# Etiology of bacterial vaginosis and polymicrobial biofilm formation

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## Abstract

Microorganisms in nature rarely exist in a planktonic form, but in the form of biofilms. Biofilms have been identified as the cause of many chronic and persistent infections and have been implicated in the etiology of bacterial vaginosis (BV). Bacterial vaginosis is the most common form of vaginal infection in women of reproductive age. Similar to other biofilm infections, BV biofilms protect the BV-related bacteria against antibiotics and cause recurrent BV. In this review, an overview of BV-related bacteria, conceptual models and the stages involved in the polymicrobial BV biofilm formation will be discussed.

Keywords: biofilm, BVAB, BV, *Gardnerella vaginalis*, pathogenesis

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## Introduction

The human vagina is a dynamic, yet balanced ecosystem that consists of different

microbes called the vaginal microbiota (Hyman et al. 2005; Kim et al. 2009).  The

vagina contains a complex population of aerobic and anaerobic bacteria that can reach

up to $10^9$ colony forming units (CFU) per ml of vaginal fluid in healthy women (Bartlett

et al. 1977; Bartlett & Polk 1984).  In most women, the normal vaginal microbiota are

typically dominated by *Lactobacillus* spp. (Kim et al. 2009), which produce lactic acid,

hydrogen peroxide ($H_2O_2$) (Hawes et al. 1996) and bacteriocins to inhibit the

colonization of other pathogenic microorganisms (Aroutcheva, Simoes, Behbakht, et al.

2001).  Lactobacilli promote a healthy vaginal environment by acidifying the vaginal

environment with lactic acid to a pH of 3.5 to 4.5 (O'Hanlon et al. 2013).  It is also

known that lactobacilli enhance the barrier function of the epithelial cells and stimulate

the host innate immunity (Karczewski et al. 2010).  A disturbed vaginal microbiota due

to hormonal changes, douching and sexual activity may lead to vaginal colonization

with potentially pathogenic bacteria, typically those causing bacterial vaginosis (BV)

(Hawes et al. 1996), aerobic vaginitis (AV) (Donders et al. 2002) and sexually

transmitted infections (STIs) (Cherpes et al. 2008).

Bacterial vaginosis is the most common reproductive tract infection in women of reproductive age worldwide (Koumans et al. 2007; Kenyon et al. 2013). Bacterial vaginosis could either be symptomatic or asymptomatic and the rate of symptomatic BV may range from 10% to 66% (Begum et al. 2003; Klebanoff et al. 2004; Schwebke & Desmond 2007). Women with symptomatic BV may experience symptoms of vaginal discomfort (including vaginal or perineal itching) and an increased amount of thin, foul-smelling, homogenous discharge after unprotected sexual intercourse or at the time of menstruation (Klebanoff et al. 2004). In contrast, women with asymptomatic BV report no typical BV symptoms and are not generally treated (Klebanoff et al. 2004; Schwebke & Desmond 2007).

Bacterial vaginosis is clinically important, because of the many adverse health sequelae associated with this condition (Hillier et al. 1995; Ralph et al. 1999; Wilson et al. 2002; Haggerty et al. 2004; Ness et al. 2005). The microbiota associated with BV produce several immunomodulatory [hemolysins, volatile (VFAs) and non-volatile fatty acids (NVFAs), proteases and sialidases] (Mattsby-Baltzer et al. 1998; Cauci et al. 2002; Mirmonsef et al. 2012) and proinflammatory substances (lipopolysaccharides) that induce the production of proinflammatory cytokines [such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)], which may consequently result in preterm birth in pregnant women (Yeganegi et al. 2009). Sialidase activity may inhibit serum immunoglobulin A (sIgA), which allows cytotoxins to induce premature rupture of membranes (PROM) (Cauci et al. 2002). Finally, sialidase and prolidase may also contribute to the degradation and detachment of vaginal epithelium and mucins, as well as the increase of IL-1β, IL-8 and TNF-α that makes the host more susceptible to human

immunodeficiency virus 1 (HIV-1) (Sturm-Ramirez et al. 2000; Cauci et al. 2008) and herpes simplex virus 2 (HSV-2) (Cherpes et al. 2003).

Microbiologically, BV is typically characterized by a reduction of $H_2O_2$-producing lactobacilli and an overgrowth of *Gardnerella vaginalis* and other anaerobic bacteria like *Atopobium vaginae*, *Bacteroides* spp., *Mobiluncus* spp. and *Prevotella* spp. (Fredricks et al. 2005; Ling et al. 2010). These anaerobic bacteria have synergistic interactions during BV infection and form a polymicrobial biofilm, which is considered as one of the factors contributing to recurrent BV (Swidsinski et al. 2008).

Biofilm formation is one of the effective strategies of microorganisms to survive in unfavorable environments (Sauer et al. 2002; Sapi et al. 2012). Biofilms can be formed everywhere, either on biotic or abiotic surfaces, including living or dead tissues or medical devices like urinary catheters (Ganderton et al. 1992; Kubota et al. 2008; Lee et al. 2008). Biofilms can play an important pathologic role during the disease process, either by preventing full penetration of antibiotics and/or by allowing microorganisms to escape host defense mechanisms (Singh et al. 2010; Thurlow et al. 2011). The incomplete eradication of biofilms by antibiotics and host defenses allows pathogenic bacteria to develop resistance to antibiotic treatment and finally lead to recurrent infections (Swidsinski et al. 2008; Swidsinski et al. 2011).

This review will provide an overview of the bacteria associated with BV (BVAB and BV-related bacteria), currently available conceptual models and theories, and the processes involved in the formation of a polymicrobial BV biofilm in three main sections. In this review, the term "BV-related bacteria" or "BV-related anaerobes" will be used to distinguish other bacteria associated with BV from "BV-associated bacteria

1, 2 and 3 (BVAB1, BVAB2 and BVAB3)" that was identified and named by Fredricks et al. (2005).

**Bacteria associated with BV and their roles in BV etiology**

Bacterial vaginosis is a polymicrobial syndrome that involves multiple facultative anaerobic and strict anaerobic bacteria (Hillier et al. 1993; Fredricks et al. 2005). Frequently described BV-related anaerobes include *A. vaginae*, *Bacteroides* spp., *G. vaginalis*, *Mobiluncus* spp., *Mycoplasma hominis* and *Prevotella* spp. (Hillier et al. 1993; Fredricks et al. 2005). The development of culture-independent molecular techniques like broad-range 16S ribosomal DNA (rDNA) PCR (Fredricks et al. 2005), quantitative real-time PCR (qPCR) assays (Zozaya-Hinchliffe et al. 2010), denaturing gradient gel electrophoresis (DGGE) and 454 pyrosequencing (Verstraelen et al. 2004; Ling et al. 2010) have improved the detection of uncultivable anaerobes, such as BVAB1, BVAB2, BVAB3, *Eggerthella* spp., *Megasphaera* type 1, *Leptotrichia* spp. and *Sneathia* spp. from BV-positive women. BV-related bacteria may have a synergistic relationship with each other during BV development and may also participate in the formation of a polymicrobial BV biofilm, which plays an important role in BV etiology (Alves et al. 2014; Castro & Cerca 2015).

*Gardnerella vaginalis*

*Gardnerella vaginalis* is a facultative anaerobic, Gram-negative to Gram-variable, non-motile, small pleomorphic rod (Greenwood & Pickett 1980). Although *G. vaginalis* may not grow at pH 4 (Greenwood & Pickett 1979; Breshears et al. 2015), *G. vaginalis* can tolerate a vaginal environment with a high oxidation-reduction (redox) potential and can adhere to vaginal epithelial cells at a vaginal pH between 4 to 5, unlike strict BV

anaerobes like *Prevotella* spp. (Sobel et al. 1981; Udayalaxmi et al. 2012; Muzny &

Schwebke 2013). *Gardnerella vaginalis* possesses several virulence factors, including

biofilm formation, bacteriocins, vaginolysins, sialidases and proteases (Harwich et al.

2010; Yeoman et al. 2010; Udayalaxmi et al. 2011) (See Table 1 for more details).

There is no doubt that *G. vaginalis* is associated with BV and is involved in the

formation of the polymicrobial BV biofilm (Swidsinski et al. 2005; Machado, Jefferson,

et al. 2013). However, some *G. vaginalis* strains can be isolated from the vagina of

"healthy" women and sexually inexperienced women and do not necessarily cause BV

(Aroutcheva, Simoes, Behbakht, et al. 2001; Fethers et al. 2012). This phenomenon

may be explained by a genetic difference between commensal and pathogenic strains of

*G. vaginalis*, as reported by several functional and genomic studies (Harwich et al.

2010; Yeoman et al. 2010; Castro et al. 2015). The pathogenic strains show greater

cytotoxicity, greater adherence and biofilm formation ability than the commensal strains

(Harwich et al. 2010; Yeoman et al. 2010; Patterson et al. 2010; Castro et al. 2015).


*Atopobium vaginae*

*Atopobium vaginae* is a facultative anaerobic, non-motile, small elongated Gram-

positive coccus that occur as either single cells, in pairs, or in short chains (Jovita et al.

1999). *Atopobium vaginae* was first isolated and described in 1999 when it was

identified as the cause of an abscess in the fallopian tube and the ovary of a woman who

had undergone transvaginal oocyte recovery (Jovita et al. 1999; Geißdörfer et al. 2003).

An association of *A. vaginae* with BV was first reported in 2004 (Ferris et al.

2004; Verstraelen et al. 2004). A study using DGGE by Ferris et al. (2004)

demonstrated that *A. vaginae* was detected in 55% (12/20) of BV-positive patients,

while only 8.3% (2/24) was detected in BV-negative patients. In the same year,

Verstraelen et al. (2004) reported similar findings using 16S rDNA cloning and sequencing, detecting *A. vaginae* in 61.3% (8/13) of disturbed vaginal microbiota and in 3.4% (3/87) of normal vaginal microbiota. *Atopobium vaginae* is suggested as a marker for abnormal vaginal microbiota and BV as *A. vaginae* is present in more than 80% of BV cases (Bradshaw et al. 2006; De Backer et al. 2007; Marconi et al. 2012). Women with *A. vaginae* are 2.6 times [95% confidence intervals (CI): 0.6 – 11.4] and 19.2 times (95% CI: 3.7 – 98.7) more likely to be categorized as BV by using the Amsel's criteria and the Nugent scoring system (Haggerty et al. 2009). In addition, *A. vaginae* is rarely detected in BV patients without *G. vaginalis* and this co-existence is also detected in patients with recurrent BV (Verhelst et al. 2004; Bradshaw et al. 2006; Trama et al. 2008). However, *A. vaginae* is also commonly found in women with normal vaginal microbiota – this may also be explained by *A. vaginae* strain diversity as recently reported (Mendes-Soares et al. 2015).

Studies have found that *A. vaginae* is one of the dominant bacterial species (along with *G. vaginalis*) in the polymicrobial BV biofilm and may represent 1% to 54.1% of the biofilm mass (Swidsinski et al. 2005; Swidsinski et al. 2008; Hardy et al. 2016). This biofilm may allow *A. vaginae* to be resistant to metronidazole treatment, as metronidazole does not fully eradicate *A. vaginae* in the biofilm and this resistance may explain why *A. vaginae* often reemerges in recurrent BV (Swidsinski et al. 2008; McMillan et al. 2011; Mayer et al. 2015).

### *Bacterial vaginosis-associated bacteria (BVAB)*

In 2005, three bacterial species were newly identified in the vaginal fluid of BV-positive women and were named as BVAB1, BVAB2 and BVAB3 (Fredricks et al. 2005). These bacteria were related to bacteria in the phylum *Clostridium* (the order

*Clostridiales*), but were detected only in the vaginal fluid of women with BV (Fredricks et al. 2005). Fluorescence *in situ* hybridization (FISH) revealed that BVAB1 appear as thin, curved bacteria; short, straight rods for BVAB2 and long, wide and straight rods for BVAB3 – these BVAB were attached to vaginal epithelial cells in a similar appearance as "clue cells" (Fredricks et al. 2005). A recent study by Muzny et al. (2014) suggested that BVAB1 could be the dominant curved rods in vaginal smears with BV and not *Mobiluncus* spp., which are often identified as curved rods in Nugent scoring. In addition, BVAB1 is associated with a positive whiff test (fishy odor) and are thus thought to produce polyamines (putrescine, cadaverine and trimethylamine), which are associated with the fishy odor (Wolrath et al. 2001; Srinivasan et al. 2012). To date, only BVAB3 have been cultured and biochemically characterized (Austin et al. 2015) and the remaining two BVAB (BVAB1 and BVAB2) have not yet been isolated by culture. The species name of BVAB3 was proposed as *Mageeibacillus indolicus* (Austin et al. 2015). The involvement of BVAB in the formation of a polymicrobial BV biofilm has not yet been confirmed and consequently, the role of BVAB in BV etiology has not been sufficiently investigated.

### *Other BV-related bacteria*

Along with the above-mentioned bacteria, other BV-related bacteria may contribute to BV development. Other bacteria associated with BV may include *Anaerococcus* spp., *Bacteroides* spp. (*B. levii*, *B. ureolyticus*), *Dialister* spp., *Eggerthella* spp., *Finegoldia* spp., *Fusobacterium nucleatum*, *Leptotrichia* spp., *Megasphaera* spp., *Mobiluncus* spp., *Peptococcus* spp., *Peptoniphilus* spp., *Peptostreptococcus* spp. (*P. anaerobius*, *P. prevotii*, *P. tetradius*), *Prevotella* spp. (*P. amnii*, *P. bivia*, *P. buccalis*, *P. corporis*, *P. disiens*), *Mycoplasma hominis*,

*Sneathia* spp., *Ureaplasma urealyticum* and viridans streptococci (*S. acidominimus*, *S. constellatus*, *S. intermedius*, *S. mitis*, *S. morbillorum*, *S. mutans*, *S. sanguis* II) (Hillier et al. 1993; Zozaya-Hinchliffe et al. 2010; Srinivasan et al. 2012). The list of bacteria associated with BV may become longer as new culture-independent techniques identify additional bacterial species associated with BV, as was shown in a recent study (Xia et al. 2016).

In order to assist with BV development, BV-related bacteria may produce amino acids, metabolic products (such as VFAs and NVFAs and polyamines) and enzymes (Chen et al. 1979; Briselden et al. 1992; Al-Mushrif et al. 2000), and possibly contribute in polymicrobial BV biofilm formation (Patterson et al. 2010; Machado, Jefferson, et al. 2013) (Table 1). The VFAs produced by BV-related bacteria as a result of the fermentation of lactic acid include formic and acetic acids produced by *Actinomyces* spp., *Eggerthella lenta*, *Mobiluncus* spp. and *Prevotella* spp.; propionic acids produced by *Megasphaera* spp. and *Propionibacterium* spp.; butyric, isobutyric, valeric and isovaleric acids by *Mobiluncus* spp. and *Prevotella* spp. (Kageyama et al. 1999; Al-Mushrif et al. 2000; Wang et al. 2012). Similarly, NVFAs like succinic acid may be produced by *Actinomyces* spp., anaerobic Gram-positive cocci, *Eggerthella* spp., *Mobiluncus* spp. and *Prevotella* spp. (Pybus & Onderdonk 1998; Kageyama et al. 1999; Al-Mushrif et al. 2000). *Mobiluncus* spp. can also produce malic acid (NVFA) that causes irritation of vaginal mucous membranes (Spiegel & Roberts 1984). Gram-negative anaerobes like *Bacteroides* spp., *Leptotrichia* spp., *Megasphaera* spp. and *Prevotella* spp. are sialidase producers (Briselden et al. 1992; Marconi et al. 2013; Doerflinger et al. 2014). In addition to sialidase, glycosulfatase, collagenase and fibrinolysins produced by *Prevotella* spp. and *Sneathia* spp. (Roberton et al. 2005;

Lewis & Lewis 2012), and the glycosidase and proteinase produced by anaerobic Gram-negative bacteria are examples of enzymes that degrade mucin in the vaginal epithelial cells and promotes vaginal sloughing (Olmsted et al. 2003). *Bacteroides* spp., *Finegoldia* spp., *Peptostreptococcus* spp. and *Propionibacterium* spp. may also produce 5-nitroimidazole reductase from the *nim* genes, which promotes metronidazole resistance (Lubbe et al. 1999; Theron et al. 2004).

*Peptoniphilus* spp. may help the biofilm formation of *G. vaginalis* during initial adhesion (Patterson et al. 2010; Castro et al. 2013; Machado, Jefferson, et al. 2013). The genus *Peptoniphilus* was proposed in 2001 (Ezaki et al. 2001) and has been associated with persistent BV as *Peptoniphilus* spp. were found in 87% (27/31) of persistent BV cases in a study by Marrazzo et al. (2008) and the risk for persistent BV was significantly higher in women if *Peptoniphilus* spp. were detected at baseline (before treatment) [risk ratio (RR) 1.7; 95% CI: 0.8 – 8.3].  In the same study, the risk for persistent BV was higher for women if *P. lacrimalis* were detected before treatment [RR 3.5; 95% CI: 1.6 – 15.5] (Marrazzo et al. 2008)**.**

**Current conceptual models and new theories in BV etiology**

Many conceptual models and theories have been proposed for the etiology of BV, yet the exact mechanism of how BV is triggered remains controversial (Schwebke, Muzny, et al. 2014).  There are currently three major conceptual models that are proposed for the etiology of BV, i.e. the "*Lactobacillus* depletion model", the "primary pathogen model" and the "polymicrobial pathogen model" (Table 2) (Srinivasan & Fredricks 2008; Muzny & Schwebke 2013).  These models share characteristic features like the reduction of lactobacilli, the exposure and overgrowth of BVAB and BV-related bacteria, and the increase in the vaginal pH (Srinivasan & Fredricks 2008; Muzny &

Schwebke 2013).  In the "*Lactobacillus* depletion model", the depletion of $H_2O_2$-producing lactobacilli and a simultaneous rise in the vaginal pH have to occur first to trigger the overgrowth of the introduced BV-related anaerobic bacteria, which then results in BV (Srinivasan & Fredricks 2008).  In the "primary pathogen model" and the "polymicrobial pathogen model", the exposure to pathogen/s leads to the displacement of vaginal lactobacilli that in turn leads to BV (Muzny & Schwebke 2013).  The formation of a polymicrobial biofilm is thought to play a key role in BV etiology, regardless of which conceptual model is followed (Swidsinski et al. 2005; Swidsinski et al. 2013; Muzny & Schwebke 2013).  In the following subsections, three conceptual models and one new theory in BV etiology will be discussed.

### *Lactobacillus depletion model*

Lactobacilli have been considered to be a part of normal vaginal microbiota since 1892, when Albert Döderlein first isolated and described an organism ("Döderlein's bacillus", identified later as *Lactobacillus acidophilus*) from the vaginal fluids of asymptomatic pregnant women (Döderlein 1892; Thomas 1928).  In 1997, Pavlova et al. (1997) suggested that the reduction of vaginal lactobacilli must be induced by external factors to trigger a rise in the vaginal pH (>4.5) and the overgrowth of BV-related bacteria.

In some women, the absence of $H_2O_2$-producing lactobacilli strains is associated with the acquisition of BV (Hawes et al. 1996; Cherpes et al. 2008). Studies show that vaginal lactobacilli possess multiple control mechanisms to maintain the "healthy" vaginal environment, such as: (i) vaginal acidification *via* lactic acid production (Boskey et al. 2001); (ii) interference of adhesion and growth of pathogenic bacteria (Velraeds et al. 1996; Coudeyras et al. 2008; Castro et al. 2013); (iii) production of

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H$_2$O$_2$ and bacteriocins (Klebanoff et al. 1991; Aroutcheva, Simoes, & Faro 2001) and (iv) biofilm formation (Terraf et al. 2014; Ventolini et al. 2015). Major disturbances of the vaginal lactobacilli may occur due to excessive douching (Pavlova & Tao 2000), use of spermicides (nonoxynol-9) (Hooton et al. 1991), antibiotics (Vallor et al. 2001) and natural causes like phages (Pavlova et al. 1997; Tao et al. 1997; Kiliç et al. 2001). Frequent douching is significantly associated with the lack of the H$_2$O$_2$-producing lactobacilli (≥1 times per month: OR 1.6; 95% CI: 1.2 − 2.1 and ≥2 times per month: OR 2.5; 95% CI: 1.1 − 6.0) (Ness et al. 2002; Beigi et al. 2005) and the acquisition of BV (douching for hygiene: RR 2.1; 95% CI: 1.0 − 4.3; p = 0.05 and douching within the past two months: adjusted OR 2.9; 95% CI: 1.5 − 5.6) (Hawes et al. 1996; Holzman et al. 2001). The usage of spermicides (such as nonoxynol-9) may also possibly reduce H$_2$O$_2$-producing lactobacilli, as the H$_2$O$_2$-producing strains of lactobacilli are more susceptible to nonoxynol-9 than non-producing strains (Hooton et al. 1991). Alternatively, bacteriophages may reduce vaginal lactobacilli (Pavlova et al. 1997; Tao et al. 1997; Kiliç et al. 2001). Pavlova et al. (1997) and Kiliç et al. (2001) found that the proportion of lysogenic phages is higher among *Lactobacillus* strains isolated from BV patients and that some of the lysogenic phages may become virulent (the lytic stage) in other vaginal lactobacilli strains due to genetic mutations that may eliminate lactobacilli in the vagina (Pavlova et al. 1997; Kiliç et al. 2001). However, Martín et al. (2009) proposed that the lysogeny of bacteriophages in lactobacilli does not play a significant role in the elimination of lactobacilli, as less than half of the 15 prophages that were studied by them lacked genes for the development of a lytic cycle. No further study has confirmed this finding in the context of vaginal lactobacilli.

In the "*Lactobacillus* depletion model", the vaginal pH becomes higher (>4.5) once the normal vaginal microbiota (vaginal lactobacilli) are disturbed, as the main source for acidity in the vagina is the lactic acid-producing lactobacilli (Boskey et al. 1999; Boskey et al. 2001; O'Hanlon et al. 2013). Furthermore, the BVAB and other BV-related bacteria like *G. vaginalis*, *Mobiluncus* spp., *Peptostreptococcus anaerobius* and *Prevotella* spp. may utilize lactic acid to produce butyric acids (VFA), succinic acid (NVFA) and other acids to modulate immune responses and to inhibit the killing activity of the remaining lactobacilli (Pybus & Onderdonk 1996; Al-Mushrif et al. 2000; Macklaim et al. 2013), while *Prevotella amnii*, *Dialister* spp. and *Megasphaera* spp. may produce polyamines (putrescine and spermidine) to relieve the excess acidity due to lactic acid (Chen et al. 1979; Macklaim et al. 2013).

The major concern against the "*Lactobacillus* depletion model" is that this model is based on the notion that women are colonized by *Lactobacillus* spp. to maintain the "healthy" vaginal environment. According to a large-scale US cross-sectional study, 27% of "healthy" women harbored vaginal microbiota that lack a large number of lactobacilli and included facultative or strict anaerobic bacteria [community state types IV (CST IV)] (Ravel et al. 2011). Smaller-scaled studies also confirmed this finding (Zhou et al. 2004; Gajer et al. 2012). As lactic acid-producing strains of *Atopobium* spp., *Leptotrichia* spp. and *Megasphaera* spp. are found in some women who still maintained "healthy" vaginal environments, the presence of non-*Lactobacillus* vaginal microbiota and the lack of vaginal lactobacilli may not necessarily cause BV (Zhou et al. 2004; Gajer et al. 2012).

*Primary pathogen model*

The "primary pathogen model" originated from a study by Gardner and Dukes in 1955, in which *Haemophilus vaginalis* (*G. vaginalis*) was successfully isolated from a BV patient and was proposed as the sole etiological agent for BV (Gardner & Dukes 1955).  Other studies have also confirmed the existence of *G. vaginalis* (up to 96%) from the vaginal fluid of women with BV (Marrazzo et al. 2008; Srinivasan et al. 2012) and the causation of BV symptoms like vaginal sloughing by *G. vaginalis* in an *in vivo* murine model (Gilbert et al. 2013).

In contrast to the "*Lactobacillus* depletion model", the "primary pathogen model" suggests that the sexual introduction of *G. vaginalis* into the vaginal environment creates the "intermediate microbiota" with a reduced number of lactobacilli, which causes a decrease in the redox potential of the vagina (increase in the vaginal pH) and triggers the overgrowth of vaginal anaerobic bacteria (Muzny & Schwebke 2013).  An extensive version of this model was proposed by Schwebke et al. (2014) that suggests *G. vaginalis* as the key organism in BV etiology.  According to Schwebke et al. (2014), *G. vaginalis* is not part of the normal vaginal microbiota, but is introduced through sexual activity.  Schwebke et al. (2014) also added that *G. vaginalis* may survive in the male urethra and semen, which may later be transmitted back to female partners through sexual contact.

According to the "primary pathogen model", *G. vaginalis* first tolerates a high oxidation-reduction (redox) potential vaginal environment (pH 4 to 5) and adheres to vaginal epithelial cells (Sobel et al. 1981; Udayalaxmi et al. 2012; Muzny & Schwebke 2013).  *Gardnerella vaginalis* may form biofilms during this stage (Udayalaxmi et al. 2012) and may produce antagonistic substances to displace lactobacilli (Nagy et al. 1991).  *Gardnerella vaginalis* produces amino and keto acids that enhance the growth

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of BV anaerobes (Chen et al. 1979), in order to create the "intermediate vaginal microbiota (IVM)".  The overgrowth of the "IVM" (including *P. bivia*) raises the vaginal pH (>4.5), as amino acids like arginine serve as a precursor for the production of ammonia and polyamines, such as putrescine, cadaverine and tyramine (Wolrath et al. 2001; Srinivasan et al. 2015), which further stimulates the growth of *G. vaginalis* and other BV-related anaerobes and confirms the establishment of the BV microbiota (Pybus & Onderdonk 1997; Pybus & Onderdonk 1998; Muzny & Schwebke 2013).

The "primary pathogen model" is not free of argument against it, as *G. vaginalis* can still be commonly found in the vaginal microbiota of women without BV (Hyman et al. 2005; Schwebke, Flynn, et al. 2014) and even in sexually inexperienced women (Fethers et al. 2012).  These findings may indicate that *G. vaginalis* can be a common inhabitant of the vaginal microbiota in some women without causing BV (Fethers et al. 2012; Schwebke, Flynn, et al. 2014).  The possible explanation for this can be the existence of different genotypic strains (Lopes Dos Santos Santiago et al. 2011; Jayaprakash et al. 2012) and biotypes (Piot et al. 1984) – especially biotype 5 (Piot's biotype) is significantly associated with the normal vaginal microbiota (Aroutcheva, Simoes, Behbakht, et al. 2001).

*Polymicrobial pathogen model*

The "polymicrobial pathogen model" is similar to the "primary pathogen model", but is different in that the sexual exposure of BV-related bacteria (including *G. vaginalis*; both from men and women) to the vaginal microbiota directly leads to the simultaneous displacement of lactobacilli and a BV condition without having "intermediate microbiota" (Muzny & Schwebke 2013).  Instead of a single primary pathogen like *G. vaginalis* in the "primary pathogen model", a consortium of BV-

related anaerobes may show antibiosis against lactobacilli or produce amino acids and other virulence factors to enhance the growth of BV-related anaerobes (Nagy et al. 1991; Pybus & Onderdonk 1996). According to a study by Nagy et al. (1991), some strains of *M. curtisii*, *Peptostreptococcus* spp., *Prevotella bivius* and *P. disiens* can inhibit the growth of lactobacilli, possibly indicating the ability of BV-related anaerobes to displace lactobacilli. *Mobiluncus* spp. may also produce cytotoxins that could damage vaginal epithelial cells similar to *G. vaginalis* (Taylor-Robinson et al. 1993). Furthermore, *P. bivia* and *Bifidobacterium* spp. can produce amino acids to enhance the growth of other anaerobic bacteria like *Eggerthella lenta* and *G. vaginalis* (Pybus & Onderdonk 1996; Africa et al. 2014).

The "polymicrobial pathogen model" is supported by the fact that BV involves multiple anaerobic bacteria and that the BV microbiota are taxonomically more diverse than normal vaginal microbiota (Ling et al. 2010; Srinivasan et al. 2012). Even the original study by Gardner & Dukes (1955), where the authors suggested *G. vaginalis* as the sole etiological agent for BV, reported that one or more other bacteria than *G. vaginalis* were isolated from 56 BV-positive women (56/141; 39.7%) and that eleven women developed BV without the presence of *G. vaginalis*. Furthermore, Gardner & Dukes (1955) reported that the inoculation of women with vaginal fluid was more successful in causing BV symptoms than inoculation with a *G. vaginalis* pure culture (11/15; 73% versus 1/13; 8%) (Gardner & Dukes 1955).

The involvement of a polymicrobial biofilm in BV is evident, as the BV biofilms found on the vaginal epithelium do not only contain *G. vaginalis*, but also a variety of BV-related bacteria, such as *A. vaginae*, *Bacteroides* spp., *Streptococcus* spp. and *Veillonella* spp. (Swidsinski et al. 2005). In fact, a recent study highlighted the

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synergy between *A. vaginae* and *G. vaginalis* in the formation of a BV biofilm, indicating the polymicrobial nature of this biofilm (Hardy et al. 2016). Furthermore, *Mobiluncus* spp. may also possibly participate in the formation of a polymicrobial BV biofilm, as *Mobiluncus* spp. (*M. curtisii* and *M. mulieris*) are able to form a biofilm (De Boer & Plantema 1988; Costello et al. 2007).

Similar to the above-mentioned two conceptual models, there also exists a controversy in this model. As previously demonstrated by several studies (Patterson et al. 2010; Machado, Jefferson, et al. 2013; Alves et al. 2014), many BV-related bacteria may not be as virulent as pathogenic strains of *G. vaginalis*, in terms of cytotoxicity, adherence and biofilm formation. Therefore, more evidence is needed to prove that the primary trigger that initiates BV is indeed a polymicrobial consortium of BV-related bacteria.

### *Internalization of BV-related bacteria in the vaginal epithelial cells: a new escaping mechanism?*

The internalization theory of the BV-related bacteria was introduced only in 2012 and so far, there are only two studies that support this theory (Marrs et al. 2012; Fichorova et al. 2013). Marrs et al. (2012) suggested that *G. vaginalis* may use the internalization mechanism into vaginal epithelial cells as a strategy to escape the host defenses and antibiotics like clindamycin and metronidazole. According to their study, the uptake of live *G. vaginalis* cells by the VK2 vaginal epithelial cells was 21.8-fold higher than the uptake of *G. vaginalis* cells killed by heat. Furthermore, the internalization process of *G. vaginalis* was inhibited when the VK2 vaginal epithelial cells were pre-treated with cytochalasin-D, an inhibitor of actin polymerization in the cell cytoskeleton (Marrs et al. 2012). However, a recent study found that *G. vaginalis*

tends to exist in a dispersed form or forms an extracellular biofilm to escape immune

responses and was less efficiently internalized by dendritic cells and T lymphocytes

than lactobacilli (Bertran et al. 2016).  The internalization mechanism of *G. vaginalis*

into the vaginal epithelial cells thus requires further investigation.

Additionally, *A. vaginae* and *P. bivia* can maintain viability by internalizing into

the vaginal epithelial cells (Fichorova et al. 2013).  According to Fichorova et al. (2013),

*A. vaginae* and *P. bivia* can persist in high numbers on *Trichomonas vaginalis*-infected

vaginal epithelial cells and that these bacteria could use the internalization mechanism

as protection against antibiotics and attacks by *T. vaginalis*.  This mechanism warrants

further investigation as it may possibly be one of the factors leading to BV and also

recurrent BV.

*Additional theories for the development of BV*

Sexual activity is not the sole determinant for the occurrence of BV and as such

BV can be classified as a sexually associated infection rather than a sexually transmitted

infection (Leppäluoto, 2011). The alkaline buffering action of semen decreases the

acidity of the vagina (increase in pH) for several hours after intercourse (Boskey et al.,

1999) which, combined with the effects of vaginal coital transudate and vaginal

neurogenic transudate, could directly result in a physiological post-coital condition (i.e.

BV) for protection of ejaculated spermatozoa (Leppäluoto, 2011). This post-coital

condition is characterized by 'pure' *Gardnerella* flora replacing *Lactobacillus*; a

polymicrobial form of BV is suggested to be autoinfection of the post-coital

physiological *G. vaginalis* flora, mixed with *M. hominis* and anaerobic bacteria at lower

vaginal acidity (Leppäluoto, 2011).

In addition, genetic polymorphisms within a population may be contributing factors in the development of BV and its associated complications (Turovskiy et al., 2011; Witkin, 2007). An example may include the increased risk of women of African ethnicity to develop BV; however, the exact reasons for the predisposition of this population to BV have yet to be elucidated (Danielsson et al., 2011). Research on genetic polymorphisms and BV has up to now been confined to the innate immune system (Turovskiy et al., 2011); polymorphisms in genes coding for components of the innate immune system (e.g. IL-1$\beta$) have been shown to influence the quantitative bacterial composition of the vagina (Witkin, 2007). However, Turovskiy et al. (2011) have pointed out the inconsistent findings of the few studies that investigated the link between genetic polymorphisms and BV and indicated that the role of intrinsic host factors in the etiology of BV is still unclear.

**Biofilm formation in bacterial vaginosis**

It is well known that the polymicrobial biofilm in BV plays a key role in its pathogenesis (Muzny & Schwebke 2015). The importance of biofilms in BV was first highlighted in 2005 when Swidsinski et al. (2005) demonstrated that only *G. vaginalis* and *A. vaginae* produce a characteristic "adherent" biofilm in women with BV. This dense "adherent" biofilm found in BV patients contained short rods (*G. vaginalis* and *A. vaginae*) that were tightly attached to each other on the vaginal epithelial surface, "resembling brickwork" (Swidsinski et al. 2005). A polymicrobial BV biofilm may ensure the survival of *G. vaginalis* and other BV-related bacteria (Schwebke, Muzny, et al. 2014). Patterson et al. (2007) observed a greater tolerance to 5-fold higher concentrations of $H_2O_2$ and 4- to 8-fold higher concentrations of lactic acid in

*G. vaginalis* biofilms as compared to planktonic cells, which may suggest that biofilm formation contributes to the survival of *G. vaginalis*.

Biofilm formation is a dynamic and complex process that involves multiple interactions between single or multiple bacterial species (Whittaker et al. 1996; Machado, Jefferson, et al. 2013). To date, the exact process of the polymicrobial biofilm formation in BV remains unknown (Machado, Jefferson, et al. 2013). However, it can be hypothesized that the BV biofilm follows the same route as the formation of oral biofilms, which is a well-described example of a polymicrobial biofilm (Machado, Jefferson, et al. 2013). In the following subsections, the current review will attempt to discuss each stage of polymicrobial biofilm formation in the context of BV, namely: (i) adhesion; (ii) coaggregation (microcolony formation); (iii) maturation and (iv) dispersion.

### *Adhesion*

As the vaginal lactobacilli may interfere with the adhesion of BV-related bacteria by colonizing the vaginal epithelium (Velraeds et al. 1996; Coudeyras et al. 2008; Castro et al. 2013), it is possible that the BV-related bacteria first need to inhibit and to displace the colonized lactobacilli in order to initiate the biofilm formation process. In fact, through a yet unknown mechanism, BV-related anaerobes, such as *P. bivius*, *P. disiens*, *G. vaginalis*, *M. curtisii*, *M. mulieris* and *Peptostreptococcus* spp. may inhibit the growth of lactobacilli and may displace lactobacilli (except *L. iners*) (Nagy et al. 1991; Castro et al. 2013). It is, however, also possible that *G. vaginalis* may first need to initiate the biofilm formation process, as only *G. vaginalis* is able to strongly adhere to vaginal epithelial cells in the presence of *L. crispatus* and to form a biofilm regardless of the pH (Udayalaxmi et al. 2012; Machado, Salgueiro, et al. 2013).

As several reports indicate that *G. vaginalis* may act as an early colonizer species in the polymicrobial BV biofilm (Patterson et al. 2010; Machado, Salgueiro, et al. 2013; Alves et al. 2014), the current review will focus on the "primary pathogen model" in an attempt to explain the adhesion process of a polymicrobial BV biofilm.

Bacterial adhesion to vaginal epithelial cells may be mediated by interactions between cell appendages (pili, fimbriae or flagella), carbohydrates and cell surface adhesins (Peeters & Piot 1985; McMillan et al. 2012; Terraf et al. 2014). *Gardnerella vaginalis* harbors genes encoding type I, II and IV fimbriae/pili, as well as a biofilm-associated protein (BAP) family gene (*bap*L) (Harwich et al. 2010; Yeoman et al. 2010). The presence of pili was confirmed by an earlier electron microscopy study (Johnson & Davies 1984). The BapL protein is a BAP found in *Listeria monocytogenes* that is involved in surface attachment during biofilm formation (Jordan et al. 2008). According to Patterson (2010), the *bap*L gene, which encodes the BapL protein of *G. vaginalis*, is highly expressed only during biofilm formation, indicating the involvement of this protein in biofilm formation. In addition, partial genome and amino acid sequence analyses of the BapL have showed that the BapL contain nine Rib domains and a cell-wall anchoring motif (LPxTG) (Patterson 2010). The name "Rib domains", originated from the Rib cell surface protein (resistance to proteases, immunity and group B) of group B streptococci that contains repetitive elements (Stålhammar-Carlemalm et al. 1993). A whole genome sequencing study by Harwich et al. (2010) confirmed that the BapL protein of *G. vaginalis* contains Rib domains, which is highly repetitive in the central repeat region. Harwich et al. (2010) showed that the BV isolates of *G. vaginalis* (strains AMD, 101 and 551) tended to produce thicker biofilms than non-BV isolates (strains 5-1 and 465). It was proposed that the

number and distribution of Rib domain repeats within the central repeat region might have played a role in biofilm formation capacity, as the distribution of Rib domains differed between a BV isolate (strain AMD) and a non-BV isolate (strain 5-1) (Harwich et al. 2010).

Lactobacillus iners and Peptoniphilus spp. may assist G. vaginalis during the initial adhesion process (Castro et al. 2013; Machado, Jefferson, et al. 2013). Even though lactobacilli are known as indicator organisms for normal vaginal microbiota, L. iners is proposed as a transitional species indicating the vaginal microbiota shifting to the BV microbiota (Ferris et al. 2007; Yoshimura et al. 2011). Indeed, a study by Castro et al. (2013) revealed that L. iners enhanced the adherence of G. vaginalis to epithelial cells rather than inhibiting the bacteria. Peptoniphilus spp. show similar adherence ability as G. vaginalis on ME-180 vaginal epithelial cells (bacteria attached on 75% to 100% of ME-180 cells in all viewed microscopic fields) (Patterson et al. 2010). However, Peptoniphilus spp. have received almost no attention in research, despite the bacteria's good adherence ability and virulence potential in BV (Africa et al. 2014). Therefore, more studies confirming the virulence potential and the role of Peptoniphilus spp. during the initial adhesion process of BV biofilm formation would be beneficial in delineating the etiology of BV.

### Microcolony formation and coaggregation

After adhesion, G. vaginalis may first develop microcolonies before biofilm maturation, as demonstrated by Muli & Struthers (1998) and Hymes et al. (2013). Thin sections of the G. vaginalis biofilms grown in a Sorbarod biofilm system contained numerous microcolonies that were 30 μm to 50 μm in diameter (Muli & Struthers 1998). In addition, a study by Hymes et al. (2013) showed that G. vaginalis forms smaller

microcolonies with no extracellular polymeric substance (EPS) matrix when

*G. vaginalis* strains were grown on a glass surface in the presence of DNase, while

*G. vaginalis* formed a fully developed three-dimensional biofilm containing the EPS

matrix when grown without DNase.  This finding may thus suggest that the

microcolony formation of *G. vaginalis* may occur before the production of the EPS

matrix.

In oral biofilms, the attachment of one species is followed by the attachment of a

second colonizing species (coaggregation) (Nagaoka et al. 2008; Okuda et al. 2012).

The coaggregation may occur through two different mechanisms: (i) the second

colonizing species starts the coaggregation cascade first in the suspension and the

formed microcolonies subsequently bind on the surface of developing biofilms and/or

(ii) individual cells of a second colonizing species in the suspension recognize and

adhere to the bacterial cells of the early colonizing species, forming an aggregate in

developing biofilms (Rickard et al. 2003).  It is not known whether second/third

colonizing species in BV use the first or second mechanism of coaggregation; however,

it is possible that *A. vaginae, F. nucleatum* and *Mobiluncus* spp. may coaggregate with

*G. vaginalis* (De Boer & Plantema 1988; Patterson et al. 2010).  *Mobiluncus* spp. may

likely follow the first route, as *Mobiluncus* spp. can form microcolonies on its own (De

Boer & Plantema 1988).  Another possibility of coaggregation can be between the

uropathogenic bacteria like *Escherichia coli* and *Enterococcus faecalis* and the

*G. vaginalis* biofilms as the presence of *E. coli* and *E. faecalis* in the *G. vaginalis*

biofilms could enhance the growth of *G. vaginalis* (Castro et al. 2016).

The possible candidate bridge species between early and late colonizers may be

*F. nucleatum* (Patterson et al. 2010).  *Fusobacterium nucleatum* was originally

described as the causative organism of periodontal infections (e.g. gingivitis and chronic periodontitis), but this organism can also reside in the vagina (in both BV and non-BV cases) (Nikolaitchouk et al. 2008; Oakley et al. 2008; Cassini et al. 2013).

*Fusobacterium nucleatum* is well known as the bridge species between early and late colonizers in oral biofilms (Kaplan et al. 2009; Okuda et al. 2012). Although *F. nucleatum* does not adhere well on vaginal epithelial cells (Patterson et al. 2010), *F. nucleatum* is known to express receptors that facilitate coaggregation of *Prevotella* spp. and *Bifidobacterium* spp. (Nagaoka et al. 2008; Okuda et al. 2012). Alternatively, the following candidate bacteria may coaggregate as late colonizers as these bacteria have shown a synergistic interaction in a dual-species biofilm model: *Actinomyces neuii*, *Bacillus firmus*, *Brevibacterium ravenspurgense*, *Corynebacterium* spp., *Enterococcus faecalis*, *Escherichia coli*, *Nosocomiicoccus ampullae*, *P. bivia*, *Propionibacterium acnes*, *Staphylococcus* spp. and *Streptococcus* spp. (Castro & Cerca 2015).

### *Biofilm maturation*

After successful microcolony formation/coaggregation, *G. vaginalis* may release extracellular DNA (eDNA) that stimulates the EPS matrix production (Hymes et al. 2013). It is possible that the EPS production of BV-related bacteria may be controlled by quorum sensing molecules (molecules used in a special cell-to-cell signaling/communication mechanism, such as cyclic-di-GMP observed in *Pseudomonas aeruginosa*) (Borlee et al. 2010), though the quorum sensing in a polymicrobial BV biofilm has not yet been studied. The fully matured *in vivo* BV biofilm may appear like a "brickwork", a highly organized structure without spaces between bacterial cells (Swidsinski et al. 2005).

Extracellular DNA is thought to originate from lysed cells and provides structural integrity and stability to biofilms in *Bacillus cereus*, *L. monocytogenes* and *S. aureus* (Whitchurch et al. 2002). A study by Hymes et al. (2013) showed that the *G. vaginalis* biofilms contain the eDNA and that DNase treatment (which degrades eDNA) with a concentration of 100 μg/mL on *G. vaginalis* biofilms resulted in up to 80% reduction in biofilm mass. In addition, the authors also showed that the release of eDNA was maximal during the early exponential growth phase, which may indicate that eDNA is actively involved in the biofilm formation of *G. vaginalis* (Hymes et al. 2013).

In addition to eDNA, the pathogenic strains of *G. vaginalis* (317 and 594) may encode glycosyltransferases (GT) (family I, II and IV) that seem to be involved in EPS production (Yeoman et al. 2010). Glycosyltransferases are essential for the biosynthesis of EPS in bacteria, such as *E. faecalis* (Theilacker et al. 2011) and *Xanthomonas campestris* (Tao et al. 2010) and are thought to be essential in *G. vaginalis* as well (Yeoman et al. 2010).

### *Dispersion*

Dispersion is an essential step in biofilm formation that contributes to bacterial survival and disease transmission (Stoodley et al. 2001). The exact dispersal mechanism of polymicrobial BV biofilm is not known as it has not yet been studied. However, it can be hypothesized that both active and passive dispersion might occur during BV. Active dispersion might occur when the vaginal environment becomes favorable during menstruation or through the overgrowth of BV-related bacteria, as the vaginal pH is increased by menstrual blood (pH 7.32) (Hovhannisyan & Grigoryan 2014) and polyamines (Wolrath et al. 2001). In contrast, passive dispersion (erosion and sloughing) might possibly occur when the biofilm is exposed to the shear forces of

vaginal sloughing induced by sialidase, glycosulfatase, glycosidase, proteinase, collagenase and fibrinolysins (Olmsted et al. 2003; Roberton et al. 2005; Lewis & Lewis 2012), and when the biofilm is exposed to the shear forces of menstrual flow.

## Conclusion

The formation of a polymicrobial biofilm is essential in the etiology of BV as it increases the chances of survival of BV-related bacteria during BV infection (Swidsinski et al. 2014).  The current review suggests that BV-related bacteria can cooperate and form synergistic relationships with each other during BV and during the formation of a polymicrobial BV biofilm (Pybus & Onderdonk 1997; Machado, Jefferson, et al. 2013).  There is evidence suggesting *G. vaginalis* as an early colonizer species and other BV-related bacteria as second/third colonizer species during the development of a polymicrobial BV biofilm (Patterson et al. 2010; Machado, Jefferson, et al. 2013; Alves et al. 2014); however, there is still a possibility that another BV-related anaerobe like *Peptoniphilus* spp. may initiate the biofilm process (Patterson et al. 2010).

The limitation of BV biofilm research is that suitable *in vivo* models are lacking (Machado & Cerca 2015).  The *in vitro* studies can provide some evidence on how BV could be caused; however, the information obtained from *in vitro* studies might not guarantee the understanding or improve results *in vivo* (i.e. in the vagina).  Conversely, *in vivo* models like the murine vaginal epithelium may also not be successful in studying BV biofilms, as the murine vaginal epithelium does not resemble the *in vivo* environment of the human vagina (such as keratinized epithelial cells and the higher pH than that of the human vagina) (Breshears et al. 2015; Herbst-Kralovetz et al. 2016).  Therefore, we might need to use every available *in vitro* and *in vivo* system, or use a

combination of both systems to decipher the complex interactions involved in BV biofilms (Herbst-Kralovetz et al. 2016). The choice of which model to use for such studies will depend on the research question being asked (Herbst-Kralovetz et al. 2016). In future studies, the following research gaps could be essential to better understand and to define the etiology of BV, such as: (i) the dispersal mechanism in a polymicrobial BV biofilm; (ii) transcriptomic studies to study gene expression during the formation of a polymicrobial BV biofilm and (iii) quorum sensing in a polymicrobial BV biofilm.

## Declaration of interest

## Funding

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**Tables with captions**

**Table 1:** Virulence factors produced by bacteria associated with BV and their roles in BV etiology

| Bacterial species | First association with BV was reported in | Virulence factors | Role of virulence factors in BV etiology |
|---|---|---|---|
| *Atopobium vaginae* | 2004 (Ferris et al. 2004; Verstraelen et al. 2004) | • Elicit proinflammatory responses (e.g. IL-1β, IL-6, IL-8 and TNF-α) in the vaginal epithelium (Libby et al. 2008; Doerflinger et al. 2014) | • Negatively affect the barrier function of epithelial cells (Doerflinger et al. 2014) |
| | | • Adherence and biofilm formation (coaggregation) (Swidsinski et al. 2005; Hardy et al. 2016) | • Allows bacteria to persist BV treatment (Swidsinski et al. 2015) |
| | | • Production of VFAs and NVFAs[*] | • Induce alterations in the shape and growth of eukaryotic cells and apoptosis of human splenic B-cells (Kurita-Ochiai et al. 1998)<br>• Inhibit chemotaxis (Botta et al. 1985; Al-Mushrif et al. 2000) |
| | | • Internalization into the vaginal epithelial cells (Fichorova et al. 2013) | • Allows bacteria to escape antibiotic treatment (Fichorova et al. 2013) |
| BV-associated bacteria 1, 2 and 3 (BVAB1, BVAB2, BVAB3) | 2005 (Fredricks et al. 2005) | • Production of polyamines (BVAB1) (Srinivasan et al. 2012) | • Decreases the redox potential and increases the vaginal pH (Srinivasan et al. 2015) |
| *Gardnerella vaginalis* | 1954 (Gardner & Dukes 1954; Gardner & Dukes 1955) | • Biofilm formation (Swidsinski et al. 2005) | • Allows to persist BV treatment (Swidsinski et al. 2008; Swidsinski et al. 2011; Swidsinski et al. 2015) |
| | | • Production of amino and keto acids (Chen et al. 1979) | • Enhances growth of BV-related bacteria like *P. bivia* and *P. anaerobius* (Pybus & Onderdonk 1997; Pybus & Onderdonk 1998)<br>• Increases ammonia and VFA production by *P. bivia* (Pybus & Onderdonk 1997) |
| | | • Production of sialidase, phospholipase C and protease (Yeoman et al. 2010; Udayalaxmi et al. 2011) | • Promotes vaginal sloughing ("clue cells") (Yeoman et al. 2010)<br>• Inhibit secretory immunoglobulin (sIgA) (Lewis et al. 2012) |
| | | • Production of vaginolysin (cytolysin) | • Induces pore formation and lysis of vaginal epithelial cells (cytotoxic activity) (Randis et al. 2013) |
| | | • Internalization into the vaginal epithelial cells (Marrs et al. 2012) | • Escape immune responses and the effects of antibiotic therapy (Marrs et al. 2012) |

[*]NVFAs: non-volatile fatty acids; VFAs: volatile fatty acids

**Table 1:** Virulence factors produced by bacteria associated with BV and their roles in BV etiology (continued)

| Bacterial species | First association with BV was reported in | Virulence factors | Role of virulence factors in BV etiology |
|---|---|---|---|
| *Mobiluncus* spp. | 1984 (Spiegel & Roberts 1984) | • Production of VFAs (acetic, formic, butyric, isobutyric, valeric and isovaleric acids) and NVFAs (succinic acid) (Al-Mushrif et al. 2000) | • Same as in *A. vaginae* |
| | | • Production of malic acid (Spiegel & Roberts 1984) | • May cause vaginal irritation (Spiegel 1991) |
| | | • Production of polyamines (trimethylamine) | • Same as in BVAB |
| | | • Biofilm formation (coaggregation) (De Boer & Plantema 1988; Costello et al. 2007) | • Same as in *A. vaginae* |
| Gram-positive anaerobic rods (*Eggerthella* spp., *Eubacterium* spp.) | 1983 (Spiegel et al. 1983) | • Production of VFAs (acetic and formic acids) and NVFAs (succinic acid) (Kageyama et al. 1999) | • Same as in *A. vaginae* |
| Gram-negative anaerobic rods (*Bacteroides* spp., *Fusobacterium nucleatum, Prevotella* spp. *Leptotrichia*/*Sneathia* spp.) | 1980 (Spiegel et al. 1980) | • Produce a large amount of amino acids and ammonia (Kapatral et al. 2005) | • Enhances growth of *G. vaginalis* and *P. bivia* (Pybus & Onderdonk 1997)<br>• Increases the vaginal pH to a very high alkaline pH (Pybus & Onderdonk 1997) |
| | | • Production of butyric acid (VFA) (Kapatral et al. 2005) and succinic acid production (NVFA) (Pybus & Onderdonk 1996) | • Same as in *A. vaginae* |
| | | • Sialidase and glycosidase production (Briselden et al. 1992; Olmsted et al. 2003; Marconi et al. 2013) | • Same as in *G. vaginalis* |
| | | • Production of hemolysin (*Sneathia* spp.) (Harwich et al. 2012) | • Cytotoxic to vaginal epithelial cells (Harwich et al. 2012) |
| | | • Production of the 5-nitroimidazole reductase (the *nim* genes) (Lubbe et al. 1999; Theron et al. 2004) | • Promotes metronidazole resistance (Lubbe et al. 1999; Theron et al. 2004) |
| Anaerobic cocci (*Anaerococcus* spp., *Finegoldia* spp., *Megasphaera* spp., *Peptococcus* spp., *Peptoniphilus* spp., *Peptostreptococcus* spp., *Veillonella* spp) | 1980 (Spiegel et al. 1980) & 1982 (*Veillonella* spp.) (Piot et al. 1982) 2005 (Fredricks et al. 2005) | • Production of propionic and butyric acids (VFA) and succinic acid (NVFA) (Pybus & Onderdonk 1998) | • Same as in *A. vaginae* |
| | | • Production of sialidase | • Same as in *G. vaginalis* |
| | | • Production of the 5-nitroimidazole reductase (the *nim* genes) (Lubbe et al. 1999; Theron et al. 2004) | • Same as above |
| | | • Biofilm formation (except *Peptoniphilus* spp.) | • May stimulate the growth of *Actinomyces* spp. and *F. nucleatum* (Periasamy & Kolenbrander 2009; Donelli et al. 2012) |

**Table 1:**      Virulence factors produced by bacteria associated with BV and their roles in BV etiology (continued)

| Bacterial species | First association with BV was reported in | Virulence factors | Role of virulence factors in BV etiology |
|---|---|---|---|
| Mollicutes (*Mycoplasma hominis*, *Ureaplasma urealyticum*) | 1973 (McCormack et al. 1973) | • Production of IgA protease A, phospholipase C and urease (*Ureaplasma urealyticum*) (Robertson et al. 1984; De Silva & Quinn 1986; Blanchard et al. 1988) | • Degrades human IgA (Robertson et al. 1984)<br>• Hydrolyze phospholipids of vaginal epithelial cells (De Silva & Quinn 1986)<br>• Hydrolyze urea to ammonia (Masover et al. 1977) |

**Table 2:** Conceptual models in BV etiology

| Conceptual models | Main features | Supported by | Not supported by |
|---|---|---|---|
| *Lactobacillus* depletion model | • Removal of lactobacilli occurs first before the rise in the vaginal pH<br>• Absence of lactic acid-producing lactobacilli leads to the rise in the vaginal pH<br>• Rise in the vaginal pH promotes the overgrowth of BV-related bacteria<br>• Provides a scientific basis for a restoration strategy using probiotics and acidification agents | • The findings that lactobacilli produce $H_2O_2$ and other antibacterial substances to maintain normal vaginal microbiota (Klebanoff et al. 1991; Aroutcheva, Simoes, & Faro 2001)<br>• The finding that BV-related bacteria are inhibited by low vaginal pH (Boskey et al. 2001) | • The findings that some women maintain a "healthy" vaginal environment without lactobacilli (Zhou et al. 2004; Gajer et al. 2012)<br>• Some strains of *Atopobium* spp., *Leptotrichia* spp. and *Megasphaera* spp. may produce lactic acid (Zhou et al. 2004; Gajer et al. 2012) |
| Primary pathogen model | • Introduction of a single pathogen (*G. vaginalis*) by sexual activity to the vaginal environment<br>• *G. vaginalis* creates a lower redox potential environment to create intermediate microbiota<br>• Intermediate microbiota promote the overgrowth of BV bacteria<br>• Provides a scientific basis for *G. vaginalis* biofilm disruptors as treatment strategy | • The findings that *G. vaginalis* outcompetes other BV-related bacteria by possessing many virulence factors including biofilm formation (Patterson et al. 2010; Alves et al. 2014)<br>• The findings that *G. vaginalis* have ability to persist in low pH, while others not<br>• The findings that *G. vaginalis* may produce antagonistic substances to displace lactobacilli (Nagy et al. 1991) | • The findings that some *G. vaginalis* strains are present in normal microbiota and in sexually inexperienced women (Fethers et al. 2012; Schwebke, Flynn, et al. 2014) |
| Polymicrobial pathogen model | • Introduction of a BV consortium of bacteria by sexual activity to the vaginal environment<br>• The overgrowth of BV-related bacteria and removal of lactobacilli take place simultaneously<br>• Provides a scientific basis for polymicrobial biofilm disruptors as treatment strategy | • High bacterial diversity in BV (Ling et al. 2010; Srinivasan et al. 2012)<br>• The involvement of the polymicrobial biofilm formation (Swidsinski et al. 2013)<br>• The finding that other BV-related bacteria than *G. vaginalis* can inhibit lactobacilli (Nagy et al. 1991) | • Other BV-related bacteria than *G. vaginalis* are not as virulent as *G. vaginalis* (Patterson et al. 2010; Machado, Jefferson, et al. 2013; Alves et al. 2014) |