

The influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis.

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Abstract

Bacterial vaginosis (BV) is the worldwide leading vaginal disorder in women of reproductive age. BV is characterized by the replacement of beneficial lactobacilli and the augmentation of anaerobic bacteria. *Gardnerella vaginalis* is a predominant bacterial species, however, BV is also associated with other numerous anaerobes, such as *Atopobium vaginae*, *Mobiluncus mulieris*, *Prevotella bivia*, *Fusobacterium nucleatum* and *Peptoniphilus* sp.. Currently, the role of *G. vaginalis* in the etiology of BV remains a matter of controversy. It is however known that, in BV patients, a biofilm is usually formed on the vaginal epithelium and *G. vaginalis* is typically the predominant species. So, the current paradigm is that the establishment of a biofilm plays a key role in the pathogenesis of BV. This review provides background on the influence of biofilm formation by *G. vaginalis* and other anaerobes in the polymicrobial etiology of BV, through its initial adhesion until biofilm formation and discusses the commensal and synergic interactions established between them to understand the phenotypic shift of *G. vaginalis*' biofilms into BV establishment.

Keywords: Bacterial vaginosis; *Gardnerella vaginalis*; Initial adhesion; Biofilm; BV-associated anaerobes

Etiology of bacterial vaginosis

Bacterial vaginosis (BV) is the most common vaginal disorder in women of reproductive age and the cause of several symptoms, such as homogeneous malodorous vaginal discharge, overgrowth of anaerobes and increased production of amines (putrescines, cadaverines and trimethylamine) that contribute to a higher vaginal pH and presence of clue cells (1). BV is responsible for more than 60% of vulvovaginal infections and has been linked to serious public health consequences including pelvic inflammatory disease (2), postoperative infections (3), acquisition and transmission of the human immunodeficiency virus (HIV) (4), preterm birth and several adverse outcomes in pregnancy (5).

The healthy vaginal epithelium is generally dominated by hydrogen peroxide and lactic acid producing lactobacilli (6), acting as a protective surfactant layer that leads to an acidic pH (7) that inhibits the adhesion and the growth of other bacteria, including opportunistic pathogens on the vaginal epithelium (8). During the development of BV, the normal vaginal microbiota composition changes and it is characterized by a decrease in these lactobacilli species and an increase in the numbers of several pathogenic bacteria, mainly anaerobes (9). Currently, the initial steps in this microbial shift are still a matter of debate and two main theories try to explain the events leading to BV. Some evidence points to the fact that certain anaerobes are capable of acting as primary pathogens in the vaginal microbiota and displace the protective lactobacilli layer (6), but there are also claims that the anaerobe overgrowth could be a symptom of the infection rather than specifically related to BV etiology (10).

The lack of basic information about the etiology of BV has led to an ongoing debate between two main hypotheses, known as the polymicrobial and the single pathogen hypotheses. The single pathogenic agent hypothesis defends that a single pathogenic species is responsible to induce the microbial switch and to cause BV (11). The polymicrobial hypothesis assumes that BV is caused by a mixture of anaerobic pathogenic bacteria. This hypothesis is strongly supported by the fact that multiple bacteria are often found in BV (12) and recently culture-independent methods showed that this polymicrobial community may be even more complex than previously thought (13). However, the presence of a bacterium in BV has been rarely supported by microbiological functional studies, demonstrating the virulence of such species. Furthermore, epidemiological data gathered in multiple studies suggest a sexual transmission role in BV and raises doubts to the polymicrobial causative nature of BV (14).

Anaerobes involved in bacterial vaginosis

Although current knowledge about BV etiology remains scarce, the common consensus is that BV is always associated with the overgrowth of numerous bacterial species. As reviewed by Livengood, conventional medium cultivation of the vaginal microbiota from BV women identified a typical spectrum of anaerobes, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus mulieris*, *Prevotella bivia*, *Fusobacterium nucleatum*, *Ureaplasma urealyticum* and *Mycoplasma hominis* (15). With the advances in culture-independent molecular techniques, BV spectrum of anaerobes was greatly expanded adding *Eggerthella*, *Megasphaera*, *Leptotrichia*, *Dialister*, *Bifidobacterium*, *Slackia* and other bacteria related to *Arthrobacter*, *Caulobacter* and *Butyrivibrio* (12). Also, the Vaginal Human Microbiome Project has detected several newly described bacteria in the *Clostridiales* order that are currently designated BVAB1, BVAB2, and BVAB3 (16). Interestingly, differences in the BV vaginal microbiome between American women and women of European ancestry were found being 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 colonized by *M. hominis*, *Dialister micraerophilus* and *Gemella* species (16). Despite of this likely secondary anaerobic colonizer diversity in women with different ancestry and geographical localization, *G. vaginalis* remains a central bacteria species in the BV vaginal microbiome.

It has been hypothesized that the establishment of a biofilm plays a key role in the pathogenesis of BV (17). Swidsinski and colleagues have demonstrated that a biofilm was present in 90% of the epithelial surfaces from BV vaginal biopsy specimens, being *G. vaginalis* the major component of these multispecies communities (9). The role of biofilms in BV is also supported by the work of Patterson and colleagues, which demonstrated that *G. vaginalis* biofilms exhibited higher tolerance to hydrogen peroxide and lactic acid, when compared to planktonic cells. They suggested that the biofilm increased resistance against lactobacilli probiotic properties would present an advantage for *G. vaginalis* growth in biofilms (18). On a follow up study, O'Hanlon and colleagues confirmed the bactericidal potential of lactic acid in BV-associated bacteria (19) although providing evidence that the role of hydrogen peroxide is minor due to its interactions with vaginal fluid and semen (20). Patterson and colleagues also showed that, when compared with *A. vaginae*, *M. mulieris*, *P. bivia* and *Peptoniphilus* sp., *G. vaginalis* was the only anaerobe to exhibit three key virulence determinants in BV, more exactly, adherence to vaginal epithelial cells, biofilm-producing capacity and cytotoxic activity (21). These findings were recently confirmed by Alves and colleagues, where they tested *G. vaginalis* virulence against other 29 bacteria species associated with BV, such as *M. hominis*, *Corynebacterium tuscaniense*, *Aerococcus christensenii*, *Gemella haemolysins* and *Mycoplasma hominis* (22). Hence, all these findings suggest that biofilm forming *G. vaginalis* plays a key role in BV pathogenesis.

***G. vaginalis* biofilm formation and BV establishment**

Generally, the biofilm life-cycle includes 3 main stages: initial adhesion, accumulation and dispersal (Figure 1A). Therefore, the initial adhesion to the vagina epithelium is a crucial step for the development of BV biofilms. It was postulated that *G. vaginalis* requires the help of others anaerobes to start BV development (23). Earlier studies by Mardh and colleagues tested the ability of multispecies inoculation with *G. vaginalis* and *Mobiluncus* spp. to cause BV in monkeys. They showed that increasing

the anaerobic bacteria concentrations in the monkeys' vaginal epithelium was not sufficient to induce BV development (24).

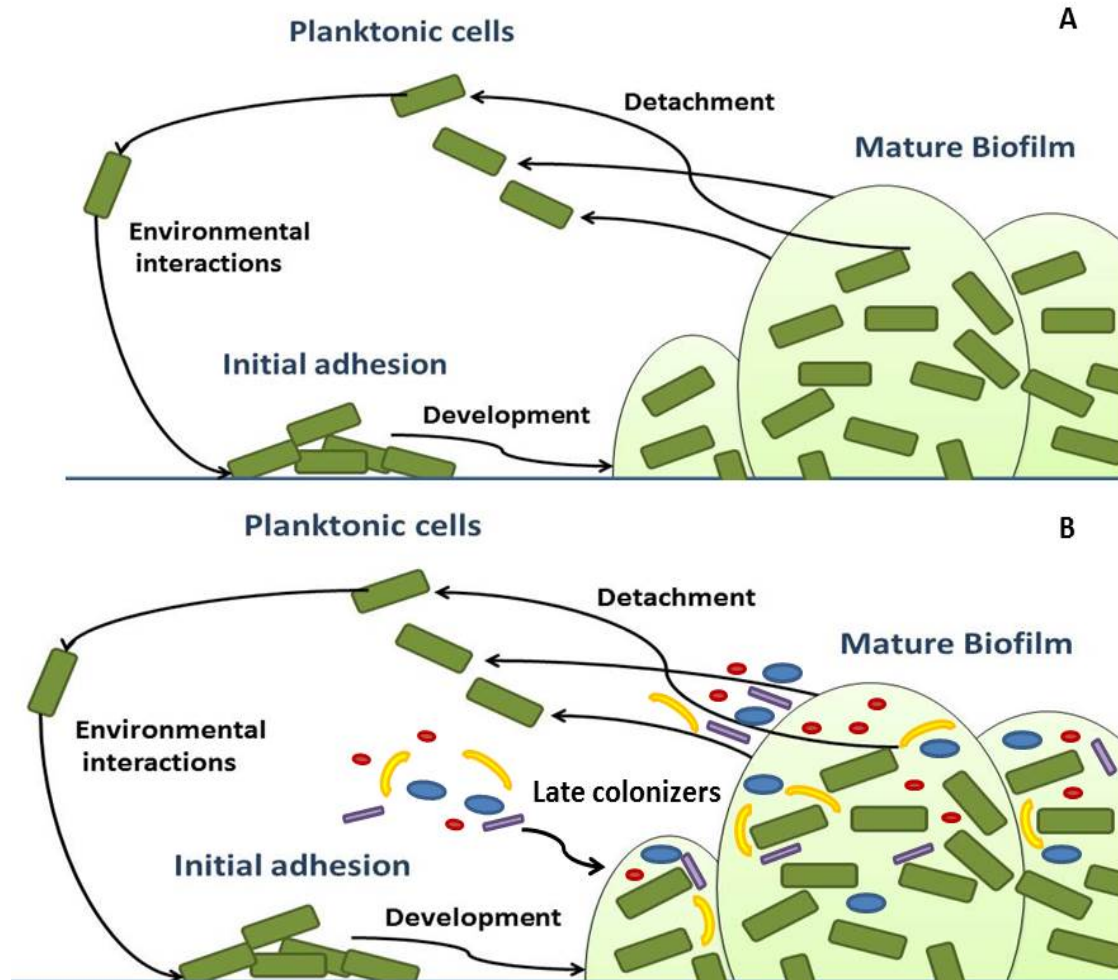


Figure 1 | Conceptual monospecies and multispecies models about the development of biofilm formation. (A) Conceptual monospecies biofilm lifecycle: when the environmental conditions are appropriate, planktonic cells will adhere to a surface and grow into a multi-layer structure composed of bacteria and extracellular components. Eventually, bacteria will detach from the biofilm and the resulting cells will be able to restart the biofilm cycle in a different location. (B) Polymicrobial biofilms, such as in BV, secondary bacteria will incorporate the biofilm after the initial colonizer species has already adhered to the surface. Synergetic relationship can then be formed, allowing the biofilm to prosper.

However, at that time, the association of biofilms with BV was unknown and these researchers were not able to explore the biofilm phenotype in BV development. With the studies of Swidsinski and colleagues, clue cells were finally understood as biofilm-coated epithelial cells desquamated from the epithelial surface (9). Although the biofilm was shown to contain high concentrations of a variety of bacterial groups, *G. vaginalis* was found to be the predominant constituent. Many follow up studies validated their findings (25,26) and it's now accepted that biofilms in BV are strongly associated with *G. vaginalis* (17). Using a novel multiplex PNA-FISH approach (27), it was recently showed that *G. vaginalis* was able to adhere and displace pre-coated protective lactobacilli from vaginal epithelial cells, while other BV-

associated anaerobes, such as *A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum*, revealed to be less virulent (28). Although other tested anaerobes were also able to adhere in high numbers, such as *A. vaginae* and *M. mulieris*, they were easily outcompeted by *L. crispatus* (28). Those results suggest that *G. vaginalis* plays a central role in the early adhesion stages that could lead to the formation of clue cell, as previously postulated by Swidsinski and Patterson (17,21). This new evidence raises an interesting question: can the overall community of bacteria found in BV mixed species biofilms be mainly composed by opportunistic secondary colonizers? Interestingly, Swidsinski and colleagues demonstrated that *G. vaginalis* was able to incorporate other bacterial groups into its biofilms (17). Later, Alves and colleagues demonstrated that more than 20 BV associated species showed a natural tendency to grown as biofilms, despite they are apparently unable to establish a primary biofilm in the vaginal epithelium (22).

Bacterial interaction in multispecies BV biofilms

Despite the growing evidence that *G. vaginalis* has an higher virulence potential than any other BV associated bacteria tested so far, an enduring enigma is whether *G. vaginalis* alone is capable of causing BV or whether *G. vaginalis* needs the interaction with others anaerobic species. Interestingly, with the advancement of molecular techniques, it has been shown that *G. vaginalis* is capable to establish different interactions with other BV-associated anaerobes (29,30). It was recently demonstrated that a pre-formed *G. vaginalis* biofilms was able to establish symbiotic relationship with other BV associated anaerobes. Regardless of the secondary species, *G. vaginalis* biofilm growth was promoted by the presence of the additional species. This effect was more prominent in the presence of *P. bivia* and *M. mulieris*, but all tested BV-associated anaerobes were able to enhance *G. vaginalis* growth (30). Furthermore, Datcu and colleagues have shown that amino acids produced by *G. vaginalis* may promote the growth of *P. bivia* and *F. nucleatum* (29). In a study on multispecies oral biofilms, Foster and Kolenbrander (31) demonstrated that *F. nucleatum* was capable of co-aggregating with pathogenic bacteria and becoming a dominant member of the oral multispecies biofilm after several days of incubation. However, *F. nucleatum* commonly failed to grow by itself in biofilms. Interestingly, *F. nucleatum* was shown to be able to join an initial biofilm and eventually establishes a symbiotic relationship with *G. vaginalis* (30). It is well-known that *F. nucleatum* expresses receptors that adhere to a large variety of other bacterial species, acting as intermediate colonizer between early and late colonizers (32).

Other studies that highlight the benefits for synergistic relationships in BV development have been reported. *A. vaginae* is commonly isolated from cases of BV (9) and has been associated with its potential to induce an inflammatory response during late stage of BV. However, alone, this bacterium did not demonstrate any specific virulence factors (33). It has also been described that, during BV, *Mobiluncus* species is known to produce proteolytic activity through proline aminopeptidase (34) and was postulated that the available peptides and aminoacids could facilitate an anaerobic growth in the vaginal epithelium (35). In fact, Pybus and Onderdonk directly demonstrated that these peptides are able to stimulates the in vitro growth of *G. vaginalis* and *P. bivia* (36). Thus, the increased availability of peptides by *M. mulieris* could act as a factor promoting the development of multispecies biofilm initiated by *G. vaginalis* during BV establishment. Furthermore, *Peptoniphilus* sp. had been recently associated with persistent cases of BV. In 2008, Marrazzo and colleagues isolated this organism from 36 % of persistent cases of BV (37). In addition, Patterson and colleagues demonstrated the robust adherence of *Peptoniphilus* sp. to vaginal epithelial cells but a lower biofilm formation (21), postulating that *Peptoniphilus* sp. could be an earlier colonizer but would require incorporation in the *G. vaginalis* biofilm during cases of BV. Finally, several other anaerobes had been also associated to BV microbiota, such as *Veillonella* sp. and *Peptostreptococcus* sp., although without demonstrating a noteworthy adhesion to epithelial cells, biofilm formation and cytotoxicity properties (21). It is therefore feasible that the overgrowth of these weak colonizers in the vagina epithelium could directly depend on *G. vaginalis* biofilm development. However, this hypothesis still needs to be experimental determined. All these data support the plausible role of *G. vaginalis* as the BV initial colonizer, enabling other BV-associated anaerobes to colonize the vagina after the initial biofilm development (Figure 1B). Importantly, *G. vaginalis* can also be found in healthy women, despite it is only present in lower amount in the vaginal epithelium (17). So, it was suggested that *G. vaginalis* biofilm induction is strictly needed to induce BV in women. Nevertheless,

understanding why *G. vaginalis* is also present in healthy women is a question that still remains to be answered.

The dilemma of vaginal colonization by *G. vaginalis* in healthy women

In the past decades, the presence of *G. vaginalis* in healthy women has been contradictory to the role of *G. vaginalis* as the main pathogen on BV (38). Back in 1955, Gardner and Dukes aimed to confirm Koch's postulates in BV by transferring *G. vaginalis* into the healthy vagina but BV did not develop in these cases (39). However, when they transferred the vaginal discharge from a BV patient, that nowadays we know to contain biofilms in clue cells, BV was then observed (39). Therefore, the association of the biofilm phenotype to *G. vaginalis* mediated BV development raised an important question: do all *G. vaginalis* possess the ability to develop a biofilm? In other biofilm-associated diseases, it has been widely demonstrated that not all strains within the same species have the ability to grow as biofilms (reviewed in 40). So, it's highly probable that not all *G. vaginalis* will be able to develop the BV associated biofilm in the woman vaginal epithelium, but this needs to be further explored. An important insight providing evidence that not all *G. vaginalis* have the same virulence potential was derived from a recent work by Swidsinski and colleagues. They highlighted the importance of *G. vaginalis* biofilms when they observed that only biofilm forming *G. vaginalis* were present in the partners of women with BV (41). They proposed that the mere presence of loosely adherent *G. vaginalis* on the vaginal epithelium was of lesser clinical significance and BV was sexually transmissible only in the presence of high density clusters of *G. vaginalis*. Castro and colleagues recently provided in vitro evidence that supports Swidsinski hypothesis (42). By comparing 7 BV and 7 non-BV isolates of *G. vaginalis*, they observed that only the BV isolates were able to adhere in high density clusters to a HeLa cell line, a condition necessary to foster biofilm development.

The genetic characterization of *G. vaginalis* virulence properties could be a possibility to confirm the existence of pathogenic and non-pathogenic *G. vaginalis* subspecies and consequently to distinguish between them. Harwich and colleagues were the first to sequence the whole genomes of BV and non-BV *G. vaginalis* isolates and compared their pathogenic potential properties (43). In addition, they also quantified, in vitro, their cytotoxic activities, adhesion ability to epithelial cells and biofilm formation. Their study revealed the differences in the *vly* genes between the BV-associated and the non-BV strains, showing differences in the proteins encoded by these genes and their promoters. Also, the differences in cytotoxicity appeared to be related to the adherence function, in which BV-associated isolate adhered more noticeably to the epithelial cells than non-BV isolate (43). Finally, these two isolates also showed differences in biofilm forming ability related to the biofilm associated protein (BAP) family gene, such as, central repeat region, its number and distribution of the Rib domains (43). Meanwhile, another comparative genomic study between *G. vaginalis* strains from BV and healthy patients revealed that BV isolates encode numerous proteins not found in healthy isolates (44). These proteins include enzymes enabling mucin degradation, a trait typically correlated with BV. On a subsequent study, Jayaprakash and colleagues attempted to classify *G. vaginalis* subspecies through a phylogenetic tree of *cpn60* universal target sequences analysis (45). This subdivision revealed the presence of 4 subgroups representing *G. vaginalis* subspecies with different extension of virulent properties, specially sialidase presence, and they proposed that only 1 of the 4 subgroups would be involved in BV (45). Sialidase is usually associated in the degradation of several key mucosal protective factors, such as mucins, as well as contributing to exfoliation and detachment of vaginal epithelial cells (46). All this data supported the hypothesis that certain *G. vaginalis* subspecies are unable to induce BV while other strains are suited to establish *G. vaginalis*'s biofilm and eventually to elicit BV, but this clearly needs to be further studied. So, genomic sequencing and bacterial interaction studies on BV initial adhesion and biofilm development are essential to clarify BV etiology.

The contribution of in vivo models to understand BV

Despite some earlier human studies (39,47), not many in vivo studies have been reported regarding BV. Many of the bacterial species associated with BV have evolved to exist only in humans and consequently a reliable animal model for multispecies BV development does not exist. In vivo studies are limited in its complexity and lacks host-specific factors, such as, anti-inflammatory cytokines, mucosal

immune response, nutrient environment conditions, types and proportion of lactobacilli species in the vaginal epithelium (48).

Recently, Gilbert and colleagues proposed a murine model for vaginal infection with *G. vaginalis* (49). However, this animal model was only tested with *G. vaginalis* and no further studies have been performed with other BV-associated anaerobes or human host-specific factors were evaluated, excepting for clue cell formation. Nevertheless, they were able to demonstrate an association between *G. vaginalis* and the occurrence of BV sign on mice, such as the presence of sialidase activity and thus exfoliated epithelial cells with adherent bacteria (49). Unfortunately, this animal model showed absence of histological inflammation or any other host-specific factor. Similar problems occur in in vitro models that fail to mimic several factors associated with BV infection, such as local cytokine production and proinflammatory immune response of the human host (50). As is, due to the absence of reliable animal models for multispecies BV development studies, we still need to rely in the best possible accurate in vitro models, in order to evaluate the virulence capacities of *G. vaginalis* and other BV-associated anaerobes.

Conclusions and future directions

Even taking in consideration the limitations in the study of multispecies biofilms in BV, current data suggest that certain anaerobic species can cooperate and establish differently types of bacterial relationships with *G. vaginalis* biofilms, during BV development. *G. vaginalis* appears to be the most virulent BV-associated anaerobe, demonstrating greater adherence to vaginal cells, cytotoxicity and biofilm-producing capacity. These virulence factors suggest that *G. vaginalis* plays a key role in BV biofilms development. Its great ability to adhere to vaginal epithelial cells and produce a biofilm may help to initiate BV establishment as well as facilitate the adherence and growth of other BV-associated anaerobes. Increasing evidence makes it plausible that *G. vaginalis* acts as the primary colonizer in the vaginal epithelium and eventually establish symbiotic relationships in the presence of potential intermediate anaerobes. The role of this secondary species is the question that now needs to be addressed in future research into *G. vaginalis* biofilms analysis. Also, further comparative genomic studies should be performed in *G. vaginalis* isolated from BV or from healthy woman, for a better clarification of their interactions with other bacterial species and properties against human host-specific factors. These complex and diverse bacterial-host interactions may explain BV symptoms, such as the sloughing of clue cells and vaginal inflammatory immune responses. In future studies, the transcriptomic and metabolic analysis of the interactions between BV-associated anaerobes, such as co-aggregation, co-adherence to vaginal cells and growth in a multispecies biofilm, could be the key to better understand BV etiology.

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Conflict of interests

The authors have a patent registered at the Portuguese patent office (INPI – process number 20121000090117) related to the molecular diagnose of BV by a PNA-FISH multiplex probe.

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