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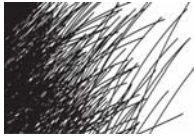
Characterization of the vaginal microflora

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List of abbreviations

AFLP   Amplified Fragment Length Polymorphism
ARDRA   Amplified Ribosomal DNA Restriction Analysis
bp   Base pairs
DGGE   Denaturing Gradient Gel Electrophoresis
DNA   Deoxyribonucleic acid
G+C content   Guanine + Cytosine content
GBS   Group B Streptococcus (*Streptococcus agalactiae*)
HPV   Human Papilloma Virus
PCR   Polymerase Chain Reaction
RAPD   Randomly Amplified Polymorphic DNA analysis
rep-PCR   repetitive element sequence-based PCR
rDNA   rRNA gene
RNA   Ribonucleic Acid
rRNA   Ribosomal RNA
STD   Sexual Transmitted Disease
tDNA-PCR   tRNA intergenic length polymorphism PCR
T-RFLP   Terminal Restriction Fragment Length Polymorphism
TRF   Terminal Restriction Fragment
tRNA   Transfer RNA
TTGE   Temporal Temperature Gradient Electrophoresis

List of bacterial species

A. christensenii	*Actinomyces christensenii*
A. neuii	*Actinomyces neuii*
A. vaginalis	*Anaerococcus vaginalis*
A. vaginae	*Atopobium vaginae*
A. minutum	*Atopobium minutum*
A. parvulum	*Atopobium parvulum*
A. rimae	*Atopobium rimae*
B. ureolyticus	*Bacteroides ureolyticus*
[*B. biavatii*]	[*Bifidobacterium biavatii*]
B. bifidum	*Bifidobacterium bifidum*
B. breve	*Bifidobacterium breve*
B. dentium	*Bifidobacterium dentium*
B. infantis	*Bifidobacterium infantis*
B. longum	*Bifidobacterium longum*
C. albicans	*Candida albicans*
E. coli	*Escherichia coli*
F. magna	*Finegoldia magna*
L. acidophilus	*Lactobacillus acidophilus*
L. amylovorus	*Lactobacillus amylovorus*
L. casei	*Lactobacillus casei*
L. coleohominis	*Lactobacillus coleohominis*
L. delbrueckii	*Lactobacillus delbrueckii*
L. crispatus	*Lactobacillus crispatus*
L. gallinarum	*Lactobacillus gallinarum*
L. gasseri	*Lactobacillus gasseri*
L. fermentum	*Lactobacillus fermentum*
L. helveticus	*Lactobacillus helveticus*
L. iners	*Lactobacillus iners*
L. jensenii	*Lactobacillus jensenii*
L. johnsonii	*Lactobacillus johnsonii*
L. kalixensis	*Lactobacillus kalixensis*
L. kitasatonis	*Lactobacillus kitasatonis*
L. mucosae	*Lactobacillus mucosae*
L. nagelii	*Lactobacillus nagelii*
L. oris	*Lactobacillus oris*
L. pontis	*Lactobacillus pontis*
L. rhamnosus	*Lactobacillus rhamnosus*
L. reuteri	*Lactobacillus reuteri*
L. salivarius	*Lactobacillus salivarius*
L. vaginalis	*L. vaginalis*
[*L. amnionii*]	[*Leptotrichia amnionii*]
G. vaginalis	*Gardnerella vaginalis*
M. curtisii	*Mobiluncus curtisii*
M. mulieris	*Mobiluncus mulieris*
P. micros	*Peptostreptococcus micros*
P. bivia	*Prevotella bivia*
P. buccalis	*Prevotella buccalis*
S. agalactiae	*Streptococcus agalactiae*
S. sanguinegens	*Sneathia sanguinegens*
T. vaginalis	*Trichomonas vaginalis*
V. cambriense	*Varibaculum cambriense*

I. General introduction

Microbiologists have been constrained in their efforts to describe the compositions of complex microbial communities when using traditional methods because few microorganisms have sufficiently distinctive morphology to be recognized by microscopy and because culture-dependent methods are biased, since a microorganism can be cultivated only after its physiological niche is perceived and duplicated experimentally.

The introduction of DNA based molecular biology techniques in bacteriology has increased taxonomic insights (127) and has made possible detecting fastidious and cultivation-resistant organisms in complex microbial communities (198). It has been estimated, by simple retrieval and comparison of DNA sequences with known sequences, that more than 99% of microorganisms present in the environment are not cultivable by using standard techniques (4).

Comparably, in the human colon, possibly hundreds of bacterial species form a bacterial community in which some members number about 10^{11} cells per gram (wet weight) of contents. Molecular genetic tools have indicated that 60 to 80% of the organisms in the human intestinal microflora have not been cultivated - even when excellent bacteriological culture methods are used - perhaps because of their fastidious requirement for anaerobiosis, but more likely due to their complex nutritional requirements (149, 271). These studies clearly confirmed that culture based approaches have essentially biased our view on the biodiversity of microbial life.

Also the vaginal ecosystem is complex, especially when disturbed. It includes several microorganisms that are difficult to cultivate or identify and that are therefore unfamiliar to many clinicians and microbiologists. Because of the recognized role of the vaginal microflora in complications of pregnancy and infections of the upper genital tract, it is important to gain a better understanding of the composition and the regulation of this system.

This doctoral thesis comprises a culture-independent analysis of the composition of the vaginal microbial community, using a common but distinctive bacterial chromosomal region, the 16S ribosomal RNA-gene, and compares the results with those obtained by culture.

II. Objectives

The main objectives of this work were to obtain a more complete view of the composition of the normal vaginal microbial flora and the bacterial vaginosis microflora, and to develop a fast PCR-based technique for the culture-independent characterization of the vaginal microflora.

Specific objectives were:

1. Molecular biological characterization of the microbial diversity of the vaginal microflora of pregnant and non-pregnant women by cloning and Terminal Restriction Fragment Polymorphism Analysis (T-RFLP) in comparison with culture and microscopy.

2. Determination of the specific microbiological differences between the normal vaginal microflora, the bacterial vaginosis microflora and the intermediate microflora.

3. Analysis of the temporal variations of the vaginal microflora during pregnancy.

III. Overview of the literature

III.1. The vaginal ecosystem

In healthy women, the vagina is a balanced ecosystem, containing 10^9 bacterial colony-forming units per gram of vaginal fluid. The most abundant isolates in a normal microflora are lactobacilli (Döderleins' bacilli), which metabolize glycogen – which is stored and secreted almost exclusively by vaginal epithelial cells – into lactic acid, resulting in acidification (pH 3.8-4.5) of the vaginal environment. In combination with bacteriocin and hydrogen peroxide production by some lactobacilli this contributes to colonization resistance, i.e. reduction of the ability of other microorganisms – including sexually transmissible pathogens – to colonize the female genital tract (1, 13, 63, 101, 114, 115, 276, 279, 306).

The remainder of the normal vaginal microflora varies among women in terms of both the kinds and abundances of species present. Investigators have demonstrated the occurrence of coagulase-negative staphylococci, group B Streptococci, and species of *Corynebacterium*, *Prevotella*, *Peptostreptococcu*s, *Gardnerella*, enteric bacteria, and various other taxa (195). The typical abundance of these populations ranges from 10^4 to 10^6 colony-forming units per ml of vaginal fluid.

A disturbed vaginal microflora is characterized as vaginitis. The most common etiologies in adults resulting in symptoms of vaginitis include infection with *Candida albicans,* infection with *Trichomonas vaginalis,* and bacterial vaginosis. Prevalences vary depending on the population studied. Bacterial vaginosis accounts for 40–50% of cases, vulvovaginal candidiasis accounts for 20–25% of cases, and trichomonal vaginitis accounts for 15–20% of cases (17, 139, 252).

Proneness to disturbance of the vaginal ecosystem is determined by three factors which influence each other mutually: i) genetic factors determining the innate immune response (225, 226, 297), ii) environmental factors, including behavioral factors (162) and iii) the composition of the vaginal microflora.

It can be stated that women with a certain genetic background and certain lifestyles will be more prone to acquire a disturbed vaginal microflora. However, the immune properties of an individual cannot be changed and changing lifestyles is in many cases just as impossible. Moreover, interventions to reduce behaviorally determined risk factors have been shown to be largely ineffective (32, 120). As a consequence, a better understanding of the underlying microbiology of the vaginal ecosystem seems to be the most straightforward road leading to

better treatment of the disturbed vaginal ecosystem and to prevention of pregnancy complications and upper genital tract infection (150, 162).

III.2. The normal vaginal microflora

III.2.1. Vaginal lactobacilli

Lactobacilli are Gram-positive, catalase-negative, non-sporing rods or coccobacilli with a G+C content ranging from 33 to 55 mol% (264). They are strictly fermentative, aerotolerant or anaerobic and have complex nutritional requirements. Lactobacilli can be found where rich, carbohydrate-containing substances are available (94).

The genus *Lactobacillus*, proposed in 1901 by Beijerinck (21), is one of the most important genera within the group of the lactic acid bacteria and currently comprises more than 80 species. Lactic acid bacteria are a group of bacteria that are functionally related through their ability to produce lactic acid, either as a single product (homofermentative metabolism) or together with other products such as organic acids and ethanols (heterofermentative metabolism). The primary ecological niches of lactic acid bacteria are the oral cavity, the intestinal mucous membranes and the vagina of humans and animals. Bifidobacteria, also encountered in the vaginal ecosystem and closely related to *G. vaginalis*, have properties similar to those of the lactic acid bacteria and are often listed along with the lactic acid bacteria, although phylogenetically this genus belongs to the *Actinomyces* subdivision of the Gram positive bacteria (comprising also *Atopobium*) and therefore is only quite distantly related to the genuine lactic acid bacteria, which belong to the *Clostridium* subdivision of the Gram-positive bacteria (282).

III.2.2. Taxonomy of vaginal lactobacilli: major taxonomic changes

For decades, bacterial taxonomy has relied on microscopy, morphology and determination of physiological properties to classify bacteria according to their phenotypic properties. Discrepancies between these traditional phenotypic tests (classical taxonomy) and the present phylogenetic insights (molecular taxonomy) resulted in a large number of nomenclatural revisions. This issue is especially significant in the study of vaginal microflora, since many of the species harboured in that ecosystem are difficult – if not impossible – to identify through other than molecular genetic methods.

As such, determination of the species to which Döderleins bacillus belongs has been a long and complicated process. In 1928 Thomas interpreted the dominant species in the vagina as being *L. acidophilus* (274). Despite its industrial importance and its role in the vaginal microflora, the taxonomy of *L. acidophilus* remained confusing for a long time. Based on

fermentation profiles *L. acidophilus* was found to be a species belonging to the group of obligately homofermentative lactobacilli.

The first indications of its heterogeneity were obtained from serological data (59) and from electrophoretic analysis of lactate dehydrogenases (82). Later, based on DNA-DNA hybridization studies (42, 78, 134, 154), the *L. acidophilus*-group was shown to be composed of at least seven species: *L. acidophilus* (96), *L. gasseri* (155), *L. crispatus* (42, 181), *L. amylovorus* (186), *L. gallinarum*, *L. johnsonii* (78) and *L. helveticus* (134). Recently three species were added to the *L. acidophilus*-group: *Lactobacillus amylolyticus* (26, 79), *Lactobacillus iners* (66, 79) and *Lactobacillus kitasatonis* (182).

In 1975 Carlsson reported, with the help of phenotypic methods, that *L. acidophilus* and another *Lactobacillus* species belonging to the obligately homofermentative *L. delbrueckii*-group, namely *L. jensenii* (83), were the dominant residents of the healthy human vagina (40). In 1987, using genotypic studies of isolates from 27 women, Giorgi *et al.* reported the dominance of *L. crispatus, L. gasseri* and *L. jensenii,* as well as an unidentified species (85). In 1999, two DNA-based studies of respectively 97 vaginal specimens from Japan (259) and 215 from the United States (8) gave similar results. The study of Antonio *et al.* was the first to identify the four species, namely *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, which are known today as the most frequently occurring vaginal lactobacilli (8).

III.3. Bacterial vaginosis

The syndrome now known as bacterial vaginosis has undergone several name changes as more knowledge of the syndrome and its associated microflora was acquired.

The name 'non-specific vaginitis' was originally used to distinguish this syndrome from the specific vaginitides associated with *T. vaginalis* and *C. albicans*. Gardner and Dukes postulated in 1955 that *Haemophilus vaginalis*, now known as *Gardnerella vaginalis* and affiliated to the family *Bifidobacteriaceae* (41, 90, 205), was the sole cause of non-specific vaginitis, because *G. vaginalis* was recovered from the vaginas of 92% of patients with non-specific vaginitis (81) and they changed the name of the syndrome to *Haemophilus vaginalis* vaginitis. Later, the term 'vaginosis' was introduced to indicate that, in bacterial vaginosis, unlike the specific vaginitides, there is an increased discharge without significant inflammation, as indicated by a relative absence of polymorphonuclear leukocytes (121). The term 'bacterial vaginosis' was adopted to indicate that bacteria rather than fungi or parasites apparently cause this syndrome, but that the identities of these bacteria have not been fully delineated. Because much of the vaginosis-associated microflora is anaerobic, the term 'anaerobic vaginosis' has been suggested (22). In the late 1980s, the name 'vaginal bacteriosis' has been recommended as being grammatically correct (110, 129, 251) however, the syndrome remains generally referred to as bacterial vaginosis.

Over the past 50 years, researchers have attempted to describe the pathobiology, etiologic factors, clinical characteristics, diagnostic methods, pathologic sequelae, and effective treatments for bacterial vaginosis. Before 1980, bacterial vaginosis was commonly regarded as a 'nuisance' infection, and mostly not recognized or ignored by physicians. However, bacterial vaginosis is increasingly recognized as directly related to a number of serious obstetrical and gynecological complications. Antimicrobial agents that act against anaerobes – metronidazole and clindamycin – are currently the preferred treatment for bacterial vaginosis recommended by the Centers for Disease Control and Prevention. Although standard treatment with metronidazole or clindamycin is usually efficient, with a cure rate of 80-90% within one week, 15-30% of the women will relapse within three months, a condition known as recurrent bacterial vaginosis (52, 102, 307). There is a poor understanding of how to optimally treat recurrent bacterial vaginosis, which may be primarily due to our limited understanding of the microbiologic cause of this syndrome (see III. 3.6).

III.3.1. Definition

Bacterial vaginosis is a polymicrobial syndrome whereby a *Lactobacillus* dominated microflora is replaced by a mixture of organisms which generally includes the following: *Gardnerella vaginalis* (41, 81, 90), Gram-negative anaerobic rods such as *Prevotella* species (formerly *Bacteroides* spp. (243)), Gram-positive anaerobic cocci such as *Peptostreptococcus* sp., the genital mollicutes *Mycoplasma hominis* and *Ureaplasma urealyticum*, and sometimes the curved Gram-positive anaerobic rods, *Mobiluncus* sp. (108). In a bacterial vaginosis microflora concentrations of aerobes and anaerobes are generally elevated respectively 100- and 1000- fold compared to levels in normal microflora (261).

III.3.2. Epidemiology

Bacterial vaginosis is the most prevalent vaginal disorder in adult women worldwide (62). It is present in 10% to 20% of white, non-Hispanic women and 30% to 50% of African American women, and it has been found in up to 85% of female sexual workers studied in Africa (62). Additional epidemiologic studies reveal that the prevalence depends on the subject population. Accordingly, 5% to 26% of pregnant women worldwide (86) and 24% to 37% of women attending STD clinics (92, 249) have been found to be bacterial vaginosis positive. Even higher prevalence rates have been found in lesbian women (24%–51%) (163). A recent study in young women entering the military in the United States found an overall prevalence of 27% (311). Most studies in North America have indicated that prevalence is significantly increased in African American women compared with non-Hispanic white women. Even when controlling for a variety of behavioral risk factors, race was independently associated with bacterial vaginosis, intermediate microflora, and a lack of hydrogen peroxide positive lactobacilli (11, 189), correlating with the presence of bacterial vaginosis-associated anaerobic and facultative aerobic microflora (189). These epidemiologic studies also reveal that bacterial vaginosis is less likely to occur in women who have used oral contraceptives and condoms consistently. Over the last 2 decades, most epidemiologic studies have been fairly consistent in identifying additional risk-associated factors (62, 189, 249). These factors include numbers of sexual partners in the prior 12 months, douching, smoking, and low socio-economic conditions (189).

III.3.3. Pathophysiology

The pathogenesis of bacterial vaginosis remains poorly understood. Bacterial vaginosis has no known single causative agent – although *G. vaginalis* is present in up to 95% of cases (112). Rather, it is a complex polymicrobial disorder characterized by decreased lactobacilli and

increased colonization by several facultative or strictly anaerobic microorganisms (62, 275). The role of *G. vaginalis* in bacterial vaginosis became less clear as investigators began to report a 30 to 40% prevalence of *G. vaginalis* among normal women. In addition, bacteria other than *G.* were positively associated with vaginosis (250), and evidence of a possible endogenous source of bacteria due to anal–vaginal transfer emerged (122).

The actual cause of bacterial vaginosis is unknown. Is decline of hydrogen-producing lactobacilli the initial event? And why do they decline? Alternatively, *Lactobacillus* loss may be secondary to the massive overgrowth of bacterial vaginosis associated bacteria. Little progress has been made in understanding the sequence of pathologic events (Figure 1).

Figure 1: The inter-relation between lactobacilli, vaginal pH, and bacterial overgrowth in the etiology of bacterial vaginosis.
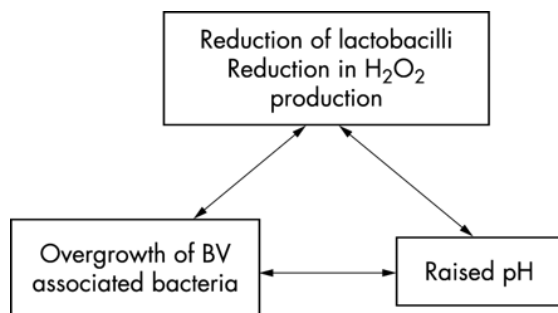
III.3.3.1. Hydrogen peroxide production

The presence of hydrogen peroxide producing strains of lactobacilli has been positively associated with being white, being aged over 20 years, using barrier contraception, and with low frequency of bacterial vaginosis and gonorrhoea (8). A cohort study showed that lack of hydrogen peroxide producing lactobacilli gave a twofold risk of acquiring bacterial vaginosis and that the absence of lactobacilli gave a fourfold risk (101). Klebanoff *et al.* showed *in vitro* that combining myeloperoxidases with hydrogen peroxide and a halide produced a potent oxidant, which was toxic to bacterial vaginosis associated bacteria (144). Myeloperoxidase activity has been found in vaginal fluid and cervical mucus, and chloride is present in cervical mucus in amounts in excess of that required for this system. *In vitro* testing showed that hydrogen peroxide producing lactobacilli in high concentration (but compatible with the levels found in the vagina) were toxic to *G. vaginalis* and *Prevotella bivia*. This toxicity was inhibited by catalase, indicating that hydrogen peroxide was the toxic agent. Another indication supports hydrogen peroxide as the toxic agent: when the concentration of hydrogen peroxide producing lactobacilli was lowered so that growth of other bacteria was no longer inhibited, the addition of myeloperoxidase and chloride reinstated the toxicity of the hydrogen peroxide producing lactobacilli through the production of HOCl from H_2O_2 (144). The toxic effect of this hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system was rapid, with a 3-log reduction in numbers of *G. vaginalis* at 15 minutes and complete loss of viability at 60 minutes.

III.3.3.2. Change in pH

The low pH of the vagina is attributable to the production of lactic acid by vaginal lactobacilli, and by the conversion of glycogen to lactic acid by estrogenized vaginal epithelial cells (28). Since bacterial cells produce both D- and L-lactate, whereas human cells produce only L-lactate, Boskey *et al.* (28) measured the relative amounts of D- and L-isoforms of lactate in vaginal secretions and found that vaginal bacteria produce about 80% of the lactic acid present in the vagina, *In vitro* lactobacilli acidify their growth medium to a pH of 3.2–4.8 (that is, similar to normal vaginal pH) (29). At that pH a steady state of equilibrium develops where the acidity becomes autoinhibitory. Anaerobes grow poorly at pH 4.5 or less: the optimum pH for *Prevotella* spp. and *G. vaginalis* growth is 6–7. *In vitro* studies show that the concentrations of these bacteria increase with increasing pH, and that both *G. vaginalis* and *Prevotella* spp. are susceptible to low pH (29). McClean and McGroarty (173) found that lactic acid and low pH had a greater inhibitory effect on *G. vaginalis* than hydrogen peroxide. The *in vitro* experiments by Klebanoff *et al.* (144) showed that the hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system had maximum toxicity at a pH of between 5 and 6. This suggests that pH has an additional effect to the hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system. At pH 4.5, the growth of *G. vaginalis* was inhibited, and the addition of hydrogen peroxide producing lactobacilli produced no extra inhibition. The hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system did have some additional inhibitory effect on growth, but less so than at pH 5–6 when there was a highly significant reduction in *G. vaginalis* (144). One interpretation of these findings is that at pH 4.5 or less the hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system is less important as the low pH has itself an inhibitory effect on bacterial growth. However, at times of a rise in vaginal pH, such as after intercourse and during menses, when bacterial overgrowth could occur, the hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system rapidly kicks in to inhibit bacterial growth. The rate of acid production in the vagina has not been directly observed, but Masters *et al.* (166) demonstrated that the alkaline buffering action of the ejaculate abolishes vaginal acidity for several hours after intercourse and that the reacidification rate of the vagina after intercourse is about 0.5 pH units per hour.

A low pH also appears to be important for *Lactobacillus* adherence to the epithelial cells. *In vitro* testing showed a mean of 5.5 *Lactobacillus* cells adhered per vaginal cell at pH of 4.4, compared with 1.4 bacterial cells at a pH of 6.2 (7).

III.3.3.3. Overgrowth of bacterial vaginosis associated organisms

The massive overgrowth of vaginal anaerobes is associated with increased production of proteolytic carboxylase enzymes, which act to break down vaginal peptides to a variety of amines, especially trimethylamine, which, in high pH, become volatile and malodorous. The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating the typical discharge (211). In conditions of elevated pH, *G. vaginalis* more efficiently adheres to the exfoliating epithelial cells, creating clue cells (204, 255). Amines further provide a suitable substrate for *M. hominis* growth (248).

III.3.3.4. Reduction in lactobacilli

It is not known what causes the reduction in hydrogen peroxide producing lactobacilli in bacterial vaginosis. Pavlova *et al.* (202) suggest that as bacterial vaginosis associated organisms are sensitive to lactic acid and hydrogen peroxide, suppression of lactobacilli must come before overgrowth of bacterial vaginosis associated bacteria. Phage mediated lysis of lactobacilli may cause such a reduction. These authors demonstrated that phages from one woman can infect lactobacilli from a different woman suggesting that phages could be the sexually transmitted agent (24, 202), which would explain the lack of benefit of treatment of the male partner with antibiotics (95, 179).

However, some studies suggest that hydrogen peroxide producing lactobacilli may not totally protect against bacterial vaginosis. A study in pregnant women revealed that 63% with bacterial vaginosis had hydrogen peroxide producing lactobacilli isolated from the vagina, yet despite these they still had bacterial vaginosis (227). These authors suggest that other bacteria start to appear and increase before disappearance of hydrogen peroxide producing lactobacilli, and that bacterial vaginosis may develop in some women despite the presence of hydrogen peroxide producing lactobacilli.

The initial work by Gardner and Dukes (81) showed that bacterial vaginosis can be induced by inoculating vaginal secretions from women with bacterial vaginosis into a healthy vagina. *G. vaginalis* alone caused bacterial vaginosis in only one of 13 women, but when vaginal secretions from women with bacterial vaginosis were inoculated, 11 of 15 women developed bacterial vaginosis. This suggests that the interrelation between the different groups of bacteria is important for overgrowth. *P. bivia* and *G. vaginalis* have a symbiotic association since growth of *G. vaginalis* is enhanced by ammonia that is produced by *P. bivia* and since amino acids are produced during *G. vaginalis* growth, which are stimulatory to the growth of *P. bivia* (210).

A microbiological study throughout the menstrual cycle showed that in women with or without bacterial vaginosis the rate of recovery of lactobacilli increased over the cycle and the concentration of non-*Lactobacillus* species was higher at menses, suggesting instability of the vaginal microflora at that time with the potential for bacterial overgrowth (64).

III.3.4. Clinical features

Symptomatic bacterial vaginosis, which accounts for approximately 60% of all cases (143), typically causes an unpleasant, 'fishy-smelling', white, thin and homogeneous discharge. The increased vaginal discharge is a consequence of degradation of the cervicovaginal mucus by bacterial sialidases and prolidases and the odour is a consequence of the production of volatile amines (like triethylamine) by the metabolism of the anaerobic bacteria (162).

III.3.5. Complications

Bacterial vaginosis is associated with serious sequelae related to the upper genital tract, increasing the risk of preterm delivery (87, 113, 118, 175), first trimester miscarriage in women undergoing *in vitro* fertilization (213), amniotic fluid infections (246), chorioamnionitis (117), postpartum and postabortal endometritis (298), and postabortal pelvic inflammatory disease (PID) (153). In non-pregnant women (266), bacterial vaginosis increases the risk of posthysterectomy infections (152, 260) and PID (113, 304), and risk of acquiring *Neisseria gonorrhoeae* (101, 165, 305). Bacterial vaginosis itself may cause endocervical inflammation that manifests as mucopurulent cervicitis (240). Finally, among women with HIV infection, the quantity of HIV shed in vaginal secretions from women with bacterial vaginosis is increased nearly sixfold relative to women without bacterial vaginosis (54) and high vaginal pH (> 5.5) is associated with nearly twice the quantity of HIV shed (241). Bacterial vaginosis may also enhance women's likelihood of sexual acquisition of HIV (165, 230, 268, 299).

III.3.6. Diagnosis

It should be noted that the diagnosis of bacterial vaginosis differs from that of most infectious diseases. Most types of infectious disease are diagnosed by culture, by showing the presence of antigens or RNA/DNA from the microbe, or by serodiagnosis to determine the presence of antibodies to the microbe. This is not the case for bacterial vaginosis, since the ultimate cause of the disease is not yet known. Nevertheless, the abundant literature, addressing bacterial vaginosis, has largely focused on a few microorganisms, which are thought to play a pivotal role in the pathology of bacterial vaginosis.

III.3.6.1. Clinical diagnosis by Amsel criteria

In clinical practice, the most widely used method for rapid diagnosis of bacterial vaginosis is based on the Amsel criteria (5), which entail observation of vaginal discharge, performance of an amine test, pH measurement, and a wet mount microscopy. A positive diagnosis requires that the patient satisfies three of the following criteria: (*a*) an adherent grayish-white vaginal discharge, (*b*) a positive sniff/whiff test on addition of 10% potassium hydroxide to the vaginal discharge, (*c*) an elevated vaginal pH greater than 4.5, and (*d*) the presence of clue cells on microscopy (Figure 2).

Figure 2, (250). High-power micrograph of a clue cell. Clue cells are large, asymmetrical, mature vaginal epithelial cells with a small nucleus. In cases where a large number of bacteria adhere to the cell surface it is difficult to distinguish the edge of the epithelial cell and the epithelial cells become obscure. Some of the older literature states that there must be at least 20% clue cells to fulfill the clue cell criterion, but this is not mentioned in the original Amsel definition (5).

These clinical criteria are imprecise for various reasons: recent intercourse, douching, menstruation, or contamination by cervical mucus can affect vaginal pH (167). Additionally, the inherent difficulty is that these criteria with exception of the pH, are subjective (appearance of discharge, whiff test) or are potentially technically difficult (appearance of clue cells in the saline preparation of vaginal fluid examined by microscopy).

Often diagnosis of bacterial vaginosis is made using variants of the Amsel criteria. Since the characteristic appearance of the discharge is the most subjective criterion, this property is sometimes skipped. A bacterial vaginosis diagnosis will then require that all three other criteria are satisfied. The Center for Disease Control (CDC) in the United States suggested a few years back that it would suffice to use the pH and whiff test criteria (43), i.e. two of two should be satisfied, but this modification has never been fully validated. There are even

studies where only one of the Amsel criteria, the presence of clue cells in microscopic examinations, was used (151, 206).

Although several reports pointed out that the use of the Amsel criteria is responsible for underdiagnosis of bacterial vaginosis (236), others recommend that the Amsel criteria should be the fundamental method for practicing clinical diagnostics of bacterial vaginosis since no other well-validated point-of-care methods have been published (75).

III.3.6.2. Commercial point-of-care tests

Several rapid point-of-care diagnostic tests for bacterial vaginosis were developed, although none of these tests is being widely used.

III.3.6.2.1. pH measurement

Self-test pH glove

In this screening procedure one finger of a medical examination glove has an attached pH indicator paper, which the patient can introduce into her vagina and then read the measured result. If the pH level is high, it is recommended that the patient consults a physician for further evaluation. The glove is reported to be useful in studies from German populations (126). More data should be collected from other populations to determine the glove's efficacy as a general screening test.

III.3.6.2.2. Presence of trimethylamine

Electronic sensor array: 'electronic nose'

The idea of using sensor arrays coupled with software interpretation of the resulting signals ('electronic nose') for the diagnosis of bacterial vaginosis is based on the assumption that the signal pattern thus detected might be an electronic counterpart to the human sensory sensation of smell and has attracted some attention. The results so far are generally disappointing (44, 103).

III.3.6.2.3. pH measurement and trimethylamine presence

This point-of-care test, FemExam® test card (Cooper Surgical, Shelton, Connecticut), is based on determining pH and trimethylamine levels in vaginal fluid for the diagnosis of bacterial vaginosis, however, the few published studies are inconclusive as to the usefulness of the test (209, 217).

III.3.6.2.4. Sialidase activity

Detection of sialidase activity from the dominant mixed anaerobic microflora in bacterial vaginosis has been proposed as a point-of-care test and validated against Amsel's clinical

criteria and Nugent scores (see III.3.5.3.1) in otherwise healthy women of childbearing age (70, 184). Acceptable-to-good results for specificity, sensitivity and predictive values were reported. In combination with pH measurement, this method is the point-of-care test of greatest value (253).

III.3.6.2.5. Proline aminopeptidase activity
Promising results in detecting proline aminopeptidase activity of anaerobes, especially *G. vaginalis* in vaginal discharge for the diagnosis of bacterial vaginosis were reported with respect to a possible point-of-care test (Pip Activity TestCard™, Litmus Concepts, Inc., Santa Clara, Ca) (235, 303).

III.3.6.2.6. DNA probe for *G. vaginalis* rRNA
The Affirm™ VP III *G. vaginalis* DNA hybridization assay (Becton Dickinson, Sparks, MD, USA) is a DNA hybridization test that is positive only for concentrations of *G. vaginalis* in excess of 2×10^5 bacterial cells per ml of vaginal fluid (31) and therefore should be positive most often in women with bacterial vaginosis and rarely in women with normal vaginal microflora. The Affirm system also can detect the presence of *Candida* spp. and *T. vaginalis* in the same specimen, making it quite attractive as a tool for evaluating women with vaginal discharge. The procedure requires between 30 and 45 minutes to complete. The test could be done in an office setting but this is not very efficient and it is usually better done in a laboratory setting. Nonetheless, the rapid turn-around time permits return of results to clinicians within 24 hours.

Promising results have been reported with this approach in two populations, one of pregnant women and one of otherwise healthy women of childbearing age (31, 217). Both studies concluded that the probe can be used as a supplement to the Amsel en Nugent methods (see 3.5.3.1).

III.3.6.3. Microscopy

III.3.6.3.1. Scoring of Gram stained vaginal smears according to Nugent
The Amsel criteria method has been greatly favored since the diagnosis can be carried out in the clinic. The examination of Gram-stained specimens of vaginal secretions for diagnosis, research and classification of vaginal pathology has a long and sometimes confusing history (23). Spiegel's method (Figure 3), introduced in 1983, was the first diagnostic method on the basis of Gram-stained vaginal smears that has been formally compared with clinical, microbiological, and biochemical criteria (262).
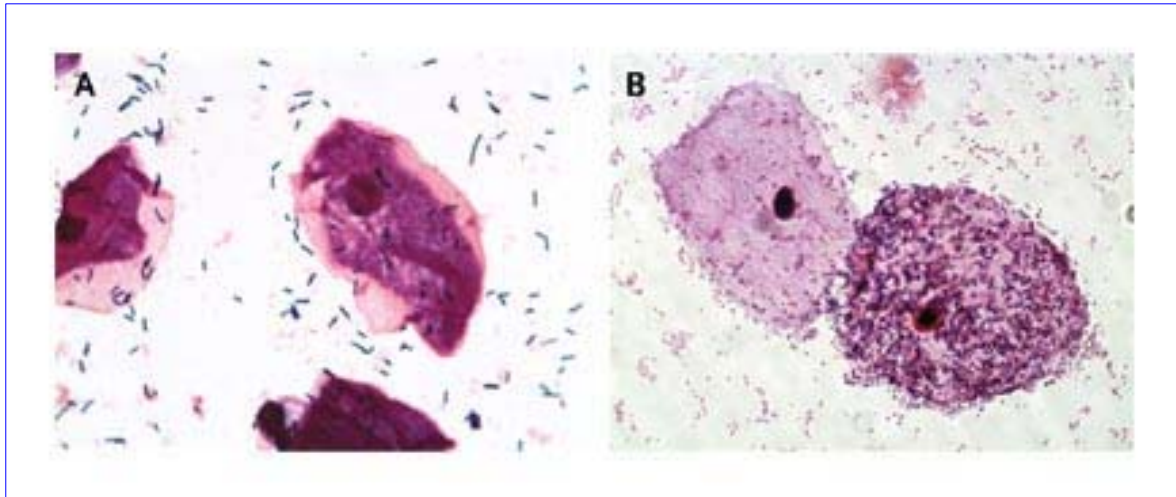
Figure 3. Gram stain of a vaginal fluid smear from a woman with *Lactobacillus* predominant microflora (Panel A) and from a woman with bacterial vaginosis (Panel B). The vaginal squamous cell on the right in Panel B is a clue cell (111).

The bacteria are grouped into 'morphotypes': elongated bacteria are called *Lactobacillus* 'morphotypes' and small, Gram-variable coccobacteria are called *Gardnerella* 'morphotypes'. Nugent's scoring system is a further development of Spiegel's method and includes validation of the categories of observable bacterial cell types (191) (Table 1). This requires an experienced clinical diagnostician.

Table 1: The Nugent scoring system. If more than 30 lactobacilli are observed in the visual field, the score is 0 points; if no lactobacilli are observed, the score will be 4 points. If no *Gardnerella*-like bacteria are observed, the score is 0 points; if more than 30 are observed, the score will be 4 points. The presence of curved rods – *Mobiluncus* – can add 2 points. All the points are added together to obtain the final score: 0–3 points indicates normal lactobacillar microflora (grade I), 4–6 intermediate microflora (grade II), and 7 or higher results in a diagnosis of bacterial vaginosis (grade III).

Points	Number of *Lactobacillus* 'morphotypes' per field	Number of *Gardnerella*/ *Bacteroides* 'morphotypes' per field	Number of Curved bacteria (*Mobiluncus*) per field
0	>30	0	0
1	5-30	<1	1-5
2	1-4	1-4	>5
3	<1	5-30	
4	0	>30	

Several studies compared the sensitivity and specificity of Amsel and Nugent criteria for diagnosis of bacterial vaginosis. Schwebke *et al*. (236) reported the sensitivity and specificity of Amsel criteria compared to Nugent score to be 70% and 94%, respectively, in a cohort of

nonpregnant women. However, in two pregnant cohorts the sensitivity of Amsel criteria compared to Gram stain was only 35% and 46% (89, 269). In a cohort of women with HIV infection, the sensitivity and specificity of Amsel criteria compared to Nugent were 37% and 99%, respectively (242).

Although the specificity of the Amsel criteria compared to Nugent score is repeatedly found to be high, a large variation in sensitivity was observed. While variability in interpretation by the clinician might be an important factor, the possibility that the sensitivity of the Amsel criteria is different in women with HIV infection and/or in pregnant women, cannot be excluded.

III.3.6.3.2. Scoring of Gram stained vaginal smears according to Hay/Ison

The Hay/Ison system (105) is based on the observation of Gram stains to estimate the ratios of the observed cellular types rather than the exact number of the bacteria. Originally the observations were divided into three categories – normal, intermediate or bacterial vaginosis – but in order to obtain a more precise classification, two additional categories have been introduced as compared to Nugent scoring (131). The new groups define those preparations that contain no bacteria at all (grade 0) and those that contain large amounts of Gram-positive cocci, such as *Streptococcus* or *Staphylococcus*. These 'morphotypes' had previously been included in the intermediate microflora classification but make up a class of their own in this scoring system (grade IV).

III.3.6.3.3. Wet smear criteria

In this scoring system, based on phase-contrast microscopy of wet mounts, the number of lactobacillary 'morphotypes' is weighed against the number of small bacterial 'morphotypes' regarded as typical of bacterial vaginosis (57, 151, 232). Wet smear interpretation of vaginal fluid resembles Nugent scoring in that it ranks the quantities of lactobacilli and cocci in the same way (Table 2). These criteria are simple to perform and interpret, and can be used as a point-of-care test. The wet smear criteria of Schmidt have been validated for diagnosis of bacterial vaginosis in primary care populations (231).

Score	Number of Lactobacillus 'morphotypes' per field*	Number of small bacterial 'morphotypes' per field*
0	>30	0
1	16-30	1-5
2	6-15	6-15
3	1-5	16-30
4	0	>30

Table 2: Scoring system for evaluation of bacterial 'morphotypes' in wet-mount preparations.

Bacterial 'morphotype' score (BMS) = lactobacillary 'morphotype' score + small bacterial 'morphotype' score. A bacterial 'morphotype' score of 7–8 is indicative of bacterial vaginosis.

* Number of morphotypes is the average number observed at x400 magnification by phase-contrast microscopy of wet mounts.

III.3.6.4. Conclusion

An international study of the interobserver variation between interpretations of Nugent scores, Ison/Hay scores and wet smear scores showed that the robustness of the Gram staining criteria of Hay & Ison and the wet mount criteria of Schmidt was comparable to that of the criteria of Nugent and that it makes no difference which of the criteria are used (76).

Although the diagnosis of bacterial vaginosis by Amsel criteria is simple, it is relatively insensitive. Nevertheless, the Amsel criteria remain recommended as standard method for clinical diagnosis of bacterial vaginosis (75).

III.3.7. Treatment

The current treatment strategy focuses on eradication of the organisms responsible for the abnormal overgrowth (Figure 4).

The 2002 CDC recommendations for treatment of bacterial vaginosis include metronidazole, 500 mg orally twice a day for 7 days; metronidazole gel, 0.75% intravaginally daily for 5 days; or clindamycin cream, 2% intravaginally for 7 days (43). Alternative therapy includes metronidazole, 2 g single dose orally, or clindamycin, 300 mg orally twice a day for 7 days (43). Clinical cure rates vary from 70% to 90% at 21 to 28 days after therapy (104, 136). There seems to be equal effectiveness of the oral and topical routes of therapy, and nitroimidazole and clindamycin regimens appear to be equally efficacious. The main advantage of the topical regimen is reduced gastrointestinal symptomatology, however vaginal therapy is more inconvenient and is associated with a high risk for vaginal candidiasis (10%–30%), even though 3-day suppositories of clindamycin are now available. Tinidazole, a second-generation nitroimidazole, recently approved for use in trichomoniasis, can be used for bacterial vaginosis but is not yet approved for this indication. Several studies have

indicated at least equivalent therapeutic efficacy compared with metronidazole and clindamycin, with some evidence of possible superior activity (19, 176). However, additional studies will be required to support this claim.

The most important complication of bacterial vaginosis is undoubtedly recurrence of the clinical syndrome. While short-term response to standard treatment regimens is acceptable, symptomatic bacterial vaginosis persists or recurs in 11 to 29% of women at one month and in 50 to 70% by three months (27, 97, 116, 159, 254). Long-term recurrence rates may approach 80% (62). All the aforementioned regimens are associated with a high recurrence rate. The cause of recurrent bacterial vaginosis is largely unknown. Theories include i) failure of vaginal acidification - although strategies aimed at vaginal acidification have not reduced the recurrence rate (162), ii) failure to reduce numbers of *G. vaginalis* and anaerobic microflora, iii) failure to recolonize the vagina with protective *Lactobacillus* spp., iv) persistence of the initial unrecognized pathogen, v) reinfection or recolonization with the unrecognized pathogen or vi) antimicrobial resistance of overgrowing microorganisms. Regarding the latter hypothesis, clindamycin resistance has been seen in vaginal anaerobic microflora, although Beigi *et al.* (20) showed recently that less than one percent of the cultivable vaginal anaerobic bacteria is resistant to metronidazole. With regard to reinfection, most studies have failed to show any benefit in the treatment of male partners of women who have recurrent bacterial vaginosis (50, 95, 179).

Management of recurrent bacterial vaginosis is controversial and largely unexplored. Most practitioners repeat therapy, avoiding short-course regimen and possibly switching from one class of antimicrobials to another (19).

Based on the hypothesis that loss of protective lactobacilli precedes the overgrowth of anaerobic vaginal organisms, a long-term solution to treatment of bacterial vaginosis contains restoration of the healthy protective lactobacillus-dominant microflora (Figure 4).

Efforts to artificially restore an unbalanced microflora with the use of probiotics have met with mixed results but research aimed at selecting *Lactobacillus* strains on a scientific basis could well provide a reliable alternative treatment and preventive regimen in the future (9).

Figure 4: The therapeutic options in the prevention of recurrent bacterial vaginosis.
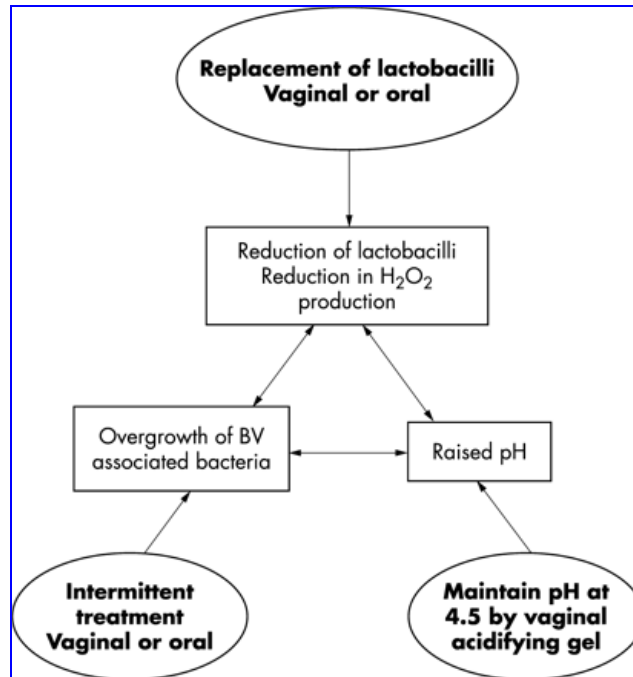
III.4. Microbial analysis of the vaginal microflora

The establishment of standard scoring criteria to define bacterial vaginosis (as described in the previous section) and research to classify vaginal microflora were conducted simultaneous.

Until 2004, essentially all our knowledge of the vaginal microflora has been obtained from isolating organisms by culture and subsequently identifying them by phenotypic means. This approach is still the mainstay for studies on the human vaginal ecosystem. However, cultivation of microbes as a means to characterize microbial communities in a natural ecosystem has major shortcomings, as it is recognized that many microbes in different ecosystems cannot be cultivated by standard culture techniques (296). Moreover, isolation techniques used prior to 1970 resulted in a gross underestimation of the importance of anaerobic bacteria as major constituents of the normal microflora of the female genital tract (150). Failure to use appropriate transport systems as well as failure to use optimal media and anaerobic culture techniques have compromised the results of many studies with regard to the delineation of the bacterial constituents present. Despite the limitations, culture techniques are very powerful and absolutely essential to obtain a complete picture of the diversity and role of the vaginal microbial ecosystem. However, to study such a complex ecosystem, it has been advocated that the combination of both culture and PCR-based non-culture techniques is required (200).

III.4.1. Culture

The essence of culturing techniques involves plating out fresh vaginal material on either selective or non-selective media and incubating at 37°C under aerobic or anaerobic conditions.

III.4.1.1. Non-selective culture methods

Non-selective media are generally used to estimate total numbers of both aerobic and anaerobic organisms. Several non-selective media have been used for vaginal cultures. Facultative anaerobic organisms are usually isolated with tryptic soy base–5% sheep blood agar, MacConkey's agar, and mannitol-salt agar. A medium frequently used for recovery of obligate anaerobes is BMB (prereduced Brucella base blood agar containing 5% sheep blood, hemin, and menadione). Fastidious organisms are often isolated on chocolate agar.

It should be emphasized that while these media contain no known selective power, they do inherently select against some bacteria from the human vagina. Specifically, bacteria that require extra requirements and also microbes that could grow on the media but may be in a physiological state which may not be conducive to culturing directly from vaginal material

will be missed (18, 138). For example, it is possible that some bacteria depend on specific factors present in the vaginal fluid, factors which are diluted too far in the second and third isolation zones on a solid culture medium to support growth of these bacteria.

III.4.1.2. Semi-selective culture methods

Analysis of complex and variable biological specimens, such as the vaginal microflora, requires an accurate means of discrimination between species and even genera. Enumeration of specific bacterial genera is generally achieved by plating onto selective or semi-selective media.

Lactobacillus species are the most numerically dominant bacteria in a normal vaginal microflora and therefore can be isolated without selective agents added to the media. However, lactobacilli are commonly specifically cultivated from vaginal samples using selective media. A number of media have been developed for this purpose (49), including Rogosa agar (224), de Man, Rogosa and Sharpe medium (MRS) (56), LBS (*Lactobacillus* selection agar, (177)) and LAMVAB (*Lactobacillus* anaerobic MRS agar with vancomycin and bromocresol green, (99)). MRS and Rogosa are the most commonly used semi-selective agars. The high acetate concentration and low pH of these agars theoretically permit growth of lactobacilli but suppress many other strains. These media support the growth of lactobacilli but are not absolutely selective, permitting the growth of other lactic acid bacteria (e.g. *Pediococcus* and *Leuconostoc*) or other genera (e.g. *Bifidobacterium* (132)).

In 1982 human bilayer Tween (HBT) agar was developed for the isolation of *Gardnerella* (*Haemophilus*) *vaginalis* (277). This medium also supports the growth of the more fastidious strains of *Lactobacillus*, such as *L. iners*, but *Lactobacillus* spp. can be differentiated from *G. vaginalis* by the lack of hemolysis.

A7 medium is used for the isolation of *Mycoplasma* and *Ureaplasma* species (Northeast Laboratory, Waterville, Maine).

Trichomonas vaginalis organisms are detected with Diamond's medium (PML Microbiologicals).

All these selective media are valuable tools for analysis of the ecology of the vaginal microflora. However, they all have the inherent disadvantages of not absolute selectivity and of toxicity against certain strains within the genus, resulting in false positive and false negative results. In addition, all culture media fail to cultivate organisms that are in a physiological state that is not conducive to growth, often termed a 'non-culturable' state. These findings strongly illustrate the potential errors where culture methods alone are used.

For example, before 1991, *L. iners*, a *Lactobacillus* species unable to grow on MRS and Rogosa agar and now recognized as being present in more than 60% of women (11, 77), was not reported as a member of the vaginal microflora because in earlier culture based studies MRS and Rogosa agar were used almost exclusively to study the vaginal lactobacilli.

III.4.1.3. Phenotypic identification of cultured microorganisms

Bacterial culture, followed by enumeration of colonies of specific morphology, is a technique commonly used to determine genus or species diversity in complex biological samples. Whereas clinical samples usually require additional biochemical and serological testing, many experimental studies rely solely on colony morphology and microscopy for bacterial identification. This can compromise the accuracy of the study, firstly because colony description can be subjective and secondly because strain differences and minor changes in culture conditions can influence the physical appearance of colonies (170, 245). We noticed that in several cases, colonies with a very different morphology were identified as being the same species. This was especially the case for *L. crispatus*.

Upon isolation of colonies it is necessary to confirm the genus identity and also to further characterize to the species level. This characterization requires a battery of classical morphological and biochemical tests. The confidence level of the species identification will increase with the more tests that are carried out. Herein lies the greatest disadvantage of classical tools for identification of organisms, as even the most sophisticated array of tests can often lead to uncertainties in the classification of isolates. Moreover, biochemical identification of all cultivable species present in a complex microflora is laborious, making it impractical for large-scale identification of isolates.

An additional problem is the lack of biochemical identification schemes for the lactobacilli and anaerobes present in the vaginal microflora. For example, Boyd *et al.* (30) evaluated the API 50 CH carbohydrate fermentation identification system (bioMérieux, France) for the identification of 97 strains of commensal lactobacilli belonging to the species *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. iners* and *L. vaginalis*. Eighteen different phenotypic patterns were found for 18 *L. crispatus* isolates, 18 different patterns for 20 *L. gasseri* isolates, 15 different patterns for 20 *L. vaginalis* isolates and 11 different patterns for 19 *L. jensenii* isolates. Moreover, all of the *L. iners* isolates and three of the *L. vaginalis* isolates were nonreactive for all of the tests in the API 50 CH system. This system agreed with the species-level identification for none of the 7 reference strains and only 4 of 90 vaginal isolates identified using whole-chromosomal DNA probes. The system misidentified 33 of 97 isolates as either

L. acidophilus or *L. fermentum*. Of the *L. jensenii* and *L. gasseri* isolates, 59% were identified as *L. acidophilus*. The API database also identified 35% *L. vaginalis* isolates as *L. fermentum*. Over half of the 97 isolates yielded an uninterpretable or doubtful API profile. The authors concluded that phenotypic identification was not useful because of the high level of phenotypic variability observed among commensal lactobacilli and because the limited database available for these species. However, until recently this identification method has been used, for example, to study the relationship of vaginal lactobacilli to local and systemic immunity and to bacterial vaginosis (3).

III.4.1.4. DNA-based identification of cultured microorganisms.

DNA-based techniques have as an advantage that the genomic material of an organism is investigated in a direct way, independent of culture conditions, making the process of identification more reliable.

III.4.1.4.1. PCR-independent DNA-based identification of cultured organisms

DNA-DNA hybridization is a method in genetics to measure the degree of genetic similarity between two closely related species. The DNA from the two species to be compared is extracted, purified and cut into small pieces. The DNA double strand fragments are separated by heating into two single strands, which are allowed to anneal with the DNA pieces of the other species. The more similar the DNA, the more of the pieces will anneal and form a hybrid double strand. Strands with a high degree of similarity will bind more firmly, and the energy required to separate them is determined.

In 1987, Giorgi *et al.* (85) were the first to identify *L. gasseri, L. jensenii,* and *L. crispatus,* not *L. acidophilus,* as the predominant vaginal *Lactobacillus* species colonizing asymptomatic women, using DNA-DNA hybridization for vaginal lactobacilli cultured on Rogosa and MRS agar. In 1999, using the same methodology, Song *et al.* (259) identified *Lactobacillus* isolates from Japanese women and newborn infants and found that the predominating lactobacilli were *L. crispatus* and *L. gasseri* in the women's vaginas and the newborns' intestines and *L. gasseri* and *Lactobacillus fermentum* in the women's intestines. Also in 1999, Antonio *et al.* (8) performed a culture-based study with identification by DNA-DNA hybridization of vaginal specimens cultured on Rogosa and blood based agar. As such, this study was the first to identify *L. iners* as a member of the vaginal microbial community and to report the species specificity of H_2O_2-production by lactobacilli.

III.4.1.4.2. PCR-based identification methods of cultured organisms

Amplification can be performed by random (301), species specific or universal primers. After universal amplification, further analysis of the amplification product is needed to catalogue the unknown isolate, i.e. to assign it to an established group of strains (= taxon, usually a species). The maximum information is obtained by total sequence analysis of the fragment. However, this technique remains expensive and laborious, making it impractical and too slow for large-scale identification of isolates. Several other methods are applied to investigate fragments for sequence diversity, making the process of identification either cheaper, less laborious and/or faster. If the obtained amplification products of different species have different sizes, determination of the fragment lengths leads to identification (133, 302). If the fragment has always the same length but the sequence is variable, the sequence polymorphism can be revealed by either hybridization based techniques, sequence dependent electrophoresis or restriction digestion. Sequence dependent systems are single-stranded conformational polymorphism (SSCP) (180), denaturing gradient gel electrophoresis (DGGE) (183), temperature gradient gel electrophoresis (TGGE) (107) and temporal temperature gradient gel electrophoresis (TTGE) (285).

Since mutations can lead to the appearance or disappearance of restriction sites, changing the restriction pattern, restriction digestion can also be used to reveal sequence polymorphism (e.g. ARDRA (284), AFLP (294)).

Recently, some PCR-based methods for the identification of cultured *Lactobacillus* species, including species belonging to the highly related *L. acidophilus* group, were described.

Kullen (147) and Tarnberg (272) used sequencing of the V1-V3 region of the 16S rRNA gene for accurate identification of bacteria in the *L. acidophilus* group. Gancheva *et al.* (79) demonstrated that RAPD and AFLP generated patterns could differentiate all species belonging to this group. A recent study has shown that it is also possible to distinguish vaginal lactobacilli by using two other molecular genetic methods: TTGE and multiplex-PCR (286). Another identification method comprises amplification of the V2-V3 region of the 16S rDNA followed by denaturing gradient gel electrophoresis (DGGE) in order to separate the fragments with different sequences. (e.g. (273, 295)).

We opted to use a technique that not only allows to unambiguously identify *Lactobacillus* species but also most cultivable species present in the vaginal microflora.

tRNA-intergenic spacer length polymorphism PCR (tDNA-PCR) allows rapid, discriminative and low cost identification of most cultivable bacteria except *Mycobacterium* spp., *Corynebacterium* spp. and *Neisseria* spp. (283, 302).

Baele *et al.* (15) applied tDNA-PCR using universal primers, followed by separation of the PCR products with capillary electrophoresis, in order to evaluate the discriminatory power of this technique within the genus *Lactobacillus* and were able to discriminate 21 of 37 tested species. Although not all species belonging to the *L. acidophilus*-group showed distinguishable patterns, the most frequently occurring vaginal lactobacilli, i.e. *L. crispatus*, *L. gasseri* and *L. jensenii*, could be identified unambiguously.

III.4.1.4.3. Principle of tRNA intergenic polymorphism length analysis (tDNA-PCR)
tDNA-PCR makes use of a single pair of universal primers – i.e. primers complementary to conserved regions in the tRNA-genes of most bacterial groups – that are directed outwardly to amplify the spacers in between the tRNA-genes (Figure 5). The tRNA intergenic spacers are multiple per genome and have different lengths, such that amplification of the spacers from a bacterial genome results in a DNA-fingerprint consisting of multiple DNA-fragments immediately after amplification and electrophoresis (302). The obtained patterns are highly similar for the different strains within the same species, but differ between species.
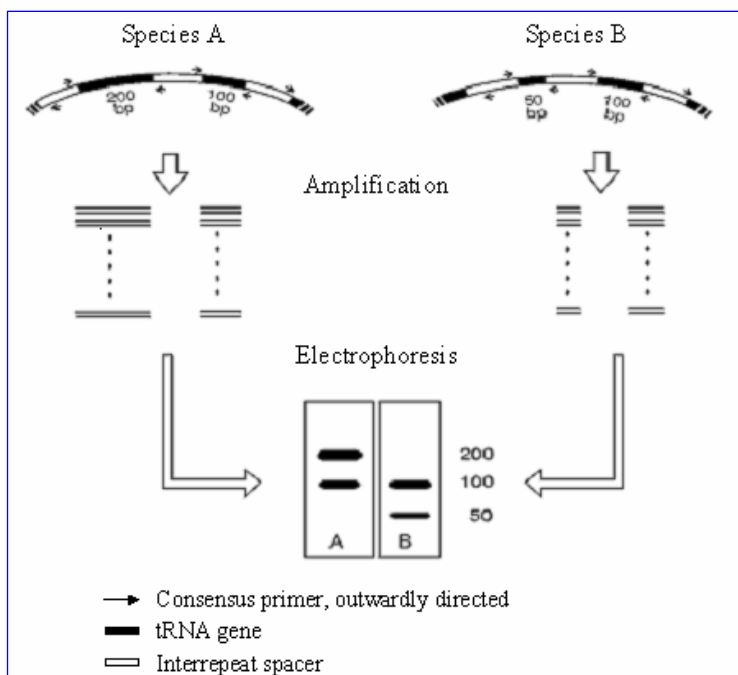
Figure 5: The principle of tRNA intergenic polymorphism length analysis (tDNA-PCR).

As such, amplification of tRNA-intergenic spacers, combined with high resolution automated electrophoresis, as is possible on e.g. the ABI310 capillary electrophoresis system, makes it possible to construct a library with tDNA-PCR fingerprints of reference strains. Identification of unknown cultured strains can then be obtained by comparing the tDNA-fingerprint of the unknown with those present in the library. Special software has been developed to do so with a minimum of hands on time (14). We have developed such a library, covering most human

bacterial species and several veterinary and environmental species. For most of the cultivable vaginal species, we have determined the corresponding tDNA-PCR patterns (288, 289).

III.4.2. DNA-based culture-independent methods

The identification methods described in the previous section rely on the ability to isolate and cultivate bacteria. Because these culture-dependent approaches are tedious and very laborious their use for the analysis of large numbers of samples is impractical and costly. Moreover, such methods are further limited due to the reliance on selective media, and because many bacterial species are refractory to cultivation (4), 292). Consequently, the obtained isolates may not always truly reflect the microbial composition of the sample and therefore, since the early 1990s culture-independent methods are being developed for the analysis of complex microbial communities (287).

III.4.2.1. Advantages of the molecular microbiologic approach

PCR-based methods, such as 16S rDNA amplification, offer several advantages. First, PCR can be performed directly on samples without cultivation, avoiding cultivation biases. Many microbes probably fail to replicate within the limited boundaries of temperature, carbon dioxide, oxygen, pH, and metabolic substrate concentrations that laboratory cultivation entails. The limitation of culture-based methods for the characterization of complex microflora has been described as 'the great plate count anomaly' (263) because only a small fraction of microorganisms that are present in a population can be cultured. In contrast, PCR can be performed on any microbe if nucleic acids are intact. Second, all bacteria possess 16S rRNA genes, and thus, all bacteria have the potential to be detected by amplification of this target. Bacterial 16S rRNA genes have regions of sequence similarity that can be used as templates for broad-range primers. These primers will amplify 16S rDNA from most bacterial species. Third, bacterial 16S rRNA genes have regions of sequence dissimilarity in between the broad-range PCR priming sites, allowing one to identify bacteria based on this sequence polymorphism. In general, unique bacterial species have unique 16S rDNA sequences in these variable regions, though exceptions occur such as identical 16S rDNA sequences among *Escherichia coli* and *Shigella* species. Knowing the collection of 16S rDNA sequences in a sample allows one to determine the community of bacteria that are present. Fourth, the 16S rRNA gene has characteristics of a molecular clock, with increasing dissimilarity between two sequences corresponding largely with increased evolutionary distance. The 16S rRNA gene is thus useful for establishing phylogenetic relationships among bacteria - cultured and uncultured.

The most startling result of the many microbial diversity studies that have employed 16S rRNA gene culture-independent methods is the richness of the uncultured microbial world. As of April 1, 2004, GenBank contained 21 466 16S rRNA gene sequences from cultured prokaryotes and 54 655 from uncultured prokaryotes, according to the search terms described by Rappe & Giovannoni (214), whereby many of those from uncultured organisms affiliate with phyla that contain no cultured members at all. When Woese (309) originally proposed a 16S rRNA-based phylogeny, 12 bacterial phyla were recognized, each with cultured representatives. Since then, 14 additional phyla with cultured representatives and 26 candidate phyla that have no known cultured representatives, have been identified (214). In summary, at present half of the known microbial phyla have no cultured representatives.

Table 3 shows an overview of the molecular techniques used for the characterization of the vaginal microflora, discussed further on.

Table 3. Overview of the molecular techniques used for the characterization of the vaginal microflora

Technique	Principle	Workload	Discriminatory Power	Reproducibility
Culture and PCR-independent				
FISH	In situ hybridisation using labeled probes	H	L	H
Multi Probe Hybridisation (checkerboard)	Hybridisation using a large set of labeled probes	H	M	H
PCR-based				
16S rRNA gene or *cpn60* gene cloning	Universal amplification of 16S rRNA genes or chaperonin-60 genes Separation by cloning in *E. coli* cells Sequencing of clones	H	H	H
16S rRNA gene DGGE	Universal amplification Separation based on sequence dependent electrophoretic differences	M	M	M
16S rRNA gene T-RFLP	Universal amplification Separation based on sequence differences in terminal restriction fragments	M	M	H
Species specific PCR	Species specific amplification	H	L	H

(L: Low, M: Moderate, H: High)

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III.4.2.2. PCR-independent and culture-independent techniques

Probing techniques are based on the hybridisation of synthetically prepared oligonucleotides to specific target sequences on bacterial DNA - as it is the case for PCR primers - but instead of being intended for amplification of DNA, they are linked to a radioactive or fluorescent label which enables the visual detection of the target after hybridisation under controlled conditions. The most frequently applied method using probes is Fluorescent in Situ Hybridisation (FISH) making use of fluorescence microscopy for the visualisation and quantification of fluorescently labeled bacteria (4). Recently, this technique was applied to study the bacterial community structure and the spatial organization of the microflora on the epithelial surfaces of vaginal biopsy specimens (267) and to confirm that newly recognized bacteria detected by PCR corresponded to specific bacterial cell types visible in vaginal fluid (77).

A method allowing for simultaneous detection of multiple organisms is the checkerboard method (257, 258). In this case, DNA of the complex microflora is extracted, spotted in separate lanes on a membrane and hybridized with multiple labeled whole genomic DNA probes. Recently, a checker-board for the simultaneous detection of *G. vaginalis*, *P. bivia*, *B. ureolyticus* and *M. curtisii* was evaluated and it was concluded that hybridization was significantly associated with Gram-stain results since more species were detected by DNA hybridization as the Nugent score progressed from a normal to a disturbed microflora (25). Another method that allows both quantitative and qualitative analysis of samples is flow cytometric analysis. Bacteria in a liquid sample or suspension are fluorescently labeled using one or more specific dyes or probes after which the labeled solution is run through a flow cytometer or cell sorter, determining the identity and quantity of bacteria. Flow cytometric analysis has been used for analysis of bacteria in clinical samples, such as blood, urine, and feces (2, 244, 247).

The main disadvantage of these probe-based methods is their high workload, preventing fast analysis. In addition, these techniques can detect only species for which DNA probes have been prepared. Thus, previously unknown species cannot be detected by this method. Also, the use of probes restricts the number of possible applications because of the limited number of bacteria targeted. An advantage is that hybridization occurs without amplification, overcoming biases in quantification imposed by PCR amplification procedures and thus providing quantitative data.

III.4.2.3. PCR-based culture-independent techniques

As the limitations of culture methods became clear many different PCR-based techniques to amplify genes of interest directly, and without a culture bias, from complex microbial samples were developed.

Some of the PCR-based methods applied for the identification of cultured isolates (as discussed in III.4.1.4.2) can also be applied to assess the bacterial composition of complex microflora. These tools produce a pattern or profile of nucleic acids amplified from a sample and that pattern reflects the microbial community diversity. When amplifying DNA directly from clinical samples amplification is mostly performed by universal primers. When dealing with a highly complex microflora (e. g. faecal microflora) one can opt to use genus or group specific primers to amplify only certain species of interest. Species-specific primers are mostly used to confirm results obtained by the amplification of universal primers or for diagnostic purposes.

After universal or genus/group specific amplification, analysis of the amplification product is needed to catalogue the unknown isolate to assign it to an established group of strains (= taxon, usually a species). If the fragment has always the same length but the sequence is variable differences can be revealed by either cloning and sequencing (233), by restriction or by sequence dependent electrophoresis – whereof DGGE ((183), Figure 6) is the most widely applied.
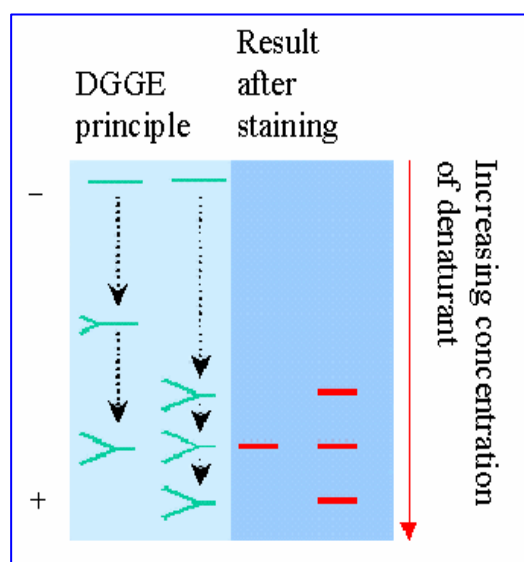
Figure 6: The separation principle of denaturing gradient gel *electrophoresis* (DGGE) is based on the melting (denaturation) properties of DNA in solution. DNA molecules melt in discrete segments called melting domains, when the denaturant concentration is raised. The melting temperature (Tm) of a melting domain depends on its nucleotide sequence. As DNA fragments are electrophoresed through a linear gradient of increasing denaturing concentration, the separation of double-stranded DNA into single-stranded segments increases. This causes the DNA segment to form a less uniform three-dimensional structure that moves through a polyacrylamide matrix at a reduced speed.

Several groups applied this technique for the characterization of the vaginal microflora (11, 33, 36, 71).

As for the identification of cultured isolates, sequence differences in PCR fragments obtained by universal or genus/group specific amplification can also be revealed by

restriction analysis (ARDRA). However, in order to simplify the obtained restriction patterns obtained from complex microflora, only one of the PCR primers is labeled resulting in the visualisation of only the terminal restriction fragment after capillary electrophoresis. Therefore, this technique has been named Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP, (158)). Only two research groups yet applied this technique to assess the composition of the vaginal microflora ((53), manuscript III and IV). The principle of the techniques used in this doctoral thesis, namely cloning of 16S rRNA genes (manuscript II and IV), T-RFLP (manuscript III and IV) and species specific PCR (manuscript II, III, V) are explained below.

III.4.2.3.1. Principle of cloning

The concept of cloning 16S rRNA-genes amplified directly from a sample was initially suggested by Pace (199) and first implemented by Schmidt *et al.* (233).

In general, DNA is extracted from samples and then PCR amplified to produce a mixture of 16S rRNA gene fragments from most species present. These are separated by ligation into vector plasmids and transformation of into *Escherichia coli* cells (cloning), whereafter the individual 16S rRNA clones are then sequenced and compared to a database with all published sequences present in the GenBank. The more clones per sample that are sequenced, the more the less abundant species can be retrieved. For example, assuming that the density of bacteria in the vaginal microflora is 10^8 bacteria per gram of vaginal fluid, characterization of 100 clones from a woman means that microorganisms present at concentrations of 10^6 or fewer colony-forming units per gram of vaginal fluid are unlikely to be detected by the cloning approach. Sequencing a large number of clones is however very costly and labor intensive, therefore some investigators opt to screen clones by ARDRA (284) prior to sequencing (77, 288). Recently, several cloning studies assessing the composition of the vaginal microflora were published (35, 77, 109, 130, 288, 313).

III.4.2.3.2. Principle of T-RFLP

This method, developed by Liu *et al.* in 1997 (141, 158, 164, 196) yields a community 'fingerprint' that reflects the kind and relative abundance of the numerically dominant species in a complex microflora and allows to investigate similarities and differences among microbial communities in a simplified manner.

T-RFLP fingerprints are obtained by PCR-based amplification of 16S rRNA genes, using universal bacterial primers of which one is fluorescently labelled and yielding a mixture

of amplicons of the same length with a fluorescent label at one end. After digestion with a restriction enzyme the obtained fragments of different sizes are separated by high-resolution gel electrophoresis on a capillary electrophoresis machine (e.g. ABI PRISM 310, Applied Biosystems, Foster City, Ca.). As such, only the fluorescent terminal restriction fragments will be visualized and a fingerprint based on fragment lengths is generated (Figure 7). When T-RFLP is carried out on a mixture of bacterial species, as e.g. present in the vagina, most species will be represented in the final fingerprint by a single fragment with a characteristic length, depending on the position of the first restriction site in the 16S rRNA gene. As such, the different peaks in a T-RFLP pattern reflect the bacterial diversity of the sample, and enable to identify most of the different species present by comparing the different fragment lengths of the T-RFLP pattern with a previously established reference library of T-RFLP patterns.
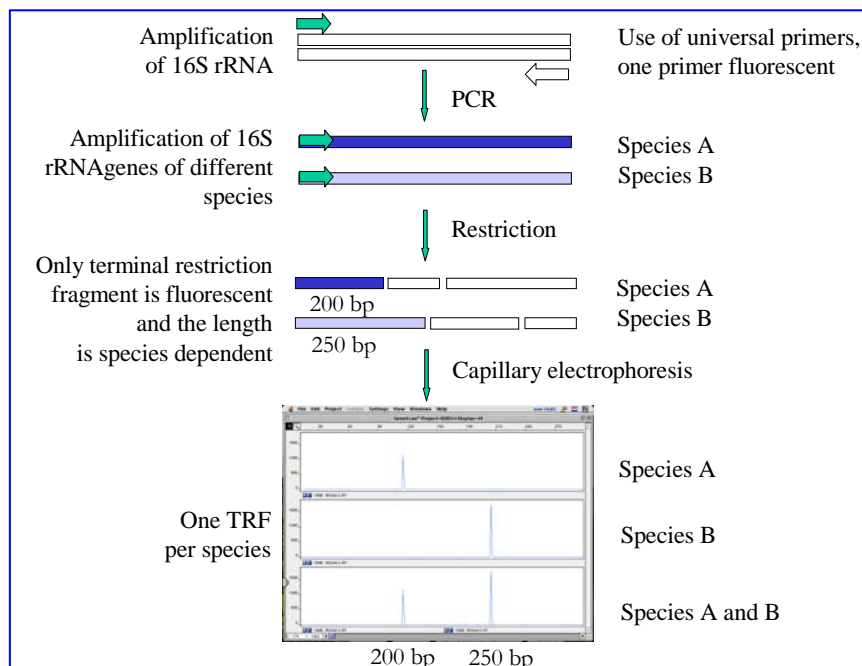
Figure 7: Overview of the T-RFLP procedure used to analyse microbial communities.

T-RFLP is already valued as an important tool in microbial ecology. This technique has been used for assessing the diversity and structure of complex bacterial communities in various environments (for reviews: (6, 12, 310)). T-RFLP also has been successfully applied to study the composition of the microflora of the human colon (228) and oral cavity (123, 229) and to assess bacterial diversity in cases of lung infection in cystic fibrosis patients (223), and has become a rapid diagnostic method for *Lactobacillus* bacteremia (46, 47).

T-RFLP has some advantages compared to sequence dependent electrophoresis techniques like DGGE: it has much higher reproducibility (196), higher throughput and a

comparable discriminatory power, which strongly depends on the restriction enzyme used (178). Above all, the immediate digitization of the fingerprints is a major advantage since this makes possible the construction of libraries of fingerprints, such that immediate identification of the different species becomes possible. Unfortunately, when a TRF fragment occurs for which no representative is present in the database, this TRF cannot be further identified, while DGGE allows hybridization analysis or sequencing of excised bands for further identification.

Selection of amplification primers

Although the above studies demonstrate the broad applicability of T-RFLP, much of the possibilities of the technique are determined by the choice of the PCR primers. The choice of universal primers allows any bacterial community to be analysed, although in case of an ecosystem with a relatively high bacterial diversity only the dominant microflora will be amplified (314).

Also, 'universal' primers for the amplification of bacterial 16S rRNA genes may not be so universal, as was noted already in previous studies (e.g. (16, 74)). In total community DNA samples, differential sequence complementarity to primers between taxa will lead to a significant bias in the amplification efficiency. Incorporation of multiple bases at degenerate positions and the use of inosine residues have been used effectively to provide 'universal specificity', but excess use of these bases has been reported to have biased template-to-product ratios (208).

Construction of the T-RFLP library

The T-RFLP pattern obtained from a mixed sample consists of the 5' terminal restriction fragments obtained from amplified rDNA of the different species present. Theoretically the number of peaks (TRFs) reflects the number of different species present in a sample. Identification of the peaks in a T-RFLP pattern, in other words assignation of a species name to each TRF, is based on comparison with a library composed of T-RFLP patterns of well-identified species. Such library T-RFLP patterns consist of a single TRF, since they are obtained from performing T-RFLP on pure cultures of a single species or from cloned 16S rDNA. The TRF of a single species can be confirmed by carrying out computer assisted (i.e. virtual) restriction analysis of published 16S rRNA sequences. The peak values in the library entries are the averages of the peak values obtained after testing different strains or cloned 16S rRNA genes of each species.

The choice of the restriction enzyme used is important. We chose *Bst*UI, based on *in silico* analysis of 16S rRNA genes (MICA: Virtual Digest) and on literature (61), indicating that this restriction enzyme was well suited for maximal differentiation between *Lactobacillus* species based on the length of the terminal 5' restriction fragment of their 16S rDNA, i.e. their TRF. Nevertheless, several species turned out to have the same TRF. For example, after restriction with *Bst*UI, the species *G. vaginalis*, *B. bifidum*, *B. breve*, *B. dentium* and *Propionibacterium acnes* were shown to have a TRF with a length between 223.9 and 225.7 bp. This means that the occurrence of this TRF in a T-RFLP pattern does not allow differentiation between these different species.

Data analysis
T-RFLP patterns were obtained as table files from the Genescan Analysis software (ABI PRISM 310, Applied Biosystems, Foster City, Ca.) and were analysed using BaseHopper, a software program developed at our university (14). Using these sample files containing TRF lengths (peak values) in base pairs, this program enabled us to assign a species name to each TRF by comparing each TRF of a T-RFLP fingerprint separately with the library.

III.4.2.3.3. Species specific PCR
The fastest and usually most specific and sensitive culture-independent approach for the genus, species or strain specific detection in a complex microflora is the use of a PCR assay applying specific primers on bacterial DNA extracted from the sample. However, with an increasing degree of microbial complexity in the sample, several PCR primers are needed in order to detect the different species present, thereby substantially increasing workload. Perhaps the main disadvantage of this approach is the fact that only 'expected' microorganisms will be detected, making such PCR assay of limited value in the analysis of complex microflora with a variable and unknown species composition. However, the data obtained by using broad-range 16S rDNA PCR-techniques is often confirmed by species specific PCR, and as such can result in a diagnostic assay.

In the past, several species-specific PCR assays have been developed for sensitive detection of vaginal bacteria that are either characteristic for a normal microflora or bacterial vaginosis related. Several assays for the detection of *G. vaginalis*, targeting respectively the 16S-23S rRNA spacer region (192, 280, 312) or the 16S rRNA gene (187) were designed. Also the fastidious anaerobic *Mobiluncus* spp. (192), (237) and *Mycoplasma* spp. (312) were interesting species for specific detection, the latter also because this species is not detected by Gram stain.

Recently, several research groups applied species specific PCR for *A. vaginae* (34, 72, 77, 288) in order to confirm the data obtained by using the different broad-range 16S rDNA PCR-techniques, discussed above.

Although these PCR assays proved to be highly sensitive for bacterial vaginosis detection, none of these individual assays gained a footing in the diagnosis of bacterial vaginosis.

IV. Experimental work

In manuscript I (15) tDNA-PCR was applied for the identification of *Lactobacillus* species. Although not all species belonging to the *L. acidophilus*-group showed distinguishable patterns, the most frequently occurring vaginal lactobacilli that were tested, i.e. *L. crispatus*, *L. gasseri* and *L. jensenii*, could be identified unambiguously. We used this technique to identify most cultivable species present in the vaginal microflora (manuscript II, III and V).

Manuscript II (288) describes the composition of the vaginal microbial community as assessed by cloning of the 16S rRNA genes obtained after direct amplification of microorganisms present on vaginal swab samples from eight non-pregnant women.
This cloning study revealed that a thus far unrecognized species, *Atopobium vaginae*, was abundant in four out of the five disturbed vaginal fluids and urged the development of a species specific PCR for *A. vaginae.*

Manuscript III (290) evaluates the added value of T-RFLP for characterization of the vaginal microflora by comparing the composition of the vaginal microflora of 100 pregnant women at three time points in pregnancy using culture and culture-independent techniques.

Manuscript IV (292) highlights the association of *A. vaginae* with bacterial vaginosis discovered by the culture-independent analysis of the vaginal microflora.

Finally, based on comparison of traditional and culture-independent approaches, it was concluded that morphological subcategorization of normal vaginal microflora was possible and useful for diagnostic purposes (manuscript V, (289)).

Summarized, the composition of the vaginal microbial flora was characterized using
1) Gram stained smears from vaginal swabs,
2) tDNA-PCR-based identification of cultured isolates obtained after anaerobic culture,

3) two culture-independent PCR-based methods, i.e. cloning and T-RFLP analysis of the 16S rRNA gene of culturable and unculturable bacteria, allowing the direct identification of most species present in the complex vaginal microflora, and

4) species specific PCR for *A. vaginae* and *G. vaginalis* (Figure 8).

Figure 8. Overview of the strategy used to characterize the vaginal microflora
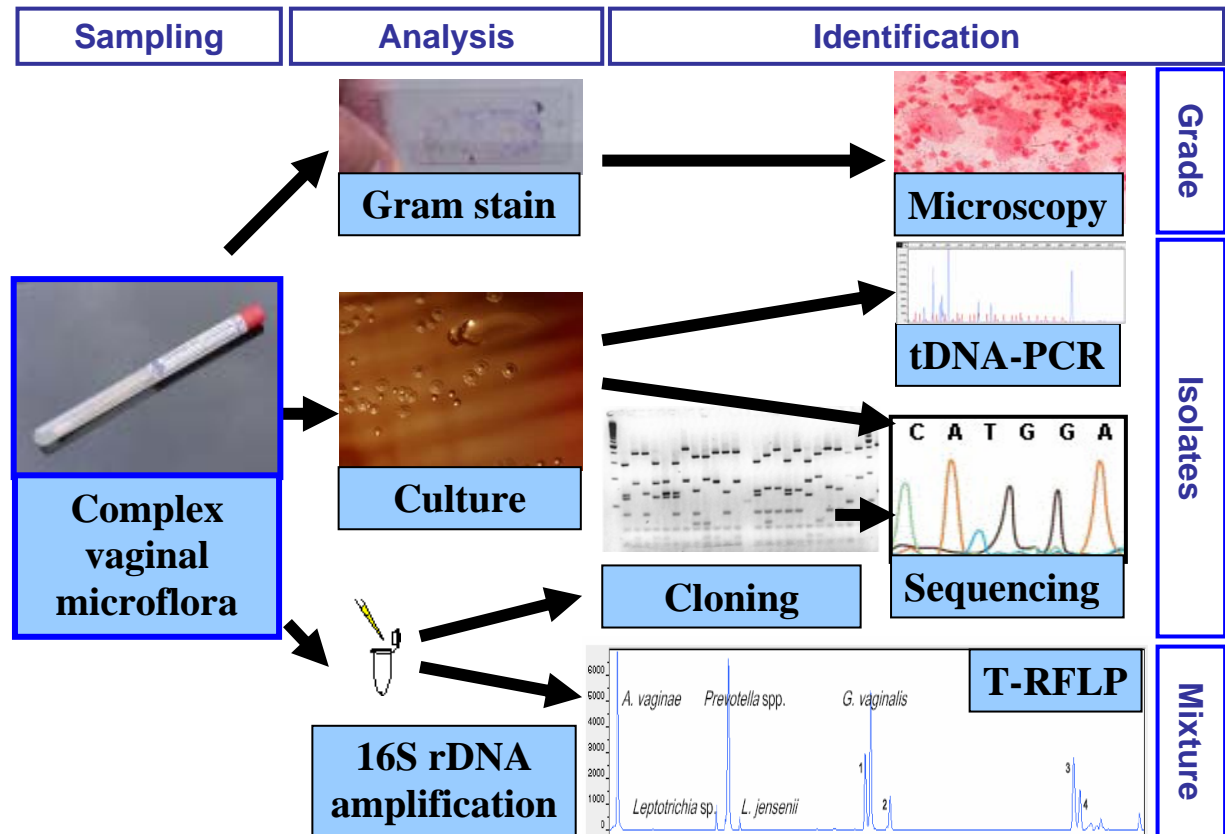
IV.1. PCR-based identification method of cultured organisms

Baele M, Vaneechoutte M, Verhelst R, Vancanneyt M, Devriese LA, Haesebrouck F.

Identification of *Lactobacillus* species using tDNA-PCR.

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Identification of *Lactobacillus* species using tDNA-PCR

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Abstract

tDNA intergenic spacer PCR (tDNA-PCR) using consensus primers complementary to the conserved edges of the tRNA genes can amplify the intergenic spacers. Separation of the PCR products with capillary electrophoresis enables discrimination between fragments differing only one basepair in length. This method was applied to a collection of 82 *Lactobacillus* strains belonging to 37 species in order to evaluate the discriminatory power of this technique within this genus. Twenty-one species could be distinguished to species level on the basis of a unique tDNA fingerprint pattern. The other species grouped by two (e.g. *L. fermentum* and *L. cellobiosus*) or three (*L. acidophilus*, *L. gallinarum* and *L. helveticus*). Inclusion of the resulting fingerprints in a numerical database containing fingerprints of numerous other Gram-positive and Gram-negative species makes the identification of unknown strains possible. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: tDNA-PCR; Lactobacillus; Identification

1. Introduction

Lactobacilli are commonly found in foods like dairy products, beverages, fruits, vegetables and fermented meat. They are also important members of the intestinal and urogenital microbiota of humans and animals. Several *Lactobacillus* strains are now being used as probiotics in commercially available food products. Strains which are studied thoroughly and are accepted as probiotics using established criteria belong to the species *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. reuteri* and *L. fermentum* (Reid, 1999). Although lactobacilli are not pathogenic for healthy individuals, several reports have described *Lactobacillus* bacteremia and endocarditis in patients with underlying clinical conditions (Husni et al., 1997).

The genus *Lactobacillus* contains over 60 species which are classified in three major groups: the obligate homofermentative lactobacilli, which ferment hexoses to lactic acid; the facultative heterofermentative lactobacilli, which ferment hexoses to lactic acid

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Table 1
Collection strains used in this study together with a list of peak values representing the tDNA spacer fragment lengths (in bp) reproducibly observed for each species

Species	Strain	Original no.	Source	Fragment lengths[a]
Lactobacillus	LMG 9433[T]	ATCC 4356	Human	66.1, 145.7, 157.8, 179.8
acidophilus (A)	LMG 11428	ATCC 832	White rat, faeces	
	LMG 11430	ATCC 43578	Human	
Lactobacillus	LMG 9186[T]	CCUG 31450	Municipal sewage	149.8, 171.8
agilis (Sal)	LMG 11398	DSM 20508	Municipal sewage	
	LMG 13085	NCFB 2334	Municipal sewage	
Lactobacillus	LMG 9187[T]	ATCC 29643	Marinated fish product	68.2, 159.5, 181.7, 265.9
alimentarius (P)	LMG 9188	ATCC 29647	Marinated fish product	
Lactobacillus	LMG 6900[T]	CCUG 30137	Swine waste–corn fermentation	62.3, 147.7, 169.4, 230.1
amylophilus (A)	LMG 11400	DSM 20534	Swine waste–corn fermentation	62.3, 147.7, 169.4, 234.9
Lactobacillus	LMG 9496[T]	ATCC 33620	Cattle waste–corn fermentation	66.1, 145.7, 159, 181
amylovorus (A)	LMG 13135	ATCC 33621	Cattle waste–corn silage	
Lactobacillus	LMG 9843[T]	ATCC 35046	Baboon, dental plaque	160, 182
animalis (Sal)				
Lactobacillus	LMG 9845[T]	ATCC 35409	Blown Dutch cheese	75, 145.3, 167.6
bifermentans (Bi)	LMG 11431	NCFB 1231	Cheese	
	LMG 11432	NCFB 1232	Cheese	
Lactobacillus	LMG 6892[T]	ATCC 4005	Tomato pulp	93.8, 151.8, 173.9, 192.5
buchneri (Bu)	LMG 11439	ATCC 9460	Unknown	
Lactobacillus	LMG 6904[T]	ATCC 393	Cheese	57, 149.4, 171, 186, 198, 245.7, 332.2, 363, 384
casei (C)				
Lactobacillus	LMG 9846[T]	ATCC 11739	Saliva	57, 164.2, 175.5, 187.2, 253
cellobiosus (R)				
Lactobacillus	LMG 9194[T]	ATCC 27612	Fermenting apple juice	93.8, 151.8, 173.9, 192.5
collinoides (Bu)				
Lactobacillus	LMG 9479[T]	ATCC 33820	Eye	66.1, 145.7, 159, 181
crispatus (A)	LMG 11415	NCIMB 8821	Human, saliva	
	LMG 11440	NCFB 5	Pregnant woman, vagina	
Lactobacillus curvatus	LMG 9198[T]	ATCC 25601	Milk	69.2, 167.1, 189.3
subsp. *curvatus* (Sak)	LMG 12008	NCFB 1970	Unknown	
	LMG 17299	CCUG 31333	Raw sausage	
Lactobacillus	LMG 6901[T]	ATCC 11842	Bulgarian yoghurt	66.2, 77.5, 145.7, 185.6
delbrueckii subsp.	LMG 12168		Homemade yoghurt	
bulgaricus (A)				
Lactobacillus	LMG 6401	ATCC 7830	Used as starter for fermented food products	66.2, 77.5, 145.7, 185.6
delbrueckii				
subsp. *lactis* (A)				
	LMG 13136	ATCC 4797	Unknown	
Lactobacillus	LMG 9189	DSM 20182	Marinated meat product	156.3, 178.3
farciminis (P)				
	LMG 9200[T]	ATCC 29644	Sausage	
Lactobacillus	LMG 6902[T]	ATCC 14931	Fermented beets	57, 164.2, 175.5, 187.2, 253
fermentum (R)	LMG 8900	ATCC 11976	Eight day old breast-fed infant, intestine	
	LMG 17551	ATCC 23271	Human, intestine	
Lactobacillus	LMG 9201[T]	ATCC 8288	Unknown	57, 149.4, 171, 386.5
fructivorans (Bu)	LMG 9202	NCFC 2166	Unknown	
	LMG 18879		Unknown	

Table 1 (*continued*)

Species	Strain	Original no.	Source	Fragment lengths[a]
Lactobacillus gallinarum (A)	LMG 9435[T]	ATCC 33199	Chicken, crop	66.1, 145.7, 157.8, 179.8
	LMG 14751	CCUG 31412	Chicken, faeces	
	LMG 14752	JCM 8783	Chicken, faeces	
Lactobacillus gasseri (A)	LMG 9203[T]	ATCC 33323	Human	156.3, 178.3
	LMG 11413	NCIMB 8819	Human, saliva	
	LMG 13134	ATCC 9857	Vaginal tract	
Lactobacillus graminis (Sak)	LMG9825[T]	DSM 20719	Grass silage	69.5, 166.1, 189.3
Lactobacillus hamsteri (A)	LMG 10754[T]	ATCC 43851	Hamster, faeces	145.8, 151.3, 173.7
Lactobacillus helveticus (A)	LMG 6113[T]	ATCC 15009	Swiss Emmental cheese	66.1, 145.7, 157.8, 179.8
	LMG 11445	ATCC 521	Unknown	
	LMG 11446	JCM 1004	Unknown	
Lactobacillus hilgardii	LMG 6895[T]	ATCC 8290	Wine	
Lactobacillus intestinalis (A)	LMG 11462	NCFB 2176	Unknown	66.3, 153, 175.2
	LMG 14196T	ATCC 49335	Rat, intestine	
Lactobacillus jensenii (A)	LMG 6414T	ATCC 25258	Human, vaginal discharge	180, 228
Lactobacillus johnsonii (A)	LMG 9436T	ATCC 33200	Human, blood	158.5, 180
	LMG 9437	ATCC 11506	Unknown	
	LMG 11468	ATCC 332	Unknown	
Lactobacillus murinus (Sal)	LMG 14189T	ATCC 35020	Rat, digestive tract	160, 182
Lactobacillus oris (R)	LMG 9848T	ATCC 49062	Human, saliva	178.7, 233.5, 250.5
Lactobacillus parabuchneri (Bu)	LMG 11457T	ATCC 49374	Human, saliva	94, 156.8, 179
	LMG 12001	NCFB 2749	Human, saliva	
Lactobacillus paracasei (C)	LMG 13717	Patarata 3	Young red table wine	56.4, 150, 172.3, 186, 246.8, 331.2, 363
Lactobacillus paracasei subsp. *paracasei* (C)	LMG 10774	CCUG 27320	Cerebrospinal fluid	56.4, 150, 172.3, 186, 246.8, 331.2, 363
	LMG 11459	ATCC 11974	Dental caries	
	LMG 13087T	ATCC 25302	Unknown	
Lactobacillus plantarum (P)	LMG 6907T	ATCC 14917	Pickled cabbage	75.7, 184
	LMG 9206	ATCC 11924	Dental caries	
	LMG 9212	NCIMB 8827	Human, saliva	
Lactobacillus reuteri (R)	LMG 13090	NCFB 2656	Rat	183, 234.7, 257
	LMG 13091	NCFB 2655	Rat	
	LMG 9213T	ATCC 23272	Adult, intestine	
Lactobacillus rhamnosus (C)	LMG 6400T	ATCC 7469	Unknown	56.2, 150.8, 173, 187.5, 246.7, 333.3, 364.7, 386.5
	LMG 8153	CCUG 17659-61	Healthy adult female, urethra	
	LMG 10775	CCUG 27333	Hip punction	
Lactobacillus ruminis (Sal)	LMG 10756T	ATCC 27780	Bovine, rumen	163.6, 186.3, 343.1, 367.1
	LMG 11461	ATCC 27781	Bovine, rumen	
Lactobacillus sakei subsp. *carnosus* (Sak)	LMG 17301	CCUG 8045	Human, blood	69.5, 166.1, 189.3
	LMG 17306	CCUG 32584	Human with endocarditis, blood	
Lactobacillus salivarius (Sal)	LMG 14476	DEVRIESE 94/438	Cat with myocarditis	155.5, 177.5
	LMG 14477	DEVRIESE 94/428	Parakeet with sepsis	

Table 1 (*continued*)

Species	Strain	Original no.	Source	Fragment lengths[a]
Lactobacillus salivarius subsp. *salivarius* (Sal)	LMG 9477T	ATCC 11742	Saliva	155.5, 177.5
Lactobacillus vaginalis (R)	LMG 12891T	ATCC 49540	Patient suffering from trichomoniasis, vagina	161.3, 184.1, 231.7, 274.8

Letters between brackets refer to the phylogenetic group whereto the species belongs: A: *Lactobacillus acidophilus* group; P: *Lactobacillus plantarum* group; Sal: *Lactobacillus salivarius* group; Bi: *Lactobacillus bifermentans* group; Bu: *Lactobacillus buchneri* group; C: *Lactobacillus casei* group; Sak: *Lactobacillus sakei* group; R: *Lactobacillus reuteri* group.

  [a] Values represent peaks that ought to be present in the fingerprint of an unknown strain in order to be identified as a certain species.

only or to lactic acid together with acetic acid, ethanol and formic acid under glucose limitation; and the obligate heterofermentative lactobacilli, fermenting hexoses to lactic acid, acetic acid, ethanol and CO_2, and pentoses to lactic acid and acetic acid (Pot et al., 1994). These groups, however, do not reflect the phylogenetic relations between species. Using 16S rDNA sequences, lactobacilli have been classified into five phylogenetic groups (Schleifer and Ludwig, 1995): the *L. acidophilus* group, the *L. salivarius* group, the *L. reuteri* group, the *L. buchneri* group and the *L. plantarum* group. This study did not confirm the earlier phylogenetic classification in three clusters by Collins et al. (1991), based on reverse transcriptase sequencing of 16S rRNA. The identification of lactobacilli using biochemical methods is notoriously difficult because several uncommonly used fermentation tests are required and because the high number of species complicates this approach (Hammes and Vogel, 1995). SDS-PAGE of whole cell proteins has proved to be a valuable method for the identification of lactobacilli (Pot et al., 1993), but this reference method cannot be used for routine identification. M13 bacteriophage repeat selective restriction fragment hybridization has been applied for certain *Lactobacillus* species and is possibly discriminative for other *Lactobacillus* species as well (Miteva et al., 1992). Recently, PCR amplification of the V2–V3 region of the 16S rDNA using universal primers, followed by denaturing gradient gel electrophoresis (DGGE) has been applied to detect and identify gastrointestinal *Lactobacillus* strains (Walter et al., 2000). However, none of these techniques seems to be applicable for rapid identification of lactobacilli in diagnostic laboratories.

tDNA-PCR, which consists of the amplification of the spacer regions in between tRNA genes and which has been shown to yield mainly species-specific DNA fingerprints, was described in 1991 by Welsh and McClelland (1991) and has been applied for *Acinetobacter* (Ehrenstein et al., 1996; Wiedmann-Al-Ahmad et al., 1994), staphylococci (Maes et al., 1997), streptococci (De Gheldre et al., 1999) and *Legionella* (De Gheldre et al., 2001). In combination with capillary electrophoresis, tDNA-PCR has been applied for *Listeria* species (Vaneechoutte et al., 1998), enterococci (Baele et al., 2000) and streptococci (Baele et al., 2001). This last paper also confirmed the interlaboratory reproducibility of this method.

Here, we evaluated the applicability of tDNA-PCR in combination with capillary electrophoresis for rapid identification of lactobacilli.

2. Materials and methods

2.1. Bacterial strains

Eighty-two well-characterized *Lactobacillus* strains from different origins, identified by whole cell protein analysis using SDS-PAGE, were obtained from the BCCM™-LMG bacterial collection. The strains were isolated from humans, animals or food products and are listed in Table 1.

2.2. tDNA-PCR

tDNA-PCR and electrophoresis were carried out as described before (Baele et al., 2000, 2001). DNA

was extracted from single colonies, grown on MRS (de Man, Rogosa, Sharpe) agar (Lab M, Bury, England) under anaerobic conditions, by simple alkaline lysis. tDNA-PCR was carried out using the outwardly directed tRNA gene consensus primers T5A (5′ AGTCCGGTGCTCTAACCAACTGAG) and T3B (5′ AGGTCGCGGGTTCGAATCC), as described by Welsh and McClelland (1991). Reactions were carried out in a 10-µl volume, containing 9.1 µl High Fidelity Mix 1.1 × (Invitrogen Life Technologies, Merelbeke, Belgium). Primers were added at a final concentration of 0.1 µM. Primer T3B consisted of a mixture of 1/5 fluorescent TET-labeled oligonucleotides and 4/5 nonlabeled oligonucleotides (Perkin-Elmer Applied Biosystems, Foster City, CA). A volume of 0.7-µl sample DNA was added (the template was diluted 15 times). After 2 min at 94 °C, reaction mixtures were cycled 30 times in a Perkin-Elmer Cetus 9600 thermocycler with following conditions: 30 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C. Final extension was 30 min at 72 °C. Reaction vials were then cooled to 10 °C and kept on ice until used in electrophoresis.

The amplified tDNA spacer regions were denatured by heating in formamide and the length of the fragments was determined by means of automated fluorescent capillary electrophoresis on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems). Electropherograms contained peaks for which peak values (i.e. fragment lengths) were calculated after interpolation with an internal size standard. The peak values cannot be expressed as exact numbers of basepairs because of reproducible small variations in electrophoresis migration between equally sized fragments due to variations in nucleotide sequence. For reproducibility, several strains were analysed (DNA extraction, PCR and electrophoresis) multiple times. The fingerprints were compared electronically with a database of fingerprints (available on request), using an in-house software program (available on request).

3. Results

Strains *L. delbrueckii* LMG 13136 and *L. gallinarum* LMG 9435[T] were tested three times in tDNA-PCR. Both species showed four reproducible peaks in

their pattern. The peak values (in bp) obtained for these peaks are shown in Table 2.

Twenty-one species showed distinguishable patterns, consisting of between two and nine tRNA-spacer fragments (Table 1) and could be easily identified. *Lactobacillus acidophilus*, *L. gallinarum* and *L. helveticus*, however, were indistinguishable from each other, as was the case for respectively *L. crispatus* and *L. amylovorus*, *L. buchneri* and *L. collinoides*, *L. gasseri* and *L. farciminis*, *L. fermentum* and *L. cellobiosus*, *L. sakei* and *L. graminis*, and *L. animalis* and *L. murinus*. The two *L. amylophilus* strains tested showed three identical peaks in their tDNA fingerprint, but both had a different additional peak. Clustering analysis with the Unweighted Pair Group Method with Arithmetic mean (UPGMA) was applied to all fingerprints and the resulting dendrogram is shown in Fig. 1. All strains belonging to the same species or subspecies clustered together. The species showing similar patterns were also found in the same cluster. The *L. hamsteri* strain clustered together with *L. buchneri* and *L. collinoides*, because they had two peaks in common, but *L. hamsteri* was distinguishable from *L. buchneri*/*L. collinoides* on the basis of a third peak.

For most species tested with tDNA-PCR in this study, a dendrogram was constructed using the 16S rDNA sequences of the type strains, available from GenBank (Fig. 2). On the basis of this phylogenetic tree, eight phylogenetic groups can be distinguished, as indicated in Fig. 2.

Table 2
Peak values (in bp) obtained by repeated tDNA-PCR testing of strains *Lactobacillus delbrueckii* LMG 13136 (a) and *Lactobacillus gallinarum* LMG 9435[T] (b)

	Sample 1	Sample 2	Sample 3	Range
(a)				
Peak 1	66.14	65.99	66.19	0.20
Peak 2	77.55	77.65	77.51	0.14
Peak 3	145.65	145.77	145.76	0.12
Peak 4	185.58	185.16	185.67	0.51
(b)				
Peak 1	65.33	65.94	65.92	0.61
Peak 2	145.92	145.48	145.62	0.45
Peak 3	158.38	157.15	157.6	1.23
Peak 4	178.75	179.43	180	1.25

L. gallinarum
L. acidophilus
L. helveticus
L. amylovorus
L. crispatus
L. johnsonii
L. delbrueckii
L. intestinalis
L. vaginalis
L. hilgardii
L. bifermentans
L. plantarum
L. alimentarius
L. animalis
L. murinus
L. amylophilus
L. reuteri
L. farciminis
L. gasseri
L. parabuchneri
L. jensenii
L. oris
L. casei
L. agilis
L. paracasei
L. fructivorans
L. rhamnosus
L. hamsteri
L. collinoides
L. buchneri
L. salivarius
L. cellobiosus
L. fermentum
L. ruminis
L. curvatus
L. graminis
L. sakei

0.1

Fig. 1. Dendrogram obtained from tDNA-PCR fingerprints after similarity calculation with in-house software and clustering with UPGMA using Neighbor software. Bar, difference of 10%.

% dissimilarity
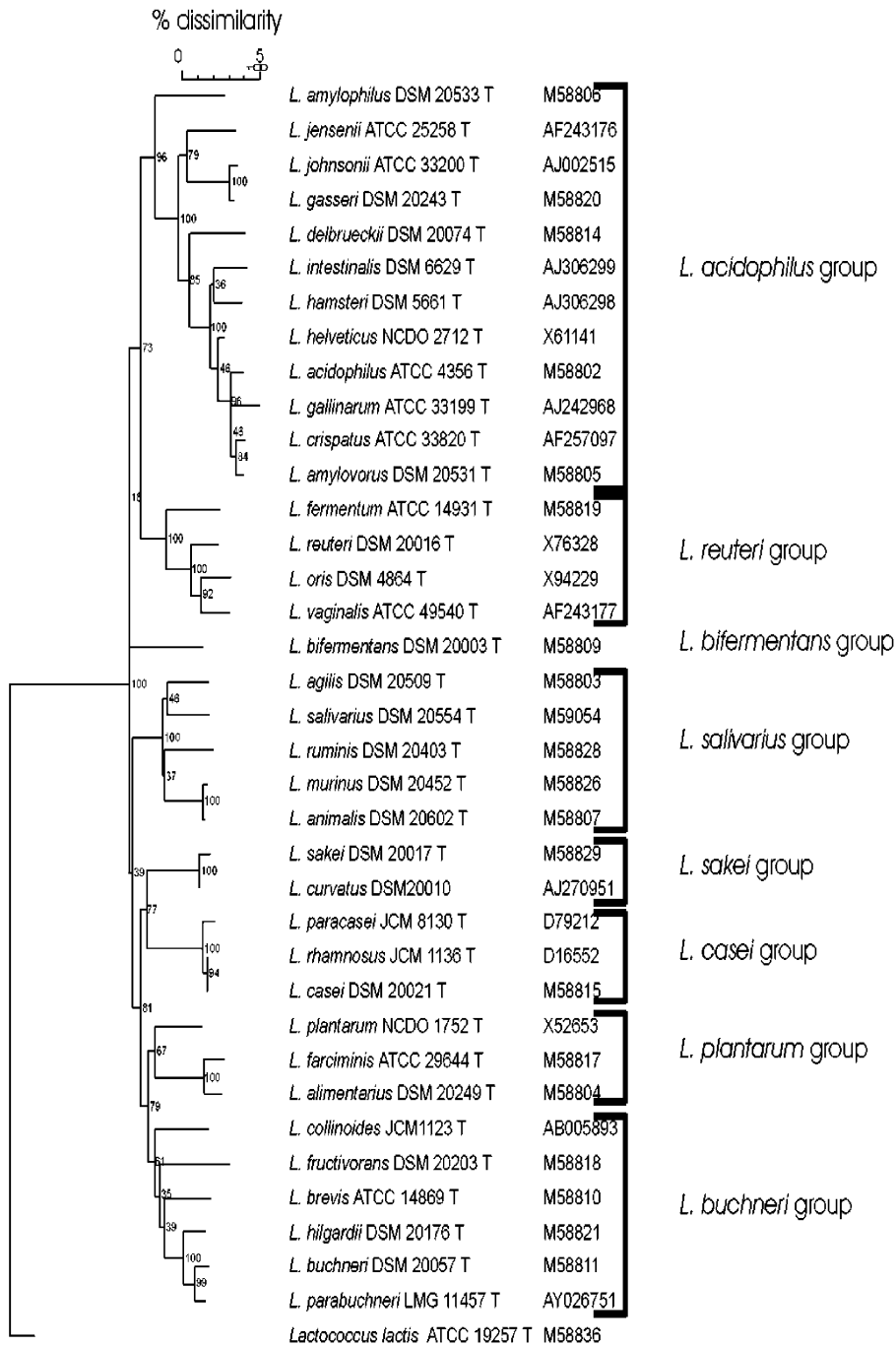
Fig. 2. Phylogenetic tree showing the relationships among *Lactobacillus* strains based on 16S rDNA sequence analysis. The tree was constructed by neighbour-joining analysis of a distance matrix from a multiple-sequence alignment. *L. lactis* was used as the outgroup and bootstrap values are indicated at the branch-points (100 trees resampled). Accession numbers are given.

4. Discussion

Earlier studies have shown that tDNA-PCR can be used to differentiate bacterial species belonging to a wide range of genera (McClelland and Welsh, 1992; Maes et al., 1997; Welsh and McClelland, 1992; Vaneechoutte et al., 1998). Automated capillary electrophoresis, the construction of exchangeable tDNA-PCR fingerprint databanks and easy-to-use data-analysis software have rendered this method suitable for fast identification of large collections of strains (Baele et al., 2000, 2001). Moreover, knowing the genus identity of the bacteria is not a prerequisite as the method is applicable for any type of bacterium.

Reproducibility testing of two *Lactobacillus* strains showed that peak values lie within a range of 1.5 bp, confirming earlier extensive reproducibility testing of tDNA-PCR on streptococci (Baele et al., 2001). These results imply that a peak position tolerance of 0.7 bp can be used for similarity calculation.

For most *Lactobacillus* strains, the obtained tDNA fingerprint was species specific. However, some *Lactobacillus* species were not distinguishable from each other using tDNA-PCR. The species with identical tDNA-PCR patterns all belonged to the same phylogenetic group as determined by 16S ribosomal DNA sequencing (Fig. 2) (Schleifer and Ludwig, 1995), except for the species *L. farciminis*, belonging to the *L. plantarum* group, and *L. gasseri*, which belongs to the *L. acidophilus* group. *L. acidophilus*, *L. gallinarum* and *L. helveticus* were indistinguishable by tDNA fingerprinting, as was the case for *L. amylovorus* and *L. crispatus*. All these species belong to the *L. acidophilus* group and their phenotypic characteristics are very similar. Thus far, differentiation between these species is possible only by DNA–DNA hybridization, 16S rDNA sequencing and SDS-PAGE whole cell electrophoresis (Kandler and Weiss, 1986; Fujisawa et al., 1992; Pot et al., 1994). *L. buchneri* and *L. collinoides* and *L. animalis* and *L. murinus*, which group together in tDNA-PCR, are phylogenetically related as determined by 16S ribosomal DNA sequencing. *L. brevis* has not been included in this study because the taxonomy of this species and its relatedness to other species in the *L. buchneri* species group needs further elucidation, as has been stated previously (Sohier et al., 1999).

The dendrogram shown in Fig. 1 is based on similarities between tDNA-PCR fingerprints and the obtained clustering does not always correspond with phylogenetic relations between species as deduced from 16S rDNA sequences. This kind of data handling can be useful to recognize groups of strains belonging to not yet described species, or to species which are not included in the database. Because of the presence of common peaks between related species, some fingerprints can cluster together, as is the case for several species of the *L. acidophilus* group, which have a peak in common at about 66 bp. However, many species grouping together on the basis of their 16S rDNA sequence, are not situated in the same tDNA cluster.

Even in those cases where the tDNA pattern obtained is not characteristic of a single named species, the array of tests needed to reach species identification is considerably narrowed.

In conclusion, the tDNA fingerprints obtained from lactobacilli are sufficiently characteristic to differentiate lactobacilli from over 180-Gram positive and 170 Gram-negative species, currently found in clinical samples (Baele et al., 2000, 2001; Maes et al., 1997; Vaneechoutte et al., 1998); and unpublished data [available on request]) and enable or largely facilitate differentiation between lactobacilli, when capillary electrophoresis equipment is present. The interlaboratory exchangeability of these capillary electrophoresis based fingerprints has been shown extensively in a previous study (Baele et al., 2001).

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IV.2. Cloning of 16S rRNA genes of normal and disturbed vaginal microflora

Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, De Ganck C, Temmerman M, Vaneechoutte M.

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Research article

Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis

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Abstract

Background: The pathogenesis of bacterial vaginosis remains largely elusive, although some microorganisms, including *Gardnerella vaginalis*, are suspected of playing a role in the etiology of this disorder. Recently culture-independent analysis of microbial ecosystems has proven its efficacy in characterizing the diversity of bacterial populations. Here, we report on the results obtained by combining culture and PCR-based methods to characterize the normal and disturbed vaginal microflora.

Results: A total of 150 vaginal swab samples from healthy women (115 pregnant and 35 non-pregnant) were categorized on the basis of Gram stain of direct smear as grade I (n = 112), grade II (n = 26), grade III (n = 9) or grade IV (n = 3). The composition of the vaginal microbial community of eight of these vaginal swabs (three grade I, two grade II and three grade III), all from non-pregnant women, were studied by culture and by cloning of the 16S rRNA genes obtained after direct amplification. Forty-six cultured isolates were identified by tDNA-PCR, 854 cloned 16S rRNA gene fragments were analysed of which 156 by sequencing, yielding a total of 38 species, including 9 presumptively novel species with at least five species that have not been isolated previously from vaginal samples. Interestingly, cloning revealed that *Atopobium vaginae* was abundant in four out of the five non-grade I specimens. Finally, species specific PCR for *A. vaginae* and *Gardnerella vaginalis* pointed to a statistically significant co-occurrence of both species in the bacterial vaginosis samples.

Conclusions: Although historically the literature regarding bacterial vaginosis has largely focused on *G. vaginalis* in particular, several findings of this study – like the abundance of *A. vaginae* in disturbed vaginal microflora and the presence of several novel species – indicate that much is to be learned about the composition of the vaginal microflora and its relation to the etiology of BV.

Background

Bacterial vaginosis (BV) is considered to be the most common cause of vaginal inflammation among both pregnant and non-pregnant women and prevalences between 4.9% and 36.0% have been reported from European and American studies [1]. The etiology of this condition remains largely unknown.

Nonetheless, the abundant literature, addressing bacterial vaginosis and ascending genital tract infection, has largely focused on the bacterial component and, in particular, on a few microorganisms, which are thought to play a pivotal role in the pathology of bacterial vaginosis. Although probably rather complex microbial community dynamics are involved, culture-dependent studies indicate that a limited number of bacterial species, including *Mobiluncus* spp., *Gardnerella vaginalis*, *Bacteroides* spp., *Prevotella* spp. and *Mycoplasma hominis*, along with the relative absence of *Lactobacillus* spp., can be used as a sensitive index for bacterial vaginosis. Consequently, according to Nugent *et al.* [2], the standard diagnosis of BV relies on the quantification of only three cellular types (*Lactobacillus*, *Gardnerella*, and *Mobiluncus*) on a Gram stained vaginal smear.

While conventional microbiological techniques are useful as screening tools to identify women with BV, they do not enable prediction of the clinical burden associated with bacterial vaginosis. It has therefore been stated that better understanding of the composition and dynamics of the vaginal microflora along with the factors associated with the pathology of bacterial vaginosis is essential to improve our predictive abilities [1].

The analysis of complex bacterial communities has been hampered by conventional culture-dependent methods and by biochemical identification methods, since it leaves many bacteria uncultured and unidentified. This may prove especially true for vaginal microflora, both under normal circumstances as well as in the setting of bacterial vaginosis, a condition primarily characterized by overgrowth of anaerobic and fastidious microorganisms. For example, until recently, several important species, like *Lactobacillus crispatus*, *L. gasseri* and *L. iners*, were all lumped together into the *L. acidophilus* complex, while present molecular techniques make it possible to differentiate between these closely related species [3-9]. Here we combined culture with tDNA-PCR [4,10,11] and sequencing of the 16S rRNA-gene for the identification of cultured organisms.

Detailed information of complex microbial communities can also be acquired from the phylogenetic analysis of 16S rDNA sequences obtained directly from samples by PCR amplification, cloning, and sequencing, so that uncultivable species are also included [12,13], although this approach may be biased as well [14,15], as becomes also apparent from this study.

Here we report on the cloning and sequencing of 16S rRNA gene fragments, amplified directly from vaginal swabs, to examine the microbial diversity in the vaginal fluid of healthy women with different grades of vaginal microflora patterns as defined previously [16,17]. Finally, the data obtained by anaerobic culture and with culture independent techniques, lead us to carry out species specific PCR for *A. vaginae* and *G. vaginalis*, such that the presence of both species in differently graded samples could be established.

Results

Grading of the Gram stained smears of the vaginal samples for which cloning was carried out, according to the criteria of Ison and Hay [17] assigned three specimens (W1-W3) to grade I, two (W4-5) to grade II and three (W6-8) to grade III. Table 1 lists the species identified by cloning and sequencing of cloned bacterial 16S rDNA-fragments, as well as by bacterial culture and tDNA-PCR from the vaginal microflora of these 8 healthy women.

Identification of cultured isolates from 8 healthy women by tDNA-PCR and 16S rRNA gene sequencing

Forty six isolates were obtained after anaerobic culture of the vaginal fluid from the 8 women in the cloning part of the study. Twenty nine isolates, belonging to the species *Bacteroides ureolyticus*, *Enterococcus faecalis*, *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri*, *L. vaginalis*, *Peptoniphilus* sp., *Prevotella bivia*, *Streptococcus anginosus* group and *S. mitis* group were identified by tDNA-PCR using a library containing tDNA-PCR fingerprints of well-identified strains. Six isolates belonging to the species *Aerococcus christensenii*, *Anaerococcus tetradius*, *Anaerococcus vaginalis*, *Lactobacillus iners*, *Mobiluncus mulieris* and *Peptostreptococcus anaerobius* were identified by sequencing because they produced a tDNA pattern that initially was not present in the database. These species were characterized by a specific tDNA-PCR pattern that allowed unambiguous identification after the new patterns were added to the database. In summary, 40 of 46 isolates (87.0%) were correctly identified by tDNA-PCR to the species level, including the species within the *L. acidophilus* complex. Of the six isolates that could not be identified, one isolate, with a sequence that was 99% identical to that of a *Peptostreptococcus* sp. strain (CCUG 42997, AJ277208), could not be identified unambiguously by tDNA-PCR because its pattern was identical to that of *P. micros*, one isolate – that was identified by sequencing as *Atopobium vaginae* – did not yield a tDNA-pattern and another four isolates (8.7%) were tDNA-PCR negative and also 16S PCR negative.

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Table 1: Cloning and culture^a results for 8 healthy females with different grades of vaginal microflora.

Grade	I	I	I	II	II	III	III	III
Sample designation	BVS30	BVS62	BVS63	BVS36	BVS34	BVS59	BVS61	BVS44
Subject code	W1	W2	W3	W4	W5	W6	W7	W8
Age	51	34	38	49	41	46	28	44
Number of clones	124	118	107	69	72	125	169	70
Species								
Lactobacillus crispatus [AF257097][b]	<u>66.1</u>c		<u>99.1</u>					
Lactobacillus gasseri [AF243144]	<u>18.5</u>	99.2						
Lactobacillus jensenii [AF243159]			<u>0.9</u>					
Lactobacillus vaginalis [AF243177]		<u>0.8</u>						
Atopobium vaginae [AF325325]				1.4	41.7	36.0	80.5	
Atopobium vaginae 97.6%^d [AF325325]						<u>0.0</u>		
Gardnerella vaginalis^e [M58744]				<u>0.0</u>		<u>0.0</u>	<u>4.1</u>	
Lactobacillus iners [Y16329]				84.1	1.4		<u>12.4</u>	
Mobiluncus mulieris [AJ427625]						<u>5.6</u>	3.0	
Peptostreptococcus anaerobius [L04168]					<u>0.0</u>	<u>1.6</u>		<u>68.6</u>
Peptoniphilus sp. [D14147]						<u>0.0</u>		14.3
Prevotella bivia 91% [L16475]				7.2	22.2			
Prevotella buccalis 96.6% [L16476]	1.6				22.2	31.2		
Sneathia (Leptotrichia) sanguinegens [L37789]				1.4	1.4	6.4		
Uncultured *Megasphaera* sp. clone [AY271937]				1.4		4.8		
Uncultured *Actinobacteridae* clone 86% [AB089070]				2.9		0.8		
Unidentified clone 1^f [AY207059]					6.9	4.0		
Atopobium rimae [AF292371]	0.8							
Fusobacterium nucleatum [AJ006964]	6.5							
Peptostreptococcus sp. [AJ277208]	<u>0.8</u>							
Pseudoramibacter alactolyticus [AB036761]	0.8							
Streptococcus anginosus group [AF104676]	<u>0.0</u>							
Treponema sp. clone [AF023055]	0.8							
Porphyromonas levi clone 94% [L16493]	1.6							
Unidentified clone 2 [AF371910]	2.4							
Aerococcus christensenii [Y17318]						<u>3.2</u>	<u>0.0</u>	
Anaerococcus tetradius [AF542234]							<u>0.0</u>	
Anaerococcus vaginalis [AF542229]					<u>1.4</u>			
Bacteroides ureolyticus [L04321]							<u>0.0</u>	
Bifidobacterium biavatii (urinalis) [AJ278695]								1.4
Dialister sp. [AF473837]								2.9
Enterococcus faecalis [AJ420803]					<u>1.4</u>			
[Leptotrichia amnionii] [AY078425]					1.4			
Prevotella bivia [L16475]				<u>0.0</u>	<u>0.0</u>			
Prevotella ruminicola 87% [L16476]								4.3
Streptococcus sp. oral strain [AY005041]								<u>8.6</u>
Unidentified clone 3 [AF371693]						6.4		
Ureaplasma urealyticum [AF073455]				1.4				

^a Four isolates were cultured which were negative for amplification of the 16S rRNA gene and for tDNA-PCR. ^b The accession number of the Genbank sequence with the highest similarity is indicated within brackets. ^c Underlined figures indicate that this species was also cultured. ^d Percentage of 16S rRNA gene sequence similarity, given only for values below 98%. ^e Low clone numbers of *G. vaginalis* are caused by the usage of one primer with 3 mismatches for this species (see Methods). ^f The 16S rRNA gene sequences of these clones showed highest similarity with entries in the Genbank listed as being obtained from uncultured bacteria.

Identification of 16S rRNA gene clones from 8 healthy women by sequencing and culture results

The ARDRA pattern of 854 clones was analysed and enabled to establish the relative frequency of the different species present (Table 1). For 130 clones, belonging to 33 ARDRA types, on average 447 bp of the 5' end of the 16S rRNA gene was sequenced. In case of grade I vaginal smears, the most predominant species were *L. crispatus*

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and *L. gasseri*. Smaller numbers of *L. jensenii* and *L. vaginalis* were also present. All females with grade I smears (W1-W3) were colonized by two *Lactobacillus* species. W2 (age 34) and W3 (age 38) were colonized by *Lactobacillus* species only, with one species – respectively *L. gasseri* and *L. crispatus* – represented by more than 99% of the clones. In the vaginal fluid of W1 (age 51) the most abundant species were *L. crispatus* (66.1%), *L. gasseri* (18.6%) and *Fusobacterium nucleatum* (6.5%). An additional 8 species were found in low numbers, six only by cloning and one only by culture. Three of these species, representing 5.6% of the clones, showed less than 98% similarity with previously published sequences. Culture results coincided largely with cloning for the grade I samples, except for the non *Lactobacillus* species of W1 of which only two were cultured.

For the two grade II smears (W4 and W5) cloning revealed four species, *A. vaginae* (resp. 1.5% and 41.6%), *L. iners* (resp. 84.1% and 1.4%), a *P. bivia*-like species (resp. 7.3% and 22.2%) and *Sneathia sanguinegens* (both 1.4%), that were present in both samples. *P. bivia* was also present in both grade II microflora but it was found by culture only. Several other species were found by cloning only in either W4 or W5. For example, *[Leptotrichia amnionii]* was found only in W5. *G. vaginalis* and *Peptostreptococcus anaerobius* were found respectively in W4 and W5 by culture only and two species, *Anaerococcus vaginalis* and *Enterococcus faecalis*, were found by both cloning and culture in the microflora of W5.

Two women (W6 and W7) with a grade III vaginal smear were colonized predominantly by *A. vaginae* (resp. 36.0 and 80.5%), *L. iners* (absent and 12.4%), *G. vaginalis* (absent and 4.1%), *M. mulieris* (5.6 and 3.0%) and a *Prevotella buccalis*-like species (31.2% and absent), according to cloning. As for grade II samples, there was limited correspondence with culture. For W6, an *A. vaginae*-like organism was cultured, but the sequence of this isolate showed less than 98% similarity to the sequence of the clones. Furthermore, a large number of colonies of *G. vaginalis* and *Peptoniphilus* sp. were present after anaerobic culture but no clones were obtained.

The most abundant species obtained by cloning the third grade III vaginal sample (W8) were *Peptostreptococcus anaerobius* (68.6%)(also cultured), *Peptoniphilus* sp. (14.3%) and an unidentified *Streptococcus* sp. (8.6%)(also cultured).

Total number of species identified and comparison between cloning and culture
Of the 38 species, 18 were discovered by cloning only, 5 by culture only, and 8 by both cloning and culture. Three species were shown by both culture and cloning in some

samples, but only by culture in the remaining samples, another three species were shown by both cloning and culture in some samples, but only by cloning in the remaining samples and one species was found once by culture only and once by cloning only. The presence of all four grade I *Lactobacillus* sp. was shown in grade I samples by both cloning and culture, whereas *L. iners* was found only in three non-grade I samples, in all three by cloning – even abundantly in W4, but only in W7 by culture. *G. vaginalis* was cultured three times, but was found by cloning only once. *A. vaginae* was shown in four samples by cloning, but cultured only once. The *P. bivia*-like species and *P. buccalis*-like species were found in respectively two and three samples by cloning but not by culture.

Possible novel species and genera
Of the 38 species that were distinguished by culture and cloning in this study, only 76.3% demonstrated more than 98% identity with previously known bacterial species (Table 1). Five of the eight vaginal samples contained previously unidentified species.

Anaerobic culture of the microflora of W6 revealed an *Atopobium* species that showed only 97.6% similarity with previously reported *A. vaginae* sequences (see below).

Two presumptively novel species within the genus *Prevotella* were found. For W4 and W5 (grade II), respectively 7.3% and 22.2% of the clones had 91% similarity with a *P. bivia* sequence. For W1 (grade I), W5 and W6 (grade III), respectively 1.6%, 22.2% and 31.2% of the clones showed 96.6% similarity with a *P. buccalis* sequence. Respectively 2.9% and 0.8% of the clones of W4 (grade II) and W6 (grade III) were identical and had only 86% similarity with an uncultured termite Actinobacteridae bacterium (accession number AB089070).

Variation in **Atopobium** sequences
Sixteen *A. vaginae* sequences are present in GenBank, one from a clinical isolate [18], one from the type strain [19] and 14 from cloned 16S rDNA fragments (Zhou, unpublished) (Figure 1). Another 17 sequences were obtained in this study, one from an isolate from patient W6, two from isolates from other patients (BVS38 and PB9) and 14 from clones from four different patients, i.e. W4 (grade II), W5 (II), W6 (III) and W7 (III) (Figure 1).

The sequence of all 7 clones from W5 was different from that of all other isolates and from the clones and the Genbank sequences, except from one Genbank clone sequence (AY269023). The sequence of all clones from the three other patients and from the two isolates from samples BVS38 and PB9 were identical to the previously published clinical isolate [18] and the type strain [19]. The sequence of the cultured isolate from patient W6 was

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Genbank   Study                           Sequence
AF325325 [15]                             ATACTCCATA TTTGTCGCAT GGCGAATATG GGAAAGCTCC GGCGGCAAAG
Y17195 (Type strain) [16]                 .......... ...N...... .......... .......... ..........
ato167f*                                             ......... .......... .
ato154f*                                      ... .......... .......
AJ585206 this study, W6 isolate 5         .......... .A.A...... .AT.T..... .......... .A........
AJ585208 this study, W6 clone 1           .......... .......... .......... .......... ..........
AJ585209 this study, W7 clone 4           .......... .......... .......... .......... ..........
AJ585212 this study, W7 clone1            .......... .......... .......... .......... ..........
AJ585213 this study, W7 clone9            .......... .......... .......... .......... ..........
AJ585210 this study, W5 clone 41          .......... .CCAC..... .ATAG..... .......... ..........
AJ585211 this study, W5 clone 47          .......... .CCA...... .ATAG..... .......... ..........
AY269023 (Zhou, unpublished)              .......... .CCA...... .ATAG..... .......... .........-.
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Figure 1
***Atopobium vaginae* 16S rDNA sequences** * Designations of the forward primers specific for *A. vaginae* and their position in the 16S rRNA gene.

Table 2: Amplification results with *A. vaginae* and *G. vaginalis* specific primers obtained for 150 vaginal samples of different grades

Grade	n	A+[a] G_{OY}+/G_Z+	A+ G_{OY}-/G_Z-	A- G_{OY}+/G_Z+	A- G_{OY}-/G_Z-
I	112	2/11	20/11	3/21	87/69
II	26	3/4	6/5	5/9	12/8
III	9	7/7	0/0	0/1	2/1
IV	3	0/0	0/0	0/1	3/2
total	150	12/22	26/16	8/32	104/80

[a] A: *A. vaginae* amplified with ato167f, G_{OY}: *G. vaginalis* amplified with Obata-Yasuoka primer set [25], G_Z: *G. vaginalis* amplified with Zariffard primer set [26].

somewhat intermediate between both above sequences, although the sequence of the clones of the same patient were identical to that of the type strain. Specific amplification with both the ato167f primer and the ato154f primer gave positive signals for the vaginal sample of W6, indicating that indeed both types of sequence were present.

***Species-specific PCR for* A. vaginae *and* G. vaginalis**
To substantiate the results obtained by cloning, an additional series of 142 vaginal samples obtained by swab from healthy pregnant (n = 115) and non-pregnant (n = 27) women, attending our out-patient clinic, were selected for culture and for PCR with 16S rRNA gene based primers specific for *A. vaginae* and *G. vaginalis* (Table 2). Of the 150 subjects in total, 38 (of which 5 non-pregnant) presented with non-grade I microflora, of which 26 were assigned grade II, 9 grade III and 3 grade IV.

After amplification with the ato167f *A. vaginae* primer set, respectively 19.6% of grade I, 34.6% of grade II, 77.8% of grade III and 0.0% of grade IV samples showed an amplicon (Table 2). Of the 37 samples that showed an amplicon after amplification with the ato167f primer only 23 were positive after amplification with ato154f. *A. vaginae* was cultured only three times from these 150 samples.

The number of positive samples for *G. vaginalis* specific PCR varied depending on the primer set that was used. The difference was greatest for grade I samples, with respectively 4.5% of grade I samples that were positive after amplification with the G_{OY} primer set and 28.6% with the G_Z primer set (Table 2).

Comparative ROC analysis of the four indicators (ato154f, ato167f, G_Z, and G_{OY} respectively) in discriminating normal and disturbed vaginal microflora showed that the overall discriminative value of qualitative PCR-based detection of *A. vaginae* or *G. vaginalis* as such is actually rather low with AUCs of 0.627 (95%CI: 0.544–0.704), 0.625 (95%CI: 0.543–0.703), 0.633 (95%CI: 0.551–0.711), and 0.675 (95%CI: 0.594–0.749).

Detection of the simultaneous presence of *A. vaginae* (ato167f) and *G. vaginalis* (G_{OY}) in a vaginal swab specimen with the purpose of assessing true bacterial vaginosis (grade III) had an accuracy of 87.8% (AUC = 0.878, 95%CI = 0.714, 1.041, p < 0.001), a sensitivity of 0.78 (95%CI = 0.40, 0.96), a specificity of 0.98 (95%CI = 0.91, 1.00), a positive predictive value of 0.78 (95%CI = 0.40, 0.96), and a negative predictive value of 0.98 (95%CI = 0.91, 1.00). When the co-existence of *A. vaginae* (ato167f)

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and *G. vaginalis* was assessed using the G_Z-primer set, the overall performance of the assay was significantly lower.

Discussion

To our knowledge, no cloning study addressing the composition of the microflora of vaginal samples and bacterial vaginosis samples has been published thus far. By means of culture and by cloning and sequencing of 16S rRNA genes, amplified directly from the vaginal samples, we studied the vaginal microflora of 8 healthy women with different grades of bacterial vaginosis according to Nugent [2], as modified by Ison and Hay [16,17].

The frequency of the different clones could be assessed by performing ARDRA of the cloned 16S rRNA genes and counting the number of clones belonging to each ARDRA type. Representative clones for each ARDRA type were subsequently sequenced to determine the species identity. Five bacterial species, i.e. *Atopobium rimae, Bifidobacterium biavatii (urinalis), Dialister* sp., *[Leptotrichia amnionii]* and *Sneathia sanguinegens*, had never been recovered from the vagina. Twenty two percent of the clones, belonging to 9 putative species, showed less than 98% homology to any of the 16S rRNA gene sequences present in GenBank, indicating that several bacterial species, indigeneous to the female genital tract, remain to be characterized.

In this study, cloning confirmed that the vaginal microflora of healthy women is dominated by a limited number of *Lactobacillus* species. For the women with grade I microflora, the two species *L. crispatus* and *L. gasseri*, alone or in combination, accounted for 85–99% of the clones. The two younger women, 34 and 38 years, had almost pure cultures of *L. crispatus* resp. *L. gasseri*, in association with low numbers of resp. *L. jensenii* (< 1%) and *L. vaginalis* (< 1%) and without any other bacteria. In our ongoing studies we find this to be the case for 61.5% of the grade I samples (unpublished data). The finding of monocultures of *L. crispatus* and *L. gasseri* is also in correspondence with recent reports, which point to the predominance of only three to four *Lactobacillus* species in normal vaginal microflora [7,20]. Antonio *et al.* [3] considered *L. crispatus, L. jensenii* and *L. gasseri* as the indicator species for normal vaginal microflora, also because most strains of these species are hydroxyperoxide producers in opposition to other *Lactobacillus* spp. It should be noted in this context that another group could not substantiate any protective effect of hydroxyperoxide producing lactobacilli [21].

In the 51-year-old woman (W1), with a combination of both *L. crispatus* and *L. gasseri*, some additional non *Lactobacillus* species were present in low numbers, with *Fusobacterium nucleatum* as the most predominant (i.e. 6.5%). This may be in accordance with another observation,

namely that postmenopausal healthy women frequently present with BV-like microscopy [5,22].

Of the five women with non-grade I microflora (two with grade II and three with grade III), one presented with predominant *Peptostreptococcus* clones (68%), a picture that was very different from that observed in the other women. However, most striking was the observation that for the other four patients between 1.5% and 80.0% of the clones were identified as *Atopobium vaginae*, a species previously known only from a single isolate from the vagina of a healthy woman [19] and from a clinically important isolate, described as the causative agent in a case of a pelvic inflammatory disease (PID) following transvaginal oocyte recruitment [18]. Interestingly, Zhou *et al.* [20] reported dominating *Atopobium* sp. in a woman, judged to carry a normal vaginal microflora. The authors concluded that *Atopobium* may be present as part of the normal microflora of the vagina [23]. This warrants a more detailed discussion of this recently described species.

The genus *Atopobium* was introduced to accommodate the species *Lactobacillus minutus, Lactobacillus rimae* and *Streptococcus parvulus* [24]. *Atopobium* species have been described to produce major amounts of lactic acid [19], a characteristic reminiscent of lactobacilli, although no special reference to this property was made in the description of *A. vaginae* [19]. *Atopobium* species are anaerobic, Gram-positive elliptical cocci or rod-shaped organisms occurring singly, in pairs or as short chains. The variable cell morphology of *A. vaginae* makes that this species may reside perfectly camouflaged and as a consequence undetectable among the mixture of other species present in grade II and III bacterial communities. Also the fact that *A. vaginae* is fastidious and forms small pinhead colonies can explain why this species was not yet established as part of the vaginal microflora, when using classical microbiology. In this study, we could culture the species from only three out of 150 vaginal specimens.

Based on our findings with *A. vaginae* specific PCR, which recovered this species in 19.6% of the 112 grade I specimens, it appears as if *A. vaginae* may be a constituent – presumably in low numbers – of the human vagina, possibly attaining replicative dominance in association with decreasing lactobacillary grading. This hypothesis could be substantiated with quantitative PCR and the development of selective culture media.

The striking fact that *Gardnerella vaginalis*, predominantly present in bacterial vaginosis samples [e.g. [25,26]], was not found by cloning as carried out in this study, can be explained as the consequence of a methodological bias, because the forward primer used for cloning (10f) contained 3 mismatches for *G. vaginalis*. On the other hand,

the use of these non *G. vaginalis* 'universal' primers may have facilitated to establish the high prevalence of *A. vaginae*. To assess the relative importance of both species, cloning should be carried out with different sets of primers. Amplification of grade I, grade II and grade III samples with species specific primers indicated that *G. vaginalis* was present in respectively 28.6%, 50.0 % and 88.9% of the samples, while *Atopobium* was present in respectively 19.6%, 34.6% and 77.8% of the vaginal microflora.

Since any pairwise comparison of the four ROC plots did not show any significant differences between the AUCs, it is also apparent that each of the four indicators under study (ato154f, ato167f, G_Z, and G_{OY} respectively) presents with a comparably limited accuracy in discriminating normal and disturbed vaginal microflora. This may be explained by the fact that, although both species are present in about 80% of the true bacterial vaginosis samples, their presence in grade I samples is not uncommon. This lack of accuracy for *G. vaginalis* was also previously assessed in culture-dependent studies [27,28].

The co-existence of *A. vaginae* and *G. vaginalis* in women with grade III microflora together with their simultaneous absence in grade I samples (Table 2) is striking. The simultaneous presence of both species therefore is highly predictive for BV.

Lactobacillus iners, was present among the clones in three of the five non-grade I samples, even in large numbers, and absent from the three grade I samples. This species was only recently recognized as a separate *Lactobacillus* species [29]. In the original description of this relatively asaccharolytic *Lactobacillus* species, the only vaginal isolate among the 9 strains from females came from vaginal discharge [29]. This species may have been largely overlooked in culture based studies [30-32] since it does not grow on *Lactobacillus* selective media, including MRS and Rogosa-Sharp medium [29]. Using molecular techniques *L. iners* was reported as one of the most frequently encountered vaginal *Lactobacillus* species [8,9] present in normal and BV microflora [5].

Leptotrichia sp. are slow-growing, gram-negative anaerobic organisms of the oral cavity and genital tract. [*L. amnionii*] is an extremely fastidious organism, which most likely explains why this organism was hitherto virtually undetectable by conventional culture-based microbiological techniques. Of interest is that this particular *Leptotrichia* species was isolated by Shukla *et al.* [33] from the amniotic fluid of a woman with second trimester fetal loss, which led the authors to conclude that [*L. amnionii*] is presumably indigenous to the genital tract and acts as an opportunist under particular clinical conditions, includ-

ing pregnancy. This study demonstrates for the first time the presence of [*L. amnionii*] in the vagina.

Sneathia sanguinegens (formerly *Leptotrichia sanguinegens*) was found in this study to be present in three patients with non-grade I vaginal microflora, in moderate numbers. *S. sanguinegens* has been isolated from human blood and amniotic fluid [34] and has been associated with several cases of pregnancy-associated bacteraemia, i.e. postpartum fever in four patients and neonatal sepsis in two patients [35]. This study demonstrates the presence of *S. sanguinegens* in the vagina for the first time.

One strain from W6 and 14.3% of the clones from W8 were identified as *Peptoniphilus indolicus* based on more than 98% similarity with a Genbank sequence with accession number D14147 corresponding with the type strain CCUG 17639 of this species. This type strain however has two Genbank entries (also AY153430) with low similarity [36]. Unfortunately our isolate was not stored so further phenotypic identification was not possible. Because of the ambiguous Genbank sequences, we chose to designate our isolate and clones as *Peptoniphilus* sp.

Thus far, identification of organisms cultured from complex microbial biota like the vagina, especially under the condition of vaginosis, has also been hampered by the limitations of conventional biochemical and phenotypic identification methods. However, using a DNA-based method, namely tDNA-PCR [11], it is convenient to appropriately identify most of these organisms, once a library, based on tDNA-PCR of well characterized organisms, has been constructed. Also, newly unknowns can be first identified by 16S rRNA gene sequence determination, whereafter it is possible to use the corresponding tDNA fingerprint for future identification of the organisms of the same species.

tDNA-PCR, which consists of the amplification of the spacer regions between tRNA genes, has been shown to yield mainly species-specific DNA fingerprints [10]. Combined with capillary electrophoresis, tDNA-PCR has been shown to be a semi-automated, digital DNA-fingerprinting method that enables rapid and discriminatory identification of species from very diverse phylogenetic groups, including *Lactobacillus* [4,11].

Conclusions

In summary, the use of tDNA-PCR based identification of cultured organisms in combination with cloning of 16S rRNA genes, amplified directly from vaginal swabs, enabled us to characterize the vaginal microflora in a detailed manner, and to compare the composition of the normal vaginal microflora with the bacterial vaginosis microflora. The presence of *A. vaginae* in 4 of the 5 women with bac-

terial vaginosis grade II-III microflora is an unexpected and previously not reported finding, which may shed new light on the etiology of this condition. There is also the ambiguous position of *L. iners*, which apparently is not indicative of a normal microflora, but may point to some intermediate condition.

Furthermore, our findings warrant more detailed studies of the ability of species like *L. iners* and *A. vaginae* to produce lactic acid, hydrogen peroxide and of their cellular and colonial morphology and biochemical characteristics, as well as their interaction with lactobacilli and *G. vaginalis*. A selective medium for these organisms might be a welcome tool for further studies, given the fact that until now they have been largely overlooked by culture methods.

Finally, the discrepancies between culture, cloning and specific PCR, together with the diversity of the recovered species, their unequal distribution over different vaginal samples, the elucidation of several species – including presumptively new species – previously not associated with the vagina, indicate that much is to be learned about the composition of the vaginal microflora and its relation to the etiology of BV.

Methods
Study population, sample collection and grading
For a total of 150 healthy women of reproductive age, attending our out-patient clinic, of which 115 were pregnant, the health condition with regard to the composition of the vaginal bacterial community was assessed microscopically after Gram stain, according to the criteria of Hay and Ison [17] and vaginal samples were taken. Briefly, specimens were considered grade I (normal) when only *Lactobacillus* morphotypes were present, grade II (intermediate) when both *Lactobacillus* and other morphotypes were present, grade III (BV) when only non *Lactobacillus* morphotypes were seen and grade IV when only Gram positive cocci were seen.

Sampling was carried out as follows. After placement of a non-lubrificated speculum two sterile cotton swabs were inserted into the vaginal vault. The swabs were rotated against the vaginal wall at the midportion of the vault and were carefully removed to prevent contamination with the vulva and introitus microflora. One swab was returned to a sterile tube (dry swab) (Copan, Brescia, Italy), for the purpose of DNA-extraction. The other swab was placed into Amies transport medium (Nuova Aptaca, Canelli, Italy) and was used for making a smear for the purpose of grading according to the Hay and Ison criteria [17] and for anaerobic culture. Both swabs were processed in the microbiology laboratory within 4 h.

Based on smear results, eight non-pregnant women (mean age 41.4 years, range 28–51 years) from the studied population, attending our out-patient clinic for a routine gynaecological visit, were selected for cloning of the 16S rRNA genes present in the vaginal microflora. The culture results are reported only for these eight women.

To substantiate the results obtained by cloning and culture, PCR with species specific primers for *A. vaginae* and *G. vaginalis* and culture were carried out for the complete population.

Culture and identification of cultured isolates by tDNA-PCR
For eight non-pregnant women, the swab on Amies transport medium was streaked onto tryptic soy agar supplemented with 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ) and incubated anaerobically at 37°C upon arrival at the microbiology laboratory. After 4 days of incubation, all the isolates with different colony morphology were selected for identification. DNA was extracted by simple alkaline lysis: one colony was suspended in 20 μl of 0.25% sodium dodecyl sulfate-0.05 N NaOH, heated at 95°C for 15 min and diluted with 180 μl of distilled water. tDNA-PCR and capillary electrophoresis were carried out as described previously [4,11]. The species to which each isolate belonged was determined by comparing the tDNA-PCR fingerprint obtained from each isolate with a library of tDNA-PCR fingerprints obtained from reference strains, using an in-house software program [11]. The library of tDNA-PCR fingerprints and the software are available on request.

DNA extraction of vaginal swab samples
For DNA extraction from the dry vaginal swabs, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications. The dry swab specimen from each patient was swirled for 15 s in 400 μl of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton). Fifty units of mutanolysin (25 U/μl) (Sigma, Bornem, Belgium) were added and the samples were incubated for 30 min at 37°C. After the addition of 20 μl Proteinase K (20 mg/ml) and 200 μl AL buffer (Qiagen), samples were incubated for 30 min at 56°C. Next, 200 μl of ethanol was added and DNA was purified by adding the lysate to the Qiagen columns as described by the manufacturer. Finally, the total bacterial DNA was eluted with 100 μl of AE buffer (Qiagen). DNA-extracts were stored at -20°C and were used for the purpose of cloning experiments and species specific PCR.

Cloning of amplified mixtures of 16S rDNA
To amplify the 5' part of the bacterial 16S rRNA genes by PCR, primers 10f (5' AGTTTGATCCTGGCTCAG) and

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534r (5' ATTACCGCGGCTGCTGG) [37], which target the domain *Bacteria*, were used. It should be noted that the forward primer contains mismatches for *G. vaginalis* at positions 1 (A/G), 5 (T/C) and 9 (C/T). A 50 μl PCR mixture contained 0.1 μM of each primer, 25 μl of Promega master mix (Promega, Madison, WI), 5 μl of DNA extract and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 50°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 50°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Cloning was done using the Qiagen PCR Cloning Kit (UA-cloning, Qiagen), whereby the purified amplicons were ligated into the pDrive cloning vector and transformed into Qiagen EZ Competent *E. coli* Cells, as specified by the manufacturer.

Screening for clones with different 16S rRNA gene inserts by ARDRA

From each sample, 100 to 200 ampicillin-resistant transformants, recognizable as white colonies on Luria-Bertani (LB) agar containing IPTG (Roche, Basel, Switzerland), X-Gal (Roche) and 100 μg ampicillin/ml after overnight incubation at 37°C, were selected for further analysis. Single colonies were picked from the agar plates and transferred with sterile tips to the wells of a 96 well plate filled with LB Broth supplemented with 100 μg ampicillin/ml and incubated for 3 h. To avoid sequencing of all clones, the 16S rRNA gene inserts were differentiated initially from each other by means of Amplified rDNA Restriction Analysis (ARDRA)[38]. One μl of bacterial culture was added to a final volume of 20 μl PCR mix, containing 0.2 μM of two plasmid-targeted primers, QF (5'TACGTATCG-GATCCAGAATTC) and QR (5'CGAGAAGCTTGTCGAC-GAATT), 10 μl of Promega master mix and distilled water. The position of primers QF and QR was chosen as such that their distance towards the insert was the same in order to result in the same ARDRA pattern regardless the orientation of the insert in the cloning vector. Cycling conditions were the same as described above for the 16S rRNA gene. Ten microliter of the amplified products were digested with 10 U of the restriction endonuclease *Bst*UI in the appropriate enzyme buffer and incubated for 3 h at 60°C. The DNA restriction fragments were separated in a 2.5% agarose electrophoresis gel, containing 2% Methaphor (FMC Bioproducts, Rockland, ME) and 0.5% MP agarose (Roche, Basel, Switzerland) in the presence of ethidium bromide (50 ng/ml). The gels were photographed and the obtained ARDRA fingerprints were compared visually. For each ARDRA type, the 16S rRNA gene insert of at least one representative clone was sequenced, thus minimizing the number of sequence reactions that had to be carried out.

Sequencing of 16S rRNA genes from isolates and clones

For 8 healthy women, the 16S rRNA gene was amplified and sequenced from selected clones and from cultured isolates that could not be identified by tDNA-PCR, using primers 10f and 534r, as described above. For the clones, 1 μl of the QF-QR amplified PCR product was diluted 1000 times with distilled water and re-amplification of the 16S rRNA gene was done with the primers 10f and 534r. For the cultured isolates, a 5 μl aliquot of the alkaline lysate was added to a 50 μl PCR mixture. The amplification products were then purified with the Qiaquick PCR purification kit, according to the manufacturer's instructions. Sequencing was done using the ABI Big Dye cycle sequencing reaction kit with Ampli*Taq* FS DNA polymerase (Applied Biosystems, Foster City, CA.) with primer 534r. Sequencing reaction products were analyzed on an ABI 310 genetic analyzer (Applied Biosystems). Inspection of the electropherograms was done with Chromas http://www.technelysium.com.au/chromas14x.html and with the BioEdit package [39]. Comparison of the sequences of the inserts and isolates to the 16S rRNA gene sequences in GenBank was done using the BLAST software [40]. Clones or isolates with DNA sequences sharing more than 98% identity with known sequences were assigned to that phylotype.

Species specific PCR for **Gardnerella vaginalis**

G. vaginalis species-specific primers as designed by Zariffard *et al.* (G$_Z$)[26] and Obata-Yasuoka *et al.* (G$_{OY}$) [25] were used. Briefly, a 20 μl PCR mixture contained respectively 0.05 and 0.4 μM primers, 10 μl of Promega master mix (Promega, Madison, WI), 2 μl of Qiagen DNA-extract of the samples and distilled water. Thermal cycling with G$_Z$ primers consisted of an initial denaturation of 10 min at 94°C, followed by 50 cycles of 5 s at 94°C, 45 s at 55°C and 45 s at 72°C, and a final extension of 10 min at 72°C. Thermal cycling with the G$_{OY}$ primers was performed by an initial denaturation of 1 min at 94°C was followed by 40 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 70°C and a final extension of 7 min at 72°C. During the first ten cycles the annealing temperature was lowered by 0.5°C per cycle. Five microliter of the amplified product of each PCR was visualized on a 2% agarose gel.

Species specific PCR for **Atopobium vaginae**

Two primer sets that allowed amplification of the 16S rRNA gene of *A. vaginae* and that lacked homology with non-target bacteria as determined by searching the Gene Bank database using BLAST software [40], were designed. The selected primers, ato167f 5' (GCGAATAT-GGGAAAGCTCCG), ato154f 5' (ATATTTGTCGCAT-GGCGAAT) and ato587r 5' (GAGCGGATAGGGGTTGAGC), were analysed for secondary structures using NetPrimer (Premier Biosoft Inter-

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national, Palo Alto, CA). A 20 µl PCR mixture contained 0.2 µM of primers (respectively ato167f or ato154f and ato587r), 10 µl of Promega master mix (Promega, Madison, WI), 2 µl of Qiagen DNA-extract of the samples and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 58°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 58°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. Five microliter of the amplified product was visualized on a 2% agarose gel. The primers amplified a DNA-fragment of respectively 420 and 433 basepairs from *A. vaginae* and showed no cross reactivity to other organisms, including *A. rimae* (data not presented).

Statistics

To assess the relative accuracy of PCR-based detection of *G. vaginalis* or *A. vaginae*, we applied comparative receiver-operating-characteristic (ROC) analysis under the non-parametric assumption and compared the accuracies of the isolated markers (amplification with ato154f, ato167f, G_{OY} and G_Z) in allocating subjects to a particular Gram stain category (grades I to IV).

Subsequently, we compared the accuracy of PCR-based combined detection of *G. vaginalis* (using the two different primer sets designated G_Z and G_{OY}) and *A. vaginae* (using ato167f) to assess the vaginal microflora status (according to Gram stain category) by non-parametric ROC-analysis.

Accuracy in these analyses is expressed as the area-under-the-curve (AUC) in the ROC-plot, the 95% confidence interval (CI) to the AUC, and the p-value to the 95% CI. We also calculated the estimated sensitivity, specificity, positive and negative predictive values (PPV and NPV) and the 95% confidence intervals to these measures. Statistical significance was accepted at the $\alpha = 0.05$-level.

Analyses were carried out using the statistical software packages EpiCalc2000 v.1.02 and SPSS v.11.0.

Nucleotide sequence accession numbers

Out of 156 16S rRNA gene sequences, obtained from 26 isolates and from 130 clones, two sequences of *A. vaginae* (-like) species and 23 sequences of uncultured bacterium clones were submitted to GenBank and were assigned accession no. AJ585206 to AJ585213 and no. AJ619698 to AJ619714.

Authors' contributions

RV, GC, GV and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. JD participated in the analysis and interpretation of the data and in the writing of the report. LVS and CDG participated in the analysis of the study samples. All authors read and approved the final manuscript.

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IV.3. Characterization of vaginal microflora at three time points in pregnancy by Gram stain, culture and T-RFLP

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Characterization of vaginal microflora at three time points in pregnancy by Gram stain, culture and T-RFLP identifies *L. gasseri* as the *Lactobacillus* species most present in bacterial vaginosis.

Scoring of Gram stained vaginal smears according to Claeys

In this manuscript Gram stained smears from vaginal swabs were scored according to the criteria of Claeys. These criteria are modified criteria of Ison and Hay (see III.3.6.3.2) and are explained in manuscript V (289).

Briefly, samples were categorized as grade Ia when only *Lactobacillus crispatus* cell types (plump, mostly short rods) were present, as grade Ib when only other *Lactobacillus* cell types were present (smaller or more elongated and less stained than in Ia smears), as grade Iab when both *L. crispatus* and other lactobacilli were present, as grade I-like when Gram positive rods, either quite small and short or otherwise irregularly shaped with clubbing, curved edges and irregular staining and often arranged like Chinese letters ('diphteroid cell types'), as grade II (intermediate) when both *Lactobacillus* and *Gardnerella* or *Bacteroides-Prevotella* cell types were present and as grade III (bacterial vaginosis) when *Lactobacillus* cell types were absent and only *Gardnerella*, *Bacteroides-Prevotella* or *Mobiluncus* cell types were present and as grade IV when Gram positive cocci were predominantly present.

VI.3.1. Abstract

Background

Accurate, informative and rapid laboratory methods to diagnose bacterial vaginosis are needed as several reports have shown that bacterial vaginosis during pregnancy predisposes to preterm labor. To evaluate the added value of terminal restriction fragment length polymorphism analysis (T-RFLP) for characterization of the vaginal microflora, we determined the composition of the vaginal microflora of 100 pregnant women at three time points in pregnancy using several approaches: 1) Gram stained smears from vaginal swabs, scored following the criteria of Claeys; 2) molecular identification of cultured isolates obtained after anaerobic culture, 3) T-RFLP of the 16S rDNA, a culture-independent DNA fingerprinting method that allows the identification of the different species present in a complex microflora, based on comparison with a library of T-RFLP fingerprints of known bacteria and 4) species specific PCR for *Atopobium vaginae* and *Gardnerella vaginalis*.

Results

Changes in the composition of vaginal microflora during gestation could be detected by T-RFLP resulting in algorithms with a high correlation between T-RFLP based classification and smear based grading. The presence of *Lactobacillus crispatus* was predictive for a stable normal microflora, while the presence of *Gardnerella vaginalis* and *Atopobium vaginae* was associated with a disturbed microflora. Most striking was the presence of *L. gasseri* in 59.3% of the women who had a TRF fingerprint with the TRF *G. vaginalis/Bifidobacterium*.

Conclusion

T-RFLP is a useful culture-independent technique to characterize the most abundant species present in normal and disturbed vaginal microflora enabling the follow up of changes in the microflora.

Our results also indicate that different approaches contribute to establishing the composition of a microbial population, with only partial overlap and that Gram stain should be combined with PCR-based identification for appropriate characterization of bacterial vaginosis in a research setting.

Lactobacillus gasseri could be cultured from approximately 60% of samples with *Gardnerella vaginalis* and seems to be associated more clearly with disturbed vaginal microflora than other lactobacilli.

IV.3.2. Background

Accurate, informative and rapid laboratory methods to diagnose bacterial vaginosis are warranted as several reports have shown that bacterial vaginosis during pregnancy predisposes to preterm labor [1-4]. Several authors reported the spontaneous disappearance of bacterial vaginosis, at least in part of the women, with advancing gestational age [e.g. 5-9]. In a recent review it was stated that there is a need for more longitudinal studies among pregnant women to assess the changes in vaginal microflora and in prevalence of bacterial vaginosis during gestation [10].

Culture-independent molecular techniques, frequently based on the detection of 16S rRNA genes, have been developed with the aim of covering also nonviable, uncultivable and fastidious bacteria. These techniques have been used recently to study the bacterial vaginal microflora and include cloning [11-14], sequence dependent electrophoresis techniques, like denaturing gradient gel electrophoresis (DGGE) [15-18] and temporal temperature gradient gel electrophoresis (TTGE) [19], and fluorescent in situ hybridization (FISH) [14, 20]. Some of these studies have recently revealed an association between *Atopobium vaginae*, a difficult-to-culture anaerobe, and bacterial vaginosis [12, 16, 21, 22]. The comparison of these culture-independent techniques with Gram stain substantiates the idea that in some situations vaginal microflora cannot adequately be classified as either normal or bacterial vaginosis positive using standard clinical and/or Gram stain criteria. Although PCR-generated information may be biased as well, it seems to provide a more objective and informative assessment of the vaginal microflora [16].

In a former study that compared Gram stain and culture in combination with PCR-based identification of the cultured isolates, we were able to refine classical Gram stain interpretation [23]. We showed that *L. crispatus* can be recognized as such on Gram stain and we established the existence of a separate additional category – grade I-like, characterized by the low prevalence of *L. crispatus* and by the abundance of bifidobacteria.

The aim of the present study was to further assess the composition of the vaginal microflora as well as its changes during gestation, by combining Gram stain and culture with an informative and rapid culture-independent PCR-based technique. This study comprises the characterization of the vaginal microflora of one hundred unselected pregnant women, enrolled as a prospective cohort, at three time points during pregnancy using i) Gram stain based grading, according to the criteria of Claeys [23], ii) culture in combination with PCR-based identification of cultured organisms, iii) terminal restriction

fragment length polymorphism analysis (T-RFLP)[24] carried out directly on DNA extracts from vaginal swabs and iv) species specific PCR for *Gardnerella vaginalis* and *Atopobium vaginae*. We chose to use T-RFLP analysis rather than sequence dependent electrophoresis techniques [15-18] for its reproducibility [25], for its high throughput and for the ability to easily compare the digital fingerprints produced by this approach. T-RFLP has become a diagnostic [26-28] and screening method and has been successfully applied to study the composition of the microflora of the human colon [29] and oral cavity [30, 31].

IV.3.3. Methods

Study population, sample collection and grading

A total of 300 vaginal swabs were collected by sampling 100 pregnant women attending our out-patient clinic, each at three time points during pregnancy. The swabs were obtained at mean gestational ages of 8.6 +/- 1.4 weeks, 21.2 +/- 1.3 weeks and 32.4 +/- 1.7 weeks, respectively.

Sampling was carried out by insertion of three sterile cotton swabs into the vaginal vault, after placement of a non-lubricated speculum. The swabs were rotated against the vaginal wall at the midportion of the vault and were carefully removed to prevent contamination with microflora of the vulva and introitus. The first swab was used to prepare a smear on a glass slide for making a Gram stain. The second swab was returned to a sterile tube (Copan, Brescia, Italy), for the purpose of DNA extraction (dry swab). The third swab was placed into Amies transport medium (Nuova Aptaca, Canelli, Italy) for anaerobic culture. The unstained smear and both swabs were sent to the microbiology laboratory and were processed within 4 hours.

Grading of samples

Gram stained smears from vaginal swabs were scored according to the criteria of Claeys [23]: Samples were categorized as grade Ia when only *Lactobacillus crispatus* cell types (plump, mostly short rods) were present, as grade Ib when only other *Lactobacillus* cell types were present (smaller or more elongated and less stained than in Ia smears), as grade Iab when both *L. crispatus* and other lactobacilli were present, as grade I-like when Gram positive rods, either quite small and short or otherwise irregularly shaped with clubbing, curved edges and irregular staining and often arranged like Chinese letters ('diphteroid cell types'), as grade II (intermediate) when both *Lactobacillus* and *Gardnerella* or

Bacteroides-Prevotella cell types were present and as grade III (bacterial vaginosis) when *Lactobacillus* cell types were absent and only *Gardnerella*, *Bacteroides-Prevotella* or *Mobiluncus* cell types were present and as grade IV when Gram positive cocci were predominantly present.

Culture and identification of cultured isolates by tDNA-PCR

For 100 women, the swab on Amies transport medium was streaked onto Schaedler agar enriched with 5% sheep blood, vitamin K, hemin and sodium pyruvate (Becton Dickinson, Franklin Lakes, NJ) and incubated anaerobically at 37°C upon arrival at the microbiology laboratory. After 4 days of incubation, all the isolates with different colony morphology were selected for identification. DNA was extracted by simple alkaline lysis: one colony was suspended in 20 µl of 0.25% sodium dodecyl sulfate-0.05 N NaOH, heated at 95°C for 15 min and diluted with 180 µl of distilled water. tDNA-PCR and capillary electrophoresis were carried out as described previously [32, 33]. The species to which each isolate belonged was determined by comparing the tDNA-PCR fingerprint obtained from each isolate with a library of tDNA-PCR fingerprints obtained from reference strains, using an in-house software program [32]. The library of tDNA-PCR fingerprints is available at our website and the software can be obtained upon request [34].

DNA extraction of vaginal swab samples

For DNA extraction from the dry vaginal swabs, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications. The dry swab specimen from each patient was swirled for 15 s in 400 µl of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton). Fifty units of mutanolysin (25 U/µl) (Sigma, Bornem, Belgium) were added and the samples were incubated for 30 min at 37 °C. After the addition of 20 µl Proteinase K (20 mg/ml) and 200 µl AL buffer (Qiagen), samples were incubated for 30 min at 56°C. Next, 200 µl of ethanol was added and DNA was purified by adding the lysate to the Qiagen columns as described by the manufacturer. Finally, the total bacterial DNA was eluted with 100 µl of AE buffer (Qiagen). DNA extracts were stored at – 20 °C and were used for the purpose of T-RFLP analysis and species specific PCR.

T-RFLP analysis

The forward primers 10f (5' TET-AGTTTGATCCTGGCTCAG), GV10f (5' TET-GGTTCGATTCTGGCTCAG) and the reverse primer 534r (5'

ATTACCGCGGCTGCTGG) [35], which target the 16S rRNA gene of the domain *Bacteria*, were used to amplify part of the 16S rDNA by PCR. Two 15 µl PCR mixtures contained respectively primer set 10f-534r and GV10f-534r (0.1 µM of each primer) and 7.5 µl of Promega master mix (Promega, Madison, WI), 1.5 µl of sample and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 50°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 50°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. A 20 µl restriction mixture, containing 0.5 µl of both PCR-products, 1 µl of *Bst*UI (Westburg, Leiden, The Netherlands) and 4 µl of the appropriate buffer, was incubated at 60°C during 3 h. Five microliters of the restriction reaction was purified by ethanol precipitation. The obtained pellet was resolved in 13.1 µl deionized formamide (AMRESCO, Solon, Ohio), 0.1 µl ROX500 and 0.3 µl HD400 GeneScan size standards (Applied Biosystems, Foster City, CA) followed by denaturation at 96°C for 2 min and immediate cooling on ice. The fluorescently labeled terminal restriction fragments (TRFs) were electrophoresed on an ABI PRISM 310 (Applied Biosystems). TRFs with a peak height less than 10% of the highest peak were excluded from the analysis, since such peaks rarely corresponded with any of the species shown to be present by cloning.

Construction of the T-RFLP library

The T-RFLP pattern obtained from a mixed sample consists of the 5' terminal *Bst*UI restriction fragments obtained from amplified rDNA of the different species present. Theoretically the number of peaks (TRFs) reflects the number of different species present in a sample. Identification of the peaks in a T-RFLP pattern, in other words assignation of a species name to each TRF, is based on comparison with a library composed of T-RFLP patterns of well-identified species. Such library T-RFLP patterns consist of a single TRF, since they are obtained from performing T-RFLP on pure cultures of a single species or from cloned 16S rDNA. The TRF of a single species can be confirmed by carrying out computer assisted (i.e. virtual) restriction analysis of published 16S rRNA sequences. The peak values in the library entries are the averages of the peak values obtained after testing different strains or cloned 16S rRNA genes of each species.

The choice of the restriction enzyme used is important. We chose *Bst*UI, based on in silico analysis of 16S rRNA genes [36] and on literature [37], indicating that this restriction enzyme was well suited for maximal differentiation between *Lactobacillus* species based on the length of the terminal 5' restriction fragment of their 16S rDNA, i.e.

their TRF. Nevertheless, several species turned out to have the same TRF. For example, after restriction with *Bst*UI, the species *G. vaginalis*, *B. bifidum*, *B. breve*, *B. dentium* and *Propionibacterium acnes* were shown to have a TRF with a length between 223.9 and 225.7 bp. This means that the occurrence of this TRF in a T-RFLP pattern does not allow differentiation between these different species.

Data analysis

T-RFLP patterns were obtained as table files from the Genescan Analysis software and were analyzed using BaseHopper, a software program developed at our university [32]. Using these sample files containing TRF lengths (peak values) in base pairs, this program enabled us to assign a species name to each TRF by comparing each TRF of a T-RFLP fingerprint separately with the library. Only TRFs with a length between 0 bp and 500 bp and with a peak height that was more than 10% of the highest peak were assigned a species name.

Species specific PCR for *Gardnerella vaginalis*

G. vaginalis species specific primers as designed by Zariffard *et al.* (G_Z) were used [38]. Briefly, a 20 µl PCR mixture contained respectively 0.05 and 0.4 µM primers, 10 µl of Promega master mix (Promega, Madison, WI), 2 µl of Qiagen DNA extract of the samples and distilled water. Thermal cycling with G_Z primers consisted of an initial denaturation of 10 min at 94°C, followed by 50 cycles of 5 s at 94°C, 45 s at 55°C and 45 s at 72°C, and a final extension of 10 min at 72°C. Five microliter of the amplified product was visualized on a 2% agarose gel.

Species specific PCR for *Atopobium vaginae*

A primer set that allowed amplification of the 16S rRNA gene of *A. vaginae* and that lacked homology with non-target bacteria was used as described earlier [12]. Briefly, a 10 µl PCR mixture contained 0.2 µM each of the primers ato167f (5' GCGAATATGGGAAAGCTCCG) and ato587r (5' GAGCGGATAGGGGTTGAGC), 5 µl of Promega master mix (Promega, Madison, WI), 1 µl of Qiagen DNA extract of the samples and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 58°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 58°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. Five microliter of the amplified product was visualized on a 2% agarose gel. The primers amplified a DNA fragment of 420 base

pairs from *A. vaginae* and showed no cross reactivity to other organisms, including *A. rimae* and *A. parvulum* (data not presented).

IV.3.4. Results

Vaginal microflora grade assessment of 100 pregnant women based on Gram stain of vaginal fluid

A total of 300 vaginal swabs were collected by sampling 100 pregnant women at three time points during pregnancy. Grading of the Gram stained vaginal smears assigned 79 specimens to grade Ia (26.3%), 40 to grade Iab (13.3%), 115 to grade Ib (38.3%), 23 to grade I-like (7.7%), 26 to grade II (8.7%), nine to grade III (3.0%), seven to grade IV (2.3%) and one to grade 0 (0.3%), according to the criteria of Claeys [23]. Because of the low number of grade IV and grade 0 samples, the findings for these samples are only presented in the tables, but not or only briefly discussed in the text.

Characterization of the vaginal microflora based on T-RFLP analysis

In a previous study we found that the 16S rDNAs of the different species present in vaginal microbial communities were not equally efficiently amplified when the 'universal' primer set 10f/534r was used [12]. The 10f primer was found to contain three mismatches for *G. vaginalis*. In this study, we applied an additional primer set, designated GV10f/534r, which enables efficient amplification of *G. vaginalis*. After separate amplification the two amplification mixtures were combined, prior to restriction digestion. For T-RFLP, we chose digestion of the amplified 16S rRNA genes with the restriction enzyme *Bst*UI, based upon *in silico* analysis of available 16S rRNA gene sequences, since T-RFLP with *Bst*UI results in the largest number of terminal restriction fragments (TRFs) with unique lengths for lactobacilli and as such enables the maximum number of *Lactobacillus* species to be identified. A T-RFLP library, whereby each entry is composed of the name of a species and its corresponding TRF length, was created by the digestion with *Bst*UI of the amplified 16S rDNA obtained from pure cultures of single species or by digestion with *Bst*UI of cloned and sequenced 16S rRNA genes, as obtained in a previous study [12].

The 23 different TRFs that were encountered during this study are presented in Table 1. Several species had identical TRF lengths. This was for instance the case for *G. vaginalis* and several *Bifidobacterium* species, with a TRF length of 225 bp. This TRF is therefore designated as the TRF *G. vaginalis*/*Bifidobacterium*. By comparison with the TRF lengths present in the library, the different TRFs present in each of the T-RFLP fingerprints

obtained from the vaginal swabs could be assigned to the respective species they represented. As such, the different species present in each swab could be identified to some degree without culture.

In total, 39 different T-RFLP fingerprints were found in the 300 vaginal swabs (Table 2). The T-RFLP fingerprints were not complex, with a maximum of 7 TRFs per sample (not taking into consideration peaks resulting from partial digestion). The most complex fingerprints were found in grade I-like, grade II and grade III smears, with respectively 69.6%, 100% and 100% of the samples having two or more TRFs, whereas the most encountered TRF fingerprint (67.9%) for grade I (a, ab and b) was composed of only one peak (Table 3 and Figure 1).

The TRF fingerprint composed of only the TRF for *L. crispatus* was most frequently encountered (Figure 1A). It was found in 101 (33.7%) of the samples, of which 100 were grade I smears and one was a grade I-like smear. This TRF fingerprint was noticed for 80% of 79 grade Ia samples, but only for 50% of 40 grade Iab and for 15% of 115 grade Ib samples. The second most encountered T-RFLP fingerprint, i.e. 65 times, was one for which only the TRF of *L. gasseri/L. iners* was present (Figure 1.C). It was found in all grades, except in grades II and III, and was mostly present in grade Ib samples (46.9%).

The TRFs of *Lactobacillus* species were present in 100%, 78%, 96% and 66% of respectively grade I (a+ab+b), grade I-like, grade II and grade III specimens. In respectively 3.3%, 71.3%, 21.7% and 3.7% of 300 samples none, one, two or three *Lactobacillus* TRFs were present (Table 4).

The TRF fingerprints of all 59 samples in which the TRF *G. vaginalis/Bifidobacterium* occurred, had more than one TRF present. Most strikingly, the TRF *G. vaginalis/Bifidobacterium* was associated with the TRF *L. gasseri/L. iners* in 48 cases (81.3%), but only in 9 cases (15.3%) with the TRF of *L. crispatus*, although the total number of samples with TRFs of *L. gasseri/L. iners* and *L. crispatus* was comparable, respectively 152 and 147. A TRF fingerprint with both the TRF *G. vaginalis/Bifidobacterium* and the TRF *L. gasseri/L. iners* present was found only in 2.5% of grade Ia, in 7.5 % of grade Iab and in 9.5% of grade Ib samples, whereas it was found in 30.4% of grade I-like, in 73.1% of grade II and in 77.8% of grade III samples.

The TRF *G. vaginalis/Bifidobacterium* and the TRF *A. vaginae* were present together in 15 samples, of which only once in 234 grade I samples (0.4%) and 4/23, 3/26 and 7/9 times in respectively grade I-like (17.4%), grade II (11.5%) and grade III (77.8%) samples.

It should be noted that all grade III samples (n = 9) had a TRF for *G. vaginalis*, of which 7 had the TRF for *A. vaginae* in addition. Also the 23 grade I-like samples compared well to the grade II and grade III samples by the presence of the TRF *G. vaginalis/Bifidobacterium* sp. in 12 samples, of which 4 had both the TRFs for *G. vaginalis* and *A. vaginae*.

The following algorithms gave a strong correlation between T-RFLP based classification and smear based grading: algorithm 1: 'Samples with only *Lactobacillus* TRFs are grade I' was correct in 207 of 219 cases (94.5%) and algorithm 2: 'Samples with *Lactobacillus* TRFs in the absence of the TRF *G. vaginalis/Bifidobacterium* and of the TRF *A. vaginae* are grade I' was correct in 213 of 229 cases (93.0%). The algorithm: 'Samples with the TRF *G. vaginalis/Bifidobacterium* and/or the TRF *A. vaginae* are non grade Ia' was correct in 62 of 64 cases (96.9%). The algorithm 'Samples with at least one TRF other than *Lactobacillus* TRFs are non grade Ia' was correct in 78 of 81 cases (96.3%). The algorithm 'Samples with the TRF *L. gasseri/L. iners* only are grade Ib' was correct in 54 of 65 cases (83.1%).

Summarized, the presence of only *Lactobacillus* TRFs was predictive for a grade I vaginal microflora (94%), the presence of the TRF *G. vaginalis/Bifidobacterium* and/or the TRF *A. vaginae* was predictive for a non grade Ia smear (96.9%). It was difficult to evaluate the usefulness of T-RFLP for the prediction of grade III smears, due to the limited number of grade III samples. Nevertheless, the simultaneous presence of the TRFs for *G. vaginalis* and *A. vaginae* seems characteristic for grade III samples (observed in 7 of 9 cases) - confirming results with specific PCR (8/9), compared to 1 case of 234 grade I samples and 7 cases of 49 grade I-like and grade II samples. The prediction of grade I-like and grade II samples was difficult on the basis of T-RFLP, due to the high variability in TRF fingerprints associated with these grades.

Identification of cultured isolates from 100 pregnant women by tDNA PCR and 16S rRNA gene sequencing

Following anaerobic incubation at 37°C during 4 days on a Schaedler based blood agar individual colonies of the numerically dominant colony types were selected for identification by tDNA-PCR and eventually 16S rDNA sequence analysis when no definite identification could be reached by means of tDNA-PCR. From the 300 samples included, 609 isolations were made of which 76 could not be identified by tDNA-PCR due to lack of amplification. The remaining 533 isolates belonged to 58 species according

to tDNA-PCR and 16S rDNA sequencing (Table 5). Each bacterial species was counted only once per sample for the analysis. In several cases, colonies with a very different morphology were identified as being the same species. This was especially the case for *L. crispatus*.

Sixteen different *Lactobacillus* species were cultured, whereof two that previously had not been isolated from the vagina: *L. nagelii* was cultured from a grade II sample and a possibly new *Lactobacillus* species, that had only 94% similarity with *L. pontis*, was isolated from one woman with a grade Ib microflora. The lactobacilli most abundant in grade I (a+b+ab) smears were: *L. crispatus* (47.4%), *L. jensenii* (27.7%), *L. iners* (20.9%), *L. gasseri* (20.5%), *L. vaginalis* (9.8%) and *L. coleohominis* (5.1%). While *L. crispatus* was absent from grades II and III, *L. jensenii*, *L. gasseri* and *L. iners* could be isolated from respectively 46.2%, 65.4% and 23.1% of grade II samples and from respectively 11.1%, 44.4% and 11.1% of grade III specimens. Only *L. jensenii* and *L. gasseri* were present in all grades, whereas *L. jensenii*, *L. gasseri*, *L. iners* and *L. rhamnosus* were the only lactobacilli cultured from grade III smears. None, one, two, three or four *Lactobacillus* species were isolated from respectively 11.3%, 48.0%, 36.3%, 3.7% and 0.7% of the samples (Table 4).

G. vaginalis was cultured in all grades but grade Ia and grade IV. It was cultured in only 3 out of 234 (1.3%) grade I samples but in 6 out of 9 grade III smears (66.7%).

Six *Bifidobacterium* species were cultured from 22 samples in total. While *Bifidobacterium* isolates were found in only 3 out of 234 (1.3%) grade I samples, they were isolated in 11 out of 23 (47.8%) grade I-like smears. The probiotic *B. bifidum* was cultured only once, from a grade I sample. *B. breve* was most frequently present, found in six grade I-like samples, in four grade II samples and in two grade III samples. Other bifidobacteria present in grade I-like specimens were *B. dentium, B. longum* and [*B. biavatii*].

To our knowledge, the following species were cultured for the first time from the human vagina: *Arthrobacter albus* (once in grade II), previously isolated from blood [39], *Clostridium colicanis* (once in grade Iab), previously isolated from canine faeces [40], *Pediococcus pentosaceus* (once in grade I-like), an industrially important lactic acid bacterium and *Varibaculum cambriense* (once from a grade III sample), previously isolated from abscesses and intrauterine contraceptive devices [41].

One isolate that was cultured from a grade III vaginal smear had less than 90% similarity with *Helcococcus* sp. and may represent a new genus.

Correspondence between T-RFLP and culture

Only 7 of the 23 TRFs that were encountered in this study were representative for only one species (i.e. there were only 7 unique TRF lengths) and *G. vaginalis*, *A. vaginae* and some of the most abundantly present *Lactobacillus* species (*L. crispatus*, *L. gasseri* and *L. iners*) do not present with a unique TRF. For example, the TRF of *L. gasseri* is identical to that of *L. iners*, the TRF of *G. vaginalis* has the same length as that of *Bifidobacterium* spp. and *Propionibacterium acnes* and the TRF length of *A. vaginae* is identical to that of *A. parvulum* and of a species of the genus *Peptoniphilus* (Table 1). Therefore, T-RFLP analysis was compared with the results obtained after anaerobic culture on a Schaedler based blood agar, and with species specific PCR for *G. vaginalis* and *A. vaginae*, to evaluate its specificity.

L. gasseri and *L. iners* have the same TRF length, which was found in 152 samples (Table 6). Of these, *L. gasseri* was cultured in 60 cases, *L. iners* in 48 cases, both in 11 cases and none in 33 cases.

In vaginal specimens from which *L. crispatus* could be cultured (112), *L. crispatus* was also reliably detected by T-RFLP in 108 (96.4%), i.e. there was 96.4% agreement of T-RFLP with culture-positive specimens (Table 6). Among vaginal specimens from which *L. crispatus* was not cultured (188), *L. crispatus* was still detected by T-RFLP in 39 (20.7%), i.e. 79% agreement of T-RFLP with culture-negative specimens. Compared to culture, T-RFLP consequently has a comparable specificity for the detection of *L. crispatus*, but a significantly higher estimated sensitivity (97.4 versus 74.1%) (p = 0.005).

In contrast, in vaginal specimens from which *L. jensenii* could be cultivated (82), *L. jensenii* was detected by T-RFLP in 52 (63.4%), i.e. 46.6% percent agreement of T-RFLP with culture-positive specimens (Table 6). Among the 218 vaginal specimens from which *L. jensenii* was not cultured, *L. jensenii* was detected by T-RFLP in 10 (4.6%), i.e. 95.4% agreement of T-RFLP with culture-negative specimens. T-RFLP therefore presents with a comparable specificity but with a significantly lower estimated sensitivity (67.1 versus 89.1% for culture) for the detection of *L. jensenii* (p < 0.0001).

Five *Lactobacillus* species were cultured which could not be detected by T-RFLP (Table 5).

The TRF 23.5-25.6 (*A. vaginae*, *A. parvulum* or *Peptoniphilus* sp.) was present in 20 samples. Based on differences in the length of the restriction fragments resulting from partial digestion – being approximately 220 bp for *A. vaginae* and 120 bp for *Peptoniphilus* sp. – we noted that in 9 samples (three each of grade I-like and III, two of

grade II and one of grade Ib) both *Atopobium* sp. and *Peptoniphilus* sp. were present, while only *Peptoniphilus* sp. was present in one sample (grade I-like) and only *Atopobium* sp., in three samples (two grade II, one grade III). In the 7 remaining samples no partial peaks were observed (due to low peak intensity).

The correspondence between the presence of the TRF *G. vaginalis*/*Bifidobacterium* sp. and the true presence of *G. vaginalis* is presented in the section 'species specific PCR'.

Species specific PCR for *A. vaginae* and *G. vaginalis*

Since the TRF for *A. vaginae* and *G. vaginalis* can correspond to several species (Table 2) and since amplification with universal 16S rRNA gene primers may be biased towards abundantly present species or species of which the 16S rRNA gene is more efficiently amplified, species specific PCR for *A. vaginae* and *G. vaginalis* was carried out to verify the obtained results with T-RFLP.

G. vaginalis

The sensitivity of the 16S rDNA primer set GV10f-534r, used for T-RFLP, and of the rRNA spacer primer set G_Z [12, 38] was evaluated using the *G. vaginalis* reference strain LMG 7832^T. The PCRs were positive when respectively 1800 and 200 colony-forming units of *G. vaginalis* were present in the DNA extract from the vaginal swab (i.e. when respectively 180 and 20 cfu were present in the PCR reaction).

G. vaginalis species specific PCR was positive for 113 samples belonging to 64 women. Of these, 26 were grade Ia (i.e. 32.9% of grade Ia samples), 39 grade Ib (33.9%), 13 grade Iab (32.5%), nine grade I-like (39.1%), 16 grade II (61.5%) and nine grade III (100%). Eleven women were positive at each time point tested, of which six always had a grade I vaginal smear, whereas the other five women had at least two non grade I vaginal smears.

Of the 59 samples that had the TRF 223.9-225.7, which represents *G. vaginalis*, *B. breve*, *B. bifidum*, *B. dentium* or *P. acnes* or which can result from partial digestion of *B. longum*, 16 were negative for the *G. vaginalis* species specific PCR, which is more sensitive than T-RFLP. Neither could *G. vaginalis* be cultured from these 16 PCR negative samples. For six of these PCR negative samples (three each of grade I-like and II) *B. breve* and/or *B. dentium* were cultured and from one grade IV sample [*B. biavatii*] was isolated.

A. vaginae

The sensitivity of both the universal and specific 16S primer sets 10f-534r and ato167f-ato587r was evaluated using the *A. vaginae* reference strain CCUG 38953[T]. Both PCRs were positive when 400 colony-forming units of *A. vaginae* were present in the DNA extract from the vaginal swab (i.e. when 40 cfu were present in the PCR reaction).

Fifty-nine of the 300 vaginal samples were positive when PCR with *A. vaginae* species specific primers was carried out. Only 11 of these, of which respectively two grade Ib samples, three grade II and six grade III samples, were also positive with T-RFLP, which uses the universal primer set. Nine of the 20 samples that showed the TRF 23.5-25.6, representing *A. vaginae*, *A. parvulum* or *Peptoniphilus* sp., were negative after PCR with *A. vaginae* species specific primers.

Of the 59 positive samples, belonging to 41 women, 16 were scored as grade Ia (20.2% of grade Ia samples), 7 as grade Iab (17.5%), 19 as grade Ib (16.5%), one as grade I-like (4.3%), six as grade II (23.1%), eight as grade III (88.9%) and one as grade IV. Of these 59, 41 samples from 32 women were also positive after amplification with the *G. vaginalis* specific primer set. Twelve of 13 double positive grade Ia smears (16.5%) belonged to women who had a grade I smear at the three time points while only one woman with a double positive PCR at the third time point had a grade II smear at the previous time point. While the percentage double positive PCRs was 88.9% for grade III smears, it was less than 16.5% for all other grades, including grade I-like (4.3%) and grade II (11.5%) smears. Respectively 10, 13 and 18 women were positive for both species specific PCRs in the first, second and third trimester of their pregnancy. Only two of 100 women scored positive for both species specific PCRs at each time point. The vaginal smears of one of these two women were all scored as grade I (Ib or Iab), while the other woman had a grade III smear in all trimesters.

Co-colonization of *L. gasseri* and *G. vaginalis*

In general, when the TRF *G. vaginalis/Bifidobacterium* was present (59 samples), *L. gasseri* was cultured 35 times (59.3%), but *L. iners* only 13 times (22.0%) and *L. crispatus* only 4 times (6.8%). In addition, *L. gasseri* was statistically more frequently cultured from samples with the TRF *G. vaginalis/Bifidobacterium* spp. and *L. gasseri/L. iners* (38/48, 79.2%) than in samples with only the *L. gasseri/L. iners* TRF (37/104) (p < 0.001) (Table 7). To the opposite, *L. iners* was statistically less frequently cultured from samples with the TRF *G. vaginalis/Bifidobacterium* spp. and *L. gasseri/L. iners* (12/48,

25%) than in samples with only the *L. gasseri/L. iners* TRF (47/104)(p < 0.025) (Table 8), as is even more the case for *L. crispatus*.

The composition of the microflora in relation to advancing gestational age

According to Gram stained smear, 73 of 100 women had the same microflora during all three trimesters, of which 68 had grade I, one had grade I-like, two had grade II, one had grade III and one had grade IV at each time point (Table 9). Fourteen of 23 women (60.9%) who had a non grade I smear at the first time point had a grade I smear towards the end of gestation. While only one smear each of women with grade Ia or Iab smears at the beginning had a non grade I smear at the end of gestation, 7 smears of women who started with a grade Ib smear changed towards non grade I and another 4 Ib smears changed back and from between grade I and non grade I.

Based on T-RFLP results, women colonized by *L. crispatus* in the first trimester (n = 42) were found more often to have a smear scored as grade I (a, ab or b) during the last trimester (39/42, PPV = 0.93 (95%CI = 0.79, 0.98) in contrast to women colonized by *L. gasseri* or *L. iners* but not *L. crispatus* (34/47, PPV = 0.74 (95%CI = 0.60, 0.85)) whereas the presence of the TRF *G. vaginalis*/*Bifidobacterium* and/or the TRF of *A. vaginae* at the first time point (23 women) resulted in 69.5% chance of having a non grade I microflora at the third time point. In accordance, culture results revealed that only 3.7% of the 27 women that harbored *L. crispatus* in the first trimester had a non grade I microflora in the third pregnancy trimester, while respectively 25.0% of 32 women and 17.4% of 23 women harboring *L. gasseri* and *L. iners* at the first time point had a non grade I microflora at the end of gestation.

IV.3.5. Discussion

Introduction

Current knowledge on bacterial vaginosis-associated disease in pregnancy is largely based on vaginal microflora assessment at a single point in time, most often during the mid-trimester [10]. To our knowledge, this is one of the few studies which includes sampling during each trimester of pregnancy. Our study indicates that in about 70% of pregnancies sampling at a single time point may be appropriate, but also that for 30% of pregnant women the composition of the microflora fluctuates during pregnancy, in accordance with previous reports [5, 7, 9].

Furthermore, the comparison of culture-independent techniques with Gram stain and culture, as carried out in this study, substantiates the idea that in some situations vaginal microflora cannot adequately be classified using standard clinical and/or Gram stain criteria [16]. In this study we evaluated whether terminal restriction fragment length polymorphism analysis (T-RFLP) provides a more objective and informative assessment of the vaginal microflora.

Finally, our data enable to refine the notion that more precise identification of the different *Lactobacillus* species is essential for a better understanding of the vaginal micro-ecology and its role in preterm birth.

T-RFLP and other culture-independent methods for the characterization of the vaginal microflora

The characterization of species colonizing the vagina by T-RFLP using the universal primers 10f, GV10f and 534r results in fingerprints with few peaks for normal and disturbed vaginal microflora. This is comparable with the limited number of different bands observed by 16S rDNA-DGGE [16, 17], but in contrast with the several different species found by culture [e.g. 42, 23 and this study] and by cloning studies [12-14, 43]. This difference may be explained in several manners. First, T-RFLP and DGGE profiles are primarily a reflection of the numerically most abundant species present in a complex microflora because – assuming equal amplification efficiency – the most abundantly present species will be amplified preferentially.

Second, another problem of T-RFLP, also possibly resulting in an underestimation of the species diversity of the vaginal microbial community, is the presence of species with a TRF of the same length. DGGE suffers from a comparable drawback. In this study, this problem was encountered primarily for *G. vaginalis*, for which the TRF has the same length as that of most *Bifidobacterium* species. To assess the true presence of *G. vaginalis* in the 59 samples for which this TRF was observed we compared the results of T-RFLP, with those of culture and of a *G. vaginalis* species specific PCR.

The presence of *G. vaginalis* as assessed by T-RFLP was confirmed for 43 samples (72.9%) by species specific PCR and 14 samples (23.7%) were found to harbor *Bifidobacterium* spp., based on culture, whereas nine samples (15.3%) possibly harbored another species with the same TRF as *G. vaginalis* and *Bifidobacterium* spp., since they were negative for both *G. vaginalis* specific PCR and *Bifidobacterium* culture.

Third, 'universal' primers for the amplification of bacterial 16S rRNA genes may not be so universal, as was noted already in previous studies [e.g. 12, 14, 44, 46]. In the present study we tried to circumvent this primer problem by using mixtures of primers ensuring more complete coverage of the different bacterial groups.

Despite these limitations, T-RFLP offers a good compromise between sample throughput and information obtained for each sample. The value of culture-independent techniques becomes apparent from the fact that different groups using different culture-independent methods and/or different universal primers [11-14, 16, 17, 20, 43] established a comparable composition of the vaginal microflora.

Correspondence between T-RFLP analysis and Gram staining

In a previous study we showed that more refined criteria for the categorization of Gram stained vaginal smears corresponded well with culture and species specific PCR (for *G. vaginalis* and *A. vaginae*) results. As a result, it can be stated that smears categorized as grade Ia (with short plump lactobacilli) primarily contain *L. crispatus*, whereas grade Ib smears (with long tender lactobacilli) primarily contain *L. gasseri* and/or *L. iners*.

In this study we showed that there is a good correspondence between T-RFLP results and Gram stain based grading, whereby 94% of the grade I samples contain TRFs of lactobacilli only – and whereby indeed grade Ia categorized smears mainly contain the TRF *L. crispatus* whereas grade Ib smears mainly contain the TRF *L. gasseri/L. iners*. Also, grade II and III samples frequently contain the TRFs for *A. vaginae* and *G. vaginalis*, frequently in combination with TRFs of lactobacilli – mostly the TRF *L. gasseri/L. iners*. In addition, 5 of the 7 grade IV samples are characterized by the presence of the TRF for group B streptococci (*S. agalactiae*) and grade I-like samples, mostly contain the TRF *G. vaginalis/Bifidobacterium*.

Although more than 39 different T-RFLP fingerprints were distinguished, two algorithms each for the prediction of respectively grade I and non grade I smears could be established. In summary, the presence of only *Lactobacillus* TRFs was predictive for a grade I vaginal microflora (94%), while the presence of the TRF *G. vaginalis/Bifidobacterium* and/or the TRF *A. vaginae* was predictive for a non grade Ia smear (96.9%). Grade I subgrade-specific patterns were also found: most grade Ia and grade Iab smears are characterized by

either the presence of the TRF *L. crispatus* only (or in combination with the TRF *L. jensenii* only), and grade Ib smears are usually characterized by the presence of the TRF *L. gasseri/L. iners* only (or in combination with the TRF *L. jensenii* only). Due to the limited number of grade I-like, grade II and grade III smears, no grade-specific T-RFLP patterns could be definitely established and it remains to be confirmed whether the presence of both the TRF *G. vaginalis/Bifidobacterium* and the TRF *L. gasseri/L. iners* might predict grade II smears while the presence of these TRFs together with the TRFs of *A. vaginae* and *P. bivia* might be predictive for grade III smears.

Correspondence between T-RFLP and culture

Although molecular-based tools were developed in part to avoid the bias of culture-based techniques, it has been suggested that 16S rDNA-based molecular techniques may overemphasize the same organisms because bacteria with high rRNA gene copy number are also most easily cultured [45, 47]. We could not confirm this statement for the rather fastidious *L. crispatus* and the easily cultured *L. jensenii*. To the contrary, while *L. crispatus* is a slowly growing organism on Schaedler based agar and often appears as satellite colonies around other lactobacilli, 96.4% of 112 cultured isolates were confirmed by T-RFLP and an additional 39 women were found to harbor *L. crispatus* as assessed by T-RFLP. Therefore, this technique might better represent the true presence of this species in vaginal microflora. On the other hand also, the presence of *L. jensenii,* producing large colonies on Schaedler based agar, might be overestimated based on culture since only 63.4% of 82 cultured isolates were confirmed by T-RFLP and since only 10 additional *L. jensenii* harboring women were found by T-RFLP.

Correspondence between T-RFLP and species specific PCR

The results with sensitive *G. vaginalis* specific PCR (able to detect 200 cfu per swab) show that this species is present in approximately 30% of grade I smears, but goes largely undetected by smear based grading and by T-RFLP. This confirms that *G. vaginalis* may be stably present in low numbers in normal vaginal microflora [38, 48, 49]. From our data, it also follows that the sensitivity of this primer set may be too high to be useful as a diagnostic tool. T-RFLP was better suited for diagnostic purposes since with this technique (able to detect 1800 cfu of *G. vaginalis* per swab) only two women with a grade Ia microflora were found to harbor *G. vaginalis*.

Although both the species specific PCR and T-RFLP PCRs have equal sensitivity for detection of *A. vaginae* starting from pure cultures, more *A. vaginae* positive vaginal

microflora were found with species specific PCR (19.7%) than with T-RFLP (6.7%). This study confirms that in the vaginal microflora of the majority of women with bacterial vaginosis *A. vaginae* is present in high concentrations [12, 21, 50]. However, our present results indicate that – in contrast to the results of these previous studies – that not only *G. vaginalis* and other bacterial vaginosis-associated organisms, such as *Peptostreptococcus* spp. and gram-negative anaerobes, but also *A. vaginae* may be stably present in low numbers in normal vaginal microflora. Nevertheless, detection with species specific PCR of the presence of *A. vaginae* with the purpose of assessing true bacterial vaginosis (grade III) is more accurate (0.83, 95%CI = 0.78, 0.87) than detection of the presence of *G. vaginalis* (0.65, 95%CI = 0.60, 0.71), because *G. vaginalis* is more frequently present in non grade III samples than *A. vaginae*.

As is the case for the *G. vaginalis* specific primer used in this study, the sensitivity of the *A. vaginae* specific primer set may be too high to be useful as a diagnostic tool and T-RFLP might be better suited for diagnostic purposes since no women with a grade Ia microflora were found to harbor *A. vaginae*, according to T-RFLP. However, because species specific PCR confirmed in only 55.0% of the cases that the TRF observed for *A. vaginae* was indeed caused by the presence of this species, and since the TRF of *A. vaginae* is a rather small fragment, possibly resulting in loss during precipitation of the restriction reaction mixture before capillary electrophoresis, T-RFLP conditions may have to be optimized for the detection of *A. vaginae*.

Colonizing lactobacilli with advancing gestation as assessed by T-RFLP

According to the data presented here and in literature [51, 52, 58-60], better understanding of vaginal micro-ecology and the role of vaginal microflora in preterm birth also depends on the ability to differentiate between the different *Lactobacillus* species. T-RFLP confirmed the presence of lactobacilli in non grade I microflora, a finding that has been reported earlier in both culture based [48, 53, 54] and culture-independent studies [14, 17, 38, 49]. Most striking was the presence of the TRF *L. gasseri/L. iners* in 81% of the TRF fingerprints with the TRF *G. vaginalis/Bifidobacterium*. Unfortunately *L. gasseri* and *L. iners* are few of the *Lactobacillus* species that cannot be differentiated by restriction with *Bst*UI, but our culture results confirmed that *L. gasseri* was present in 59.3% of the women that had a TRF fingerprint with the TRF *G. vaginalis/Bifidobacterium* present, while *L. iners*, *L. jensenii* and *L. crispatus* were isolated from respectively only 22.0%, 22.0% and 6.7% of these women. Also, the fact that *L. gasseri* could be cultured

significantly more from TRF *G. vaginalis/Bifidobacterium* positive samples than from TRF *G. vaginalis* negative samples, in opposition to *L. iners*, is a semi-quantitative indication that *L. gasseri* is present in higher numbers when *G. vaginalis* is present. These results confirm a previous culture-independent study that demonstrated that in 19 postmenopausal women under hormone replacement therapy only 9% of the samples containing either *L. crispatus* or *L. iners* contained also *G. vaginalis* while 67% of the samples containing *L. gasseri* also contained *G. vaginalis* [17]. However, in another culture-independent study *L. iners* was the only *Lactobacillus* species present in bacterial vaginosis microflora [14].

Our results may explain the at first sight contradictory observation of a previous report that the quantitative counts for *Lactobacillus* (without further specification to species level) did not vary significantly among the three defined groups of vaginal microflora [53]. Possibly an explanation for our findings may be in accordance with the reports of previous studies which showed that *L. crispatus* (and *L. jensenii*) are stronger hydrogen peroxide producers than *L. gasseri* and *L. iners* [51, 55] and studies which demonstrated that there did appear to be a decrease in the number of H_2O_2-producing *Lactobacillus* in the bacterial vaginosis group [48, 53, 56]. Others reported that 30% of the women with a normal vaginal microflora are colonized with non hydrogen peroxide producing lactobacilli [57].

Indeed, while *L. crispatus* was clearly the *Lactobacillus* species associated with a grade I vaginal microflora (93.2%), this was less the case for *L. gasseri* and *L. iners* (71.1%). Moreover, these latter species occurred in 21 of 26 grade II (80.8%) and in 7 of 9 of grade III (85.6%) samples, whereas *L. crispatus* was never observed in grade III samples and in only 19.2% of grade II samples.

While *L. iners* is known as the weakest H_2O_2-producer [51, 55] it was surprising that *L. gasseri* was the lactobacillus that was most accompanied by the TRF *G. vaginalis/Bifidobacterium.* Moreover, follow-up during pregnancy revealed that a woman that was colonized by *L. gasseri* in the first pregnancy trimester was more likely to obtain a non grade I microflora later in pregnancy. These findings suggest that presence of *L. gasseri* does not correspond with a normal vaginal microflora or at least that *L. gasseri* confers only little colonization resistance.

Already in 1991, Påhlson and Larsson [58] pointed to the presence of different species of lactobacilli and their different association with different clinical symptoms. For example, a common mild form of vaginitis, known as cytolysis, was characterized by massive

overgrowth of homogeneous long or homogeneous short lactobacilli, which never produce hydrogen peroxide. Another form consisted of curved lactobacilli, which always produced hydrogen peroxide, but could be mistaken for *Mobiluncus*.

Horowitz *et al.* [59] previously described a form of vaginal discharge as 'vaginal lactobacillosis', a condition whereby extremely long lactobacilli (60 µm on average) were observed in the smears of 37 women, suffering from thick, white, creamy or curdy vaginal discharge, associated with vaginal itching, burning and irritation. These symptoms recurred each cycle 7 to 10 days before menses, reaching a peak shortly before menses and abating after menses, with an average length of duration of 23 months prior to inclusion in the study. The vaginal pH was 4.5. Fungal cultures remained mostly negative although prior diagnosis was candidiasis for 30 of the 37 women.

Platz-Christensen *et al.* [60] hypothesized that the longest type of lactobacilli encountered in their study, designated fusoform type 4 and identified by these authors as *L. gasseri*, corresponded to the lactobacilli observed by Horowitz *et al.* [59], although the cell length of the bacilli observed by Platz-Christensen *et al.* [60] was determined to measure between 8 and 20 µm.

Other studies have shown that *L. gasseri* more frequently colonizes the rectum, whereas *L. crispatus, L. jensenii,* and *L. iners* colonize predominantly the vagina [51, 61].

Most intriguing is the recent observation that the healthy vaginal microflora of Nigerian women is dominated by *L. crispatus* in only 4% of the subjects, while 64% were colonized by *L. iners*, followed by 8.3% colonized by *L. gasseri* [18]. This absence of *L. crispatus* in the majority of women might be a possible explanation for the high prevalence of bacterial vaginosis among African women [62]. If this association between the wrong lactobacilli and higher risk for bacterial vaginosis can be confirmed, and given the fact that bacterial vaginosis has been shown to increase the infection risk with HIV [57], one can hypothesize that the development and generalized use of vaginal probiotic lactobacilli might contribute to decreasing the risk of acquisition of HIV and other STDs in developing countries.

Co-colonization of *Lactobacillus* species as assessed by T-RFLP

Since it has been shown that women harboring both H_2O_2-producing and non H_2O_2-producing lactobacilli are at increased risk for preterm delivery [63], it might be important to have an estimate of the degree of co-colonization of lactobacilli.

Our estimate of co-colonization varied according to the technique: using T-RFLP we established 71.3% of the samples with only one species of *Lactobacillus*, whereas culture revealed 48.0%. The rather high percentage of samples that harbored more than one *Lactobacillus* species, respectively 25.4% as estimated by T-RFLP and 40.7% by culture, is in agreement with previous culture-independent studies [13, 14, 43] but are in contradiction with previous culture based [19, 48, 51] and a culture-independent study [11], that reported that a minority of women were co-colonized by more than one *Lactobacillus* species.

In a previous study, by cloning the 16S rRNA genes of the vaginal microflora of eight women we found that all three women with a grade I microflora harbored more than one *Lactobacillus* species and we noted a large difference in concentration of the *Lactobacillus* species present [12]. These data are consistent with previous findings that found that the totality of the *Lactobacillus* species on the vaginal epithelium is more complex than reported thus far [13, 14, 43].

IV.3.6. Conclusions

In conclusion, in this study we used a non culture based technique, T-RFLP, to corroborate the findings reported previously, whereby we refined Gram stain based grading of vaginal smears by comparison with culture [23]. Although T-RFLP and culture results were not entirely in agreement, both techniques corresponded rather well, and reinforced the value of Gram stain based grading of smears according to the criteria of Claeys [23].

tDNA-PCR-based identification allows a more accurate identification of lactobacilli and most other vaginal bacterial species, but relies on culture to obtain pure isolates, whereas T-RFLP allows a more rapid, non-culture dependent characterization of the complex vaginal microflora. Therefore, the latter technique can be used to process large sets of samples.

Each technique contributes to establishing the composition of complex microbial populations. Both culture and molecular techniques have their advantages and limitations when studying the composition of the complex vaginal microflora. When performed under proper incubation conditions and on the appropriate (selective) media, culture may be more sensitive than (universal) molecular techniques. Even though molecular techniques have allowed the identification of species as *L. iners* and *A. vaginae*, one must

continue searching for selective media for fastidious species so that extensive characterization will become possible and diagnosis can be improved.

The association of *L. gasseri* with *G. vaginalis* observed by comparing molecular methods and detailed microscopic study of vaginal smears has implications for the basic understanding of the vaginal microflora and bacterial vaginosis. This finding indicates the importance of combining Gram-stain with PCR-based identification for the diagnosis of bacterial vaginosis when carrying out comparative studies, when evaluating therapy outcome or when studying the etiology of bacterial vaginosis.

Notwithstanding that the sample size of this study does not allow to make an association between observed TRF fingerprints and pregnancy outcome, the techniques presented in this study allow to perform more studies among pregnant women to assess the changes in vaginal microflora and in prevalence of bacterial vaginosis during gestation.

IV.3.7. Authors' contributions

RV, GC, GV, EDB and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. LVS and CDG participated in the analysis of the study samples. All authors read and approved the final manuscript.

IV.3.8. Acknowledgements

IV.3.9. References

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IV.3.10. Figure and Tables

Figure 1. Examples of T-RFLP fingerprints of samples classified into different grades according to the criteria of Claeys (Verhelst 2005)

T-RFLP analysis following PCR amplification of the 16S rRNA genes of total vaginal bacterial communities obtained by swab, show the culture-independent relative abundance of distinct bacterial species within the vaginal microflora according to Gram stain category. The TRF *L. crispatus* is present in the majority of grade Ia microflora (panel A). Grade Iab smears contain TRFs of several *Lactobacillus* species (panel B). Grade Ib microflora are dominated by the TRF *L. gasseri/L. iners* (panel C). Grade II and grade III microflora are primarily represented by the TRF *G. vaginalis/Bifidobacterium and the TRF L. gasseri/L. iners* (panel D and E). Grade IV contains a TRF characteristic for *S. agalactiae* (group B streptococci) (panel F). Species names between brackets represent restriction fragments resulting from partial digestion.
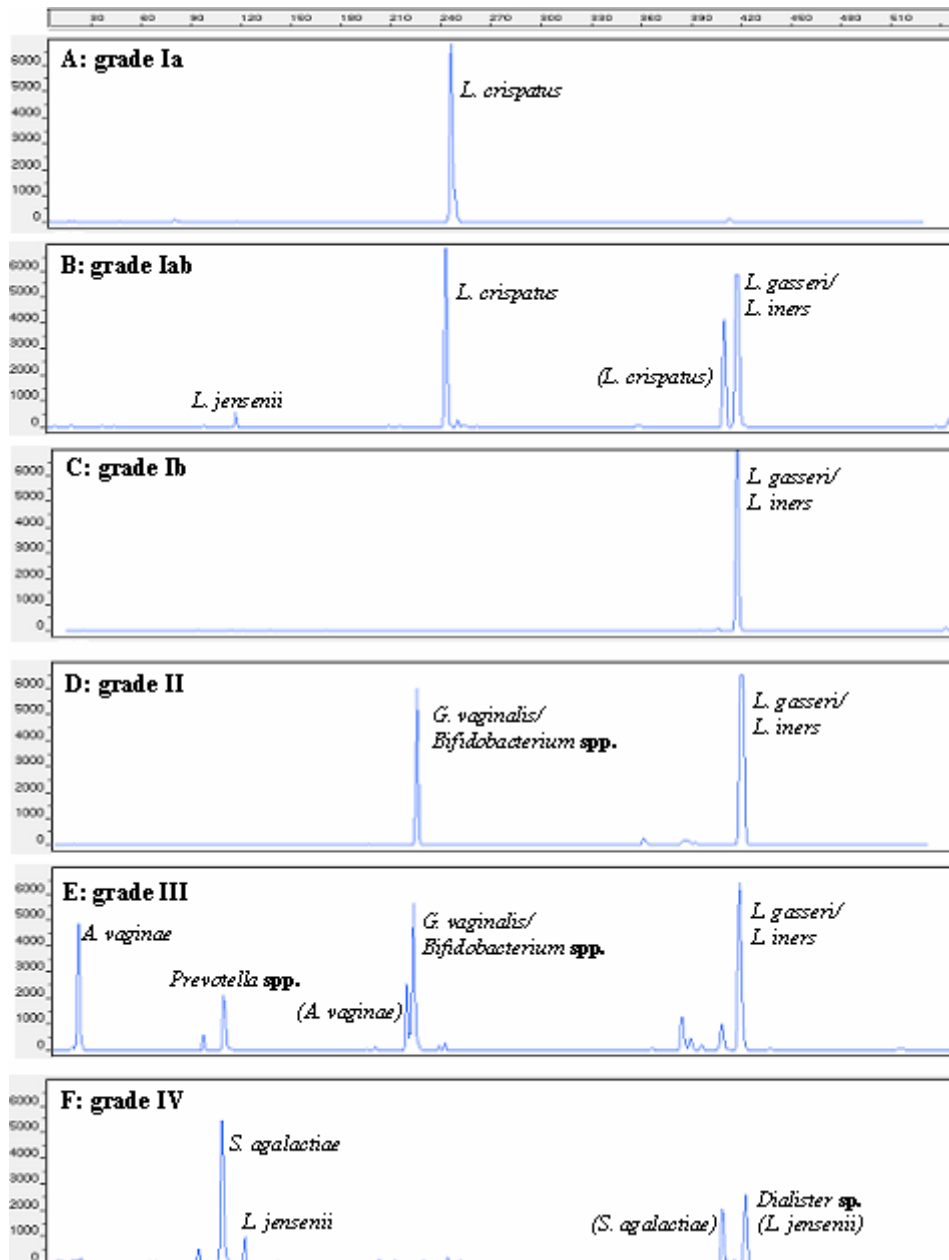
Table 1. T-RFLP library with terminal restriction fragment lengths (TRFs) representative of vaginal bacterial species

Range of TRF-lengths (bp)	n[a]	Species
23.5-25.6	3	*Atopobium vaginae*,[b] *A. parvulum*, *Peptoniphilus* sp.
53.2-54.4	3	*Lactobacillus coleohominis*, *L. fermentum*, *L. mucosae*
106.1-106.5	1	*Streptococcus* sp.
107.6-110.2	9	*Streptococcus agalactiae*, *Bifidobacterium biavatii*, *Streptococcus* sp., *Veillonella* sp.
112.0-112.7	4	*Prevotella bivia*, *P. buccalis* 96.6%,[c] *P. bivia* 91%, uncultured *Megasphaera* sp. clone
115.2-116.1	1	*Streptococcus anginosus* group
120.3-120.9	4	*Anaerococcus tetradius*, *Finegoldia magna*, *Pseudoramibacter alactolyticus*, *Peptoniphilus* sp.[d]
122.2-123.6	1	*Lactobacillus jensenii*
205.6-207.0	1	*Bifidobacterium longum*
219.8-220.7	5	*Corynebacterium* sp., *Dermabacter hominis*, *Propionibacterium avidum*, *A. vaginae*, *S. agalactiae*
229.3-231.9	6	*Staphylococcus* spp., *Varibaculum cambriensis*
223.9-225.7	6	*Gardnerella vaginalis*, *Bifidobacterium breve*, *B. bifidum*, *B. dentium*, B. longum, *Proponibacterium acnes*
241.2-243.2	3	*Enterococcus faecalis*, *Lactobacillus casei*, *L. nageli*
244.6-245.1	3	*Lactobacillus delbrueckii*, *L. salivarius*
245.8-248.2	3	*Lactobacillus crispatus*, *L. kalixensis*, *L. kitasatonis*
248.7-250.3	1	*Lactobacillus rhamnosus*
257.2-258.4	2	*Lactobacillus vaginalis*, *L. oris*
388.0-388.8	1	*Escherichia coli*, G. vaginalis
395.9-397.1	2	*Aerococcus christensenii*, *Treponema* sp.
411.0-411.7	3	*Dialister* sp., L. crispatus, L. jensenii
415.8-419.7	2	*Lactobacillus gasseri*, *L. iners*
420.4-421.1	1	*Lactobacillus reuteri*
422.9	1	*Lactobacillus pontis* 94%

a. Number of species within this TRF length range that are present in the T-RFLP library composed of 81 vaginal species.

b. Bold species names show the species most frequently found by culture from samples with this TRF.

c. Percentages indicate the percentage of 16S rRNA gene sequence similarity with the closest match in GenBank.

d. Underlined species names indicate that the corresponding TRF-length is the result of partial digestion.

Table 2. T-RFLP fingerprints in relation to Gram stain based grading of 300 vaginal samples from 100 pregnant women

Species						Grade								Total
L. crispatus	L. jensenii	L. gasseri/ L. iners	G. vaginalis/ Bifidobacterium	A. vaginae	Other	Ia 79	Iab 40	Ib 115	I-like 23	II 26	III 9	IV 7	0 1	300
x						63	20	17	1					101
x	x					8	9	2	1					20
x		x				1	2	2	1					6
x	x	x				1	1	2						4
x		x			L. vaginalis			1						1
x		x			L. vaginalis, L. delbrueckii			1						1
	x							2						2
x	x					1		15		1				17
		x				2	1	54	5			2	1	65
		x			L. vaginalis			1						1
		x			L. reuteri			1						1
x		x				1		1	1	2				5
	x	x						1		1				2
		x			P. bivia				1					1
x		x						1		5				6
		x	x			1	3	7	1	8	1			21
			x	x	Other				1					1
		x	x	x	S. agalactiae, other				2					2
			x	x	Other				1		1			2
			x	x	P. bivia, other					2	4			6
		x	x	x				1			1			2
			x	x	L. vaginalis				1					1
	x		x	x	L. casei, other				1	1				2
			x	x					1		1			2
			x	x	Staphylococcus sp.					1				1
			x	x	S. agalactiae, other					1	1	1		3
			x	x	Other				2					2
				x	L. nagelii					1				1
		x						2						2
	x			x	S. agalactiae, L. nagelii			1		1				2
		x	x		L. vaginalis, other		2	1						3
		x	x		S. agalactiae, other					1				1
			x	x	Other	1				1				2
x		x			E. coli		1							1
x		x			S. anginosus		1							1
		x		x	S. agalactiae, other				1			4		5
					L. vaginalis, L. delbrueckii, other			1						1
			x		S. agalactiae, other				1					1
					Other			1						1
					No bacteria				1					1

Table 3. Terminal Restriction Fragments (TRFs) present in relation to Gram stain based grading of 300 vaginal samples from 100 pregnant women

	Vaginal smears graded by Gram stain							
	Ia	**Iab**	**Ib**	**I-like**	**II**	**III**	**IV**	**0**
Number of samples	79	40	115	23	26	9	7	1
Range of number of TRFs/ T-RFLP fingerprint	1-3	1-4	1-4	0-6	2-6	2-7	1-3	1
One TRF only per TRF fingerprint (%)	82.3	52.5	64.3	30.4	0.0	0.0	28.6	100.0
Only TRF *L. crispatus* present (%)	79.7	50.0	14.8	4.3	0.0	0.0	0.0	0.0
TRF *G. vaginalis/Bifidobacterium* present (%)	2.5	7.5	12.2	52.2	80.8	100.0	14.3	0.0
TRF *G. vaginalis/Bifidobacterium* and TRF *L. gasseri/L. iners* present (%)	2.5	7.5	9.6	30.4	65.4	77.8	0.0	0.0

Table 4. Number of *Lactobacillus* species detected per sample in relation to method used for 300 vaginal samples

Number of *Lactobacillus* species detected	Method			
	T-RFLP		**Culture**	
	n	**%**	**n**	**%**
0	10	3.3	34	11.3
1	214	71.3	145	48.3
2	65	21.7	109	36.3
3	11	3.7	11	3.7
4	0	0.0	2	0.7

Table 5. Number of samples from which the different species were cultured, in relation to grade of smear

Species	Vaginal smears graded by Gram stain								
	Ia	Ib	Iab	I like	II	III	IV	0	Total
	79	115	40	23	26	9	7	1	300
***Lactobacillus* spp.**									
Lactobacillus casei		1		1					2
Lactobacillus coleohominis	1	9	2						12
Lactobacillus crispatus	67	13	31	1			1		113
Lactobacillus delbrueckii		1		1					2
Lactobacillus fermentum	1	1	2						4
Lactobacillus gasseri	5	35	8	13	17	1	2		81
Lactobacillus iners		46	3	4	6	4			63
Lactobacillus jensenii	19	26	20	3	12	1	1		82
Lactobacillus mucosae					1				1
Lactobacillus nagelii					1				1
Lactobacillus oris					1				1
Lactobacillus pontis 94%		1							1
Lactobacillus reuteri		2		1					3
Lactobacillus rhamnosus		1		3	2	1	1		8
Lactobacillus salivarius		1							1
Lactobacillus vaginalis	8	12	3	3	2		1		29
***Bifidobacterium* spp.**									
[*Bifidobacterium biavatii*]				2					2
Bifidobacterium bifidum		1							1
Bifidobacterium breve				6	4	2			12
Bifidobacterium dentium				3					3
Bifidobacterium longum	1	1		2		1			5
Bifidobacterium sp.					1				1
Bacterial vaginosis related									
Actinomyces neuii					1	1			2
Aerococcus christensenii					2				2
Anaerococcus vaginalis				1					1
Atopobium vaginae					1				1
Bacteroides ureolyticus					1				1
Dialister sp.						2			2
Finegoldia magna			1			1			2
Gardnerella vaginalis		2	1	1	6	6			16
Gemella morbillorium					1				1
Peptoniphilus sp.	1	3		1	1				6
Prevotella bivia				1					1
Varibaculum cambriense						1			1
Other organisms									
Arthrobacter albus					1				1
Clostridium colicanis		1							1
Clostridium sp.						1			1
Corynebacterium amycolatum				1					1
Corynebacterium pseudogenitalium	1								1
Corynebacterium sp.		1							1
Enterococcus faecalis	2	5	2						9
Escherichia coli		1		1					2
Haemophilus influenzae					1				1
Helcococcus sp.						1			1
Pediococcus pentocaseus						1			1
Propionibacterium acnes	3								3
Propionibacterium avidium				1					1
Serratia sp.		1							1
Staphylococcus aureus		1			1				2
Staphylococcus epidermidis		5	1	1	1	1			9
Staphylococcus haemolyticus			1						1
Staphylococcus hominis		1							1
Streptococcus agalactiae	2	5		3		1	4		15
Streptococcus anginosus group	2	3	1	1					7
Streptococcus mitis					1				1
Streptococcus salivarius		1							1
Veilllonella atypica	3	2		1					6
Veillonella sp.					1				1
Unidentified isolates									76

Table 6. Correspondence between culture and T-RFLP

	Culture negative and TRF present	Culture positive and TRF present	Culture positive and TRF absent
L. crispatus	39	108	4
L. jensenii	10	52	30
L. gasseri/ L. iners [a]	33	119	13
G. vaginalis/Bifidobacterium spp. [b]	34	25	7
A. vaginae	19	1	0
S. agalactiae	8	7	8
Prevotella sp.	8	0	1

Legend:
a. *L. gasseri* and *L. iners* have the same TRF length.
b. *G. vaginalis* and *Bifidobacterium* spp. have the same TRF length.

Table 7. Presence of *Lactobacillus gasseri* in samples with the terminal restriction fragment (TRF) *L. gasseri/L. iners* present with or without the TRF *G. vaginalis/Bifidobacterium*.

L. gasseri	TRF GI +[a] TRF GV +	TRF GI +[b] TRF GV -	Total
Cultured	38	37	75
Not cultured	10	67	77
Total	48	104	152

Legend:
a. TRF *L. gasseri/L. iners* and TRF *G. vaginalis/Bifidobacterium* present
b. TRF *L. gasseri/L. iners* present and TRF *G. vaginalis/Bifidobacterium* absent

Table 8. Presence of *L. iners* in samples with the terminal restriction fragment (TRF) *L. gasseri/L. iners* present with or without the TRF *G. vaginalis/Bifidobacterium*.

L. iners	TRF GI + TRF GV +	TRF GI + TRF GV -	Total
Cultured	12	47	59
Not cultured	36	57	93
Total	48	104	152

Legend:
a. TRF *L. gasseri/L. iners* and TRF *G. vaginalis/Bifidobacterium* present
b. TRF *L. gasseri/L. iners* present and TRF *G. vaginalis/Bifidobacterium* absent

Table 9. Changes in the vaginal microflora with advancing gestation as assessed by Gram staining

First trimester	Third trimester						Total 1st
	Ia or Iab	Ib	I-like/II	III	IV	0	
Ia or Iab	28	4	2				34
Ib	12	23	8				43
I-like	2	1	1			1	5
II	3	3	5				11
III	1	2		1			4
IV		1	1		1		3
Total 3rd	46	34	17	1	1	1	100

IV.4. The unrecognized association of <u>Atopobium vaginae</u> with bacterial vaginosis

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Culture-independent analysis of vaginal microflora: the unrecognized association of *Atopobium vaginae* with bacterial vaginosis.

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ELSEVIER

Culture-independent analysis of vaginal microflora: The unrecognized association of *Atopobium vaginae* with bacterial vaginosis

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Although the pathogenesis of bacterial vaginosis remains elusive, a few microorganisms, such as *Gardnerella vaginalis*, are considered markers on Gram stain or culture. Culture-independent analysis of vaginal microflora using 16S rDNA cloning and sequencing of total bacterial communities reveals the gram-positive *Atopobium vaginae* as a predominant species in disturbed vaginal flora. © 2004 Elsevier Inc. All rights reserved.

Bacterial vaginosis (BV) is a polymicrobial infestation of the human vagina, involving a shift from a lactobacilli-dominated microflora towards overgrowth of gram-negative anaerobes.[1] Although not considered a sexually transmitted infection (STI), BV may act as a cofactor to the acquisition of major STIs, such as HIV-1 and *Chlamydia trachomatis*. In pregnancy, BV predisposes to adverse pregnancy outcome, presumably through an ascending infection pathway.[1] The pathogenesis of BV is still elusive, and it remains to be determined which microorganisms are essential to the microbial shift.[1] The human vagina harbors a complex flora, including many fastidious bacteria. Hence, culture-dependent analysis is likely to overestimate the role of cultivable bacteria while uncultivable species may remain overlooked.[2]

We assessed the vaginal species complexity of healthy and disturbed microflora by culture-independent analysis of total vaginal bacterial populations.

Material and methods

Detailed information of microbial communities can now be acquired from the phylogenetic analysis of 16S rDNA sequences obtained from clinical samples by polymerase chain reaction (PCR) amplification.[2] 16S rDNA gene fragments were amplified from a vaginal swab by PCR with universal 16S rDNA primers. The PCR mixture obtained represents all species constituting the vaginal microflora. Within the mixture, each 16S rDNA fragment is unique to a bacterial species, and yields a genomic fingerprint on further processing.[2] Species identification was performed either by cloning

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panel A: Normal flora (grade I flora on gram stain) in a 34-year old women

panel B: Intermediate flora (grade II flora on gram stain) in a 41-year old women

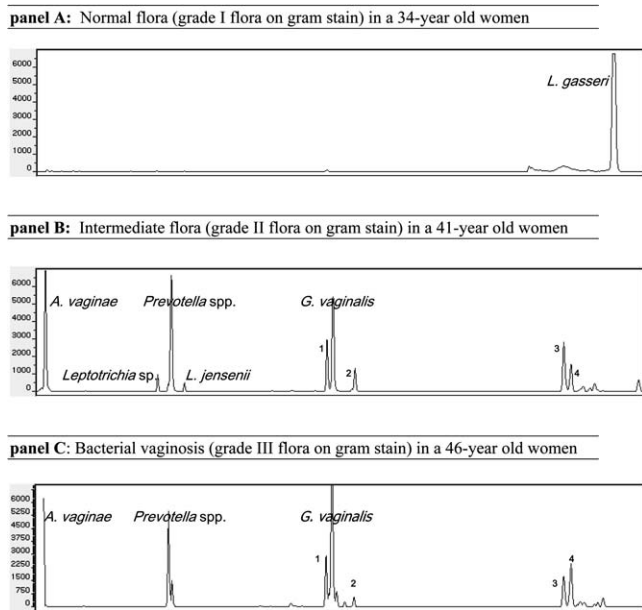panel C: Bacterial vaginosis (grade III flora on gram stain) in a 46-year old women

Figure Terminal restriction fragment length polymorphism (T-RFLP) analysis of total vaginal bacterial communities. T-RFLP analysis after PCR amplification of the 16S rDNA of total vaginal bacterial communities obtained by swab show the culture-independent relative abundance of distinct bacterial species within the vaginal microflora according to Gram-stain category. Grade I flora are dominated by lactobacilli such as *L gasseri* (**A**). Grade II and garde III flora are primarily represented by *Atopobium vaginae*, *Prevotella* spp., and *Gardnerella vaginalis* (**B** and **C**).

1, 2, 3, and 4: partial endonuclease digestion 16S rDNA T-RFLP fragments for A vaginae, P indolicus, A vaginae, and G vaginalis, respectively.

and sequencing the 16S rDNA, or by determining the terminal 16S rDNA fragment length after endonuclease restriction.[3]

The Ghent University Ethics Commission approved the protocol.

Results

Eight healthy nonpregnant women (mean age 41.4 years, range 28 to 51 years) attending our outpatient clinic were selected based on a vaginal Gram stain smear according to Hay et al.[4] We included 3 women with healthy (grade I) flora, and 5 women with intermediate (grade II) flora, or with overt bacterial vaginosis (grade III). 16S rDNA was cloned by inserting each 16S rDNA fragment in an *Escherichia coli* plasmid vector, yielding 100 clones on average (range 70 to 169 clones per patient). Subsequently, the 16S rDNA gene inserts were sequenced and compared with the 16S rDNA gene sequences in the GenBank.

Lactobacilli were predominant in healthy microflora. In disturbed vaginal flora, a single gram-positive anaerobe of the *Coriobacteriaceae* family, recently identified

as *Atopobium vaginae*, was a predominant species.[5] This species has not been reported before in culture-dependent studies.[1,4] Yet, *A vaginae* was present in 4/5 disturbed flora, and numerically dominant in 3, while absent from the 3 normal vaginal samples. We hypothesized that *A vaginae* may represent a previously unrecognized marker of altered vaginal flora (Figure).

To substantiate this, we randomly selected a series of 100 vaginal bacterial communities obtained by swab from healthy pregnant (n = 65) and nonpregnant (n = 35) women attending our outpatient clinic on the occasion of a routine antenatal or gynecologic visit. The prevalence of bacterial vaginosis (grade III) and bacterial vaginosis-like (grade II) microflora was 12.3% (8/65) and 14.3% (5/35), respectively. Genetic fingerprints of these samples were obtained after terminal restriction fragment length polymorphism (T-RFLP) analysis, an established culture-independent technique for the analysis of complex microbial mixtures.[3]

A vaginae was present in 61.3% of disturbed flora (8/13), while uncommon (3.4%) to healthy microflora (3/87) ($P < .0001$). A statistically significant co-occurrence of *A vaginae* and *G vaginalis* was observed, regardless of microscopy grading. Detection of *A vaginae* alone had a sensitivity of 62% (95% CI 0.32-0.85), a specificity of 97% (95% CI 0.90-0.99), and an accuracy of 97% (95% CI 0.90-0.99) for the diagnosis of disturbed microflora.

Comment

By cloning and sequencing 16S rDNA we identified a series of species that have not been described before as (common) members of the vaginal community, including *Atopobium rimae*, *Bifidobacterium biavatii* (*urinalis*), *Dialister* sp., *Leptotrichia amnionii*, and *Sneathia sanguinegens*.

In this sample of women of reproductive age, culture-independent analysis also points at a strong correlation between *A vaginae* and perturbation of the vaginal niche. Phenotypic characterization of this species of previously unknown clinical relevance should further unravel its exact etiologic role, eg, whether *A vaginae* is capable of inhibiting lactobacilli, altering the vaginal environment, and how this species interferes with *G vaginalis*. These ecologic observations may be clinically relevant both from the point of view of pathogenesis and of the diagnosis of bacterial vaginosis.

The pathogenicity of a microorganism does not only depend on the intrinsic virulence of the microorganism, but also on its relative quantitative dominance. Therefore, while the literature on bacterial vaginosis has largely focused on *G vaginalis* in particular, the abundance of *A vaginae* in disturbed vaginal microflora warrants further scrutiny.

Our data suggest that nonculture-dependent molecular techniques, identifying and quantifying known as well as previously unrecognized species like *A vaginae,* may improve our basic knowledge of vaginal ecology. Culture-independent analysis of vaginal microflora may also enhance the clinical management of bacterial vaginosis—associated disease.

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IV.5. *Comparison between Gram stain and culture for the characterization of vaginal microflora*

Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, De Ganck C, De Backer E, Temmerman M, Vaneechoutte M.

Comparison between Gram stain and culture for the characterization of vaginal microflora: Definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora.

Research article

Comparison between Gram stain and culture for the characterization of vaginal microflora: Definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora

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Abstract

Background: The microbiological diagnosis of bacterial vaginosis is usually made using Nugent's criteria, a useful but rather laborious scoring system based on counting bacterial cell types on Gram stained slides of vaginal smears. Ison and Hay have simplified the score system to three categories and added a fourth category for microflora with a predominance of the *Streptococcus* cell type. Because in the Nugent system several cell types are not taken into account for a final score, we carried out a detailed assessment of the composition of the vaginal microflora in relation to standard Gram stain in order the improve the diagnostic value of the Gram stain. To this purpose we compared Gram stain based categorization of vaginal smears with i) species specific PCR for the detection of *Gardnerella vaginalis* and *Atopobium vaginae* and with ii) tDNA-PCR for the identification of most cultivable species.

Results: A total of 515 samples were obtained from 197 pregnant women, of which 403 (78.3%) were categorized as grade I microflora, 46 (8.9%) as grade II, 22 (4.3%) as grade III and 8 (1.6%) as grade IV, according to the criteria of Ison and Hay. Another 36 samples (7.0%) were assigned to the new category 'grade I-like', because of the presence of diphtheroid bacilli cell types. We found that 52.7% of the grade I-like samples contained *Bifidobacterium* spp. while *L. crispatus* was present in only 2.8% of the samples and *G. vaginalis* and *A. vaginae* were virtually absent; in addition, the species diversity of this category was similar to that of grade II specimens.

Based on the presence of different *Lactobacillus* cell types, grade I specimens were further characterized as grade Ia (40.2%), grade Iab (14.9%) and grade Ib (44.9%). We found that this classification was supported by the finding that *L. crispatus* was cultured from respectively 87.0%

and 76.7% of grade Ia and Iab specimens while this species was present in only 13.3% of grade Ib specimens, a category in which *L. gasseri* and *L. iners* were predominant.

Conclusion: Further refinement of Gram stain based grading of vaginal smears is possible by distinguishing additional classes within grade I smears (Ia, Iab and Ib) and by adding a separate category, designated grade I-like. A strong correlation was found between grade Ia and the presence of *L. crispatus* and between grade I-like and the presence of bifidobacteria. This refinement of Gram stain based scoring of vaginal smears may be helpful to improve the interpretation of the clinical data in future studies, such as the understanding of response to treatment and recurrence of bacterial vaginosis in some women, and the relationship between bacterial vaginosis and preterm birth.

Background

Currently the criteria as defined by Nugent *et al.* [1] are considered as the standard procedure to score vaginal smears by Gram stain [2]. This method scores the smears in a standardized manner by quantification of some of the cell types present – designated as *Lactobacillus*, *Gardnerella vaginalis*, *Bacteroides* and *Mobiluncus* 'morphotypes'. However, the Nugent scoring system conflates women with potentially very different vaginal microflora in a single category [3]. Since the method requires considerable time and skill, simpler versions have been described which assess the categories in a more qualitative manner [4-6]. Recent developments in our knowledge of the vaginal microflora – including the observation of different *Lactobacillus* species producing different amounts of hydrogen peroxide [7-9] with a potential effect on pregnancy outcome [10,11] urge to refine the Gram stain criteria in an effort to increase the agreement between Gram stain and the true composition of the vaginal microflora. In addition, a strong association of the metronidazole resistant fastidious anaerobic coccobacillus *Atopobium vaginae* with bacterial vaginosis [12-14] might have important implications in the pathophysiology of bacterial vaginosis related preterm labour and birth. The more accurate allocation of subjects according to vaginal microflora status, as assessed by Gram stain, may enhance the validity of studies on the etiology of bacterial vaginosis, and help to better understand response to treatment and recurrence in some women, as well as its relation to preterm birth.

Here we report our findings obtained by studying a total of 515 vaginal samples by Gram stain, by DNA-based techniques – like cloning and sequencing of amplified 16S rRNA-genes [13-15] and species specific PCR [12,15-18] – which make it possible to detect fastidious bacteria like *A. vaginae* [13,16,17] and by culture in combination with tDNA-PCR [20,21], which allows the rapid identification of large numbers of cultured isolates, including isolates from different *Lactobacillus* species [22]. Based on these findings, we propose refined criteria to categorize the status of the microflora of vaginal smears.

Results

We studied the composition of the vaginal microflora of 515 vaginal swabs from a prospective cohort of 197 unselected pregnant women at three time points during pregnancy using i) Gram stain based grading according to modified Ison & Hay criteria [6] – which will be further denoted here as the criteria of Claeys, ii) culture in combination with molecular identification of cultured organisms by tDNA-PCR and iii) species specific PCR for *G. vaginalis* and *A. vaginae*.

Detailed observation of the Gram stained vaginal smears in combination with specific PCR and tDNA-PCR based identification of cultured isolates led to subdivision of grade I samples and the recognition of a separate category, designated grade I-like: Grade I specimens were characterized as grade Ia when only *Lactobacillus crispatus* cell types (plump, mostly short rods) were present (Figure 1a – 1b), as grade Ib when only other *Lactobacillus* cell types were present (smaller or more elongated and less stained than in Ia smears)(Figure 1c – 1d) and as grade Iab when both *L. crispatus* and other lactobacilli were present (Figure 1e – 1f). Furthermore a number of samples were designated as grade I-like because of the presence of Gram positive rods, either quite small and short or otherwise irregularly shaped with clubbing, curved edges and irregular staining and often arranged like Chinese letters ('diphtheroid cell types') (Figure 1g – 1h). To corroborate that grade I-like samples indeed represent a separate class, cloning was carried out for two samples that had been categorized as grade I-like. For completeness, figures 1i – 1j represent grade II vaginal smears and figures 1k – 1l represent grade III vaginal smears.

Comparison between Gram stain and culture

Using the criteria of Claeys, 162 vaginal smears were scored as grade Ia, 181 as grade Ib, 60 as grade Iab, 36 as grade I-like, 46 as grade II, 22 as grade III and eight as grade IV (Table 1).

We cultured 1108 isolates anaerobically out of the 515 vaginal swabs and identified these with tDNA-PCR. A

124
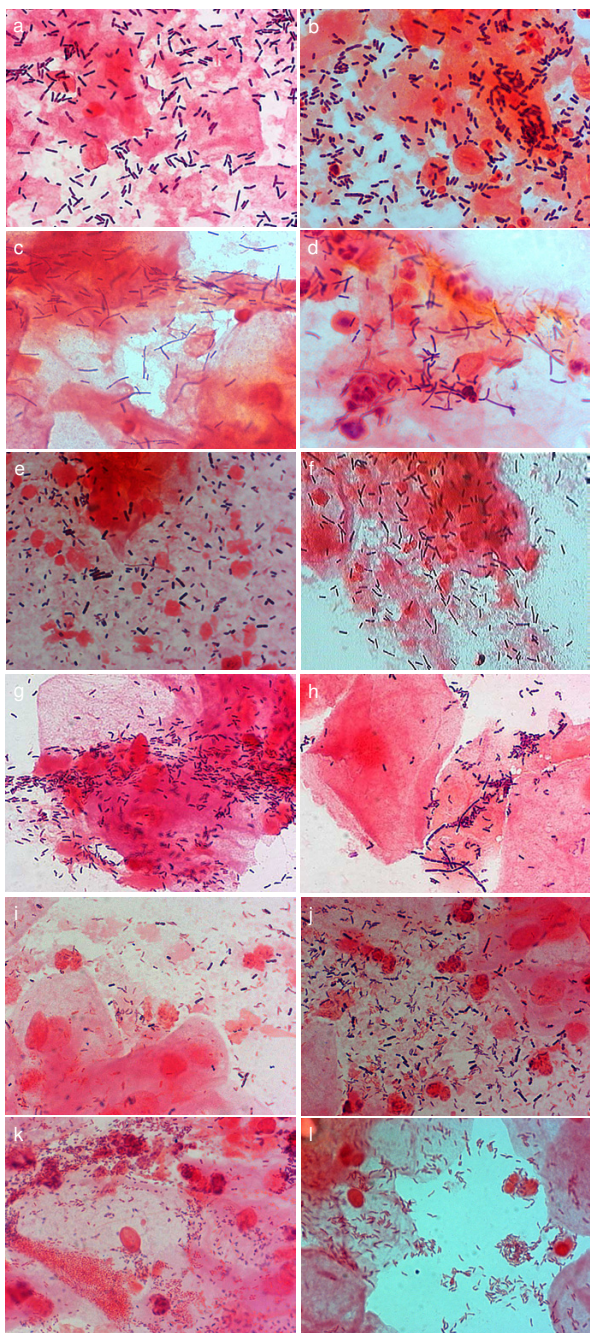
Figure 1
Microscopic image (100 ×) of Gram-stained vaginal smears illustrating the different categories of vaginal microflora described:. a, b: grade Ia, i.e. mainly *Lactobacillus crispatus* cell types, plump quite homogeneous lactobacilli. c, d: grade Ib, i.e. non-*L. crispatus* cell types, long or short, thin lactobacilli. e, f: grade Iab, i.e. containing mixtures of *L. crispatus* and non-*L. crispatus* cell types. g, h: grade I-like, i.e. irregular-shaped Gram positive rods. i, j: grade II, i.e. mixture of *Lactobacillus* cell types and bacterial vaginosis-associated bacteria (*Gardnerella*, *Bacteroides-Prevotella* and *Mobiluncus* cell types). k, l: grade III, i.e. bacterial vaginosis.

total of 136 isolates remained unidentified, since no corresponding tDNA-PCR fingerprint could be found in the database or because no amplification was obtained. A total of 72 species were identified, of which 17 belonged to the genus *Lactobacillus* and six to the genus *Bifidobacterium* (Table 1). The most common species recovered from grade Ia, Ib and Iab specimens were lactobacilli. *L. crispatus* (87.0%) and *L. jensenii* (22.2%) were the most abundant bacteria in grade Ia samples, whereas *L. gasseri* (32.0%) and *L. iners* (39.8%) were the most frequently present species in grade Ib specimens. Grade I-like specimens were found to contain mostly bifidobacteria (54.9%) and *L. gasseri* (52.8), while *L. crispatus* was almost absent (2.8%). In 19.8% of grade I-like specimens bifidobacteria were present while lactobacilli were absent. Bifidobacteria were more frequent in grade I-like samples than in other samples (χ^2 = 120.6, p < 0.001, Table 2).

L. crispatus was present in 87.0% of grade Ia, 76.7% of grade Iab and 37.5% of grade IV samples but in less than 13.3% in all other grades. *L. crispatus* was the only *Lactobacillus* species that was linked to a single grade, namely grade Ia (χ^2 = 186.3, p < 0.001), while the other lactobacilli were more evenly distributed over all samples (Table 3, 4, 5, 6). *L. jensenii* was the second most abundant species in grade Ia (22.2%), but was also frequent in most other grades, for example in 47.8% of grade II. *L. vaginalis*, the third most abundant species in grade Ia (9.3%) was absent from grade III and present in less than 20% of all other grades. *L. gasseri* and *L. iners* were more abundant in grade Ib (32.0 and 39.8%), grade I-like (52.8 and 19.4%), grade II (54.3 and 26.1%) and grade III (9.1 and 31.8%) than in grade Ia (6.8 and 3.7%).

The most characteristic cultured organisms in grade II and grade III specimens were *G. vaginalis* (respectively 21.7% and 72.7%) (χ^2 = 120.6, p < 0.001, Table 7), *Actinomyces neuii* (respectively 6.5% and 9.1%), *Aerococcus christensenii* (respectively 4.3% and 22.7%), *A. vaginae* (respectively 4.3% and 13.6%), *Finegoldia magna* (respectively 2.2% and 9.1%) and *Varibaculum cambriense* (respectively 2.2% and 13.6%). These were virtually absent from grade I and grade IV, although *G. vaginalis* was present in approximately 2.0% of grade I samples. *L. jensenii* (47.8%) and *L. gasseri* (54.3%) were the most common lactobacilli in grade II specimens. Furthermore, whereas *L. crispatus* and *L. vaginalis* were never cultured from grade III specimens, *L. iners* (31.8%) was the lactobacillus mostly present in grade III. *Mobiluncus curtisii* and *Peptostreptococcus* sp. were cultured from grade III specimens only (both 4.5%). *Dialister* sp. (22.7%) and *Prevotella* spp. (22.6%) were frequently cultured from grade III specimens and only sporadically from other specimens.

Table 1: Detailed composition of the vaginal microflora of 515 vaginal swab samples, as determined by culture and tDNA-PCR based identification

Species	Ia	Ib	Iab	I-like	II	III	IV	Total
Grade	Ia	Ib	Iab	I-like	II	III	IV	Total
Number of samples	162	181	60	36	46	22	8	515
***Lactobacillus* spp.**								
Lactobacillus crispatus	87.0[a]	13.3	76.7	2.8	10.9		37.5	42.7
Lactobacillus jensenii	22.2	24.3	43.3	13.9	47.8	18.2	12.5	26.8
Lactobacillus gasseri	6.8	32.0	25.0	52.8	54.3	9.1	25.0	25.6
Lactobacillus iners	3.7	39.8	8.3	19.4	26.1	31.8		21.2
Lactobacillus vaginalis	9.3	12.7	15.0	11.1	6.5		20.0	1.7
Lactobacillus casei		1.1	1.7	2.8	2.2	4.5	12.5	1.4
Lactobacillus coleohominis	1.2	5.5	5.0		2.2			3.1
Lactobacillus delbrueckii		0.6		5.6	2.2			0.8
Lactobacillus fermentum	0.6	1.1	3.3			4.5		1.2
Lactobacillus kalixensis		0.6						0.2
Lactobacillus mucosae					4.3			0.4
Lactobacillus nagelii					2.2			0.2
Lactobacillus oris					4.3			0.4
Lactobacillus pontis 94%		0.6						0.2
Lactobacillus reuteri		1.7	1.7	5.6				1.2
Lactobacillus rhamnosus		0.6		8.3	4.3	4.5	12.5	1.6
Lactobacillus salivarius		0.5						0.2
***Bifidobacterium* spp.**								
Bifidobacterium biavatii		0.6		5.6			12.5	0.8
Bifidobacterium bifidum		0.6		2.8		4.5		0.6
Bifidobacterium breve		0.6		25.0	10.9	9.1		3.3
Bifidobacterium dentium	0.6			8.3		4.5		1.0
Bifidobacterium longum	0.6	0.6		5.6		4.5		1.0
Bifidobacterium sp.		0.6		5.6				0.6
Bacterial vaginosis-related anaerobe organisms								
Actinomyces neuii					6.5	9.1		1.0
Aerococcus christensenii					4.3	22.7		1.4
Anaerococcus tetradius[b]					2.2			0.2
Anaerococcus vaginalis[b]				2.8				0.2
Atopobium vaginae		0.6			4.3	13.6		1.2
Bacteroides ureolyticus	0.6			2.8	2.2	9.1		1.0
Dialister sp.						22.7	12.5	1.2
Finegoldia magna[b]	0.6	0.6			2.2	9.1		1.0
Gardnerella vaginalis	1.2	2.8	1.7	2.8	21.7	72.7		6.8
Gemella morbillorium[b]					2.2			0.2
Mobiluncus curtisii						4.5		0.2
Mycoplasma hominis					4.3			0.4
Peptoniphilus sp.[b]	3.1	1.7		2.8	6.5	9.1		2.7
Peptostreptococcus sp.						4.5		0.2
Prevotella bivia		0.6		2.8		13.6		1.0
Prevotella ruminicola						4.5		0.2
Prevotella sp.						4.5		0.2
Varibaculum cambriense					2.2	13.6		0.8
Other species								
Actinomyces europaeus 96%	0.6							0.2
Actinomyces urogenitalis						4.5		0.2
Arcanobacterium bernardiae	0.6							0.2
Arthrobacter albus					4.3			0.4
Atopobium parvulum						4.5		0.2
Clostridium colicanis			3.3					0.4
Clostridium sp.						4.5		0.2
Corynebacterium amycolatum				2.8				0.2
Corynebacterium coyleae		0.6						0.2
Corynebacterium pseudogenitalium	0.6							0.2

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Table 1: Detailed composition of the vaginal microflora of 515 vaginal swab samples, as determined by culture and tDNA-PCR based identification *(Continued)*

Corynebacterium sp.		0.6			4.3			0.6
Enterococcus faecalis	2.5	4.4	3.3		2.2	4.5		3.1
Escherichia coli		0.6	3.3	2.8		9.1		1.2
Haemophilus influenzae					2.2			0.2
Helcococcus sp.						4.5		0.2
Pediococcus pentosaceus				2.8		4.5		0.4
Propionibacterium acnes	1.9							0.6
Propionibacterium avidium				2.8				0.2
Serratia sp.		0.6						0.2
Staphylococcus aureus	0.6	0.6			2.2			0.6
Staphylococcus capitis		0.6						0.2
Staphylococcus epidermidis	1.2	5.5	1.7	5.6	4.3			3.3
Staphylococcus haemolyticus			1.7		2.2			0.4
Staphylococcus hominis		1.1		2.8	2.2			0.8
Streptococcus agalactiae	1.9	4.4	1.7	11.1		4.5	75.0	4.5
Streptococcus anginosus group	3.1	3.9	1.7	5.6	4.3	9.1		3.7
Streptococcus gallolyticus			1.7					0.2
Streptococcus mitis				2.8	2.2			0.4
Streptococcus salivarius	0.6	1.7						0.8
Veilllonella atypica	1.2	0.6		2.8				0.8
Veillonella sp.	0.6	1.1			2.2			0.8

[a] Numbers represent percentage of samples from which the species was cultured.

[b] Formerly known as *Peptostreptococcus*.

The average number of species cultured per sample ranged from 1.5 for grade Ia specimens to 3.6 for grade III specimens (Table 8). Overall, the species diversity of the grade I-like category was higher (0.83) than that of the grade I subcategories (0.17, 0.21 and 0.30 for grades Ia, Ib, and Iab respectively) and comparable to that of the grade II category (0.76). The grade III category had the highest species diversity (1.50) (Table 8).

Comparison between Gram stain and species specific PCR for *Gardnerella vaginalis* and *Atopobium vaginae*

The series of 515 vaginal samples were analyzed by PCR with 16S rRNA gene based primers specific for *A. vaginae* and 16S–3S spacer primers specific for *G. vaginalis*.

After amplification with the ato167f *A. vaginae* primer set, respectively 14.7% of grade I, 8.3% of grade I-like, 28.3% of grade II, 86.4% of grade III and 12.5% of grade IV samples showed an amplicon. The percentage of positive samples for *G. vaginalis* specific PCR was respectively 28.9%, 19.4%, 47.8%, 86.4% and 12.5%.

The simultaneous presence of *A. vaginae* and *G. vaginalis* in a vaginal swab specimen had an accuracy of 90% [95% CI: 86–92%], a sensitivity of 82% [95% CI: 59–94%], a specificity of 90% [95% CI: 87–92%], a positive predictive value of 26% [95% CI: 17–39%] and a negative predictive value of 99% [95% CI: 98–100%] in assessing bacterial vaginosis (defined as a grade III smear).

Comparison between culture and cloning of grade I-like samples

Cloning of two grade I-like samples from trimesters 1 and 2 of the same patient, revealed the presence of *Bifidobacterium breve* (respectively 33.1 and 53.5%), *Lactobacillus delbrueckii* (64.8 and 13.3%) and *L. gasseri* (2.1 and 33.1%) clones. This was in agreement with the culture results which revealed the presence of *B. breve* in both trimesters, *L. delbrueckii* only in the first and *L. gasseri* only in the second.

In general, grade I-like samples were found by culture to contain more frequently *Bifidobacterium* (19/36 samples) and more different *Bifidobacterium* species (6) than samples from all other categories. Of the *Bifidobacterium* species, *B. breve* was most clearly associated with grade I-like, grade II and grade III.

Discussion
The importance of correct diagnosis of bacterial vaginosis and of more detailed characterization of the vaginal microflora

Although not causing a vaginal inflammatory response, bacterial vaginosis is considered to be the most common cause of vaginitis in pregnant and non-pregnant women and prevalences between 4.9% and 36.0% have been reported from European and American studies [23]. Several studies suggest the possibility that bacterial vaginosis increases the risk of acquiring HIV [24,25] and that the

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Table 2: Presence of *Bifidobacterium* spp. in grade I like samples versus other samples.

Bifidobacterium spp.	Grade I-like	Other grades	Total
Cultured	19	18	37
Not cultured	17	461	478
Total	36	479	515

Table 3: The presence of *Lactobacillus* species in grade Ia and grade Ib samples.

	Grade Ia	Grade Ib	Total
Total	**162**	**181**	**343**
L. crispatus	141	24	165
L. jensenii	36	44	80
L. gasseri	11	58	69
L. iners	6	72	78

bacterial flora associated with bacterial vaginosis increases genital-tract HIV shedding [26]. A recent meta-analysis by Leitich *et al*. [27] established an odds ratio of 8 for preterm birth in association with bacterial vaginosis during early pregnancy. Spontaneous preterm birth occurs in 7–11% of pregnancies but accounts for three quarters of perinatal morbidity and mortality and for half of long term neurological impairment in children [28,29].

Bacterial vaginosis is characterized by the replacement of the normal vaginal microflora of lactobacilli by *Gardnerella vaginalis* and anaerobic organisms. Recently, different groups showed that the strict anaerobe *Atopobium vaginae* is another organism that is strongly associated with bacterial vaginosis [12,13,16,17]. The association between *A. vaginae* and bacterial vaginosis might help explain why some women suffer from recurrent bacterial vaginosis. For example, a recent study pointed to great *in vitro* efficacy of metronidazole, since this antibiotic inhibited growth of 99% of the vaginal isolates from bacterial vaginosis samples [30], but most likely overlooked the fastidious metronidazole resistant *A. vaginae*, shown in this study to be present in 86.4% of bacterial vaginosis samples when detected with species specific PCR.

Given the possibility that certain not yet characterized subgroups within the presumably heterogenic clinical entity of women with bacterial vaginosis could identify a group at higher risk for preterm birth than women with bacterial vaginosis as a whole and that adequate treatment of women from this higher risk group may allow for more targeted preterm birth prevention, better understanding of the composition and dynamics of the vaginal microflora and accurate diagnosis of bacterial vaginosis are warranted. Also, our data indicate that refined characterization of vaginal microflora may be necessary for more accurate interpretation of the results of clinical studies. For example, thus far *Atopobium vaginae* has been overlooked in clinical studies and furthermore, the fact that different *Lactobacillus* species may confer different strengths of colonisation resistance [10,11] has not been taken into account, partly because most laboratories lack

the access to rapid and accurate methods for the identification of lactobacilli to the species level. In other words, several studies concerning the relation between the status of the vaginal microflora and different gynecologic and obstetric diseases and their treatments thus far may have reached biased conclusions due to insufficiently precise characterization of the microflora.

Criteria for microbiological categorization of vaginal microflora status

Spiegel *et al*. [31] defined a scoring system based on some of the bacterial cell types that can be seen in Gram stained smears of vaginal secretion. This was later refined by Nugent et al. [1], who provided a scoring system that evaluates the changes in vaginal microflora, from the normal condition to bacterial vaginosis status, as a continuum. Although the Nugent criteria have gained wide acceptance for the evaluation of the condition of the vaginal microflora [2,32], further refinement is warranted for several reasons. First, no definite criteria have been described to distinguish the *Lactobacillus* cell types from the *Gardnerella* and *Bacteroides-Prevotella* cell types. In practice and in our experience, 'morphotypes' are often difficult to assign to one of these groups. Also, some genera and species that are clearly associated with bacterial vaginosis, like *Peptostreptococcus* spp. [32] and *A. vaginae* [12,17,13] are not included in the Nugent score. Furthermore, Forsum *et al*. [2] found major discrepancies in scoring when the lactobacillary cell types were few in number and Larsson *et al*. [33] reported several problems in the interpretation of smears. For example, using the Nugent criteria, the presence of different *Lactobacillus* cell types in smears from patients with bacterial vaginosis can lead to assignation to grade II, whereas patients without bacterial vaginosis but with smears with more than 300–500 pleomorphic *Lactobacillus* cells may be regarded as containing *G. vaginalis*, also because some of these cells are very small. Additionally, the Nugent scoring system conflates women with potentially very different vaginal microflora in a single category [3].

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Table 4: Presence of *L. gasseri* or *L. iners* in grade II and grade III samples versus presence in other samples.

L. gasseri or *L. iners*	Grades II and III	Other grades	Total
Cultured	41	184	225
Not cultured	27	263	290
Total	68	447	515

Table 5: Number of samples with lactobacilli in grade Ia versus the other grades.

Species	Grade Ia	Other grades	Total
L. crispatus	185	35	220
L. jensenii	36	102	138
L. gasseri	11	123	132
L. iners	6	103	109

In this study, the clinical microbiologist (GC) could not grade some of the smears due to the presence of cell types not scored in the system developed by Nugent [1] and classified these samples as grade I-like. Further detailed observation lead to the splitting up of grade I samples into subcategories designated grade Ia, grade Ib and grade Iab. After blind grading of the vaginal smears into grades Ia, Ib, Iab, I-like, II, III and IV, this classification was compared with the culture results and with species specific PCRs.

Grade Ia and Iab: Agreement with the presence of L. crispatus

From this comparison it became obvious that it is possible to recognize the presence of *L. crispatus* by means of Gram stain, since this species was cultured in 81.9% of the grade Ia samples and 76.7% of the grade Iab samples. Nevertheless, *L. crispatus* was not cultured from 21 of the 162 grade Ia samples. This may be explained by the fact that *L. crispatus* is not as easily cultured as other lactobacilli. Indeed, *L. crispatus* colonies were quite often observed as satellites of other bacteria and in some cases no growth at all was observed in samples with numerous *L. crispatus*-like lactobacilli on Gram stain. Using non culture dependent t-RFLP-analysis (data not presented) the Ia samples negative for *L. crispatus* culture were tested and 16 were positive for *L. crispatus*, bringing the agreement between Gram stain grading as grade Ia and the presence of *L. crispatus* to 96.9%. Similarly, when taking into account t-RFLP-analysis results, the agreement between categorization as grade Iab and t-RFLP-analysis positive for *L. crispatus* was 92.9% whereas *L. crispatus* was detected by t-RFLP-analysis only in 27.3%, 20.0%, 22.5% and 0% for grades Ib, I-like, II and III, respectively. These results indicate that – for a trained microbiologist – it is possible to recognize *L. crispatus* bacteria upon cell morphology, a finding that is of importance since this species is clearly associated with healthy microflora, and possibly better ensures stable healthy microflora than other lactobacilli [9]. Samples were scored as grade Ib when no *L. crispatus* cell types were observed, but other *Lactobacillus* cell types were predominant. The agreement with culture results was high: only 13.3% contained *L. crispatus* upon culture, whereas *L. gasseri*, *L. iners*, and *L. jensenii* were present in respectively

32.0, 39.8, and 24.3% of the grade Ib samples. These were clearly grade I samples since bacterial vaginosis-associated organisms were mostly absent.

The colonisation resistance conferred differs between Lactobacillus *species*

Overall the frequency of isolation of all *Lactobacillus* species together was comparable for the different grades in our population, since lactobacilli were cultured from 96.9% of grade Ia, 94.5% of grade Ib, 96.7% of grade Iab, 78.9% of grade I-like, 93.5% of grade II, 59.1% of grade III and 62.5% of grade IV samples. This is in agreement with previous reports [32,34]. However, we observed a clear difference with regard to the *Lactobacillus* species frequency distribution for the different grades. While *L. crispatus*, known as a strongly H_2O_2-producing species [7,8], was cultured from 87.0% of grade Ia specimens, it was absent in grade III specimens and only present in 2.8% of grade I-like specimens. In contrast, *L. iners*, reported as a weakly H_2O_2-producing species [7,8], was present in only 3.7% of Ia specimens but in 39.8% of grade Ib and 31.8% of grade III specimens. Whether it is the hydrogen peroxide production by *L. crispatus* that confers colonisation resistance remains a matter of debate, since a correlation between the presence of hydrogen peroxide production and the type of vaginal microflora was found by some [35], though not by others [36]. Possibly other species specific characteristics, present in *L. crispatus*, but absent in species like *L. gasseri* and *L. iners*, confer colonisation resistance. It has also been hypothesized that the onset of perturbation leading to bacterial vaginosis may be due to competition between *Lactobacillus* species [36], a situation possibly reflected by grade Iab specimens.

Grade I-likes: a separate category of vaginal microflora status

A number of samples were initially difficult to score because the predominant cell types could not be categorized as *Lactobacillus*, *Gardnerella*, *Bacteroides-Prevotella* or *Mobiluncus* cell types. These samples were considered as belonging to a separate category because of the presence of Gram positive rods, either quite small and short or oth-

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Table 6: Number of samples with lactobacilli in grade I versus the other grades.

Species	Grades I a + Iab + Ib	Other grades	Total
L. crispatus	211	9	220
L. jensenii	106	32	138
L. gasseri	84	48	132
L. iners	83	26	109

Table 7: Presence of *G. vaginalis* in grade II and grade III samples versus presence in other samples.

G. vaginalis	Grades II and III	Other grades	Total
Cultured	26	9	35
Not cultured	42	438	480
Total	68	447	515

erwise irregularly shaped with clubbing, curved edges and irregular staining and often arranged like Chinese letters ('diphtheroid cell types'). Since it is likely that most microbiologists would score this cell type as '*Lactobacillus*-like' and that therefore it would be scored in most cases as grade I, we designated it as 'grade I-like'. Culture and species specific PCR confirmed that indeed these samples represent a separate kind of vaginal microflora. This is reflected by the increased species diversity of 0.83, which is much higher than that for grades Ia, Ib and Iab (0.15–0.30) and which is comparable to that of grade II (0.76), but even more so by the virtual absence of *L. crispatus* (cultured from only one of 36 samples) as well as of *G. vaginalis* and *A. vaginae* (cultured from respectively 1 and 0 samples) and the presence of *Bifidobacterium* spp. in 19 of 36 samples, a much higher prevalence than in samples from all other grades. This was confirmed by cloning of two grade I-like samples, which contained only *L. delbrueckii*, *L. gasseri* and *B. breve*.

Rosenstein *et al*. [34] mentioned a category of vaginal smears with aberrant morphology, which they designated as grade I revertants. At first sight, their category shows resemblance with the category we describe here as I-like, because of low numbers of *G. vaginalis* and increased numbers of bifidobacteria, but on the other hand they reported even more bifidobacteria in their grade II and grade III samples and they designated this category as grade I revertants because the vaginal microflora of all 41 women with such smears reverted to grade I, which was not the case in our study (data to be presented elsewhere).

Importantly, since Gram stain based categorizing can result in the interpretation of grade I-like samples as genuine grade I samples (whereof their designation), this class of samples may jeopardize – and probably has done so in the past – the interpretation of the results of clinical studies.

Grade II: a microbiologically intermediate stage between healthy microflora and bacterial vaginosis
Our results confirm that grade II samples represent a microbiologically clearly intermediate status between

grade I and III. *L. crispatus* is still present in 10.9% of the samples (compared to 59.0% of grade I and 0% of grade III samples), whereas the number of samples with *L. gasseri* (54.3%) is increased compared to grade I (21.3%) and grade III (9.1%). Species diversity of grade II is intermediate between that for grade I and grade III and species typically associated with bacterial vaginosis, like *A. neuii*, *A. christensenii*, *A. vaginae*, *B. ureolyticus*, *F. magna*, *G. vaginalis*, *Peptoniphilus sp*. and *V. cambriense*, are present, but again in a lower number of samples than in grade III specimens.

Grade III: Characterization of bacterial vaginosis -related organisms
The following species are generally considered as bacterial vaginosis related anaerobe organisms: *Anaerococcus (Peptostreptococcus) tetradius*, *A. (Peptostreptococcus) vaginalis*, *Atopobium vaginae*, *Bacteroides ureolyticus*, *Finegoldia (Peptostreptococcus) magna*, *G. vaginalis*, *Gemella (Peptostreptococcus) morbillorum*, *Mobiluncus curtisii*, *Mycoplasma hominis*, *Peptoniphilus* sp., *Peptostreptococcus* sp., *Prevotella bivia*, *Prevotella ruminicola* and *Prevotella* sp. [37,38]. Using tDNA-PCR we were able to identify 87.8% of the cultured isolates to the species level and found our results to be largely in agreement, but in addition we cultured *Actinomyces neuii*, *Aerococcus christensenii*, *Dialister* sp. and *Varibaculum cambriense*, whereas *Mobiluncus* spp., *Mycoplasma hominis* and *Ureaplasma urealyticum* were not or only sporadically cultured from grade II and grade III specimens. The absence of the latter species in our study can be explained by the fact that we did not use the specific culture methods for these fastidious organisms.

In this study we confirmed the strong association, as established previously [12,13,17], between *A. vaginae* and bacterial vaginosis.

Conclusion
In summary, our characterization of the vaginal microflora by means of detailed Gram stain interpretation and by culture in combination with genotypic identification helps to refine our understanding of normal and disturbed vaginal microflora. We showed that *L. crispatus* can be recognized as such on Gram stain, we established the

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Table 8: Numbers of species cultured per patient and species diversity indices

Grade	Number of species cultured		Diversity	
	Average	Range	Number of Species/ Number of Samples (Index)	Simpson's Diversity Index
Ia	1.5	1–6	27/162 (0.17)	0.6
Ib	1.7	1–7	38/181 (0.21)	0.9
Iab	2.0	1–7	18/60 (0.30)	0.8
I-like	2.3	1–8	30/36 (0.83)	0.9
II	2.7	1–6	35/46 (0.76)	0.9
III	3.6	2–8	33/22 (1.50)	0.9
IV	2.0	1–3	5/8 (0.63)	0.8

existence of a separate additional category, characterized by the absence of *L. crispatus* and the abundance of bifidobacteria and we confirmed the association of *Atopobium vaginae* with bacterial vaginosis.

These data have implications for the basic understanding of the vaginal microflora and bacterial vaginosis; in addition, they may add to the value of Gram smear based diagnosis of bacterial vaginosis because of better defined Gram stain criteria.

Methods
Study population and sample collection
A total of 515 vaginal swabs were collected by sampling 197 pregnant women attending our out-patient clinic, each at three time points during pregnancy (respectively 197, 171 and 147 first, second and third trimester samples were collected). The swabs were obtained during the first, second and third pregnancy trimester, at mean gestational ages of 9.1 +/- 3.2 weeks, 20.4 +/- 2.3 weeks and 32.2 +/- 1.7 weeks, respectively.

Sampling was carried out by insertion of three sterile cotton swabs into the vaginal vault, after placement of a non-lubricated speculum. The swabs were rotated against the vaginal wall at the midportion of the vault and were carefully removed to prevent contamination with microflora of the vulva and introitus. The first swab was used to prepare a smear on a glass slide for the purpose of grading according to the criteria of Claeys (this study). The second swab was returned to a sterile tube (Copan, Brescia, Italy), for the purpose of DNA-extraction (dry swab). The third swab was placed into Amies transport medium (Nuova Aptaca, Canelli, Italy) for anaerobic culture. The unstained smear and both swabs were sent to the microbiology laboratory and were processed within 4 hours.

Grading of slides
Smears were dried, Gram stained (Mirastainer, Merck-Belgolabo, Overijse, Belgium) and examined under oil immersion at a magnification of 1000. Gram stained smears from vaginal swabs were all scored by one clinical microbiologist (GC) according to Ison & Hay criteria [5,6]: samples were categorized as grade I when only *Lactobacillus* cell types (large Gram positive rods) were present, as grade II (intermediate) when both *Lactobacillus* and *Gardnerella* or *Bacteroides-Prevotella* cell types were present, as grade III (bacterial vaginosis) when *Lactobacillus* cell types were absent and only *Gardnerella*,*Bacteroides-Prevotella* or *Mobiluncus* cell types were present and as grade IV when Gram positive cocci were predominantly present. Further subdivision of grade I samples into categories Ia, Iab and Ib and the description of a separate category, designated grade I-like, is presented in the Results section.

Culture and identification of cultured isolates by tDNA-PCR
For 515 specimens collected from 197 women, the swab on Amies transport medium was streaked onto Schaedler agar enriched with 5% sheep blood, vitamin K, hemin and sodium pyruvate (Becton Dickinson, Franklin Lakes, NJ) and incubated anaerobically at 37°C upon arrival at the microbiology laboratory. After 4 days of incubation, all the isolates with different colony morphology were selected for identification. DNA was extracted by simple alkaline lysis: one colony was suspended in 20 µl of 0.25% sodium dodecyl sulfate-0.05 N NaOH, heated at 95°C for 15 min and diluted with 180 µl of distilled water. tDNA-PCR and capillary electrophoresis were carried out as described previously [20,22]. The species to which each isolate belonged was determined by comparing the tDNA-PCR fingerprint obtained from each isolate with a library of tDNA-PCR fingerprints obtained from reference strains, using an in-house software program

[20]. The library of tDNA-PCR fingerprints is available at our website [39] and the software can be obtained upon request.

DNA extraction of vaginal swab samples

For DNA extraction from the dry vaginal swabs, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications, as described previously [13]. DNA-extracts were stored at -20°C and were used for the purpose of species specific PCR and cloning experiments.

Species specific PCR for Gardnerella vaginalis

G. vaginalis species specific primers as designed by Zariffard *et al.* (G_Z) [19] were used. Briefly, a 20 µl PCR mixture contained respectively 0.05 and 0.4 µM primers, 10 µl of Promega master mix (Promega, Madison, WI), 2 µl of Qiagen DNA-extract of the samples and distilled water. Thermal cycling with G_Z primers consisted of an initial denaturation of 10 min at 94°C, followed by 50 cycles of 5 s at 94°C, 45 s at 55°C and 45 s at 72°C, and a final extension of 10 min at 72°C. Five microliter of the amplified product was visualized on a 2% agarose gel.

Species specific PCR for Atopobium vaginae

A primer set that allowed amplification of the 16S rRNA gene of *A. vaginae* and that lacked homology with non-target bacteria was used as described earlier [13]. Briefly, a 10 µl PCR mixture contained 0.2 µM each of the primers ato167f (5' GCGAATATGGGAAAGCTCCG) and ato587r (5' GAGCGGATAGGGGTTGAGC), 5 µl of Promega master mix (Promega, Madison, WI), 1 µl of Qiagen DNA-extract of the samples and distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 58°C and 1 min at 72°C, followed by 35 cycles of 20 sec at 94°C, 1 min at 58°C and 1 min 72°C, with a final extension of 10 min at 72°C, and cooling to 10°C. Five microliter of the amplified product was visualized on a 2% agarose gel. The primers amplified a DNA-fragment of 420 base pairs from *A. vaginae* and showed no cross reactivity to other organisms, including *A. rimae* and *A. parvulum* (data not presented).

Cloning of amplified mixtures of 16S rDNA

Cloning and sequencing was carried out largely as described previously [13]. However, to increase the amplification efficiency of the 16S rRNA-genes of *G. vaginalis* and bifidobacteria the following mixture of primers (0.1 µM each) was used for the initial amplification of the samples prior to cloning: primers 10 f (5' AGTTTGATCCT-GGCTCAG), 534r (5' ATTACCGCGGCTGCTGG) and GV10f (5' GGTTCGATTCTGGCTCAG).

Statistical analysis

The Simpson's Diversity Index was calculated as $D = 1-\sum (n/N)^2$ where n is the number of isolates of a particular species and N is the total number of isolates. Chi square analyses were carried out using the statistical software package SPSS v.11.0.

Authors' contributions

RV, GC, GV, EDB and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. LVS and CDG participated in the analysis of the study samples. All authors read and approved the final manuscript.

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V. General discussion

V.1. Review of PCR-based studies of vaginal microflora

Recently, several research groups have applied culture-independent methods to study the bacterial microflora of the human vagina and showed - by means of cloning of the 16S rRNA-gene (35, 77, 130, 288, 313) or the chaperonin-60 gene (109), by specific PCR (34, 72, 77, 288), by DGGE of the 16S rRNA gene (11, 33, 36, 71), by T-RFLP of the 16S rRNA gene (53, 289, 292) or by FISH (77, 267) - that previously unrecognized organisms are part of the vaginal microflora.

V.1.1. 16S rDNA or chaperonin-60 cloning

Several groups have used broad-range 16S rDNA PCR with sequence analysis of cloned PCR products to assess the vaginal bacterial microflora. The advantage of this approach is detection of greater bacterial diversity relative to DGGE and T-RFLP. Unfortunately, analysis of clone libraries is laborious, expensive and time consuming.

A major limitation that compromises comparison of the obtained results is that the investigators used different inclusion criteria. While Burton *et al.* (35), Hill *et al.* (109), Fredricks *et al.* (77) and our group (288, 289) relied on objective Gram staining methods to categorize vaginal specimens as normal or disturbed, some authors, relied on self-reported symptoms (130, 313). Because about half the women with bacterial vaginosis are asymptomatic, self-reported symptoms cannot be used to define women without bacterial vaginosis (143).

Manuscript II (288) describes a study using a combination of cloning and cultivation methods to identify vaginal bacteria in eight women: three with normal microflora by Nugent score, two with intermediate microflora, and three with bacterial vaginosis. Broad-range PCR was used to amplify an approximately 500 base-pair segment of the bacterial 16S rRNA gene from vaginal samples, the PCR products were cloned, the plasmid inserts screened for sequence diversity using amplified ribosomal DNA restriction analysis (ARDRA), whereafter finally clones with unique ARDRA patterns were sequenced to identify the bacteria. In addition, 46 bacterial colonies cultivated from the eight women were identified by tDNA PCR or by sequencing their 16S rRNA genes. Analysis of the 854 clones generated by broad-range PCR and of the 46 colonies revealed the presence of 38 bacterial species. *Lactobacillus* species dominated the microflora in women with low Nugent scores, whereas women with intermediate or bacterial vaginosis microflora by Gram stain were colonized with a large

diversity of bacteria. *Atopobium vaginae* was found in a high percentage of clones generated from women with a disturbed microflora and was also associated with bacterial vaginosis by species specific PCR. *Gardnerella vaginalis* was detected in only one clone library, but this resulted from choosing a primer for broad-range PCR that poorly matched the *G. vaginalis* 16S rDNA sequence, and *G. vaginalis*-specific PCR showed this bacterium was associated with bacterial vaginosis in more than 7 of 9 women. A series of species that have not been described before as common members of the vaginal community, including *Atopobium rimae*, *A. vaginae*, [*Bifidobacterium biavatii*], *Dialister* sp., *Leptotrichia amnionii*, and *Sneathia sanguinegens* were found in clone libraries from women in all groups. There was no single pattern of bacterial colonization in women with bacterial vaginosis or intermediate microflora. Four other research groups also performed a 16S rDNA based cloning study (35, 77, 130, 313) and another group amplified and cloned bacterial chaperonin-60 genes (109).

Burton *et al.* (35) performed broad-range 16S rDNA PCR on vaginal fluid from a single woman with bacterial vaginosis as assessed by Nugent scoring, generating approximately 1500 base pair products that were cloned.

Of the 65 clones that were analyzed, 61% matched *A. vaginae* (35). Other bacteria detected in this woman were related to *Slackia, Eggerthella, Megasphaera, Dialister, Prevotella, Lactobacillus,* and some uncultivated bacterial species. They then applied an *A. vaginae* specific PCR assay to 35 postmenopausal women and found that about half the women with bacterial vaginosis were PCR positive. The limitation of this study is that broad-range 16S rDNA PCR with clone analysis was not performed on other women with bacterial vaginosis or control women without bacterial vaginosis to assess the relevance of these findings.

Zhou *et al.* (313) used broad-range PCR to amplify an approximately 900 base-pair segment of bacterial 16S rDNA from vaginal fluid samples obtained from five 'healthy' women. 979 clones were sequenced to identify the bacteria (176–250 clones/woman).

There were two to seven bacterial phylotypes (species) per woman. Two women had libraries dominated by *L. crispatus*, while *L. iners* was detected in three of the five women. Several novel phylotypes were detected in vaginal samples, including bacteria related to *Atopobium, Megasphaera* and *Leptotrichia* species.

A major limitation of this study is that the investigators used self-reported symptoms instead of objective criteria to exclude women with bacterial vaginosis. Therefore it is possible that several of these women with novel bacterial phylotypes had bacterial vaginosis.

Hyman *et al.* (130) used broad-range PCR to amplify an approximately 1400 base-pair segment of the bacterial 16S rRNA gene from vaginal samples collected on cryoloops.

Approximately 1000 clones were sequenced from both ends from twenty menarchial women, generating about 2000 reads per subject. Although all were reportedly asymptomatic, it is not clear if women with bacterial vaginosis were specifically excluded by analysis of vaginal fluid Gram stains or evaluation of clinical criteria.

Four women had clone libraries that were almost exclusively derived from *Lactobacillus* species. There was a high degree of sequence diversity within the *Lactobacillus* species, demonstrating that the vaginal lactobacilli are not clonal. Nine women had libraries with mixtures of lactobacilli and diverse other bacterial species. Seven women had clone libraries that were devoid of *Lactobacillus* species and consisted of mixed bacterial communities. Several libraries consisted of more than 50 bacterial 16S rDNA sequence types, although criteria for defining bacterial phylotypes (species) based on sequence similarity were not described.

The strengths of this study are that an almost complete portion of the 16S rRNA gene was used for phylogenetic analysis and identification, that a large number of women were studied, and that many clones were analyzed from each woman. The limitations of this study include the choice of a reverse primer with poor sequence homology to *Atopobium* species, and the lack of explicitly stated objective criteria to identify women with bacterial vaginosis. Another concern is the presence of 16S rDNA sequences in the clone libraries that have been linked previously to bacterial contamination of *Taq* polymerase, such as *Pseudomonas*, *Comamonas*, and *Stenotrophomonas* sequences, and thus may not arise from vaginal epithelium.

Fredricks *et al.* (77) used broad-range PCR to amplify an approximately 1000 base-pair segment of the bacterial 16S rRNA gene from vaginal fluid samples from nine women with bacterial vaginosis and eight controls without bacterial vaginosis. For each subject 100 clones were screened for sequence diversity using restriction fragment length polymorphism analysis with two restriction enzymes (ARDRA). Inserts with unique ARDRA patterns were sequenced to identify bacteria. Bacterial vaginosis was defined using Amsel clinical criteria and was verified by Gram stain of vaginal fluid. In addition, they obtained serial vaginal fluid samples from four women to assess the changes that ensue with incident, cured, relapsing, and persistent bacterial vaginosis.

In this study, women without bacterial vaginosis had clone libraries dominated by a few *Lactobacillus* species, particularly *L. crispatus* and *L. iners*, whereas women with bacterial vaginosis had a more diverse microflora. *L. crispatus* was not detected in libraries of clones from women with bacterial vaginosis, whereas *L. iners* was detected in most women. *G. vaginalis* was detected in all bacterial vaginosis clone libraries, *A. vaginae* in eight out of

nine, and *M. mulieris* in one library. In total, sixteen bacterial vaginosis associated species including several novel species in the *Clostridiales* order, as well as *Atopobium, Dialister, Eggerthella, Leptotrichia,* and *Megasphaera* species were recognized. Most remarkably were three newly recognized bacteria that were detected only in libraries of clones from women with bacterial vaginosis, and that were provisionally named bacterial vaginosis–associated bacteria (BVAB) 1, 2, and 3. BVAB1, BVAB2 and BVAB3 are related to bacteria in the *Clostridium* phylum but are not closely related to any bacteria with known 16S rDNA sequences. No single bacterial community was found in all women with bacterial vaginosis.

The strength of this study is the use of broad-range 16S rDNA PCR, bacterium-specific PCR, and FISH applied to samples from women who were well characterized for the presence or absence of bacterial vaginosis. Moreover, this study confirms our results indicating that many of the novel bacteria detected in 'healthy' women in previous studies (130, 313) are linked to bacterial vaginosis when bacterial vaginosis is assessed using objective criteria.

Also other universal bacterial genes than 16S rRNA genes are candidates for the amplification and cloning of most organisms present in a complex bacterial community. Hill *et al.* (109) used degenerate, universal PCR primers to amplify an approximately 555 base pair region of the universal chaperonin-60 (*cpn60*) gene, which is found in all eubacteria and eukaryotes. Library clones from 16 asymptomatic women between 19 and 35 years and with a Nugent score of less than four were sequenced, and the resulting sequences were assigned to taxonomic groups on the basis of similarity to reference sequence data.

Of the 7860 obtained clones, more than 5300 clones, representing 32 sequences, fell into *Lactobacillus* clusters and another 750 clones, representing 22 sequences, fell into the *G. vaginalis* cluster. Furthermore, single sequences were identified with similarity to the Clostridiales family, the Bacteroidetes family, and the Chlamydiales.

In this study, consistently with 16S rDNA cloning studies, most of the *cpn60* sequence libraries of women with normal vaginal microflora were dominated by sequences with strong similarity to *L. jensenii* and to the *L. acidophilus* group, specifically *L. crispatus*, *L. gasseri* and *L. iners*. Most of the libraries were found to be composed of representatives of one or two of the *Lactobacillus* clusters, frequently *L. crispatus* and *L. iners*.

A large amount of variation within the taxonomic groups was identified. For example, the six identified *Lactobacillus*-like clusters each contained from one to 12 distinct sequences and similar 'intraspecies' variation was observed in the *G. vaginalis*-like taxa.

This is the first study that identified *Chlamydophilia psittaci,* a pathogen thus far related only to human airway infections from avian origin (65), as a member of the normal vaginal microflora in three of the 16 women.

The strengths of this study are that a significant number of women were studied and that many clones were analyzed from each woman. As such this study and that of Hyman *et al.* (130) discovered a potentially biologically significant 'intraspecies' diversity that would not be apparent with the use of culture-based methods or PCR-based methods such as DGGE and T-RFLP.

V.1.2. 16S rDNA DGGE

Three groups have combined broad-range 16S rDNA PCR with DGGE to identify vaginal bacteria. Burton and Reid (36) analyzed serial samples from 20 asymptomatic postmenopausal women. At the initial evaluation, 70% of women had either an intermediate microflora or bacterial vaginosis by Nugent score. Broad-range 16S rDNA PCR with DGGE was performed on the initial vaginal fluid samples from 20 women, resulting in 27 bands that were excised from the gel and sequenced. The six women with normal vaginal microflora by Gram stain had one to two bands per sample, all from *Lactobacillus* species. The seven women with bacterial vaginosis had zero to three bands each, and sequencing indicated the presence of bacteria related to *Gardnerella, Prevotella, Peptostreptococcus* and *Bacteroides* species. The seven women with intermediate microflora by Gram stain had zero to four bands detected, and sequencing showed the presence of *Lactobacillus, Prevotella, Gardnerella, Streptococcus* and *Slackia* species. This study was the first attempt to apply broad-range molecular methods to analyze the vaginal microflora, but only a limited assessment of bacterial diversity was possible using DGGE analysis of broad-range 16S rDNA PCR products.

The same group analyzed 19 asymptomatic, premenopausal women using DGGE, and identified a dominance of vaginal lactobacilli, though some unusual bacteria related to *Arthrobacter, Caulobacter* and *Butyrivibrio* were detected (33). *Lactobacillus iners* was detected in 42% and *G. vaginalis* in 32% of patients. Three of the six women with *G. vaginalis* had bacterial vaginosis based on Nugent score.

Ferris *et al.* (71) also used broad-range bacterial PCR to amplify a 300 base pair segment of 16S rDNA from vaginal samples and DGGE to analyze the PCR products. They identified bands consistent with *Atopobium vaginae* 16S rDNA in 12 of 22 women with bacterial vaginosis and only 2 of 24 controls. Other bacteria detected in women with bacterial

vaginosis included *Gardnerella, Bifidobacterium, Mycoplasma, Prevotella* and *Lactobacillus* species, although not all bands detected by DGGE were sequenced. In a follow-up study, these investigators applied an *A. vaginae*-specific 16S rDNA PCR assay to vaginal fluid samples to confirm the results obtained by DGGE analysis (72). All 19 women without bacterial vaginosis were PCR-negative. Four of 11 women with bacterial vaginosis (but without evidence of *A. vaginae* on analysis by broad-range PCR with DGGE) were PCR-positive in the species-specific *A. vaginae* assay. These data confirm again that *A. vaginae* is associated with bacterial vaginosis.

Anukam *et al.* (11) used group specific *Lactobacillus* primers to amplify the V2-V3 region of the 16S rRNA gene and DGGE to analyze the PCR product of 185 vaginal swabs collected from healthy premenopausal Nigerian women (18 to 48 years). Most intriguing is their observation that only 4% of the healthy vaginal microflora of Nigerian women is dominated by *L. crispatus*, while *L. iners* was present in 64%, followed by *L. gasseri* in 8.3%. This observation might be a possible explanation for the high prevalence of bacterial vaginosis among African women (189) since it is known that most *L. iners* strains are very weak H_2O_2-producers and that in contrast, *L. crispatus* is known to produce large amounts of H_2O_2 and as such provides better colonization resistance (8).

V.1.3. 16S rDNA T-RFLP

In manuscript III (290), the use of T-RFLP for characterization of the vaginal microflora was evaluated. Therefore, the composition of the vaginal microflora of 100 pregnant women at three time points in pregnancy was assessed using microscopy, culture, species specific PCR for *A. vaginae* and *G. vaginalis*, in comparison with T-RFLP.

Another research group (53) also developed a T-RFLP approach to define and monitor the structure of microbial communities found in the human vagina. Sixteen bacterial strains commonly found in the human vagina were used to construct model communities that were subsequently used to develop efficient means for the isolation of genomic DNA and the optimal primers and restriction enzyme for T-RFLP analysis. These authors found that the various genera in the model community could best be resolved by digesting amplicons - obtained by using bacterial primers 8f and 926r - with *Hae*III. To demonstrate the utility of the approach, samples from five women that had been collected over a 2-month period were analyzed. Differences and similarities among the vaginal microbial communities of the women were readily apparent. The T-RFLP data suggest that the communities of three women were dominated by a single *Lactobacillus* phylotype. In contrast, the communities of

two other women included numerically abundant populations that differed from *Lactobacillus* species. The T-RFLP profiles of samples from all the women were largely invariant over time, indicating that the vaginal microflora was relatively stable throughout two menstrual cycles. The authors concluded that T-RFLP of 16S rRNA genes can be used to compare vaginal microbial communities and to gain information about the numerically dominant populations that are present.

V.1.4. Species specific PCR

The data obtained by using broad-range 16S rDNA PCR-techniques are often confirmed by species specific PCR. However, since species specific PCR assays targeting a single species are not well-established for the diagnosis of bacterial vaginosis, attempts were made to combine several species specific PCRs in order to obtain more predictive assays. Obata-Yasuoka *et al.* (192) proposed as a diagnostic test for bacterial vaginosis a multiplex PCR assay using primers specific to 16S rRNA genes of *M. mulieris* and *M. curtisii,* the *nanH* gene of *Bacteroides fragilis*, and an internal spacer region of the rDNA of *G. vaginalis*. The diagnostic sensitivity, specificity, positive predictive value and negative predictive value of multiplex PCR in comparison with Gram stain examination were 78.4%, 95.6%, 82.9% and 94.2%, respectively.

In manuscript V (289) we suggest to use a combination of the species specific PCRs for *G. vaginalis* and *A. vaginae* when diagnosing bacterial vaginosis. The simultaneous presence of *A. vaginae* and *G. vaginalis* in a vaginal swab specimen as detected by species-specific PCR had an accuracy of 90% [95% CI: 86-92%], a sensitivity of 82% [95% CI: 59-94%], a specificity of 90% [95% CI: 87-92%], a positive predictive value of 26% [95% CI: 17-39%] and a negative predictive value of 99% [95% CI: 98-100%] in assessing bacterial vaginosis (defined as a grade III smear).

Zarrifard *et al.* (242) determined the feasibility of using realtime PCR to detect and quantify *L. crispatus*, *L. jensenii*, *G. vaginalis* and *M. hominis* in the genital tract of 21 women (of which 16 were negative for bacterial vaginosis according to Amsel criteria) using stored vaginal samples. Lactobacilli were detected in all of the samples, *G. vaginalis* was detected in all but one of the samples, but in contrast, only six of the samples had detectable numbers of *M. hominis* organisms. The results show that samples from women with bacterial vaginosis that was diagnosed clinically have significantly higher numbers of *G. vaginalis*, but significantly lower numbers of lactobacilli. Moreover, there was a noticeable pattern where low numbers of lactobacilli were found in samples with high numbers of *G. vaginalis* and

conversely, low numbers of *G. vaginalis* organisms were seen in samples that had high numbers of lactobacilli. In a following study (242) this research group compared Nugent score to Amsel criteria and quantitative bacterial PCR for diagnosing bacterial vaginosis in 203 cervicovaginal lavage samples from women with Nugent scores of 7 to 10 (bacterial vaginosis group) and 203 samples from women with bacterial vaginosis Nugent scores of 0 to 3 ('no-bacterial vaginosis' group). Although there was significant overlap in the $\log_{10}$ lactobacillus counts between the two groups, their data demonstrate that quantitative bacterial PCR for *G. vaginalis*, *M. hominis* and lactobacilli significantly correlates with the Nugent Gram stain method to diagnose bacterial vaginosis. In addition they were able to identify cut off points for *G. vaginalis* and *M. hominis* that differentiated the bacterial vaginosis group from the no-bacterial vaginosis group. Utilizing all three $\log_{10}$ bacterial counts (*G. vaginalis*, *M. hominis* and lactobacilli) the sensitivity and specificity of the PCR assay were 83% and 78%, respectively, in comparison with Nugent score, while the sensitivity and specificity of the Amsel criteria compared to Nugent were 37% and 99%, respectively.

The most promising species-specific PCR approach was reported by Fredricks *et al.* (77) who applied bacterium-specific PCR for bacterial vaginosis–associated bacteria (BVAB) 1, 2, and 3 to a group of 27 women with bacterial vaginosis and 46 women without bacterial vaginosis. These uncultivated *Clostridium* species were highly specific indicators of bacterial vaginosis (> 95% specificity). Their *Atopobium* and *Megasphaera* assays were very sensitive tests for bacterial vaginosis (> 96%), but detection of *Megasphaera* was more specific for bacterial vaginosis (91%) than *Atopobium* (80%).

V.1.5. FISH

Two research groups applied FISH to assess the bacterial composition of normal and disturbed vaginal microflora. Swidsinski *et al.* (267) investigated the composition and spatial organization of bacteria associated with the vaginal epithelium in biopsy specimens from 20 patients with bacterial vaginosis and 40 normal premenopausal and postmenopausal controls using a broad range of fluorescent bacterial group-specific rRNA-targeted oligonucleotide probes. Bacterial vaginosis was associated with greater occurrence and higher concentrations of a variety of bacterial groups. However, only *G. vaginalis* developed a characteristic adherent biofilm that was specific for bacterial vaginosis.

No biofilm could be detected in 14 of 20 healthy premenopausal and 6 of 20 postmenopausal controls. In these patients, only single bacteria - long rods that positively hybridized with the

Lactobacillus probe - were dispersed over the epithelial surface at a concentration of less than 10^6 bacteria per ml.

A dense bacterial biofilm was attached to at least 50% of the intact epithelial surface in 90% of the biopsy specimens from women with bacterial vaginosis and 10% of the normal controls. Bacterial concentrations within this biofilm reached 10^{11} bacteria per milliliter. This adherent biofilm was mainly composed of 3 bacterial groups. Bacteria that positively hybridized with the *Gardnerella* probe composed 60% to 95% of the biofilm mass. Bacteria that positively hybridized with the *Atopobium* probe were homogeneously intermixed with the *Gardnerella*-positive biofilm in 70% of the biopsy specimens and composed 1% to 40% of the biofilm mass. Bacteria that positively hybridized with the *Lactobacillus* probe were found in all but one woman. However, the proportion of the *Lactobacillus*-positive bacteria reached between 1% and 5% in only 20% of the biopsy specimens with adherent biofilm.

Fredricks *et al.* (77) used FISH to localize bacteria in vaginal fluid smears and to characterize the bacterial morphology of three previously uncultivated bacterial vaginosis-associated clostridial bacteria BVAB1, BVAB2 and BVAB3. The authors demonstrated that the three uncultivated bacteria have distinct morphology and are found attached to vaginal epithelial cells in a way that is typical of the clue cells that characterize bacterial vaginosis. Interestingly, BVAB1 has a curved rod morphology that can be confused with the *Mobiluncus* morphotype. Furthermore, BVAB2 appeared to be a short, straight rod, whereas BVAB3 was a relatively long, wide, straight, lancet-shaped rod.

V.2. Limitations of the molecular microbiologic approach

Although molecular methods offer distinct advantages over cultivation for the characterization of complex microflora, they do have limitations. Previous papers (e.g. (293)) have discussed in general terms the pitfalls of PCR-based technologies. Some of these problems specifically related to broad-range 16S rDNA PCR are discussed here.

First, broad-range 16S rDNA PCR is capable of detecting low levels of bacterial DNA in many sources besides the intended sample (false positives), such as laboratory reagents, DNA extraction kits (281), and PCR reagents. For instance, the *Taq* polymerase used in PCR is known to contain contaminating bacterial 16S rDNA (234). One study provided proof that low levels of contaminating bacterial 16S rDNA fragments were present in either their ultra-pure water system or had been distributed with the purchased molecular biology reagents or disposable plastic articles (88). Identified bacteria were the water-borne bacterial genera *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Ralstonia* and *Bacillus*. Another study

reported a survey of 16S rDNA sequences that were obtained from negative extraction controls, that is, DNA extraction and purification performed without added sample, and identified 16S rDNA sequences closely related to the genera *Duganella* (formerly *Zoogloea*), *Acinetobacter*, *Stenotrophomonas*, *Escherichia*, *Leptothrix* and *Herbaspirillum* (270). Methods to monitor this problem are inclusion of 'no template' PCR controls in every run, which consist of DNA from sham samples processed through the DNA extraction method to assure that bacterial PCR products do not arise from the extraction reagents. Because removal of all bacterial DNA contamination is nearly impossible, it is advisable to limit the number of PCR cycles used in broad-range 16S rDNA PCR in order to reduce assay sensitivity and thus avoid detection of low-level background bacterial DNA. Cloning PCR products arising from the sham digest control will help reveal contaminating sequences in clone libraries. However, the abovementioned contaminating bacteria were not reported in most of the studies that used broad-range PCR to characterize the vaginal microflora, except in the study of Hyman *et al*. (130).

Second, broad-range bacterial PCR primers may not amplify 16S rDNA from some species because these primers are not 'completely' universal (16, 74, 125). Some bacteria have polymorphic nucleotides at otherwise 'conserved' positions in the 16S rRNA gene. Consequently, some broad-range PCR amplification primers may have one or more base mismatches with the 16S rRNA genes of these bacteria. The presence of a mismatch between primer and template will reduce the efficiency of priming, especially in the critical early rounds of amplification. As a consequence, in total community DNA samples, differential sequence complementarity to primers between taxa will lead to a significant bias in the amplification efficiency of the different taxa. Incorporation of multiple bases at degenerate positions and the use of inosine residues have been applied effectively to provide 'universal specificity', but excess use of these bases has been reported to induce biased template-to-product ratios (208). Furthermore, we can be even less confident that bacteria from unknown phylogenetic groups will have rRNA genes that can be efficiently amplified with the primers designed from current sequence alignments. Careful selection of the most appropriate primers for specific objectives and pooling of several PCR reactions utilising slightly different primers may significantly reduce bias and provide a more accurate understanding of microbial community structure (16, 208, 265, 293).

The positions of the broad-range 16S rDNA primers we used (resp. 10f, GV10f en 536r) are marked on a bacterial variability map of the *E. coli* 16S rRNA gene sequence (Addendum). The reverse primer 536r hybridizes with a completely conserved region, however, the

variability of the region that hybridizes with the forward primer 10f is higher and this primer contains three mismatches to the *G. vaginalis* 16S rRNA gene. For this reason a second forward primer GV10f was designed. For the purpose of T-RFLP two separate broad-range PCR reactions, i.e. respectively with the pairs 10f-536r and GV10f-536r, were performed and the reaction products were combined prior to restriction digestion.

Third, PCR bias may skew the representation of bacterial sequences detected in a sample. For instance, a 16S rRNA gene from a bacterium with high guanine plus cytosine content may result in reduced amplification efficiency compared to a low guanine plus cytosine content bacterium. PCR additives such as tetramethylammonium chloride and dimethyl sulfoxide have been used to equalize amplification efficiency across PCR templates and reduce nonspecific amplifications (146). The representation of bacterial 16S rDNA sequences in a sample can also be affected by the number of PCR cycles used, with some data suggesting that greater diversity results from the use of fewer cycles (308).

Fourth, the DNA-extraction may influence with which 16S rDNA fragments are amplified from different groups of bacteria. Gram negative and Gram positive organisms have differing susceptibilities to lysis with various DNA extraction methods. For a more efficient lysis of Gram positives present in the vaginal microflora we used mutanolysin.

Further, microorganisms differ in the number of *rrn* operons they carry in their genomes, ranging between 1 and 13 (48), thus bacteria with high rDNA copy numbers can be expected to be more efficiently amplified (68). There are currently no satisfactory methods to overcome this limitation. Candela *et al.* (37) determined that the variability in the number of *Bifidobacterium* rRNA genes ranges from one to five. While the strains of *B. infantis*, *B. breve* and *B. longum* exhibited constant *rrn* operon copy numbers at species level, i.e. three for *B. infantis* and two for *B. breve* and *B. longum*, variability was observed for the *B. bifidum* strains, with *rrn* operon copy number ranging from two to three (37).

It has been postulated that the number of *rrn* operons is positively correlated with growth rate (142) and thus that slowly-growing, difficult-to-culture bacteria will be poorly represented as well in 16S rRNA libraries generated by PCR. Consequently, molecular-based tools developed in part to avoid the bias of culture-based techniques, would overemphasize the same organisms. We could not confirm this statement for the rather fastidious *L. crispatus* and the easily cultured *L. jensenii*. To the contrary, while *L. crispatus* is a slowly-growing organism on Schaedler based agar and often appears as satellite colonies around other lactobacilli, 96.4% of 112 cultured isolates were confirmed by T-RFLP and an additional 39 women were found to harbor *L. crispatus* as assessed by T-RFLP. Therefore, T-RFLP might

better than culture represent the true presence of this species in vaginal microflora. On the other hand, the presence of *L. jensenii,* producing large colonies on Schaedler based agar, might be overestimated based on culture since only 63.4% of 82 cultured isolates were confirmed by T-RFLP and since only 10 additional *L. jensenii* harboring women were found by T-RFLP. Therefore, it seems that the efficiency of culture depends more on the availability of the appropriate culture methods than on the number of rrn operons.

Sixth, because of the conserved nature of the rRNA molecule, cross-reactions between closely related species occur. The resulting chimeras suggest the presence of organisms that do not actually exist. Several types of PCR artefacts have been reported: (i) chimeras between two different homologous molecules, (ii) deletion mutants due to stable secondary structures (128, 145, 293).

Finally, in case of an ecosystem with a relatively high bacterial diversity only the dominant species will be amplified (314). The relative abundances of organisms in any complex microbial community can vary over many orders of magnitude so that, in a total DNA preparation from the community, genomes of the most abundant organisms far outnumber those of rare organisms and will be overrepresented correspondingly in the PCR product pool. It should be noted that this will be a problem for culture-based approaches on non-selective media as well.

Furthermore, in case of T-RFLP, PCR products from different 16S rDNA templates that have a terminal restriction fragment of the same length will appear as one TRF after capillary electrophoresis and as such the diversity in the sample will be underestimated. The same problem exists for DGGE if PCR products from two different 16S rDNA templates happen to have similar mobility profiles, they appear as one product on the gel. Moreover, the limited resolution on DGGE may cause that fragments with only slightly different electrophoretic mobility are visually indistinguishable.

In our opinion, because of possible PCR amplification and cloning biases (68, 220, 265) and chimeric artifacts (128, 145), PCR-based methods should be viewed only as providing a way of sampling microbial biodiversity only in addition to cultivation and microscopy. On their own, these methods do not provide a complete and totally objective view of community composition and structure. Nevertheless, culture-independent molecular methods have made it possible to detect and identify previously overlooked but important fastidious vaginal bacteria.

V.3. The unrecognized vaginal species

The various culture-independent studies that were recently published, (see V.1) demonstrated that previously uncultivated species indeed constitute a large percentage of the vaginal microflora. The importance of some of these newly recognized species with regard to vaginal health is worthwhile to be further investigated, e.g. when these organisms are present in a large number of women (as is the case for *L. iners*) or when these microbes are found to be associated with bacterial vaginosis (as is the case for *A. vaginae*).

V.3.1. *Lactobacillus iners*

L. iners, which unlike other *Lactobacillus* spp., can be cultured only on blood agar, was first described in 1999 (66) and has only recently been shown to be part of the vaginal microflora (e.g. (8, 272, 286)). The initial isolates originated from medical care products, human urine, vaginal discharge and endometrial and cervical specimens. This species was missed in earlier studies of lactobacilli in vaginal secretions that did not include blood agar and that relied solely on MRS and/or Rogosa agar (85, 140, 202, 219, 259).

Phylogenetically, *L. iners* clusters with the *Lactobacillus acidophilus* group of species and is most closely related to *L. gasseri* and *L. johnsonii* (approx. 96% 16S rDNA sequence similarity) (66). This bacterium is biochemically readily distinguishable from all other currently described lactobacilli, by its relatively asaccharolytic nature. The 11 strains described by Falsen *et al.* (66) were biochemically homogeneous and produced acid, but no gas, only from glucose.

It was found that less than 10% of the *L. iners* strains produce hydrogen peroxide while more than 70% of the other vaginally abundant *Lactobacillus* species are hydrogen peroxide producers (8, 10, 212). This finding suggests that *L. iners* will poorly protect against the acquisition of bacterial vaginosis.

The presence of *L. iners* in the vaginal microflora reported in culture-independent studies is usually higher than that reported in culture-based studies.

One culture-based study (8) reported the presence of *L. iners* in 15% of 302 women and of these, 36% had a bacterial vaginosis microflora. In another report *L. iners* was cultured from 17% of 23 women with a normal vaginal microflora (286). We cultured *L. iners* in respectively 20%, 17%, 23% and 44% of pregnant women with respectively normal, grade I-like, intermediate and bacterial vaginosis microflora (289). Remarkably, Antonio *et al.* (10) recovered *L. iners* from the rectum in less than 1% of 290 women while *L. crispatus*, *L. jensenii* and *L. gasseri* were recovered rectally in respectively 16%, 10% and 10%.

Burton *et al.* (33) examined the vaginal bacterial microflora of 19 premenopausal women by DGGE and found that *L. iners* was the most common *Lactobacillus* present (in 42% of the women). In the cloning study of Fredricks *et al.* (77) *L. crispatus* and *L. iners* were the most abundant vaginal lactobacilli. Whereas *L. crispatus* and *L. iners* were found in respectively all (100%) and 6 (75%) of 8 women with a normal microflora, *L. iners* was the only *Lactobacillus* species detected in 5 of 9 (55%) women with bacterial vaginosis. Also in our cloning study (288) *L. iners* was the only *Lactobacillus* species present in the bacterial vaginosis microflora. However, based on the combination of culture and T-RFLP results (289) we found especially *L. gasseri* to be associated with the presence of *G. vaginalis*.

Most intriguing is the recent observation that the healthy vaginal microflora of Nigerian women is dominated by *L. crispatus* in only 4% of the subjects, while 64% were colonized by *L. iners*, followed by 8.3% colonized by *L. gasseri* (11). Given the fact that the hydrogen peroxide producing capacity of lactobacilli is believed to determine proneness to bacterial vaginosis, the occurrence of *L. iners* in absence of *L. crispatus* in the majority of women might be a possible explanation for the high prevalence (30-50%) of bacterial vaginosis among African women (62, 189).

In conclusion, it is now apparent that *L. iners* is a major component of the vaginal microflora. However, the importance of this microorganism with regard to vaginal health has yet to be determined. From our present knowledge, it seems that *L. iners* contributes little to protection against bacterial vaginosis.

V.3.2. *Atopobium vaginae*

The genus *Atopobium* was proposed by Collins & Wallbanks (51) to accommodate species formerly designated *Lactobacillus minutus* (100), *Lactobacillus rimae* (193) and *Streptococcus parvulus* (300). Phylogenetically, *Atopobium* species form a distinct group that branches off within the actinomycete line of descent (Stackebrandt & Ludwig, 1994). The closest phylogenetic relative of the genus *Atopobium* is *Coriobacterium glomerans*, isolated from the intestinal tract of red soldier bugs (*Pyrrhocoris apterus*) (91).

Atopobium species occur in human gingival crevices (*A. rimae, A. parvulum*) and colon (98, 169) and may be isolated from a range of human infections, e. g. *A. rimae* and *A. parvulum* were isolated from dental abscesses (58) and *A. minutum* from pelvic abscesses (135).

Atopobium species are anaerobic, Gram-positive elliptical cocci or rod-shaped organisms occurring singly, in pairs or as short chains. The variable cell morphology of *A. vaginae* makes that this species may reside perfectly camouflaged and as a consequence undetectable

among the mixture of other species present in grade II and III vaginal microflora (i.e. disturbed vaginal microflora). Also the fact that *A. vaginae* is fastidious and forms small pinhead colonies can explain why this species was not yet established as part of the vaginal microflora, when using classical microbiology, i.e. culture. Moreover, two studies (71, 84) reported that *A. vaginae* was misidentified as *Gemella morbillorum* using the API ID32A system. Some members of the genus *Atopobium* are known to produce large amounts of lactic acid. On this basis, some species belonging to the genus *Atopobium* were originally identified as *Lactobacillus* sp.

A. vaginae was described as a novel species only since 1999 (222) and currently only some ten strains have been isolated. In 2003 Geissforfer *et al.* (84) identified *A. vaginae* as the causative agent of a tuboovarian abscess and recently Hebb *et al.* (106) found *A. vaginae* - using culture-independent methods - in the Fallopian tube microflora of three women with salpingitis, but the clinical significance of *Atopobium* species has not been defined to date. The possible role of *A. vaginae* in the vaginal microflora is further discussed in section V.4.4.

V.4. Contributions to the increasing knowledge of the vaginal microflora and bacterial vaginosis

V.4.1. Application of a combination of culture and non-culture based methods extended the characterization of the normal and disturbed vaginal microflora

The strength of our studies on the characterization of the vaginal microflora is the combined use of classical microscopy, cultivation and PCR-based culture-independent methods to study the same samples. Each of these techniques contributes to establishing the composition of a complex microbial population. Both culture and molecular techniques have their advantages and limitations when studying the composition of the complex vaginal microflora. Although T-RFLP and culture results were not entirely in agreement, both techniques corresponded rather well.

Of the 38 species noted in the cloning study (288) of eight women, 18 (47%) were detected by cloning only, demonstrating that uncultivated species indeed constitute a large percentage of the vaginal bacterial diversity. The weaknesses of the cloning study is that the primers employed for broad-range PCR were not optimal, that some bacterial diversity may have been missed by using ARDRA to screen clones and that a limited number of clones were analyzed for each woman. Since the density of bacteria in the vaginal microflora, on the basis of culture techniques, has been estimated to range from 10^6 to 10^8 bacteria per gram of vaginal fluid, characterization of about 100 clones from each woman means that microorganisms present at concentrations of 10^6 or fewer colony-forming units per gram of vaginal fluid are unlikely to be detected by the PCR approach.

When performed under proper incubation conditions and on the appropriate (selective) media, culture may be more sensitive than broad-range PCR-based techniques. Even though molecular techniques have allowed the identification of species as *L. iners* and *A. vaginae*, the development of selective media for fastidious species is essential so that extensive biochemical characterization and investigation of their role in urogenital tract diseases will become possible.

We also showed that it is possible to differentiate between *L. crispatus* (short plump rods) and *L. gasseri*/*L. iners* (long tender rods) on the basis of Gram smear (289) and that the former is a better predictor of stable normal vaginal microflora during pregnancy (290). This may be somewhat in correspondence with a previous report (207) that considered 'long uniform'

lactobacilli as a risk factor for postoperative infection after first trimester abortion and with another manuscript (124) that described a form of vaginal discharge as 'vaginal lactobacillosis', a condition whereby extremely long lactobacilli were observed in the smears of women, suffering from thick, white, creamy or curdy vaginal discharge, associated with vaginal itching, burning and irritation.

In general, the limited detail with which the microbiology has been carried out in most studies probably is at the basis of the difference between the conclusions of many studies.

V.4.2. Refined identification of lactobacilli indicates different distribution of different *Lactobacillus* species

By means of tDNA-PCR (15, 283, 302), it was possible to identify the vaginal *Lactobacillus* species rapidly and with high discriminatory power, whereas lactobacilli remain difficult to identify with biochemical means. The poor taxonomy and the identification difficulties have hampered until very recently the study of the distribution of the different species in vaginal samples. As a consequence, thus far only the presence of lactobacilli in general was considered in most studies. However, recent publications (e.g. (8, 279)) and our findings (288-290, 292) clearly indicate that important differences exist between the different vaginal lactobacilli, also with regard to presence in normal and disturbed vaginal microflora. *L. crispatus* is clearly associated with normal vaginal microflora, in accordance with previous reports (8, 279), while *L. gasseri* and *L. iners* are present in disturbed vaginal microflora as well (manuscript III (290) and with *L. gasseri* strongly associated with *G. vaginalis* (290).

Recent research has made clear that identification of lactobacilli has to be made at the species level to better understand the etiology of the disturbance of the vaginal microflora. Indeed, one of the hypotheses regarding the etiology, put forward by different groups, is the disappearance of the 'right' lactobacilli, possibly by competition with other *Lactobacillus* species (24, 194).

V.4.3. The recognition of new vaginal microflora categories on Gram stained vaginal smears points to probable misinterpretations of the results of clinical studies in the past

One of our recent findings shows that a separate 'grade' can be distinguished on the basis of Gram stained vaginal smears. We designated this separate category as 'grade I-like', since morphologically the Gram stain can easily be misclassified as a genuine grade I sample (normal vaginal microflora), although it is microbiologically completely different: while

grade I samples are characterized by the predominance of a few *Lactobacillus* species (288, 289), grade I-like samples never contain *L. crispatus* and more than half of the samples contain predominantly bifidobacteria, a group of organisms rarely found in all other grades. Interestingly, even *G. vaginalis* and *A. vaginae* are absent from grade I-like samples, although they can be found sporadically in grade I samples. It is important to note that in all clinical studies thus far this separate class, with clearly disturbed vaginal microflora, has been considered as normal vaginal microflora and as such must have compromised to some degree the conclusions of all of these studies.

V.4.4. The association of bacterial vaginosis and the unculturable bacterium *Atopobium vaginae* urges to reconsider previous treatment studies and may explain contradictions between previous reports

Possibly the most important new insight which stems from our research efforts is the discovery that there is a strong association between bacterial vaginosis and the presence of a thus far unrecognized species, i.e. the strictly anaerobe, very fastidious Gram positive coccus *A. vaginae*, (288, 292). Another group (72) simultaneously and independently confirmed this finding. In fact, *A. vaginae* was described as a novel species only since 1999 (222) and currently only some ten strains have been isolated. Again, i) the fact that we could show that the association between *A. vaginae* and bacterial vaginosis is even stronger than that between *G. vaginalis* and bacterial vaginosis and especially ii) the observation that part of the *A. vaginae* isolates is metronidazole-resistant, urges to reconsider the conclusions of most previous studies with regard to bacterial vaginosis and preterm birth. For example, only recently an extensive study reported that 99% of the anaerobe vaginal microflora is sensitive to metronidazole and thus that this antibiotic remains first choice (20) (together with clindamycin). Evidently *A. vaginae* was not included in this study, since its importance was not yet recognized by these authors and since it is so fastidious. The assumption that this species may play an important role in the etiology of bacterial vaginosis as well as that of preterm birth stems from the following observations: i) Meta-analysis showed that metronidazole-treatment did not positively affect the preterm birth-ratio (171). ii) A very large trial (39) using metronidazole at 18-22 weeks of gestation showed no reduction of preterm birth, whereas recent studies, using clindamycin *per os* (278) or intravaginal (148) showed a clear reduction in preterm birth.

Maybe, the bacterium *A. vaginae* provides also an explanation for the observation of frequent recurrence of bacterial vaginosis, despite initial successful (metronidazole) treatment.

VI. Conclusions

The question to what is the etiology of BV has remained unanswered since the first description of the syndrome in 1955 (81). Efforts to link bacterial vaginosis to a single, cultivated bacterial pathogen, such as *G. vaginalis*, have been unconvincing until recently, when Swidsinski *et al.* (267) showed by means of FISH of vaginal biopsy specimens that the presence of an adherent vaginal biofilm that predominantly hybridizes with a *G. vaginalis* probe is sensitive and specific for detecting bacterial vaginosis. Although more studies are needed, these observations raise the possibility that the development of this biofilm may be responsible for the pathogenesis of bacterial vaginosis, rather than gradual overgrowth of resident vaginal microflora within vaginal secretions as hypothesized previously.

Whether molecular analysis of bacterial vaginosis-associated bacteria will succeed in elucidating the etiology of bacterial vaginosis remains to be seen, although tremendous progress is made during the past 5 years, through cloning studies (35, 77, 109, 130, 288, 313), species-specific PCR (34, 72, 77, 242, 288, 312), broad-range PCR techniques like T-RFLP (53, 290, 292) and DGGE (11, 33, 36, 71), and *in situ* hybridisation studies (77, 267). It is possible that bacterial vaginosis is a heterogeneous syndrome caused by different combinations of bacterial communities, as has been described for the syndrome of periodontitis (256), and that a woman's individual risk for a particular etiologic community might depend on specific practices, such as unprotected sex or vaginal douching. When bringing together these latest insights with the vast knowledge gathered during the last 50 years, our current view is that bacterial vaginosis can be considered as a disturbance of the normal vaginal microflora that depends on a combination of innate immunity status, of microbiological factors and of external factors challenging the normal lactobacillar microflora. As such, external factors as menstruation, vaginal douching and sexual intercourse cause intermittent increases in pH, which creates the opportunity for multiplication of anaerobe bacteria. Depending on the innate type of lactobacilli, i.e. depending on their ability to suppress anaerobes by bacteriocins and H_2O_2 production at high pH and on the speed with which they can produce lactic acid, pH can be restored sufficiently quickly to avoid overgrowth of anaerobes. In combination with the type of innate immunity of the women, this will determine the likelihood that the lactobacillar microflora will regain supremacy or that an anaerobe biofilm will develop and cover the vaginal epithelium.

Summary of contributions of our research group

The several new insights with regard to the composition of the vaginal microflora resulting from this doctoral thesis can be summarized as follows:

First, clear differences exist with regard to colonization resistance offered by the different lactobacilli: *L. gasseri* and *L. iners* are just as frequently present in disturbed vaginal microflora as in normal vaginal microflora, while *L. crispatus* (and *L. jensenii*) are clearly absent from disturbed vaginal microflora (289).

Second, a clearly separate class of vaginal microflora exists, predominated by *Bifidobacterium* species, which we have designated grade I-like. This class represents a form of aberrant vaginal microflora but has been confused with normal vaginal microflora, designated grade I (289).

Third, the very fastidious bacterium *Atopobium vaginae* is as strongly associated with bacterial vaginosis as is *Gardnerella vaginalis* (288, 292), but due to its fastidiousness, this organism has been overlooked thus far in bacterial vaginosis and preterm birth studies. Its resistance to metronidazole urges to reconsider past metronidazole treatment studies and the applicability of this antibiotic for treatment of bacterial vaginosis in general. Given these findings, it may be advisable to include all classes of disturbed vaginal microflora and to clearly distinguish between these, when carrying out clinical epidemiological and treatment studies.

VII. Perspectives

The new insights, highlighted in the previous sections – in combination with the improved microbiological detection and identification techniques which were recently developed, call for new clinical and *in vitro* studies, since several old and new questions can be addressed and probably better answered than thus far possible.

VII.1. Further development and validation of diagnostic tools for bacterial vaginosis and urogenital infection in general

VII.1.1. Validation study of microbial and vaginal cell types used in scoring Gram stained vaginal fluid smears for the diagnosis of bacterial vaginosis

Five years ago, an international workshop (76) brought together researchers with the goal of ending the confusion created by many different criteria for scoring Gram stained vaginal smears, some of which had similar or overlapping definitions. The workshop was undertaken on the assumption that the kind of bacterial and vaginal cell types that form the best basis of a scoring system need to be reconsidered. The results were encouraging in that good concordance was observed among most observers, although also several disagreements surfaced with regard to the scoring of microflora not related to bacterial vaginosis (i.e., altered vaginal microflora and the 'problem' of intermediate microflora (76)), indicating a strong need for further studies on how to standardize and validate the scoring systems used.

Shortly, we will participate in another international workshop, organized by Prof. Dr. U. Forsum and Dr. C. Spiegel, intended to follow up the previous workshop and to further define the scoring systems used in more detail.

The strategy of this workshop is

1. To identify possible classes of microbial and vaginal cell types
2. To determine what classes of microbial and vaginal cell types might be robust in the sense that they are unequivocally identifiable
3. To determine whether previously unrecognized patterns or associations among the organisms can be observed

Given the outcome, existing scoring schemata and possible new scoring schemata will be evaluated against diagnosis of bacterial vaginosis using the Amsel and other published standard clinical criteria. Also, the schemata should be evaluated against laboratory identification of vaginal microflora using conventional quantitative aerobic and anaerobic culture and/or quantitative nucleic acid amplification (FISH). The scoring system developed

based on the workshop data will then be used as a training set of health care and laboratory workers that are diagnosing bacterial vaginosis to explore its robustness in practice.

This workshop promises to standardize the scoring and grading effort for bacterial vaginosis worldwide. This is of utmost importance, since clinical outcome parameters critically depend on a robust scoring and grading protocol for bacterial vaginosis.

VII.1.2. Development of semi-quantitative DNA-based techniques

During this doctoral work we have developed tDNA-PCR, focused on the identification of cultured vaginal bacteria (manuscript I, III, V), T-RFLP-analysis, capable of detecting and identifying most vaginal bacteria without culture (manuscript V) and species specific PCR for *A. vaginae* and *G. vaginalis* (manuscript II, III, V). However, as with all techniques, there are some drawbacks to these approaches, like culture dependency (tDNA-PCR), limited quantification (T-RFLP), limitation to prokaryotic organisms (both tDNA-PCR and T-RFLP) or limitation to the species for which the PCR was developed (in the case of species specific PCR). Although all of these approaches are currently still in use for the characterization of the vaginal microflora in ongoing studies and certainly will be among the basic instruments for the planned studies, in some instances it may be appropriate to have a more quantifiable result. Therefore, real-time PCR will be developed for some of the most important species, i.e. *L. crispatus*, *A. vaginae*, *G. vaginalis* and the three recently discovered bacterial vaginosis-associated clostridial bacteria BVAB1, BVAB2 and BVAB3 (77).

VII.2. Understanding of the etiopathogenesis of bacterial vaginosis

Several studies have been carried out to determine the composition of normal and disturbed vaginal microflora, frequently in relationship with the pregnancy outcome. The results of these studies are often contradictory. We have good reasons to believe that these contradictory results are largely due to poor microbiological back up.

In order to address the etiology of bacterial vaginosis we suggest several clinical studies, *in vitro* microbiological studies and studies using an *in vitro* vaginal model.

VII.2.1. Clinical studies

VII.2.1.1. Follow-up study of the composition of the normal vaginal microflora

A very limited number of studies thus far have addressed the natural course of the vaginal microflora during subsequent menstrual cycles. Categorization and identification was smear based (137, 238, 239) or culture based with identification to group/genus level only (64). In view of the number of recent developments in the field of the composition of the vaginal

microflora, including our increased knowledge of the different vaginal *Lactobacillus* species and the recognition of hitherto largely unknown vaginal microflora species, and in view of our improved molecular microbiological techniques (T-RFLP, tDNA-PCR, real-time PCR) such a longitudinal study design may now allow to address the natural course and the dynamics of the vaginal microflora in a more detailed manner, with implications not only to our basic knowledge of the vaginal microflora but also to our therapeutic abilities to safeguard the maintenance of healthy vaginal microflora.

The emphasis during this study should be on the occurrence and genotypic diversity of *L. crispatus*, since one of the major conclusions of a follow-up study of pregnant women was that the presence of *L. crispatus* confers the best guarantee for a good colonization resistance (290, 292), in accordance with previous reports in non-pregnant women (8, 279). In other words, the presence of *L. crispatus* in pregnant women decreases the likelihood that other bacteria are found later in pregnancy (290) and this may decrease the risk of preterm birth (291). Therefore, in order to better understand the factors that confer the colonization resistance offered by *L. crispatus* and with regard to the planned probiotic studies (see VII.3), we aim to study this organism and its occurrence in the vagina in more detail.

VII.2.1.2. Comparison of the composition of the vaginal microflora between different categories of disturbed microflora on Gram stain

During our previous and ongoing studies several new insights were gained. First there was the discovery that *A. vaginae* plays a role in bacterial vaginosis and that *L. crispatus* is important in normal vaginal microflora, while *L. gasseri* and *L. iners* are not only frequently present in normal vaginal microflora but also in all degrees of disturbed vaginal microflora (289). As such, the well-known fact and apparently contradictory finding that lactobacilli are frequently present in almost equal numbers in normal vaginal microflora as well as in bacterial vaginosis samples, is readily explained – again as a result of more refined microbiological characterization. Later, we discovered an additional grade (grade I-like) for which the vaginal microflora appears to consist principally of bifidobacteria and coryneforms (289), and finally, our data point to the possible importance of scoring polymorphonuclear leukocytes, whatever the condition of the vaginal microflora (291).

To reconcile these insights, a new comparative study has to be set up, including the different categories of vaginal microflora, but now with foreknowledge about possibly important factors. The refined microbiological characterization should be accompanied by detailed characterization of the vaginal fluid.

VII.2.1.3. Comparison of the vaginal microflora and the vaginal milieu in bacterial vaginosis, with emphasis on clinical and recurrent bacterial vaginosis

It remains unclear why bacterial vaginosis is symptomatic in only some women (clinical bacterial vaginosis) and recurrent in only some of the women with clinical bacterial vaginosis. For a study like this, which compares clinical symptoms, thorough directed metabolite analysis and differential metabolomic analysis could be carried out. Both amines and acids of the intermediary metabolism should be analyzed, since it has been shown that bacterial metabolism and, most probably, the hosts' response to infection will interactively define the composition of the vaginal and amniotic fluids (60, 119). Amines such as tyramine, histamine, β-phenylethylamine and the polyamines putrescine, cadaverine, spermine and spermidine are secreted by bacteria in the vaginal environment. These amines are produced as a result of protein and amino acid metabolism, and some of these are the cause of malodor in bacterial vaginosis (45, 67, 211). Also, polyamines, irrespective of their endogenous or exogenous origin, are e.g. implicated in the damaging of mucosal integrity and these compounds act to facilitate the transudation of vaginal fluid and exfoliation of epithelial cells, as such also explaining vaginal discharge (211).

In case the differences between clinical and asymptomatic bacterial vaginosis have a microbiological cause, we expect that the combination of refined microbiology and detailed characterization of the vaginal milieu will expose these. In case it can be shown that recurrent bacterial vaginosis is caused by residual metronidazole resistant organisms (like *A. vaginae*), additional studies, comparing metronidazole treatment with clindamycin or azithromycin treatment can be carried out.

VII.2.1.4. Comparison of the microflora of paired rectal/vaginal samples in pregnant women

The origin of the vaginal microflora and especially of the organisms that disturb the *Lactobacillus* dominated microflora remains poorly studied. However, it is generally accepted that the rectal microflora is the most likely source, given its vicinity to the urogenital tract and given the well-documented importance of fecal microflora in urogenital tract infections. For example, fecal *E. coli* is the most important cause of urinary tract infection in women. Recently, *E. coli* has even been found to be strongly associated with preterm birth (38). At present, few studies, comparing directly vaginal and rectal microflora, have been carried out, probably also because of the difficulties to culture and identify the large number of bacterial species present in the intestinal compartment. Two studies date before 1990 (93, 122). The authors suggest that the organisms associated with bacterial vaginosis, i.e. *Mobiluncus* spp.

(93, 122), *M. hominis*, and *G. vaginalis*, are not spread sexually but colonize the vagina from an endogenous intestinal tract site (122). Recently, Song *et al.* (259) and Antonio *et al.* (10) studied lactobacilli from rectal and vaginal origin by DNA-based identification of cultured isolates. Antonio *et al.* (10) found that co-colonization of the vagina and rectum by H_2O_2-producing lactobacilli was associated with the lowest prevalence of bacterial vaginosis (5%), whereas females colonized only vaginally, only rectally, or at neither site had an increased risk of bacterial vaginosis. These authors suggest that the presence of the right *Lactobacillus* species in the rectum may contribute to the maintenance of vaginal microflora.

To clarify this matter, and to contribute to the clarification of the etiology of bacterial vaginosis, we will carry out the characterization of the vaginal microflora on paired rectal/vaginal specimens, sampled at gestation week 35, by using i) species specific real-time PCR for *A. vaginae*, *G. vaginalis* and *S. agalactiae* (GBS), ii) T-RFLP with *Bifidobacterium* specific primers (also complementary for the closely related *G. vaginalis*) and iii) T-RFLP with *Lactobacillus* specific primers for rectal and vaginal samples. In addition, T-RFLP with universal primers will be used for vaginal samples only.

Direct comparison of the vaginal microflora related bacteria present in vagina and rectum should provide an indication as to which degree the components of the disturbed vaginal microflora originate from the rectal microflora.

The presence of the different bacteria at gestation week 35 can be related to the pregnancy outcome and to the presence of GBS. As an extra, this study will result in a limited evaluation of real-time PCR for the detection of GBS, since this PCR will be carried out in comparison with the routinely performed selective culture technique. This evaluation may eventually lead to routine application of real-time PCR for screening women at 35 weeks of gestation for the presence of GBS, as a replacement of or addition to the current culture based approach.

VII.2.2. Characterization of the biochemistry of and interactions between lactobacilli, bifidobacteria, *G. vaginalis* and *A. vaginae*

The study of the biochemistry and interactions of vaginal bacteria may contribute to answer questions regarding i) the etiology of bacterial vaginosis, ii) some of the clinical symptoms of bacterial vaginosis and iii) the role of the vaginal microflora in the etiology of preterm labour/preterm birth.

i) The etiology of bacterial vaginosis boils down to the question why the beneficial *Lactobacillus* species/strains disappear. Some metabolites are known to have bactericidal effects, inhibiting the growth of other bacteria (including lactobacilli) (211). Such compounds

can be characterized and quantified, as part of the explanation of the disappearance of lactobacilli in bacterial vaginosis and recurrent bacterial vaginosis.

ii) With regard to the well-known altered characteristics of vaginal fluid as a consequence of disturbed vaginal microflora and of bacterial vaginosis in special, eventually leading to malodor (one of the Amsel criteria (5)), to date, it has not been fully resolved which organisms actually produce the volatile amines, which cause the foul smell (211). Thus, the detailed characterization of the metabolites of the different species may help to elucidate why some women have symptomatic bacterial vaginosis – e.g. in case only some bacterial vaginosis bacteria produce malodorous amines.

iii) The etiology of preterm birth as a consequence of bacterial infection comes down to the questions of how bacteria can ascend and which bacterial metabolic products are able to induce preterm birth, directly or indirectly.

VII.2.2.1. Development of a selective culture medium for *A. vaginae*

Although there are several indications that *A. vaginae* may play an important role in the development of bacterial vaginosis and possibly preterm birth, only few strains are available for study, due to the fastidiousness of this organism. In mixed samples, *A. vaginae* will be overgrown by other bacteria. There are several reasons to attempt to obtain more isolates than the approximately ten that are now available (of which 8 are present in our collection). First, our unpublished 16S rDNA sequence data on the few strains available (and obtained by cloning), indicate that there is genetic diversity at the 16S rRNA gene level, pointing to subgroups within *A. vaginae* or to the existence of other closely related species. Second, our preliminary data indicate - not in complete correspondence to the results reported by Ferris *et al.* (71) - that metronidazole resistance varies markedly among the few strains of *A. vaginae* we have tested, with some strains borderline susceptible (55). Therefore treatment failure may be caused by only some *A. vaginae* strains. This increases the need of being able to culture *A. vaginae* in order to match treatment outcome to metronidazole MIC-value of the cultured *A. vaginae* isolate. Third, to test metabolic and biochemical characteristics in general, a larger collection is needed, especially in the light of the genetic diversity that appears to be present.

VII.2.2.2. Characterization of the properties of vaginal bacteria with emphasis on *L. crispatus*, *L. gasseri*, *L. iners*, *A. vaginae* and *G. vaginalis*

Given the epidemiological differences between *L. crispatus*, *L. gasseri* and *L. iners*, detailed characterization of the biochemistry and adherence properties of these species will be undertaken in an attempt to pinpoint why *L. crispatus* apparently confers colonisation

resistance while the other *Lactobacillus* species do not (289). For *A. vaginae* and *G. vaginalis*, the two species predominantly present in disturbed vaginal microflora, biochemical characterization can help to determine whether one or both of these species are (co-) responsible for some of the clinical signs, like malodor and whether the presence of these species is merely a sign of disturbance rather than that they possess special enzymatic activity which confers to the disturbance of the normal vaginal microflora. Also, for the development of a selective medium better knowledge of the biochemistry and antibiotic susceptibility of *A. vaginae* will be useful.

A broad set of metabolic and biochemical tests will be carried out, including

1. Directed metabolic analysis: polyamines and fatty acids of intermediary metabolism
2. Classical biochemical characterization by means of API and macro tests, hydrogen peroxide production, production of enzymes like arginine deaminase, sialidase and collagenase.
3. Antibiotic susceptibility testing.

Also, phages from *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners* will be tested for activity against *L. crispatus*. In addition, the ability of vaginal fluids – comparing those of smoking vs. non-smoking women - to induce prophage expression, as described by Pavlova *et al.* (203) (see VII.2.2.3), will be tested by adding these to *L. crispatus* liquid cultures.

VII.2.2.3. Study of the intermicrobial interactions

The study of intermicrobial interactions addresses two sides of a coin. First, it is clear that *L. crispatus* confers colonisation resistance, but the exact mechanism is not really known. Although lactic acid production is clearly an important factor, many other bacteria (incl. e.g. *G. vaginalis* (28, 29), *Bifidobacterium* spp. (156) and *Atopobium* spp. (51)) and vaginal epithelial cells (28, 29) acidify the environment as well to a certain degree. Although hydrogen peroxide production has been put forward in numerous publications as a potent antibacterial mechanism since at least a decade (101, 112, 114, 279), Famularo *et al.* (67) and Pybus & Onderdonk (211) have listed several arguments to doubt about this hypothesis.

Famularo *et al.* (67) have put forward the alternative thesis that arginine deaminase production may be more important to understand colonisation resistance.

Thus, the study of intermicrobial interactions may address the question which characteristics of *L. crispatus* inhibit growth of other bacteria. Also, it may be that some strains of *L. crispatus* are better than others in protecting against disturbance.

On the other hand, also the reason why *L. crispatus* disappears is not understood (etiology of bacterial vaginosis). A major candidate is competition with other bacteria – including other lactobacilli, possibly after an initial disturbance (like increased pH), which puts the lactobacilli in a temporarily weak position (211).

Another suggestion has been that introduction by foreign phages (as free phage particles or present in exogenous lysogenic lactobacilli) – as a consequence of acquiring a new partner or of promiscuity – may extinguish the lactobacilli present (24), a condition dubbed 'phaginosis'. Kilic *et al.* (140) showed that approximately 30% of lactobacillary strains were lysogenic for prophages with a broad host range and that induction of the phages could be caused by mitogenic substances, for example like those present in cigarette smoke. Indeed, trace amounts of benzo[a]pyrene diol epoxide (BPDE), which can be found in vaginal secretions of women who smoke, significantly increased phage induction in lactobacilli, a finding that might be an explanation for the observed relationship between smoking and bacterial vaginosis (203). Thus, the study of intermicrobial interactions may also address the question of which microbial factors are of importance in inhibiting the growth of *L. crispatus* strains.

Susceptibility to and inhibition by strains of different lactobacilli and *A. vaginae* and *G. vaginalis*

Testing of the susceptibility of strains towards the culture supernatants of other strains or towards the compounds present in e.g. vaginal fluid is easily done by applying the challenging material to wells punched in an agar plate that was inoculated with the test strain prior to punching wells. Susceptibility to molecules in the culture supernatant of the challenge strains can then be read as inhibition zones around the wells. A comparable approach is the mutual antagonism test, already used to study the interaction of vaginal bacteria (160, 185). Another easy approach that is worthwhile to be used is the preconditioned culture test (69). Test strains will belong to the species *L. crispatus*, *G. vaginalis*, *B. breve* and *A. vaginae*. Challenging will be carried out with i) dilutions of vaginal fluid (of smoking vs. non-smoking women) and with ii) supernatants of strains of very different species.

Characterization of the inhibitors will be carried out by evaluating the effect of different pretreatments, as described by Fernandez *et al.* (69). The supernatants of *L. crispatus* strains that appear to be powerful inhibitors of *A. vaginae* and *G. vaginalis* will be characterized by gas chromatography/mass spectrometry.

Not only inhibition, but also possible stimulation of one bacterial strain by another will become clear from these experiments. Experiments will be carried out at pH 4.5 and at pH 6.0,

which reflect the normal and the disturbed vaginal pHs and which have been shown to have a profound effect on inhibitory capacities of different compounds (211).

VII.2.3. *In vitro* vaginal/endocervical epithelial cell model

To our knowledge, thus far no group has used *in vitro* simulations of the vaginal milieu to study the influence of different bacteria on the production of metabolites and immunobiological markers. The use of an *in vitro* vaginal/endocervical cell culture model will make it possible to vary different parameters (like pH, oestradiol supply, glycogen content, iron content, addition of bacterial vaginosis-related or of probiotic strains) to study the microbial interactions and the factors that may influence stability of a *L. crispatus* dominated microflora, and to study the influence of different vaginal microflora categories on vaginal physiology and innate immunity markers. Inclusion of the monolayered, ciliated endocervix in such a model is of interest since the endocervix provides the ultimate defense against ascending infection, is a resort of immunobiological defense and is at the same time a region that is generally accepted to be the principle location of infection for e.g. *Chlamydia trachomatis* and HIV. A very realistic model, based on Human Papilloma Virus (HPV) immortalized vaginal and endocervical cell lines has been described, but has not been used in conjunction with bacterial colonisation, although infection with *Neisseria gonorrhoeae* has been studied (73).

Fichorova *et al.* (73) have shown convincingly that these vaginal/endocervical cells, maintain their differentiation characteristics as well as their immunobiological specificities: vaginal cell lines differentiate into stratified squamous non keratinizing epithelia whereas endocervical cell lines form simple columnar epithelium and only the endocervical cell lines produce IL-6, IL-7 and RANTES upon stimulation, as is the case *in vivo*. The different cytokine patterns among both cell types are consistent with the observation that under healthy conditions the endocervical cells thrive in a sterile environment, while the vaginal epithelial cells are constantly exposed to the normal vaginal microflora, having developed cytokine-orchestrated mechanisms for down regulation of inflammatory responses to bacterial components.

VII.3. Preventive and therapeutic perspectives to restore the vaginal microflora and infection-mediated preterm birth

The ultimate goal of fundamental research and diagnosis of infections is the improvement of preventive and/or therapeutic possibilities. It is generally known that attempts at correcting the imbalance in vaginal micro-ecology by means of antibiotics have been substantially unsuccessful (67, 157, 188, 221). In light of the discovery of the strong association of the

frequently metronidazole resistant bacterium *A. vaginae* with bacterial vaginosis, also antibiotic treatment regimens need to be readdressed. Moreover, antibiotic treatment focuses on eradication of the organisms responsible for the abnormal overgrowth. However, based on the hypothesis that loss of protective lactobacilli precedes the overgrowth of anaerobic vaginal organisms, a long-term solution to treatment of bacterial vaginosis contains restoration of the healthy protective *Lactobacillus*-dominant microflora. Efforts to artificially restore an unbalanced microflora with the use of probiotics have met with mixed results but research aimed at selecting *Lactobacillus* strains based on current knowledge regarding the possible characteristics which confer colonisation resistance could well provide a reliable alternative treatment and preventive regimen in the future. The appraisal that not any *Lactobacillus* species is associated with strong colonisation resistance, but most probably only (some) *L. crispatus* strains urges to carry out probiotic studies with a carefully selected set of strains of this species.

The idea of instilling the vagina with lactobacilli preparations (probiotics) dates back from at least 1993 (172, 188). Although Giorgi *et al.* (85) published that vaginal lactobacilli were not *L. acidophilus*, but *L. crispatus*, *L. gasseri* and *L. jensenii* already back in 1987, this knowledge was largely overlooked and microbiologists continued to speak of *L. acidophilus*. Therefore, it is known only since a few years that *L. crispatus* strains are the major inhabitants of the normal vaginal microflora (8, 279, 288, 292), and therefore probiotic studies have been carried out basically with a range of other - mostly non-vaginal - *Lactobacillus* species: *L. rhamnosus* GR-1 and *L. fermentum* RC-14 by the group of Burton & Reid (80, 161, 215, 216, 218), *L. brevis* CD2, *L. salivarius* FV2 and *L. gasseri* MB335 (168), *L. delbrueckii* and *L. acidophilus* (174), *L. acidophilus* (197, 201) and *L. johnsonii* (190). McLean *et al.* (174) tested one *L. crispatus* strain, but found it to be not adhering to vaginal epithelial cells. Only recently a patent was registered for the use of an H_2O_2-generating *L. crispatus* strain as probiotic (276), and at present only one research group evaluated *L. crispatus* as a vaginal probiotic (9, 25). Interestingly, Famularo *et al.* (67) suggest that not hydrogen peroxide production but rather arginine deaminase production may be the most powerful characteristic of a good vaginal probiotic strain. Deprivation of arginine as a result of arginine deaminase activity causes i) growth retardation of bacteria since it is a basic nutrient, ii) decreases NO production - since arginine is a precursor and thus decreases inflammation and iii) decreases malodor because arginine is a precursor - via arginine decarboxylase - for polyamines.

In addition, products which add to the colonization resistance against vaginal pathogens (prebiotics) are commercially available (e.g. Lactacyd®, GSK). One can envisage that

intravaginal administration of glycogen or glucose should support growth of *L. crispatus* and promote acidification of the vaginal milieu. Vaginal glycogen concentration could be raised as well by oral intake of oestradiol. Indeed, a commercial product, Gynoflor® (Grünenthal), which contains 0.03 mg estriol and 50 mg '*Lactobacillus acidophilus*' is already available and has been evaluated (197, 201). The authors concluded that restoration of the vaginal flora can be significantly enhanced by the administration of live lactobacilli in combination with low dose oestriol.

Given these facts, characterization of a genetically diverse collection of *L. crispatus* isolates should result in strains which i) adhere well to vaginal epithelial cells – from a wide range of individual women, ii) are resistant to a wide range of bacteriophages iii) are resistant to a wide range of bacteriocins from other lactobacilli and other vaginal bacteria, iv) produce high rates of lactic acid at different glycogen/glucose concentrations, v) produce high rates of hydrogen peroxide, vi) produce themselves toxic substances for other bacteria, vii) eventually produce arginine deaminase – to reduce quickly polyamine levels (67) and vii) can be cultured well together with other selected *L. crispatus* strains.

VIII. Summary

The microbial flora of the vagina affects the health of women, their fetuses, and newborns. Conventional culture methods failed to detect some fastidious vaginal bacteria, leading to an incomplete picture of the diversity of the vaginal microflora. DNA-based identification methods have facilitated the detection and identification of bacteria without cultivation, and the advantages and limitations of this approach are described.

Recently, molecular studies of the vaginal microflora have discovered several uncultivated bacterial species. An important new insight which stems from our research efforts is the discovery that there is a strong association between bacterial vaginosis and the presence of a thus far unrecognized species, i.e. the strictly anaerobe and very fastidious Gram positive coccus *Atopobium vaginae*. Also *Dialister, Leptotrichia* and *Megasphaera* species were commonly found in women with bacterial vaginosis. Another research group (77) found that several unknown bacteria in the *Clostridiales* order were found to be highly specific indicators of bacterial vaginosis and suggested that these might be possible candidates for future PCR-based diagnosis of bacterial vaginosis.

In addition, the comparison of traditional methods, i.e. microscopy and culture, with DNA-based techniques for characterization of the vaginal microflora have allowed us to refine Gram stain based grading of vaginal smears. We pointed out the presence of sub-categories within what was designated as grade I normal microflora, pinpointed especially a distinct cell type related to *Lactobacillus crispatus* (grade Ia) and described an additional category (grade I-like) related to the predominant presence of *Bifidobacterium* spp.

Already molecular analysis of bacterial vaginosis-associated bacteria has contributed to the refined described description of normal and disturbed vaginal microflora, but it remains to be seen whether it will succeed in elucidating the etiology of bacterial vaginosis. Our study showed that culture remains essential and the most complete picture of the composition of complex microbial communities is obtained by the combination of culture-based with culture-independent approaches. From the findings during the last five years, it is already obvious that a more complete understanding of vaginal microbial populations will lead to better strategies to maintain healthy vaginal microflora and might create opportunities to explore the role of previously unknown bacteria in reproductive tract diseases.

IX. Samenvatting

De samenstelling van de vaginale microflora is bepalend voor de gezondheid van vrouwen, hun foetussen en hun baby's. Met behulp van conventionele kweekmethodes was het tot heden niet mogelijk om moeilijk kweekbare vaginale bacteriën te detecteren en studies gebaseerd op cultuur leidden aldus tot een onvolledig beeld van de diversiteit van bacteriën aanwezig in zowel de normale als de verstoorde vaginale microflora. De introductie van kweek-onafhankelijke DNA-gebaseerde identificatiemethoden in de microbiologie vereenvoudigden de detectie en identificatie van bacteriën. De voordelen en beperkingen van deze aanpak worden beschreven.

Recent werden door middel van DNA-gebaseerde technieken verschillende nog niet eerder gekweekte bacteriën in de vaginale microflora aangetoond. Onze studies leidden tot een belangrijk nieuw gegeven: de associatie van *Atopobium vaginae*, een strikt anaërobe, zeer moeilijk kweekbare Gram positieve kok, met bacteriële vaginose. Ook vonden we frequent *Dialister, Leptotrichia* and *Megasphaera* species bij vrouwen met bacteriële vaginose. Een andere onderzoeksgroep detecteerde verschillende totnogtoe ongekende bacteriën behorende tot de familie van de *Clostridiales* die zeer specifieke merkers voor bacteriële vaginose zouden zijn en suggereerden dat deze bacteriën mogelijke kandidaten zijn voor PCR-gebaseerde diagnose van bacteriële vaginose.

Dankzij de vergelijking van traditionele methoden - microscopie en kweek - met DNA-gebaseerde technieken voor de karakterisatie van de vaginale microflora konden we een nauwkeurigere methode voor de beoordeling van de Gramkleuring van vaginale uitstrijkjes op punt stellen. We toonden aan dat bij vrouwen met een normale vaginale microflora verschillende *Lactobacillus* celtypes kunnen onderscheiden worden en dat de meest gunstige *Lactobacillus* soort, *L. crispatus*, microscopisch kan herkend worden als korte, plompe staafjes. Bovendien introduceerden we een categorie ('grade I-like'), microscopisch gelijkend op graad I, maar waarbij *L. crispatus* volledig ontbreekt en *Bifidobacterium* spp., afwezig in de meeste vaginale monsters, frequent in voorkomen.

DNA-gebaseerde technieken leidden reeds tot een beter begrip van de vaginale microbiële populatiedynamiek, maar de etiology van bacteriële vaginose blijft nog onduidelijk. Onze studies toonden aan dat een volledig beeld van de samenstelling van complexe microbiële gemeenschappen enkel bekomen kan worden door de combinatie van kweek en kweek-onafhankelijke technieken. Uit de bevindingen gepubliceerd tijdens de laatste 5 jaar is het reeds duidelijk dat DNA-gebaseerde technieken zullen bijdragen tot betere strategieën voor

het herstel en het behoud van een normale vaginale microflora en tot verdere opheldering van de rol van tot op heden ongekende bacteriën in gynaecologische aandoeningen.

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XI. Addendum

Bacterial variability map (276). *E. coli* 16S rRNA gene sequence annotated with priming sites and variable regions V1–V3. The sequence is colour coded to indicate bacterial sequence variability.
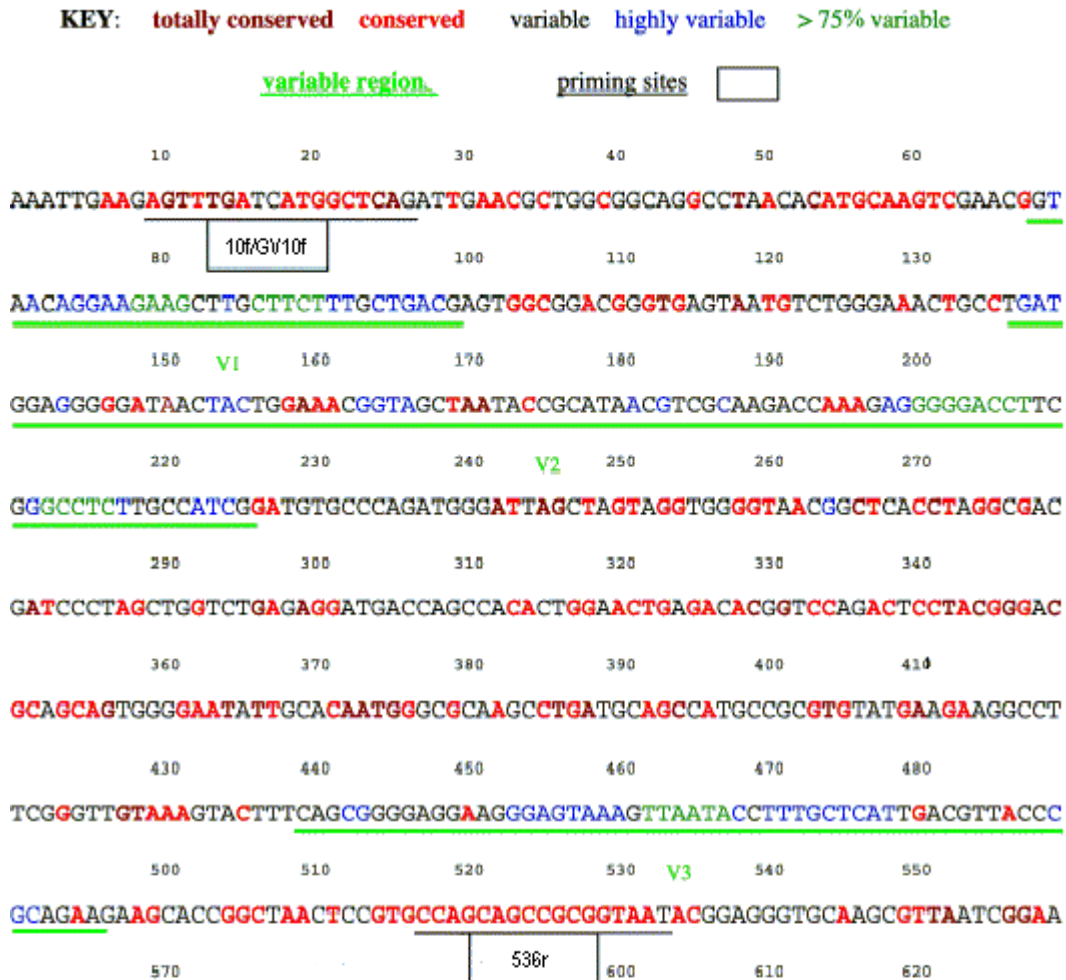
XII. Dankwoord

Het tot stand komen van een proefschrift is een lange, boeiende zoektocht die eindigt in een bergrit.

Dank aan de leden van de examencommissie voor hun opbouwende kritiek aan de eindstreep en dank aan Prof. Leroux-Roels, voorzitter van de examencommissie, om 'de laatste loodjes' in goede banen te leiden. Voor het zover kwam, werd er een lange weg afgelegd:

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Gelukkig is de wetenschap een ploegsport, met een krachtdagige manager, die zorgt voor de planning, voor de navigatie-instrumenten en voor een kritische duw in de rug bij de moeilijke beslissingen aan de vele kruispunten en die de drijvende kracht is achter de wedstrijdverslagen (Prof. Vaneechoutte).

In een zoektocht waar je een brug wil leggen tussen gespecialiseerde disciplines sla je de verkeerde weg in zonder de wijze raad en de ervaring van de kenners (Prof. Claeys, Prof. Plum, Prof. Temmerman, Prof. Verschraegen).
Zonder een kritisch oog bij het bekijken van de Gramkleuringen en bij het isoleren van de (vele!) stammetjes zou de cover en zeker de inhoud van dit proefschrift er helemaal anders uitgezien hebben (Prof. Claeys).

Zonder goed startmateriaal geen zoektocht (patiëntes en personeel van de dienst Gynecologie van het UZGent, Prof. Temmerman, Dr. Hans Verstraelen). Maar ook zonder produkten (Liliane, Nadine), zonder groeibodems (Jeannette) en zonder goed draaiende PC's (Katia) kom je niet ver.

Maar dagdagelijks zijn het uiteindelijk je ploegmaten die door hun positieve inzet en vaardigheden ervoor zorgen dat de nodige vooruitgang wordt geboekt (Catharine, Leen), dat je nog maar eens een sequentie analyse kan doen (Els, Nancy), dat er vlug nog eens een kweekje kan geïncubeerd worden (Ann en de collega's van het routinelabo bacteriologie) en dat er af en toe gepauseerd kan worden met een gezellige babbel (collega's van de immunologie).

Bij het zoeken naar nieuwe impulsen, omzeilen van obstakels en onzekerheden kan je rekenen op medereizigers, die hetzelfde pad bewandelen en met enthousiasme hun ervaringen delen (Ann, Ellen, Hans, Kim, Thierry).

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XIII. Curriculum

Rita Verhelst werd geboren op 29 mei 1970 te Hamme. Na het beëindigen van het secundair onderwijs, richting Wetenschappen B, in Onze-Lieve-Vrouw-Presentatie te Sint-Niklaas, startte zij in 1998 in de kandidatuur scheikunde aan de Universiteit Gent. In 1993 studeerde zij met onderscheiding af als licentiaat Biotechnologie.

Tot eind 1996 was zij wetenschappelijk medewerker op het AIDS-Referentielaboratorium van het UZGent en na drie jaar werkzaam te zijn in de research afdeling van Innogenetics kwam zij terug naar het wetenschappelijk onderzoek op het Laboratorium Bacteriologie Research (LBR) van het UZGent.

Rita Verhelst is auteur of mede-auteur van meerdere publicaties in internationale tijdschriften.

Rita heeft samen met Dirk Van de Vyver drie kinderen: Lotte, Yenthe en Seppe.

(a₁) artikels in tijdschriften opgenomen in Science Citation Index

Verhelst R, Verstraelen H, De Backer E, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, Temmerman M, Vaneechoutte M. Characterization of vaginal microflora at three time points in pregnancy by Gram stain, culture and T-RFLP identifies *L. gasseri* as the *Lactobacillus* species most present in bacterial vaginosis. Submitted to BMC infectious diseases.

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