

The female genital tract microbiota

Composition, relation to innate immune factors, and effects
of contraceptives

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*Photo on the front page: Gram-stained vaginal smears of normal *Lactobacillus*-dominant microbiota (left) and bacterial vaginosis (right), magnification $\times 1,000$*

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ABSTRACT

Abnormal vaginal microbiota, as in bacterial vaginosis (BV), is associated with increased risk of obstetrical and gynaecologic complications and acquisition of sexually transmitted diseases. However, very little is known about the pathogenesis of BV. In BV, the normal vaginal *Lactobacillus*-dominated biota (LDB) is replaced by anaerobic bacteria. The diagnosis of BV is based on clinical symptoms (vaginal malodorous discharge) and/or microscopy of vaginal smears, methods that do not identify specific microorganisms.

The aim was to analyse the composition of the vaginal microbiota in healthy, asymptomatic women of reproductive age, and investigate the relationship between the bacterial species and locally secreted proinflammatory cytokines and the antimicrobial secretory leucoprotease inhibitor (SLPI).

In the study of 37 women, a total of 42 bacterial species were found in vaginal secretions, by cultivation. In the women with asymptomatic BV, particularly, high numbers of the lesser-known *Atopobium vaginae*, *Peptoniphilus harei*, and *Actinomyces urogenitalis* were noted (exceeding 10^{11} bacteria per ml). The latter bacterium, together with *Lactobacillus coleohominis*, were both isolated from vaginal secretions and have been proposed as new species, based on phenotypic (biochemical testing, SDS-PAGE analysis of whole cell proteins) and phylogenetic results (16S rRNA gene sequencing).

The frequency of LDB in healthy asymptomatic women (n=313) was found to decrease with age, analysing age (years) cohorts 20-29, 30-39, and 40-49. Furthermore, the contraceptive methods used (oral hormone pills, copper- or hormone intrauterine device) were found to affect the frequency of LDB.

A non-cultivation-based, semi-quantitative, checkerboard DNA-DNA hybridisation technique (CDH), based on genomic probes from 13 selected bacterial species, was applied for analysis of vaginal and cervical secretions of 26 women. It was found that the anaerobic bacteria were more frequently detected by CDH, compared to cultivation. Correlations were found between specific bacterial species and cytokines or SLPI. For instance, the strict anaerobic species, *B. ureolyticus* and *F. nucleatum*, both correlated with vaginal IL-1 α .

By identification and quantification of bacterial species of the lower genital tract, and analysis of their relationships to host-derived innate immune factors, it will be possible to define various types of abnormal microbiota, to develop ways of assessing the risk of specific bacterial species or groups of bacteria in various clinical settings, and to treat them. CDH will be a suitable tool for the quantitative analysis of as many as 40 specific bacterial species, making it possible to investigate large numbers of women. Both age and contraceptive method need to be considered when investigating the compositions of abnormal vaginal microbiota.

Key words: bacterial vaginosis, checkerboard DNA-DNA hybridisation, cytokines, SLPI, *Lactobacillus coleohominis*, *Lactobacillus iners*, *Actinomyces urogenitalis*

Publications

This dissertation is based on the following papers, which will be referred to in the text by their Roman numbers (I-IV):

- I. **Nikolaitchouk N**, Andersch B, Falsen E, Strömbeck L, Mattsby-Baltzer I.
The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH).
APMIS. 2008 Apr;116(4):263-77.
PMID: 18397461 [PubMed - indexed for MEDLINE]
- II. **Nikolaitchouk N**, Hoyles L, Falsen E, Grainger JM, Collins MD.
Characterization of *Actinomyces* isolates from samples from the human urogenital tract: description of *Actinomyces urogenitalis* sp. nov.
Int J Syst Evol Microbiol. 2000 Jul;50 Pt 4:1649-54.
PMID: 10939672 [PubMed - indexed for MEDLINE]
- III. **Nikolaitchouk N**, Wachter C, Falsen E, Andersch B, Collins MD, Lawson PA.
Lactobacillus coleohominis sp. nov., isolated from human sources.
Int J Syst Evol Microbiol. 2001 Nov;51(Pt 6):2081-5.
PMID: 11760950 [PubMed - indexed for MEDLINE]
- IV. **Nikolaitchouk N**, Mattsby-Baltzer I, Andersch B.
The influence of age and contraceptive methods on the prevalence of *Lactobacillus*-dominant vaginal microbiota in healthy fertile women.
In manuscript.

List of abbreviations

BV	Bacterial vaginosis
CDH	Checkerboard DNA-DNA hybridisation
FGT	Female genital tract
IUD-Cu	Intrauterine device with copper
IUD-L	Levonorgestrel-releasing intrauterine device
LBD	<i>Lactobacillus</i> -dominant microbiota
OCP	Oral contraceptive pills
PID	Pelvic inflammatory disease
SLPI	Secretory leucoprotease inhibitor
TLRs	Toll-like receptors

Introduction

Lactobacilli are the predominant bacteria in the lower genital tract in women of reproductive age. The presence of these bacteria is a prerequisite for a healthy vaginal condition. Lactobacilli act by restraining the growth of pathogenic microorganisms via several mechanisms of which the lactate metabolite is considered one of the major factors, keeping the pH below 5 [1-3].

Abnormal vaginal microbiota, such as bacterial vaginosis (BV), the most prevalent vaginal disorder in women of child-bearing age, is associated with an increased risk of gynaecologic and obstetrical complications, such as postoperative infections, spontaneous abortion, and preterm birth [4-11]. BV is also associated with increased risk of acquisition of sexually transmitted infections [12-15]. BV may also be asymptomatic [16]. In addition, a disturbed non-BV microbiota has also been associated with pregnancy complications [17, 18].

In BV the *Lactobacillus*-dominated microbiota has been replaced by high numbers of anaerobic bacteria. From a microbiological point of view, BV is an enigma and the factors that initiate the transformation to an abnormal vaginal microbiota are not known.

The diagnosis of BV is based on vaginal malodorous discharge, pH, and fresh wet-mount microscopy, or microscopy of Gram-stained vaginal smears [19, 20]. The methods do not identify specific microorganisms.

In order to understand more about the mechanism behind the change of a *Lactobacillus*-dominated microbiota to an abnormal one, the bacterial community needs to be characterised, and their relation to host innate immune factors investigated.

It is necessary to understand the relationship between bacterial patterns and different clinical conditions or risks. In practice, this information can help to develop effective treatment of unwanted vaginal conditions due to abnormal microbiota and provide prophylactic screening to reduce gynaecologic and obstetrical complications.

The Female Lower Genital Tract

Anatomy

Anatomically, the female genital tract (FGT) is divided into internal and external genitalia. The internal genitalia are located in the pelvic cavity and consist of *ovaries*, *uterine tubes (Fallopian tubes)*, *uterus* and *vagina* (Figure 1). The external genitalia, or vulva includes *mons pubis*, *labia majora* and *minora*, *clitoris* with glands, and structures associated with *vestibule* (bulb of vestibule, greater vestibular glands).

The internal FGT is divided into three compartments: the lower genital tract (vagina and ectocervix), the endocervix, and the upper genital tract (endometrium and Fallopian tubes) [21].

The lower FGT in this thesis is defined by the non-sterile areas, i.e. the lower part of the endocervix in addition to the ectocervix and vagina (Figure 1).

The vagina is a thin-walled fibromuscular tube, about 8-10 cm long, extending from the vestibule to the cervix [22]. It is located between the bladder and the rectum. The upper part of the vagina surrounds the end of the cervix, and produces a vaginal recess called the vaginal fornix (posterior, lateral and anterior fornices). The fornices allow the physician to collect samples of vaginal secretions during physical examination.

The vagina plays a broad spectrum of roles: it receives the erect penis during sexual intercourse, it transports uterine secretions and menstrual flow, and it provides a passageway for delivery of a foetus during birth. The vagina and cervix form a complex and dynamic ecosystem of epithelia, secretions, microbiota and innate immunity factors that depend on the levels of steroidal hormones. These levels influence the changes in the FGT not only from the childhood to menopause but also at different stages in the monthly cycle [23].

The cavity of the cervix, called the cervical canal, forms a connection between the vagina and the uterine cavity. The cervical canal opens into the vagina via the external *orifice* and to the uterine body, via the internal *orifice* (not shown). The part of the cervical canal which protrudes into the vagina is called the ectocervix and the lining of the lumen is called the endocervix.

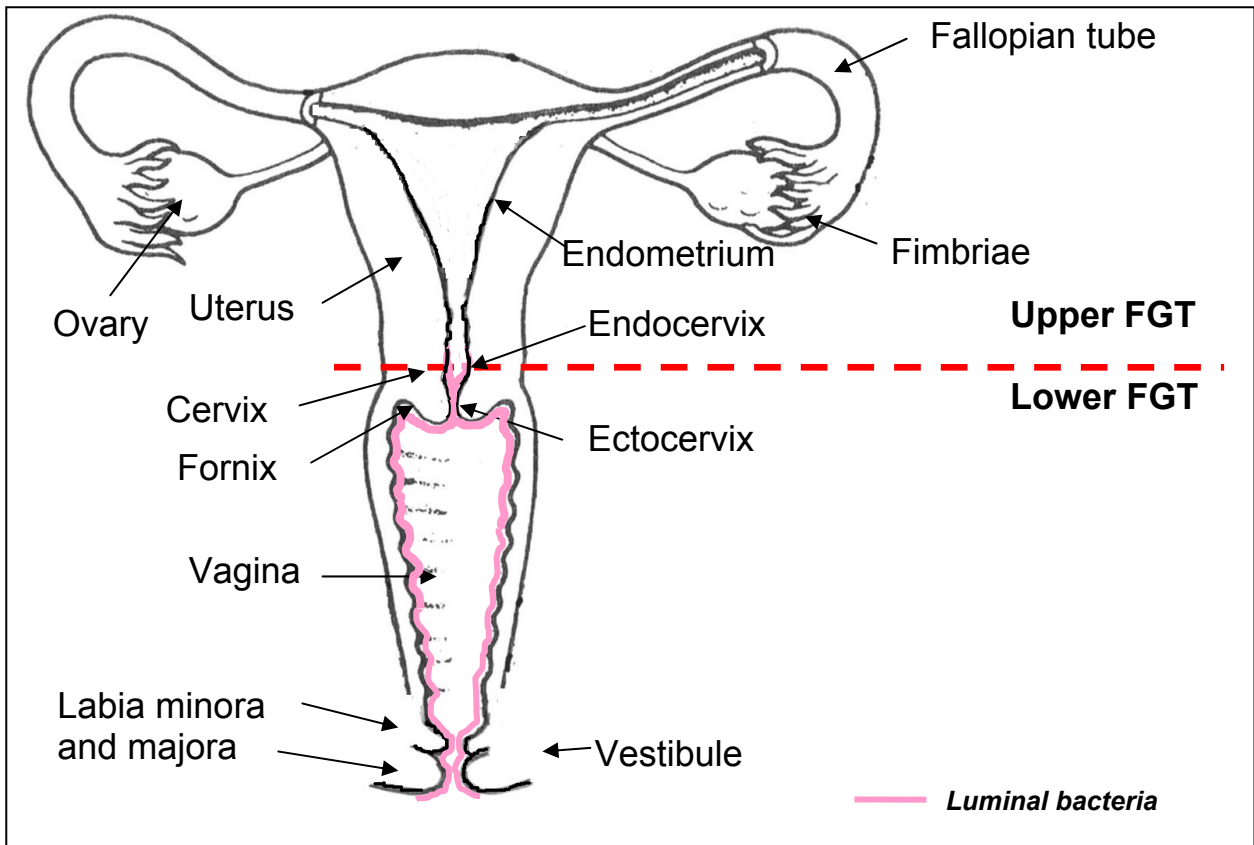


Figure 1. *Organs and the area of microbial colonization of the female genital tract*

Histology

Vagina

The vaginal wall consists of three layers. The outer layer, *the adventitia*, is composed of fibrous connective tissue interlaced with elastic fibres. The middle layer, *the muscularis*, mainly consists of smooth muscle fibres arranged in longitudinal and circular bundles. The inner layer of the vaginal wall consists of the *mucosa* and is lubricated by mucous of the cervical glands.

The vaginal lumen is lined by non-keratinized stratified squamous epithelium, some 10-30 cells deep, which rests on a thick submucosa, *lamina propria* (Figure 2B). The vaginal epithelium consists of four layers: *the basal layer* is a single row of columnar cells on the basal membrane; *the parabasal layer* is a layer of 2 to 5 cuboidal cells; *the intermediate layer* has variable thickness, cells are somewhat flattened, and contain glycogen; *the superficial layer* consists of flat cells, which do contain keratin, but does not normally form a true horny layer.

The superficial cells are constantly exfoliated. The superficial layer has variable thickness of squamous cells containing large amounts of glycogen, which is necessary for the production of lactic acid by resident bacteria. The glycogen

content of the superficial cells and some intermediate cells is regulated by oestrogen. The epithelial cells of the vagina contain large numbers of oestrogen receptors, which respond to ovarian oestrogen stimulation [24].

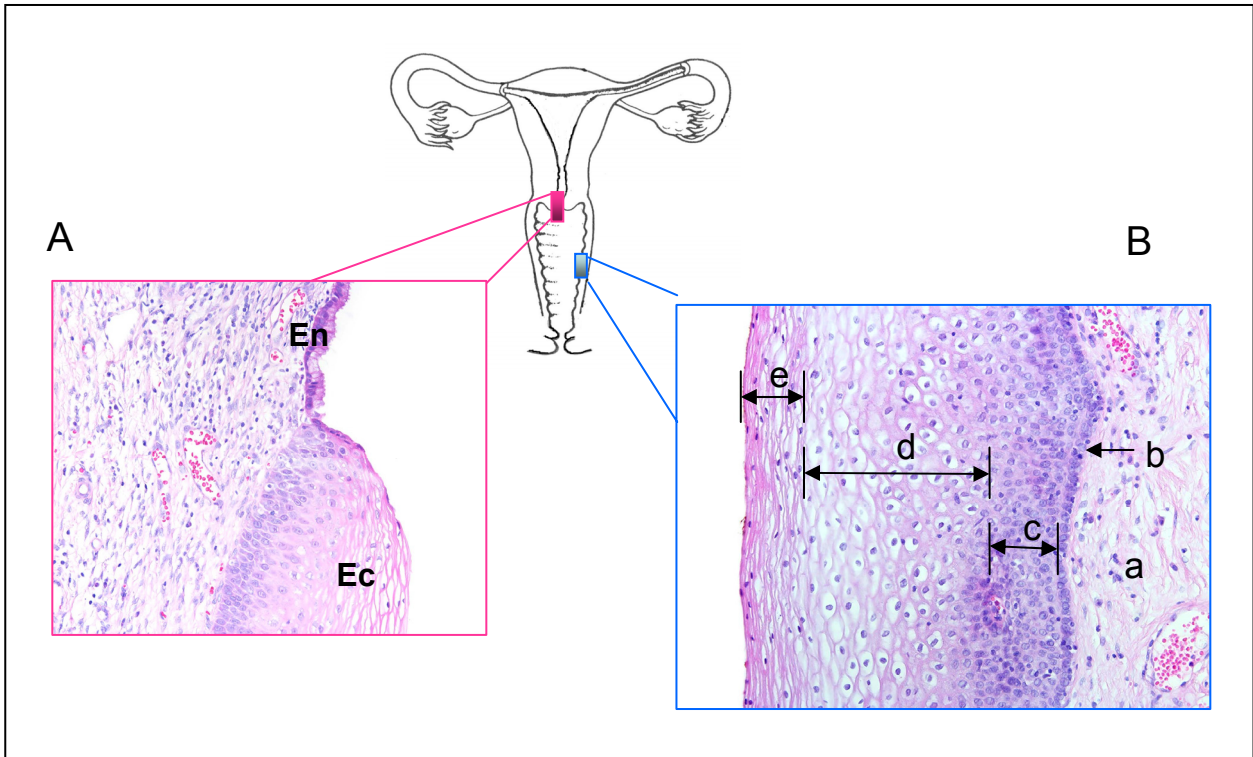


Figure 2. *Histological structure of the squamo-columnar cell junction (A) and a part of the luminal vaginal wall (B). A) Stratified squamous epithelium covers the ectocervix (Ec). The endocervix (En) is lined by a single layer of columnar mucus-secreting epithelium. B) The lamina propria (a), basal (b), parabasal (c), intermediate (d), superficial (e) layers of the normal vaginal mucosa.*

Cervix

The ectocervix is also lined by a non-keratinizing, stratified, squamous epithelium that continues into the vaginal epithelial layer. The area where the squamous epithelium of the ectocervix meets the columnar epithelium of the endocervix is called the squamo-columnar cell junction (transformation zone) (Figure 2A). This area is the most affected by disease such as dysplasia and carcinoma in situ (cervical intraepithelial neoplasia) [25].

The endocervix is lined by a mucus-secreting single cell layered columnar epithelium (Figure 2A). The endocervical tubular glands are deep invaginations (crypts) of the surface epithelium that increase the surface area of the mucus-producing cells [25]. The endocervical tubular glands secrete mucus that covers the external os and fills the cervical canal. This mucus acts as a protective barrier, blocking the spreading of bacteria from the vagina into the endometrial cavity. The secretory activity of the endocervical glands is regulated by oestrogens [24-26].

Composition of the vaginal secretion

The human vaginal epithelium prevents colonization by exogenous microbes and their entrance into deeper tissue. At the same time, it supports luminal commensal bacteria by providing suitable conditions for their growth. The vaginal surface is kept moist by a fluid that is a transudate through the vaginal epithelium and from the cervical mucus with additional fluids from the endometrium, uterine tubes, and vestibular glands [26]. The vaginal secretion is a mixture of several components, including ions (Na^+ , Ca^{2+} , Cl^-), proteins/peptides, glycoproteins, lactic acid, acetic acid, glycerol, urea, and glycogen, which vary depending on the absolute levels and ratios of oestrogens and progesterone, sexual stimulation and the status of microbiocenosis [25-27]. Additionally, the vaginal secretion contains exfoliated cells, which, under the oestrogen stimulation, is predominated by cells of the superficial layer, while, in the progesterone phase, cells of the intermediate layer become more frequent [27].

In a fertile woman, the desquamated vaginal epithelial cells release glycogen, which supplies the main bacteria (*Lactobacillus*) with nutrients. These bacteria degrade glycogen and create an acidic environment, which restricts the growth of pathogenic microorganisms [28]. Thus, at fertile age the normal pH in the vagina ranges from 3.5 to 4.5, with a typical value of 4.2 [29]. The vaginal secretion also contains antimicrobial components of the immune system and leukocytes [21, 26].

Cervical mucus, which has a pH of approximately 8.0, is the main contributor to the vaginal secretion and physically prevents microbes from attaching to the mucosal surface [30]. Mucus consists of water (92-98%), glycoproteins (mucins), ions, antimicrobial proteins and polypeptides such as lactoferrin, lysozyme, immunoglobulins, and defensins [21, 31]. The most distinctive high molecular weight constituents of the cervical secretion are gel-forming mucins, which are large polymeric molecules [32]. The mucins MUC5B, MUC5A, and MUC6, and transmembrane MUC16 and MUC1 have been identified in endocervical secretions [33-36]. The secreted mucus in the FGT is under hormonal control, and changes in viscosity can be seen during the menstruation cycle [32, 37-39]. The capacity of bacteria to degrade mucin molecules by microbial enzymes (mucinases, sialidases) is often a fundamental step in disruption of the defensive mucosal barrier, as these constitute direct interfaces between internal and external environments [32, 39].

Defence systems of the lower FGT

The immune defence against infections is based on the innate and adaptive immune systems of which the innate system provides an immediate non-specific defence. The innate immune system is capable of rapid elimination of microbes by locally secreted antimicrobial factors, phagocytosis and by activation of inflammation via

pro-inflammatory soluble proteins (cytokines and chemokines), leading to enhanced phagocytosis by local macrophages and recruitment of phagocytes (neutrophils, monocytes) [40, 41]. This innate immunity also includes cells, such as dendritic cells and natural killer (NK) cells. Bacterial cells or minute amounts of bacterial cell wall products at the site of invasion induce secretion of cytokines and chemokines from macrophages.

Macrophages are the most efficient phagocytes, while neutrophils are the most abundant ones, appearing early at sites of infection. NK cells are specialised in attacking virus-infected host cells. Dendritic cells are specialised in phagocytising bacteria or virus particles and carrying degraded pathogen antigens to peripheral lymphoid organs, where they activate the adaptive immune system. This system is triggered when the innate immune system fails to eliminate an invading pathogen.

The innate immune system not only provides the early defence against infections but also instructs the adaptive immune system to respond to different microbes [42, 43]. Both dendritic cells and macrophages are professional antigen presenting cells (APC).

The adaptive immunity is mediated by lymphocytes and their products, and leads to the development of specific cellular responses and secretion of antibodies on exposure to a pathogen [44]. APCs present antigens to these highly specialised cells, which will recognise the pathogen in a specific manner and induce processes that will eliminate and memorise the pathogen.

Each naive lymphocyte carries receptors with a unique specificity. When the lymphocyte binds to its ligand, it will start to proliferate and differentiate the effector cells. Lymphocytes are divided into T and B lymphocytes, of which the T cells are involved in cell-mediated immune responses, and B cells in humoral responses (secretion of antibodies). The T cells are either cytotoxic ($CD8^+$ T cells), killing virus-infected cells, or they activate ($CD4^+$ T cells) other cells (macrophages, B-cells).

The cervico-vaginal epithelium, together with tissue-associated phagocytes (macrophages and neutrophils), represent the first line of the cellular host defence against microbes. The epithelial cells not only provide an essential physical and chemical barrier against infections, they can also actively participate in the defence against infection by their ability to secrete cytokines and chemokines [44-47].

The most prominent intrapithelial lymphocytes in the lower FGT appear to be $CD8^+$ T lymphocytes [48]. The transformation zone (TZ) of endocervix contains the highest numbers of macrophages and T cells ($CD4^+$ - and $CD8^+$ lymphocytes). Numerous neutrophils are also present in this region. Few helper $CD4^+$ T lymphocytes, NK cells, macrophages, or dendritic cells are found within the

vaginal epithelium. Most of the CD4⁺ T cells and macrophages are located in the vaginal submucosa. The distribution of immune cells differs somewhat between the vagina and ectocervix in that more CD4⁺ T cells, NK cells, and dendritic cells are present in the epithelium of the ectocervix. Few CD4⁺- and CD8⁺ T cells are found in the epithelium and submucosa of endocervix. Macrophages are found in the epithelium, as well as the submucosa of endocervix, while dendritic cells are absent. Neutrophils are more or less present in the endocervical epithelium and can be found in high numbers. Antibodies (IgM, IgG, IgA), which are produced by local B lymphocytes are secreted into the tissue and partly transferred to the mucosal fluid. Antibodies help block attachment of microorganisms to the epithelium (secretory IgA) and neutralise and eliminate microbes and microbial toxins (IgG and IgM) in the lumen or tissue of mucosal organs.

In human cervical mucus, there are higher levels of IgG than of IgA, which determines the specific humoral defence of the mucosal surface [49, 50]. Immunohistochemical examination of tissue sections has indicated that the endocervix contains higher numbers of Ig-secreting cells than the ectocervix, fallopian tubes, and vagina [51].

Membrane associated factors

The components of the innate immune system recognise microbial-associated molecular patterns (PAMPs) by germ-line-encoded pattern recognition receptors (PRRs) [41]. These receptors can be soluble, located on the cell membrane or intracellularly. There are several different types of receptors, specific for the different structures of PAMPs. PRRs have been found on various types of cells, such as macrophages, neutrophils, fibroblasts, dendritic cells, and epithelial cells of the female reproductive tract. When binding to their ligands, PRRs, such as Toll-like receptors (TLRs) and nuclear-oligomerisation-domain (NOD)-like receptors (NLRs), induce a rapid acute response, leading to the production of pro-inflammatory mediators and other factors involved in the antimicrobial defence of the host [52, 53].

Homologous (TLR 2, 4, and 5) or heterologous (TLR1-2, 2-6) extracellular dimers of TLRs bind bacterial cell wall components, such as peptidoglycan (PG), lipoteichoic acid (LTA), lipopeptide/lipoprotein, lipopolysaccharide (LPS), and flagellin (a component of the flagellum) [3, 54, 55]. For LPS, accessory PRRs, such as CD14 and the protein MD2 are needed in order to initiate the intracellular signalling pathway [42, 53, 56, 57]. Most probably, both non-signalling co-receptors and cofactors are required to induce significant host responses [58]. The cytoplasmic NLRs (NOD1 and NOD2) recognise parts of peptidoglycan and, thus, react to intracellular bacteria [59].

Vaginal epithelial cells express TLR 1, -2, -5 and -6, and endocervical epithelial cells have been shown to express TLR 1, -2 and -6 [60].

Cytokines and chemokines

Cytokines and chemokines are soluble proteins, which are secreted by cells of both the innate and adaptive immune systems [55, 61, 62]. Macrophages are one of the main producers of local cytokines in the early response to microorganisms. However, mucosal epithelial cells may also secrete cytokines and chemokines such as TNF- α , IL-1, IL-6, and IL-8 in response to pathogens [63]. The magnitude and pattern of the cytokine response depend on the pathogen and type of epithelial cells [64-66]. The pro-inflammatory cytokines induce a local inflammatory response and secretion of antimicrobial defence factors [67, 68].

Some major pro-inflammatory cytokines and chemokines secreted by immune cells and epithelial cells of vagina and cervix in response to bacterial pathogens are shown in table 1.

Table 1. Proinflammatory cytokines produced by immune cells, and vaginal and cervical epithelial cells.

Cytokines /Chemokines	Cell source(s)	Function	Ref
TNF- α	Macrophages, NK-cells, T cells,	Local inflammation, activation of endothelial cells and neutrophils; inducing fever, septic shock and apoptosis, stimulation of synthesis of prostaglandins	[40, 42, 43, 55]
IL-1 α/β	Macrophages, epithelial cells (vaginal and cervical cells), endothelial cells, dendritic cells, NK-cells,	Activation of macrophages, stimulation of prostaglandin synthesis, T cell activation, fever	[40, 43, 45, 55, 69]
IL-6	Macrophages, epithelial cells (vaginal and cervical cells), endothelial cells, and T cells	Regulation of acute phase response and inflammation, fever, T and B cell growth and differentiation, and Ig production	[43, 45, 55, 69]
IL-8	Macrophages, epithelial cells (vaginal and cervical cells), fibroblasts, and endothelial cells,	Chemoattractant for neutrophils, stimulate the affinity and motility of the leukocyte integrins of the epithelium; activate the degranulation of neutrophils	[40, 43, 45, 69]

Antimicrobial peptides/proteins (AMPs)

In response to microbial stimuli, epithelial cells and phagocytes secrete soluble peptides/proteins, which help to eliminate pathogens and enhance both the innate and the adaptive immunity [60]. Some AMPs are constantly secreted and are suggested to provide a protective layer onto the surface of the epithelium [21]. The cervico-vaginal secretion contains AMPs, such as defensins, cathelicidin, lactoferrin, lysozyme, calprotectin, elafin and secretory leucoprotease inhibitor (SLPI) [21]. It has been suggested that defective production of AMPs can predispose the genital tract to infections [60].

AMPs disrupt the membranes of microbial pathogens at the epithelial surface. The amphipathic and cationic properties of AMPs mediate the event leading to loss of the bacterial membrane integrity [70]. Additionally, other mechanisms, such as hydrolysis of PG by lysozyme and withdrawal of the microbial growth promoting iron by lactoferrin, complement the antimicrobial arsenal of the host.

SLPI, which shows antimicrobial activity against bacteria, viruses, and fungi, is produced constitutively by cervical epithelial cells and is a major AMP in cervical secretion [21, 60]. SLPI is also a serine protease inhibitor and, thus, helps control the damage resulting from inflammation. The anti-inflammatory effect of SLPI is, however, not solely attributed to the antibacterial and anti-protease activities, since SLPI also can suppress a central transcription factor of the inflammatory response (NF κ B) [71].

High concentrations of SLPI in the vaginal fluid are associated with reduced rates of perinatal HIV-1 transmission and may contribute to natural antiretroviral defence [72]. The concentrations of SLPI in cervical tissue and in the cervical mucus plug are significantly increased during pregnancy and after delivery [73]. Low levels of SLPI in vaginal secretions have been associated with the presence of genital tract infections [60, 74, 75].

Infections of the lower FGT

Infections of the lower genital tract are classified according to the site of symptoms and clinical findings, *e.g.*, vaginitis, and cervicitis (Figure 3).

Lower genital tract complaints, such as abnormal discharge, odour, vaginal itching and vaginal burning, among women may be the results of bacterial vaginosis (BV), vulvovaginal candidiasis, Trichomoniasis, gonorrhoea and *Chlamydia* infections (Figure 3) [39, 76, 77]. In one study, symptoms of discharge and itching were shown to be more often due to BV than *Candida* vaginitis, while vaginal burning

was equally common among the two infections [78]. Furthermore, in a quite high frequency (20-34%), infections can not be identified by the symptoms given above [78].

BV

BV is the most common cause of abnormal discharge in women of reproductive age. It is a polymicrobial vaginal disorder with a heavily disturbed vaginal microbiota, where the *Lactobacillus* - predominant microbiota is replaced by an overgrowth of anaerobic bacteria. This condition is most often not associated with clinical signs of inflammation (such as vaginal wall erythema, and leukocytosis), thus the term “vaginosis” is used instead of “vaginitis” [79].

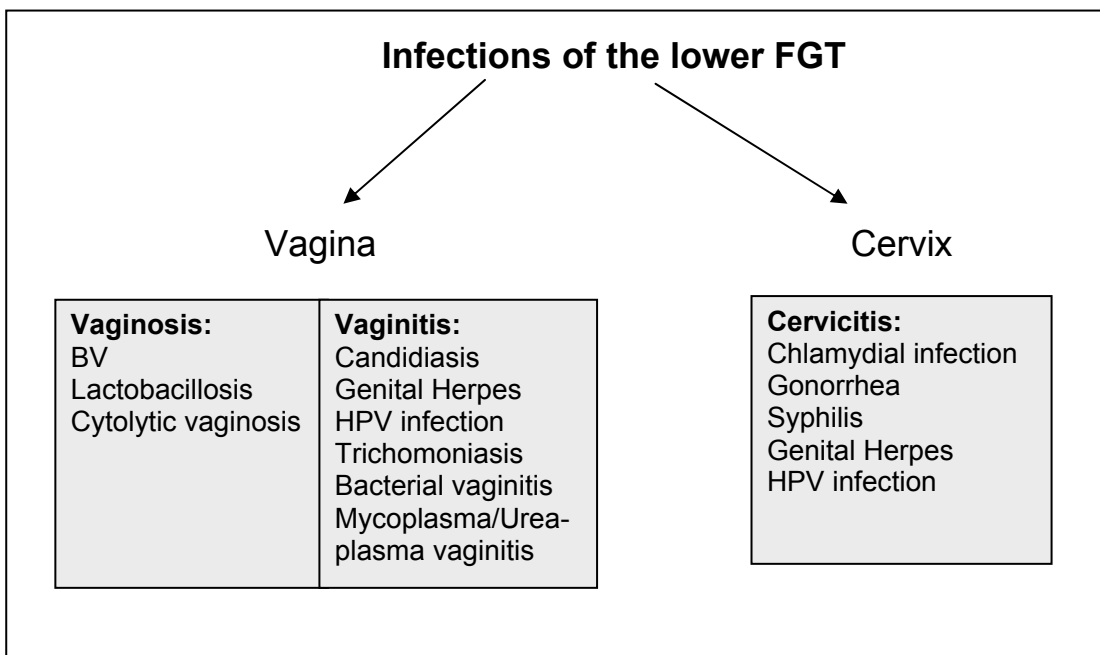


Figure 3. Infections of the lower FGT

Cytolytic vaginosis and Lactobacillosis

Cytolytic vaginosis (Döderlein’s cytolysis) and *Lactobacillus* vaginosis (Lactobacillosis) are two clinical conditions with discharge, irritation and itching, and thus, almost identical to candidiasis [80-82]. The cause of these two types of vaginosis is overgrowth of the normal *Lactobacillus* dominated biota. The differential diagnosis is based on the microscopic appearances [77, 83].

Bacterial vaginitis

Bacterial vaginitis, also called aerobic vaginitis, is not a common condition and sometimes is confused with BV [77, 83, 84]. Group B streptococci, alpha-haemolytic streptococci, *Escherichia coli* and *Staphylococcus aureus* may cause bacterial vaginitis.

Vulvovaginal candidiasis (Candida vaginitis)

Vulvovaginal candidiasis (candidosis) is the most common cause of vaginitis [85]. Most cases of acute vulvovaginal candidiasis are caused by *C. albicans* (89%) and *C. glabrata* (5%). Other species of the *Candida* genus, such as *C. tropicalis*, *C. parapsilosis*, and *C. krusei*, are estimated to constitute less than 1% of each, and *Saccharomyces cerevisiae* 1-2% [76, 77, 86-88]. This infection can be called endogenous, since *Candida* may be isolated from vaginal secretion in approximately one-fourth of asymptomatic women, although in small numbers [88]. *Candida* vaginitis has been associated with antibiotic therapy, pregnancy, use of hormonal contraceptives, oestrogen therapy, diabetes and chronic stress [86, 88-90].

Trichomoniasis

Trichomonas vaginalis is the cause of trichomoniasis, a sexually transmitted infection that can be asymptomatic in 50-75% of the cases [77, 79, 83, 91]. The infection is caused by a protozoan parasite.

Cervicitis

Cervicitis does not induce pain and becomes apparent only by discharge and contact bleeding [76]. *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *T. vaginalis*, *Herpes simplex* virus (HSV), and human papilloma virus (HPV) are frequent causes of cervicitis [39]. These pathogens can invade external stratified squamous epithelium of the ectocervix of the cervical canal, although only *C. trachomatis* and *N. gonorrhoeae* can infect the endocervical single-layered columnar epithelium [39, 48, 92].

Pelvic inflammatory disease (PID)

Ascending of microorganisms from the lower to the upper genital tract lead to PID, which is usually associated with abdominal pain and other symptoms from various organ systems [92]. PID comprises several inflammatory disorders, such as endometritis, salpingitis, tubo-ovarian abscess, pelvic peritonitis and their combinations [39]. The most common causes of PID are *N. gonorrhoeae*, *C. trachomatis*, BV-associated bacteria or highly virulent pathogens (e.g., streptococci of Group A) [4, 76].

Untreated genital tract infections during pregnancy may result in fetus loss, preterm labour (PTL), preterm delivery (PTD), preterm prelabour rupture of the membranes (pPROM), low birth weight, and eye and lung damage in the newborn [76, 93].

Bacterial vaginosis

BV is approximately twice as common as candidiasis [94]. Based on population studies, the prevalence of BV varies according to age, race or ethnicity, education, and poverty [95]. BV occurs as many as 25% of women attending gynaecologic outpatient clinics, in 10 - 40% of pregnant women and in 24 - 37% of women attending sexually transmitted disease clinics. Lesbian women, have a high rate of BV, ranging from 29 - 52% [16, 96-98]. Many women who meet the laboratory diagnostic criteria for BV are asymptomatic in approximately 50% or more of cases [16, 96].

The incidence of BV has been shown to be significantly associated with exposure to a new sexual partner or having ≥ 3 male sexual partners, or at least one female sexual partner in the past 12 months [99-102]. BV is more common among young black than white women, in women using intrauterine devices and in smokers [20, 95, 100, 101, 103-105]. Douching is a predisposing factor of BV [95, 100, 105]. In contrast, use of hormonal contraceptives or condoms consistently decreases the risk of contracting BV [99-102, 105].

BV-associated complications

BV increases the risk of acquisition and transmission of sexually transmitted infections such as HIV and HSV [9, 12-15, 106, 107]. Women with BV shed significantly more cytomegalovirus infection (CMV) in the lower genital tract than women without BV. Thus, local CMV replication and infection is facilitated by the presence of BV [108]. It has also been suggested that BV increases the susceptibility to chlamydia and gonorrhoea [109-111].

BV-associated bacteria are frequent among women with PID [4, 112]. When identified by microbial culture, a combination of BV-related microorganisms in the vagina has been shown to significantly elevate the risk of acquiring PID in a prospective study [113].

BV is associated with increased rates of obstetrical complications, such as preterm birth [6, 10, 11, 17, 114-118], preterm labour [11], low birth weight [10, 114, 115, 119], preterm premature rupture of the membranes [5, 120, 121], late miscarriage, spontaneous abortion [6, 7, 11, 17], chorioamnionitis [10, 122], intraamniotic infections [123-126], postpartum maternal infections [6] and infertility [8, 127].

Gynaecologic complications such as post-operative infections (hysterectomy, legal abortion) have also been associated with BV [128, 129].

Thus, BV is associated with serious medical complications in women.

Treatment of BV

BV is treated with oral metronidazole, which has the 4-week cure rate of 60-70%. The treatment with local vaginal metronidazole gel or vaginal clindamycin cream, gives the same cure rate [130]. This unsatisfactory cure rate means that new knowledge regarding the vaginal microbiota and local immunity needs to be sought in order to improve the treatment strategy.

Diagnostic methods

The BV diagnosis is based mostly on the clinical Amsel's criteria or grading of Gram-stained vaginal smears, according to Nugent [19, 20]. Bacterial cultivation of vaginal secretions is not used for BV diagnosis, since the infection is polymicrobial.

The diagnosis, according to Amsel's criteria [20, 131], requires, at least, three of the features listed in table 2. The Amsel's criteria remain the method used the most for diagnosis of BV by clinicians.

Table 2. *Amsel's criteria for diagnosis of BV. Three out of the four characteristics are required for a BV diagnosis.*

Amsel's criteria:
1. thin, white homogenous discharge
2. vaginal pH greater than 4.5
3. detection of "Clue cells" in vaginal wet smear
4. presence of amine odor with addition of 10% KOH to vaginal discharge on a glass-slide (positive "whiff" test)

The Nugent's scoring is a laboratory-based method and considered to be the "gold standard" in research studies. By this method, the diagnosis of BV is based on Gram-stained vaginal smears. This method was first reported by Spiegel et al. in 1983 [132], while Nugent, eight years later, proposed a scoring system for the Gram-stained vaginal smears (Table 3) [19].

Morphotypes are scored as the average number seen per oil-immersion field (magnification $\times 1,000$). Each morphotype is quantified from 1 to 4+, according to the number of morphotypes observed per field. The total score for the different

morphotypes is classified as normal microbiota (score 0 to 3), intermediate microbiota (4 to 6), and BV (7 to 10) (Figure 4).

Table 3. Scoring system of Gram-stained vaginal smears according to Nugent.

Score*	<i>Lactobacillus</i> morphotypes	<i>Gardnerella</i> and <i>Bacteroides</i> spp. morphotypes	Curved gram-variable rods
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

*score:
 0 = no morphotypes present
 1 = <1 morphotype present
 2 = 1 - 4 morphotypes present
 3 = 5 - 30 morphotypes present
 4 = ≥ 30 or more morphotypes present
 Total score= Lactobacilli + *Gardnerella* and *Bacteroides* spp. + curved rods.

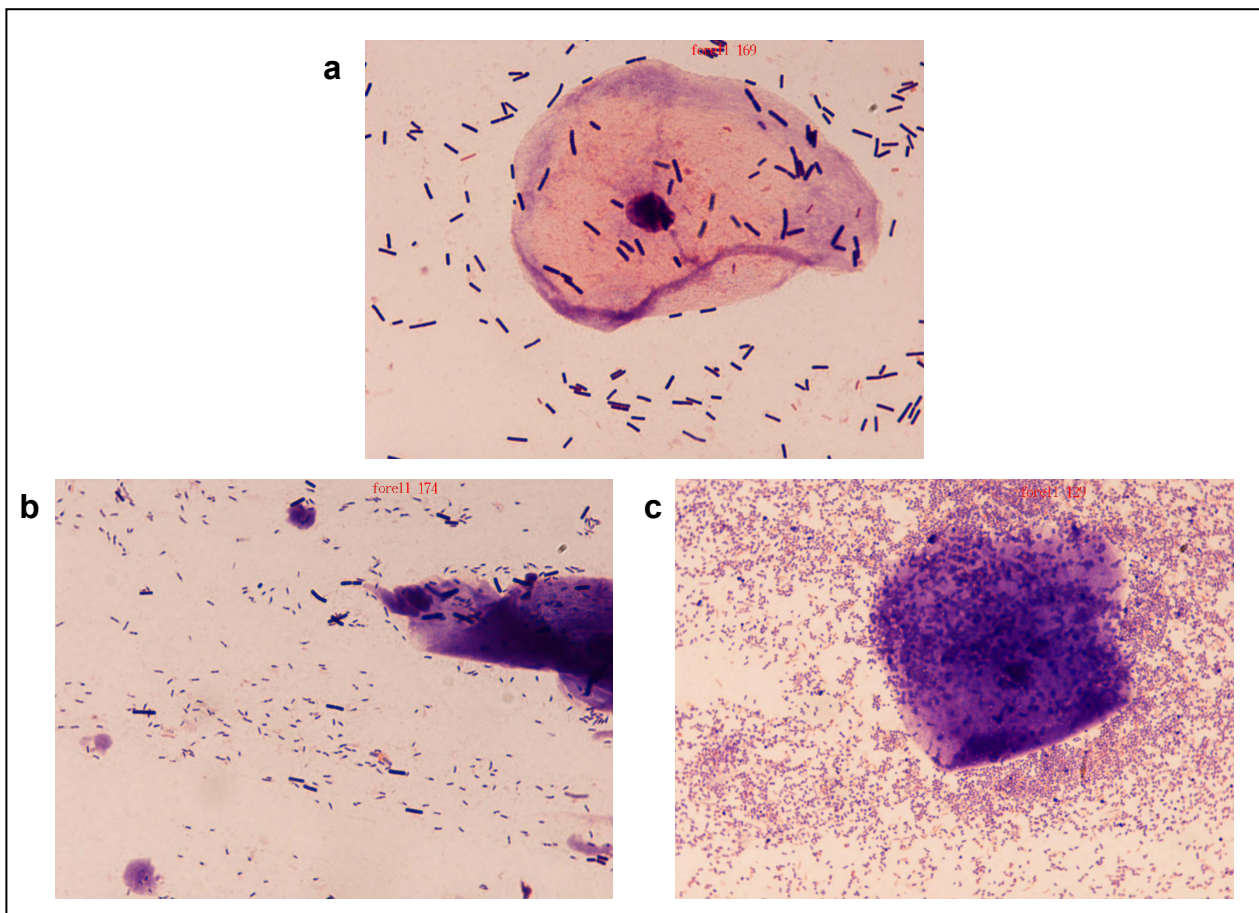


Figure 4. Gram-stained vaginal smears of a normal vaginal microbiota (a), intermediate (b), and bacterial vaginosis (c). In C, the epithelial cell is covered with bacteria, termed “clue cell”

Several microscopy-based grading systems have been proposed both prior to and after the report of Nugent scoring. However, these systems are not widely used [17, 83, 133-135]. All scoring systems have been tested for reliability and accuracy, and their weaknesses criticised [136-141].

Cultivation has been used only in research, since both aerobic, microaerophilic, and anaerobic conditions with various types of culture media are needed in order to reflect, at least partially, the diversity of the vaginal microbiota. Molecular biology techniques are being used to assess the diversity of microbial species found in the vagina, although still applied only in research.

The vaginal microbiota

The normal vaginal microbiota

The normal vaginal microbiota is a unique and dynamic system and continually fluctuates under the environmental changes and physiological conditions. The vagina of healthy fertile women harbours an extensive number of bacteria, of which lactobacilli predominate [3].

Lactobacilli

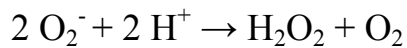
In 1894, the German obstetrician and gynaecologist, A. S. G. Döderlein (1860-1941), isolated Gram-positive, catalase-negative rods, now referred to as *Lactobacillus* spp., from the vagina of healthy pregnant women [39]. The genus *Lactobacillus* comprises a phenotypically heterogeneous group of facultative and obligate anaerobic, catalase-negative, Gram-positive, non-spore-forming, rod-shaped bacteria, which produce lactic acid as the major final product of the metabolism. *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* appear to be the most predominant species in the vagina of fertile women [142-145]. Lactobacilli differ greatly in morphology between various species, as illustrated by the vaginal isolates of the four most predominant *Lactobacillus* spp. in figure 5.

Lactobacilli suppress the growth of pathogenic microorganisms by production of lactic acid and a variety of antimicrobial compounds (Figure 6), but also by competition for adherence, combined with a general stimulation of the immune system [1, 3, 146-150].

Lactic acid production. Lactobacilli metabolise glycogen released from epithelial cells. Glycogen is degraded into glucose, which is fermented to lactic acid via

pyruvate. Lactic acid and the low pH of vaginal secretions have been shown to exert antimicrobial activity against non-resident bacteria [151].

Hydrogen peroxide production. The very toxic superoxide radicals, intermediate products in oxidative processes, are catalysed by enzymes to hydrogen peroxide, which also is a toxic compound:



H₂O₂ - generating lactobacilli are present in the normal biota of healthy women and absent in most women with bacterial vaginosis [152-154]. The lack of H₂O₂ - producing lactobacilli may predispose to overgrowth of pathogenic bacteria [155]. Furthermore, the presence of vaginal lactobacilli with high levels of hydrogenperoxide production (*L.jensenii* and *L.vaginalis*) has been shown to be associated with a reduction of preterm birth and/or inflammation of the fetal membranes [156].

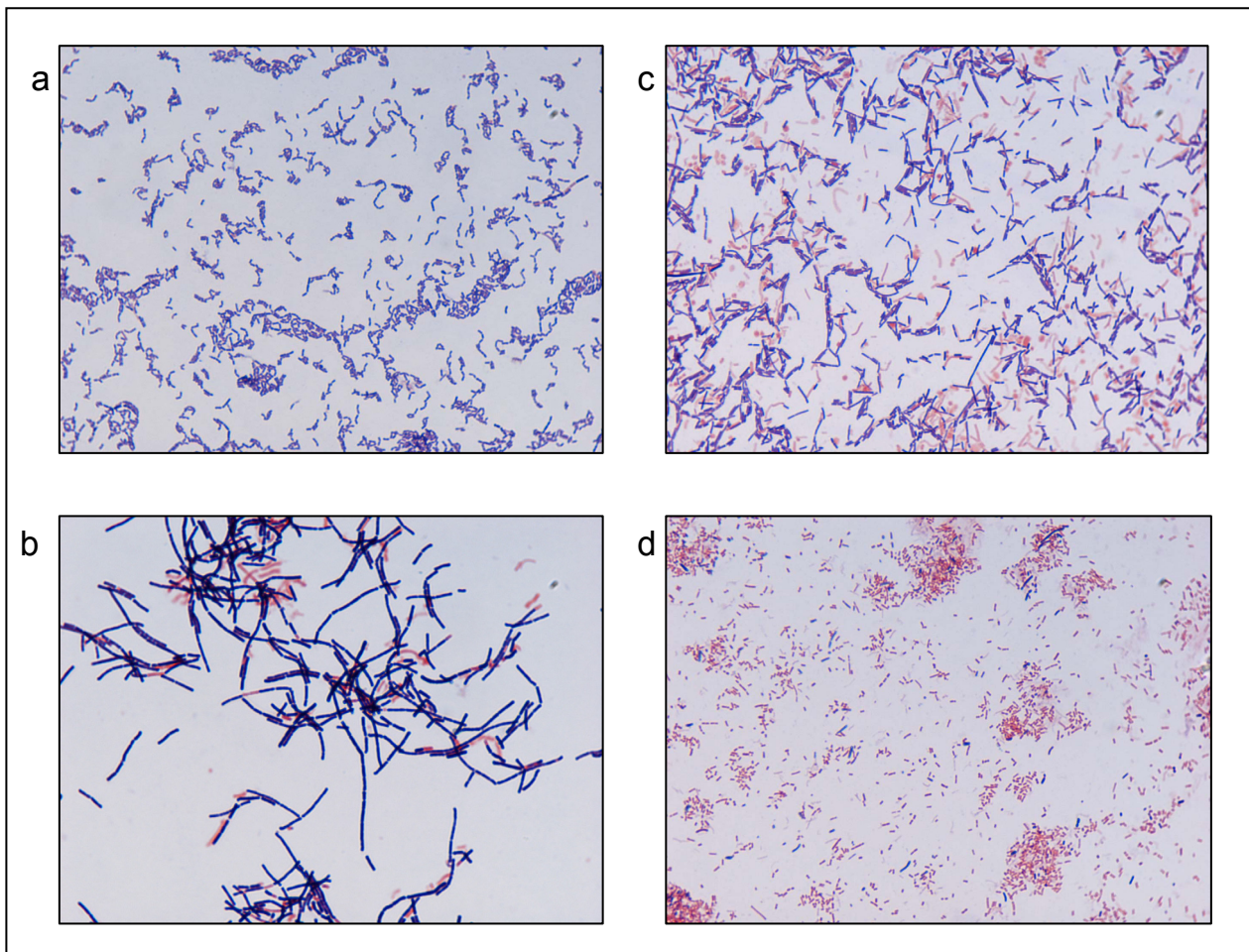


Figure 5. Morphology of Gram-stained vaginal *Lactobacillus* spp. under immersion oil, $\times 1000$ magnification. The bacterial strains were cultured under anaerobic condition at 37°C during 24 hours. *L. jensenii* (a), *L. crispatus* (b), and *L. gasseri* (c) were cultured on MRS medium. *L. iners* (d), which does not grow on MRS medium, was cultured on chocolate-GL.

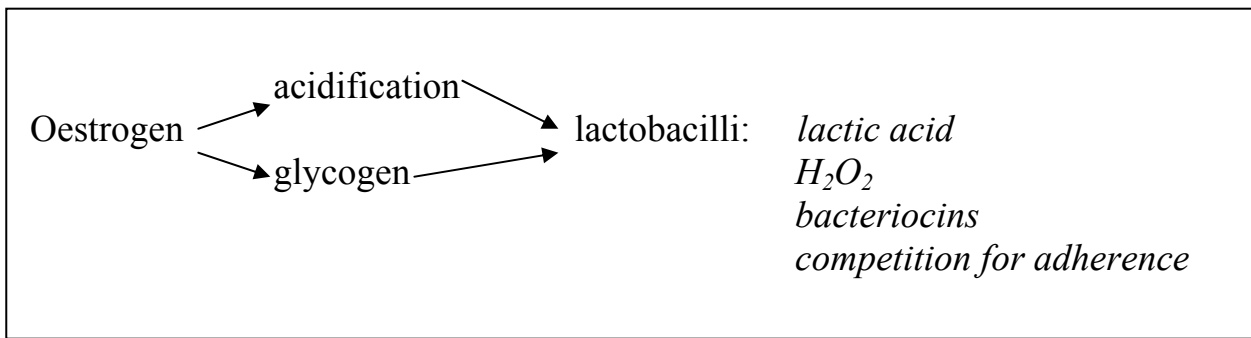


Figure 6. A link between oestrogen, vaginal lactobacilli and host protection. Oestrogen regulates the glycogen content in vaginal epithelial cells. Through metabolism of glycogen, the vagina becomes acidic and thereby promotes growth of lactobacilli. Lactobacilli enhance the acidic milieu by production of lactic acid in the presence of glycogen. The lactobacilli produce several antimicrobial factors and compete for adherence to epithelial cells.

Bacteriocin production. Lactobacilli produce a variety of bacteriocins (proteins/peptides) that inhibit the growth of BV-associated bacteria and other pathogens, such as *N. gonorrhoea*, *Escherichia coli*, and *C. albicans* [146-148]. It has been suggested that the bacteriocin-like components and H₂O₂ produced by vaginal *Lactobacillus* species act in synergy in the eradication of pathogenic microorganisms.

Competition for adherence. Lactobacilli have been shown to interfere with colonisation of pathogenic bacteria, by blocking attachment to the vaginal epithelium [149, 157]. Adherence of lactobacilli to the epithelial cells is stimulated by fluctuation of oestrogen levels, which generates movement of ions across the vaginal epithelium and alters the vaginal cell charge. This electrical potential difference could change the bacterial adherence properties and, finally, affect the bacterial composition [158].

Stimulation of growth. Reproduction of lactobacilli depends on oestrogen levels (Figure 6) [159-164]. The numbers of these bacteria may change during the menstrual cycle. Acidification by vaginal secretion stimulates the growth of lactobacilli.

Other microrganisms

Several other microorganisms are frequently found in the vagina, such as *Staphylococcus epidermidis*, *Streptococcus* spp., *Corynebacterium* spp., *E. coli*, peptostreptococci, *Bacteroides melaninogenica*, *Gardnerella vaginalis*, *Ureaplasma*, *Mycoplasma* and *C. albicans* [165, 166]. Some of the obligate and facultative anaerobic bacteria are associated with BV [167].

BV-associated bacteria

BV is characterized by overgrowth of anaerobic bacteria, usually a 100-1000 fold increase of the bacterial concentration, wherein *G. vaginalis* is the predominant microorganism (316). Other bacterial species, such as *Mycoplasma hominis*, *Bacteroides* spp., *Mobiluncus* spp., *Peptostreptococcus* spp., *Prevotella* spp., are also associated with BV [142, 168, 169].

The change of the normal *Lactobacillus*-dominated microbiota to BV alters the concentrations of vaginal and cervical cytokines. BV is associated with increased levels of IL-1 α and IL- β , and IL-8 [142, 170-172]. However, BV appears not to influence the levels of TNF- α and IL-6 [170, 173]. The levels of IL-1 β and IL-8 increase 4 to 10-fold with decreasing number of lactobacilli [174].

Factors influencing the vaginal microbiota

Endogenous and exogenous factors, including menses, douching, use of contraceptives, vaginal medication use, number of sexual partners, frequency of intercourse, methods of menstrual protection have been suggested to be associated with shifts in the vaginal microbiota [168, 175, 176].

Endogenous factors

The vaginal epithelial structures, as well as the microbiota, change considerably from childhood to menopause (Table 4) [163]. At birth, the neonatal vaginal epithelium is rich in glycogen, due to maternal oestrogen. Thus, the infant vagina is colonised by lactobacilli within the first 24 hours after birth, acquired from the maternal birth canal. Several weeks later, when the level of oestrogen has decreased, the vaginal epithelium becomes thin and atrophic with a low glycogen levels. Gram-positive cocci and bacteria other than lactobacilli become predominant, a condition, which continues until the puberty [24].

During menstruation, non-Lactobacillus species appear to increase in number, while the lactobacilli decrease or stay approximately the same number [177-181].

During pregnancy, a *Lactobacillus*-dominant microbiota is strengthened by the increased oestrogen levels [24], however, at the same time, the incidence of vulvo-vaginal candidiasis increases, compared with non-pregnant women [24, 88, 168]. The reason for the increase has been proposed to be a somewhat suppressed cell-mediated immunity in pregnant women leading to an increased susceptibility to pathogens such as *C. albicans* [88].

Table. 4 Cyclic changes in the vagina related to age.

	Oestrogen	Glycogen	pH	Microbiota
New born	+++	+++	acid	lactobacilli
Child	-	-	neutral	cocci or varied
Reproductive age	+++	+++	acid	lactobacilli
Post menopause	+ to -	+ to -	neutral	lactobacilli to varied

At menopause, the prevalence of lactobacilli is decreased, due to the low oestrogens levels, and the vaginal pH is increased [182-184]. The vaginal microbiota of postmenopausal women is similar to that in the pubertal period. Lactobacilli, yeasts and BV-associated bacteria are a less common component of the vaginal microbiota in postmenopausal women than in women of reproductive age, while *E. coli* is recovered at higher frequency [185, 186].

Thus, oestrogen has a decisive effect on the composition of the lower genital tract microbiota.

Exogenous factors

Antimicrobial agents can adversely affect the vaginal lactobacilli. Lactobacilli have variable susceptibility to cephalosporins, but sensitive to penicillin. In contrast, vancomycin, doxycycline and metronidazole are inactive against lactobacilli [156, 187]. Clindamycin vaginal cream, used for treatment of BV is also active against lactobacilli [39].

There are contradictory reports on the effects of contraceptives on the vaginal microbiota. Some studies have reported no major changes in the levels of aerobic and anaerobic bacteria in oral pill users, intrauterine device (IUD) – users and no users [165, 188-190]. In contrast, in some studies, higher incidences of anaerobic bacteria have been found in women using IUDs, as compared to those not using IUDs [159, 191]. However, culturing samples from the removed IUDs revealed growth of coagulase-negative *Staphylococcus*, *E. coli*, *E. faecalis*, and *Actinomyces*-like bacteria [192-194].

Postmenopausal women with hormone replacement therapy have the same levels of vaginal *Lactobacillus* colonisation as women of fertile age [186, 195].

Comparison of tampon use with napkin (pad) has been shown that the tampon use increases the counts of coagulase-negative staphylococci during menstruation [196].

Douching has been associated with an increased risk of PID, as well as ectopic pregnancy, preterm delivery, and other gynaecologic health problems [197-200]. However, contradictory results have been obtained concerning the effect of douching practises on the vaginal microbiota [201-205].

Aims of the study

The aims of the present study were to:

- identify and quantify bacterial species of the lower genital tract microbiota of healthy asymptomatic women, and to relate the bacterial species to proinflammatory cytokines and SLPI;
- investigate the prevalence of vaginal *Lactobacillus*-dominant microbiota in healthy fertile women with respect to age and contraceptive methods;
- apply a non-culture dependent DNA-DNA hybridisation technique for semiquantitative identification of bacterial species of the lower genital tract.

Materials and Methods

Study groups (I and IV)

The two study groups consisted of 37 (I) and 313 (IV) healthy, non-pregnant fertile women, attending the routine gynaecological health care for control or contraceptive advice at the Department of Obstetrics and Gynaecology, East Hospital, Gothenburg, Sweden.

Patients were excluded from the study if they were menstruating at the sampling occasion, if they had taken antimicrobial agents less than 4 weeks before examination, if they had genital tract complaints or signs of PID, if they had had vaginal douching or sexual intercourse during the previous 24 hours. The presence of *C. trachomatis*, *C. albicans*, and *T. vaginalis* infections resulted also in exclusion from the study. In the first study group (I) 14 (38 %) women were OCP users, 4 (11 %) had an IUD, and 19 (51%) were non-users of contraceptives. The distribution of participants (IV) in relation to age and contraceptive method in the second study group is shown in figure 7.

313 asymptomatic women				
	126 (40 %) No contraceptives	75 (24%) OCP	60 (19 %) IUD-Cu	52 (17%) IUD-L
20-29 yrs:	30	30	0	0
30-39 yrs:	51	24	30	21
40-49 yrs:	45	21	30	31

Figure 7. Distribution of participants with regard to non-users of contraceptives, oral contraceptive pills (OCP) -, copper intrauterine devices (IUD-Cu)-, and levonorgestrel-releasing IUD (IUD-L) users in three age cohorts.

Bacterial strains (I, II, III)

Three isolates of *Actinomyces* (II) and four of *Lactobacillus* (III) had all been collected at Culture Collection of the University of Gothenburg (CCUG). In table 5, sources and clinical information of the bacterial isolates are summarised.

Table 5. Sources and clinical information of bacterial strains

paper	CCUG	source	clinical information
II	28744 <i>Actinomyces</i>	urine	70 years old woman, 10×10^7 CFU/ml
	38702 <i>Actinomyces</i>	vaginal secretion	33 years old woman, abnormal vaginal discharge, IUD during 7 years
	42029 <i>Actinomyces</i>	urethra	44 years old patient
III	44007 <i>Lactobacillus</i>	vaginal secretion	32 years old woman, healthy, 4×10^9 CFU/ml, Nugent's score 0
	44087 <i>Lactobacillus</i>	vaginal secretion	29 years old woman, healthy, 2.25×10^8 CFU/ml, Nugent's score 5
	30840 <i>Lactobacillus</i>	urine	45 years old woman
	44174 <i>Lactobacillus</i>	cervix	34 years old woman, preterm labour

Thirteen bacterial strains associated with the vaginal microbiota were used in the checkerboard DNA-DNA hybridisation technique (CDH) (I) (Table 6).

Sampling (I, IV)

Informed consent was obtained from all participating women. Specimens were obtained during speculum examination of the vagina before any other vaginal examination. Separate cervical (I) and vaginal samples (I, IV) were collected for analyses. A sterile cotton swab (SARSTEDT, Sweden) was used for collecting vaginal secretion from the posterior vaginal fornix, and cervical secretion was obtained by cytobrush (Medscand Medical, Sweden). The cotton swabs were used for vaginal smears, culture, CDH (I), SLPI (I)- and cytokine (I) analyses. The cotton swab for bacterial culture was placed in Amies transport media (Sarstedt AB, Sweden). The cytobrush sample was used for CDH, SLPI- and cytokine

analyses. The cytobrush and the swab were placed in separate sterile tubes and transported to the laboratory within 2 hrs after collection (I).

Table 6. *Bacterial strains employed for the development of DNA templates*

Species	CCUG	ATCC ^a
<i>Bacteroides ureolyticus</i>	44020B T	-
<i>Escherichia coli</i>	29300 T	11775
<i>Fusobacterium nucleatum</i>	32989 T	25586
<i>Prevotella bivia</i>	9557 T	29303
<i>Prevotella disiens</i>	9558 T	29426
<i>Prevotella melaninogenica</i>	4944 T	25845
<i>Gardnerella vaginalis</i>	3717 T	14018
<i>Mobiluncus curtisii ss curtisii</i>	21018 T	35241
<i>Staphylococcus aureus ss aureus</i>	1800 T	12600
<i>Streptococcus agalactiae</i>	4208 T	13813
<i>Streptococcus anginosus</i>	27298 T	12395
<i>Atopobium vaginae</i>	43049	-
<i>Lactobacillus iners</i>	28746 T	-

^a American Type Culture Collection

At the laboratory, the swabs and the cytobrushes were submerged in 1 ml of sterile distilled water and shaken for 1 hour at 4°C followed by vortexing (I). The suspension was centrifuged at 12,000 × g for 20 min at 4°C (Hettich Zentrifugen, GmbH, Germany). The supernatant was transferred to another tube for further analysis of SLPI and cytokines. Half a ml of sterile super-Q water was added to the pellet. Both pellets and supernatants were stored frozen at -70° C.

Vaginal specimens from 34 out of the 37 women were collected for quantitative culturing, and within this group 26 random samples of each of vaginal and cervical secretions were used for CDH analysis (I).

The vaginal smears were Gram stained and evaluated by Nugent's score system. Vaginal pH was measured on the speculum by using pH-strips with a range from 3.0 to 7.0 (Merck, Germany).

Culturing (I, II, III)

Actinomyces strains were cultured at 37°C on Columbia agar (Difco) supplemented with 5% horse blood, in aerobic conditions with 5% CO₂ (II); and *Lactobacillus* strains were cultured on MRS agar, in anaerobic conditions (III).

In paper I the cotton swabs containing approximately 100 µl of vaginal secretion were placed into 1 ml of oxygen-reduced 0,06 M NaCl sterile solution and were mixed vigorously on a vortex mixer for 3-5 min. Ten serial 10-fold dilutions were prepared. Aliquots (10 µL) of all the dilutions were plated onto Columbia agar (Difco) supplemented with 5% horse blood, MRS agar, and Chocolate agar. Sets of plates were incubated aerobically, in 5% CO₂, and anaerobically (Whitley Anaerobic Cabinet, Don Whitley Scientific Ltd, Shipley, West Yorkshire, England) with 10% H₂, 5% CO₂ and 85% N₂ gas mixture for 72 hours at 37° C. All colony types were counted, isolated and characterised by morphologic features, haemolysis pattern, and Gram stain morphologic features. When the same organism was recovered from different plates, the highest count was used for determination of the final concentration. Concentrations of bacteria were expressed as CFU/ml of vaginal secretion (cotton swab). The isolated bacteria were cultured for a comparative study on different media and in different conditions.

For CDH (I), the strains were cultured on Columbia agar supplemented with 5% horse blood and Chocolate agar plates at 37° C under either aerobic or anaerobic conditions for 24-72 h.

All specimens collected in paper IV were inoculated within 4 hrs after collection on blood agar plates, Sabouraud dextrose agar, *Streptococcus*-Gbg-medium, *Staphylococcus* 110 agar (Scharlau), Drigalski agar, GBS broth (Todd Hewitt Broth, BBL) and cultivated under aerobic condition. Also, anaerobic culturing was performed on Columbia agar with 5% sheep blood by using anaerobic box (Concept, Anaerobic Work Station 400, Ruskinn Technology Limited, UK). All plates were incubated at 37°C for 48 hours before examination. When lactobacilli were the only bacteria present, or they dominated in comparison with other bacteria that were sparsely present on any other agar plate or after enrichment in medium broth, the vaginal microbiota was defined as a LDB.

Biochemical characterisation of isolated bacterial strains

Bacterial strains were biochemically characterised by using the API systems according to the manufacturer's instructions (API bioMérieux, France). The following APIs were used:

API Rapid ID32Strep, Rapid ID32A, ID32STAPH, ID32E, API ZYM, API Coryne, API50CHL, ID32C. All the APIs were used for identification of the bacterial isolates in Paper I and IV, while three - API Rapid ID32Strep, API ZYM, API Coryne were used in paper II, and two - Rapid ID32Strep and API ZYM were used in paper III.

The final interpretations of the results were determined by using the software program for bacterial examination at the CCUG.

SDS-PAGE (I, II, III)

A comparative analysis of whole-cell protein profiles by polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Pot *et al.* [206] and Vandamme *et al.* [207]. For densitometric analysis, normalisation and interpretation of protein patterns, the GelCompar 4.1 software package (Applied Maths Gent, Belgium) was used.

16S rRNA gene sequence analysis (I, II, III)

The 16S rRNA genes of the bacterial isolates were amplified by PCR and directly sequenced by using a *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems Foster City, USA) and an automatic DNA sequencer (model 373A, Applied Biosystems).

CDH (I)

Bacteria

Thirteen bacterial strains (Table 6) were used for the preparation of whole genomic DNA probes. The bacteria were harvested from plates and dispersed in Tris-EDTA buffer (10 mM Tris HCl, 1.0 mM EDTA, pH 7.6) (TE). The bacterial suspension was centrifuged at $10\,000 \times g$ 10 min, and the pellet was resuspended in TE buffer. A series of 2-fold dilutions was made and OD at 610 nm was measured in four to five different dilutions. The number of bacteria was estimated by using a Bürker chamber. A standard curve was plotted for each strain of bacteria. A bacterial stock suspension was made for each strain with a concentration of 2×10^8 bacteria/ml. This suspension was used for making standard bacterial mixtures in concentration of 10^5 and 10^6 of bacteria /ml in TE buffer/0.5 M NaOH (1:1 v/v) for each species. These mixtures were used in the DNA-DNA hybridisation.

DNA preparation and labelling

Whole genomic bacterial DNA was prepared according to Current Protocols of Molecular Biology (John Wiley & Sons, Inc, 2000) with some modifications [208]. DNA was labelled with digoxigenin by using “DIG-High Prime” (Roche, Germany). The labelling procedure was performed according to the manufacture’s protocol. The probes were stored at +4°C until used. The principle of the steps in the DNA-DNA hybridisation technique is shown in figure 8.

Hybridisation

The method was a modified version of the one described by Socransky *et al.* for dental plaque samples [209]. The samples consisting of bacterial suspensions or cervical-/vaginal secretions were dissolved in 200 µl TE buffer/NaOH. The modifications consisted of a high stringency buffer and the following steps for the antibody reaction (Anti-Digoxigenin-AP, Roche, Germany) [210].

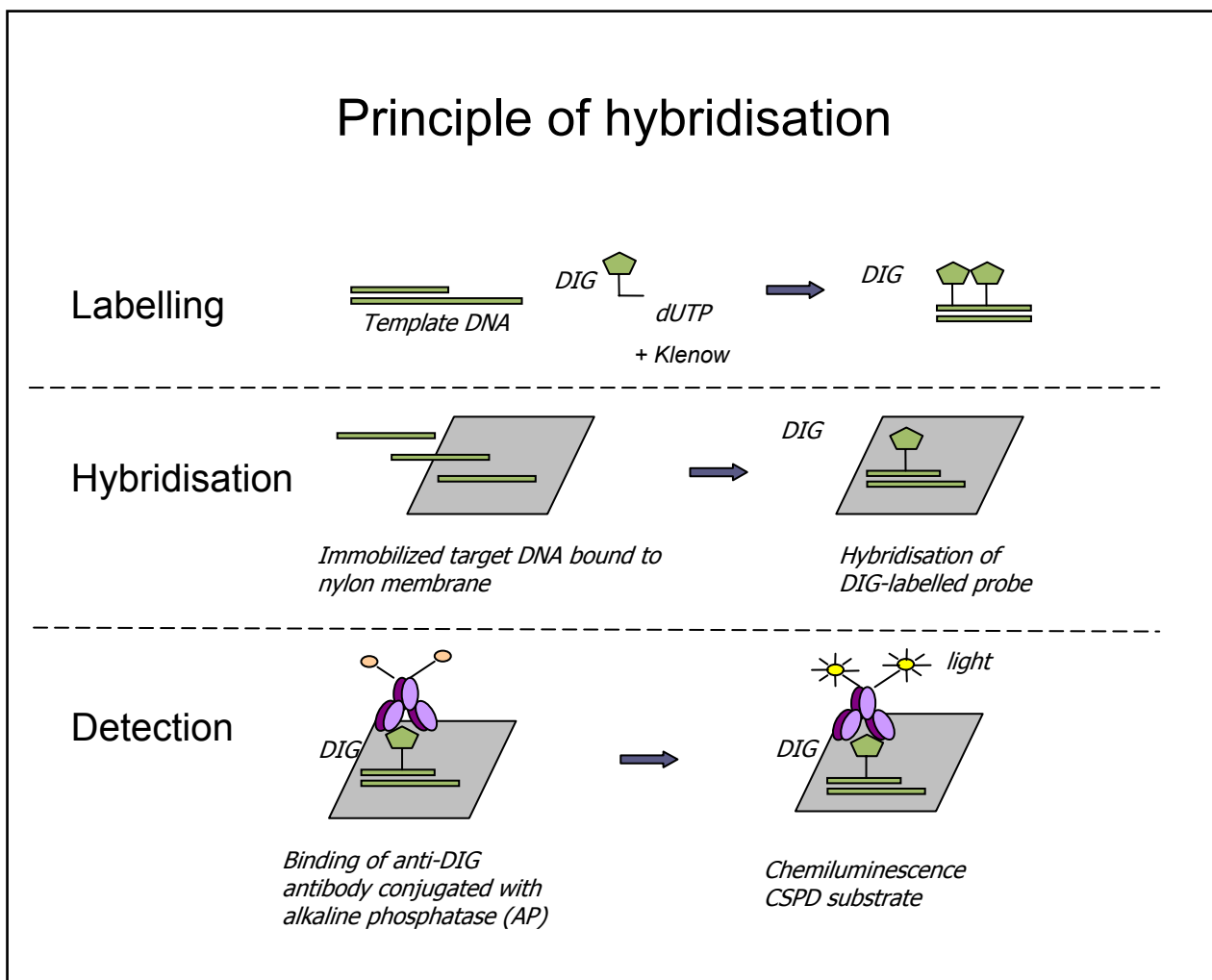


Figure 8. The principle of Checkerboard DNA-DNA hybridisation technique

Detection

The chemiluminescence signals were obtained by exposure of the membranes to an X-ray film. A semiquantitative estimation was based on the visual comparison with the mixture of bacteria containing 10^5 and 10^6 of each species per ml according to Papapanou *et al.* [211]. The quality of the DIG-labelled probes was tested and standardised by running different concentrations of DNA probes against the homologous bacterial strain and the pool of all bacterial strains used for DNA probes (10^5 and 10^6).

The scoring of the intensity of the spots were as follows:

- 0 = no reaction,
- 1 = less than 10^5 bacteria,
- 2 = equal to 10^5 ,
- 3 = more than 10^5 but less than 10^6 ,
- 4 = equal to 10^6 ,
- 5 = more than 10^6

Each membrane contained positive (bacterial standards) and negative (without DNA) control lanes. An example of a CDH membrane is shown in figure 9.

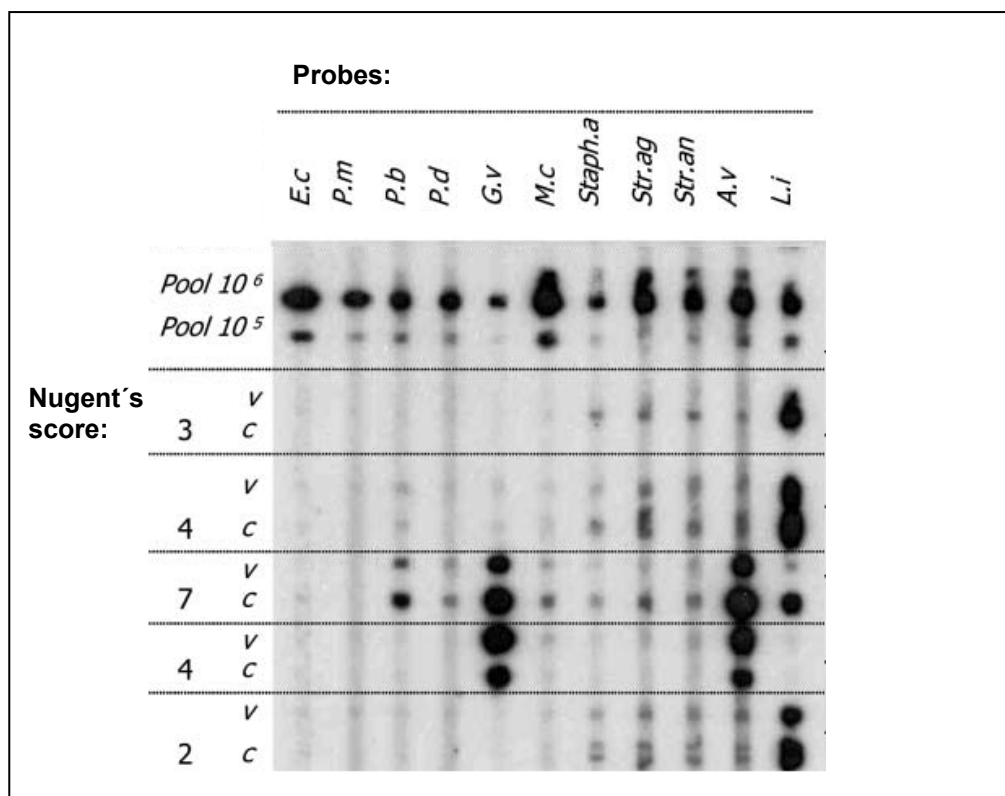


Figure 9. CDH membrane showing the chemiluminescence intensity of vaginal (v) and cervical (c) samples from five women graded according to Nugent's score (healthy = 2-3, intermediate = 4, and BV = 7). (A. vaginae, *A.v*, *E. coli*, *E.c*, *P. bivia*, *P.b*, *P. disiens*, *P.d*, *P. melaninogenica*, *P.m*, *G. vaginalis*, *G.v*, *M. curtisii*, *M.c*, *S. aureus*, *Staph.a*, *S. anginosus*, *Str.an*, *S. agalactiae*, *Str.ag*, *L. iners*, *L.i*).

Cytokine and SLPI assays (I)

The levels of cytokines and secretory leukocyte protease inhibitor (SLPI) in vaginal and cervical secretions were quantified by enzyme-linked immunosorbent assays (ELISA). The assays were based on matched anti-human IL-1 α , IL-1 β , IL-6, IL-8 and SLPI antibody pairs, and related recombinant cytokine or SLPI (R&D System Europe Ltd., UK) [212, 213]. All samples were run in duplicates diluted 1:5, 1:20 and 1:100.

Endotoxin assay (I)

The endotoxin content of the vaginal and cervical secretions was determined by the endpoint *Limulus* amoebocyte lysate test, Endochrome (Charles River Endosafe, SC USA). The analysis was performed according to the manufacturer's manual. All samples were diluted 1:100 and run in duplicates. The limit for detection was 0,6 EU/ml (corresponding to 60 pg/ml) with respect to the dilution of the sample.

Statistics

Spearman's rank-order correlation coefficient test (I, IV) and the Kruskal-Wallis with Dunn's multiple comparison tests were performed (I). Statistical analyses of frequencies were performed using Fisher's exact test (IV). One-way ANOVA with Bonferroni's multiple comparison test was used for analysis of statistical significance regarding pH and Nugent scores (IV). Also Mann-Whitney *U* test was applied (IV).

Results and discussion

The lower genital tract microbiota in relation to cytokines and SLPI (I)

Identification and quantification of the vaginal microbiota

In order to define various types of abnormal vaginal microbiota and identify risk factors for ascending infections, more information is needed regarding the numbers and identity of the bacterial species present in the normal and disturbed microbiota.

Forty-two bacterial species and 19 genera were detected in vaginal secretions of the 37 healthy women. The total number of bacteria (CFU per ml of secretion) ranged from 10^6 to 10^{12} , of which the highest concentrations were found for *Actinomyces urogenitalis* and *G. vaginalis*. Several *Lactobacillus* species (*L. crispatus* and *L. jensenii*) as well as *Peptoniphilus harei* and *Atopobium vaginae* were found in concentrations just below this limit. In previous studies the concentrations of bacterial species in vaginal secretions was reported to be approximately 100 times less than in the present study [165, 169].

The most frequent species were *G. vaginalis*, *L. gasseri*, and *L. iners*. Subsequently, the four most common *Lactobacillus* species were the three mentioned above in addition to *L. jensenii*. The results agreed with those of others [144, 145].

The presence and amount of several bacterial species (*Actinomyces neuii* ss *neuii*, *Actinomyces urogenitalis*, *Anaerococcus prevotii*, *Lactobacillus coleohominis*, *Peptoniphilus asaccharolyticus*, *Peptoniphilus harei*, *Corynebacterium amycolatum* and *Corynebacterium coyleae*) have not been reported before. Comparison with a similar non-quantitative study on the vaginal microbiota in 197 pregnant women showed that 28 bacterial species were common for both studies, and adding all the species found in both studies, a total of approximately 80 bacterial species have been identified colonising the vagina [133].

Although some of the most predominant bacterial species in asymptomatic women with BV are *G. vaginalis* and *Prevotella* spp., we have shown that other less known species such as *A. urogenitalis*, *A. vaginae*, and *P. harei* occur in concentrations exceeding 10^{11} CFU/ml of vaginal secretion. The concentrations are equal or similar to that of *G.vaginalis*.

With regard to an association of *G. vaginalis* with other bacterial species, a strong negative correlation was seen between *G. vaginalis* and *L. iners* ($r=-0.4733$, $P = 0.0047$), and a weaker one with *L. crispatus* ($r=-0.3489$, $P = 0.0431$). Despite being the most frequent *Lactobacillus* species no correlation was found between *L. gasseri* and *G. vaginalis*. In fact, *L. gasseri* was the most abundant *Lactobacillus* species in the intermediate group (6 out of 8 women). Furthermore, a negative correlation was found between *L. iners* and the total bacterial count ($r=-0.5615$, $P=0.0006$).

The intermediate grade was characterised by the highest frequency and numbers of *L. gasseri* and streptococci/staphylococci. Several women with a Nugent score of 6 appeared to have high concentrations of *G. vaginalis* and some in combinations with high numbers of *A. vaginae*. The intermediate grade has been regarded as being a transition phase either leading to BV or returning to a normal grade [215, 216]. The findings suggest, however, that this grade includes various bacterial patterns of which one may be a transition phase, while others may be entities of their own. Our culturing results are better reflected by the scoring of Gram-stained vaginal smears according to Ison and Hay, since they included two more categories as compared to Nugent scoring of which one was defined by high concentrations of streptococci or staphylococci [217]. The occurrence of *L. gasseri*, *G. vaginalis* or streptococci as the predominating species in women graded as intermediate according to Nugent scoring indicates that the method is inadequate for defining entities not being BV or typical *Lactobacillus* dominated biota. The Gram-stained morphotype of cultured *L. iners* (Figure 5d) for instance, is easily mistaken for a mixture of *Lactobacillus* and non-*Lactobacillus* morphotypes (the actual appearance in the vagina is not known).

Relationship of bacterial species with cervical cytokines and endotoxin

A cervico-vaginal increase of proinflammatory cytokines such as IL-1 and IL8 has been associated with BV [172, 173]. The presence of *Ureaplasma urealyticum* in cervical mucus has been associated with IL-6 [213]. Also endotoxin may be increased in BV [172]. In very few studies, however, single bacterial species of the vaginal microbiota has been related to the cytokine levels.

In the cervical secretions, both IL-1 α and IL-1 β were significantly correlated to the total number of bacteria ($P=0.0343$ and $P=0.0112$ respectively).

L. iners showed an inverted correlation with cervical IL-1 α ($r=-0.4733$, $P=0.0047$), while *L. gasseri* and staphylococci were positively correlated with cervical IL-1 β ($r=0.3997$, $P=0.0431$, and $r=0.4345$, $P=0.0266$). In addition, both cervical and

vaginal IL-8 correlated positively with staphylococci ($r=0.4118$, $P=0.0366$, and $r=0.4584$, $P=0.0095$ respectively). All aerobic bacteria together (*E.coli*, *Streptococcus* spp., *Streptococcus* spp., *Enterococcus faecalis*, *Corynebacterium* spp., *Brevibacterium*-like, *Aerococcus christensenii*) also correlated with cervical IL-8 ($r=0.4942$, $P=0.0103$). IL-6 was detected only in a few women, and hence no correlations.

As opposed to cervical IL-1 α , cervical SLPI correlated positively with *L. iners* ($r=0.574$, $P=0.0066$).

Despite the absence of inflammation, BV is characterised by increased vaginal cytokine levels of IL-1 and IL-8, but not with IL-6 [170, 172, 173]. Our finding of a correlation between the total CFU and cervical IL-1 α/β could be interpreted as the IL-1 levels are a consequence of the vaginal bacterial load in the non-inflammatory condition, escalating the alert status of the host.

Donders *et al.* defined a new term, aerobic vaginitis, for a group of women with clinical vaginitis carrying mainly bacteria such as *S. aureus*, group B streptococci, and *E.coli* [84]. The vaginal levels of IL-1 were significantly higher in these women than in women with BV. Our findings of correlations between the levels of cervical IL-1 or IL-8 and staphylococci, and between the combined group of aerobic bacteria and cervical IL-8, indicate that even in the absence of inflammation and BV the host responds to increased numbers of these bacteria.

Also, the finding of Hedges *et al.*, that women with an intermediate vaginal microbiota (Nugent's score) expressed increased IL-1 β levels compared with healthy women, support the importance of analysing women with a disturbed non-BV microbiota with respect to bacterial species [170].

A factor that could influence growth of bacteria in the genital tract is SLPI, which expresses antimicrobial activity [218, 219]. Of the four most common *Lactobacillus* species, a correlation was only established between *L. iners* and cervical SLPI. Interestingly, this *Lactobacillus* species in contrast to *L. crispatus* has been shown to promote the constitutive secretion of SLPI in human monocytic cells *in vitro* [212].

The prevalence of *Lactobacillus* – dominant microbiota in asymptomatic fertile women with respect to age and contraceptives (IV)

No age-related study on the frequency of LDB in fertile women representative of a female population has been reported to our knowledge. Furthermore, the effects of

contraceptive method on the vaginal microbiota are contradicting [176, 188, 220]. Therefore, it was of interest to investigate the influence of age and contraceptive method on the occurrence of LDB, vaginal pH, and Nugent score.

The participants of three age cohorts were grouped into non-users of contraceptives, oral contraceptive users (OCP), users of intrauterine device with copper (IUD-Cu), and users of IUD with levonorgestrel (IUD-L) (Figure 7).

LDB

In women using no contraceptives, a natural decrease in the LDB frequency was observed with age (χ^2 for trend $P=0.0018$) (Figure 11). Comparing the individual age groups a significant difference was found between age cohorts 20-29 and 40-49.

The LDB frequency of women using OCP did not change with age (81-87 %) (Figure 11). The LDB frequency was as high as for women in the age cohort 20-29 using no contraceptives.

Comparing the IUD-Cu users with their corresponding age groups using no contraceptives, a significant reduction of the LDB frequency was observed for IUD-Cu users at age 30-39, but not at age 40-49. The IUD-L users did not significantly differ from the women with no contraceptives. Although there was no difference between IUD-Cu and IUD-L users in age cohort 30-39, a significantly higher LDB frequency was found in the IUD-L group in age cohort 40-49. The percentage of LDB in this group was close to that of the corresponding OCP group.

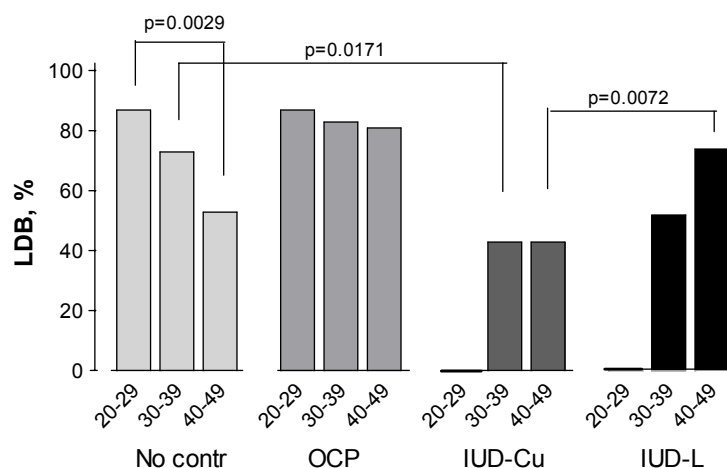


Figure 11. Frequency of LDB in relation to age and contraceptive method (non-users of contraceptives = No contr, users of oral contraceptive pills = OCP, users of intrauterine devices with copper = IUD-Cu, and of IUD-levonorgestrel = IUD-L). Statistical significant differences were calculated with Fischer's exact test.

pH

Regarding pH, the mean pH of all age cohorts for both non-contraceptive users and OCP users was fairly stable ranging between 4.09 and 4.23 (Table 7). However, the pH of both IUD-Cu- and IUD-L users of age cohort 30-39 was significantly elevated ($P<0.05$), compared with non-users.

Nugent score

In women using no contraceptives, the mean level of Nugent's score was significantly lower in age cohort 20-29 than in 40-49 ($P<0.05$), which was in contrast to the LDB frequency, where the results were inverse (Figure 11).

Also, a significantly higher mean level was observed in non-users of contraceptives compared to OCP users ($P<0.05$) in age cohort 40-49. As for the LDB frequency, a significant difference was found between IUD-Cu and IUD-L in age cohort 40-49 ($P<0.01$), but the mean level of Nugent's score was higher in the group of women using IUD-Cu than IUD-L.

Overall, the LDB frequencies correlated inversely with pH (Spearman $r = -0.8257$, $P = 0.0047$), and Nugent score (Spearman $r = -0.8781$ and $P = 0.0016$).

Age cohort	Test	Mean (SEM)			
		no-contr.	OCP	IUD-Cu	IUD-L
20-29	pH	4.13 (0.06)	4.13 (0.04)	-	-
	Nugent's score	1.1 (0.4)	0.9 (0.3)	-	-
30-39	pH	4.23 (0.04)	4.09 (0.04)	4.49 (0.10)	4.52 (0.15)
	Nugent's score	1.6 (0.3)	1.6 (0.3)	2.7 (0.5)	2.9 (0.6)
40-49	pH	4.23 (0.05)	4.21 (0.07)	4.39 (0.05)	4.47 (0.14)
	Nugent's score	2.3 (0.4)	1.0 (0.4)	3.1 (0.4)	1.3 (0.4)

Table 7. Mean of pH and Nugent score in women using no contraceptives, OCP, IUD-Cu, and IUD-L.

In women using no contraceptives, a significantly higher LDB frequency as well as a lower Nugent score were found in age cohort 20-29 compared to women in the older age cohorts. This age related pattern of the vaginal microbiota underlines its hormone-dependence [176]. In age cohort 40-49, the oestrogen levels decrease slowly and there is a corresponding change in the vaginal microbiota in favour of less acid-tolerant bacteria. The menstrual cyclic pattern also changes with more frequent irregular menstrual periods, long bleedings, ovulation bleedings and

premenstrual spotting, which give an access of proteins in favour of an anaerobic microbiota [163, 221].

Women using oral contraceptives had the same occurrence of LDB in all three age cohorts, approximately 85%, which was identical to women using no contraceptives of the youngest age cohort. The oestrogen control of the vaginal glycogen content (and thus of the acids produced by glycogen metabolism) is the major factor controlling the microbial types and population in the vagina [222]. This environment selects for acid-tolerant and glycogen metabolising bacteria (lactobacilli). Thus, a stable hormone level appears to be the primary cause of maintaining a LDB.

Regarding pH, our results are in accordance with those of Kandil *et al.* [223], who found that the mean value of vaginal pH was higher in IUD-Cu users than in women using no contraceptives. An explanation was that IUD gives access of protein secretion due to spotting and long menstrual bleedings, thus increasing the pH and thereby promoting a more disturbed vaginal microbiota. Our finding of a high pH for the hormone releasing IUD-L, even in the presence of an increased LDB and a low Nugent score could possibly be explained by leakage of a small volume of pH neutral exudate from cervix and endometrium into the vagina.

A difference was observed between IUD-Cu and IUD-L users in the oldest age cohort concerning the frequency of LDB and Nugent score. The LDB frequency was higher and the Nugent score lower in IUD-L than those of the IUD-Cu. The locally released levonorgestrel acts by thickening the cervical mucus, while Cu affects the endometrial enzymes, glycogen metabolism, and oestrogen uptake. It is possible that the local progestogen induces changes that favour the growth of lactobacilli during a diminishing production of endogenous oestrogen.

Description of new *Actinomyces* and *Lactobacillus* species of the lower FGT microbiota (II, III)

During the identification process of the vaginal microbiota, several bacterial strains were characterised as unknown organisms. After preliminary phenotypic tests, it was found that these strains possibly related to the genera *Actinomyces*, *Lactobacillus*, *Brevibacterium*, *Fusobacterium*, *Micromonas* and *Peptostreptococcus*. A polyphasic taxonomic study was carried out on two of these organisms, together with similar strains from the CCUG bacterial collection, and the new *Actinomyces urogenitalis* sp. nov. and *Lactobacillus coleohominis* sp. nov. were described.

Actinomyces urogenitalis sp. nov. (II)

The three bacterial isolates (Table 5) were gram-positive straight to slightly curved rods which were non-acid-fast and non-spore-forming (Figure 12A). The strains were catalase-negative and phenotypically closely resembled each other. The cellular morphology and biochemical reactions of the isolates were consistent with their assignment to the genus *Actinomyces*.

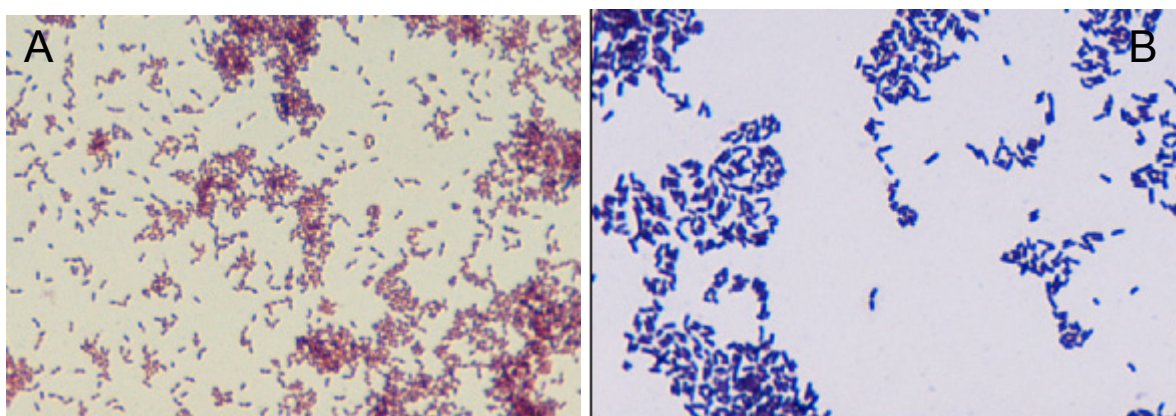


Figure 12. Gram-stained bacterial cells of *Actinomyces urogenitalis* (A), CCUG 38702, and of *Lactobacillus coleohominis* (B), CCUG 44007, (magnification $\times 1,000$).

SDS-PAGE

To assess the phenotypic resemblance of the three isolates to each other and to the reference strain of *Actinomyces* species, a comparative analysis of whole-cell protein profiles by SDS-PAGE was performed. The three isolates clustered together and formed a distinct group with a within-group correlation level of 85% or more. *Actinomyces slackii*, *Actinomyces turicensis* and *Actinobaculum schaalii* were the nearest species to the unknown isolates, joining the cluster at a correlation level of about 60% (Figure 13). The PAGE results confirmed that the three unidentified strains represent a phenotypically homogeneous group of organisms and that they are distinct from all *Actinomyces* species and close relatives described to date. The PAGE protein profiling results demonstrated that the unknown isolates represent a separate species from *Actinomyces bovis*, *Actinomyces slackii* and other close relatives.

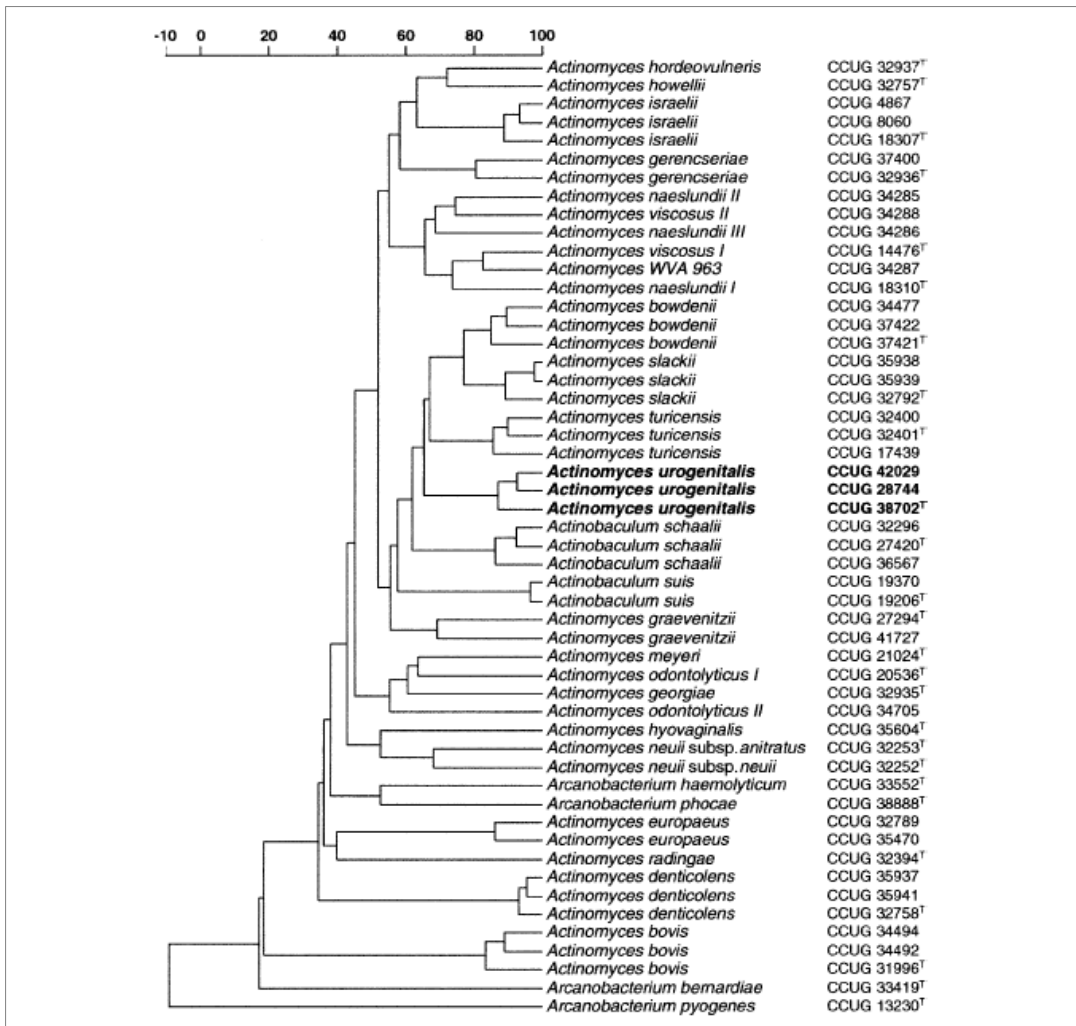


Figure 13. Similarity dendrogram based on whole-cell protein patterns of *Actinomyces urogenitalis* and related species. Levels of correlation are expressed as percentages of similarity for convenience

16S rRNA

To ascertain the phylogenetic relationships of the clinical isolates, their 16S rRNA genes were sequenced and subjected to a comparative analysis (Figure 14). The almost complete gene sequences (>1500 nucleotides) of the three strains were determined and pairwise analysis showed these to be almost identical (99.8 – 100% similarity). Sequence database searches confirmed that the unknown bacterium was most closely related to species of the genus *Actinomyces*. Highest sequence relatedness was shown with *Actinomyces bovis*, *Actinomyces bowdenii*, *Actinomyces naeslundii*, *Actinomyces viscosus* and *Actinomyces slackii*. The results of neighbour-joining analysis confirmed the association of the unknown clinical bacterium (CCUG 38702) with *Actinomyces bovis* and its near relatives.

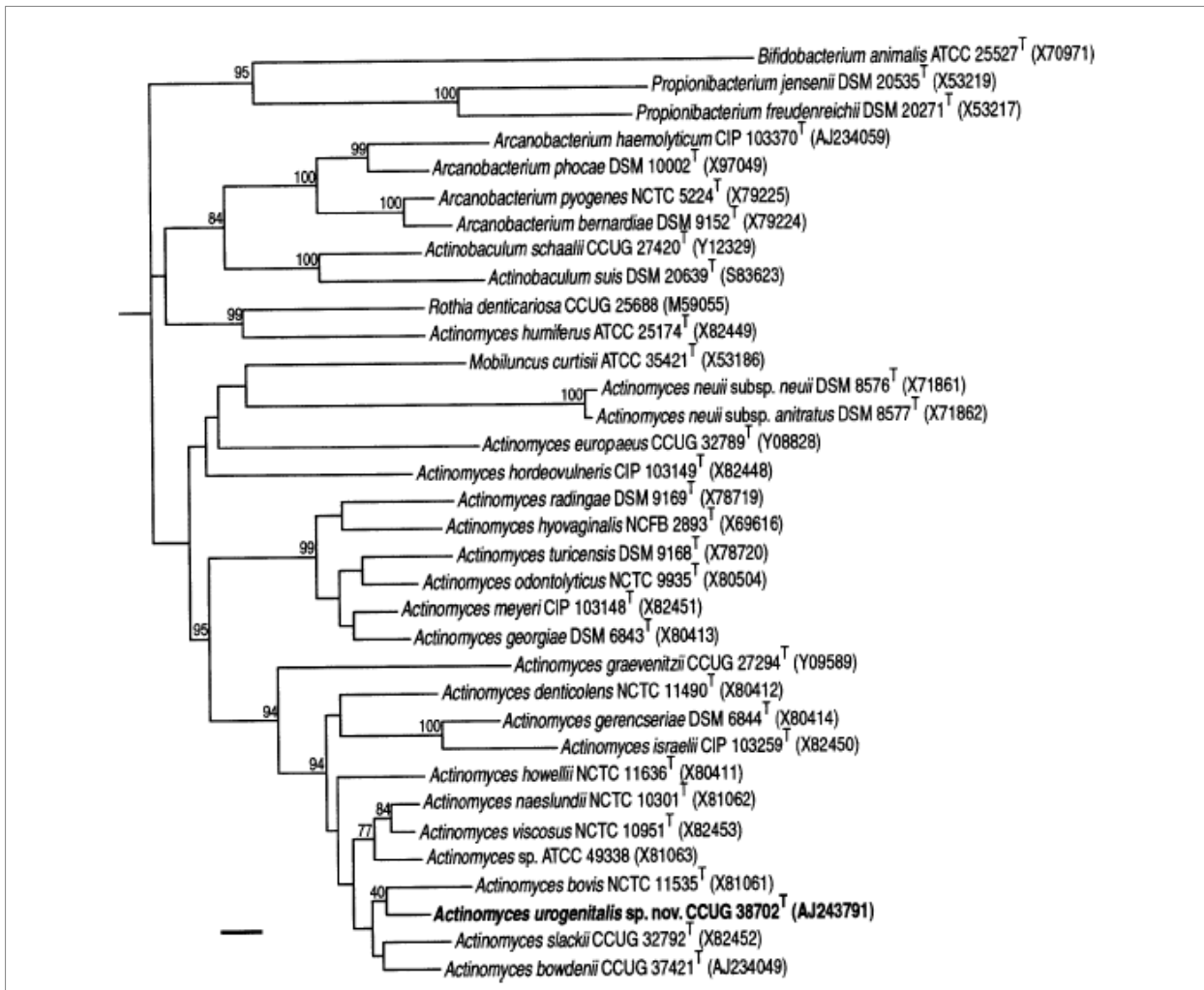


Figure 14 Unrooted tree showing the phylogenetic relationships of *Actinomyces urogenitalis* and some other high G+C containing Gram-positive bacteria.

It is clear from the present 16S rRNA study that the novel bacterium forms a distinct subline within a cluster of species which includes the type species *Actinomyces bovis* and, therefore, can be regarded as an authentic *Actinomyces* species. The observed > 3% divergence between the unknown organism and other members of the *Actinomyces bovis* cluster of species is also greater than that which may be expected between different strains of the same species. Hence the 0.2% divergence observed between the three unknown clinical isolates and > 3% divergence shown with respect to *Actinomyces bovis* and *Actinomyces slackii* is considered very significant.

Bacteria of genus *Actinomyces* have been reported to be involved in development of periodontal, urinary and genital tract infections [224-227]. This pathogen has been associated with use of an intrauterine contraceptive device [228, 229] and implicated as a cause of pelvic inflammatory disease among women using such devices [192].

The diagnostic criteria are mainly based on detection of “sulphur” granules in biopsies and observation of Gram-stained smears of secretions [225]. Culturing often fails due to the overgrowth of other concomitant microorganisms or inadequate culturing techniques. Advanced molecular technologies have been used mostly in the research. In order to effectively treat patients, it is important to identify this microorganism, taking into account, that *Actinomyces*-infection mimics neoplasm [225]. In the present work, *A. urogenitalis* was isolated from a patient with Nugent’s score 7, i.e. BV, in concentration of 10^{12} CFU/ml. The result agrees with previous studies, and shows that *Actinomyces* spp. is often present in women with a disturbed vaginal microbiota [192, 193, 230, 231].

***Lactobacillus coleohominis* sp. nov. (III)**

The isolates from human sources consisted of Gram-positive, non-spore-forming, rod-shaped cells (Figure 12B). All isolates were facultatively anaerobic, catalase-, and oxidase-negative. On the basis of the cultural and biochemical characteristics, the isolates resembled the genus *Lactobacillus* but did not correspond to any of the currently recognized species.

SDS-PAGE

The overall phenotypic resemblance of the unidentified isolates and their relationship to known members of this genus, were determined by whole-cell protein profiles. A numerical analysis of PAGE protein patterns of the four strains together with some reference species of *Lactobacillus* is shown in figure 15.

Protein profiling confirmed the high overall phenotypic homogeneity of the isolates and showed that they represent a tight cluster (intra-cluster correlation level > 83%) that is separate from other *Lactobacillus* species.

16S rRNA

The phylogenetic relationships of the unknown isolates were determined by 16S rRNA gene sequencing and comparative analysis. The almost complete gene sequences (> 1450 nucleotides) of the 4 strains were determined and pair-wise analysis showed them to be identical (100% sequence similarity), thereby demonstrating the high degree of genetic relatedness of the isolates. The results of neighbour-joining analysis, shown in figure 16, confirmed the placement of the unknown bacterium within rRNA group II of the genus *Lactobacillus*. The unknown bacterium formed a distinct subline within a small cluster of species that included *L. oris*, *L. panis*, *L. pontis* and *L. vaginalis* (94.8 – 95.8% 16S rRNA sequence similarity).

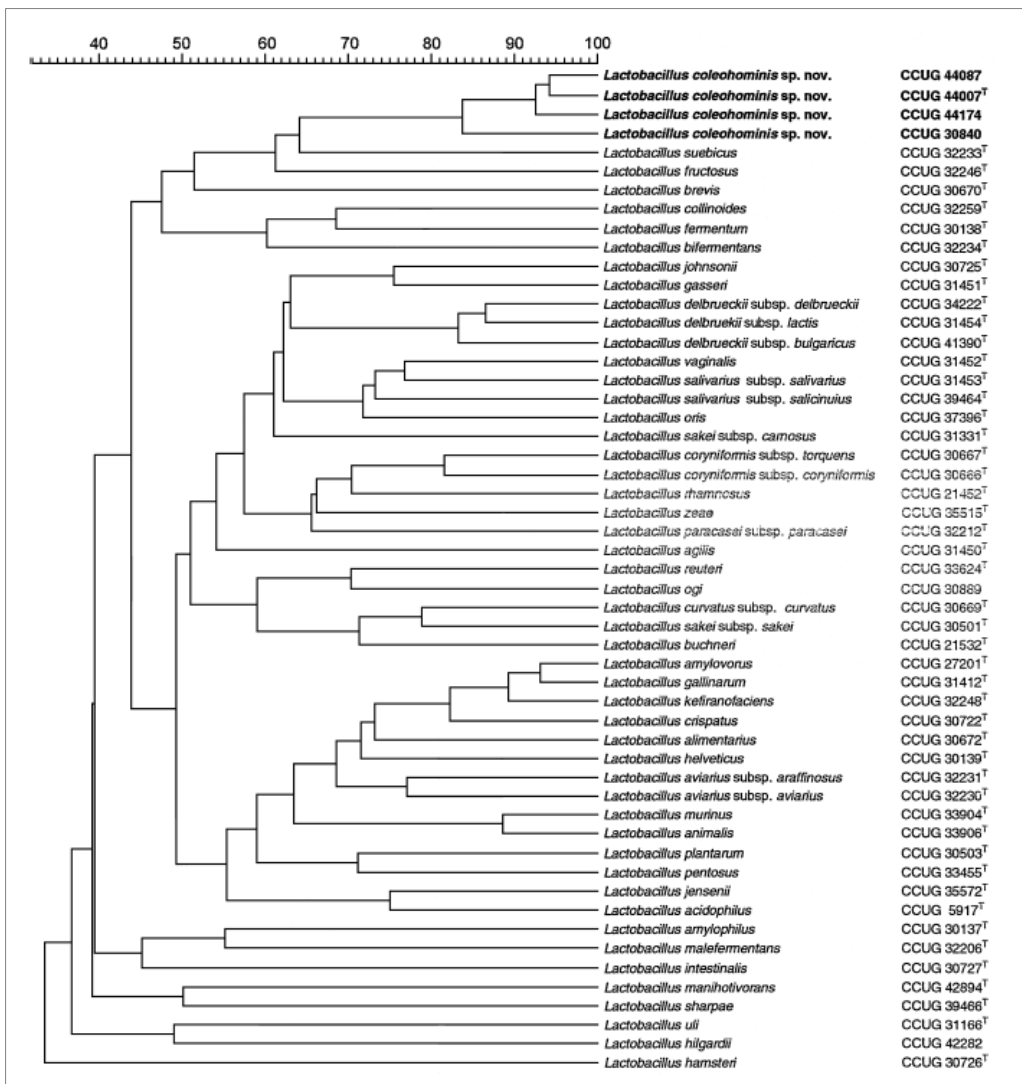


Figure 15 Similarity dendrogram based on whole-cell protein patterns of *Lactobacillus coleohominis* and some other *Lactobacillus* species. Levels of correlation are expressed as percentages of similarity.

It is clear from the investigation that the *Lactobacillus*-like isolates recovered from human sources represent a homogeneous group of organisms which are phenotypically and phylogenetically distinct from currently recognised members of the genus *Lactobacillus*. Phylogenetically, the unknown bacterium represents an unknown subline within the genus *Lactobacillus*, and the rRNA sequence divergence values of > 4% with respect to other lactobacilli demonstrate that it warrants classification as a new species.

Biochemically, the novel bacterium from humans can be readily distinguished from all described species of the genus. Therefore, on the basis of the findings presented, we proposed that the unidentified bacterium be classified in the genus *Lactobacillus*, as *L. coleohominis* sp. nov..

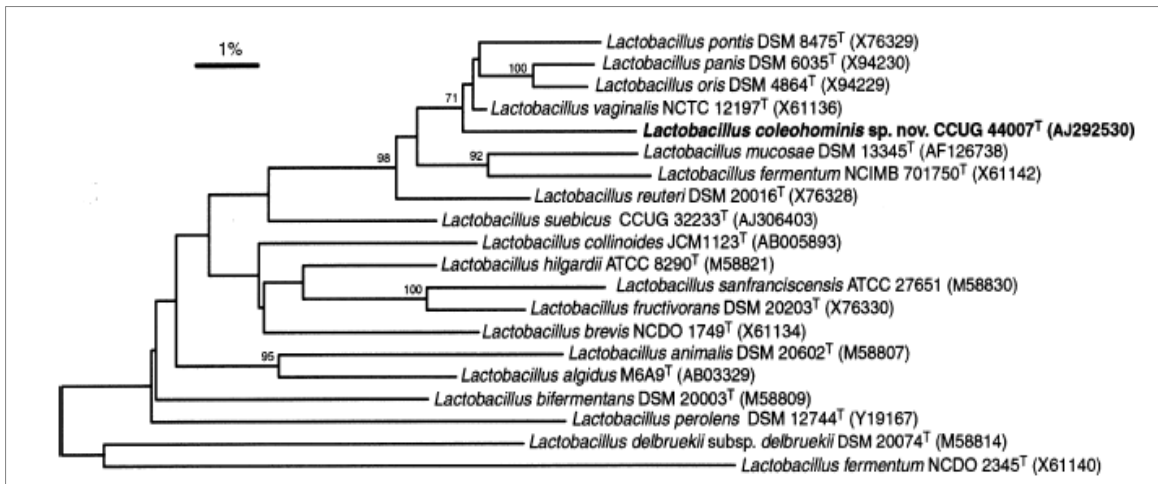


Figure 16 Unrooted tree showing the phylogenetic relationships of *Lactobacillus coleohominis*. Bar, 1% sequence divergence.

In the present study, the new *L. coleohominis* strains were isolated in high concentration from patients with normal and intermediate microbiota, with the Nugent's score 0 and 5, respectively.

Applying a semi-quantitative non-culture dependent DNA-DNA hybridisation method (CDH) for analysis of the lower FGT microbiota (I)

Comparison of CDH with culture

The frequencies of women with any of the 13 bacterial species included in the CDH assay (Table 6) were overall higher by CDH compared with culturing, except for *E.coli* (not shown). The frequency differed especially for the strict anaerobic species.

In a study on the subgingival microbiota, comparison of the checkerboard methodology with culture showed that CDH gave higher values of bacterial counts for the majority of the bacterial species analysed [211]. CDH thus seems advantageous compared to culture with regard to fastidious and strictly anaerobic bacteria. Furthermore, analysis of cervico-vaginal samples would be less affected by the problems of using suboptimal growth media for various bacterial species. With this method, the problems with reduction in viability of various species due to long periods in transport-medium can be avoided.

Concentration of bacteria in relation to Nugent's score

Out of the eight typical BV associated bacteria (Table 6) six correlated with Nugent score (*P. bivia*, *P. disiens*, *P. melaninogenica*, *G. vaginalis*, *M. curtisii*, *A. vaginae*). Four out of the six bacterial species comparing cervical samples still correlated with Nugent score (*P. bivia*, *P. disiens*, *G. vaginalis*, *A. vaginae*).

Bacterial patterns of cervical and vaginal *G. vaginalis*, *P.bivia*, *F. nucleatum* and *L. iners*

With regard to cervical bacteria, *G. vaginalis* correlated with *A. vaginae* ($r=0.6451$, $P=0.007$) and *P.bivia* with *P. disiens* ($r=0.9889$, $P<0.0001$) and *B. ureolyticus* ($r=0.05408$, $P=0.0138$). *F. nucleatum* was associated with *S. aureus* ($r= 0.5710$, $P=0.0209$) and *S. anginosus* ($r=0.7009$, $P=0.0025$). The only analysed lactobacillus species, *L. iners*, correlated with *S. aureus* ($r= 0.7360$ and $P=0.0012$).

Vaginal *G. vaginalis*, *P.bivia* and *F. nucleatum* were all significantly correlated to the five bacterial species *A. vaginae*, *M. curtisii*, *B. ureolyticus*, *P. disiens* and *P. melaninogenica*. In addition, *G. vaginalis* was significantly associated with *P. bivia*. Furthermore, *F. nucleatum* also correlated with *S. aureus*, *S. anginosus*, and *S. agalactiae*.

Fewer correlations were found in the cervical samples than in the vaginal samples. It may indicate that the host innate defence more strongly antagonizes the colonization of bacteria in the cervical canal. Also, more correlations were found by CDH than by culture with regard to the eight BV-associated bacteria due to the increased rate of anaerobes in CDH.

The correlations between the BV-associated bacteria were compatible with the correlations obtained with the Nugent score, except for *F. nucleatum* and *B. ureolyticus*. *F. nucleatum* correlated with *S. aureus*, *S. anginosus*, *S. agalactiae*, and *L. iners*, indicating an association with the intermediate and normal grades of Nugent score. The results of *F. nucleatum* and *B. ureolyticus* as not being specific BV-associated bacteria agrees with one report showing that these two species in contrast to ten other BV-associated species did not relate inversely to vaginal colonisation of H₂O₂-producing lactobacilli [153].

Correlations of bacterial species with cytokines and endotoxin

Analysis of correlations between the 13 bacterial species and cytokines showed that in both cervical and vaginal secretions, *B. ureolyticus* correlated with IL-1 α , while a correlation was observed only in cervix secretion with regard to IL-8. In addition, vaginal *F. nucleatum*, *S. agalactiae*, *S. anginosus* and *S. aureus* showed

correlations with vaginal IL-1 α . The only correlation with IL-1 β was seen with cervical *G. vaginalis*.

When all Gram-negative bacteria were combined a negative correlation was found with SLPI, and a positive one with endotoxin.

CDH confirmed the findings with culturing that staphylococci and streptococci may stimulate cytokine secretion (IL-1) in asymptomatic healthy women. Regarding the vaginal presence of *B. ureolyticus* and *F. nucleatum*, they have been reported to be associated with preterm delivery, amniotic fluid infection or elevated IL-6 levels in the amniotic fluid [118, 124].

It appears that microbial patterns or single bacterial species, including several strict anaerobic Gram-negative bacteria, need to be related to the grade of clinical and laboratory inflammatory signs in order to clarify whether there are distinct entities of abnormal vaginal microbiota that lead to somewhat different host responses.

An inverted correlation was obtained between vaginal Gram-negative bacteria and SLPI. These results suggest that the secretion of SLPI is affected by both the number and type of bacteria. Explanations could be microbial degradation of SLPI, as well as down regulation of the SLPI secretion by the host in response to a disturbed vaginal microbiota. In fact, *in vitro* studies have shown that the constitutive SLPI secretion in cervical epithelial cells can be down regulated by bacteria such as *E.coli* [212]. Our results are in agreement with the findings of Draper et al. [232], showing a reduction of vaginal SLPI in women with BV.

Conclusion

In the present study, the composition of the normal vaginal microbiota was analysed by culturing and related to cytokines and SLPI. In addition, the effects of age and use of contraceptives on the vaginal microbiota were investigated by the analysis of the frequency of LDB.

The concentrations of various bacterial species correlated to Nugent's score, the most widely used method for characterisation of vaginal microbial condition, and to cytokine levels, as well as to endotoxin and SLPI. The normal aging process and the use of different types of contraceptives were found to affect the vaginal LBD-microbiota. It is not known yet whether the reduction in LDB frequency in IUD users would lead to a similar replacement of bacterial species of the vaginal microbiota as in older fertile women.

During the isolation and identification process of bacterial species, the new *Lactobacillus coleohominis* and *Actinomyces urogenitalis* were described. In addition, several bacterial species, which were isolated by culturing, were recorded for the first time. The isolation and identification techniques worked with high precision. However, "Checkerboard" DNA-DNA hybridisation showed to be especially efficient with respect to detection of many fastidious and strictly anaerobic bacteria. This study was the first application of the CDH methodology in identification of bacteria in the FGT. By analysis of cervico-vaginal specimens with CDH the inherited bias of culturing due to not optimal media for various bacterial species can be avoided. Likewise, the effect on the viability of sometimes long periods in transport medium before the samples reach the laboratory would be of less importance for the analysis.

It thus appears, as CDH is a suitable tool for future studies on various patient groups with the aim of quantifying 30-40 separate bacterial species without the laborious work of culturing and identification.

BV is associated with an increased risk of ascending infections, spontaneous preterm delivery, spontaneous abortion, and increased susceptibility to HIV and other STDs [4, 11, 14, 15]. More information about the quantitative composition and patterns of microorganisms of the lower genital tract and their relationship to the local host inflammatory response could help understand more about the mechanisms leading to a disturbed vaginal microbiota, subclinical ascending infections, and a reduced resistance towards STDs. By defining a risk group of women based on bacteriological diagnosis and host immune factors, antibiotic treatment could be made more specific and possibly increasing the cure rate in women with a disturbed vaginal microbiota.

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