Vaginal Microbiota

6

Werner Mendling

Abstract

The knowledge about the normal and abnormal vaginal microbiome has changed over the last years. Culturing techniques are not suitable any more for determination of a normal or abnormal vaginal microbiota. Non culture-based modern technologies revealed a complex and dynamic system mainly dominated by lactobacilli.

The normal and the abnormal vaginal microbiota are complex ecosystems of more than 200 bacterial species influenced by genes, ethnic background and environmental and behavioral factors. Several species of lactobacilli per individuum dominate the healthy vagina. They support a defense system together with antibacterial substances, cytokines, defensins and others against dysbiosis, infections and care for an normal pregnancy without preterm birth.

The numbers of *Lactobacillus (L.) iners* increase in the case of dysbiosis.

Bacterial vaginosis (BV) – associated bacteria (BVAB), *Atopobium vaginae* and *Clostridiales* and one or two of four *Gardnerella vaginalis* – strains develop in different mixtures and numbers polymicrobial biofilms on the vaginal epithelium, which are not dissolved by antibiotic therapies according to guidelines and, thus, provoke recurrences.

Aerobic vaginitis seems to be an immunological disorder of the vagina with influence on the microbiota, which is here dominated by aerobic bacteria (*Streptococcus agalactiae, Escherichia coli)*. Their role in AV is unknown.

W. Mendling (✉)
Deutsches Zentrum für Infektionen in Gynäkologie
und Geburtshilfe, St. Anna Hospital,
Vogelsangstrasse, 106, 42109 Wuppertal, Germany
e-mail: w.mendling@t-online.de;
http://www.werner-mendling.de

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Vaginal or oral application of lactobacilli is obviously able to improve therapeutic results of BV and dysbiosis.

Keywords
Vaginal microbiota • Dysbiosis • Bacterial vaginosis • Aerobic vaginitis • Lactobacilli • Probiotics

6.1 A Historic Perpective

Albert Döderlein (1860–1941) was the first one to discover the importance of lactic acid producing bacteria in the vagina (Döderlein 1892). Krönig (1895), a co-worker of Döderlein, described lactobacilli as anaerobic and curved rods, which were later cultured by Curtis (1913) and named *Mobiluncus curtisii* (Spiegel and Roberts 1984). Finally, Stanley Thomas (1928) coined the term *Lactobacillus acidophilus*. In the 1980s Lauer, Helming and Kandler were able to distinguised several Lactobacillus species previously termed L. acidophilus by DNA-DNA hybridisation.

At the beginning of the last century first attempts were made to grade the vaginal microbiota. Manu af Heurlin (1914) characterised the vaginal microbiota of children, pregnant and not pregnant women and women of old age and tried to establish grades of healthines. Robert Schröder (1921) was the first one to define three bacteriologically different vaginal microbiota types termed "Reinheitsgrade" (grades of purity). Otto Jirovec (Jirovec et al. 1948) distinguished between six vaginal microbiota types (normal, abnormal, abnormal with many leucocytes, gonorrhea, trichomoniasis, candidosis).

In 1955 Herman Gardner and Charles Dukes (1955) described *Haemophilus vaginalis*, later renamed as *Gardnerella vaginalis* (Greenwood and Pickett 1980) as the main causative for bacterial vaginosis (BV), the most common disturbance of the vaginal microbiota and considered it as a sexually transmitted disease. Furthermore, they emphasised the importance of microscopy and defined "clue cells" as diagnostic marker.

By the end of the last century the hitherto valid definition of bacterial vaginosis was described as "a replacement of lactobacilli by characteristic groups of bacteria accompanied by changed properties of the vaginal fluid" (Mardh et al. 1984). With the advent of molecular and genetic technologies we had to reconsider our view and definition of a normal vaginal microbiota. New bacteria were discovered and the concepts of a bacterial biofilm and of vaginal microbiota types was introduced.

6.2 Normal Vaginal Microbiota

The outer and inner surfaces of a child born by vaginal delivery are primarily colonised by the vaginal microbiota of the mother. Futher colonisers are aquired from the skin and mouth microbiota of the mother. In the last years it became evident that mothermilk harbours a unique microbiota, mainly dominated by Lactobacilli, which is transfered to the suckling child (Martin et al. 2003). Before the menarche the vagina microbiota is a unsteady mix of skin and gut microbes, which may harbor some lactobacilli (Fettweis et al. 2012). The environmental conditions for lactobacilli become improved by estrogens and progesterone with the start and during the reproductive phase of women. Estrogens support the proliferation of the vaginal epithelium and the development of intraepithelial glycogen, while progesterone supports the cytolysis of epithelial cells, which release glycogen. Lactobacilli and other bacteria are able to metabolise this glycogen to glucose and maltose and further to lactic acid. This leads to a vaginal pH of 3.8–4.4 which is defined as normal.

Until now more than 120 *lactobacillus* species have been described (de Vos et al. 2012). Within the vaginas of women of reproductive age more

than ten different species can be found. However, a single women is usually dominated by one or two species, of which the most frequent are *L. crispatus*, *L. gasseri*, *L. jensenii and L. iners* (Vasquez et al. 2002). Several of these lactobacilli are able to produce bacteriocines, biosurfactants and coaggregating molecules to inhibit the adhesion of pathogens (Reid 2001). Another property of lactobacilli found within the vagina is the ability to produce hydrogen peroxid (H_2O_2). Lactobacilli are by definition strict anaerobic bacteria. However, they are often found in niches enriched with oxygen. To detoxify the otherwise toxic oxygen, several but not all lactobacilli are able to produce H_2O_2. The presence of H_2O_2 producing lactobacilli is negatively associated with the formation of BV (Eschenbach et al. 1989).

6.2.1 The Normal Vaginal Microbiota: A Mixture of Many Bacteria in a Balance

Currently, the dogma, that a healthy vaginal microbiota is dominated by lactobacill is faltering as by genomic sequencing over 250 species of bacteria have been identified in the vagina (Li et al. 2012). Besides Lactobacilli many other bacteria can be found in the normal or abnormal vaginal microbiota, such as *Actinomyces*, *Aerococcus*, *Allisonella*, *Alloscardovia*, *Anaerococcus*, *Arcanobacterium*, *Atopobium*, *Bacteroides*, *Balneimonas*, *Bifidobacterium*, *Blastococcus*, *Blautia*, *Bulleidia*, *Campylobacter*, *Citrobacter*, *Coriobacteriacea*, *Corynebacterium*, *Enterobacter*, *Escherichia*, *Facklamia*, *Faecalibacterium*, *Finegoldia*, *Gardnerella*, *Gemella*, *Haemophilus*, *Lachnospiracea*, *Massilia*, *Megasphera*, *Mobiluncus*, *Mollicutes*, *Moryella*, *Olsinella*, *Parvimonas*, *Peptinophilus*, *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Proteobacteria*, *Providencia*, *Rhizobialis*, *Ruminococcaceae*, *Salmonella*, *Shigella*, *Shuttleworthia*, *Sneathia*, *Solobacterium*, *Staphylococcus*, *Streptococcus*, *Veillonella*, *Ureaplasma*, and many lactobacilli species (Gajer et al. 2012).

Within the human microbiome project, the vaginal microbiome project investigated the relationship between the vaginal microbiota and various physiological and infectious conditions (Fettweis et al. 2012). Various "vagitypes" have been identified of which many are dominated by a single bacterial taxon, others by a broad spectrum of different bacteria. Interestingly the ethnic background of women has an influence on the vaginal microbiota, as white/caucasian women are dominated by *L. iners*, asian women by *L. crispatus* and black and hispanic women by *L. jenseni. However*, *a* significant group of women harbored no lactobacilli in the vagina (Ravel et al. 2011, Hickley et al. 2012).

Jespers et al. (2012) identified in Antverpen/Belgium similarly three types of vaginal microbiota in healthy premenopausal women and in women at risk for BV of a STD clinic.

One group of women was dominated by *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis* with lower counts (<30 %) of *L. gasseri* and *Atopobium vaginae*. A second group harbored preferentially *L. gasseri* and *L. vaginalis*, but less *L. jensenii*, *L. iners* or *L. crispatus*. The third group was dominated by *L. gasseri*, *A. vaginae* and *L. iners*. Whithin the third group were mainly african and asian women. These flora types underly dynamic variations during the menstrual cycle and are influenced by external circumstances, for instance sexual behavior. But they seem to be in a rather stable balance, and a healthy vaginal system can obviously be strong enough to correct disturbences from outside, as Gajer et al. (2012) demonstrated in a longitudinal study. Women were grouped according to Ravel's et al. (2011) "community state types" and vaginal swaps were taken for 16 successive weeks. Furthermore, menstruation, tampon use, vaginal, anal or oral sex, sex toys, digital penetration and lubricants were documented. It was evident, that the vaginal microbiota of several women became heavily disturbed by some of these actions, however other microbiotas showed no disturbences despite very frequent manipulations. Once again, black women were significantly different in their "community state types".

The vaginal flora is influenced by the anal and the oral flora. Petricevic et al. (2012) found in around 80 % of 30 pregnant women and in 40 % of 30 postmenopausal women one or more *Lactobacillus ssp*. in the vagina and in the rectum, and they were in 80 % resp. 40 % of the same identity. These women were also in 50 % (pregnant) and in 30 % (postmenopausal) colonised by one or more *Lactobacillus ssp*. in their mouth. A healthy vaginal, balanced microbiota protects not only against ascending infections or HIV acquisition, but also against prematurity (Hoyme and Hübner 2010; Donders et al. 2011; Lamont et al. 2011; Martin 2012; Mendling et al. 2013).

On the other hand, too many vaginal lactobacilli (Cibley and Cibley 1991) or abnormal long lactobacilli (Horowitz et al. 1994) can cause vestibular pruritus, itching and dysuria. This "cytolytic vaginosis" or "lactobacillosis" can be misdiagnosed clinically as candidosis (Demirezen 2003).

6.2.2 Gene Polymorphisms and Vaginal Immunity

The vaginal microbiota is not only influenced by the ethnic background, but also by gene polymorphisms: the individual capacity to produce low or high levels of anti- or pro-microbial factors influences the composition of the vaginal microbiota. Polymorphisms in the interleukin-1 receptor antagonist gene or the Toll-like receptor (TLR) 4, which acts in the innate recognition of Gram-negative bacteria, influence the quantity of vaginal bacteria (Rodriguez et al. 1999) and can influence individual susceptibility to pregnancy complications (Genc and Onderdonk 2011). Such polymorphisms vary between different racial groups and may be associated with the different ecosystems between different populations (Linhares et al. 2010). Interestingly, periodontal disease and BV are influenced by gene polympr-phisms and are both associated with preterm birth (Sanu and Lamont 2011).

The innate immune system of the vagina is represented by soluble factors like mannose-binding lectin (MBL), defensins, secretory leucocyte protease inhibitor, nitric oxid, and membrane-associated factors, the TLR (11 TLR have been identified) and phagocytes. Different TLR recognize lipoproteins and peptidoglucan in the surface of Gram-positive bacteria, the lipopolysaccharid of Gram-negative bacteria, flagellins, and others (Linhares et al. 2010; Mirmonsef et al. 2011). Vaginal cells release defensins with a non-specific antimicrobial activity. The production of special defensins is stimulated by estrogens and inhibited by progesterone. Bacterial vaginosis in pregnant women was associated with lower vaginal concentrations of defensin 3 (Mitchell et al. 2013). Women with MBL deficiency due to a polymorphism are more susceptible to recurrent *Candida albicans* vaginitis (Babula et al. 2003).

Toll-like receptor ligands and fatty acids, which are produced by many vaginal bacteria, have dramatic effects on the vaginal immune function: the anaerobes of BV produce bad smelling amines (putrescin, cadaverin and others), succinate, sialidases, and immunomodulatory substances such as lipopolysaccharides, lipoteichoid acids and peptidoglycans with many influences on cytokines and other immune responses (Mirmonsef et al. 2011).

6.3 Abnormal Vaginal Flora

A disturbed vaginal microbiota may be the cause for various diseases. However, within this chapter only two will be discussed, as only they are directly connected to a dysbiotic vaginal microbiota.

6.3.1 Bacterial Vaginosis (BV)

Gardner and Dukes (1955) named the vaginal disorder *Haemophilus vaginalis* vaginitis and described "clue cells". It was later characterized as bacterial vaginosis and is defined by a replacement of lactobacilli with characteristic groups of bacteria accompanied by changed properties of the vaginal fluid (Weström et al. 1984).

The first diagnostic criteria for BV were published by Amsel et al. (1983): grey-white milky discharche, pH >4.5, bad "fishy" smell, especially if 10 % KOH solution is added, and at least 20 % "clue cells". Later, Eschenbach et al. (1989) determined the lack of H_2O_2-producing lactobacilli, an overgrowth of G. vaginalis and anaerobic Gram – negative rods and anaerobic Gram – positive cocci as essential factors for the presence of BV. To improve the diagnostic analyis, Nugent et al. (1991) poposed a score (Nugent score): 0–3 = normal, 4–6 = intermediate, 7–10 = BV. It is solely based on Gram-staining criteria. However, it has been reported, that roughly 20 % of pregnant women in Germany have BV by definition, but not all suffered from symptoms (Mendling et al. 2013).

The development of BV was long associated with the presence of G. vaginalis. Currently, four different *G. vaginalis* strains have been described, of which only two produce the BV marker sialidase and only one predominated in women with BV (Jayaprakash et al. 2012). Hence, the existance of *G. vaginalis* in the vagina is no precondition of BV. In the last years it became evident, that no single strain alone is the cause of BV. Recently, BV associated bacteria (BVAB) 1, 2 and 3 have been described. Nearly all of these bacteria are unknown in clinical practice. Women, who harbor BVAB, especially *G. vaginalis* and *Leptotrichia/Snethia* or *Megasphera* in higher concentrations, develop significantly more BV ($p = 0.001$) (Marrazzo et al. 2012; Hillier et al. 2010). Additionally, Fredricks et al. (2005) demonstrated that the presence of *L. iners* is strongly associated with BV. *L. iners*, which belongs to the normal microbiota, but seems to be a "poisened apple in the basket", because its presence is strongly connected with a shift of normal to abnormal microbiota. On the other hand, Women, who harbor *L. crispatus* are significantly less at risk to develop BV than others ($p = 0.02$).

BV is influenced by environmental and genetic factors. Thus, gene polymorphisms influence the occurrence of *G. vaginalis* and *A. vaginae* (Verstraelen et al. 2009). Furthermore, decreasing estrogen levels influence the number and diversity of vaginal lactobacilli and are in some women a risk factor for urogenital infections.

Sexual practices, especially receptive oral sex and digital vaginal penetration are significant risk factors for BV (which is perhaps an explanation for a higher risk of BV in lesbian women (Marrazzo et al. 2012)), and also cigarette smoking, black race, receptive anal sex before vaginal intercourse (Cherpes et al. 2008; Manhart et al. 2012). It should be kept in mind, that Gardner and Dukes (1955) could not cause BV by transferring cultivated *G. vaginalis* from a woman with BV to a healthy woman, but if they transferred the discharge of a woman with BV to a healthy vagina, this woman got BV. Hence, not single bacteria, albeit in high numbers, is important, but a critical mixture of BVAB together with special lactobacilli, and a lack of other lactobacilli seem to play a role in the development of BV (Lamont et al. 2011). Lamont et al. (2011) discussed, "that it is whether or not the strain/species of *Lactobacillus* produces H_2O_2 that dictates whether BV is present or absent. However, given that H_2O_2 – producing *L. gasseri* are found in BV patients, albeit at lower incidence, one might also argue that in vitro production of H_2O_2 is only a biomarker of a protective species of *Lactobacillus*, not an active factor in limiting the growth of vaginal anaerobes."

6.3.2 Polymicrobial Bacterial Biofilms in BV and Sexual Transmission

A biofilm is defined as any group of microorganisms in which cells stick to each other on a surface. The first biofilm in gynecology was described in women with BV by Swidsinski et al. (2005). The epithelial cells of the vagina of healthy pre- or postmenopausal women or of children are free of bacteria. But BV is characterized by structured polymicrobial biofilms adherent to epithelial cells of the vagina. "Clue cells", which Gardner and Dukes (1955) have seen microscopically on vaginal epithelial cells of the discharge, have their origin from this biofilm – coat on the vaginal wall. The biofilm consists in

its majority of *G. vaginalis* (>50 % up to 90 %) and of *A. vaginae* (10–40 %), but also of lactobacilli and other bacteria. *G. vaginalis* formes in vitro significantly stronger biofilms, if *Fusobacterium nucleatum* or *Prevotella bivia* are added (Machado et al. 2013). No BV is equal in the composition of different bacteria and no biofilm of BV is equal.

It is unknown, whether the lactobacilli found in the biofilm are *L. iners* or other species. If treated with metronidazole, it does not get disrupted and, thus, seems to be the reason for the high recurrence rates of about 30 % after 3 months and 60 % after 6 months following therapy, respectively.

In addition to be present in the vagina, the BV-typical biofilm can be found on epithelial cells in the urine of females with BV and in the urine of their partners. Sometimes it may be found in cryopreserved donor semen, in the endometrium of non-pregnant women and in tissue of missed abortion/abortion (Swidsinski et al. 2013). If men were asked to void their urine after having pulled back the preputium, no biofilm was found, which confirms the observation that male circumcision reduces the risk for ulcerations, trichomoniasis and BV (Gray et al. 2009). Circumcision is associated with a significant change in the microbiota and with a significant decrease in putative anaerobic bacteria, especially *Clostridiales* and *Prevotellaceae* (Price et al. 2010). Women with treated BV have a higher risk for recurrence, if they have intercourse with the same partner without using condoms (Marrazzo et al. 2012; Guédou et al. 2013).

6.3.3 Aerobic Vaginitis (AV)

In 2002 Donders et al. (2002) characterised a new type of vaginitis. It is in contrast to BV dominated by aerobic bacteria, mainly *Streptococcus agalactiae* and *Escherichia coli* and named aerobic vaginitis (AV). The patients suffer from yellow-green discharge, the vagina is red by inflammation, the pH is 5.5–6.5, many toxic leucocytes, parabasal cells and a sparse coccoid flora without lactobacilli dominate the microscopic field. High levels of interleukin – 1 beta, – 6, – 8 and leukaemia inhibiting factor in contrast to BV. Severe cases resemble to desquamative inflammatory vaginitis, which is discussed to be an early form of Lichen ruber of the vagina. AV is a higher risk factor for preterm labour and preterm birth than BV (Donders et al. 2011). Some believe, that AV is primarily an immunologic disorder with secondary abnormal microbiota, or a dermatological disease in the vagina (Edwards 2010). Women with AV are at risk for low grade cervical squamous intraepithelial cell lesions (Jahic et al. 2013). About 5 % of women in reproductive age are suffering from AV, but some diagnosed it in a much higher frequence of 23 % (Fan et al. 2013). But partner treatment is without benefit for the woman with BV.

6.4 Prophylaxis and Therapy with Probiotics

Probiotics are microorganisms that provide a health benefit to the host. They act in the gastrointestinal tract and influence in various ways the immune system (Sherman et al. 2009). Although lactobacilli are in use for prophylaxis or treatment of vaginal discharge since decades, probiotic research devoleped rapidly over the last 30 years within the field of gynaecology (Spurbeck and Arvidson 2011; Reid 2012). One of the first clinical studies proposed the daily oral application of about 250 g Yoghurt containing *L. acidophilus* for 6 months to women with recurrent candida vulvovaginitis. The mean rate of recurrences in the control arm was 2.5 versus 0.38 in the yoghurt arm ($p=0.001$) (Hilton et al. 1992). Since then, several species have been tested in various studies. *L. rhamnosus Lcr 35* (Coudeyras et al.2008a, b) showed increased ability to metabolise glycogen to lactic acid and in vitro growth inhibition of *G. vaginalis* and *C. albicans*. The effect was higher after the manufactering process than compared to three other *L. rhamnosus* strains (Nivoliez et al. 2012). Lcr 35 adheres to cervicovaginal cells and is an antagonist of BVAB (Coudeyras et al. 2008a, b).

The strain *L. rhamnosus* GR-1 causes significant killing of *E. coli* in vitro and is able to

cause bacterial death in BV biofilms in vitro (McMillan et al. 2011). The two strains *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (formerly *L. fermentum*), which are traded in a vaginal tablet, inhibited in vitro the growth of *C. albicans* and upregulated inflammatory Interleukin levels in a human vaginal epithelial cell line (Martinez et al. 2009a, b). *C. albicans* lost it's metabolic activity, showed increased expression of stress-related genes and lower expression of genes involved in fluconazole resistance (Köhler et al. 2012). Similar results were demonstrated by Sanchez et al. (2008) with a different strain, *L. rhamnosus* GG, which showed in a monolayer cell culture protection against damage by *C. albicans*, modulation of immune responses and immune conditioning of the mucosal surfaces (Schaller 2012). Probiotics, here administered as a daily probiotic drink for 6 months, can also enhance the clearance of human papillomavirus-related cervical lesions significantly against placebo (Verhoeven et al. 2013).

Clinical studies had been performed with different probiotics administered vaginally or orally. *L. crispatus* CTV-05 is one of the new probiotics in gynecology and well tolerated (Hemmerling et al. 2010). Vaginal intercourse (seminal fluid), and the presence of lactobacilli of the same species during vaginal administration inhibit the colonisation (Antonio et al. 2009). There seems to be a competition of one's own and the foreign lactobacilli.

Oral administration of lactobacilli to influence the vaginal microbiota seems to be effective. The first to demonstrate this were Hilton et al. (1992) against *Candida* vaginitis and Shalev et al. (1996) against *Candida* and/or BV. Rectal lactobacilli with vaginal tropism can colonise the vagina and vice versa. Oral application of a mixture of 10^8 *L. fermentum* 57A, *L. plantarum* 57B and *L. gasseri* 57C daily for 60 days was able to colonise the rectum and the vagina between day 20 and 70 and decreased the vaginal pH, while the Nugent score improved (Strus et al. 2012). The oral administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for 30 days following treatment of BV with oral metronidazole improved the cure rate (Anukam et al. 2006). Furthermore, Bohbot and Cardot (2012) showed in a pilot study, that oral administration of *L. rhamnosus* Lcr35 is able to improve the Nugent score to normal values.

In addition to the improvement of BV symptoms the recurrences of vulvovaginal candidosis can be influenced by probiotics (Homayouni et al. 2014, Huang et al. 2013). Ehrström et al. (2010) showed improved treatment rates for women with BV and vulvovaginal candidosis by additional administration of *L. gasseri* LN40, *L. fermentum* LN99, *L. casei subsp. rhamnosus* LN113 and *P. acidilactici* LN23 for 5 days in vaginal capsules. Martinez et al. (2009a, b) improved the clinical treatment results of vulvovaginal candidosis with oral fluconazole, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 similar to Kern et al. (2012).

Prebiotics, such as inulin, glycogen, or others, which support the metabolism of probiotics are sometimes added to probiotic tablets. However, within the field of gyneacolgy hitherto clinical studies to assess their superiority over probiotics are missing.

6.5 Summary and Conclusion

The normal and the abnormal vaginal microbiota is not yet fully understood. It is an ecosystem, which is influenced by genetic, ethnic, environmental and behavioral factors. More than 100 to 200 bacterial species, commensal, transient and endogenous, colonise the vagina and are influenced by the oral, rectal and penile microbiota. Cultural methods for the determination of normal or abnormal microbiota are insufficient and detect only a small, mostly aerobic, not representative and clinically unimportant spectrum. Lactobacilli mainly dominate the vaginal microbiota and are responsible, with other bacterial species, for the creation of a pH value between 3.8 and 4.5, which is considered as normal, at least in caucasian or asian women. Together with their antibacterial properties and immunological factors lactobacilli create a defense system against dysbiosis and infections within the vagina. This system is responsible for a healthy outer and inner genital tract, for a balanced resti-

tution after intercourse and for a normal pregnancy and childbirth on time.

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