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THE ETIOLOGY OF BACTERIAL VAGINOSIS

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Abstract

Bacterial vaginosis (BV) is the most common vaginal infection among women of childbearing age. This condition is notorious for causing severe complications related to the reproductive health of women. Five decades of intense research established many risk factors for acquisition of BV, however due to the complexity of BV and due to lack of a reliable animal model for this condition, its exact etiology remains elusive. In this manuscript we use a historical perspective to critically review the development of major theories on the etiology of BV, ultimately implicating BV-related pathogens, healthy vaginal microbiota, bacteriophages and the immune response of the host. None of these theories on their own can reliably explain the epidemiological data. Instead, BV is caused by a complex interaction of multiple factors, which include the numerous components of the vaginal microbial ecosystem and their human host. Many of these factors are yet to be characterized because a clear understanding of their relative contribution to the etiology of BV is pivotal to formulation of an effective treatment for and prophylaxis of this condition.

Keywords

bacterial vaginosis; etiology; *Gardnerella vaginalis*; causes; immune response; lactobacilli; *Lactobacillus*

1. INTRODUCTION

The healthy microbiota of the lower genital tract in women predominantly consists of *Lactobacillus* spp., with *L. crispatus, L. jensenii* and *L. iners* being the most prevalent species (Pavlova *et al.* 2002; Zhou *et al.* 2004; Shi *et al.* 2009). It is generally accepted that these bacteria form a critical line of defense against potential pathogens. The symbiotic relationship between vaginal lactobacilli and their human host is modulated by the hormones circulating in a woman's body which stimulate the vaginal epithelia to produce glycogen (Hay 2005). Vaginal lactobacilli metabolize glycogen secreted by the vaginal epithelia, in turn producing lactic acid, which is largely responsible for the normal vaginal pH being acidic (<4.5) (Donati *et al.* 2010). The acidic environment of a healthy vagina is not permissive for growth of many potential pathogens (Aroutcheva *et al.* 2001a; Donati *et al.* 2010). Additionally, vaginal lactobacilli are thought to fend off pathogens through competitive exclusion via the possible formation of biofilms (Domingue *et al.* 1991) and through the production of antimicrobials such as hydrogen peroxide and bacteriocin-like substances (Aroutcheva *et al.* 2001a).

The most common vaginal infection among women of childbearing age is bacterial vaginosis (BV). This condition is characterized by replacement of vaginal lactobacilli with

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predominantly anaerobic microorganisms such as *Gardnerella vaginalis* and *Prevotella*, *Peptostreptococcus* and *Bacteroides* spp. (Forsum *et al.* 2005; Larsson and Forsum 2005; Srinivasan and Fredricks 2008; Livengood 2009). Historically, *G. vaginalis* is thought to have the leading role in the infection, making the niche suitable for colonization by strict anaerobes that are largely responsible for the clinical symptoms of BV (Swidsinski *et al.* 2005; Swidsinski *et al.* 2008; Josey and Schwebke 2008; Harwich, Jr. *et al.* 2010), although this idea has been challenged by recent findings Ultimately, BV is not caused by the mere presence of the potential pathogens (which is common) but rather by their unrestrained increase in number, often reaching cell counts that are 100–1000 fold above the normal bacterial levels of the vagina (Eschenbach 1993; Eschenbach 1994; Forsum *et al.* 2005; St John *et al.* 2007). However, the exact mechanisms and sequences of the infective processes are largely unknown due to the lack of a reflective animal model.

Epidemiological studies indicate that the risk of BV is increased in women of African ethnicity (Simhan *et al.* 2008; Cherpes *et al.* 2008; Klatt *et al.* 2010). Other risk factors include low socio-economic status, cigarette smoking, douching, antibiotic treatment for another condition, young age of coitarche, acquisition of a new sex partner and a recent history of multiple sex partners (Merchant *et al.* 1999; Yen *et al.* 2003; Verstraelen 2008; Cherpes *et al.* 2008; Fethers *et al.* 2009; Verstraelen *et al.* 2010). Conversely, a consistent use of condoms was shown to be somewhat protective against BV (see reviews by Verstraelen 2008; Fethers *et al.* 2008; Fethers *et al.* 2009; Verstraelen *et al.* 2010). The fact that many of the high risk behaviors are also well-established risk factors for acquisition of common sexually transmitted infections (STIs) suggests that BV could be transmitted sexually (Gardner and Dukes 1955; Criswell *et al.* 1969; Gardner 1980; Verstraelen 2008). However, unlike a typical STI with a single etiological agent and clear routes of infection, BV involves multiple pathogens, a great majority of which are also frequently detected (albeit in low numbers) in the vaginas of BV-free and sexually inexperienced women. The fact that there is no evidence for a decrease in the rates of BV recurrence following antibiotic treatment of men sexually involved with affected women is another distinction between BV and the common STIs (Verstraelen *et al.* 2010). In fact, many researchers prefer to view BV not as an infection but as a complex microbial imbalance, with a significant role played by the indigenous vaginal lactobacilli (Guise *et al.* 2001; Hay 2005; Schwiertz *et al.* 2006).

The diagnosis of BV in clinical settings is usually based on fulfillment of three out of four clinical criteria described by Amsel *et al.* (1983). Amsel's criteria include 1) elevated vaginal pH (>4.5) and 2) the presence of white adherent discharge that contains 3) numerous exfoliated epithelial cells with bacteria (Gram-variable polymorphic rods) attached to their surface (clue cells) that 4) has a characteristic fishy odor, especially when 10% KOH is added (whiff test). However, BV can be asymptomatic in about 50 % of women, and for that reason microbiological diagnostic methods, such as Nugent's scoring system (Nugent *et al.* 1991), are preferred in the scientific community (Schwiertz *et al.* 2006).

Aside from causing unpleasant symptoms, BV is notorious for setting off an entire array of serious gynecological and obstetric complications. BV is a risk factor for the development of postpartum and post-abortion endometritis and pelvic infection following gynecologic surgery (Watts *et al.* 1990; Lin *et al.* 1999). In pregnant women, BV has been connected to premature labor and preterm delivery that leads to high prenatal mortality (Leitich *et al.* 2003; Romero *et al.* 2004; Oakeshott *et al.* 2004; Stevens *et al.* 2004). *G. vaginalis* and *Prevotella* spp. are high risk factors for intra-amniotic infections (Goldenberg *et al.* 2000; Hashemi *et al.* 2000). BV-associated microorganisms and their toxins capable of crossing the placenta are among the major causes of brain injury for fetuses. BV is traditionally considered a risk factor for long-term neurological consequences in children, such as

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hyperactivity, academic difficulties in school, and severe handicaps such as cerebral palsy and preventricular leukomalacia (Eschenbach 1997; Grether and Nelson 2000; Ling *et al.* 2004). High concentrations of lipopolysaccharides (LPS) were found in the vaginas of women with BV (Platz-Christensen *et al.* 1993). These LPS induce damage in the dopaminenergic system in neonates (Ling *et al.* 2004).

Additionally, the physiology of the female genital tract makes women twice as sensitive to HIV as men. A disturbed vaginal ecology caused by BV creates a more permissive environment for acquiring HIV (Schmid *et al.* 2000; Schwebke 2003; Sha *et al.* 2005; Watts *et al.* 2005). Major BV-associated organisms directly up-regulate HIV replication (Al-Harthi *et al.* 1999; Hashemi *et al.* 2000; Simoes *et al.* 2001; Zariffard *et al.* 2005). *G. vaginalis* was found at high concentrations in 60% of HIV positive women (Mascellino *et al.*, 1991). *G. vaginalis* also increased the production of HIV by HIV-infected monocytoid cells and in certain T cells by as much as 77-fold (Hashemi *et al.* 1999). *P. bivia* and *Pep. assaccharolyticus* also induce HIV expression (Hashemi *et al.* 2000). Moreover, an elevated pH resulting from *Lactobacillus* spp. replacement with BV-associated microorganisms makes the vaginal environment more favorable for HIV proliferation (Taha *et al.* 1998). In addition, a recent study showed that BV increased the risk of viral shedding of genital herpes (HSV-2) by a factor of 2.3 (Cherpes *et al.* 2005).

Conventional treatment of BV with metronidazole and clindamycin (Paavonen *et al.* 2000; Sobel *et al.* 2001), as recommended by the Centers for Disease Control and Prevention, does not eradicate all BV-associated bacteria (Flores Rivera *et al.*, 1997). After treatment for BV, many women remained colonized by *G. vaginalis* or other BV-associated anaerobes (Ferris *et al.* 1995; Boris *et al.* 1997). These treatments are effective in only 60% of all cases, contributing to the BV recurrence rate of 30–40% (Colli *et al.* 1997; Bannatyne and Smith 1998; Paavonen *et al.* 2000; Eriksson *et al.* 2005). In addition, these treatments play a significant role in the expansion of drug resistance in *G. vaginalis* and *Prevotella, Bacteroides*, and *Peptostreptococcus* spp. (Lubbe *et al.* 1999; Bryskier 2001; Liebetrau *et al.* 2003).

One of the major obstacles to discovery of effective treatment and prophylaxis of BV is our limited understanding of this condition, particularly its etiology, which remains enigmatic despite decades of research (Forsum *et al.* 2005; Larsson and Forsum 2005; Larsson *et al.* 2005). In this manuscript, we review the major theories on the causes of BV, critically evaluating evidence for the role of *G. vaginalis*, endogenous vaginal lactobacilli, bacteriophages and the genetic predispositions of the host in the etiology of this condition.

2. GARDNERELLA VAGINALIS

Bacterial vaginosis was initially thought to be a sexually transmitted infection propagated by a bacterium that is now known as *Gardnerella vaginalis*. Ever since the discovery of this pathogen in the mid-1950s, its history has been intriguing and full of controversies. The elusive nature of this microorganism is even revealed by the fact that it has been renamed several times, mainly because of its unique cell wall structure and nutritional requirements. As the polymicrobial nature of BV became evident, the role of *G. vaginalis* in the etiology of this condition became less clear. As a result, general interest in *G. vaginalis* declined in the late 1980s, only to reemerge in recent years as the relationship of this microorganism to BV was reevaluated. Consequently, *G. vaginalis'* cell wall was thoroughly investigated for decades, while little is known about the microorganism's physiology and, until very recently, virtually no research was conducted on the genetics of *G. vaginalis.*

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2.1 Structure and physiology

The original discovery of *G. vaginalis* was made by Leopold (1953), who described this microorganism as a novel "*Haemophilus*-like" species associated with prostatitis and cervicitis (Catlin 1992). Gardner and Dukes (1955) were the first to describe the microorganism in relation to BV. The bacterial cell's morphology, apparent negative reaction to Gram staining, and inability to grow on agar media lacking blood convinced these researchers that they were dealing with a new *Haemophilus* species which they named, based on its origin, *Haemophilus vaginalis*. Eventually it became apparent that, unlike other members of the *Haemophilus* genus, '*Haemophilus vaginalis'* occasionally had a positive reaction to Gram staining and did not require either hemin or NAD for its growth. The microorganism was then temporarily placed into the *Corynebacterium* genus, and for some time was referred to as *Corynebacterium vaginale* (Dunkelberg, Jr. *et al.* 1970; Deane *et al.* 1972). However, the bacterium did not fit into the description of the *Corynebacterium* genus because it was catalase-negative and because it lacked arabinose in its cell wall (Catlin 1992). Finally, two large taxonomic studies evaluating multiple criteria revealed the lack of similarities between "*Haemophilus vaginalis*" and other established genera (Piot *et al.* 1980; Greenwood and Pickett 1980). As a result, a new genus named *Gardnerella* was proposed, with *Gardnerella vaginalis* being the only species in it.

The uncertainties in the taxonomic status of *G. vaginalis* fueled an almost half century-long debate about the structure of the microorganism's cell wall. The fundamental difference in the chemical structure and molecular architecture between the two types of bacterial cell walls can generally be revealed by a simple Gram staining technique. Typical Gram-negative cell walls have a complex, multilayered structure with a thin layer of peptidoglycan and an outer membrane largely composed of lipopolysaccharide. In contrast, peptidoglycan arranged into a thick amorphous matrix is the predominant component of a Gram-positive cell wall (Silhavy *et al.* 2010). *G. vaginalis*, however, is commonly described as a Gram-variable or Gram-uncertain microorganism, meaning that its reaction to Gram staining can vary from negative to positive (Catlin 1992). For instance, the cells of *G. vaginalis* can have both a Gram-negative and Gram-positive appearance in a stained preparation of a vaginal smear. Similarly, a pure culture grown on a medium containing starch appeared as Gram-variable. In contrast, the cells grown on vaginalis agar (V-agar) predominantly stained as Gram-negative, while the early exponential phase cells grown on inspissated serum medium (Zinnemann and Turner 1963; Piot *et al.* 1980) mostly stained as Gram-positive (Zinnemann and Turner 1963; Piot *et al.* 1980), indicating that the age of the culture and its growth conditions may influence the reaction to Gram staining.

Numerous attempts to study the biochemistry and ultrastructure of *G. vaginalis* cell wall have led to some conflicting results. The electron micrographs published by Reyn *et al.* (1966) revealed a single-layered but relatively thin murium which was in close association with the cytoplasmic membrane. Formation of a well-defined septum between dividing cells was clearly seen in a longitudinal section, further indicating the Gram-positive nature of the cell wall.

In contrast, Criswell *et al.* (1971, 1972) reported that, much like the reference strain of the Gram-negative bacterium *E. coli*, *G. vaginalis* has a multilayered cell wall containing a low (20%) peptidoglycan content. These multiple laminations, which are typical for cell walls of Gram-negative organisms, were also reported by Greenwood and Pickett (1980), swaying the scientific community towards the view that *G. vaginalis* has cell wall characteristics reminiscent of a Gram-negative microorganism.

The initial chemical analysis of the peptidoglycan conducted by Criswell *et al.* (1971) showed that although the matrix lacks diaminopimelate, it has a diverse amino acid profile

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common to Gram-negatives. Additionally, ribitol teichoic acid, which is almost universal to Gram-positive cell walls, was not detected in *G. vaginalis* (Criswell *et al.* 1971). Furthermore, Greenwood and Pickett (1980) reported that *G. vaginalis'* cell wall material obtained by a hot aqueous phenol extraction gave a positive reaction in a limulus amoebocyte lysate (LAL) assay, suggesting the presence of an LPS-like substance. The amino acid profile reported by Criswell *et al.* (1971) was disputed in later publications (Greenwood and Pickett 1980; Sadhu *et al.* 1989). Even more significantly, the detailed chemical analysis of the lipid extract involving tests for LPS-specific components such as heptose and hydroxy fatty acids failed to identify typical lipopolysaccharides in the cell wall of *G. vaginalis* (Greenwood and Pickett 1980; O'Donnell *et al.* 1984). Moreover, LPS could be detected neither by silver staining on a SDS-PAGE gel or by LAL assay (Sadhu *et al.* 1989). Sadhu *et al.* (1989) proposed that the previously reported positive reaction to LPS was induced by lipoteichoic acid, since the extract samples were used in very high concentrations.

At the same time, electron micrographs of ruthenium red stained cells published by Sadhu *et al.* (1989) revealed that the oblique angle of sectioning is likely responsible for the previously reported lamellar appearance of the cell wall. The absence of the outer membrane was clearly observed in images of cells sectioned at the right angle. Additionally, the absence of an outer cleavage phase in freeze-etched *G. vaginalis* cells once again demonstrated the lack of outer membrane. Although thick (up to 50 nm) peripheral cell walls were visible in a minority of cells, in most cells the cell wall appeared fibrillar and unstructured with thicknesses ranging between 8–12 nm, similar to what has been previously reported (Sadhu *et al.* 1989; Catlin 1992). Sadhu *et al.* (1989) proposed that the fluctuation in thickness of the peptidoglycan layer is responsible for the variable reaction to Gram-staining. The peptidoglycan layer in *G. vaginalis* cells is thought to become thinner as the culture ages, and the relatively thin peptidoglycan matrix cannot effectively retain the crystal violet-iodine complex which typically serves as an indicator in a Gram-staining reaction (Sadhu *et al.* 1989;Catlin 1992).

Muli *et al.* (1999) once again examined the ultrastructure of *G. vaginalis* cells grown in both conventional and biofilm systems and essentially confirmed the Gram-positive nature of the cell walls. This group described the *G. vaginalis* cell wall as a relatively thin (8–12 nm) but homogeneous fibrillar structure. Interestingly, Muli *et al.* (1999) noticed a group of uncommon cell wall particles seen in cross-section as a set of seven circles (18–20 nm in diameter) which were predominately observed in the biofilm-associated cells. It has been proposed that these fiber-like structures may function as a part of the mesosomal system or as a precursor to a developing septum in some Gram-positive bacteria (Vaniterson 1961).

Electron microscopy has also revealed fimbriae (pili) which are 3 to 7.5 nm in diameter covering the cellular surface (Scott *et al.* 1989). Ultrastructural investigation conducted by Scott *et al.* (1989) indicated that the outer fibrillar coat was predominantly responsible for the attachment of *G. vaginalis* to exfoliated vaginal epithelial cells (clue cells); conversely, fimbriae were involved in attachment of the pathogen to human red blood cells. Both fimbriae and exopolysaccharides are thought to be involved in attachment of *G. vaginalis* to the vaginal epithelium *in vivo* (Scott *et al.* 1989; Catlin 1992). Electron microscopy has also revealed that the cells are non-sporulating, they lack flagella, and they do not possess a typical capsule (Greenwood and Pickett 1980).

Overall, the cells of *G. vaginalis* are small, pleomorphic rods having average dimensions of 0.4 by 1.0–1.5 μm (Edmunds 1960; Catlin 1992); however, the length of some cells may reach up to 2–3 μm (Greenwood and Pickett 1980; Taylor-Robinson 1984). The cell size

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and morphology largely depend on their growth conditions and on their physiological state (Piot *et al.* 1980; Jolly 1983).

This bacterium is immotile, with the cells frequently occurring in clumps in vaginal smears and when grown in liquid media (Greenwood and Pickett 1980; Taylor-Robinson 1984). Strands of exopolysaccharide produced by cells can be visualized using electron microscopy and detected using ruthenium red staining (Greenwood and Pickett 1980; Greenwood 1983; van der Meijden *et al.* 1988). They are presumed to be responsible for the cell clumping effect (Catlin 1992).

Until 2010, virtually nothing was known about *G. vaginalis*' genetics. Early studies conducted using a variety of techniques indicated that the genome of *G. vaginalis* has a low (42–43.5 %) GC content (Piot *et al.* 1980; Greenwood and Pickett 1980; Vandamme *et al.* 1991). Additionally, Lim *et al.* (1994) digested chromosomal DNA of four *G. vaginalis* strains with rare restriction enzymes and analyzed the fragments using pulsed-field gel electrophoresis (PFGE), eventually concluding that the size of the *G. vaginalis* genome ranges between 1.67 Mb and 1.72 Mb. Finally, various attempts at genotyping *G. vaginalis*, as described in the next section, revealed an incredible diversity between the genomes of different isolates. Presumably, the difficulty of lysing *G. vaginalis* hindered early genetic explorations (Greenwood and Pickett 1980; Piot *et al.* 1980; Vandamme *et al.* 1991); concurrently, the interest in *G. vaginalis* faded in the 1980s–90s, right when techniques in molecular genetics were rapidly advancing.

Recently the genomes of several *G. vaginalis* strains were sequenced, providing a plethora of information about the microorganism. These studies estimated the genome of *G. vaginalis* to be 1.62–1.67 Mb with a low GC content (41–42%), thus confirming the earlier findings (Yeoman *et al.* 2010). Sequence analysis of *G. vaginalis'* 16 S rRNA conducted by Yeoman *et al.* (2010) indicated that the bacterium is most closely related to *Bifidobacterium coryneforme* and *Bifidobacterium minimum* (Gram-positive organisms).

Interestingly, the analysis of *G. vaginalis*' genome revealed that, aside from simple conversions, the microorganism lacks enzymes in biochemical pathways involved in amino acid synthesis. It was predicted that *G. vaginalis* can synthesize some but not all purine and pyrimidine bases (Harwich, Jr. *et al.* 2010). The researchers also did not find genes coding for phosphofructokinase and fructose-bisphosphate aldolase, the two enzymes essential for glycolysis; however, enzymes responsible for portions of the pentose phosphate pathway were identified. The authors suggested that the pentose phosphate pathway could potentially compensate for the deficiency in the glycolysis pathway. Finally, Yeoman *et al.* (2010) reported that most of the genes coding for enzymes involved in the TCA cycle are missing from the *G. vaginalis* genome. These recent findings explained at least some of the complex nutritional requirements that are needed for *in vitro* growth of *G. vaginalis*; they are hardly surprising, since the fastidious nature of this microorganism has been apparent from its very discovery (Gardner and Dukes 1955). The bacterium's relatively small genome size and its deficiencies in important biochemical pathways are consistent with the parasitic lifestyle of this microorganism.

Biochemical tests revealed that *G. vaginalis* is catalase-, oxidase- and β-glucosidase-negative. It can ferment starch, dextrin, sucrose, glucose, fructose, ribose, maltose and raffinose. Some strains can also ferment xylose and trehalose. Conversely, *G. vaginalis* is unable to ferment rhamnose, melibiose, mannitol, and sorbitol (Harwich, Jr. *et al.* 2010). Additionally, *G. vaginalis* can hydrolyze hippurate but not gelatin or esculin. This microorganism is also positive for α-glucosidase activity and for β-hemolysis on human blood, but not sheep's blood.

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The hemolytic activity associated with *G. vaginalis* is thought to be mainly due to secretion of vaginolysin (VLY), a cholesterol-dependent cytolycin. Vaginolysin recognizes complement regulatory molecule CD59 on the surface of its target cells, which explains this toxin's specificity towards human erythrocytes. *In vivo,* the cytolytic activity of vaginolysin is thought to increase nutrient availability for its producer strain (Yeoman *et al.* 2010).

2.2 *Gardnerella vaginalis* as the etiological cause of BV

Gardner and Dukes (1955), who were the first ones to link *G. vaginalis* to BV, reported the microorganism being isolated from the lower genital tract of BV-affected women in 92% of cases, compared to a 0% isolation rate from healthy women. In the questionable experiments that followed, Gardner and Dukes (1955) intravaginally inoculated 13 BV-free women with a pure *G. vaginalis* culture. The vaginas of two women got colonized by the microorganism but had no clinical signs of BV, while one woman (8%) developed asymptomatic BV. In a separate experiment, Gardner and Dukes (1955) used vaginal secretions from BV-affected women to inoculate the vaginas of 15 BV-free volunteers. Eleven of these volunteers (73%) developed symptomatic BV that would not resolve spontaneously within four months. Additionally, *G. vaginalis* was isolated from the urethra of 45 out of 47 (96%) men sexually involved with the BV-positive women (Gardner and Dukes 1955). These observations led the researchers to the conclusion that *G. vaginalis* is the sole etiological cause of BV (Eschenbach *et al.* 1989). Years later, the same group of researchers repeated their experiments on human volunteers, this time managing to induce colonization in 7 out of 29 pregnant women vaginally inoculated with the pure *G. vaginalis* culture (Criswell *et al.* 1969).

Eventually, advances in the formulation of media selective for *G. vaginalis* allowed for the detection of this bacterium when present in low numbers. Subsequent studies found *G. vaginalis* in the vaginas of 14–69% of BV-free women, an incidence that is considerably higher than the 0% originally reported by Gardner and Dukes (1955) (Gardner and Dukes 1955; Totten *et al.* 1982; Hill *et al.* 1984; Masfari *et al.* 1986; Eschenbach *et al.* 1988; Fredricsson *et al.* 1989; Cristiano *et al.* 1989; Mikamo *et al.* 2000).

In order to explain epidemiological data showing the common occurrence of *G. vaginalis* in healthy women, numerous studies were directed to identify specific virulent subtypes of this organism responsible for BV. Accordingly, Piot *et al.* (1984) originally distinguished between eight *G. vaginalis* biotypes based on lipase, hippurate hydrolysis, and β-galactosidase reactions. These characteristics were shown to be stable in multiple subcultures and the typing procedure was simple and reproducible. Certain biotypes were more prevalent than others, although their relative distribution was similar among the samples collected from three international cities. Although Piot *et al.* (1984) failed to link any particular *G. vaginalis* biotype to the occurrence of BV, they made other useful observations. For instance, it was shown that some women harbored multiple *G. vaginalis* biotypes. Moreover, the biotypes isolated following a week-long treatment for BV were identical to those isolated prior to the treatment. Finally, the *G. vaginalis* isolated from the vaginas of the participating women were generally of the same biotypes as the urethral isolates from their male sexual partners, possibly indicating a sexually transmitted nature of this infection.

In contrast, Briselden and Hillier (1990) reported a statistically significant association between all four lipase-positive biotypes and the manifestation of BV, suggesting that the lipase reaction is important for the pathogenesis of *G. vaginalis*. Moreover, the authors concluded that women who acquired BV during the investigation generally also acquired a new biotype of *G. vaginalis*. Some of these results, however, were later disputed because of a flaw in the detection method for lipase activity (Moncla and Pryke 2009). Additionally, the

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fact that women can harbor multiple biotypes of *G. vaginalis* (also confirmed by Briselden and Hillier (1990)) significantly complicates the analysis, since the apparent acquisition of a new biotype can simply be a reflection of a change in the ratio of existing biotypes (Piot *et al.* 1984; Moncla and Pryke 2009).

Benito *et al.* (1986) expanded the *G. vaginalis* biotyping scheme by incorporating additional tests for the fermentation of arabinose, galactose and xylose into the system. Some of the 17 newly defined biotypes were more prevalent in women with BV, although they were not identical to the BV-associated biotypes reported by Briselden and Hillier (1990), thus creating yet another discrepancy.

Aroutcheva *et al.* (2001b) reported that *G. vaginalis* which is only positive for hippurate hydrolysis (biotype 5) is predominantly isolated from asymptomatic women, allowing the researchers to suggest the possibility of using this biotype as a marker of “normal vaginal microflora”; however, additional research is needed to make any definitive conclusions. To summarize, it is still rather unclear whether any of the biochemical characteristics selected for biotyping *G. vaginalis* are linked to the virulence of the microorganism.

Attempts at serotyping *G. vaginalis* are also reported in the literature. For instance, 50 strains of *G. vaginalis* tested by Edmunds (1962) were differentiated into seven serological groups based on the precipitin assay involving 13 antisera (Catlin 1992). Ison *et al.* (1987) were able to differentiate between 20 *G. vaginalis* serotypes in a dot blotting test using polyclonal antibodies raised in rabbits. Immunoblot analyses of the whole cell lysates separated on SDS-PAGE gels suggested that the specificity of this reaction was determined by various immunodominant proteins and by a carbohydrate. Of the 91 clinical isolates tested by Ison *et al.* (1987), 79 (87%) were successfully typed using this scheme. To the best of our knowledge, however, the use of *G. vaginalis* serotyping has not yet been utilized for epidemiological studies; hence, the link between specific serotypes of *G. vaginalis* and BV is still unknown.

Genetic subtyping is frequently preferred over phenotypic methods; however, due to great variability in the DNA sequence of various *G. vaginalis* isolates, dividing this single species into a limited number of homogeneous genotypes proved to be challenging. For instance, DNA restriction profiles generated by *Bam*HI, *Eco*RI, *Pst*I and other restriction enzymes were considerably different in all 12 examined *G. vaginalis* strains. Moreover, Southern blot analysis of a specific DNA restriction fragment revealed the fragment’s length polymorphism among all the evaluated strains (Nath, 1991). Similarly, restriction endonuclease analysis (REA) involving *Bam*HI, *Eco*RI, *Cla*I, *Hae*II, *Hin*dIII, and *Msp*I restriction enzymes revealed major differences between DNA fingerprints of 20 *G. vaginalis* biotype 1 isolates (Wu *et al.* 1996).

Ingianni *et al.* (1997) used several ribotyping techniques in an attempt to differentiate between the genetic subtypes of 34 *G. vaginalis* strains. DNA profiles produced by classical ribotyping with Southern blot detection were different for all 34 strains. Conversely, the DNA fragment produced by PCR ribotyping, along with the restriction patterns of 16S–23S rRNA intergenic spacer sequences, were identical in all 34 strains. Limited success was achieved by DNA restriction analysis (ARDRA) technique; depending on the utilized restriction endonucease, 3–4 *G. vaginalis* genotypes were identified using this method (Ingianni *et al.* 1997). However, there was no link between a specific genetic subtype and the presence of BV.

Yeoman *et al.* (2010) compared the genomes of several *G. vaginalis* strains and reported that two strains originating from the vaginas of women with symptomatic BV had the capability to degrade mucins secreted by the vaginal epithelia to form a protective barrier. In

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contrast, the strain originating from a woman with asymptomatic BV (Nugent score of 9) did not have this capability. Based on this observation, Yeoman *et al.* (2010) proposed that *G. vaginalis'* ability to degrade mucins could be the decisive virulence factor determining the course of infection. This theory is feasible, although many more *G. vaginalis* isolates must be analyzed in order to make any conclusions.

Conversely, Harwich, Jr. *et al.* (2010) proposed that the key difference between virulent and commensal strains of *G. vaginalis* is in their ability to adhere to the vaginal epithelia and to form biofilms. This conclusion was reached based on a series of *in vitro* assays that compared five strains of *G. vaginalis*, three of which were BV isolates and two that were isolated from the vaginas of healthy women. Subsequent genetic analysis of a single strain from each 'set' revealed sequence differences in the gene coding for biofilm associated protein (BAP), which could potentially effect biofilm properties. Once again, although these observations are intriguing, comparison of additional strains is needed for a meaningful analysis.

To summarize, in spite of considerable research efforts, the specific virulent type(s) of *G. vaginalis* responsible for BV is yet to be identified. Alternative theories that may explain why some women who carry *G. vaginalis* develop BV while others do not are explored in the following sections. In any case, despite the plethora of epidemiological data indicating a strong association (93–100%) of *G. vaginalis* with BV, without direct experiments it has been challenging to implicate this microorganism in the etiology of BV (Pheifer *et al.* 1978; Balsdon *et al.* 1980; Symonds and Biswas 1986; Eschenbach *et al.* 1988; Borchardt *et al.* 1989; Hillier *et al.* 1990; Holst 1990; Mikamo *et al.* 2000). The direct experiments on human subjects similar to the ones performed by Gardner and Dukes (1955) and Criswell *et al.* (1969) would be unethical by the current standards outlined in The Declaration of Helsinki (Rikham 1964; Giordano 2010).

Over the past decade, multiple studies utilized PCR-based techniques for characterization of the vaginal microbiota associated with BV. This approach allowed for the identification of microorganisms (including previously unrecognized species) that were not detected through cultivation-based methods. Most notably, a number of publications reported that *Atopobium vaginae* is consistently associated with BV (Verhelst *et al.* 2004; Menard *et al.* 2008; Haggerty *et al.* 2009). Moreover, Verhelst *et al.* (2004) reported that a low frequency of detection of *A. vaginae* in BV-free individuals makes this microorganism even more specific to BV than *G. vaginalis*; however, in this study most women with BV were shown to be concurrently inhabited by both species. Additionally, three previously uncharacterized species in the *Clostridium* phylum as well as some *Leptotrichia* and *Megasphaera* species were detected with high frequency in the vaginas of women with BV (Verhelst *et al.* 2004; Haggerty *et al.* 2009; Marrazzo *et al.* 2010). In contrast, the remainder of the BV-associated microbiota varies to a great extent among affected women (see review by Srinivasan and Fredricks (2008)).

Regardless of these recent discoveries, some authors still maintain that *G. vaginalis* leads the infectious process, making the niche permissible for proliferation of strict anaerobes (Josey and Schwebke 2008; Harwich, Jr. *et al.* 2010). This view is based not only on the historic placement of the microorganism but also on its relatively superior cytotoxicity and its *in vivo* ability to form antibiotic-resistant biofilms on the vaginal epithelium (Swidsinski *et al.* 2005; Swidsinski *et al.* 2008; Harwich, Jr. *et al.* 2010). In accordance with this view, Josey and Schwebke (2008) pointed out that trichomoniasis can also be accompanied by a decrease in the numbers of vaginal lactobacilli and by an increase in the numbers of strict anaerobes which are responsible for the typical amine odor. We propose that, under favorable conditions (as discussed in subsequent sections), the infection can be seeded not

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only by *G. vaginalis* but by a group of pathogens (including *A. vaginae*) that are relatively tolerant to the healthy vaginal environment, thus allowing the more fastidious opportunists to take advantage of the created niche.

2.3 The non-existent animal model for *G. vaginalis* infection

The lack of a reflective animal model for BV is likely the major reason why the role of *G. vaginalis* in the etiology of this condition is still unclear. Several attempts to construct a reflective animal model for bacterial vaginosis were reported in the 1980s and 1990s, but they had very limited success.

For instance, the study conducted by Johnson *et al.* (1984) investigated the susceptibility of three primate species to vaginal infection with *G. vaginalis*. The researchers inoculated the lower genital tracts of four tamarins, three chimpanzees, and ten pig-tailed macaques with several strains of *G. vaginalis* and tried recovering the microorganism from these animals throughout an observation period. *G. vaginalis* did not colonize the tamarins or the chimpanzees, but did colonize all of the pig-tailed macaques for a period of 11–39 days. However, the infected animals did not display characteristic signs of bacterial vaginosis, such as an elevated vaginal pH, presence of clue cells or an increased succinate to lactate ratio in their vaginal smears. The poor resemblance of the model's condition to BV in humans is likely due to major differences in the microbiota and physiology of lower primate and human female genital tracts.

Mardh *et al.* (1984) attempted to induce BV in grivet monkeys by vaginally inoculating them with several strains of BV-associated pathogens. Thin, grey and translucent vaginal discharge was noticed in monkeys who were simultaneously infected with *G. vaginalis* and a *Mobiluncus* strain (referred to as a long curvy rods) (Catlin 1992). These symptoms appeared 5 days after the inoculation and persisted until the end of the 6-week long observation period. Both microorganisms were recovered from the primates' vaginal smears 12 days after the inoculation and the *Mobiluncus* strain persisted for at least an additional 25 days. Interestingly, monkeys infected with only one of the pathogens did not exhibit any obvious BV-like symptoms, although in one animal *Mobiluncus* spp. persisted for at least 9 months.

During routine prebreeding examinations, Salmon *et al.* (1990) isolated Gram-variable pleomorphic bacilli from the genital tracts of 4 mares. These bacilli were identified as *G. vaginalis* by various tests. A larger study that followed investigated the occurrence of *G. vaginalis* in specimens collected from the genital tracts of 93 mares (Salmon *et al.* 1991). The presence of *G. vaginalis* was reported in at least 31 of these specimens (although mostly at <50 CFU/sample); furthermore, 70 other isolates were identified as *G. vaginalis*-like organisms (GVLO). The researchers did not observe any evidence of disease in these 'infected' horses (including abnormal vaginal discharge), which prompted them to suggest that this condition might be similar to asymptomatic BV in humans. The finding of natural *G. vaginalis* infection in mares was originally perceived as very promising for the development of an animal model for BV; after all, this was the first report of *G. vaginalis* isolated from a nonhuman species (Salmon *et al.*, 1990; Caitlin 1991). To the best of our knowledge, however, the equine model for BV has never been developed any further.

Several previously reported studies confirm the rabbit vaginal model as being somewhat satisfactory for studying BV and antimicrobials active against BV-associated microorganisms (i.e. McDuffle and Gibbs 1996; Gibbs *et al.* 2004). However, a closer analysis of the literature leads us to conclude that a fully reliable animal model for studying the etiology, pathogenesis and treatment of BV will not be forthcoming any time soon.

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In the paper by McDuffie *et al.* (2002), direct surgical inoculation of *G. vaginalis* into the uterine horns of pregnant rabbits yielded positive cultures in all animal samples on day 1, but only 60% on day 4 and 50% on day 6, suggesting that infection may not have been established. Further, this does not indicate utility for vaginal inoculation or evaluation of protection from infection. The authors quote an earlier paper by Field *et al.* (1993) that used essentially the same model and concluded that *G. vaginalis* is not a maternal pathogen. The latter paper reported negative fetal effects such as 80% (infected) vs. 95% (uninfected) live birth, a 23% reduction in birth weights, and brain injury in 60% of treated fetuses. Four days after inoculation, decidual samples from all rabbits yielded positive cultures of *G. vaginalis*, although titers were not reported. *G. vaginalis*-treated animals had diffuse infiltration of the decidua and subplacental separation zone with polymorphonuclear leukocytes, consistent with histologic deciduitis.

Amniotic fluid was also positive for *G. vaginalis* in 15 of 17 rabbits. Infection was not systemic as indicated by the lack of a positive blood or peritoneal culture. The authors of this paper concluded that "*G. vaginalis* produced a clinical picture suggestive of subclinical infection-positive cultures; mild, if any, maternal symptoms". In summary, the use of this model is inherently expensive, requires surgical expertise, does not address vaginal infection and may not be appropriate for non-pregnant animals.

Finally, Yan *et al.* (1996) reported isolating 145 *G. vaginalis* strains from foxes being raised in various fox farms in China. This group later attempted to develop a vaccine against *G. vaginalis* by using cells inactivated by various chemical agents (Yan *et al.* 1997). The aluminum hydroxide gel inactivated vaccine did not induce any adverse effects in foxes and was consequently used to immunize the animals. The vaccination apparently made the animals more resistant to bacterial challenge for the duration of six months. This model is very promising, although it is unclear why such a successful animal model for BV has not been developed any further since 1997 and why, at least to the best of our knowledge, it has not been used in any additional BV-related studies.

Overall, Gelber *et al.* (2008) suggested that the failures of animal models for BV could be related to the specificity of vaginolysin produced by *G. vaginalis* (previous section). Accordingly, transgenic animals expressing CD59 would be vulnerable to *G. vaginalis* infection.

3. THE 'INCOMPETENT' VAGINAL LACTOBACILLI THEORY

3.1 Establishment of vaginal microbiota

The microbiota of a healthy human vagina undergoes numerous changes throughout the lifetime of a woman. The natural selection of bacterial strains in the vaginal milieu is modulated by numerous factors, including the host's genetic predispositions, environment and behavior; however, the overall selection process is poorly understood (Spiegel 1991; Forsum *et al.* 2005; Larsson and Forsum 2005). Nevertheless, some of the changes in the vaginal microbiota are predictable because the composition of this dynamic bacterial community is influenced by levels of circulating estrogens (Robinson and Ridgway 1994; Macsween and Ridgway 1998; Brabin *et al.* 2005). Normal changes in the vaginal bacterial flora associated with different stages of sexual maturity and a menstrual cycle are described in this section.

Normal *in utero* development of a human fetus occurs in a sterile environment. An infant's initial exposure to microorganisms takes place during childbirth. The encountered bacteria come from the mother's birth canal, hands of the caregivers, and the general surroundings (Forsum *et al.* 2005; Penders *et al.* 2006; Adlerberth 2008; Reinhardt *et al.* 2009).

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For the first four to six weeks after birth, residual maternal estrogens still have a strong influence on a female infant's vaginal tissues. As a result, the vagina of a newborn child resembles an adult vagina both morphologically and microbiologically. Aside from the observable physical characteristics, the vaginal mucosa of a newborn is relatively thick, with glycogen being secreted in abundance by epithelial cells (Spiegel 1991; Robinson and Ridgway 1994; Brabin *et al.* 2005; Farage and Maibach 2006). Within the first 24 hours of its life, the vagina of a child is normally colonized by lactobacilli (Robinson and Ridgway 1994; Farage and Maibach 2006). These facultative lactobacilli dominate the vaginal microbiota of the child until the maternal estrogens are metabolized.

Depletion of the residual estrogens is followed by significant changes in the vaginal microbiota. This early stage of sexual development is also associated with a neutral to alkaline vaginal pH (Brabin *et al.* 2005; Farage and Maibach 2006). The microorganisms found on the skin and in the enteric microbiota also dominate the vaginal microbiota of prepubescent girls. At this stage of development, *Staphylococcus epidermidis*, *Escherichia coli*, and various *Enterococci* are among the most frequently identified aerobic microorganisms in a child's vaginal microbiota (Hammerschlag *et al.* 1978b; Robinson and Ridgway 1994; Myhre *et al.* 2002). Anaerobes such as *Bacteroides melaninogenicus*, *Veillonella parvula*, and *Peptococcus*, *Peptostreptococcus* and *Propionibacterium* spp. also account for a significant component of the vaginal microbiota at this stage (Hammerschlag *et al.* 1978a; Gerstner *et al.* 1982; Hill *et al.* 1995).

Puberty is marked by a gradual rise in estrogen levels, leading to thickening of the vaginal mucosa and an increase in glycogen production. Consequently, multiple researchers who investigated the vaginal microbiota of premenarchial girls have noticed an increase in the frequency of vaginal *Lactobacillus* isolation with age, suggesting that a shift in the vaginal microbiota occurs gradually (Hammerschlag *et al.* 1978b). A study conducted by Yamamoto *et al.* (2009) also revealed that the onset of menarche does not trigger significant changes in the vaginal microbiota.

During reproductive years, the healthy vaginal microbiota is dominated by *Lactobacillus* spp. However, the incidence of non-*Lactobacillus* species such as *Gardnerella vaginalis* and *Prevotella bivia* (in low numbers) is also common, as discussed in previous sections. Recently, culture-independent methods have revealed that the vaginal microbiota of many healthy women may contain extremely low or nonexistent *Lactobacillus* counts, which does not necessarily constitute an abnormal state (Lamont *et al.* 2011). At this stage of sexual development, the relative thickness and glycogen content of the vaginal mucosa is largely influenced by steroid hormone cycling (Farage and Maibach 2006).

The effects of the menstrual cycle on the vaginal microbiota was investigated by Eschenbach *et al.* (2000), who reported that the numbers of vaginal lactobacilli had a tendency to increase over the course of the cycle. Concurrently, the incidence of heavy levels of non-*Lactobacillus* (including *Prevotella* spp.) growth tended to decrease over the cycle ($P = 0.002$). As a result, Eschenbach *et al.* (2000) concluded that *in vivo* levels of potential vaginal pathogens are highest during menstruation, making this the most vulnerable time period for development of BV. It is likely that this imbalance in the microbiota is brought about by an increase in the vaginal pH due to passage of the menses. Recovery of the *Lactobacillus* population is then facilitated by an estrogen-mediated increase in the thickness and glycogen content of the vaginal epithelia, which reaches its peak mid-cycle (Eschenbach *et al.* 2000).

Menopause is accompanied by a decrease in estrogen secretion, atrophy of the vaginal epithelia, and an elevated vaginal pH (Devillard *et al.* 2004; Farage and Maibach 2006).

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This final stage of reproductive maturation is also associated with a decline of typical vaginal microbiota (especially lactobacilli) and with an increased prevalence of coliforms in the vaginal microbiota (Hillier and Lau 1997). Interestingly, a hormone replacement therapy was shown to reestablish the dominance of lactobacilli in the vaginal microbiota of post-menopausal women as well as decrease the incidence of strains with pathogenic potential (Heinemann and Reid 2005).

3.2 The epidemiological findings

The role of vaginal lactobacilli as a primary line of defense against various vaginal pathogens has been recognized for decades. Accordingly, researchers hypothesized that women affected by recurring BV are colonized by *Lactobacillus* strains that are not particularly competent as 'defenders'. The actual evidence supporting this hypothesis, however, was not discovered until the late 1980s. Eschenbach *et al.* (1989) were the first to postulate that H_2O_2 production by vaginal lactobacilli is critical for sustainment of healthy vaginal microbiota.

The original study conducted by Eschenbach *et al.* (1989) included a population of 95 non-pregnant women, 71% of whom had BV and 29% of whom did not. This research group reported that while the vaginas of 96% of healthy women were colonized by H_2O_2-producing lactobacilli (LB+), LB+ were only isolated from 6% of women with BV ($P<0.001$). This correlation has since been confirmed by numerous studies, several of which are described below.

In a cross-sectional study involving 103 non-pregnant women, Nagy *et al.* (1991) tested 47 vaginal *Lactobacillus* strains collected from healthy women and 39 strains from women with BV for production of H_2O_2. This group reported that 79% of the strains isolated from healthy women and only 23% of the strains coming from the BV-affected women were H_2O_2 producers.

A cross-sectional study involving 275 pregnant women in their second trimester revealed that 59% (117 out of 199) of healthy women and only 13% (10 out of 76) of women with BV were vaginally colonized by H_2O_2 producing *Lactobacillus* spp. (Hillier *et al.* 1992).

In their subsequent study, Hillier *et al.* (1993) enrolled 171 pregnant women in labor at term. The vaginal microbiota of the participants was classified as normal, intermediate or typical of BV. Vaginal H_2O_2 producing *Lactobacillus* spp. were detected in 5% of women with BV, 37% of women having the intermediate flora, and in 61% of BV-free women. Additionally, the presence of many BV-related pathogens including *G. vaginalis*, *P. bivia*, *Bacteroides* and *Mobiluncus* spp. was inversely related to the presence of H_2O_2 producing *Lactobacillus* spp.

These early studies were conducted in North America and Western Europe and involved participants residing in those areas. More recent studies conducted in South America, Eastern Europe, and Asia showed the same trend, suggesting that the discovered correlation is a worldwide phenomenon (Puapermpoonsiri *et al.* 1996; Mijac *et al.* 2006; Dimitonova *et al.* 2007; Martinez *et al.* 2008).

Nonetheless, some studies failed to show a correlation between lack of vaginal H_2O_2 -producing *Lactobacillus* spp. and BV. For instance, Rosenstein *et al.* (1997) analyzed the vaginal flora of 174 pregnant women. Fifty of these women were diagnosed with BV, and out of this group 19 were shown to be vaginally colonized by lactobacilli, with cell counts reaching 10^5-10^6 CFU/ml in the vaginal secretions of six women. Rosenstein *et al.* (1997) then randomly selected 12 women from this group of 19 for further analysis. Surprisingly,

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11 (92%) of the 12 women with BV harbored vaginal H_2O_2-producing lactobacilli. Based on these results, the authors suggested that in some women the growth of BV-related pathogens can occur prior to the decline of vaginal lactobacilli.

Nevertheless, the great majority of studies established the inverse association between BV and the occurrence of vaginal H_2O_2-producing *Lactobacillus* spp. The atypical results reported by Rosenstein *et al.* (1997) could alternatively be explained by a sampling error resulting from a small population size (12 study subjects).

The results of the numerous studies described in this section make it tempting to suggest that BV is caused by the lack of vaginal H_2O_2-producing *Lactobacillus* spp.; however, the established correlation may not necessarily indicate causality (Eschenbach *et al.* 1989). Some evidence demonstrating a protective role of vaginal H_2O_2-producing lactobacilli against BV was provided by a two-year long longitudinal study conducted by Hawes *et al.* (1996). The study enrolled 182 non-pregnant female participants, 50 of whom developed BV during the two year period. The results of this study revealed that women lacking vaginal lactobacilli had a four-fold greater risk of acquiring BV than the women harboring vaginal H_2O_2-producing lactobacilli ($P<0.001$). Presence of vaginal lactobacilli incapable of producing H_2O_2 (LB−) and absence of H_2O_2-producing *Lactobacillus* spp. increased the risk of BV acquisition by 2.2 fold ($P=0.02$). Accordingly, the authors concluded that the production of H_2O_2 by vaginal lactobacilli is protective against BV (Hawes *et al.* 1996; Pybus and Onderdonk 1999).

3.3 *In vitro* studies

Multiple studies attempted to model the antagonism between vaginal H_2O_2-producing *Lactobacillus* spp. and BV-related pathogens *in vitro*. The great majority of these studies demonstrated the inhibitory and/or bactericidal properties of vaginal H_2O_2-producing lactobacilli against pathogens (Atassi *et al.* 2006; Atassi and Servin 2010); however, the relative contribution of the H_2O_2 produced by the *Lactobacillus* spp. to the overall antimicrobial effect is still a matter of debate. For instance, Klebanoff *et al.* (1991) conducted multiple co-culture experiments and reported that within one hour of incubation in liquid suspension, H_2O_2-producing *Lactobacillus* strains totally inhibited *G. vaginalis* and *P. bivia*; conversely, the pathogens were not inhibited by *Lactobacillus* isolates incapable of producing H_2O_2. The inhibitory activity was enhanced by myeloperoxidase and chloride, and it diminished following treatment with catalase (but not with heat-inactivated catalase), suggesting that the activity was specifically due to H_2O_2. McLean and McGroarty (1996), O'Hanlon *et al.* (2010), and Atassi *et al.* (2006) conducted similar studies using different vaginal isolates of H_2O_2-producing lactobacilli, however the results were the same.

The ability of vaginal H_2O_2-producing *Lactobacillus* strains to inhibit *G. vaginalis* was also demonstrated using a simultaneous antagonism (sandwich plate) assay on solid media. Additionally, the cell-free supernatants of some vaginal H_2O_2-producing *Lactobacillus* strains were able to inhibit *G. vaginalis* and *P. bivia* in deferred antagonism (well-diffusion) assays (Klebanoff *et al.* 1991; McLean and McGroarty 1996; Dimitonova *et al.* 2007). It is worth mentioning that Fontaine *et al.* (1996) reported cell-free supernatants from several H_2O_2-producing *Lactobacillus* strains of vaginal origin had only a slight inhibitory effect on *G. vaginalis* and *Mobiluncus* spp., whereas *Bact. ureolyticus* and *Prevotella melaninogenica* were not inhibited at all by these supernatants.

Interestingly, there is a strong correlation between the inhibitory activity of the cell-free supernatants and their pH but not their H_2O_2 content (McLean and McGroarty 1996; Strus *et al.* 2006). Moreover, the inhibitory activity of the supernatants against *G. vaginalis* (quantified through their zone of inhibition) decreased 60–95% after neutralization of the pH

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by sodium hydroxide and only up to 30% after the elimination of H_2O_2 by catalase (McLean and McGroarty 1996).

Some *in vitro* studies quantified the levels of H_2O_2 produced by the vaginal *Lactobacillus* spp. grown in MRS broth. As expected, the concentration of H_2O_2 in these cultures largely depended on oxygen availability. Accordingly, multiple studies reported undetectable levels of H_2O_2 in vaginal *Lactobacillus* cultures grown under anaerobic conditions (McLean and McGroarty 1996; Strus *et al.* 2006; O'Hanlon *et al.* 2010). Strus *et al.* (2006) established that under aerobic conditions, the concentration of H_2O_2 in cultures of various H_2O_2-producing *Lactobacillus* isolates would generally increase throughout the exponential and stationary growth phases, reaching its peak at the onset of death phase. H_2O_2 production varied greatly among vaginally derived *Lactobacillus* strains. The reported H_2O_2 concentrations in static aerobic cultures ranged between 1–1000 μM, reaching up to 1.8 mM under intense aeration (McLean and McGroarty 1996; Strus *et al.* 2006; Aslim and Kilic 2006; O'Hanlon *et al.* 2010).

Strikingly, Nagy *et al.* (1991) reported that commercially available hydrogen peroxide in concentrations of 0.882–88.2 mM did not inhibit the growth of *G. vaginalis, Bacteroides, Mobiluncus* and *Peptostreptococcus* spp. on solid media. Similarly, Fontaine *et al.* (1996) reported that *G. vaginalis*, *Mobiluncus* spp. *and Bact. ureolyticus* were not inhibited on solid media by 8.82 mM H_2O_2 (Pybus and Onderdonk 1999). The minimal bacteriocidal concentrations (MBCs) of H_2O_2 in liquid suspensions containing 10^8 CFU/ml of *P. bivia* and *G. vaginalis* were 1.7 mM and 3.5 mM, respectively (Strus *et al.* 2006). We are aware of only one reported vaginal isolate (*L. delbrueckii*) capable of producing H_2O_2 in concentrations greater than 1.0 mM (in MRS broth with intense aeration). The concentration of H_2O_2 in most reported cultures of vaginally derived H_2O_2-producing *Lactobacillus* strains are within the 4–350 μM range (McLean and McGroarty 1996; Strus *et al.* 2006; Aslim and Kilic 2006). The same strains are expected to produce much lower quantities of H_2O_2 *in vivo*, since the vaginal environment is mostly anaerobic (Strus *et al.* 2006; O'Hanlon *et al.* 2010). In hypoxic conditions, the metabolism of H_2O_2-producing *Lactobacillus* strains shifts away from production of H_2O_2 and towards the production of lactic acid, since the enzymes involved in these two metabolic pathways compete for NADH (McLean and McGroarty 1996).

Finally, O'Hanlon *et al.* (2010) modeled H_2O_2 production in the vagina using real cervicovaginal fluids collected from healthy female volunteers. This research group reported that under hypoxic conditions resembling an *in vivo* environment, H_2O_2 production was undetectable after 4 hours of incubation, whereas the maximum H_2O_2 concentration in the aerated samples reached 23 μM ± 5 μM within 4 hours. Commercially available H_2O_2 at twice this concentration (50 μM) was not inhibitory to *G. vaginalis*, *P. bivia, Mycoplasma hominis, Mobiluncus curtsii*, *Mobiluncus mulieris*, *Peptostreptococcus anaerobius* and *Hemophilus ducreyii*. Additionally, O'Hanlon *et al.* (2010) detected significant H_2O_2-blocking activity in both cervicovaginal fluids and semen. The H_2O_2 antagonists in these bodily fluids neutralized 1 mM and 10 mM concentrations of H_2O_2, respectively, to undetectable levels. Moreover, the H_2O_2-mediated deactivation of the vaginal pathogens by vaginal H_2O_2-producing *Lactobacillus* strains in the liquid suspension (Klebanoff *et al.* 1991) was completely inhibited by the presence of as little as 1% of cervicovaginal fluids, further indicating their antagonistic properties against H_2O_2.

To summarize, these *in vitro* studies did not provide evidence supporting the significance of H_2O_2-mediated control of the vaginal microbiota.

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3.4 Clinical trials

If BV is truly caused by the lack of H_2O_2-mediated control of the vaginal microbiota, then topical application of H_2O_2 should be at least somewhat effective in restoring the microbial balance in BV-affected individuals. Early gynecologists commonly practiced intravaginal douching with hydrogen peroxide to treat (with some degree of success) persistent vaginal discharge, which was presumed to be due to trichomonal infections (Winceslaus and Calver 1996). Bacterial vaginosis was not recognized as a separate condition at the time; however, a vaginal discharge successfully treated by this method was probably due to BV since trichomonal infections are unresponsive to peroxide (Winceslaus and Calver 1996). The discovery made by Eschenbach *et al.* (1989), linking vaginal H_2O_2-producing lactobacilli to BV prompted a few researchers to reevaluate this long forgotten approach.

The trial conducted by Winceslaus and Calver (1996) enrolled 30 women with relapsing BV who fulfilled all four of Amsel's criteria and were positive for mixed vaginal anaerobes. During the procedure, women were placed on the lithotomy couch with their pelvis lifted relative to the upper body. Then, 3% hydrogen peroxide was introduced into their vaginas using a disposable plastic bivalve speculum. After three minutes of exposure, the hydrogen peroxide was drained out by reclining the couch back into the horizontal position. At the follow-up exam three weeks post-treatment, all 23 women who completed the trial tested negative for clue cells and for mixed vaginal anaerobes. Twenty-two women (95%) had a normal vaginal pH (<4.5) and were negative for the amine test. Overall, 18 (78%) women self-reported a complete remission of the symptoms and none of the participants complained about undesirable side effects related to the treatment. Based on the results obtained in this trial, the success rate and the acceptability of the H_2O_2 treatment for BV is comparable to conventional treatments; the main shortcoming of this study, however, is that it lacked a control group.

Chaithongwongwatthana *et al.* (2003) compared the efficacy of a single H_2O_2 douche for BV treatment to a single oral metronidazole regimen, although the results were not as encouraging as the ones reported by Winceslaus and Calver (1996). This randomized, placebo-controlled trial enrolled 142 women with BV diagnosed by Amsel's criteria. The follow-up exam two weeks after treatment revealed that oral metronidazole was more effective in curing BV than a H_2O_2 douche (78.6% *vs.* 62.5%, $P = 0.036$). The trial did not have a group treated with placebo only. Therefore, it is difficult to compare a cure rate due to a single H_2O_2 douche to a spontaneous recovery rate.

We also came across a case study describing a 17-year old virginal adolescent with exceptionally persistent BV, which was completely cured by a multisession H_2O_2 douching regimen. The patient was seeking medical help due to "extraordinarily malodorous" vaginal discharge; however, her symptoms were unresponsive to conventional antibiotic treatments during the six months preceding the experimental trial. Further examination by Papanikolaou *et al.* (2002) revealed *G. vaginalis* and other anaerobes in the girl's vaginal secretions. The secretions were also positive for clue cells, an elevated pH>4.5, and the amine 'whiff' test. Following yet another unsuccessful metronidazole regimen, the patient was given a douche containing a 3% H_2O_2/15% NaCl/10% povidone iodine solution which was administered daily for ten consecutive days. Additionally, the girl's vaginal walls were cleansed during each session using small gauze, which probably assisted in removal of pathogenic biofilms. Remission of all the symptoms was documented a month after the treatment and the patient remained symptom-free during the year that followed. Repeated vaginal washouts with H_2O_2 accompanied by a mechanical removal of biofilms may potentially be an effective treatment for BV, which is worth investigating in a well-controlled clinical trial.

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In a cohort study conducted by Cardone *et al.* (2003), 3% hydrogen peroxide was vaginally administered to 58 non-pregnant women with recurrent BV on a daily basis for the duration of one week. Three months after the treatment, a great majority of the participants was no longer affected by the clinical symptoms of BV: 98% had a vaginal pH <4.5, 89% were free of malodorous leucoxanthorrhea, 97.8% had a negative amine test and 100% were negative for clue cells. Additionally, H_2O_2-producing *Lactobacillus* spp., but not the typical anaerobic strains, were isolated from vaginal smears of all participants. Accordingly, the authors concluded that H_2O_2 treatment for BV is inexpensive, fully acceptable and at least as effective as conventional antibiotic treatments.

Intravaginal administration of hydrogen peroxide is a promising treatment for BV; however, additional clinical trials are needed to evaluate the efficacy of this approach. So far, two out of the three trials evaluating this remedy produced very encouraging results, providing further evidence that H_2O_2-mediated control is significant for maintaining microbial balance in the vaginal milieu. The concentration of H_2O_2 (3%) used in these trials was much higher than the H_2O_2 concentrations expected to be produced by vaginal lactobacilli *in vivo*. However, in the healthy human vagina, H_2O_2 works in conjunction with other antimicrobials (lactic acid, bacteriocins, etc.) produced by the native vaginal microbiota. Moreover, the local concentrations of H_2O_2 at the frontline where lactobacilli 'hold their positions' against pathogens can be much higher than the total H_2O_2 concentrations measured *in vitro*. Finally, peroxidases, in combination with halide ions found in normal vaginal secretions, greatly enhance the toxicity of hydrogen peroxide to anaerobic species (Klebanoff *et al.* 1991; Tomas *et al.* 2004). Therefore, in a healthy vaginal environment this antimicrobial could be effective at much lower concentrations.

In vitro studies failed to show the significance of H_2O_2 on its own for control of the vaginal microbiota, although they confirmed its role as an integral part of the natural defenses produced by vaginal lactobacilli (Atassi and Servin 2010). Conversely, the expected levels of H_2O_2 production in the vaginal environment, which is hypoxic, are very low. Therefore, the role of this antimicrobial *in vivo* is still unclear and the causal relationship between vaginal H_2O_2-producing *Lactobacillus* spp. and BV is yet to be established.

4. PHAGE THEORY FOR THE DECLINE OF VAGINAL LACTOBACILLI

The healthy vaginal environment created by lactobacilli through the production of lactic acid, hydrogen peroxide and bacteriocins is thought to be hostile for the proliferation of *G. vaginalis* and other BV-associated pathogens (such as strict anaerobes). Some theories state that the overgrowth of pathogens characteristic to BV has to be preceded by a major disturbance within the *Lactobacillus* population (Pavlova *et al.* 1997; Blackwell 1999). The resultant decline of lactobacilli allows for a shift in the vaginal microbiota, which is similar to the 'meteor theory of dinosaur extinction' explaining how mammals became the dominant class of vertebrates on Earth. It is well accepted that excessive douching and/or use of spermicidal agents and the use of antibiotics can cause a disturbance within the vaginal *Lactobacillus* population (Tao *et al.* 1997). Alternatively, Pavlova *et al.* (1997) proposed that bacteriophages could cause a decline in vaginal lactobacilli. The involvement of bacteriophages in the etiology of BV would explain why this condition is epidemiologically similar to STIs, and yet the rate of its recurrence in women is unaffected by an antibiotic treatment of their male partners (Blackwell 1999). In their study, Pavlova *et al.* (1997) collected vaginal specimens from 37 participants, 16 of which were diagnosed with BV based on Amsel's criteria. Among the 37 *Lactobacillus* strains isolated from these specimens, seven (19%) were identified as phage carriers (lysogens). The proportion of lysogens was presumably higher among the strains derived from BV-affected women, although the number of these strains was too low to establish statistical significance. Further

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in vitro investigation revealed that some of these phages were able to infect a broad range of *Lactobacillus* species originating from different women.

A very similar but much larger study with 209 participants in the USA and Turkey was conducted by Kilic *et al.* (2001). This study confirmed the previous finding; 67 out of 209 tested vaginal *Lactobacillus* strains (32%) were induced to release phages. Most importantly, the presence of lysogens was more common among women with BV (P<0.05). Once again, all the isolated phages were infective against a wide range of vaginal *Lactobacillus* strains, including the ones that were collected on a different continent.

All the phages identified in these two studies were temperate, i.e. they would only lyse a small portion of the infected bacteria unless induced. However, once released from a bacterial cell, some of these phages would undergo a lytic cycle in a different bacterial strain (Pavlova *et al.* 1997; Kilic *et al.* 2001). Accordingly, a lysogen or a phage itself may potentially be introduced into a healthy vagina through sexual activity, causing lysis of the native *Lactobacillus* strains (Pavlova *et al.* 1997; Blackwell 1999; Pavlova and Tao 2000; Kilic *et al.* 2001).

Another interesting finding was reported by Tao *et al.* (1997), who sampled multiple commercially available probiotic products for phages capable of infecting vaginal lactobacilli. A number of such phages were isolated from yogurts. All the phages were temperate and only 20% of the tested vaginal *Lactobacillus* strains were susceptible to their infection. Nonetheless, the presence of these phages in commonly consumed foods suggests an additional route of phage infection for vaginal lactobacilli. Some scientists, however, believe that if vaginal *Lactobacillus* populations were dramatically affected by phages derived from commercial foods, BV would be far more prevalent (Blackwell 1999). Additionally, the lack of BV-related complaints from women who regularly consume yogurts indicates that there is no obvious link between ingestion of fermented milk and BV.

It has been proposed that, *in vivo*, some external factors can induce these normally temperate phages into a lytic cycle (Blackwell 1999; Kilic *et al.* 2001). Interestingly, a study conducted by Pavlova and Tao (2000) revealed that the lysogenic *Lactobacillus* strains of vaginal origin can be induced to release phages by benzol[α]pyrene diol epoxide, a metabolically activated form of benzol[α]pyrene found in cigarette smoke. The phage theory of the lactobacilli decline, along with this finding, may explain why cigarette smoking is a significant risk factor for bacterial vaginosis. Accordingly, the inhaled benzol[α]pyrene is metabolically converted to its activated form in the liver and is eventually secreted into the vagina. Previous studies reported detectable levels of cigarette smoke chemicals in the cervico-vaginal mucus of women who smoke (Pavlova and Tao 2000). Although the expected secretion levels of benzol[α]pyrene diol epoxide would be insufficient to directly kill bacteria, this known mutagen can cause bacterial cell lysis by inducing phages (Pavlova and Tao 2000). Additional external factors capable of inducing phages within the infected vaginal lactobacilli will likely be identified in the future. We speculate that these factors may include stressors produced by the host immune response.

Moreover, if bacteriophages truly play a significant role in the etiology of BV, then intravaginal installation of phage-resistant probiotic strains could be effective for treatment and prophylaxis of this condition (Blackwell 1999). We speculate that phage-mediated selective pressures would give an advantage to these extraneous resistant *Lactobacillus* strains over the endogenous vaginal lactobacilli, allowing for the highly desirable long-term colonization (strain replacement therapy).

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5. THE ROLE OF INTRINSIC HOST FACTORS IN THE ETIOLOGY OF BV

As mentioned in previous sections, BV-associated pathogens can frequently be detected in the lower genital tract of asymptomatic women. At least some of these pathogens are thought to originate from the GI tract, which is considered their natural habitat (Witkin *et al.* 2007). It remains unclear why certain women are unaffected by these pathogens while others develop BV. Furthermore, the specific factors determining the severe BV-related complications in some women but not in others are also poorly understood (Witkin *et al.* 2007). Currently, many researchers are leaning towards the idea that host immunity is a decisive factor in the equation determining the initial development and later course of BV (Forsum *et al.* 2005; St John *et al.* 2007; Witkin *et al.* 2007). Several approaches are being used to evaluate the implication of the host's immune response in the etiology of BV. For instance, multiple studies attempted to compare the vaginal concentrations of various immune mediators in women with and without BV (Mattsby-Baltzer *et al.* 1998; Genc *et al.* 2004b; St John *et al.* 2007). This methodology can establish a correlation between the signs of BV and the immune response, but not necessarily a cause-effect relationship. Therefore, *in vitro* models can additionally be used to assess the expression levels of immune mediators of cervico-vaginal epithelia in response to BV microbiota. However, to the best of our knowledge, much like with the animal models, a reliable *in vitro* model for BV is yet to be developed. That is why, in this current section, we have mainly concentrated on epidemiological studies exploring hereditary predisposition towards bacterial vaginosis. The main purpose of these studies is to relate the etiology of both BV and the associated complications to genetic variabilities (gene polymorphisms) occurring within a population. So far, research in this area primarily targeted genes coding for components of the innate immune system due to their prominent role in other infectious conditions (Genc and Schantz-Dunn 2007; Misch and Hawn 2008).

Innate immune response is triggered when pathogen-associated molecular patterns (PAMPs) are recognized by host cells. The PAMPs are biologically important and therefore largely invariant components of many microbial pathogens (Aderem and Ulevitch 2000; Ozinsky *et al.* 2000). These potential threats act as ligands for Toll-like receptors (TLRs), which belong to a family of transmembrane proteins expressed by various immune and tissue cells of the host. So far, 10 TLRs have been identified in humans (St John *et al.* 2007; Misch and Hawn 2008). Possession of multiple receptors allows the host to distinguish between major groups of pathogens and to react accordingly. For instance, double-stranded viral RNA is a ligand for TLR3, while bacterial flagellen is recognized by TLR6 (Witkin *et al.* 2007). Certain components of microbial cell walls such as peptidoglycan from Gram-positive bacteria and mannan from yeast are recognized by a combination of multiple TLRs (TLR2 in conjuction with either TLR1 or TLR6 (Witkin *et al.* 2007)). TLR activation triggers expression of pro-inflammatory mediators, such as cytokines and chemokines. These molecular signals have multiple functions, including activation and recruitment of certain immune cells, such as neutrophils, to the site of a potential infection (Forsum *et al.* 2005). The submucosal localization of immune cells is thought to be partially responsible for the observed rise of vaginal HIV concentration among BV-affected individuals (Spear *et al.* 1997; Hillier 1998; Zariffard *et al.* 2005). The TLR-mediated signaling cascade, initiated in response to BV, is also thought to directly induce expression of HIV and, further down the line, a premature myometrial contraction (Al-Harthi *et al.* 1998; Al-Harthi *et al.* 1999; Genc and Schantz-Dunn 2007).

It has been proposed that various effector molecules such as bacterial proteases and toxins produced by BV-related pathogens might inactivate TLRs on cervico-vaginal epithelia. These compounds can inactivate local immune response through a direct degradation of TLRs by interference with TLR-ligand recognition, or alternatively by inducing anti-

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inflammatory cytokines such as IL-10 (Witkin *et al.* 2007). It is known, for instance, that unsaturated fatty acids commonly accumulated in the lower genital tract of BV-affected individuals inhibit the activation of TLR2 and TLR4 in a murine monocytic cell line (Lee *et al.* 2003). The inactivation of the innate immune response, in theory, would allow unrestrained multiplication of pathogens, manifested as BV. Most importantly, it has been suggested that certain polymorphisms in genes coding for innate immune system components (i.e. TLRs) would make women susceptible to these bacterially produced mediator molecules, and therefore vulnerable to BV (Witkin *et al.* 2007).

An intriguing finding supporting the polymorphisms theory was published by Genc *et al.* (2004b). This group reported a *TLR4* variant (*TLR4* 4785A>G polymorphism) associated with a rise in vaginal pH (P=0.05), and with at least a tenfold increase in cell numbers of *G. vaginalis* (P<0.0001) and anaerobic Gram-negative rods (P=0.08). Additionally, increased vaginal levels of IL-1β were detected among 896A homozygotes who were vaginally colonized by *G. vaginalis* and anaerobic Gram-negative rods, but not among *TLR4* 896G allele carriers colonized with the same microorganisms. This observation suggested that the *TLR4* 4785A>G polymorphism reduced the host's immunological responsiveness to BV-related pathogens (Genc *et al.* 2004b; Genc and Schantz-Dunn 2007). Two hundred and thirty-eight pregnant women at 18–22 weeks gestation participated in the study; the *TLR4* 4785G allele, associated with the increased numbers of vaginal pathogens and decreased immune-responsiveness, was identified in 10.3% of the population. Surprisingly, however, there was no significant association between the *TLR4* 4785A>G polymorphism and the incidence of BV as diagnosed by Nugent's score.

Two later studies involving 885 and 144 women in mid-pregnancy also could not reveal a link between the *TLR4* 4785A>G polymorphism and BV (Goepfert *et al.* 2005; Verstraelen *et al.* 2009). The association between BV and two other *TLR4* polymorphisms, *TLR4* 5095C>T and -2026A>G (the promoter region), has not been established either. Although the univariate analysis conducted by Goepfert *et al.* (2005) suggested that women with BV were less likely to have a polymorphism at *TLR4* 5095 loci, the association lost its significance when the race variable was taken into account since the participants were predominantly African-American women. Indeed, if the structurally Gram-positive *G. vaginalis* is the 'pioneer colonizer' of the niche, leading the way for the other BV-related pathogens, then it is not surprising that TLR4, an endotoxin receptor, is not the key player in BV etiology (Verstraelen *et al.* 2009).

It is worth mentioning that multiple studies assessed the implication of the *TLR*4 896A >G polymorphism in premature deliveries among pregnant women; however, the results were conflicting, as such association was reported in one study (Lorenz *et al.* 2002) but not in two others (Ferrand *et al.* 2002; Hartel *et al.* 2004).

Goepfert *et al.* (2005) also looked for associations between BV occurring in mid-pregnancy and polymorphisms in various genes coding for cytokines, including interleukins (ILs) and tumor necrosis factor-α (TNF-α). Multivariate analysis, controlled for maternal race, revealed that both IL8 –845 T>C and IL1β Exon 5 +3954 T>C polymorphisms were protective against BV, while IL6 –174 G>C polymorphism increased the risk of developing this condition. The significance of these findings is yet to be established.

Increased vaginal levels of the pro-inflammatory cytokine interleukin-1β (IL-1β) have been associated with BV by numerous studies (Mattsby-Baltzer *et al.* 1998; Genc *et al.* 2004b; St John *et al.* 2007). The interleukin-1 receptor antagonist (IL-1ra) acts as a competitive inhibitor of IL-1β by binding the IL-1β receptor without initiating a signaling cascade (Azevedo *et al.* 2010). Differences within a microsatellite region in intron 2 of *IL-1RN*, the

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gene coding for IL-1ra, give rise to five alleles. Three of these alleles (variants 3, 4 and 5) occur rarely, accounting for <5% of the allele's frequency (Genc and Schantz-Dunn 2007). Of the remaining two alleles, *IL-1RN**2 has been associated with the elevated expression of IL-1ra and simultaneously with a decreased expression of IL-1β by human monocytes *in vitro* (Danis *et al.* 1995; Vamvakopoulos *et al.* 2002). The same *IL-1RN**2 allele has also been linked to multiple chronic inflammatory conditions; therefore, it was logical to suspect the involvement of this polymorphism in BV.

Genc *et al.* (2004a) reported that among African-American women, but not among other ethnicities, BV based on Nugent's score was more prevalent in *IL-1RN**2 carriers than in non-carriers (60% vs. 15%, $P=0.04$). Additionally, Genc *et al.* (2004a) reported association of this allele with a many-fold increase in the numbers of anaerobic Gram-negative rods, mycoplasma, and peptostreptococci ($P<0.05$), and with a decrease in the numbers of lactobacilli ($P<0.001$) in the lower genital tract of African-American women. Surprisingly, the lack of this allele was associated with the higher rate of *G. vaginalis* and *Peptostreptococcus* spp. isolation ($P=0.02$) in Hispanic women, and this allele did not have any effect on vaginal flora in Caucasian women. Within the entire study population (212 pregnant women in their mid-trimester), this polymorphism was linked to an elevated vaginal pH ($P<0.006$) and to a decreased IL-1β response to a few BV-associated pathogens, including *G. vaginalis* and some anaerobic Gram-negative rods. Conversely, Genc *et al.* (2004a) reported exaggerated local IL-1β response to BV-associated pathogens among the *IL1RN**1 homozygotes, who were also at a significantly higher risk of giving preterm birth. Accordingly, Genc and Schantz-Dunn (2007) proposed that IL-1β hypo-responders (*IL1RN**2 carriers) are at a higher risk of developing BV because of their inability to mount an appropriate immune response to a potential threat. In contrast, IL-1β hyper-responders (*IL1RN**1 homozygotes) are less predisposed to BV; however, they are more likely to develop obstetric complications due to this condition. The theory proposed by Genc and Schantz-Dunn (2007) is supported by the fact that previous studies (Genc *et al.* 2004c; Genc and Schantz-Dunn 2007) linked the elevated vaginal levels of IL-1β measured during pregnancy to subsequent spontaneous preterm birth.

Cauci *et al.* (2007) evaluated the same *IL-1RN* polymorphisms in relation to BV in a cohort consisting of 570 non-pregnant Italian women, thereby eliminating the race factor. In this study, women with BV were recruited by Amsel's criteria, with the condition also being confirmed by a Nugent's score. Three out of the five *IL-1RN* variants were observed within the study population, and only the rare *IL-1RN**3 allele had a tendency to be protective against BV ($P=0.049$). The authors pointed out that the distribution of this rare allele somehow deviated from a Hardy–Weinberg equilibrium, therefore questioning their own results. It is worth noting that Cauci *et al.* (2007) did not evaluate isolation rates and quantities of BV-related pathogens. Additionally, all the participants were Caucasian; therefore, it is difficult to make a comparison between these results and those obtained by Genc *et al.* (2004a).

Genc *et al.* (2007) also conducted an epidemiological study involving 203 pregnant women at 18 to 22 weeks gestation to assess the contribution of a *TNFA* -308G>A polymorphism (promoter region of the gene) to BV etiology. *TNFA* codes for Tumor Necrosis Factor-α (TNF-α), a pro-inflammatory cytokine with numerous regulatory functions. Previous studies revealed that among the two alleles, *TNFA* -308A had a higher transcriptional activity. However Genc *et al.* (2007), reported that the *TNFA* 308G>A polymorphism was not linked to BV as diagnosed by Nugent's score. There was also no association between this polymorphism with either vaginal presence or quantity of BV-associated pathogens or with the elevation of vaginal pH. The same conclusion was also reached by Goepfert *et al.* (2005) in an even larger study involving 946 pregnant women with various ethnic backgrounds,

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suggesting that this polymorphism has no influence on the rate of BV. Interestingly, Genc *et al.* (2007) reported that bacterial vaginosis induced a more than six-fold increase (P=0.02) in vaginal TNF-α levels among the *TNFA*-308A carriers, but not among the *TNFA*-308G homozygotes. This amplified TNF-α response may explain certain obstetric complications caused by BV in some women. Accordingly, Genc *et al.* (2007) reported that pregnant women with BV who carry this hyperexpressive TNFA-308A allele have a six-fold higher risk to deliver prematurely than the TNFA-308G homozygotes with BV. Strikingly, Macones *et al.* (2004) reported that among pregnant women with BV, TNFA-308A carriers (hyper-responders) had a six-fold higher risk to deliver prematurely than TNFA-308G homozygotes. It is worth noting that the same study also reported that symptomatic BV (diagnosed by Amsel's criteria), on its own, did not increase the risk for preterm delivery when other known risk factors such as ethnicity, genital infections, and *TNFA* genotype were taken into account. These results, however, were met with criticism because clinical criteria as opposed to microbiological criteria were used to diagnose BV. Genc and Schantz-Dunn (2007) provides a detailed review on genetic predispositions to premature birth.

Mannose Binding Lectin (MBL) is another component of innate immunity suspected in being involved in BV etiology. This protein, shown to be localized in vaginal mucosa, binds carbohydrate moieties on cell surfaces of invading microbial agents, thereby activating the complement system. MBL insufficiency has been linked to some infectious conditions. Moreover, SNPs in the first exon (at codons 52, 54, and 57) of the gene coding for MBL (*MBL*2) were correlated to low serum levels of this protein (Eisen and Minchinton 2003). However, the epidemiological study involving 322 Caucasian Italian women failed to link any of the three *MBL*2 alleles along with the MBL deficiency to recurrent BV diagnosed by a Nugent's score (Milanese *et al.* 2008). Lack of association between the same *MBL*2 polymorphisms and BV was also observed in a cohort containing 201 Caucasian Italian women.

To summarize, the evidence linking specific genetic polymorphisms to BV is scarce, and it has not always been consistent across the various studies. As a result, the role of intrinsic host factors in the etiology of BV is still unclear, requiring more research to be conducted. Additionally, the research in this area has so far mainly targeted the components of the non-specific immune response. Aside from the immune mediators, other hereditary host factors such as the ones responsible for selection of healthy vaginal microbiota should also be considered.

With that said, most of the inconsistencies can be resolved by considering a larger and more homogenous study population. Stringent control for ethnic background and other known risk factors would eliminate many variables (Cauci *et al.* 2007). It is also likely that any particular allele has only a minor effect on the vaginal microbiota that would only be revealed within a large cohort. Ultimately, predisposition to BV is probably determined not by a single allele but by a specific allelic combination known as a haplotype (Cauci *et al.* 2007). We anticipate that future research exploring specific human haplotypes in relation to BV will bring promising results. Nevertheless, it is also important to bear in mind that although heredity may predispose a woman towards BV, the condition itself is caused by interaction of intrinsic host factors with the environment.

6. DISCUSSION

Our interaction with microorganisms begins at birth, and continues throughout our lives and even after death as our bodies decompose. Numerous factors, including our genetic make-ups and our behaviors, facilitate these dynamic interactions. It has been widely accepted that symbiosis between vaginal lactobacilli and their human hosts is imperative for human

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reproductive health. The importance of this symbiotic relationship is reflected by the fact that bacterial vaginosis, a condition resulting from disturbances in the healthy vaginal microbiota, has serious gynecological and obstetric complications.

In this article we critically evaluated four major theories explaining the etiology of bacterial vaginosis. The epidemiological findings, along with numerous *in vitro* studies, indicate that the symptoms of BV and the related complications arise from a drastic increase in the numbers of vaginal anaerobes, with *G. vaginalis* leading the way. However, BV is not a typical sexually transmitted infection, even though various sexual activities are well-known risk factors for acquisition of this condition. Instead, BV is a microbial imbalance among the constituents of the vaginal microbiota. Most likely this imbalance has a complex etiology which involves interactions between pathogenic species, endogenous vaginal microbiota, the host, and possibly bacteriophages. The relative contributions of these factors are unknown. Additionally, the interactions between these factors are modulated by a woman's behavior and her environment, further complicating the overall 'equation'. This inherent complexity, along with the lack of a reliable animal model, is the likely reason why the etiology of BV remains a mystery after decades of research. Nonetheless, a clear understanding of the pivotal factors involved in the etiology of BV along with their interactions is imperative to finding an effective treatment for this condition.

A probiotic treatment could potentially be used to replenish the healthy vaginal microbiota of women with BV. If the incompetence of vaginal lactobacilli is central to the etiology of recurrent BV, then a successful strain replacement therapy would, in theory, cure this condition. However, the long-term colonization by a probiotic strain is a complex process and, so far, the results of clinical trials investigating the use of probiotics for treatment of BV are rather conflicting (Reid 2008; Senok *et al.* 2009; Reid *et al.* 2009; MacPhee *et al.* 2010). Nevertheless, this approach may have potential with the use of carefully selected probiotic strains.

Alternatively, the topical application of the purified antimicrobials produced by vaginal lactobacilli can potentially be used to restore microbial balance in the vaginal ecosystem through selective inhibition of BV-related pathogens (Klebanoff *et al.* 1991; Winceslaus and Calver 1996; Papanikolaou *et al.* 2002; Cardone *et al.* 2003; see also thereview by Dover *et al.* 2008). Moreover, immunomodulators could potentially be used to compensate for the hereditary deficiencies that make some women vulnerable to BV. Finally, if bacteriophages play a significant role in the etiology of BV, then bacterial strain replacement therapy using phage-resistant *Lactobacillus* strains could potentially be effective for prevention and treatment of this condition.

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