Bacterial Vaginosis

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Bacterial vaginosis (BV) has been reported in one-third of women worldwide at different life stages, due to the complex balance in the ecology of the vaginal microbiota. It is a common cause of abnormal vaginal discharge and is associated with other health issues. Since the first description of anaerobic microbes associated with BV like *Gardnerella vaginalis* in the 1950s, researchers have stepped up the game by incorporating advanced molecular tools to monitor and evaluate the extent of dysbiosis within the vaginal microbiome, particularly on how specific microbial population changes compared to a healthy state. Moreover, treatment failure and BV recurrence rate remain high despite the standard antibiotic treatment. Consequently, researchers have been probing into alternative or adjunct treatments, including probiotics or even vaginal microbiota transplants, to ensure successful treatment outcomes and reduce the colonization by pathogenic microbes of the female reproductive tract.

bacterial vaginosis | microbiome | probiotics | Lactobacillus | Gardnerella | VMT

1. Introduction

The colonization of the vaginal microbiome begins to occur at birth, just like the gut or skin microbiome, and is subject to variation depending on delivery mode (i.e., vaginal birth or cesarean section) [1][2][3]. Just as the morphology and physiology of the vagina change throughout a woman's life, the vaginal microbiome is also greatly affected by factors such as the onset of puberty and hormonal changes during the menstrual cycle, menopause as well as pregnancy [4][5][6]. Therefore, it is very important to bear in mind that just as the vaginal microbiota can affect the host's reproductive physiology, the microbial composition can also be influenced by host physiology.

Being the most common cause behind vaginal discharge with a foul odour, bacterial vaginosis (BV) can occur when there is an imbalance in the vaginal microbiome (i.e., reduction in *Lactobacillus* spp. abundance) and overgrowth of certain microbial populations(s) in the vagina [7][8]. BV-affected women may also encounter itch/burning sensation and discomfort around the intimate area [7][9]. While many individuals may not display symptoms during BV, one of the main reasons contributing to the poor health-seeking behaviour of vaginal discharge is shame and fear of judgment by others, which accentuates the need to increase the awareness of BV [10].

2. Pathogenesis of BV and the Identification of Causative Agents

BV occurs due to microbial dysbiosis, presenting a highly diverse vagina microbiome (including *Gardnerella* spp. and *Atopobium* spp.) as opposed to the healthy form, which is mainly dominated by *Lactobacillus* spp. The predominant symptom is the presence of grayish-white thin homogenous discharge with an unpleasant odor or "fishy smell," which is more apparent during menses or after sexual intercourse due to the increased production of amines by anaerobic bacteria. The invasion of normal, healthy microflora by pathogens disrupt the host physiology via multiple routes, including depleting nutrients essential for the growth of other residents within the vagina, destroying the vaginal barrier via hydrolytic enzymes (e.g., sialidase and prolidase), and promoting the release of pro-inflammatory chemokines and cytokines (IL-6, IL-8, IL-1α, IL-1β, TNF-α) [11][12]. With the depletion of *Lactobacillus* spp., the vaginal pH fails to be maintained at the normal range (i.e., 3.8–4.5), which successively creates a cascade of undesirable events in the host, including persistent infection, potentially caused by a mixture of difficult-to-treat pathogens, especially when some of them possess the ability to form biofilms [12][13][14][15][16].

Along with developing diagnostic tools and treatment plans for BV, multiple attempts can be seen throughout these years to monitor changes in the microbial composition while identifying potential causative agent(s) [17]. Recent advancements in molecular tools greatly accentuated their position as a better tool with higher resolution power as compared to traditional cultivation methods and biochemical tests, which may be difficult to be applied to fastidious organisms as well as to differentiate microbes at the species-strain level [18][19][20][21][22][23]. Several reviews have been published over the past few decades, summarizing the tools available for studying microbial composition and vaginal microbiome transition during BV [20][24][25]. One of the infamous bacteria that is highly implicated in the literature associated with BV is *G. vaginalis.* The first description of this Gram-negative facultative anaerobe was made in 1953 by Leopold as a novel microbe from patients with cervicitis and prostatitis, which later demonstrated by two researchers—Gardner and Charles D. Dukes to be associated with BV [26][27][28]. Previously, scientists have been contemplating the causative roles of *G. vaginalis* in BV as this microbe exists in women without BV, yet Janulaitiene et al. found that only particular clades of *G. vaginalis* are pathogenic, which might be due to the presence of gene coding for sialidase [29]. As we know, the first step of invasion is adherence to the host cell. As demonstrated by Patterson and the team, the virulence of *G. vaginalis* can be attributed to several features: (a) the production of a cytolysin known as vaginolysin, which targets human cell specifically and activates the cell death pathways via binding to the complement regulatory molecule CD59, (b) strong adherence to host cells which avoid clearance by the host, and (c) ability to form biofilms [30]. On top of its capability in producing hydrolytic enzyme-like sialidase, which damages vaginal mucosal surfaces and thus increases the level of pro-inflammatory cytokines, *G. vaginalis* has the propensity to form dense biofilms, allowing it to persist in the environment and serve as a scaffold that supports the growth of other opportunistic pathogens [11][30][31][32][33][34][35]. The formation of a "new community" within the biofilm subsequently competes with *Lactobacillus* spp. as those living within the biofilms of *G. vaginalis* have a higher tolerance against lactic acid and hydrogen peroxide produced by *Lactobacillus* spp.; this increases their survival chances and failure to purge these opportunistic pathogens from the environment will eventually tilt the balance of the entire vaginal ecosystem [36]. Many efforts have been poured into the investigation of *G. vaginalis* biofilm, allowing identification of other opportunistic bacteria such as *Mobiluncus* spp., *Atopobium vaginae*, *Prevotella bivia*, *Mycoplasma hominis*, *Peptostreptococcus* spp., *Porphyromonas* spp., *Sneathia* spp., *Ureaplasma urealyticum*, *Leptotrichia* spp., *Candidatus Lachnocurva vaginae* (formerly known as BVAB1),

Mageeibacillus indolicus (formerly known as BVAB3) and BV-associated bacterium type 2 (BVAB2) [11][36][37][38][39][40][41].

Having said that, members of the biofilm community often work hand in hand to ensure they thrive in the vaginal ecosystem. *P. bivia* is another commonly detected microbe found in vaginal swabs of BV patients [42][43][44][45][46]. Working adjacent with the action of *G. vaginalis*, *Prevotella* spp. including *P. bivia* and *Prevotella disiens* can produce collagenase and fibrinolysin, which neutralize the mucosal protective factors and facilitate adhesion to the host cells [47][48]. Likewise, Pybus and Onderdonk uncovered a special commensal relationship between *G. vaginalis* and *P. bivia* while studying how nutrient utilization contributes to the pathogenesis of BV [42]. Through a series of tests, it was then found out that *P. bivia* stimulates the growth of *G. vaginalis* by supplementing the latter with ammonia. Apart from that, the interactions between *P. bivia* and other anaerobic bacteria associated with BV, such as *Peptostreptococcus anaerobius* are worthwhile to be investigated, particularly for the assimilation of amino acids and other supplements which favors the growth of pathogenic microbes within the vaginal ecosystem [49][50].

Initially isolated as a novel strain in 1999, the facultative anaerobic *A. vaginae* was recovered from the vagina microflora of a healthy person [51]. Its role in BV has always been the centre of the argument until lately, whereby researchers unveiled that the bacterium is indeed an important focal component in the abnormal microflora of BV [52][53][54][55][56][57][58]. As demonstrated through in vitro experiments, *A. vaginae* triggered the host's innate immune response via vaginal epithelial cells, which later increased the localized production of IL-6 and IL-8 and an antimicrobial β-defensin peptide, mediated through the toll-like receptor 2 [59]. These immune responses observed were consistent with clinical features of BV; thus, researchers suggested that *A. vaginae* may possibly contribute to the pathogenesis of BV via alteration on the host immune system. A study by Ferris and team in 2004 studied the association between *A. vaginae* and BV using a molecular approach—performing 16S rRNA gene-targeted PCR and denaturing gradient gel electrophoresis (DGGE) before comparing the microbe's presence among BV patients as well as healthy controls [53]. As predicted, only samples from patients with BV displayed *A. vaginae* amplicons with distinct band patterns in DGGE analysis, but neither amplicons nor bands appeared in healthy control samples. Another investigation done by a Belgium team revealed a high bacterial load of *A. vaginae* within the *G. vaginalis*-dominated biofilm, emphasizing the potential interactions between these two microbes [60]. An interesting article by Castro et al. explained a potential reason why *A. vaginae* rarely triggers BV alone but often in conjunction with *G. vaginalis* and its associated biofilm [58]. Nearly 92% of *A. vaginae* died within 48 h under mono-species planktonic culture conditions, but this bacterium remained viable when co-cultured with *Gardnerella* spp. or after a pre-conditioning step with cell-free supernatant of *Gardnerella* spp. cultures. There is still a lot to be studied regarding the primary roles of *A. vaginae* in BV, but accumulating evidence is showing that rather than being an "initiator" in BV pathogenesis, this anaerobe is most likely a "secondary colonizer" and symbiont of *G. vaginalis* [56][61][62]. While *G. vaginalis*-dominated biofilm provides protection and growth support upon anaerobes like *A. vaginae*, one of the reasons that urged the scientific community to investigate this bacterium thoroughly is owing to its antimicrobial resistance (AMR) pattern and strong association with recurrent BV [61][63]. Apart from displaying resistance towards metronidazole (which is a commonly used drug to treat BV), several clinical strains of *A. vaginae* also displayed resistance towards nalidixic acid and colistin [52][64][65][66]. Undeniably, the emergence of multi-drug resistant microbes has caused a substantial dilemma among the scientific community [31][63][65][67][68].

AMR can be acquired between microbes via several routes, including mutation, transduction, conjugation, and transposons; this topic has been heavily reviewed in the past 40 years or so [68][69][70][71][72][73][74]. The ability of *A. vaginae* to live and adapt within the *G. vaginalis*-dominated biofilms constitutes another threat in terms of ensuring successful treatment and complete remission of BV [75][76]. Members living within the biofilm often exchange genetic material, including those encoding for AMR and biofilm development. Using the next-generation sequencing method, Bostwick et al. performed a case-control study to study AMR genes' prevalence within BV patients. Their results showed that AMR genes corresponding to all class of antibiotics were detected including macrolides, (35.2%), lincosamides (35.6%), tetracyclines (21.8%), aminoglycosides (streptomycin, gentamicin, and tobramycin, 5.2% each), 5-nitroimidazoles (0.3%) and triazoles (18.7%) [65]. Moreover, a significantly higher frequency of AMR genes was detected in BV pathogens as compared to healthy control (i.e., macrolides: 58.2% versus 12.3%, lincosamides: 58.9% versus 12.3%, tetracyclines, 35.6 versus 8.0%). The monitoring of AMR in BV is crucial in assisting clinicians in choosing the best treatment for patients with BV and preventing antimicrobials misuse, which can give rise to more multi-drug resistant microbes. As a matter of fact, *G. vaginalis* has recently been shown to produce membrane vesicles that are cytotoxic against vaginal epithelial cells uncovered by a team in India [77]. As membrane vesicles are proposed to be another mechanism that promotes horizontal gene transfer, including AMR genes, these findings collectively show the dire need for an effective treatment plan to treat a polymicrobial infection, as seen in BV.

3. Diagnostics Methods of BV

Vaginal cultures for BV diagnosis generally lack positive predictive value or diagnostic value as the infection itself usually is polymicrobial in nature [20][78][79]. Also, bearing in mind that performing conventional bacteria culture can be challenging to observe fastidious microbes and is time-consuming, resulting in misinterpretation or underdiagnosis. One of the standard methods for clinical diagnosis of BV is based on the presence of three out of four Amsel's criteria in most clinical settings (Table 1) [80]. The four Amsel's criteria include: (a) presence of thin grayish-white homogenous discharge, (b) vaginal pH above 4.5, (c) potassium hydroxide (KOH) test (which is also known as the positive whiff-amine test), and (d) at least 20% clue cells (which are exfoliated vaginal epithelial cells heavily coated with the less favorable microbes) can be observed on a saline wet mount [81]. Alternatively, two other methods can be used to diagnose BV—the Spiegel criteria and Nugent's criteria. The only similarity between the three tests is the investigation of vaginal smears, though as to the interpretation process, Spiegel's criteria and Nugent's criteria focus on morphological observation of microbial cells without considering the presence of clue cells [82][83]. The Nugent's criteria, also known as the Gram stain diagnosis method, is considered the gold standard for diagnosing BV based on a 10-point scale using microscopic observation of Gram-stained vaginal smears under the oil immersion method [84]. The score is given as a weighted score calculated from the average number of different morphotypes seen per oil immersion field. Usually, there are three morphotypes described: *Lactobacillus* spp. morphotype (decrease in number scored as 0 to 4), *Gardnerella* or *Bacteroides* spp. morphotype (small gram-variable rods or gram-negative rods; scored as 0 to 4), and curved Gram-variable rods (scored as 0 to 2). The BV diagnosis is confirmed with a score of ≥7; a scoring of 4–6 indicates intermediate flora, and a score of 0–3 is classified as normal flora. Essentially, the scoring system of Nugent's criteria is derived from

the Spiegel criteria, which was described in the early 1980s. However, the Nugent's criteria present as a better, standardized scoring system with higher intercenter reliability (r = 0.82) than Spiegel criteria (r = 0.61), which led to its adoption as the gold standard for the diagnosis of BV. Additionally, the results from a study by Moussavi and Behrouzi sparked the discussion on whether Amsel's or Nugent's criteria are superior to another. The duo studied the sensitivity and specificity of each criterion of Amsel before comparing it to the Nugent's criteria [85]. Using pH criteria alone showed the lowest sensitivity (61%), while the presence of vaginal discharge alone showed moderate sensitivity (63%) with the highest specificity (80%) among all four criteria when evaluated individually for BV. Equally, the examination of clue cells alone also reflected low sensitivity (67%). Altogether, these findings indicated that the use of tests in Amsel's criteria showed lower diagnostic validity when used alone compared to Nugent's criteria, hence further strengthening the need of fulfilling three of the four described criteria described by Amsel et al. for the diagnosis of BV [81][85][86]. The Nugent's criteria versus Amsel's criteria have comparable diagnostic value for BV as discussed by many research groups, with specificity and sensitivity of Amsel's criteria reaching as high as 95.2% and 91%, respectively, when compared with Nugent's criteria [85][86][87][88]. As the Nugent's criteria require proper laboratory equipment and experienced technical staff, several studies highlighted that Amsel's criteria could be used to diagnose BV to ensure patients receive the necessary treatment promptly [86][89][90][91]. At the same time, there is another simpler classification known as Hay/Ison criteria, which classified vaginal microbiome into three different categories: normal (Group 1), intermediate (Group 2), and BV (Group 3), depending on the relative amount of *Lactobacillus* morphotypes as compared to *Gardnerella* morphotypes [92]. During the first introduction of this classification, Ison and Hay successfully diagnosed BV in 83 out of 162 patients, and the method showed high sensitivity (97.5%), specificity (96%), and predictive value for a positive (94.1%) and negative (96%) test, kappa index = 0.91, when compared with the Amsel's criteria. A few years later, Chawla et al.'s report also compared Hay/Ison classification and Nugent's criteria [93]. Their analysis successfully diagnosed 70 BV cases (32.86%) by Nugent's method and 87 (40.85%) BV cases by Hay/Ison classification. Based on their calculation, Hay/Ison classification's sensitivity and specificity were determined as ≥97.2% and ≥88.1%, respectively. Collectively, these results implied the suitability of the Hay/Ison classification to be utilized as an alternative diagnosis method when there is a lack of time or expertise [92][93][94]. A team in Italy also suggested the potential of automation using the WASP® automatic system (BioMérieux diagnostics) to analyze samples collected in LMB ESwab® (BioMérieux diagnostics). The introduction of advanced technology helps increase reliability and shorten sampling time while ensuring timely diagnosis and treatment [95].

Table 1. Comparison between Amsel's, Nugent's, and Hay/Ison criteria (BV: bacterial vaginosis) (Adapted from Hainer and Gibson [171]).

	Amsel's criteria	Nugent's criteria*	Hay/Ison criteria
Type	Clinical and laboratory diagnosis	Laboratory diagnosis	Laboratory diagnosis

Diagnosis duration	Fast	Long	Long
Expertise requirement	Clinicians	Experienced laboratory technicians and pathologist	Experienced laboratory technicians
Laboratory requirement	Low	High	Moderate
Grading system	Diagnosis is confirmed when three out of four criteria are fulfilled. (a) presence of thin grayish-white homogenous discharge (b) vaginal pH > 4.5 (c) potassium hydroxide (KOH) or the positive whiff-amine test (d) at least 20% clue cells observed on a saline wet mount	Score 0 – 4: Normal flora Score 4 – 6: Intermediate Score ≥7: BV	Group 1: Normal flora (*Lactobacillus* only) Group 2: Intermediate (*Lactobacilli* = *Gardnerella*) Group 3: BV (*Lactobacilli* < *Gardnerella*)

*_Lactobacillus_ spp. morphotype (decrease in number scored as 0 to 4), _Gardnerella_ or _Bacteroides_ spp. morphotype (small gram-variable rods or gram-negative rods; scored as 0 to 4), and curved Gram-variable rods (scored as 0 to 2).

Besides using Amsel's criteria as a point-of-care test, there are other commercially available diagnostic kits for BV [96][97][98]. The OSOM BV Blue test (SEKISUI Diagnostics, MA, USA) is a rapid chromogenic diagnostic kit based on bacterial enzyme activity, sialidase [96][99]. Bypassing the need for laboratory equipment and experts for the interpretation of wet mount, the detection of sialidase activity is simple–placing the vaginal swab into the BV test vessel and mixing gently before adding the developing solution as indicated by the manufacturer's instructions [97]

[100]. Another optional diagnostic kit that would be used is the FemExam card (developed initially by Litmus Concepts, Santa Clara, CA, USA, now available from Cooper Surgical, Shelton, Conn) [80][101]. The kit comprises two cards: card 1 for pH and amines and card 2 for proline iminopeptidase (PIP) activity. The easy-to-understand feature makes it an attractive diagnostic kit; two swabs are provided with the kit, and each caters for one card. The vaginal fluid collected with the first cotton swab should be applied onto the pH test site and amine test within 2 min to induce a colorimetric reaction. A blue sign on each site indicates a pH of 4.7 or greater and the presence of trimethylamine. Another swab containing vaginal fluid is used on card 2 containing a *G. vaginalis* PIP activity test site. In the presence of PIP, enzymatic and colorimetric reactions would take place after rubbing the swab onto the test site containing a chromogen (Fast Red) and a PIP substrate (l-propyl-β-naphthylamide). A PIP-positive sample results in a pink color change on the swab tip within 5 min of the test. Although this kit does offer high sensitivity and specificity as compared to conventional clinical diagnosis, the FemExam two-card method comes at a high cost as West et al. estimated the cost per patient and cost per true case detected at US $8.32 and US $18.49, respectively [101][102]. Lowering the cost for these kits may improve the accessibility in developing countries or even allow its usage as home self-examination using kits, but it remains controversial due to the possible overdiagnosis [103][104].

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