>Lactobacillus</strong> prevent the growth of bacteria associated with BV</th>
</tr>
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<tbody>
<tr>
<td><strong>Lactobacillus spp. and Gardnerella spp., Mobiluncus spp., Peptostreptococcus</strong></td>
<td><strong>Lactobacillus</strong> inhibited the growth of <strong>Peptostreptococcus</strong>, <strong>M. curtisii</strong>, <strong>Gardnerella</strong> spp., and other</td>
<td>The inhibition by <strong>Lactobacillus</strong> was influenced by the pH of the growth medium</td>
<td>The interactions between <strong>Lactobacillus</strong> and other bacteria may regulate the microbiological ecosystem of the vagina</td>
</tr>
</tbody>
</table>

*(Castro, Machado and Cerca 2016)  
(Cox et al. 2016)  
(Gilbert et al. 2019)  
(Castro, Machado and Cerca 2019)  
(Skarin and Sylwan 1986)  
(Nagy, Petterson and Mardh 1991)
<table>
<thead>
<tr>
<th>Bacteroides spp., Bacteroides spp.</th>
<th>Anaerobes</th>
<th>Production of acids and hydrogen peroxide (H₂O₂) by lactobacilli</th>
<th>Lactobacilli would prevent colonization by other bacteria associated with BV (McLean and Rosenstein 2000)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactobacillus spp. and Gardnerella spp., Bacteroides spp., P. bivia</strong></td>
<td><em>Lactobacillus</em> inhibited the growth of bacteria</td>
<td>Production of a bacteriocin by <em>L. acidophilus</em></td>
<td>Lactobacilli, by the production of bacteriocins, have the capacity to prevent the growth of pathogenic bacteria (Aroutcheva, Simoes and Faro 2001)</td>
</tr>
<tr>
<td><strong>Lactobacillus acidophilus and Gardnerella spp.</strong></td>
<td><em>L. acidophilus</em> produced a bacteriocin that inhibited the growth of <em>Gardnerella</em> spp. isolates</td>
<td>The antagonistic activity is due to the compounds produced by <em>L. helveticus</em></td>
<td><em>L. helveticus</em> is a potential probiotic strain (Atassi et al. 2006a)</td>
</tr>
<tr>
<td><strong>Lactobacillus helveticus and Gardnerella spp. and P. bivia</strong></td>
<td><em>L. helveticus</em> inhibited the growth and viability of <em>Gardnerella</em> spp. and <em>P. bivia</em> and also decreased the capacity of adhesion of <em>Gardnerella</em> spp., to HeLa cells</td>
<td>Production of H₂O₂ and proteolytic enzyme-resistant compounds by <em>Lactobacillus</em> spp.</td>
<td><em>Lactobacillus</em> can control the vaginal microbiota and compete with other organisms for the adherence to epithelial cells (Atassi et al. 2006b)</td>
</tr>
<tr>
<td><strong>Lactobacillus spp. and Gardnerella spp. and P. bivia</strong></td>
<td><em>Lactobacillus</em> strains isolated from vaginas of healthy women showed antagonistic activity against <em>Gardnerella</em> spp. and <em>P. bivia</em> in co-culture and also inhibited viability and adhesion of bacteria to HeLa cells</td>
<td>The production of H₂O₂ by some <em>Lactobacillus</em> strains seems to be the primary effect, however for some non-producer strains the production of biosurfactants, bacteriocins and signalling molecules may have effect on the displacement and viability of <em>Gardnerella</em> spp. growing as biofilm</td>
<td><em>Lactobacillus</em> strains have the ability to disrupt biofilms that occur during BV and potentially reduce the need to antibiotics. Indigenous lactobacilli may have a restorative function to maintain a healthy vaginal microbiota (Saunders et al. 2007)</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus and Gardnerella spp. and P. bivia</td>
<td>Lactobacillus showed bactericidal activity against Gardnerella spp. and P. bivia</td>
<td>It probably includes the production of hydrogen peroxide, lactic acid, and antibacterial compounds by Lactobacillus</td>
<td>L. rhamnosus is considered a probiotic strain - a promising candidate for use in BV therapy (Coudeyras et al. 2008)</td>
</tr>
<tr>
<td>Lactobacillus spp. and Gardnerella spp., P. bivia, Mobiluncus spp., and Bacteroides fragilis</td>
<td>Lactobacillus species inhibited the growth of Gardnerella spp., P. bivia, and Mobiluncus spp., but did not show effect against B. fragilis</td>
<td>Production of lactic acid, H₂O₂, and bacteriocins by Lactobacillus spp.</td>
<td>Potential role of lactobacilli against BV pathogens (Matu et al. 2010)</td>
</tr>
<tr>
<td>Lactobacillus johnsonii, Lactobacillus gasseri and Gardnerella spp.</td>
<td>Lactobacilli inhibited the growth of Gardnerella spp.</td>
<td>Possibility of lactobacilli preventing BV development by their heat stable molecules</td>
<td>The main metabolites of Lactobacillus spp. act cooperatively to kill BV-associated bacteria (Atassi and Servin 2010)</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus, Lactobacillus reuteri and Gardnerella spp.</td>
<td>The secreted products of L. rhamnosus and L. reuteri inhibited BV biofilms and caused bacterial cell death</td>
<td>Possible production of acid, bacteriocins or biosurfactant-like substances by L. rhamnosus and L. reuteri</td>
<td>Lactobacilli can induce a return to a normal microbiota from a BV state (McMillan et al. 2011)</td>
</tr>
<tr>
<td>Lactobacillus spp. and Gardnerella spp.</td>
<td>Lactobacillus showed antagonistic activity against Gardnerella spp.</td>
<td>Unknown</td>
<td>Success in the BV development depends on the presence of Lactobacillus species (Teixeira et al. 2012)</td>
</tr>
<tr>
<td>Lactobacillus crispatus and Gardnerella spp.</td>
<td>L. crispatus produced lactic acid and inhibited the growth of Gardnerella spp. on an ex vivo porcine vaginal mucosal model</td>
<td>Production of antimicrobial compounds by L. crispatus</td>
<td>A stable L. crispatus colonization of live vaginal mucosa is able to prevent colonization of Gardnerella spp. in a pH-dependent manner (Breshears et al. 2015)</td>
</tr>
<tr>
<td>L. acidophilus, L. rhamnosus,</td>
<td>Lactobacillus were able to inhibit the growth of both</td>
<td>The effect could be due to the production of lactic acid, H₂O₂, and bacteriocins</td>
<td>L. acidophilus alone or combined with L. rhamnosus can be used in probiotic products to (Bertuccini et al. 2017)</td>
</tr>
</tbody>
</table>
and *Gardnerella* spp. and *A. vaginae*

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Interaction</th>
<th>Mechanism</th>
<th>Effect in host</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gardnerella</em> spp., other BV-associated bacteria and <em>Chlamydia trachomatis</em></td>
<td>Cooperative interactions between <em>Gardnerella</em> spp., other BV-associated bacteria, and <em>C. trachomatis</em></td>
<td>Production of sialidase and other glycosides by <em>Gardnerella</em> spp., which can potentially alter mucosal integrity and facilitate infection with genital pathogens</td>
<td>Detrimental changes to the mucosal barrier</td>
<td>(Wiesenfeld <em>et al.</em> 2003)</td>
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<tr>
<td><em>Gardnerella</em> spp. and <em>Neisseria gonorrhoeae</em></td>
<td><em>Gardnerella</em> spp. and other BV-bacteria are associated with an increase acquisition of <em>N. gonorrhoeae</em> colonization</td>
<td>Production of sialidase and other glycosides by <em>Gardnerella</em> spp., which can potentially alter mucosal integrity and facilitate infection with genital pathogens</td>
<td>Detrimental changes to the mucosal barrier</td>
<td>(Wiesenfeld <em>et al.</em> 2003)</td>
</tr>
<tr>
<td><em>Gardnerella</em> spp., other vaginal pathogens and HSV-2</td>
<td>Common bacteria found in vaginal dysbiosis are associated with increased acquisition of HSV</td>
<td>BV-associated bacteria propagate viral replication and vaginal shedding of HSV, thereby further enhancing spread of this STI</td>
<td>Viral replication and vaginal shedding of HSV</td>
<td>(Cherpes <em>et al.</em> 2005)</td>
</tr>
<tr>
<td><em>Gardnerella</em> spp., other vaginal pathogens and HPV</td>
<td><em>Gardnerella</em>, other BV-associated bacteria which produce mucin-degrading enzymes, and HPV</td>
<td>Mucin-degrading enzymes present in <em>Gardnerella</em> spp. might degrade the gel layer coating the cervical epithelium, causing micro- abrasions or alterations of epithelial cells</td>
<td>Detrimental changes to the mucosal barrier</td>
<td>(Gillet <em>et al.</em> 2011)</td>
</tr>
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</table>

**Table 4.** Bacterial interactions occurring between *Gardnerella* spp. and other STIs-associated microbes.
| **Gardnerella spp. and Trichomonas vaginalis** | Cooperative interactions between *Gardnerella* spp., bacteria belonging to CST-IV, and *T. vaginalis* | *Gardnerella* spp. induced higher chemokine responses (namely to IL-8 and RANTES) and amplified the pro-inflammatory responses to both Lipophosphoglycan/ceramide-phosphoinositol-glycan core | Inflammatory damage accompanied by recruitment of CD4 cells; and weakened antiviral barrier | (Fichorova *et al.* 2013) |
| **Gardnerella spp. and HIV** | *Gardnerella* spp. and other common bacteria found in vaginal dysbiosis are associated with increased acquisition of HIV | APCs use Toll-like receptor-4 signalling to respond to LPS, which activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), | Genital inflammation and recruitment of lymphocytes by chemokine production | (Anahtar *et al.* 2016) |
| **Gardnerella spp., other vaginal pathogens and HIV** | *Gardnerella* spp. and other common bacteria found in vaginal dysbiosis are associated with increased acquisition of HIV | Mucus and cytoskeleton alterations, increasing lactate dehydrogenase A/B as markers of cell death, increasing proteolytic activity, altered antimicrobial peptide balance, increasing proinflammatory cytokines, and decreasing immunoglobulins | Cervicovaginal inflammation and other detrimental changes to the mucosal barrier | (Borgdorff *et al.* 2016) |
| **Gardnerella spp. and HIV** | *Gardnerella* spp. and other anaerobes are associated with increased acquisition of HIV | Higher activation of CD4+ HIV target cells | Increase HIV risk acquisition by inducing mucosal HIV target cells | (Gosmann *et al.* 2017) |
| **Gardnerella spp., other vaginal pathogens, HIV and HSV-2** | High-diversity CSTs, *Gardnerella* spp., and *P. bivia* were strongly associated with cervicovaginal inflammatory cytokines | Genital inflammation is a key determinant of HIV transmission and may increase HIV-susceptible target cells and alter epithelial integrity | Genital microbiota and HSV-2 infection may influence HIV susceptibility through independent biological mechanisms | (Shannon *et al.* 2017) |
Gardnerella spp. and Chlamydia trachomatis

- Gardnerella spp. infections may act as a chlamydial reservoir contributing to the transmission of C. trachomatis in the population
- Incorporation of C. trachomatis on a Gardnerella spp. biofilm
- Typical chlamydial inclusions observed in HeLa cells monolayers

Gardnerella spp., other CST-IV bacteria, and Trichomonas vaginalis

- Cooperative interactions between Gardnerella spp., bacteria belonging to CST-IV and T. vaginalis
- Enhancement of the paracellular permeability of the cervicovaginal epithelium by disturbing the integrity of the tight junction complex
- Damage on cervicovaginal epithelium

<table>
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<tr>
<th>Table 5. Common and proposed alternative or preventive treatment strategies used against vaginal infections.</th>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>BACTERIAL VAGINOSIS</td>
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<td><strong>Recommended antibiotics</strong></td>
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<td><strong>Proposed alternative approaches</strong></td>
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<td><strong>VULVOVAGINAL CANDIDIASIS</strong></td>
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<tr>
<td><strong>Proposed alternative approaches</strong></td>
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<td><strong>TRICHOMONIASIS</strong></td>
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<td><strong>Proposed preventive therapy</strong></td>
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<td><strong>CHLAMYDIA</strong></td>
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<td><strong>Proposed preventive therapy</strong></td>
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<td><strong>GONORRHEA</strong></td>
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<td><strong>Proposed preventive therapy</strong></td>
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<td><strong>VIRAL VAGINITIS</strong></td>
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