Gardnerella and vaginal health: the truth is out there

Aliona S. Rosca[a], Joana Castro[a], Lúcia G. V. Sousa and Nuno Cerca[#]

Centre of Biological Engineering (CEB), Laboratory of Research in Biofilms Rosário Oliveira (LIBRO), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Running Title: Interactions within the vaginal microbiota

Keywords: bacterial vaginosis; STIs; *Gardnerella* spp.; vaginal biofilms; microbial interactions; antimicrobial tolerance

[#] **Corresponding author details**: Nuno Cerca. Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Tel: +351-253-60443; Fax: +351-253-678-986;
E-mail address: nunocerca@ceb.uminho.pt

[a] A.R. and J.C. contributed equally to this work.

Total Word Count: 10702 (from abstract to acknowledgements)

TABLE OF CONTENTS

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 Wijgert 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 Marrazzo 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 *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 *Gardnerella* spp. was able to adhere to epithelial cells and displace pre-coated *L. crispatus*, while other BV-associated species, including *A. vaginae*, *M. mulieris*, *F. nucleatum*, and *P. bivia* were outcompeted by the protective lactobacilli (Machado *et al.* 2013). A subsequent study confirmed that *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 *Gardnerella* spp. alone is able to trigger BV or whether *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 oncogenes 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. vagina*e 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 (Algburi, Volski and Chikindas 2015). Afterwards, Thellin and colleagues

demonstrated that concentrations of 600 µgmL^{-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

This work was supported by the Portuguese Foundation for Science and Technology. AR and NC are funded by the individual Grants [PD/BD/128037/2016] and the strategic funding of unit [UID/BIO/04469/2019], respectively. JC and LS are funded by the research project [PTDC/BIA-MIC/28271/2017], under the scope of COMPETE 2020 [POCI-01-0145-FEDER-028271].

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of the Portuguese Foundation for Science and Technology.

Conflict of interest: none declared.

REFERENCES

Africa CW. Efficacy of methods used for the diagnosis of bacterial vaginosis. *Expert Opin Med Diagn* 2013;**7**:189–200.

Aggarwal A, Shier RM. Recalcitrant *Trichomonas vaginalis* infections successfully treated with vaginal acidification. *J Obstet Gynaecol Can* 2008;**30**:55–8.

Agostini A, Beerli M, Franchi F *et al. Garnerella vaginalis* bacteremia after vaginal myomectomy. *Eur J Obstet Gynecol Reprod Biol* 2003;**108**:229.

Algburi A, Volski A, Chikindas ML. Natural antimicrobials subtilosin and lauramide arginine ethyl ester (LAE) synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli. *Pathog Dis* 2015;**73**:ftv018.

Algburi A, Zehm S, Netrebov V *et al.* Benzoyl peroxide inhibits quorum sensing and biofilm formation by *Gardnerella vaginalis* 14018. *Infect Dis Obstet Gynecol* 2018;**2018**:1426109.

Algburi A, Zhang Y, Weeks R *et al.* Gemini cationic amphiphiles control biofilm formation by bacterial vaginosis pathogens. *Antimicrob Agents Chemother* 2017;**61**:e00650-17.

Allsworth JE, Lewis VA, Peipert JF. Viral sexually transmitted infections and bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. *Sex Transm Dis* 2008;**35**:791–6.

Allsworth JE, Peipert JF. Severity of bacterial vaginosis and the risk of sexually transmitted infection. *Am J Obstet Gynecol* 2011;**205**:113.e1-113.e6.

Alves P, Castro J, Sousa C *et al. Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *J Infect Dis* 2014;**210**:593–6.

Amabebe E, Anumba DOC. The vaginal microenvironment: the physiologic role of lactobacilli. *Front Med* 2018a;**5**:1–11.

Amabebe E, Anumba DOC. Psychosocial stress, cortisol levels, and maintenance of vaginal health. *Front Endocrinol (Lausanne)* 2018b;**9**:568.

Amaya RA, Al-Dossary F, Demmler GJ. *Gardnerella vaginalis* bacteremia in a premature neonate. *J Perinatol* 2002;**22**:585–7.

Amjadi F, Salehi E, Mehdizadeh M *et al.* Role of the innate immunity in female reproductive tract. *Adv Biomed Res* 2014;**3**:1.

Amsel R, Totten PA, Spiegel CA *et al.* Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am J Med* 1983;**74**:14–22.

Anahtar MN, Byrne EH, Doherty KE *et al.* Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 2016;**42**:965–76.

Anderson DJ, Marathe J, Pudney J. The structure of the human vaginal stratum corneum and its role in immune defense. *Am J Reprod Immunol* 2014;**71**:618–23.

Anukam K, Osazuwa E, Ahonkhai I *et al.* Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial. *Microbes Infect* 2006;**8**:1450–4.

Anukam KC, Reid G. Effects of metronidazole on growth of *Gardnerella vaginalis* ATCC 14018, probiotic *Lactobacillus rhamnosus* GR-1 and vaginal isolate *Lactobacillus plantarum* KCA. *Microb Ecol Health Dis* 2008;**20**:48–52.

Aroutcheva AA, Simoes JA, Faro S. Antimicrobial protein produced by vaginal *Lactobacillus acidophilus* that inhibits *Gardnerella vaginalis*. *Infect Dis Obstet Gynecol* 2001;**9**:33–9.

Atashili J, Poole C, Ndumbe PM *et al.* Bacterial vaginosis and HIV acquisition: a meta-

analysis of published studies. *AIDS* 2008;**22**:1493–501.

Atassi F, Brassart D, Grob P *et al. In vitro* antibacterial activity of *Lactobacillus helveticus* strain KS300 against diarrhoeagenic, uropathogenic and vaginosis-associated bacteria. *J Appl Microbiol* 2006a;**101**:647–54.

Atassi F, Brassart D, Grob P *et al. Lactobacillus* strains isolated from the vaginal microbiota of healthy women inhibit *Prevotella bivia* and *Gardnerella vaginalis* in coculture and cell culture. *FEMS Immunol Med Microbiol* 2006b;**48**:424–32.

Atassi F, Servin AL. Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain *Lactobacillus johnsonii* NCC933 and vaginal strain *Lactobacillus gasseri* KS120.1 against enteric, uropathogenic and vaginosis-associated pathog. *FEMS Microbiol Lett* 2010;**304**:29–38.

Austin MN, Rabe LK, Srinivasan S *et al. Mageeibacillus indolicus* gen. nov., sp. nov.: a novel bacterium isolated from the female genital tract. *Anaerobe* 2015;**32**:37–42.

De Backer E, Dubreuil L, Brauman M *et al. In vitro* activity of secnidazole against *Atopobium vaginae*, an anaerobic pathogen involved in bacterial vaginosis. *Clin Microbiol Infect* 2010;**16**:470–2.

De Backer E, Verhelst R, Verstraelen H *et al.* Antibiotic susceptibility of *Atopobium vaginae*. *BMC Infect Dis* 2006;**6**:51.

Backus KV, Muzny CA, Beauchamps LS. *Trichomonas vaginalis* treated with boric acid in a metronidazole allergic female. *Sex Transm Dis* 2017;**44**:120.

Bagnall P, Rizzolo D. Bacterial vaginosis: a practical review. *J Am Acad Physician Assist* 2017;**30**:15–21.

Bahar H, Torun MM, Öçer F *et al. Mobiluncus* species in gynaecological and obstetric infections: antimicrobial resistance and prevalence in a Turkish population. *Int J Antimicrob Agents* 2005;**25**:268–71.

Balkus JE, Richardson BA, Rabe LK *et al.* Bacterial vaginosis and the risk of *Trichomonas vaginalis* acquisition among HIV-1–negative women. *Sex Transm Dis* 2014;**41**:123–8.

Bamias G, Arseneau KO, Cominelli F. Cytokines and mucosal immunity. *Curr Opin Gastroenterol* 2014;**30**:547–52.

Bautista CT, Wurapa EK, Sateren WB *et al.* Association of bacterial vaginosis with chlamydia and gonorrhea among women in the U.S. Army. *Am J Prev Med* 2017;**52**:632–9.

Berardi-Grassias L, Roy O, Berardi JC *et al.* Neonatal meningitis due to *Gardnerella*

vaginalis. *Eur J Clin Microbiol Infect Dis* 1988;**7**:406–7.

Bertuccini L, Russo R, Iosi F *et al.* Effects of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* on bacterial vaginal pathogens. *Int J Immunopathol Pharmacol* 2017;**30**:163–7.

Bilardi J, Walker S, McNair R *et al.* Women's management of recurrent bacterial vaginosis and experiences of clinical care: a qualitative study. *PLoS One* 2016;**11**:e0151794.

Bodean O, Munteanu O, Cirstoiu C *et al.* Probiotics - a helpful additional therapy for bacterial vaginosis. *J Med Life* 2013;**6**:434–6.

Boisvert AA, Cheng MP, Sheppard DC *et al.* Microbial biofilms in pulmonary and critical care diseases. *Ann Am Thorac Soc* 2016;**13**:1615–23.

Borgdorff H, Gautam R, Armstrong SD *et al.* Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. *Mucosal Immunol* 2016;**9**:621–33.

Borgdorff H, Van Der Veer C, Van Houdt R *et al.* The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. *PLoS One* 2017;**12**:1–17.

Boselli F, Petrella E, Campedelli A *et al.* Efficacy and tolerability of fitostimoline (vaginal cream, ovules, and vaginal washing) and of benzydamine hydrochloride (tantum rosa vaginal cream and vaginal washing) in the topical treatment of symptoms of bacterial vaginosis. *ISRN Obstet Gynecol* 2012;**2012**:183403.

Boskey ER, Telsch KM, Whaley KJ *et al.* Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. *Infect Immun* 1999;**67**:5170–5.

Bradshaw CS, Morton AN, Hocking J *et al.* High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* 2006a;**193**:1478–86.

Bradshaw CS, Sobel JD. Current treatment of bacterial vaginosis-limitations and need for innovation. *J Infect Dis* 2016;**214 Suppl 1**:S14-20.

Bradshaw CS, Tabrizi SN, Fairley CK *et al.* The association of *Atopobium vaginae* and *Gardnerella vaginalis* with bacterial vaginosis and recurrence after oral metronidazole therapy. *J Infect Dis* 2006b;**194**:828–36.

Bradshaw CS, Walker SM, Vodstrcil LA *et al.* The influence of behaviors and relationships on the vaginal microbiota of women and their female partners: the

WOW health study. *J Infect Dis* 2014;**209**:1562–72.

Braga CP, Dal Sasso M, Culici M *et al.* Inhibitory activity of thymol on native and mature *Gardnerella vaginalis* biofilms: *in vitro* study. *Arzneimittelforschung* 2011;**60**:675–81.

Breshears LM, Edwards VL, Ravel J *et al. Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol* 2015;**15**:276.

Brotman RM, Bradford LL, Conrad M *et al.* Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women. *Sex Transm Dis* 2012;**39**:807–12.

Brotman RM, Shardell MD, Gajer P *et al.* Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection. *J Infect Dis* 2014;**210**:1723–33.

De Brucker K, Tan Y, Vints K *et al.* Fungal β-1,3-glucan increases ofloxacin tolerance of *Escherichia coli* in a polymicrobial *E. coli*/*Candida albicans* biofilm. *Antimicrob Agents Chemother* 2015;**59**:3052–8.

Buggio L, Somigliana E, Borghi A *et al.* Probiotics and vaginal microecology: fact or fancy? *BMC Womens Health* 2019;**19**:1–6.

Bump RC, Buesching WJ. Bacterial vaginosis in virginal and sexually active adolescent females: evidence against exclusive sexual transmission. *Am J Obstet Gynecol* 1988;**158**:935–9.

Burgess SP, Daramola O, Lacey S. *Gardnerella vaginalis* causing tubo-ovarian abscess. *J Obstet Gynaecol* 1997;**17**:90–1.

Caliendo AM, George KS, Allega J *et al.* Distinguishing cytomegalovirus (CMV) infection and disease with CMV nucleic acid assays. *J Clin Microbiol* 2002;**40**:1581–6.

Calvert LD, Collins M, Bateman JRM. Multiple abscesses caused by *Gardnerella vaginalis* in an immunocompetent man. *J Infect* 2005;**51**:E27-9.

Cardone A, Zarcone R, Borrelli A *et al.* Utilisation of hydrogen peroxide in the treatment of recurrent bacterial vaginosis. *Minerva Ginecol* 2003;**55**:483–92.

Casadevall A, Kontoyiannis DP, Robert V. On the emergence of *Candida auris*: climate change, azoles, swamps, and birds. *MBio* 2019;**10**:7e01397-19.

Castro J, Alves P, Sousa C *et al.* Using an *in-vitro* biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis *Gardnerella vaginalis*

isolates. *Sci Rep* 2015;**5**:11640.

Castro J, Cerca N. BV and non-BV associated *Gardnerella vaginalis* establish similar synergistic interactions with other BV-associated microorganisms in dual-species biofilms. *Anaerobe* 2015;**36**:56–9.

Castro J, França A, Bradwell KR *et al.* Comparative transcriptomic analysis of *Gardnerella vaginalis* biofilms vs. planktonic cultures using RNA-seq. *NPJ Biofilms Microbiomes* 2017;**3**:3.

Castro J, Henriques A, Machado A *et al.* Reciprocal interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on initial adherence to epithelial cells. *Int J Med Sci* 2013;**10**:1193–8.

Castro J, Jefferson K, Cerca N. Innate immune components affect growth and virulence traits of bacterial vaginosis-associated and non-BV associated *Gardnerella vaginalis* strains similarly. *Pathog Dis* 2018;**76**:fty089.

Castro J, Machado D, Cerca N. *Escherichia coli* and *Enterococcus faecalis* are able to incorporate and enhance a pre-formed *Gardnerella vaginalis* biofilm. *Pathog Dis* 2016;**74**:ftw007.

Castro J, Machado D, Cerca N. Unveiling the role of *Gardnerella vaginalis* in polymicrobial bacterial vaginosis biofilms: the impact of other vaginal pathogens living as neighbors. *ISME J* 2019;**13**:1306–17.

Catlin W. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clin Microbiol Rev* 1992;**5**:213–37.

Centers for Disease Control and Prevention. STDs during pregnancy - CDC fact sheet. *Natl Cent HIV/AIDS, Viral Hepatitis, STD, TB Prev* 2016:1–2.

Cerca N, Vaneechoutte M, Guschin A *et al.* Polymicrobial infections and biofilms in women's health Gahro Expert Group Meeting Report 2017. *Res Microbiol* 2017;**168**:902–4.

Chen KCS, Forsyth PS, Buchanan TM *et al.* Amine content of vaginal fluid from untreated and treated patients with nonspecific vaginitis. *J Clin Invest* 1979;**63**:828–35.

Cherpes TL, Hillier SL, Meyn LA *et al.* A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sex Transm Dis* 2008;**35**:78–83.

Cherpes TL, Melan MA, Kant JA *et al.* Genital tract shedding of herpes simplex virus

type 2 in women: effects of hormonal contraception, bacterial vaginosis, and vaginal group B Streptococcus colonization. *Clin Infect Dis* 2005;**40**:1422–8.

Coleman JS, Gaydos CA. Molecular diagnosis of bacterial vaginosis: an update. *J Clin Microbiol* 2018;**56**:e00342-18.

Cone RA. Vaginal microbiota and sexually transmitted infections that may influence transmission of Cell-Associated HIV. *J Infect Dis* 2014;**210**:S616–21.

Cook RL, Reid G, Pond DG *et al.* Clue cells in bacterial vaginosis: immunofluorescent identification of the adherent gram-negative bacteria as *Gardnerella vaginalis*. *J Infect Dis* 1989;**160**:490–6.

Cooper J. Disorders are different from diseases. *World Psychiatry* 2004;**3**:24.

Coudeyras S, Jugie G, Vermerie M *et al.* Adhesion of human probiotic *Lactobacillus rhamnosus* to cervical and vaginal cells and interaction with vaginosis-associated pathogens. *Infect Dis Obstet Gynecol* 2008;**2008**:549640.

Cox C, Watt AP, McKenna JP *et al. Mycoplasma hominis* and *Gardnerella vaginalis* display a significant synergistic relationship in bacterial vaginosis. *Eur J Clin Microbiol Infect Dis* 2016;**35**:481–7.

Criswell BS, Ladwig CL, Gardner HL *et al. Haemophilus vaginalis*: vaginitis by inoculation from culture. *Obstet Gynecol* 1969;**33**:195–9.

Dan M, Yampolsky E, Poch F. Serum concentrations and *ex vivo* inhibitory/bactericidal activity of clindamycin after administration of two oral dosages. *Chemotherapy* 1997;**43**:227–31.

Danielsson D, Teigen PK, Moi H. The genital econiche: focus on microbiota and bacterial vaginosis. *Ann N Y Acad Sci* 2011;**1230**:48–58.

Datcu R, Gesink D, Mulvad G *et al.* Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR. *BMC Infect Dis* 2013;**13**:480.

Denning DW, Kneale M, Sobel JD *et al.* Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* 2018;**18**:e339–47.

Dingens AS, Fairfortune TS, Reed S *et al.* Bacterial vaginosis and adverse outcomes among full-term infants: a cohort study. *BMC Pregnancy Childbirth* 2016;**16**:278.

Döderlein A. *Das Scheidensekret Und Seine Bedeutung Für Das Puerperalfieber*. 1892.

Donders GG, Bosmans E, Dekeersmaecker A *et al.* Pathogenesis of abnormal vaginal bacterial flora. *Am J Obstet Gynecol* 2000;**182**:872–8.

Donders GGG, Bellen G, Grinceviciene S *et al.* Aerobic vaginitis: no longer a stranger. *Res Microbiol* 2017;**168**:845–58.

Donders GGG, Vereecken A, Bosmans E *et al.* Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. *BJOG* 2002;**109**:34–43.

Doss M, White MR, Tecle T *et al.* Human defensins and LL-37 in mucosal immunity. *J Leukoc Biol* 2010;**87**:79–92.

Dover SE, Aroutcheva AA, Faro S *et al.* Natural antimicrobials and their role in vaginal health: a short review. *Int J Probiotics Prebiotics* 2008;**3**:219–30.

Drell T, Lillsaar T, Tummeleht L *et al.* Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. *PLoS One* 2013;**8**:e54379.

Edwards JL, Jennings MP, Seib KL. *Neisseria gonorrhoeae* vaccine development: hope on the horizon? *Curr Opin Infect Dis* 2018;**31**:246–50.

Edwards T, Burke P, Smalley H *et al. Trichomonas vaginalis*: clinical relevance, pathogenicity and diagnosis. *Crit Rev Microbiol* 2016;**42**:406–17.

Edwards VL, Smith SB, McComb EJ *et al.* The cervicovaginal microbiota-host interaction modulates *Chlamydia trachomatis* infection. *MBio* 2019;**10**:e01548-19.

Esber A, Vicetti Miguel RD, Cherpes TL *et al.* Risk of bacterial vaginosis among women with herpes simplex virus type 2 infection: a systematic review and meta-analysis. *J Infect Dis* 2015;**212**:8–17.

Eschenbach DA. History and review of bacterial vaginosis. *Am J Obstet Gynecol* 1993;**169**:441–5.

Eschenbach DA, Hillier S, Critchlow CM *et al.* Diagnosis and clinical manifestations of bacterial vaginosis. *Am J Obstet Gynecol* 1988;**158**:819–28.

Falconi-McCahill A. Bacterial vaginosis: a clinical update with a focus on complementary and alternative therapies. *J Midwifery Womens Health* 2019, DOI: 10.1111/jmwh.13013.

Farage MA, Miller KW, Gerberick GF *et al.* Innate immunity in the lower female mucosal tract. *J Steroids Horm Sci* 2011;**2**:106.

Fazeli A, Bruce C, Anumba DO. Characterization of Toll-like receptors in the female reproductive tract in humans. *Hum Reprod* 2005;**20**:1372–8.

Ferris MJ, Masztal A, Aldridge KE *et al.* Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infect Dis* 2004;**4**:5.

Ferris MJ, Norori J, Zozaya-hinchliffe M *et al.* Cultivation-independent analysis of

changes in bacterial vaginosis flora following metronidazole treatment. *J Clin Microbiol* 2007;**45**:1016–8.

Fethers KA, Fairley CK, Hocking JS *et al.* Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clin Infect Dis* 2008;**47**:1426–35.

Fethers KA, Fairley CK, Morton A *et al.* Early sexual experiences and risk factors for bacterial vaginosis. *J Infect Dis* 2009;**200**:1662–70.

Fichorova RN, Buck OR, Yamamoto HS *et al.* The villain team-up or how *Trichomonas vaginalis* and bacterial vaginosis alter innate immunity in concert. *Sex Transm Infect* 2013;**89**:460–6.

Filardo S, Di Pietro M, Tranquilli G *et al.* Biofilm in genital ecosystem: a potential risk factor for *Chlamydia trachomatis* infection. *Can J Infect Dis Med Microbiol* 2019;**2019**:1672109.

Flemming HC, Wingender J, Szewzyk U *et al.* Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 2016;**14**:563–75.

Flórez C, Muchada B, Nogales MC *et al.* Bacteremia due to *Gardnerella vaginalis*: report of two cases. *Clin Infect Dis* 1994;**18**:125.

Forney LJ, Foster JA, Ledger W. The vaginal flora of healthy women is not always dominated by *Lactobacillus* species. *J Infect Dis* 2006;**194**:1468–9.

Forsum U, Holst E, Larsson PG *et al.* Bacterial vaginosis - a microbiological and immunological enigma. *APMIS* 2005;**113**:81–90.

Fosch SE, Ficoseco CA, Marchesi A *et al.* Contraception: influence on vaginal microbiota and identification of vaginal lactobacilli using MALDI-TOF MS and 16S rDNA sequencing. *Open Microbiol J* 2018;**12**:218–29.

Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med* 2005;**353**:1899–911.

Fredricks DN, Fiedler TL, Thomas KK *et al.* Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol* 2007;**45**:3270–6.

Frobenius W, Bogdan C. Diagnostic value of vaginal discharge, wet mount and vaginal pH - an update on the basics of gynecologic infectiology. *Geburtshilfe Frauenheilkd* 2015;**75**:355–66.

Gajer P, Brotman RM, Bai G *et al.* Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;**4**:132ra52.

Gallo MF, Macaluso M, Warner L *et al.* Bacterial vaginosis, gonorrhea, and chlamydial infection among women attending a sexually transmitted disease clinic: a

longitudinal analysis of possible causal links. *Ann Epidemiol* 2012;**22**:213–20.

Gardner HL, Dukes CD. *Haemophilus vaginalis* vaginitis. *Am J Obstet Gynecol* 1955;**69**:962–76.

Gibbs RS. Asymptomatic bacterial vaginosis: is it time to treat? *Am J Obstet Gynecol* 2007;**196**:495–6.

Gilbert NM, Lewis WG, Li G *et al.* *Gardnerella vaginalis* and *Prevotella bivia* trigger distinct and overlapping phenotypes in a mouse model of bacterial vaginosis. *J Infect Dis* 2019;**220**:1099-1108.

Gillet E, Meys JF, Verstraelen H *et al.* Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC Infect Dis* 2011;**11**:10.

Godha K, Tucker KM, Biehl C *et al.* Human vaginal pH and microbiota: an update. *Gynecol Endocrinol* 2018;**34**:451–5.

González-Bello C. Antibiotic adjuvants – a strategy to unlock bacterial resistance to antibiotics. *Bioorg Med Chem Lett* 2017;**27**:4221–8.

Gosmann C, Anahtar MN, Handley SA *et al.* *Lactobacillus*-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in young South African women. *Immunity* 2017;**46**:29–37.

Gottschick C, Szafranski SP, Kunze B *et al.* Screening of compounds against *Gardnerella vaginalis* biofilms. *PLoS One* 2016;**11**:e0154086.

Graham S, Howes C, Dunsmuir R *et al.* Vertebral osteomyelitis and discitis due to *Gardnerella vaginalis*. *J Med Microbiol* 2009;**58**:1382–4.

Grenier Capoci IR, Souza Bonfim-Mendonça P, Arita GS *et al.* Propolis is an efficient fungicide and inhibitor of biofilm production by vaginal *Candida albicans*. *Evidence-Based Complement Altern Med* 2015;**2015**:287693.

Haddad LB, Wall KM, Kilembe W *et al.* Bacterial vaginosis modifies the association between hormonal contraception and HIV acquisition. *AIDS* 2018;**32**:595–604.

Hafner LM, Timms P. Development of a *Chlamydia trachomatis* vaccine for urogenital infections: novel tools and new strategies point to bright future prospects. *Expert Rev Vaccines* 2018;**17**:57–69.

Hardy L, Cerca N, Jespers V *et al.* Bacterial biofilms in the vagina. *Res Microbiol* 2017;**168**:865–74.

Hardy L, Jespers V, Abdellati S *et al.* A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated

biofilm. *Sex Transm Infect* 2016;**92**:487–91.

Hardy L, Jespers V, Dahchour N *et al.* Unravelling the bacterial vaginosis-associated biofilm: a multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence *in situ* hybridization assay using peptide nucleic acid probes. *PLoS One* 2015;**10**:e0136658.

Harmanli OH, Cheng GY, Nyirjesy P *et al.* Urinary tract infections in women with bacterial vaginosis. *Obs Gynecol* 2000;**95**:710–2.

Harriott MM, Noverr MC. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. *Antimicrob Agents Chemother* 2009;**53**:3914–22.

Hauser AR, Mecsas J, Moir DT. Beyond antibiotics: new therapeutic approaches for bacterial infections. *Clin Infect Dis* 2016;**63**:89–95.

Hay P. Life in the littoral zone: lactobacilli losing the plot. *Sex Transm Infect* 2005;**81**:100–2.

Heczko PB, Tomusiak A, Adamski P *et al.* Supplementation of standard antibiotic therapy with oral probiotics for bacterial vaginosis and aerobic vaginitis: a randomised, double-blind, placebo-controlled trial. *BMC Womens Health* 2015;**15**:115.

Hickey DK, Patel M V, Fahey J V *et al.* Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J Reprod Immunol* 2011;**88**:185–94.

Hilbert DW, Smith WL, Chadwick SG *et al.* Development and validation of a highly accurate quantitative real-time PCR assay for diagnosis of bacterial vaginosis. *J Clin Microbiol* 2016;**54**:1017–24.

Hill GB. The microbiology of bacterial vaginosis. *Am J Obstet Gynecol* 1993;**169**:450–4.

Hill J, Albert AYK., VOGUE Research Group. Resolution and co-occurrence patterns of *Gardnerella leopoldii*, *Gardnerella swidsinskii*, *Gardnerella piotii* and *Gardnerella vaginalis* within the vaginal microbiome. *Infect Immun* 2019, DOI: 10.1128/IAI.00532-19.

Hillebrand L, Harmanli OH, Whiteman V *et al.* Urinary tract infections in pregnant women with bacterial vaginosis. *Am J Obstet Gynecol* 2002;**186**:916–7.

Hinderfeld AS, Phukan N, Bär A-K *et al.* Cooperative interactions between

Trichomonas vaginalis and associated bacteria enhance paracellular permeability of the cervicovaginal epithelium by dysregulating tight junctions. *Infect Immun* 2019;**87**:e00141-19.

Hoarau G, Bernard S, Pavese P *et al. Gardnerella vaginalis* as a rare cause of prosthetic joint infection. *J Clin Microbiol* 2012;**50**:4154–6.

Hodge TW, Levy CS, Smith MA. Disk space infection due to *Gardnerella vaginalis*. *Clin Infect Dis* 1995;**21**:443–5.

Holst E, Svensson L, Skarin A *et al.* Vaginal colonization with *Gardnerella vaginalis* and anaerobic curved rods. *Scand J Urol Nephrol Suppl* 1984;**86**:147–52.

Holzman C, Leventhal JM, Qiu H *et al.* Factors linked to bacterial vaginosis in nonpregnant women. *Am J Public Health* 2001;**91**:1664–70.

Homayouni A, Bastani P, Ziyadi S *et al.* Effects of probiotics on the recurrence of bacterial vaginosis: A review. *J Low Genit Tract Dis* 2014;**18**:79–86.

Hsu DC, O'Connell RJ. Progress in HIV vaccine development. *Hum Vaccines Immunother* 2017;**13**:1018–30.

Huang B, Fettweis JM, Brooks JP *et al.* The changing landscape of the vaginal microbiome. *Clin Lab Med* 2014;**34**:747–61.

Hymes SR, Randis TM, Sun TY *et al.* DNase inhibits *Gardnerella vaginalis* biofilms *in vitro* and *in vivo*. *J Infect Dis* 2013;**207**:1491–7.

Isik G, Demirezen Ş, Dönmez H *et al.* Bacterial vaginosis in association with spontaneous abortion and recurrent pregnancy losses. *J Cytol* 2016;**33**:135–40.

Jakobsson T, Forsum U. *Lactobacillus iners*: a marker of changes in the vaginal flora? *J Clin Microbiol* 2007;**45**:3145.

Jerse AE, Deal CD. Vaccine research for gonococcal infections: where are we? *Sex Transm Infect* 2013;**89**:63–9.

Johnson AP, Boustouller YL. Extra-vaginal infection caused by *Gardnerella vaginalis*. *Epidemiol Infect* 1987;**98**:131–7.

Johnston C, Gottlieb SL, Wald A. Status of vaccine research and development of vaccines for herpes simplex virus. *Vaccine* 2016;**34**:2948–52.

Jones BM, Geary I, Alawattegama AB *et al. In-vitro* and *in-vivo* activity of metronidazole against *Gardnerella vaginalis*, *Bacteroides* spp. and *Mobiluncus* spp. in bacterial vaginosis. *J Antimicrob Chemother* 1985;**16**:189–97.

Josey WE, Schwebke JR. The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. *Int J STD AIDS* 2008;**19**:152–4.

Jung H-S, Ehlers MM, Lombaard H *et al.* Etiology of bacterial vaginosis and polymicrobial biofilm formation. *Crit Rev Microbiol* 2017;**43**:651–67.

Kaul R, Prodger J, Joag V *et al.* Inflammation and HIV transmission in Sub-Saharan Africa. *Curr HIV/AIDS Rep* 2015;**12**:216–22.

Kenyon C, Colebunders R, Crucitti T. The global epidemiology of bacterial vaginosis: a systematic review. *Am J Obstet Gynecol* 2013;**209**:505–23.

Khan S, Voordouw MJ, Hill JE. Competition among *Gardnerella* subgroups from the human vaginal microbiome. *Front Cell Infect Microbiol* 2019, DOI: 10.3389/fcimb.2019.00374.

King CC, Jamieson DJ, Wiener J *et al.* Bacterial vaginosis and the natural history of human papillomavirus. *Infect Dis Obstet Gynecol* 2011;**2011**:319460.

Kinghorn GR, Jones BM, Chowdhury FH *et al.* Balanoposthitis associated with *Gardnerella vaginalis* infection in men. *Sex Transm Infect* 1982;**58**:127–9.

Kline KA, Lewis AL. Gram-positive uropathogens, polymicrobial urinary tract infection, and the emerging microbiota of the urinary tract. *Microbiol Spectr* 2016;**4**:1–54.

Koch R. The etiology of anthrax, based on the life history of *Bacillus anthracis*. *Contrib to Biol plants* 1876;**2**:277–310.

Kolenbrander PE, Palmer RJ, Periasamy S *et al.* Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 2010;**8**:471–80.

Kondo S, Tabe Y, Yamada T *et al.* Comparison of antifungal activities of gentian violet and povidone-iodine against clinical isolates of *Candida* species and other yeasts: a framework to establish topical disinfectant activities. *Mycopathologia* 2012;**173**:21–5.

Kot B, Sytykiewicz H, Sprawka I. Expression of the biofilm-associated genes in methicillin-resistant *Staphylococcus aureus* in biofilm and planktonic conditions. *Int J Mol Sci* 2018;**19**:e3487.

Kovachev S. Defence factors of vaginal lactobacilli. *Crit Rev Microbiol* 2018;**44**:31–9.

Kremleva EA, Sgibnev A V. Proinflammatory cytokines as regulators of vaginal microbiota. *Bull Exp Biol Med* 2016;**162**:75–8.

Kriebel K, Hieke C, Müller-Hilke B *et al.* Oral biofilms from symbiotic to pathogenic interactions and associated disease - connection of periodontitis and rheumatic arthritis by peptidylarginine deiminase. *Front Microbiol* 2018;**9**:53.

Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int*

Rev Immunol 2011;**30**:16–34.

Lagacé-Wiens PRS, Ng B, Reimer A *et al. Gardnerella vaginalis* bacteremia in a previously healthy man: case report and characterization of the isolate. *J Clin Microbiol* 2008;**46**:804–6.

Lam MH, Birch DF, Fairley KF. Prevalence of *Gardnerella vaginalis* in the urinary tract. *J Clin Microbiol* 1988;**26**:1130–3.

Larsson P, Brandsborg E, Forsum U *et al.* Extended antimicrobial treatment of bacterial vaginosis combined with human lactobacilli to find the best treatment and minimize the risk of relapses. *BMC Infect Dis* 2011;**11**:223.

Larsson PG, Bergström M, Forsum U *et al.* Bacterial vaginosis. Transmission, role in genital tract infection and pregnancy outcome: an enigma. *APMIS* 2005;**113**:233–45.

Le KY, Park MD, Otto M. Immune evasion mechanisms of *Staphylococcus epidermidis* biofilm infection. *Front Microbiol* 2018;**9**:359.

Lebeaux D, Ghigo J-M, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol Rev* 2014;**78**:510–43.

Leccese Terraf MC, Juarez Tomás MS, Rault L *et al. In vitro* effect of vaginal lactobacilli on the growth and adhesion abilities of uropathogenic *Escherichia coli*. *Arch Microbiol* 2017;**199**:767–74.

Lee JE, Lee S, Lee H *et al.* Association of the vaginal microbiota with human papillomavirus infection in a Korean twin cohort. Medeiros R (ed.). *PLoS One* 2013;**8**:e63514.

Lee KWK, Periasamy S, Mukherjee M *et al.* Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. *ISME J* 2014;**8**:894–907.

Legrand JC, Alewaeters A, Leenaerts L *et al. Gardnerella vaginalis* bacteremia from pulmonary abscess in a male alcohol abuser. *J Clin Microbiol* 1989;**27**:1132–4.

Leppäluoto PA. Bacterial vaginosis: what is physiological in vaginal bacteriology? An update and opinion. *Acta Obstet Gynecol Scand* 2011;**90**:1302–6.

Li J, McCormick J, Bocking A *et al.* Importance of vaginal microbes in reproductive health. *Reprod Sci* 2012;**19**:235–42.

Liang TJ. Hepatitis B: the virus and disease. *Hepatology* 2009;**49**:S13–21.

Lin L, Song J, Kimber N *et al.* The role of bacterial vaginosis in infection after major gynecologic surgery. *Infect Dis Obstet Gynecol* 1999;**7**:169–74.

Liu CM, Hungate BA, Tobian AAR *et al.* Penile microbiota and female partner bacterial vaginosis in Rakai, Uganda. *MBio* 2015;**6**:17–20.

Livengood CH. Bacterial vaginosis: an overview for 2009. *Rev Obstet Gynecol* 2009;**2**:28–37.

Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. *Genome Med* 2016;**8**:51.

Lopes G, Cools P, Verstraelen H *et al.* Longitudinal study of the dynamics of vaginal microflora during two consecutive menstrual cycles. *PLoS One* 2011;**6**:e28180.

Lopes SP, Ceri H, Azevedo NF *et al.* Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection. *Int J Antimicrob Agents* 2012;**40**:260–3.

López D, Vlamakis H, Kolter R. Biofilms. *Cold Spring Harb Perspect Biol* 2010;**2**:a000398.

Luong M, Libman M, Dahhou M *et al.* Vaginal douching, bacterial vaginosis, and spontaneous preterm birth. *J Obstet Gynaecol Can* 2010;**32**:313–20.

Ma B, Forney LJ, Ravel J. The vaginal microbiome: rethinking health and diseases. *Annu Rev Microbiol* 2012;**66**:371–89.

Machado A, Castro J, Cereija T *et al.* Diagnosis of bacterial vaginosis by a new multiplex peptide nucleic acid fluorescence *in situ* hybridization method. *PeerJ* 2015;**3**:e780.

Machado A, Cerca N. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *J Infect Dis* 2015;**212**:1856–61.

Machado A, Jefferson KK, Cerca N. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 2013;**14**:12004–12.

Machado A, Salgueiro D, Harwich M *et al.* Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. *Anaerobe* 2013;**23**:1–4.

Machado D, Castro J, Palmeira-de-Oliveira A *et al.* Bacterial vaginosis biofilms: challenges to current therapies and emerging solutions. *Front Microbiol* 2016;**6**:1528.

Machado D, Gaspar C, Palmeira-de-Oliveira A *et al. Thymbra capitata* essential oil as potential therapeutic agent against *Gardnerella vaginalis* biofilm-related infections. *Future Microbiol* 2017;**12**:407–16.

Macklaim JM, Clemente JC, Knight R *et al.* Changes in vaginal microbiota following

antimicrobial and probiotic therapy. *Microb Ecol Health Dis* 2015;**26**:27799.

Macklaim JM, Fernandes AD, Di Bella JM *et al.* Comparative meta-RNA-seq of the vaginal microbiota and differential expression by *Lactobacillus iners* in health and dysbiosis. *Microbiome* 2013;**1**:12.

Macklaim JM, Gloor GB, Anukam KC *et al.* At the crossroads of vaginal health and disease, the genome sequence of *Lactobacillus iners* AB-1. *Proc Natl Acad Sci U S A* 2011;**108**:4688–95.

Manavathu EK, Vager DL, Vazquez JA. Development and antimicrobial susceptibility studies of *in vitro* monomicrobial and polymicrobial biofilm models with *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. *BMC Microbiol* 2014;**14**:53.

Marrazzo JM. Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused? *Anaerobe* 2011;**17**:186–90.

Marrazzo JM, Dombrowski JC, Wierzbicki MR *et al.* Safety and efficacy of a novel vaginal anti-infective, TOL-463, in the treatment of bacterial vaginosis and vulvovaginal candidiasis: a randomized, single-blind, phase 2, controlled trial. *Clin Infect Dis* 2019;**68**:803–9.

Marrazzo JM, Thomas KK, Agnew K *et al.* Prevalence and risks for bacterial vaginosis in women who have sex with women. *Sex Transm Dis* 2010a;**37**:335–9.

Marrazzo JM, Thomas KK, Fiedler TL *et al.* Risks for acquisition of bacterial vaginosis among women who report sex with women: a cohort study. *PLoS One* 2010b;**5**:e11139.

Martin DH. The microbiota of the vagina and its influence on women's health and disease. *Am J Med Sci* 2012;**343**:2–9.

Matu MN, Orinda GO, Njagi ENM *et al. In vitro* inhibitory activity of human vaginal lactobacilli against pathogenic bacteria associated with bacterial vaginosis in Kenyan women. *Anaerobe* 2010;**16**:210–5.

McCool RA, DeDonato DM. Bacteremia of *Gardnerella vaginalis* after endometrial ablation. *Arch Gynecol Obstet* 2012;**286**:1337–8.

McKinnon LR, Achilles SL, Bradshaw CS *et al.* The evolving facets of bacterial vaginosis: implications for HIV transmission. *AIDS Res Hum Retroviruses* 2019;**35**:219–28.

McLean NW, Rosenstein IJ. Characterisation and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. *J Med Microbiol* 2000;**49**:543–52.

McMillan A, Dell M, Zellar MP *et al.* Disruption of urogenital biofilms by lactobacilli. *Colloids Surf B Biointerfaces* 2011;**86**:58–64.

Mehriardestani M, Aliahmadi A, Toliat T *et al.* Medicinal plants and their isolated compounds showing anti-*Trichomonas vaginalis* - activity. *Biomed Pharmacother* 2017;**88**:885–93.

Menard JP. Antibacterial treatment of bacterial vaginosis: current and emerging therapies. *Int J Womens Health* 2011;**3**:295–305.

Mendes-Soares H, Suzuki H, Hickey RJ *et al.* Comparative functional genomics of *Lactobacillus* spp. reveals possible mechanisms for specialization of vaginal lactobacilli to their environment. *J Bacteriol* 2014;**196**:1458–70.

Merchant RM, Asch DA, Crutchley P *et al.* Evaluating the predictability of medical conditions from social media posts. *PLoS One* 2019;**14**:e0215476.

El Mezouar I, Abourazzak FE, Aradoini N *et al.* Reactive arthritis induced by *Gardnerella vaginalis*. *Egypt Rheumatol* 2014;**36**:151–2.

Miljkovic V, Arsic B, Bojanic Z *et al.* Interactions of metronidazole with other medicines: a brief review. *Pharmazie* 2014;**69**:571–7.

Mitchell C, Marrazzo J. Bacterial vaginosis and the cervicovaginal immune response. *Am J Reprod Immunol* 2014;**71**:555–63.

Moncla BJ, Chappell CA, Mahal LK *et al.* Impact of bacterial vaginosis, as assessed by Nugent criteria and hormonal status on glycosidases and lectin binding in cervicovaginal lavage samples. *PLoS One* 2015;**10**:e0127091.

Moons P, Michiels CW, Aertsen A. Bacterial interactions in biofilms. *Crit Rev Microbiol* 2009;**35**:157–68.

Morris MC, Rogers PA, Kinghorn GR. Is bacterial vaginosis a sexually transmitted infection? *Sex Transm Infect* 2001;**77**:63–8.

Mullish B, Williams HR. *Clostridium difficile* infection and antibiotic-associated diarrhoea. *Clin Med* 2018;**18**:237–41.

Murray L, Halpin J, Casserly B *et al.* A pyo-hydropneumothorax with sepsis, secondary to *Gardnerella vaginalis* infection in a post-partum female. *Respir Med Case Reports* 2019;**26**:189–92.

Muzny CA, Blanchard E, Taylor CM *et al.* Identification of key bacteria involved in the induction of incident bacterial vaginosis: a prospective study. *J Infect Dis* 2018;**218**:966–78.

Muzny CA, Lensing SY, Aaron KJ *et al.* Incubation period and risk factors support

sexual transmission of bacterial vaginosis in women who have sex with women. *Sex Transm Infect* 2019a, **95**:511-15.

Muzny CA, Schwebke JR. *Gardnerella vaginalis*: still a prime suspect in the pathogenesis of bacterial vaginosis. *Curr Infect Dis Rep* 2013;**15**:130–5.

Muzny CA, Schwebke JR. Pathogenesis of bacterial vaginosis: discussion of current hypotheses. *J Infect Dis* 2016;**214**:S1-5.

Muzny CA, Taylor CM, Swords WE *et al.* An updated conceptual model on the pathogenesis of bacterial vaginosis. *J Infect Dis* 2019b;**220**:1399-05

Nagy E, Petterson M, Mardh P. Antibiosis between bacteria isolated from the vagina of women with and without signs of bacterial vaginosis. *APMIS* 1991;**99**:739–44.

Nasu K, Narahara H. Pattern recognition via the Toll-like receptor system in the human female genital tract. *Mediators Inflamm* 2010;**2010**:1–12.

Nelson DB, Macones G. Bacterial vaginosis in pregnancy: current findings and future directions. *Epidemiol Rev* 2002;**24**:102–8.

Neri P, Salvolini S, Giovannini A *et al.* Retinal vasculitis associated with asymptomatic *Gardnerella vaginalis* infection: a new clinical entity. *Ocul Immunol Inflamm* 2009;**17**:36–40.

Ness RB, Kip KE, Hillier SL *et al.* A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *Am J Epidemiol* 2005;**162**:585–90.

Nguyen P V, Kafka JK, Ferreira VH *et al.* Innate and adaptive immune responses in male and female reproductive tracts in homeostasis and following HIV infection. *Cell Mol Immunol* 2014;**11**:410–27.

Nightingale LM, Eaton CB, Fruehan AE *et al.* Cephalhematoma complicated by osteomyelitis presumed due to *Gardnerella vaginalis*. *JAMA* 1986;**256**:1936–7.

Novakov Mikic A, Budakov D. Comparison of local metronidazole and a local antiseptic in the treatment of bacterial vaginosis. *Arch Gynecol Obstet* 2010;**282**:43–7.

Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 1991;**29**:297–301.

Nugeyre M-T, Tchitchek N, Adapen C *et al.* Dynamics of vaginal and rectal microbiota over several menstrual cycles in female cynomolgus macaques. *Front Cell Infect Microbiol* 2019;**9**:188.

Nygren P, Fu R, Freeman M *et al.* Evidence on the benefits and harms of screening and

treating pregnant women who are asymptomatic for bacterial vaginosis: an update review for the U.S. Preventive Services Task Force. *Ann Intern Med* 2008;**148**:220–33.

O'Hanlon DE, Moench TR, Cone RA. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BMC Infect Dis* 2011;**11**:200.

O'Hanlon DE, Moench TR, Cone RA. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One* 2013;**8**:e80074.

Olmsted SS, Meyn LA, Rohan LC *et al.* Glycosidase and proteinase activity of anaerobic Gram-negative bacteria isolated from women with bacterial vaginosis. *Sex Transm Dis* 2003;**30**:257–61.

Onderdonk AB, Delaney ML, Fichorova N. The human microbiome during bacterial vaginosis. *Clin Microbiol Rev* 2016;**29**:223–38.

Orfanelli T, Jayaram A, Doulaveris G *et al.* Human epididymis protein 4 and secretory leukocyte protease inhibitor in vaginal fluid: relation to vaginal components and bacterial composition. *Reprod Sci* 2014;**21**:538–42.

Oscáriz JC, Pisabarro AG. Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. *Int Microbiol* 2001;**4**:13–9.

Paavonen J, Mangioni C, Martin MA *et al.* Vaginal clindamycin and oral metronidazole for bacterial vaginosis: a randomized trial. *Obstet Gynecol* 2000;**96**:256–60.

Palmeira-de-Oliveira R, Palmeira-de-Oliveira A, Martinez-de-Oliveira J. New strategies for local treatment of vaginal infections. *Adv Drug Deliv Rev* 2015;**92**:105–22.

Papanikolaou EG, Tsanadis G, Dalkalitsis N *et al.* Recurrent bacterial vaginosis in a virgin adolescent: a new method of treatment. *Infection* 2002;**30**:403–4.

Patterson JL, Stull-Lane A, Girerd PH *et al.* Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 2010;**156**:392–9.

Patton DL, Thwin SS, Meier A *et al.* Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle. *Am J Obstet Gynecol* 2000;**183**:967–73.

Perez AC, Pang B, King LB *et al.* Residence of *Streptococcus pneumoniae* and *Moraxella catarrhalis* within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence *in vivo*. *Pathog Dis* 2014;**70**:280–8.

Van de Perre P, Segondy M, Foulongne V *et al.* Herpes simplex virus and HIV-1: deciphering viral synergy. *Lancet Infect Dis* 2008;**8**:490–7.

Petersen EE, Genet M, Caserini M *et al.* Efficacy of vitamin C vaginal tablets in the treatment of bacterial vaginosis: a randomised, double blind, placebo controlled clinical trial. *Arzneimittelforschung* 2011;**61**:260–5.

Petrosky E, Bocchini JA, Hariri S *et al.* Use of 9-valent human papillomavirus (HPV) vaccine: updated HPV vaccination recommendations of the advisory committee on immunization practices. *MMWR Morb Mortal Wkly Rep* 2015;**64**:300–4.

Pirotta M V, Gunn JM, Chondros P. Not thrush again! Women's experience of post-antibiotic vulvovaginitis. *Med J Aust* 2003;**179**:43–6.

Pointer BR, Boyer MP, Schmidt M. Boric acid destabilizes the hyphal cytoskeleton and inhibits invasive growth of *Candida albicans*. 2015;**32**:389–98.

Van Der Pol B, Daniel G, Kodsi S *et al.* Molecular-based testing for sexually transmitted infections using samples previously collected for vaginitis diagnosis. *Clin Infect Dis* 2019;**68**:375–81.

Polatti F, Rampino M, Magnani P *et al.* Vaginal pH-lowering effect of locally applied vitamin C in subjects with high vaginal pH. *Gynecol Endocrinol* 2006;**22**:230–4.

Prabhurajeshwar C, Chandrakanth RK. Probiotic potential of lactobacilli with antagonistic activity against pathogenic strains: an *in vitro* validation for the production of inhibitory substances. *Biomed J* 2017;**40**:270–83.

Pritchard H. A case of pyelonephritis with bacteremia caused by *Gardnerella vaginalis* in a man. *Infect Dis Clin Pract* 2018;**26**:e61–3.

Pybus V, Onderdonk AB. Evidence for a commensal, symbiotic relationship between *Gardnerella vaginalis* and *Prevotella bivia* involving ammonia: potential significance for bacterial vaginosis. *J Infect Dis* 1997;**175**:406–13.

Pybus V, Onderdonk AB. A commensal symbiosis between *Prevotella bivia* and *Peptostreptococcus anaerobius* involves amino acids: potential significance to the pathogenesis of bacterial vaginosis. *FEMS Immunol Med Microbiol* 1998;**22**:317–27.

Pybus V, Onderdonk AB. Microbial interactions in the vaginal ecosystem, with emphasis on the pathogenesis of bacterial vaginosis. *Microbes Infect* 1999;**1**:285–92.

Pyles RB, Vincent KL, Baum MM *et al.* Cultivated vaginal microbiomes alter HIV-1 infection and antiretroviral efficacy in colonized epithelial multilayer cultures.

PLoS One 2014;**9**:e93419.

Ralph ED, Clarke JT, Libke RD *et al.* Pharmacokinetics of metronidazole as determined by bioassay. *Antimicrob Agents Chemother* 1974;**6**:691–6.

Ravel J, Gajer P, Abdo Z *et al.* Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci* 2011;**108**:4680–7.

Reid G. Is bacterial vaginosis a disease? *Appl Microbiol Biotechnol* 2018;**102**:553–8.

Reid G. The need to focus on therapy instead of associations. *Front Cell Infect Microbiol* 2019;**9**:327.

Ribet D, Cossart P. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect* 2015;**17**:173–83.

Rickard AH, Gilbert P, High NJ *et al.* Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 2003;**11**:94–100.

Romero-Lastra P, Sánchez MC, Ribeiro-Vidal H *et al.* Comparative gene expression analysis of *Porphyromonas gingivalis* ATCC 33277 in planktonic and biofilms states. *PLoS One* 2017;**12**:1–18.

Romero R, Hassan SS, Gajer P *et al.* The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2014;**2**:4.

von Rosenvinge EC, O´May GA, Macfarlane S *et al.* Microbial biofilms and gastrointestinal diseases. *Pathog Dis* 2013;**67**:25–38.

Safrit JT, Fast PE, Gieber L *et al.* Status of vaccine research and development of vaccines for HIV-1. *Vaccine* 2016;**34**:2921–5.

Saikali P, Koff A, Missick S *et al.* An unusual case of *Gardnerella vaginalis*. *Infect Dis Clin Pract* 2017;**25**:282–3.

Salah RM, Allam AM, Magdy AM *et al.* Bacterial vaginosis and infertility: cause or association? *Eur J Obstet Gynecol Reprod Biol* 2013;**167**:59–63.

Salo J, Pietikäinen A, Söderström M *et al.* Flow-tolerant adhesion of a bacterial pathogen to human endothelial cells through interaction with biglycan. *J Infect Dis* 2016;**213**:1623–31.

Saunders S, Bocking A, Challis J *et al.* Effect of *Lactobacillus* challenge on *Gardnerella vaginalis* biofilms. *Colloids Surf B Biointerfaces* 2007;**55**:138–42.

Schwebke JR. Bacterial vaginosis-more questions than answers. *Genitourin Med* 1997;**73**:333–4.

Schwebke JR. Asymptomatic bacterial vaginosis: response to therapy. *Am J Obstet*

Gynecol 2000;**183**:1434–9.

Schwebke JR. Abnormal vaginal flora as a biological risk factor for acquisition of HIV infection and sexually transmitted diseases. *J Infect Dis* 2005;**192**:1315–7.

Schwebke JR, Desmond R. Risk factors for bacterial vaginosis in women at high risk for sexually transmitted diseases. *Sex Transm Dis* 2005;**32**:654–8.

Schwebke JR, Weiss HL. Interrelationships of bacterial vaginosis and cervical inflammation. *Sex Transm Dis* 2002;**29**:59–64.

Schwiertz A, Taras D, Rusch K *et al.* Throwing the dice for the diagnosis of vaginal complaints? *Ann Clin Microbiol Antimicrob* 2006;**5**:4.

Scott TG, Curran B, Smyth CJ. Electron microscopy of adhesive interactions between *Gardnerella vaginalis* and vaginal epithelial cells, McCoy cells and human red blood cells. *J Gen Microbiol* 1989;**135**:475–80.

Scully JL. What is a disease? *EMBO Rep* 2004;**5**:650–3.

Seña AC, Bachmann LH, Hobbs MM. Persistent and recurrent *Trichomonas vaginalis* infections: epidemiology, treatment and management considerations. *Expert Rev Anti Infect Ther* 2014;**12**:673–85.

Setzer MS, Byler KG, Ogungbe IV *et al.* Natural products as new treatment options for trichomoniasis: a molecular docking investigation. *Sci Pharm* 2017;**85**:e5.

Sgibnev A V, Kremleva EA. Vaginal protection by H_2O_2-producing lactobacilli. *Jundishapur J Microbiol* 2015;**8**:4–8.

Sha BE, Chen HY, Wang QJ *et al.* Utility of Amsel criteria, Nugent score, and quantitative PCR for *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Lactobacillus* spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *J Clin Microbiol* 2005;**43**:4607–12.

Shah NN, Nanjappa S, Greene JN. Osteomyelitis and hip abscess caused by *Gardnerella vaginalis*. *Infect Dis Clin Pract* 2017;**25**:162–4.

Shannon B, Gajer P, Yi TJ *et al.* Distinct effects of the cervicovaginal microbiota and herpes simplex type 2 infection on female genital tract immunology. *J Infect Dis* 2017;**215**:1366–75.

Sharami SH, Afrakhteh M, Shakiba M. Urinary tract infections in pregnant women with bacterial vaginosis. *J Obstet Gynaecol* 2007;**27**:252–4.

Sherrard J, Donders G, White D *et al.* European (IUSTI/WHO) guideline on the management of vaginal discharge, 2011. *Int J STD AIDS* 2011;**22**:421–9.

Sherrard J, Wilson J, Donders G *et al.* 2018 European (IUSTI/WHO) International

Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge. *Int J STD AIDS* 2018;**29**:1258–72.

Shipitsyna E, Roos A, Datcu R *et al.* Composition of the vaginal microbiota in women of reproductive age - sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS One* 2013;**8**:e60670.

Silva D, Henriques A, Cereija T *et al.* Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in Portuguese women and association with risk factors for bacterial vaginosis. *Int J Gynecol Obstet* 2013:2–3.

Sivadon-Tardy V, Roux A-L, Piriou P *et al. Gardnerella vaginalis* acute hip arthritis in a renal transplant recipient. *J Clin Microbiol* 2009;**47**:264–5.

Skarin A, Sylwan J. Vaginal lactobacilli inhibiting growth of *Gardnerella vaginalis*, *Mobiluncus* and other bacterial species cultured from vaginal content of women with bacterial vaginosis. *Acta Pathol Microbiol Immunol Scand B* 1986;**94**:399–403.

Sobel JD. Vulvovaginal candidosis. *Lancet* 2007;**369**:1961–71.

Sobel JD. Recurrent vulvovaginal candidiasis. *Am J Obstet Gynecol* 2016;**214**:15–21.

Sobel R, Sobel JD. Metronidazole for the treatment of vaginal infections. *Expert Opin Pharmacother* 2015;**16**:1109–15.

Sosto F, Benvenuti C, CANVA Study Group. Controlled study on thymol + eugenol vaginal douche versus econazole in vaginal candidiasis and metronidazole in bacterial vaginosis. *Arzneimittelforschung* 2011;**61**:126–31.

Spiegel CA. Susceptibility of *Mobiluncus* species to 23 antimicrobial agents and 15 other compounds. *Antimicrob Agents Chemother* 1987;**31**:249–52.

Spiegel CA, Amsel R, Holmes KK. Diagnosis of bacterial vaginosis by direct gram stain of vaginal fluid. *J Clin Microbiol* 1983;**18**:170–7.

Spiegel CA, Davick P, Totten PA *et al. Gardnerella vaginalis* and anaerobic bacteria in the etiology of bacterial (nonspecific) vaginosis. *Scand J Infect Dis Suppl* 1983;**40**:41–6.

Srinivasan S, Hoffman NG, Morgan MT *et al.* Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* 2012;**7**:e37818.

Stapleton AE, Au-Yeung M, Hooton TM *et al.* Randomized, placebo-controlled phase 2 trial of a *Lactobacillus crispatus* probiotic given intravaginally for prevention of

recurrent urinary tract infection. *Clin Infect Dis* 2011;**52**:1212–7.

Stewart L, Sinha S, Madsen PJ *et al.* Spinal epidural abscess caused by *Gardnerella vaginalis* and *Prevotella amnii*. *Infect Dis Clin Pract* 2018;**26**:237–9.

Stoyancheva G, Marzotto M, Dellaglio F *et al.* Bacteriocin production and gene sequencing analysis from vaginal *Lactobacillus* strains. *Arch Microbiol* 2014;**196**:645–53.

Stubbendieck RM, Vargas-Bautista C, Straight PD. Bacterial communities: interactions to scale. *Front Microbiol* 2016;**7**:1234.

Sturm-Ramirez K, Gaye-Diallo A, Eisen G *et al.* High levels of tumor necrosis factor-alpha and interleukin-1beta in bacterial vaginosis may increase susceptibility to human immunodeficiency virus. *J Infect Dis* 2000;**182**:467–73.

Sturm AW. *Gardnerella vaginalis* in infections of the urinary tract. *J Infect* 1989;**18**:45–9.

Sturm AW, de Leeuw JH, de Pree NT. Post-operative wound infection with *Gardnerella vaginalis*. *J Infect* 1983;**7**:264–6.

Sumati A, Saritha N. Association of urinary tract infection in women with bacterial vaginosis. *J Glob Infect Dis* 2009;**1**:151.

Sutton M, Sternberg M, Koumans EH *et al.* The prevalence of *Trichomonas vaginalis* infection among reproductive-age women in the United States, 2001-2004. *Clin Infect Dis* 2007;**45**:1319–26.

Sutyak Noll K, Prichard MN, Khaykin A *et al.* The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, and ε-poly-L-lysine against bacterial vaginosis-associated pathogens but not human lactobacilli. *Antimicrob Agents Chemother* 2012;**56**:1756–61.

Svare JA, Schmidt H, Hansen BB *et al.* Bacterial vaginosis in a cohort of Danish pregnant women: prevalence and relationship with preterm delivery, low birthweight and perinatal infections. *BJOG* 2006;**113**:1419–25.

Swidsinski A, Doerffel Y, Loening-Baucke V *et al. Gardnerella* biofilm involves females and males and is transmitted sexually. *Gynecol Obstet Invest* 2010;**70**:256–63.

Swidsinski A, Loening-Baucke V, Mendling W *et al.* Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). *Histol Histopathol* 2014;**29**:567–87.

Swidsinski A, Loening-Baucke V, Swidsinski S *et al.* Polymicrobial *Gardnerella*

biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. *Arch Gynecol Obstet* 2015;**291**:605–9.

Swidsinski A, Mendling W, Loening-Baucke V *et al.* Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 2005;**106**:1013–23.

Swidsinski A, Mendling W, Loening-Baucke V *et al.* An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 2008;**198**:1–6.

Swidsinski A, Verstraelen H, Loening-Baucke V *et al.* Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS One* 2013;**8**:4–8.

Tachedjian G, Aldunate M, Bradshaw CS *et al.* The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Res Microbiol* 2017;**168**:782–92.

Tachedjian G, O'Hanlon DE, Ravel J. The implausible "*in vivo*" role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota. *Microbiome* 2018;**6**:3–7.

Taherali F, Varum F, Basit AW. A slippery slope: on the origin, role and physiology of mucus. *Adv Drug Deliv Rev* 2018;**124**:16–33.

Tankovic J, Timinskas A, Janulaitiene M *et al. Gardnerella vaginalis* bacteremia associated with severe acute encephalopathy in a young female patient. *Anaerobe* 2017;**47**:132–4.

Teixeira GS, Carvalho FP, Arantes RME *et al.* Characteristics of *Lactobacillus* and *Gardnerella vaginalis* from women with or without bacterial vaginosis and their relationships in gnotobiotic mice. *J Med Microbiol* 2012;**61**:1074–81.

Teixeira GS, Soares-Brandão KLK, Branco KMGR *et al.* Antagonism and synergism in *Gardnerella vaginalis* strains isolated from women with bacterial vaginosis. *J Med Microbiol* 2010;**59**:891–7.

Thellin O, Zorzi W, Zorzi D *et al.* Lysozyme as a cotreatment during antibiotics use against vaginal infections: an *in vitro* study on *Gardnerella vaginalis* biofilm models. *Int Microbiol* 2016;**19**:101–7.

Thulkar J, Kriplani A, Agarwal N. A comparative study of oral single dose of metronidazole, tinidazole, secnidazole and ornidazole in bacterial vaginosis. *Indian J Pharmacol* 2012;**44**:243–5.

Tikkinen KAO, Leinonen JS, Guyatt GH *et al.* What is a disease? Perspectives of the public, health professionals and legislators. *BMJ Open* 2012;**2**:e001632.

Tjabringa GS, Vos JB, Olthuis D *et al.* Host defense effector molecules in mucosal

secretions. *FEMS Immunol Med Microbiol* 2005;**45**:151–8.

Togni G, Battini V, Bulgheroni A *et al. In vitro* activity of nifuratel on vaginal bacteria: could it be a good candidate for the treatment of bacterial vaginosis? *Antimicrob Agents Chemother* 2011;**55**:2490–2.

Torcia M. Interplay among vaginal microbiome, immune response and sexually transmitted viral infections. *Int J Mol Sci* 2019;**20**:266.

Turovskiy Y, Cheryian T, Algburi A *et al.* Susceptibility of *Gardnerella vaginalis* biofilms to natural antimicrobials subtilosin, ε -poly-L-lysine, and lauramide arginine ethyl ester. *Infect Dis Obstet Gynecol* 2012;**2012**:284762.

Turovskiy Y, Ludescher RD, Aroutcheva AA *et al.* Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*. *Probiotics Antimicrob Proteins* 2009;**1**:67–74.

Turovskiy Y, Noll KS, Chikindas ML *et al.* The etiology of bacterial vaginosis. *J Appl Microbiol* 2011;**110**:1105–28.

Uchihashi M, Bergin L, Bassis CM *et al.* Influence of age, reproductive cycling status, and menstruation on the vaginal microbiome in baboons (*Papio anubis*). *Am J Primatol* 2015;**77**:563–78.

Valenti P, Rosa L, Capobianco D *et al.* Role of lactobacilli and lactoferrin in the mucosal cervicovaginal defense. *Front Immunol* 2018;**9**:376.

Vallor AC, Antonio MAD, Hawes SE *et al.* Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. *J Infect Dis* 2001;**184**:1431–6.

Vaneechoutte M. The human vaginal microbial community. *Res Microbiol* 2017;**168**:811–25.

Vaneechoutte M, Guschin A, Van Simaey L *et al.* Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella*. *Int J Syst Evol Microbiol* 2019;**69**:679–87.

Verstraelen H, Swidsinski A. The biofilm in bacterial vaginosis: implications for epidemiology, diagnosis and treatment. *Curr Opin Infect Dis* 2013;**26**:86–9.

Verstraelen H, Verhelst R, Vaneechoutte M *et al.* The epidemiology of bacterial vaginosis in relation to sexual behaviour. *BMC Infect Dis* 2010;**10**:81.

Vieira-Baptista P, Lima-Silva J, Pinto C *et al.* Bacterial vaginosis, aerobic vaginitis,

vaginal inflammation and major Pap smear abnormalities. *Eur J Clin Microbiol Infect Dis* 2016;**35**:657–64.

Vodstrcil LA, Twin J, Garland SM *et al.* The influence of sexual activity on the vaginal microbiota and *Gardnerella vaginalis* clade diversity in young women. *PLoS One* 2017;**12**:1–15.

Vodstrcil LA, Walker SM, Hocking JS *et al.* Incident bacterial vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. *Clin Infect Dis* 2015;**60**:1042–53.

Watson CJ, Grando D, Fairley CK *et al.* The effects of oral garlic on vaginal candida colony counts: a randomised placebo controlled double-blind trial. *BJOG* 2014;**121**:498–506.

Watts DH, Fazzari M, Fazarri M *et al.* Effects of bacterial vaginosis and other genital infections on the natural history of human papillomavirus infection in HIV-1-infected and high-risk HIV-1-uninfected women. *J Infect Dis* 2005;**191**:1129–39.

Weeks RM, Moretti A, Song S *et al.* Cationic amphiphiles against *Gardnerella vaginalis* resistant strains and bacterial vaginosis-associated pathogens. *Pathog Dis* 2019:ftz059.

Wei Q, Fu B, Liu J *et al. Candida albicans* and bacterial vaginosis can coexist on Pap smears. *Acta Cytol* 2012;**56**:515–9.

Weissenbacher T, Walter C, Mylonas I *et al.* Interleukin-6, interleukin-10 and interleukin-12 in vaginal fluid from women with bacterial vaginosis. *Arch Gynecol Obstet* 2010;**281**:77–80.

Wertz J, Isaacs-Cosgrove N, Holzman C *et al.* Temporal shifts in microbial communities in nonpregnant African-American women with and without bacterial vaginosis. *Interdiscip Perspect Infect Dis* 2008;**2008**:181253.

Wewalka G, Stary A, Bosse B *et al.* Efficacy of povidone-iodine vaginal suppositories in the treatment of bacterial vaginosis. *Dermatology* 2002;**204**:79–85.

Wiesenfeld HC, Hillier SL, Krohn MA *et al.* Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin Infect Dis* 2003;**36**:663–8.

Wiggins R, Hicks SJ, Soothill PW *et al.* Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. *Sex Transm Infect* 2001;**77**:402–8.

van de Wijgert JHHM. The vaginal microbiome and sexually transmitted infections are

interlinked: consequences for treatment and prevention. *PLoS Med* 2017;**14**:e1002478.

van de Wijgert JHHM, Verwijs MC. Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs. *BJOG* 2019, DOI: 10.1111/1471-0528.15870.

Wilson J. Managing recurrent bacterial vaginosis. *Sex Transm Infect* 2004;**80**:8–11.

Wira CR, Patel M V, Ghosh M *et al.* Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am J Reprod Immunol* 2011;**65**:1–23.

Wolrath H, Forsum U, Larsson PG *et al.* Analysis of bacterial vaginosis-related amines in vaginal fluid by gas chromatography and mass spectrometry. *J Clin Microbiol* 2001;**39**:4026–31.

Wood BA, Monro AM. Pharmacokinetics of tinidazole and metronidazole in women after single large oral doses. *Br J Vener Dis* 1975;**51**:51–3.

Workowski K, Bolan G. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm Rep* 2015;**64**:1–137.

World Health Organization Regional Office for Europe. *Infections and Infectious Diseases - A Manual for Nurses and Midwives in the WHO European Region*. 2001.

Xie YT, Gao JM, Wu YP *et al.* Recombinant α-actinin subunit antigens of *Trichomonas vaginalis* as potential vaccine candidates in protecting against trichomoniasis. *Parasit Vectors* 2017;**10**:1–12.

Xu S, Cavera VL, Rogers MA *et al.* Benzoyl peroxide formulated polycarbophil/carbopol 934P hydrogel with selective antimicrobial activity, potentially beneficial for treatment and prevention of bacterial vaginosis. *Infect Dis Obstet Gynecol* 2013;**2013**:909354.

Xu X, Zhang Y, Li Q. Characteristics of herpes simplex virus infection and pathogenesis suggest a strategy for vaccine development. *Rev Med Virol* 2019:1–12.

Yarbrough VL, Winkle S, Herbst-Kralovetz MM. Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. *Hum Reprod Update* 2015;**21**:353–77.

Yoon HJ, Chun J, Kim J-H *et al. Gardnerella vaginalis* septicaemia with

pyelonephritis, infective endocarditis and septic emboli in the kidney and brain of an adult male. *Int J STD AIDS* 2010;**21**:653–7.

Younes JA, Lievens E, Hummelen R *et al.* Women and their microbes: the unexpected friendship. *Trends Microbiol* 2018;**26**:16–32.

Zeron Mullins M, Trouton KM. BASIC study: is intravaginal boric acid non-inferior to metronidazole in symptomatic bacterial vaginosis? Study protocol for a randomized controlled trial. *Trials* 2015;**16**:315.

Zhang L, Gallo RL. Antimicrobial peptides. *Curr Biol* 2016;**26**:R14–9.

Zhou X, Bent SJ, Schneider MG *et al.* Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiology* 2004;**150**:2565–73.

Zozaya M, Ferris MJ, Siren JD *et al.* Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome* 2016;**4**:16.

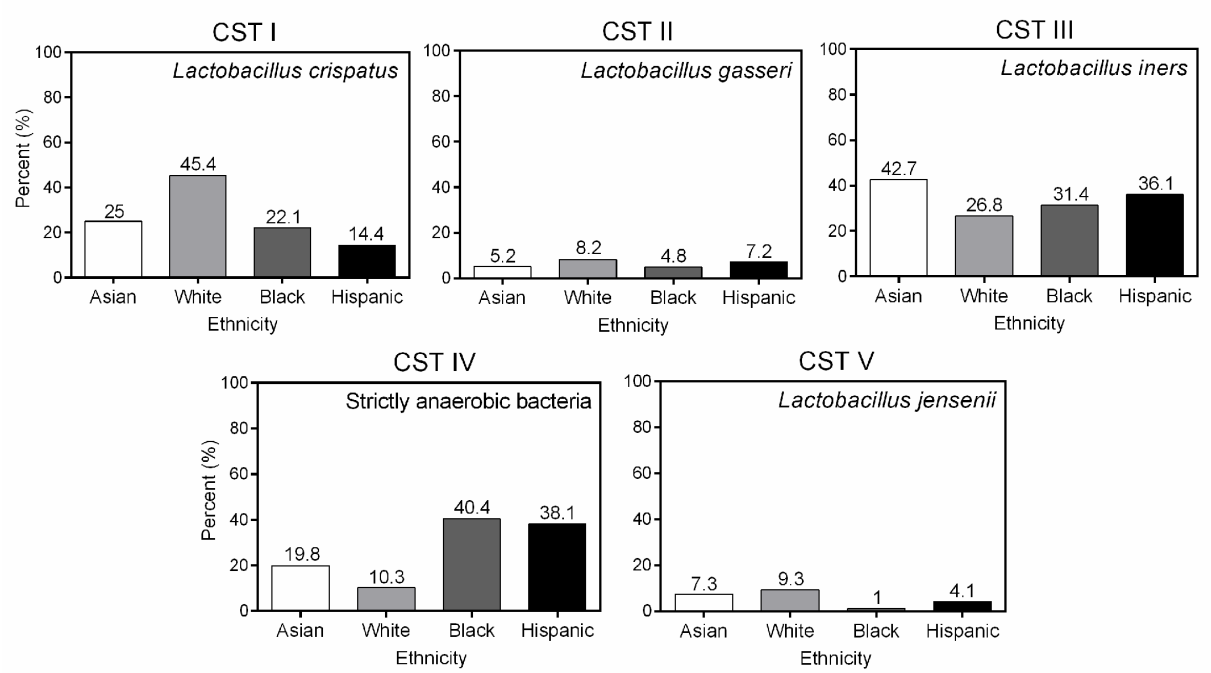

Figure 1. Representation of the different ethnic groups of women within each community state type (CST) proposed by Ravel *et al*. 2011. The study cohort consisted of 96 Asian women, 97 white women, 104 black women, and 97 Hispanic women, showing the relationship between ethnic background and vaginal bacterial community composition.

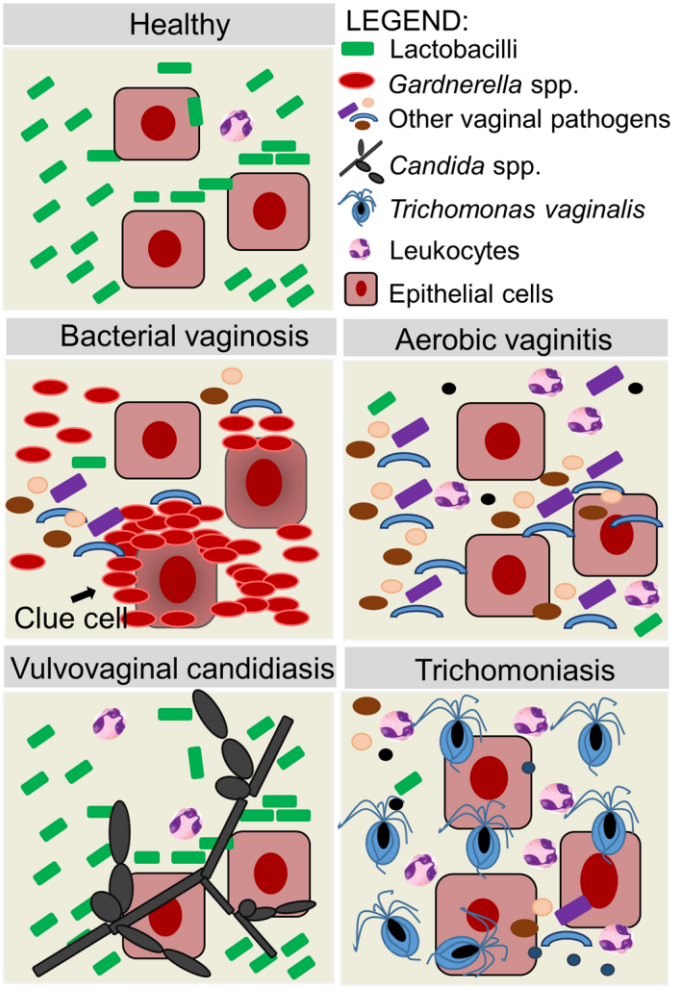

Figure 2. Schematic representation of the healthy and unbalanced vaginal microbiota according to the most common vaginal infections.

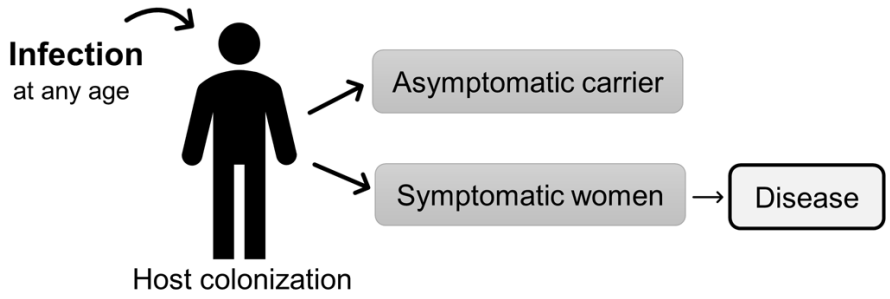

Figure 3. Representation of the putative model of BV infection.

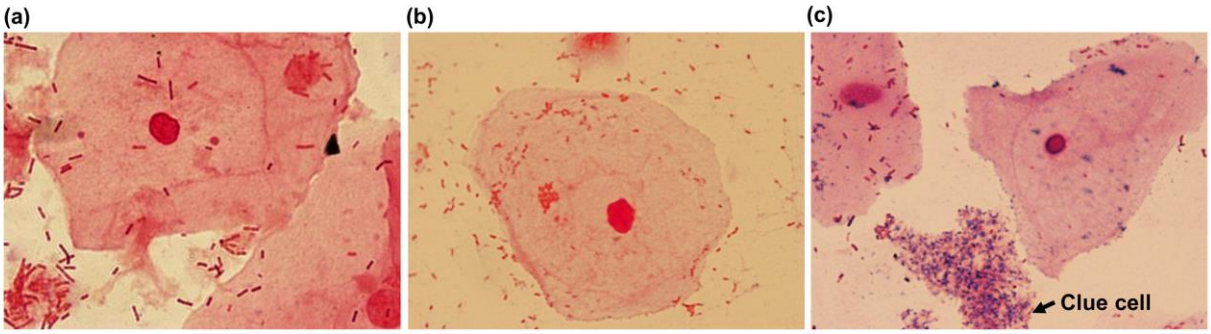

Figure 4. Gram-staining vaginal smears illustrate the vaginal microbiota. (a) Normal vaginal epithelial cells. (b) Intermediate vaginal microbiota. (c) BV-associated microbiota, showing a vaginal clue cell, which corresponds to vaginal squamous epithelial cells coated with *Gardnerella* spp. and other anaerobic bacteria. Original magnification: 1000 times.

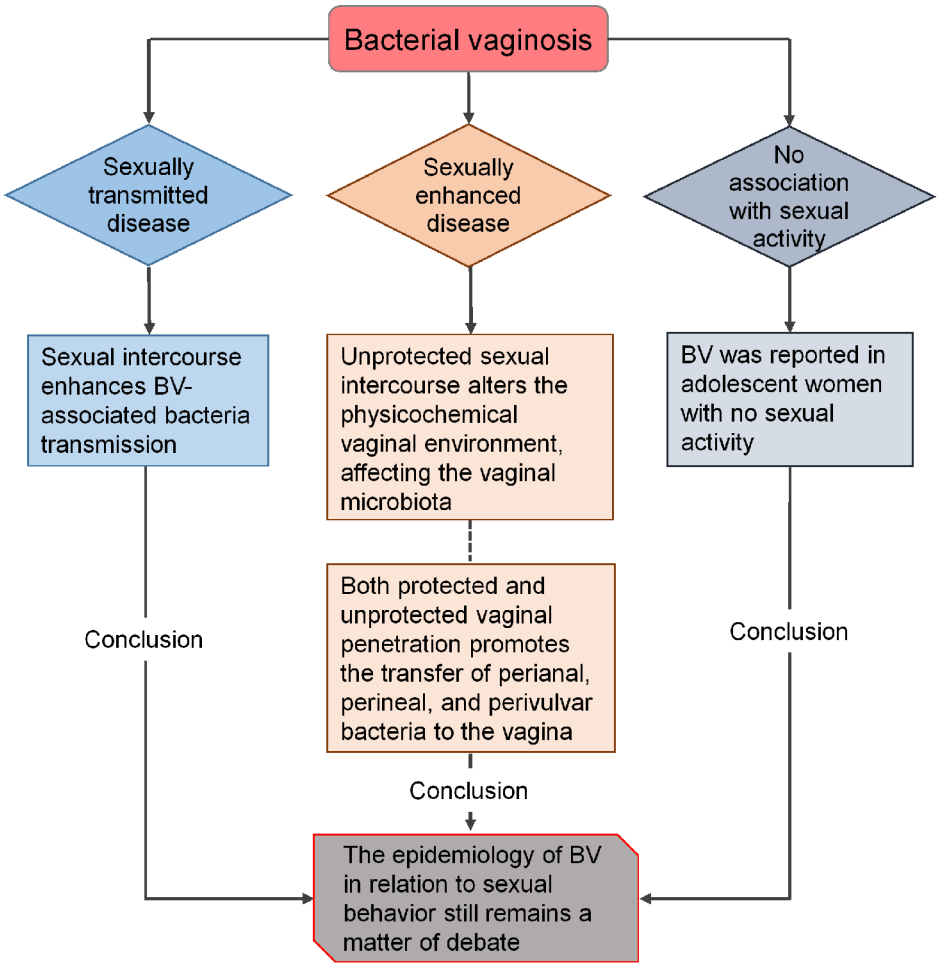

Figure 5. Representation of the epidemiological profile of BV in relation to sexual behavior. This figure was created based on the information presented in the article by Verstraelen *et al.* 2010.

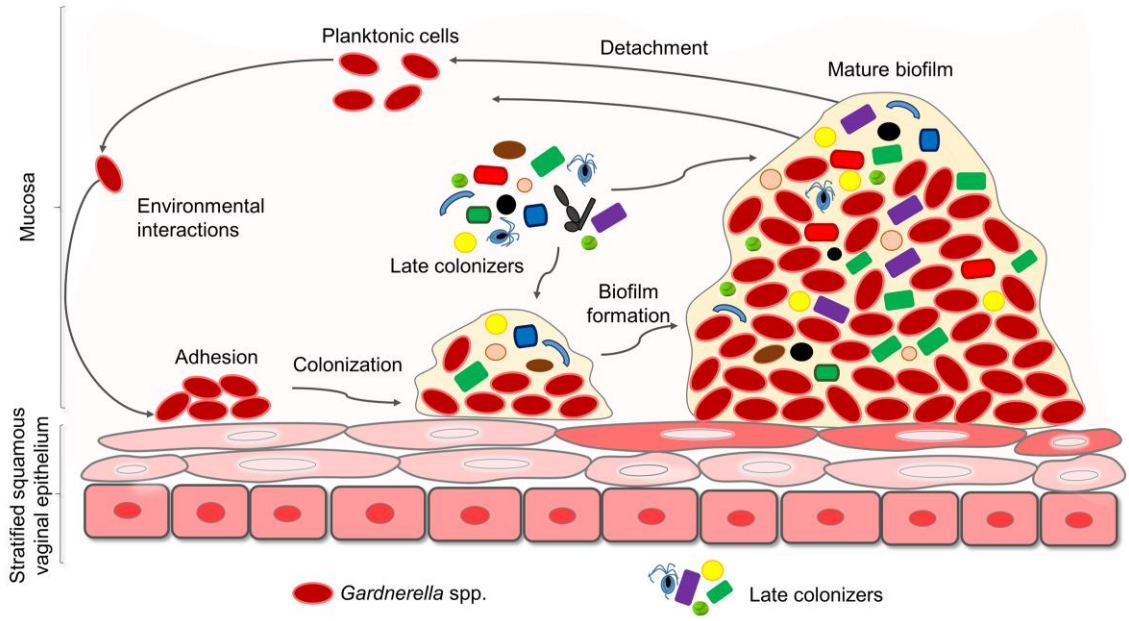

Figure 6. Conceptual multi-species model of the BV-associated biofilm development. In multi-species BV-related biofilms, secondary pathogens are able to incorporate the initially formed biofilm by *Gardnerella* spp. which is already adhered to the vaginal epithelium. Following, a synergistic relationship can be formed, allowing the biofilm to prosper.

Table 1. ***Main features of the normal vaginal microbiota and the most common vaginal infections.***

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

Table 2. ***Association of BV with other vaginal infections.***

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

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.***

Score	***Lactobacillus* Morphotypes**	***Gardnerella* and *Bacteroides* spp. Morphotypes**	**Curved Gram-Variable Rods**
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

VAGINAL MICROBIOTA DIAGNOSIS BY NUGENT SCORE SYSTEM	
Total score [a]	**Interpretation**
0 – 3	Normal vaginal microbiota
4 – 6	Intermediate vaginal microbiota
7 – 10	Bacterial vaginosis in vaginal microbiota

[a] Morphotypes are scored as the average number see 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 *Mobiluncu*s spp. Adapted from Nugent, Krohn and Hillier 1991.

Table 2. Koch's postulates and Gardner and Duke´s conclusions.

Koch's postulates (Koch 1876)	**Gardner and Duke observations** (Gardner and Dukes 1955)
1. The etiologic microbe should be found in every case of the disease	**1.** 92% of patients with a primary diagnosis of BV were found to have *H. vaginalis* infection
2. The bacterium must be isolated from a diseased organism and grown on pure culture	**2.** This was accomplished in each of the 141 cases with positive *H. vaginalis* cultures
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	**3.** A patient, known to be free of disease, was inoculated with *H. vaginalis.* The patient developed clinical manifestations of the disease and the organism was recovered in pure culture
4. The etiologic microbe should be re-isolated from the experimentally inoculated host	**4.** This requirement was fulfilled since pure cultures of the bacterium were successfully obtained from the patient's culture material

Table 3. ***Bacterial interactions occurring in the context of BV and their predictive ecological effects.***

Microbes	Interaction	Mechanism	Effect in host	References
SYNERGISM WITHIN MICROBES				
Gardnerella* spp. and *Prevotella bivia	*P. bivia* produced ammonia which was utilized by *Gardnerella* spp. which produced amino acids that were utilized by *P. bivia*	Ammonia and amino acids cycle	Presence of high vaginal pH	(Pybus and Onderdonk 1997)
Peptostreptococcus anaerobius* and *P. bivia	Amino acids accumulation in *P. bivia* culture supernatants and subsequent growth of *P. anaerobius* in the conditioned supernatants	*P. anaerobius* was able to grow in vaginal defined medium with *P. bivia*, but not in pure culture. Amino acids serve as a source for *P. anaerobius* growth	Increased risk for female pelvic infections, adverse pregnancy outcome, and intra-amniotic infection	(Pybus and Onderdonk 1998)
Gardnerella* spp. and *Atopobium vaginae	*A. vaginae* was homogeneously intermixed with *Gardnerella* spp. in BV-associated biofilms	Unknown	Presence of clue cells	(Swidsinski *et al.* 2005)
Gardnerella* spp. and *P. anaerobius	*Gardnerella* spp. strains were able to enhance the growth of *P. anaerobius*	Production of synergistic compounds by *Gardnerella* spp.	Bacterial interactions present an important role in the ecology of the vaginal microbiota	(Teixeira *et al.* 2010)
Gardnerella* spp. and *Eggerthella, Dialister* sp. type 2, *A. vaginae*, and *Aerococcus christensenii	Metabolic co-dependencies between these bacteria	Unknown	Possible contribution to increase the incidence of BV	(Srinivasan *et al.* 2012)
***A. vaginae* and *Prevotella* spp.**	Both bacterial species could have metabolic co-dependencies	Unknown	A combination of *Prevotella* spp. and/ or *A. vaginae* seems to diagnose BV with high accuracy	(Datcu *et al.* 2013)

Gardnerella* spp. and *Fusobacterium nucleatum, Mobiluncus mulieris, A. vaginae* or *P. bivia	*In vitro* dual-species biofilms of *Gardnerella* spp. derived a growth benefit from the addition of a second species, regardless of the species. *Gardnerella* spp. biofilms enhanced the growth of *P. bivia* and to a minor extent of *F. nucleatum*	*F. nucleatum* was shown to be able to join an initial *Gardnerella* spp. biofilm (intermediate colonizer)	The symbiotic relationships established between *Gardnerella* spp. and other anaerobes in BV biofilms could contribute to the progression of BV	(Machado, Jefferson and Cerca 2013)
Gardnerella* spp. and *Actinomyces neuii, Brevibacterium ravenspurgense, Corynebacterium amycolatum, Corynebacterium tuscaniense, Staphylococcus saprophyticus, Enterococcus faecalis, Nosocomiicoccus ampullae, Staphylococcus simulans, Staphylococcus warnerii, Streptococcus anginosus, Propionibacterium acnes* or *Escherichia coli	These bacterial species were able to cause an increase of the biomass of a pre-established *Gardnerella* spp. biofilm	Unknown	Could be associated with a high number of clue cells	(Castro and Cerca 2015)
Gardnerella* spp. and *A. vaginae	*Gardnerella* spp. and *A. vaginae* are important constituents of the vaginal biofilm	Unknown	Presence of clue cells	(Hardy *et al.* 2016)

Gardnerella* spp. and *E. coli* or *E. faecalis	*E. coli* and *E. faecalis* were able to incorporate and enhance a pre-formed *Gardnerella* spp. Biofilm	In dual-species biofilms, these bacterial species seem to be able to co-aggregate	Uropathogens can associate in BV biofilm	(Castro, Machado and Cerca 2016)
Gardnerella* spp. and *Mycoplasma hominis	Strong association between *Gardnerella* spp. and *M. hominis* were found in women with BV	A potential quorum sensing-like interaction or co-response to an environmental stimulus	The transmission of one of these bacteria could trigger the outgrowth of the other and start a process leading to BV	(Cox *et al.* 2016)
Gardnerella* spp. and *P. bivia	*Gardnerella* spp. facilitated uterine infection by *P. bivia*	The presence of *Gardnerella* spp. enhanced the invasive potential of *P. bivia*, facilitating its ascension into the uterus	BV bacteria may actively inhibit inflammatory responses	(Gilbert *et al.* 2019)
Gardnerella* spp. and *A. vaginae, A. neuii, C. tuscaniense, M. mulieris, S. anginosus, P. bivia, C. amycolatum, N. ampullae, P. acnes, B. ravenspurgense, E. faecalis, S. saprophyticus, S. simulans, S. hominis, S. warnerii	Despite all BV-associated species were able to increase the cell number of a pre-established *Gardnerella* spp. biofilm, not all bacterial species enhanced the *Gardnerella* spp. virulence according to transcriptomic findings	Increased expression of genes associated with cytotoxicity, biofilm formation, antimicrobial resistance, and evasion of immune response by *Gardnerella* spp. in presence of specific BV-associated bacteria in dual-species biofilms	Bacterial interactions between co-infecting bacteria can profoundly affect the progress of BV and its clinical outcome	(Castro, Machado and Cerca 2019)
ANTAGONISM WITHIN MICROBES				
***Lactobacillus* spp. and *Gardnerella* spp., *Mobiluncus* spp., *Bacteroides*, and anaerobic cocci**	*Lactobacillus* inhibited the growth of bacteria isolated from women with BV	The capacity of *Lactobacillus* to acidify the medium with a consequent decrease of pH and inhibition of growth	*Lactobacillus* prevent the growth of bacteria associated with BV	(Skarin and Sylwan 1986)
Lactobacillus* spp. and *Gardnerella* spp., *Mobiluncus* spp., *Peptostreptoc	*Lactobacillus* inhibited the growth of *Peptostreptococcus, M. curtisii, Gardnerella* spp., and other	The inhibition by *Lactobacillus* was influenced by the pH of the growth medium	The interactions between *Lactobacillus* and other bacteria may regulate the microbiological ecosystem of the vagina	(Nagy, Petterson and Mardh 1991)

***occus* spp., *Bacteroides* spp.**	anaerobes			
Lactobacillus* spp. and *Gardnerella* spp., *Bacteroides* spp., *P. bivia	*Lactobacillus* inhibited the growth of bacteria	Production of acids and hydrogen peroxide (H_2O_2) by lactobacilli	Lactobacilli would prevent colonization by other bacteria associated with BV	(McLean and Rosenstein 2000)
***Lactobacillus acidophilus* and *Gardnerella* spp.**	*L. acidophilus* produced a bacteriocin that inhibited the growth of *Gardnerella* spp. isolates	Production of a bacteriocin by *L. acidophilus*	Lactobacilli, by the production of bacteriocins, have the capacity to prevent the growth of pathogenic bacteria	(Aroutcheva, Simoes and Faro 2001)
Lactobacillus helveticus* and *Gardnerella* spp. and *P. bivia	*L. helveticus* inhibited the growth and viability of *Gardnerella* spp. and *P. bivia* and also decreased the capacity of adhesion of *Gardnerella* spp., to HeLa cells	The antagonistic activity is due to the compounds produced by *L. helveticus*	*L. helveticus* is a potential probiotic strain	(Atassi *et al.* 2006a)
Lactobacillus* spp. and *Gardnerella* spp. and *P. bivia	*Lactobacillus* strains isolated from vaginas of healthy women showed antagonistic activity against *Gardnerella* spp. and *P. bivia* in co-culture and also inhibited viability and adhesion of bacteria to HeLa cells	Production of H_2O_2 and proteolytic enzyme-resistant compounds by *Lactobacillus* spp.	*Lactobacillus* can control the vaginal microbiota and compete with other organisms for the adherence to epithelial cells	(Atassi *et al.* 2006b)
***Lactobacillus* spp. and *Gardnerella* spp.**	*Lactobacillus* have the capacity to displace and kill *Gardnerella* spp. growing as biofilm	The production of H_2O_2 by some *Lactobacillus* 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	*Lactobacillus* 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)

		viability of *Gardnerella* spp.		
Lactobacillus rhamnosus* and *Gardnerella* spp. and *P. bivia	*Lactobacillus* showed bactericidal activity against *Gardnerella* spp. and *P. bivia*	It probably includes the production of hydrogen peroxide, lactic acid, and antibacterial compounds by *Lactobacillus*	*L. rhamnosus* is considered a probiotic strain - a promising candidate for use in BV therapy	(Coudeyras *et al.* 2008)
Lactobacillus* spp. and *Gardnerella* spp., *P. bivia*, *Mobiluncus* spp., and *Bacteroides fragilis	*Lactobacillus* species inhibited the growth of *Gardnerella* spp., *P. bivia*, and *Mobiluncus* spp., but did not show effect against *B. fragilis*	Production of lactic acid, H_2O_2, and bacteriocins by *Lactobacillus* spp.	Potential role of lactobacilli against BV pathogens	(Matu *et al.* 2010)
***Lactobacillus johnsonii*, *Lactobacillus gasseri* and *Gardnerella* spp.**	Lactobacilli inhibited the growth of *Gardnerella* spp.	Production of lactic acid, H_2O_2, and heat-stable molecules by lactobacilli	The main metabolites of *Lactobacillus* spp. act cooperatively to kill BV-associated bacteria	(Atassi and Servin 2010)
***Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *Gardnerella* spp.**	The secreted products of *L. rhamnosus* and *L. reuteri* infiltrated BV biofilms and caused bacterial cell death	Possible production of acid, bacteriocins or biosurfactant-like substances by *L. rhamnosus* and *L. reuteri*	Lactobacilli can induce a return to a normal microbiota from a BV state	(McMillan *et al.* 2011)
***Lactobacillus* spp. and *Gardnerella* spp.**	*Lactobacillus* showed antagonistic activity against *Gardnerella* spp.	Unknown	Success in the BV development depends on the presence of *Lactobacillus* species	(Teixeira *et al.* 2012)
***Lactobacillus crispatus* and *Gardnerella* spp.**	*L. crispatus* produced lactic acid and inhibited the growth of *Gardnerella* spp. on an *ex vivo* porcine vaginal mucosal model	Production of antimicrobial compounds by *L. crispatus*	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)
***L. acidophilus*, *L. rhamnosus*,**	*Lactobacillus* were able to inhibit the growth of both	The effect could be due to the production of lactic acid, H_2O_2, and bacteriocins	*L. acidophilus* alone or combined with *L. rhamnosus* can be used in probiotic products to	(Bertuccini *et al.* 2017)

and *Gardnerella* spp. and *A. vaginae*	*Gardnerella* spp. and *A. vaginae*		prevent bacterial infections	

Table 4. ***Bacterial interactions occurring between* Gardnerella *spp. and other STIs-associated microbes.***

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

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	(Filardo *et al.* 2019)
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	(Hinderfeld *et al.* 2019)

Table 5. ***Common and proposed alternative or preventive treatment strategies used against vaginal infections.***

Treatment		**Reference**
BACTERIAL VAGINOSIS		
Recommended antibiotics	Metronidazole, Clindamycin, Tinidazole	(Workowski and Bolan 2015)
Proposed alternative approaches	Povidone iodine	(Wewalka *et al.* 2002)
	Hydrogen peroxide	(Cardone *et al.* 2003)
	Lactocin 160	(Turovskiy *et al.* 2009)
	Octenidine hydrochloride/ phenoxyethanol	(Novakov Mikic and Budakov 2010)
	Thymol	(Braga *et al.* 2011)
	Silicon-coated tablets containing 250 mg vitamin C	(Polatti *et al.* 2006; Petersen *et al.* 2011)
	Mixture of thymol and eugenol	(Sosto, Benvenuti and CANVA Study Group 2011)
	Nifuratel	(Togni *et al.* 2011)
	Benzydamine hydrochloride	(Boselli *et al.* 2012)
	Glycerol monolaurate	(Sutyak Noll *et al.* 2012)
	Lauramide arginine ethyl ester	(Turovskiy *et al.* 2012)
	Benzoyl peroxide formulated polycarbophil/ carbopol 934P hydrogel	(Xu *et al.* 2013)
	Subtilosin	(Cavera, Volski and Chikindas 2015)
	Boric acid	(Zeron Mullins and Trouton 2015)
	Thymbra capitata essential oil Benzoyl peroxide	(Machado *et al.* 2017) (Algburi *et al.* 2018)
	Dequalinium chloride	(Sherrard *et al.* 2018)
	Probiotics	(Homayouni *et al.* 2014; van de Wijgert and Verwijs 2019)
	TOL-463 (boric acid-based vaginal anti-infective with enhanced antibiofilm activity)	(Marrazzo *et al.* 2019)

	Cationic amphiphiles	(Weeks *et al.* 2019)
VULVOVAGINAL CANDIDIASIS		
Recommended antifungal drugs	Clotrimazole, Miconazole, Tioconazole, Butoconazole, Terconazole, Fluconazole	(Workowski and Bolan 2015)
Proposed alternative approaches	Povidone iodine	(Kondo *et al.* 2012)
	Garlic pills	(Watson *et al.* 2014)
	Propolis	(Grenier Capoci *et al.* 2015)
	Boric acid	(Pointer, Boyer and Schmidt 2015)
	Probiotics	(Buggio *et al.* 2019)
	TOL-463 (boric acid-based vaginal anti-infective with enhanced antibiofilm activity)	(Marrazzo *et al.* 2019)
TRICHOMONIASIS		
Recommended antibiotics	Metronidazole, Tinidazole	(Workowski and Bolan 2015)
Proposed alternative approaches	Boric acid	(Aggarwal and Shier 2008; Backus, Muzny and Beauchamps 2017)
	Medicinal plants	(Mehriardestani *et al.* 2017)
	Phytochemicals	(Setzer *et al.* 2017)
Proposed preventive therapy	Vaccines	(Xie *et al.* 2017)
CHLAMYDIA		
Recommended antibiotics	Azithromycin, Doxycycline	(Workowski and Bolan 2015)
Proposed preventive therapy	Vaccines	(Hafner and Timms 2018)
GONORRHEA		
Recommended antibiotics	Ceftriaxone, Azithromycin	(Workowski and Bolan 2015)
Proposed preventive therapy	Vaccines	(Jerse and Deal 2013; Edwards, Jennings and Seib 2018)
VIRAL VAGINITIS		
Proposed preventive therapy	Vaccines	(Petrosky *et al.* 2015; Hsu and O'Connell 2017; Xu, Zhang and Li 2019)