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MUCOSAL IMMUNE RESPONSE AND MICROBIAL FACTORS IN BACTERIAL VAGINOSIS

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SUMMARY

Bacterial vaginosis (BV) is a polymicrobial syndrome afflicting about 20% of women in developed countries and more than 40% of African women. The synergy among different microorganisms causing the setting and the persistence of an altered vaginal flora and the role of host response are still to be understood. The better characterised microbial factors present in BV are the *Gardnerella vaginalis* haemolysin (Gvh), a pore-forming toxin which triggers a specific IgA response in about 50% of patients with BV and the sialidase activity which is detectable in 75% of women with BV. The absence of anti-Gvh IgA response in BV is associated with high levels of sialidase activity and IgA cleavage. *G. vaginalis* colonisation could be harmless until the host is able to counteract its toxin. When growth of other anaerobic bacteria occurs hydrolytic enzymes impair the local defence machinery of the host allowing Gvh and other microbial factors to elicit all their virulence. Increasing evidence points to synergistic relationships between *G. vaginalis* and anaerobic bacteria as pathologic core of BV.

INTRODUCTION

Bacterial vaginosis still represents an ecological mystery. BV is a very common cause of vaginal discharge in women of reproductive and postmenopausal age (*Eschenbach*, 1993). It is a polymicrobial syndrome characterised by an imbalance of vaginal flora, due to a marked reduction of the normal Lactobacilli replaced by large numbers of *Gardnerella vaginalis*, anaerobes including *Bacteroides*, *Prevotella*, and *Mobiluncus* species, and *Mycoplasma* (*Hill*, 1993). BV infrequently produces severe symptoms; the main complaint of women is malaodour mostly perimenstrual or postcoital. Several studies have shown that BV is implicated in serious obstetric and gynecologic conditions, such as preterm labour, preterm birth (*Gravett* et al., 1986), low birth weight (*Hillier* et al., 1995), premature rupture of membranes, histologic chorioamnionitis (*Hillier* et al., 1988), and pelvic inflammatory disease (PID) (*Soper* et al., 1994). Recent reports documented the correlation of BV with susceptibility to HIV infection (*Cohen* et al., 1995; *Sewankambo* et al., 1997).

Clinically, the diagnosis of BV is based on four factors, the so called Amsel criteria (*Amsel* et al., 1983), including the presence of a milky homogeneous white discharge, a vaginal pH>4.5, a positive amine odour test (release of a fishy amine odour when vaginal fluid is mixed with 10% KOH),

and the identification of clue cells (vaginal epithelial cells heavily coated with bacilli) seen on microscopic examination. Since the original study by *Spiegel* et al. in 1983, several other studies have evaluated the reliability of Gram-stained vaginal smears for diagnosing BV (*Mazzulli* et al., 1990; *Nugent* et al., 1991), presently the Gram-evaluation according to Nugent score is considered the gold standard (*Schwebke* et al., 1996). The Gram-stain of vaginal fluid from patients with a clinical diagnosis of bacterial vaginosis has a characteristic appearance: It shows many small Gram-negative or Gram-variable organisms resembling *G. vaginalis* (which comprise *Bacteroides*, *Prevotella*, and *Porphyromonas* spp.), curved Gram-variable rods (*Mobiluncus* spp.) in the absence of *Lactobacillus* species (large Gram-positive rods). The three morphotypes are quantitated and summed to yield a score 0-3 for normal flora, 4-6 for intermediate abnormal flora, 7-10 for BV (scores 9 and 10 are attained only if curved rods are present) (*Nugent* et al., 1991). Frequently clinicians make empirical diagnosis without the aid of microscopy and/or vaginal swab specimens are often sent to the diagnostic microbiology laboratory for culture test of *G. vaginalis*, but as small numbers of *G. vaginalis* can be detected also in healthy women, BV is frequently misdiagnosed.

Many basic questions regarding BV remain unanswered. It is not known what triggers the shift of the vaginal flora from lactobacilli colonisation to facultative and anaerobic overgrowths. About half of the patients having BV have no symptoms. One third of pregnant women spontaneously recover from BV in the late pregnancy. Although the epidemiological association between BV and preterm delivery has been well documented, it is still questioned if treatment during pregnancy prevents adverse outcomes. In the non-pregnant patients debate is still open on the influence of sexual habits, contraceptive methods, hormonal status on setting and persistence or recurrence of BV. Questions regarding treatment of BV as a means to prevent PID and sexual transmission of HIV are also still open.

For most clinicians the BV diagnosis is still doubtful and a simple, objective and inexpensive diagnostic test should be highly helpful. A reliable criterion for selecting patients who really need antibiotic treatment has not been proposed so far.

GARDNERELLA VAGINALIS CYTOLYSIN

G. vaginalis is a small, non-motile, catalase negative, pleomorphic bacillus. Although *G. vaginalis* has a Gram-positive organisation of the cell wall, it stains frequently like a Gram-negative or Gram-variable coccobacillus (*Catlin*, 1992).

Gardner and Dukes identified the bacterium in 1955 as the aetiological agent of the so-called "non-specific" vaginitis later defined bacterial vaginosis because of the absence of inflammatory signs (*Gardner* and *Dukes*, 1955). *G. vaginalis* adheres to exfoliated epithelial cells forming the so-called 'clue cells' which constitute the main diagnostic marker for BV. The role of *G. vaginalis* in BV has been long debated, in fact it is the only bacterium invariably present in high numbers in BV, but rarely it is the only microbial species colonising patients. The lack of inflammatory reaction

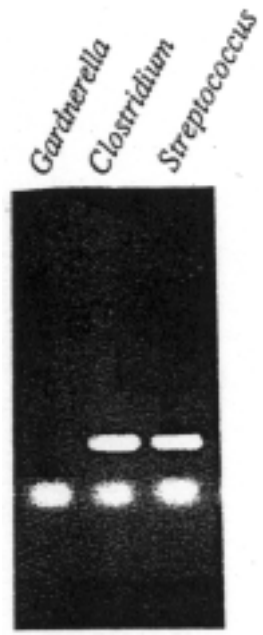

Figure 1: PCR amplification of *Gardnerella vaginalis*, *Clostridium perfringens*, *Streptococcus pyogenes* DNAs obtained with synthesised degenerated primers corresponding to conserved regions upstream and downstream the consensus undecapeptide sequence ECTGLAWEWWR.

and the detection, although in low amounts, in healthy women of *G. vaginalis* portrayed this bacterium as non-invasive and of low virulence.

Exponentially growing *G. vaginalis* releases in the culture broth a haemolysin (Gvh) which is the haemolytic factor responsible for the β-haemolysis of the bacterium on human blood agar plates. The toxin provokes a swelling of human erythrocytes which precedes haemoglobin release and ghost formation. Cell lysis occurs by osmotic unbalance due to the formation of transmembrane pores on the target plasma membrane (*Cauci* et al., 1993a). The protein is hydrophobic and requires ammonium acetate for preservation of activity. Very interestingly protein stability is essentially constant in the pH range 5-7 (pH values found in vaginal fluids of women with BV) whereas a dramatic loss of toxin activity is achieved by storing it at pH 4 (pH value of healthy women). Haemolysis occurs with a sigmoid dose dependence profile indicating that a co-operative aggregation of toxin monomers on the target membrane forms the effective pores. The lytic process exhibits a marked temperature dependence with maximal efficiency at 37°C. At variance with other pore-forming agents the best pH value for Gvh lysis is around 5 (*Cauci* et al., 1993b). So the toxin appears to elicit its maximal activity in conditions present in the vaginal niche during BV. Gvh binds avidly to lipid vesicles comprised of cholesterol and phospholipids especially if a negatively charged phospholipid as phosphatidyl serine is present. Overall features of Gvh resemble those of the cholesterol-binding toxins produced by Gram-positive bacteria belonging to the genera *Bacillus*, *Clostridium*, *Listeria*, and *Streptococcus*. These toxins form a family of antigenically related membrane-damaging proteins

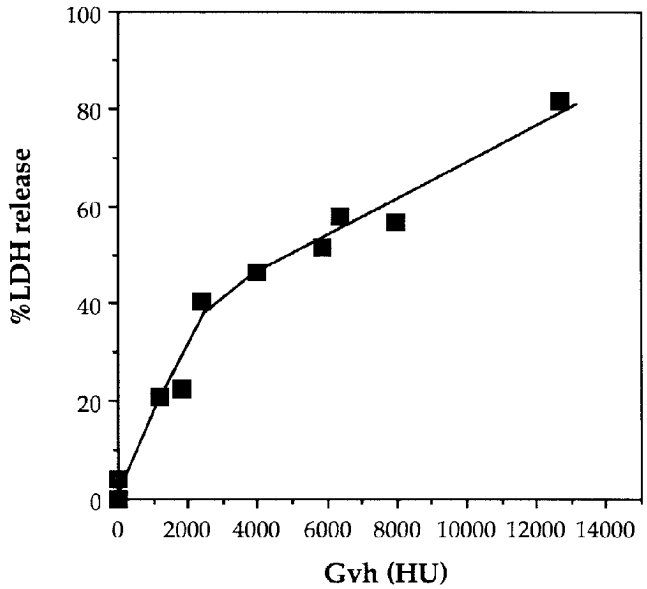

Figure 2: Release of lactate dehydrogenase (LDH) from human amnion cells in primary culture after 15 min incubation at 37°C with increasing amounts (expressed in haemolytic units, HU) of the *Gardnerella vaginalis* toxin (Gvh).

having cholesterol-anchoring properties. All these toxins (perfringolysin O (PFO), streptolysin O (SLO), listeriolysin O (LLO), alveolysin (ALV), pneumolysin (PLY)) share homologies in the protein sequence. In particular they contains a conserved undecapeptide sequence (ECTGLAWEWWR) in the C-terminal part region which is functionally important for activity (*Alouf* and *Geoffroy*, 1991). To verify if also Gvh sequence contains the conserved undecapeptide we synthesised degenerated primers corresponding to upstream and downstream conserved regions and made PCR amplification using *G. vaginalis*, or *Clostridium perfringens*, or *Streptococcus pyogenes* DNA, obtaining a good amplification in the case of *Clostridium* and *Streptococcus* but not in that of *Gardnerella* (Figure 1). This finding indicates that Gvh sequence must diverge from that of the cholesterol-binding toxins. Moreover Gvh is not antigenically related to such proteins as it is not recognised by an anti-SLO serum, nor is sulphydryl-activated as are these toxins being instead deactivated by reducing agents (*Cauci* et al., 1993a). Although in many respects Gvh action resembles PFO, a member of the cholesterol-binding, sulphydryl-activated cytolysins, the *G. vaginalis* toxin is an unique cytolysin suitable to act in the vaginal ecosystem.

Gvh could damage host cells other than erythrocytes perturbing host defence. In fact high doses of Gvh are cytolytic for human umbilical vein endothelial cells and for human leukocytes (*Rottini* et al., 1990). Gvh could be involved in the damage to the epithelial cells, which after desquamation form the clue cells.

We have measured Gvh cytotoxic effects also on amnion cells in primary culture (Figure 2) by monitoring the release of lactate dehydrogenase (LDH) after 15 min incubation at 37°C. This finding supports the hypothesis of a di-

rect role of the toxin in the intrauterine niche and in obstetric complications.

At low doses Gvh causes chemotaxis inhibition of human polymorphonuclear leukocytes *in vitro* (*Shubair* et al., 1993), so the toxin could play a role in the inhibition of inflammatory response in patients with BV in concert with other inhibitors of leukocyte functions as succinate produced by obligately anaerobic rods.

ENZYMATIC MICROBIAL FACTORS IN BACTERIAL VAGINOSIS

Extracellular mucolytic enzymes, including mucinases and sialidases have been found in vaginal fluids of patients with BV (*McGregor* et al., 1994). Mucins play a relevant role in the homeostasis of the female reproductive tract and are important in the mucosal host defence against various microorganisms as bacteria, protozoa and viruses, and cytotoxic substances. Particularly in pregnancy the mucin plug is considered to prevent the entry of microorganisms into the uterine cavity. Bacterial mucinases play complex roles in the physical defence of the host, in cell recognition and in microbial pathogenesis.

Sialidases, or neuraminidases, have been implicated in the pathogenesis of many diseases. They catalyse the removal of terminal sialic acid residues from various glycoconjugates as mucins, and play roles in bacterial nutrition, cellular interactions and immune response evasion (*Corfield*, 1992; *Pilatte* et al., 1993; *Taylor*, 1996). Bacteria involved in urogenital infections are able to produce sialidases. *Briselden* et al. (1992) demonstrated increased levels of sialidase activity in vaginal wash samples obtained from women with BV and they correlated sialidase activity mainly with the presence of high titres of *Prevotella* spp. and *Bacteroides* spp. Sialidase activity found in vaginal fluids exhibits maximal activity in the pH range from 4.5 to 5.5 (typical pH of women with BV) whereas a drastic reduction is observed at lower pHs (values found in healthy women). An average 80% reduction was measured at pH 3.5 (*Cauci* et al., 1996). This observation could be of relevance in the strategies adopted to eradicate bacterial vaginosis by local therapy.

Noteworthy sialidase activity was present in all women who had recurrent BV 1 month after oral therapy with metronidazole or ampicillin (*Briselden* et al., 1992). Persistence or recurrence of sialidase activity in vaginal fluids of pregnant women with BV treated with 2% clindamycin cream or placebo correlate with an increased risk of preterm birth and low birth weight, whereas mucinase activity did not (*McGregor* et al., 1994).

Proline aminopeptidase activity has been identified in the vaginal fluids of women with BV. Prolidase is presumed to be produced by *G. vaginalis* and *Mobiluncus* spp. Other genera known to produce prolidases include *Peptostreptococcus*, *Streptococcus*, *Actinomyces*, *Propionibacterium* (*Schoonmaker* et al., 1991). The prolidase assay shows an excellent correlation with Gram-stain diagnosis, and has been proposed as a diagnostic test for BV (*Hillier*, 1993), but unfortunately it requires many hours of incubation so it is more suitable for research purposes than as doctor office test. Additional work is needed to better identify the microorganism(s) responsible for prolidase activity and clarify the role of this enzymatic activity in BV.

Several other bacterial enzymes including phospholipases have been suggested as potential virulence determinant

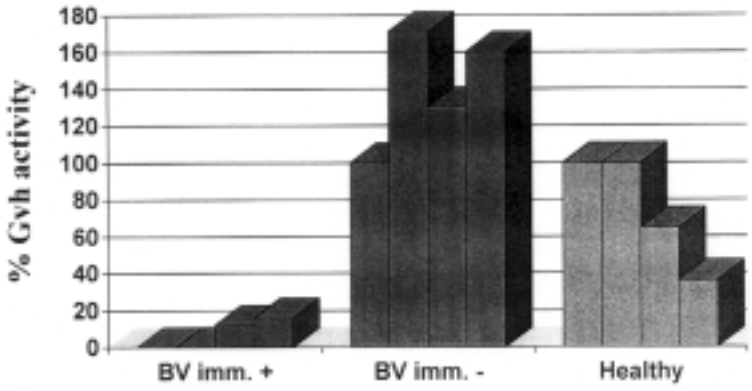

Figure 3: Haemolytic activity of Gvh after *in vitro* 1:1 incubation with vaginal washings from four patients having BV positive for anti-Gvh IgA (BV Immune+), four patients with BV and no detectable anti-Gvh IgA (BV Immune-), and four healthy women without anti-Gvh IgA.

in BV but production of such enzymes has been proved only by testing the *in vitro* release from cultured isolates. Proteases producing microorganisms include *Prevotella melaninogenica*, *Prevotella bivia*, *Bacteroides fragilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Ureaplasma urealyticum*.

Production of virulence factors such as sialidases, collagenases, prolidases and proteases by microorganisms implicated in BV may play key roles in the perturbation of host defences, allowing ascent of microorganisms into the upper genital tract and/or favouring subsequent microbial or viral infections (*McGregor* et al., 1986; *McGregor* et al., 1994).

ANTI-HAEMOLYSIN IgA RESPONSE AND SIALIDASE ACTIVITY

A specific IgA response against Gvh has been documented in the vaginal secretions showing the involvement of this toxin in the *in vivo* colonisation by *G. vaginalis* (*Cauci* et al., 1996) and the ability of the bacterium to activate the immune response in spite of the paucity of inflammatory signs. The purified toxin is a suitable antigen to evaluate the host response to *G. vaginalis* independently of the particular strain of the bacterium harboured by the woman. Previous attempts to evaluate the host immune response in serum of BV patients demonstrated that using the whole bacterium as antigen the immune response is detectable only if the *G. vaginalis* strain colonising the woman is employed (*Ghione* et al., 1989). Anti-Gvh IgA levels are significantly higher in vaginal fluids of women with BV than in the normal controls: anti-haemolysin antibodies are detectable in about half of the women with BV, 20% of women with intermediate microflora, and in 9% of healthy women (*Cauci* et al., 1996). Vaginal fluids of women with BV having anti-Gvh IgA (Immune+) are able to completely inactivate *in vitro* the cytolysin, whereas those of women with BV without anti-Gvh IgA (Immune-) are inactive; some of the vaginal fluids of healthy women (negative for anti-Gvh IgA) are able to partially deactivate the toxin likely through aspecific mechanisms (Figure 3). It is to

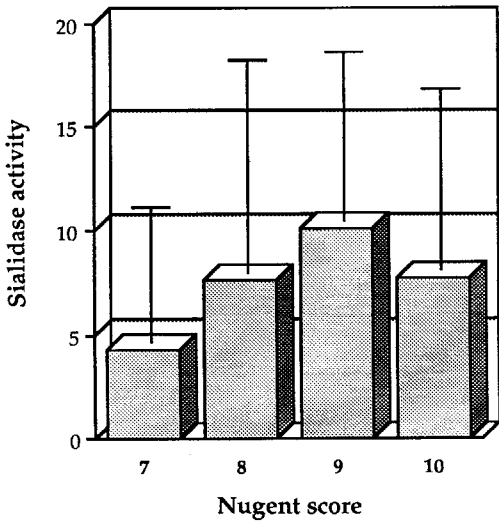

Figure 4: Average values (error bars indicate standard deviations) of sialidase activity in vaginal fluids of 57 women with BV having different Nugent scores. No subset was statistically different from each of the others (using Student t test for unpaired observations, $p>0.05$).

be underlined that the absence of an anti-Gvh response in BV patients colonised by *G. vaginalis* is not due to the inability of the strains harboured by the patients to secrete the haemolysin and that the two subgroups of BV patients (Immune+ and Immune-) are indistinguishable by the normal criteria used to diagnose BV: pH, amine odour, discharge, clue cells, Nugent score.

Sialidase activity has been reported to be present in 84% (*Briselden* et al., 1992), 45% (*McGregor* et al., 1994) and 75% (*Cauci* et al., 1998a) of the vaginal fluids of women with BV. Sialidase activity is absent in vaginal fluids of women with *Candida* vaginitis (without concurrent BV), and very low values are detectable in only 5% of healthy women. Interestingly sialidase levels of clinical diagnosed BV are not increasing with the value 9 and 10 of the Nugent score (Figure 4). This finding confirms previous *in vitro* observations on the inability of *Mobiluncus* spp. to produce sialidases (*Briselden* et al., 1992). As very few isolates if any of *G. vaginalis* secrete sialidases, anaerobic Gram-negative rods such as *Prevotella* spp., and *Bacteroides* spp. are the most probable source of sialidases in BV.

When the two subgroups of women with BV (Immune+ and -) were examined 87% of the women Immune- were positive for sialidase, whereas 59% of women Immune+ were positive. Moreover women with BV and no detectable anti-Gvh response show a five-fold higher sialidase mean value than women with BV positive for anti-Gvh response (*Cauci* et al., 1998a). This study demonstrated that high sialidase activity is associated with the absence of an IgA local immune response to *G. vaginalis* cytolysin.

Recently the existence of two main different subgroups of BV patients has been further substantiated by analysis of immunoglobulin integrity in vaginal fluids: the subgroup of patients without anti-Gvh response shows extensive degradation of IgA and IgM (*Cauci* et al., 1997; *Cauci* et al., 1998b).

SYNERGY BETWEEN *GARDNERELLA VAGINALIS* AND ANAEROBIC BACTERIA

Synergistic mechanisms among microorganisms involved in bacterial vaginosis have been already hypothesised in the past (*Pheifer* et al., 1978; *Chen* et al., 1979; *Spiegel* et al., 1980). Very recent studies support this hypothesis: a symbiotic relationship between *G. vaginalis* and *P. bivia* involving ammonia utilisation by the former has been described by *Pybus* et al. (1997); the combination of *G. vaginalis* and anaerobic bacteria and/or *M. hominis* has been proposed as pathologic core of BV on the basis of microbiologic epidemiology by *Thorsen* et al. (1998).

The only virulence factor so far characterised of *G. vaginalis* is its cytolysin, Gvh. The toxin is suitable to work in the vaginal niche but it is largely inactivated at pH 4, becoming more active at pH around 5 that can result from amine release by anaerobes.

It is conceivable that Gvh contributes to survival of *G. vaginalis* by providing metabolites, but the supply of nutrients especially of iron could also favour colonisation of strictly iron requiring species as *Bacteroides* and *Prevotella* spp.

The detection of a specific IgA response against Gvh induce to hypothesize that *G. vaginalis* colonization is relatively inoffensive until the host immune system is able to counteract the cytotoxic effects of the toxin. This is in keeping with the observation that colonization by *G. vaginalis* in patients without anaerobic overgrowth may frequently be asymptomatic and transient. When synergistic growth of anaerobic bacteria occurs, microbial factors as hydrolytic enzymes and/or exoproducts impair the immune response of the host permitting the toxin (and other virulence determinants) to elude clearance and explicate all the virulence potential.

Sialidases, mainly produced by anaerobes, may promote virulence not only by destroying the mucins and enhancing adhesion of bacteria but also by impairing the specific IgA immune response against virulence factors as cytolysins. Cleaving the sialic acid residues off the IgA molecules, makes them more accessible to protease degradation (*Kilian* and *Russel*, 1994; *Mattu* et al., 1998). Other mechanisms as the modification of the carbohydrate complexes involved in cellular recognition events and in aspecific antibacterial functions of S-IgA may be compromised. Sialidases are often one of several virulence factors secreted by anaerobic bacteria which are able to produce an array of proteases.

Bacterial proteases (as prolidases) have been detected in cervical secretions of BV patients but until now their pathologic role is still hypothetical. Our data on the *in vivo* cleavage of mucosal IgA and IgM give insights on the failure of the host to counteract bacterial proteases. In fact IgAs are considered intrinsically resistant to proteolysis and the secretory component of mucosal immunoglobulins enhances this resistance. Moreover the release of protease inhibitors in human secretions and the production of a specific immune response against bacterial proteases, should protect immunoglobulins from *in vivo* degradation. Our study, to our knowledge, is the first demonstration of the *in vivo* IgA and IgM degradation in human mucosal secretions as a consequence of microbial pathogens colonisation (*Cauci* et al., 1998b). The immunoglobulin degradation pattern, which includes several low molecular weight bands and the involvement of IgM in the degradation exclude that the cleavage is entirely due to known pro-

teases, as IgA1 proteases (*Mulks* and *Shoberg*, 1994). It remains to identify which microorganism(s) produces the proteolytic enzymes responsible for the extensive cleavage of the vaginal secretory immunoglobulins.

It has to be reminded that IgA dimers are thought to have a pivotal role in the protection of the vaginal tract. A very recent paper has shown that vaginal IgAs but not IgGs together with systemic cell-mediated immunity have a main role in the protection from viral transmission (*Mazzoli* et al., 1997). Thus the development of any HIV vaccine that should enhance the host protective mucosal immunity against penetration of viruses must take into account that a very common women disease such as BV can compromise the immunoglobulins integrity. If future studies will demonstrate that the patients with compromised local immune response show a higher risk of prematurity, bacterial invasion of the amniotic cavity, upper genital tract infections, bacterial vaginosis recurrence, susceptibility to HPV or HIV infection, sialidase activity (or others hydrolytic enzymatic activities) could become a valuable diagnostic marker for predicting the severity of the disease and helping in the choice of the chemotherapeutic treatment.

CONCLUSIONS

Much remains to be learned about the interactions among members of the vaginal microflora and factors of the human mucosal defence. Bacterial inhabitants of ecological "niches" in the female genital tract are dynamic and can vary in number and composition also in the same host. Complex interrelationships among invading microorganisms themselves and between microbial virulence determinants and host defence factors are increasingly evidenced. In particular the role of hydrogen peroxide producing Lactobacilli present in healthy women is under active investigation. Some synergistic mechanisms in which *G. vaginalis* is implied have been clarified increasing our understanding of BV pathogenesis. More extensive studies of the mechanisms involved in the homeostasis of the vaginal ecosystem should indicate new avenues for prevention of preterm labour, low birth weight, transmission of STD and susceptibility to sexual infection by HIV.

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