Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

BIOLOGIA CELLULARE, MOLECOLARE E INDUSTRIALE
PROGETTO 3: MICROBIOLOGIA E BIOTECNOLOGIE INDUSTRIALI

Ciclo XXIV

Settore Concorsuale di afferenza: 03/D1

Settore Scientifico disciplinare: CHIM/11

THERAPEUTIC STRATEGIES FOR MODULATION OF THE VAGINAL MICROBIOTA

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Esame finale anno 2012

ABSTRACT

Abstract

The vaginal microbiota of healthy women consists of a wide variety of anaerobic and aerobic bacteria, dominated by the genus *Lactobacillus*. The activity of lactobacilli is essential to protect women from genital infections and to maintain the natural healthy balance of the vaginal ecosystem. This role is particularly important during pregnancy because vaginal infection is one of the most important mechanisms for preterm birth. The most common vaginal disorder is bacterial vaginosis (BV). BV is a polymicrobial disorder, characterized by a depletion of lactobacilli and an increase in the concentration of other bacteria, including *Gardnerella vaginalis*, anaerobic Gram-negative rods, anaerobic Gram-positive cocci, *Mycoplasma hominis*, and *Mobiluncus* spp. An integrated molecular approach based on real-time PCR and PCR-DGGE was used to investigate the effects of two different therapeutic approaches on the vaginal microbiota composition. (i) The impact of a dietary supplementation with the probiotic VSL#3, a mixture of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains, on the vaginal microbial ecology and immunological profiles of healthy women during late pregnancy was investigated. The intake was associated to a slight modulation of the vaginal microbiota and cytokine secretion, with potential implications in preventing preterm birth. (ii) The efficacy of different doses of the antibiotic rifaximin (100 mg/day for 5 days, 25 mg/day for 5 days, 100 mg/day for 2 days) on the vaginal microbiota of patients with BV enrolled in a multicentre, double-blind, randomised, placebo-controlled study was also evaluated. The molecular analyses demonstrated the ability of rifaximin 25 mg/day for 5 days to induce an increase of lactobacilli and a decrease of the BV-associated bacteria after antibiotic treatment, and a reduction of the complexity of the vaginal microbial communities. Thus, confirming clinical results, it represents the most effective treatment to be used in future pivotal studies for the treatment of BV.

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INTRODUCTION

1. The vaginal microbiota

1.1 Overview

The female genital tract is composed of a sequence of cavities. The external genital tract leads into the vagina that connects in succession to the endocervix, the uterus and then to the Fallopian tubes. This passageway allows for the migration of the mature fetus and menstrual flow to the exterior, and for the movement of spermatozoa to the interior. This exposure of the female genital tract to the external environment carries with it the risk of potential infections in this area. Among the defense mechanisms, one of the most important is the composition of the microbial communities that colonize the vagina (Witkin *et al*, 2007-a). The microbial inhabitants of the human vagina constitute a finely balanced ecosystem, with the vaginal environment controlling the colonizing bacteria and the microbiota in turn controlling the vaginal environment. This dynamic microbial community plays a key role in preventing colonization by undesirable organisms.

Historically, studies of the components of the vaginal ecosystem relied first on microscopic evaluation and then on identification of specific bacteria by culture techniques (Larsen and Monif, 2001). In 1892, Professor Albert Döderlein published a monograph, *Das Scheidensekret* ("vaginal secretions"), reporting the first descriptions and images of the vaginal bacillus, subsequently called the "Döderlein's bacillus". He divided the bacterial communities of pregnant women into normal (dominated by the vaginal bacillus) and abnormal (containing numerous other organisms), and recorded that, in the normal secretions, vaginal bacilli were able to promote conditions, including acidity, that could inhibit the growth of pathogens *in vitro* and *in vivo* (Döderlein, 1892). In 1928, Stanley Thomas renamed "Döderlein's bacillus" as *Lactobacillus acidophilus*, and defined it as a characteristic group of related species, or a species that underwent a remarkable transformation (Thomas, 1928). Subsequent improvements in culture techniques and in biochemical characterization of microorganisms led to the conclusion that *L. acidophilus* was not a single organism but a heterogeneous group of lactobacilli with distinct characteristics (Hunter *et al*, 1959; Johnson *et al*, 1980; Rogosa and Sharpe, 1960). The use of quantitative culture, improved transport media, and anaerobic incubation greatly expanded the survey of vaginal microbes. Gram-positive rods, staphylococci and streptococci, and Gram-negative enteric organisms, as well as a variety of cultivable anaerobes, such as *Prevotella* spp., and *Fusobacteria* spp., could all be found in vaginal samples (Larsen and Monif, 2001).

Much of our knowledge about the composition of the vaginal microbial communities comes from qualitative and semi-quantitative descriptive studies using cultivation-dependent techniques (Chow and Bartlett, 1898; Johnson *et al*, 1985; Larsen and Monif, 2001; Marrazzo *et al*, 2002-b; Stahl and Hill, 2009). However, utilization of culture media for the comprehensive identification of bacterial ecosystem diversity is now recognized as being incomplete and fragmentary. In most instances, this is because readily cultivated populations represent a small fraction of the existing community (McCaig *et al*, 1999). In recent years, the development and introduction of cultivation-independent molecular-based techniques have revolutionized bacterial detection and provided new information about the phylogenetic diversity of microorganisms composing the vaginal microflora. The microbiota of the vagina have been revealed as considerably more dynamic and complex, comprising previously undetected bacterial vaginal inhabitants (Fredricks *et al*, 2005; Verhelst *et al*, 2004; Zhou *et al*, 2004).

The vaginal ecosystem of healthy women typically shows a predominance of *Lactobacillus* species, but a diverse array of other bacteria and microorganisms, such as *Staphylococcus*, *Ureaplasma*, *Corynebacterium*, *Streptococcus*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Mycoplasma*, *Enterococcus*, *Escherichia*, *Veillonella*, *Bifidobacterium* and *Candida* (Hyman *et al*, 2005; Larsen and Monif, 2001; Marrazzo *et al*, 2002-b; Redondo-Lopez *et al*, 1990) can be present in much lower amounts. Lactobacilli are involved in promoting a healthy vaginal environment by preventing overgrowth of pathogenic and opportunistic organisms, and by producing lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins (Aroutcheva *et al*, 2001-a; Hawes *et al*, 1996; Rönnqvist *et al*, 2006; Skarin and Syl, 1986). On the other hand, *Lactobacillus*-deficient conditions are associated with the development of numerous infectious conditions, such as bacterial vaginosis (BV), candidiasis (CA) and aerobic vaginitis (AV), and promote the transmission of sexually transmitted diseases, such as Gonorrhoea, Chlamydia, Syphilis, Trichomoniasis, HIV, and HPV which may lead to cervical cancer (Donders, 2007; Fredricks *et al*, 2005; Myer *et al*, 2005; Sobel, 2002; Watts *et al*, 2005; Witkin *et al*, 2007-a). In 1921, Schröder was the first to classify the vaginal microflora into three “Lactobacillary grades” (Schröder, 1921). Lactobacillary grade I, corresponding to a “healthy” or “normal” microflora, had predominant lactobacillary morphotypes of variable size (Figure 1A). Lactobacillary grade III, or “abnormal” microflora, represented a condition wherein the lactobacillary morphotypes were completely replaced by other bacterial morphotypes (Figure 1D and 1E). Lactobacillary grade II, corresponding to an “intermediate” microflora, was characterized by the partial replacement of the lactobacilli by other bacteria (Figure 1B and 1C). Afterwards, Donders refined grade II and subdivided this group into slightly disturbed, fairly normal (IIa) and moderately disturbed, rather abnormal (IIb) lactobacillary microflora

(Donders, 1999). In order to diagnose such abnormal lactobacillary grades, the use of the wet mount was preferred to the Gram stain due to its superior accuracy (Donders *et al*, 2000-b) and better correlation with vaginal lactate (Donders *et al*, 1998), accepted by most as the best functional test for lactobacillary defense function (Forney, 2004).

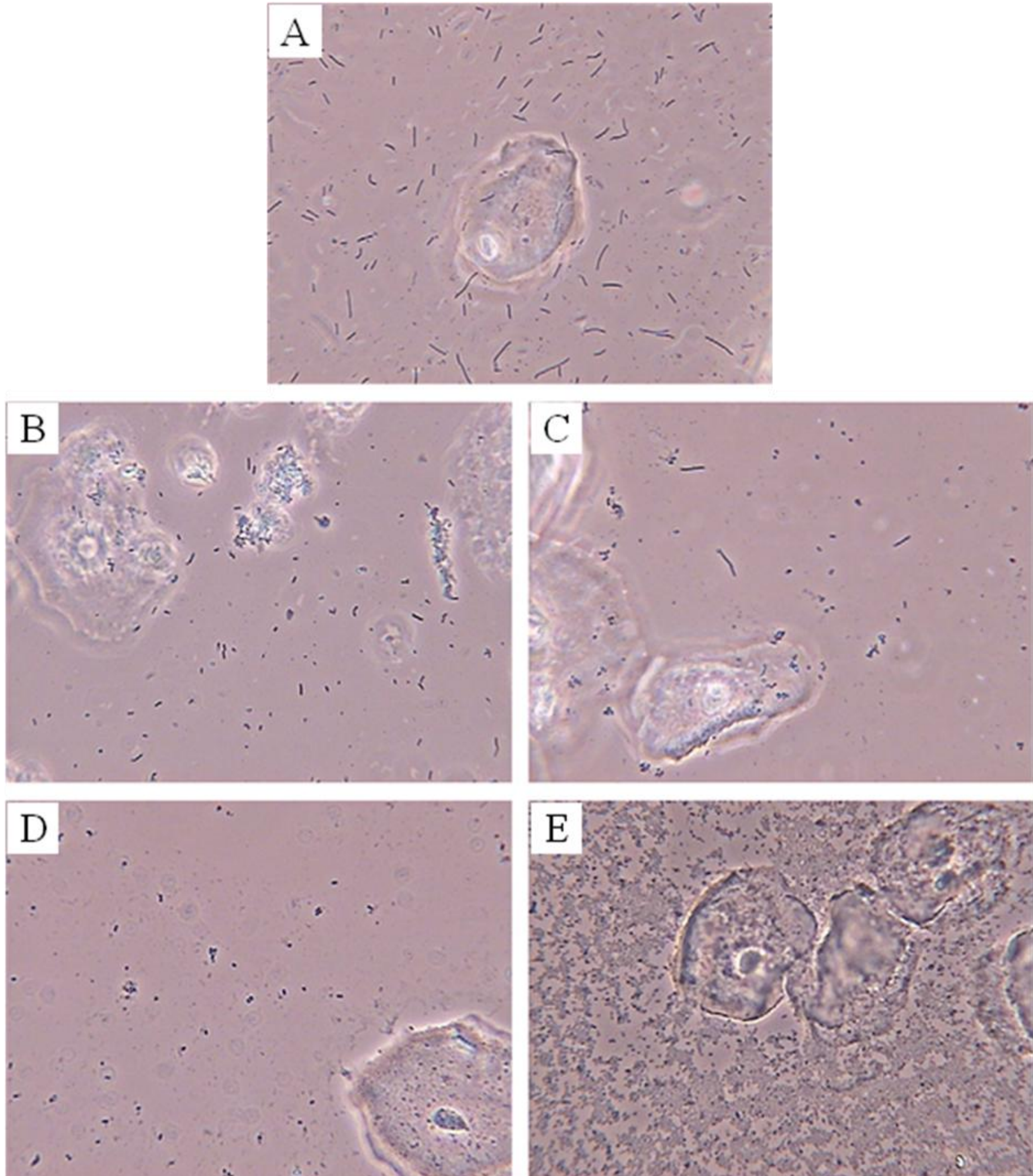

Figure 1. "Lactobacillary grades": (A) Lactobacillary grade I, normal vaginal microflora; (B) Lactobacillary grade IIa, lactobacilli prominent, but mixed with some other bacteria; (C) Lactobacillary grade IIb, lactobacilli still present, but more bacteria of other types present; (D) Lactobacillary grade III: coccoid aerobic vaginitis (AV) microflora; (E) Lactobacillary grade III, bacterial vaginosis (BV) microflora. (Donders, 2007).

1.2 Normal vaginal microbiota

As previously stated, the dominant species in the vagina of healthy premenopausal women was initially identified as *Lactobacillus acidophilus*. This turned out to be an oversimplification, however. There are many different strains of lactobacilli present in the vagina, the most frequent being *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii*, and there is a wide variation in species and relative numbers of species according to the population studied (Pavlova *et al*, 2002; Tärnberg *et al*, 2002). Using molecular-based techniques, we now know that the healthy vaginal microbiota does not contain high numbers of many different species of *Lactobacillus*. Rather, a single species among the most frequent, or a group of closely related species are dominant, whereas other species are rare, lower in titer, and tend to be novel phylotypes (Biagi *et al*, 2009; Burton and Reid, 2002; Shi *et al*, 2009; Song *et al*, 1999; Thies *et al*, 2007; Vásquez *et al*, 2002; Verhelst *et al*, 2004; Vitali *et al*, 2007; Wilks *et al*, 2004; Yamamoto *et al*, 2009; Zhou *et al*, 2004; Zhou *et al*,2007). The rare coexistence of multiple species of lactobacilli in vaginal communities could be caused by competitive exclusion of one species by another, pre-emptive colonization by a particular species or host factors that strongly influence which species are able to colonize the environment (Zhou *et al*, 2004). Despite there is a wide variation in species and relative numbers of species according to the population studied, the principle of numerical dominance persists and may be an important defense mechanism.

Interestingly, apparently healthy vaginal ecosystems are maintained in a significant proportion (7–33%) of women in the absence of a *Lactobacillus*-dominant vaginal microbiota (Anukam *et al*, 2006-b; Hyman *et al*, 2005; Verhelst *et al*, 2004; Zhou *et al*, 2004). Lactobacilli may be replaced by other lactic acid-producing bacteria, such as *Atopobium vaginae*, *Megasphaera* spp., and *Leptotrichia* spp. (Ravel *et al*, 2011; Rodriguez *et al*, 1999; Zhou *et al*, 2004 ; Zhou *et al*, 2007). Although the structure of the vaginal communities may differ between populations, health can be maintained provided that the function of the microbiota is conserved, that is the production of lactic acid and maintenance of a low pH environment that precludes the colonization and growth of pathogens and other undesirable microorganisms (Schwebke, 2001; Yamamoto *et al*, 2009; Zhou *et al*, 2004). Consequently, the absence of lactobacilli or the presence of certain organisms such as *Gardnerella vaginalis*, or species of *Peptostreptococcus*, *Prevotella*, *Pseudomonas*, and/or *Streptococcus*, does not always constitute an abnormal state (Hyman *et al*, 2005).

Several bacterial populations recovered using molecular-based techniques are not readily cultivated and may have been overlooked in culture-dependent studies. *L. iners* does not grow on the selective media commonly used for the isolation of *Lactobacillus* spp., namely MRS and

Rogosa media (Falsen *et al*, 1999). Likewise, *A. vaginae*, *Megasphaera* spp. and *Leptotrichia* spp. are strict anaerobes, require specialized media and often grow slowly. The finding of these microorganisms as members of normal vaginal microflora illustrates how culture-independent techniques have revolutionized bacterial detection. Their application has clarified the identification of the most common *Lactobacillus* spp., demonstrated that lactobacilli are not always the dominant microbial species in apparently healthy women, and has identified previously undetected bacterial vaginal inhabitants (Fredricks *et al*, 2005; Verhelst *et al*, 2004; Zhou *et al*, 2004). Although cultivation-independent techniques may show greater diversity, they are limited by their tendency to sample only the most prevalent bacteria in a community, such that low-abundance or minority species are likely to be missed (Hillier, 2005). This being the case, despite their limitations, cultivation studies remain an important part of vaginal microbiology, and should be used in combination with cultivation-independent techniques (Donachie *et al*, 2007).

The advent of next-generation ultra-high-throughput sequencing technologies has removed an important quantitative barrier in molecular analysis, by increasing the number of reads from a gene or genome by orders of magnitude in a single run (Schellenberg *et al*, 2009). The recent transition from low-throughput clone library sequencing studies to deep sequencing of PCR amplicons has led to a rapid accumulation of data regarding human associated microbial communities and has been crucial in furthering our understanding of the microbiota colonizing the genital tract (Rampersaud *et al*, 2012). The most detailed investigation to date used pyrosequencing of barcoded 16S rRNA genes to probe the vaginal microbiota in women of childbearing age. The study by Ravel et al developed an in-depth characterization of the vaginal microbial communities in a cohort of 396 North American women equally representing four ethnic backgrounds (Asian, White, Black, and Hispanic) (Ravel et al, 2011). In the study the depth of coverage for each bacterial community was sufficient to detect taxa that constitute ~0.1% of the community. The low-abundance or "rare" taxa are only rare in the context of sampling depth, but they could play major roles in the ecology of a community, whereas undetected members may constitute a "seed bank" of species whose numbers increase under conditions that favor their growth. The analysis, in which the vaginal bacterial communities were grouped according to community composition, revealed five major groups, which is reminiscent of previously published studies on microbial diversity in the human vagina (Zhou et al, 2009). Four of the five groups, accounting for 73% of the participants, were dominated by one or more species of *Lactobacillus*, that constituted >50% of all sequences obtained. Communities in group I (26.2% of the women), were dominated by L. crispatus, whereas groups II (6.3%), III (34.1%), and V (5.3%) were dominated by L. gasseri, L. iners, and L. jensenii, respectively (Fig 2). The remaining community (group IV), found in 27% of the women, formed a

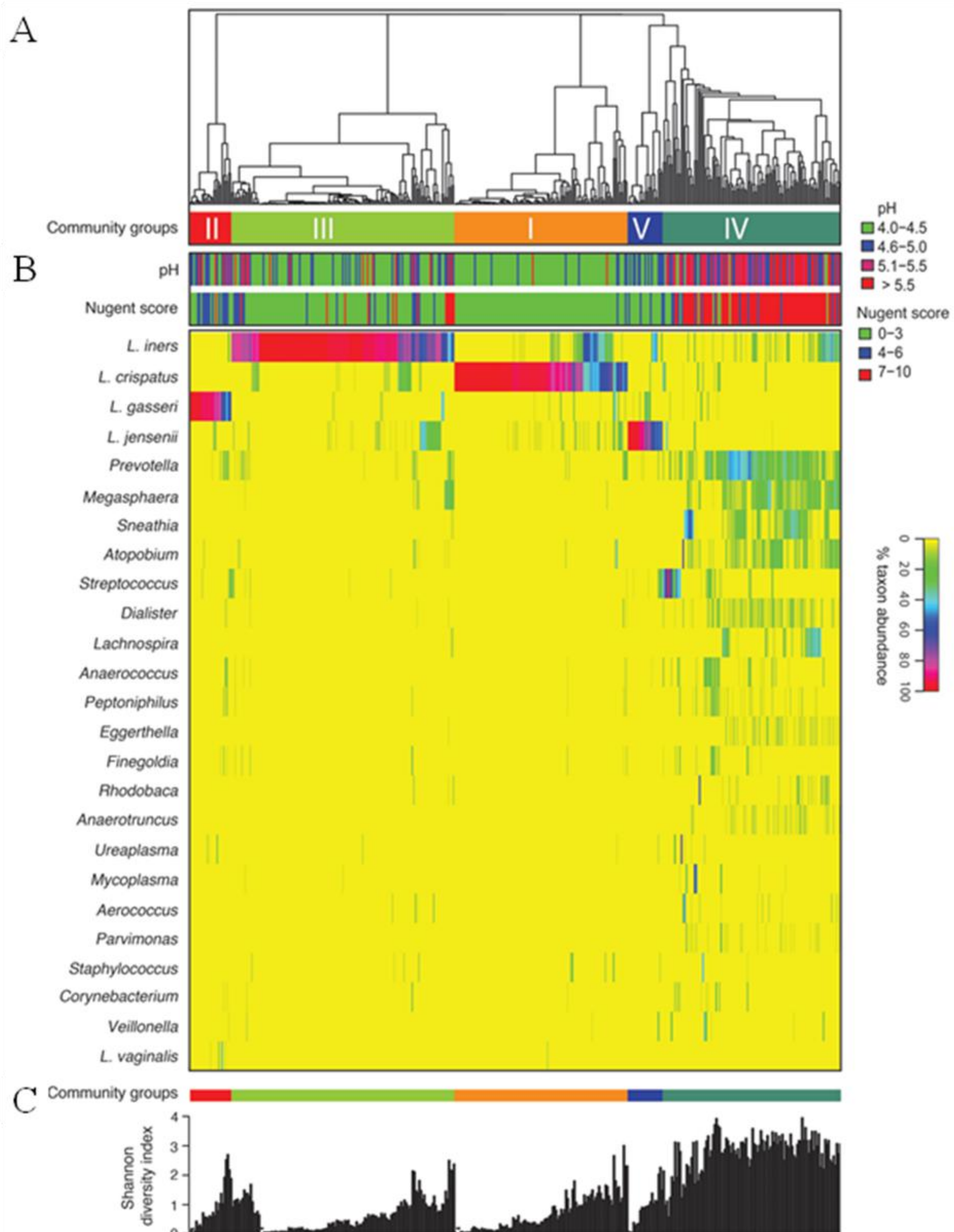

Figure 2. Heat map showing the distribution of microbial taxa found in the vaginal microbial communities of 394 reproductive-age women: (A) Complete linkage clustering of samples based on species composition and abundance in communities. (B) Nugent scores and pH measurements for each of the 394 samples. (C) Shannon diversity indices calculated for each of the 394 vaginal communities (two singletons were excluded) (Ravel et al, 2011). Adapted with permission from Proceedings of the National Academy of Sciences of the United States of America (Brotman, 2011).

large heterogeneous group and was characterized by a more equally represented abundance of strictly anaerobic bacteria, including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia*, and *Mobiluncus*. Interestingly, all communities contained members that have been assigned to genera known to produce lactic acid, including *Lactobacillus*, *Megasphaera*, *Streptococcus*, and *Atopobium*. This suggests that the production of lactic acid may be conserved among communities despite differences in the species composition. Although this high-throughput technique has provided more detailed information, the findings are consistent with those of previous studies wherein the species composition of vaginal communities was investigated by cloning and sequencing of 16S rRNA genes (Ferris *et al*, 2004; Ferris *et al*, 2007; Fredricks *et al*, 2005; Srinivasan and Fredricks, 2008; Verhelst *et al*, 2004; Verstraelen *et al*, 2004; Zhou *et al*, 2007; Zhou *et al*, 2009). Somewhat unexpectedly, *Prevotella* spp. were detected in more than 50% of the samples, therefore their prevalence may have been previously underappreciated. Although their role in the community is unknown, it should be noted that *Prevotella* spp. positively affect the growth of the species linked to bacterial vaginosis *G. vaginalis* and *Peptostreptococcus anaerobius*, by producing key nutrients for these species, such as ammonia and aminoacids. Hence the wide distribution of *Prevotella* spp. in the vaginal microbiota might be a factor that facilitates bacterial vaginosis (Pybus and Onderdonk, 1999).

One objective of studies on the human vaginal microbiota is to determine whether there is a core set of microbial species associated with the vaginal ecosystems of all healthy women. It is postulated that changes to this “core microbiome” may be correlated with risk to disease. The results from pyrosequencing and molecular-based studies (Fredricks *et al*, 2005; Ravel *et al*, 2011; Zhou *et al*, 2007; Zhou *et al*, 2009) suggest that for the human vagina there is no single “core microbiome”. Instead, it seems there are multiple “core microbiomes” that can be defined by the community groups I-V detected in the study by Ravel *et al* (Ravel *et al*, 2011). Despite differences in their species composition, it may be hypothesized that core functions are conserved among communities (Konopka, 2009).

Conversely, the small number of different kinds of vaginal communities is somewhat surprising given that these communities are probably assembled independently after birth. The repeatability of community assembly suggests that a host exerts strong selection for a rather limited number of different kinds of bacteria. This is especially evident in the limited number of *Lactobacillus* phylotypes and other lactic acid-producing bacteria that are abundant in these communities. It can be supposed that vaginal microbial communities exist in a state of dynamic equilibrium and that homeostatic mechanisms exist to provide resilience. Given the fundamental differences in the species composition of these communities, one can speculate that they will differ

in terms of relative resistance and resilience of each community type to disturbances. Hay *et al* stated that lactobacilli are in a continuous effort to acidify the vaginal milieu after the disturbing events that may occur in this dynamically changing environment. Whether and how easily they succeed in maintaining predominance, providing colonization resistance to anaerobes, depends on the combination of the ease and speed with which the lactobacilli can acidify/colonize the vaginal econiche after a disturbing event and the number of disturbing events they are confronted with (Hay, 2005). This capability appears to be strongly different between individual women, depending on genotypic differences between their vaginal lactobacilli (different *Lactobacillus* species or strains, and differences in production of lactic acid, hydrogen peroxide and bacteriocins), between women (differences in hormone metabolism and innate immunity), and between the match of certain strains to certain hosts (Santiago *et al*, 2011). Then invasive species, including both opportunistic and pathogens, are more likely to become established in communities that exhibit low stability (Hobbs and Huenneke, 1992).

1.2.1. Dynamics of the vaginal microbiota

The composition of the vaginal ecosystem is not static but changes rapidly over time and in response to endogenous and exogenous influences (Eschenbach *et al*, 2000; Eschenbach *et al*, 2001; Priestley *et al*, 1997; Schwebke *et al*, 1999). Variables include age, host genetic background, diet and nutritional status, hormonal status/phase of menstrual cycle, pregnancy, use of contraceptive agents, sexual behaviors, hygiene practices, and utilization of antibiotics or other medications with immune or endocrine activities.

One of the main variable factors is the age, which is closely related to the estrogen levels. The vaginal microbiota changes in a typical manner during the female life cycle. At birth, the vagina is sterile. After only a few days, estrogen from the mother induces colonization by lactobacilli (Granato, 2003). The growth of lactobacilli is the consequence of the estrogen-induced increase in glycogen content in vaginal epithelial cells, since glucose derived from the metabolism of glycogen constitutes the main nutritional factor for lactobacilli, which convert it to lactic acid, lowering the vaginal pH (Boskey *et al*, 1999; Eschenbach *et al*, 2000; Hillier *et al*, 1993-a). On the other hand, the availability of glucose potentially could also promote the growth of other microorganisms (Mårdh, 1991). During childhood, skin commensals and bowel bacteria colonize and dominate the microbial content of the vagina. At the time of menarche, the rise in estrogen increases glycogen deposition in the vaginal epithelial cells, which is a prerequisite for the development of the adult vaginal microbiota. This ecosystem is predominant until menopause, when it is replaced with a microflora similar to that found prior to the menarche, unless hormonal replacement therapy (HRT)

is started (Cauci *et al*, 2002). Postmenopausal status involves major vaginal environment changes that are not confined to the glycogen content of epithelial cells. In spite of the impressive decrease in *Lactobacillus* spp. colonization, increase in vaginal pH, increase in levels of Gram-positive cocci and increase in coliform colonization (Hillier and Lau, 1997), the prevalence of BV is significantly lower than in fertile women (Cauci *et al*, 2002).

Due to fluctuations of estrogen levels, the vaginal econiche is also subject to cyclical changes during the menstrual cycle, whereby the normal domination of the lactobacilli is frequently challenged (Hay, 2005). The cyclic fluctuation of estrogen and progesterone levels affects the vaginal epithelial cell surface receptor expression, the amount and viscosity of cervical mucus, the amount of vaginal transudate, the glycogen level, vaginal oxygen and carbon dioxide tension, the reduction-oxidation potential, the pH, and the vaginal innate immune response (Hill *et al*, 2005; Wagner and Ottesen, 1982; Wira *et al*, 2010). Several studies indicated that *Lactobacillus* spp. growth increases throughout the menstrual cycle, but decreases during the menses, when the concentration of non-*Lactobacillus* species, such as *G. vaginalis*, Gram-positive cocci and *Candida albicans* is increased (Eschenbach *et al*, 2000; Keane *et al*, 1997; Santiago *et al*, 2011; Srinivasan *et al*, 2010). Recent molecular studies pointed out that *G. vaginalis* is a natural constituent of the vaginal microflora (De Backer *et al*, 2007; Hummelen *et al*, 2010; Santiago *et al*, 2011), but it may produce transient dominance in healthy women as a result of perturbations, such as an increase in pH, during menstruation (Srinivasan *et al*, 2010). During this period, the increase in *G. vaginalis* levels is accompanied by decreased quantities of *L. jensenii* and *L. crispatus*; the latter being the species that is most negatively affected by the menses (Santiago *et al*, 2011). The growth of *G. vaginalis* may be tied to the availability of iron during menstruation. Iron enhances the replication of many pathogens and is an essential growth factor for most bacteria (Litwin and Calderwood, 1993; Miethke and Marahiel, 2007; Weinberg, 2009); one of these microorganisms is *G. vaginalis*, that cannot grow in iron-limiting conditions and is well adapted to harvest iron from the environment (Jarosik *et al*, 1998). During menses, concentrations of *L. iners* tended to increase along with levels of *G. vaginalis*, in fact both the species are easily cultivated on blood agar medium (Srinivasan *et al*, 2010). Besides *G. vaginalis*, also Gram-positive cocci, especially the *Streptococcus anginosus* group, *P. anaerobius* and *Peptostreptococcus asaccharolyticus*, were predominant during non-infectious conditions that alter the normal vaginal pH, such as menses, recent sexual intercourse and use of antibiotics (Santiago *et al*, 2011). In general, the increased presence of Gram-positive cocci seems to be a characteristic feature of the disturbances of the vaginal ecology, and could be explained as an absolute increase of the Gram-positive cocci loads or

as the already present Gram-positive cocci becoming more easily detectable, due to the disappearance of the lactobacilli.

Racial variation and geographical area are also important (Pavlova *et al*, 2002), and different racial groups within the same geographical region have significant differences in what is the dominant vaginal organism (Anukam *et al*, 2006-b). In a recent pyrosequencing study, Ravel *et al* demonstrated that vaginal bacterial communities of Asian and White women are dominated by species of *Lactobacillus*, while communities not dominated by *Lactobacillus* spp. are more common in Hispanic and Black women; for this reason the latter are characterized by higher median pH values (Ravel *et al*, 2011). Thus, the previous acceptance that the occurrence of high numbers of lactobacilli and pH < 4.5 is synonymous with "healthy" suggests that a significant proportion of asymptomatic Hispanic and Black women are "unhealthy", a notion that seems implausible. Bacterial communities not dominated by species of *Lactobacillus* are common and appear normal in Black and Hispanic women (Ravel *et al*, 2011; Zhou *et al*, 2007; Zhou *et al*, 2009). Hence, the enhanced diagnosis of bacterial vaginosis in Black women as opposed to White women might merely reflect an increased likelihood that bacteria other than lactobacilli typically predominate in the former population, and not that these women have an "abnormal" microflora. The reasons for these differences among ethnic groups are unknown, but it can be hypothesized that the species composition of vaginal communities could be governed by environmental as well as genetic differences between hosts, such as differences in innate and adaptive immune systems, the composition and quantity of vaginal secretions, and ligands on epithelial cell surfaces (Schwebke, 2009-a). Alternatively, diet might influence the *Lactobacillus* species resident in the gastrointestinal tract, and hence the vagina, as the lactobacilli of the gut varies between women from different geographic locations, such as Japanese and Western women (Ahrné *et al*, 1998).

As regards sexual and vaginal hygiene practices, they have been associated with differences in *Lactobacillus* spp. colonization and vaginal microbial composition (Schwebke *et al*, 1999; Vallor *et al*, 2001). Identified risk factors for a lack of H_2O_2-producing lactobacilli, and thus for BV, include douching (particularly frequent and recent) (Hawes *et al*, 1996; Ness *et al*, 2002), having had multiple sex partners during the preceding year (Beigi *et al*, 2005), and frequent sexual intercourse (Vallor *et al*, 2001). Sexual intercourse may cause a disruption of the local vaginal microbiological and/or inflammatory milieu; therefore, more frequent and/or new partners may accentuate these proposed changes. As regards the distribution of bacterial species among lesbian and bisexual women, different studies reported a decreased quantity of protective H_2O_2-producing lactobacilli and a higher likelihood of colonization with *G. vaginalis*, causing a major risk of BV (Marrazzo *et al*, 2009; Mitchell *et al*, 2011). Sexual practices involving receptive vaginal sex with a

toy and receptive digital-vaginal sex were associated with a higher likelihood of acquiring an "abnormal" vaginal flora (Bradshaw *et al*, 2005; Mitchell *et al*, 2011). This could potentially be due to more contact with the perianal skin and rectal bacteria and suggests that BV could be a sexually facilitated condition.

A major impact on the composition of vaginal microbial communities was also observed by usage of antimicrobial agents. Assuming that the antibiotics also affect the rectal microbiota, this might suggest that antibiotic treatment on its own is insufficient to restore a woman's vaginal microbiota and that additional therapeutics, such as probiotics with vaginal lactobacilli, might be useful for restoring a *Lactobacillus*-dominated microflora, as indicated by several studies (Martinez *et al*, 2009; Mastromarino *et al*, 2009; Reid, 1999; Rossi *et al*, 2010; Santiago *et al*, 2011).

1.3 Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive, low GC content, acid-tolerant and non-spore-forming rods or coccobacilli (Hugenholtz, 1998). They generally lack catalase, although in rare cases pseudocatalase can be found. They are chemo-organotrophic and grow only in complex media. Fermentable carbohydrates are used as energy sources and are degraded to lactate (homo-fermentatives) or to lactate and additional products such as acetate, ethanol, carbon dioxide, formate or succinate (hetero-fermentatives).

LAB might be the most numerous group of bacteria linked to humans. Their natural habitat varies widely, from food, plants and sewage, to the oral, genital and gastrointestinal tracts of humans and animals (Hammes and Vogel, 1995). LAB play an essential role in food technology, because of preservative action due to acidification, and/or enhancement of flavor, texture and nutrition (Jay, 1996; Stiles, 1996). They are used for industrial fermentation of dairy products, meats, and vegetables, and they are also critical for the production of wine, coffee, silage, cocoa, and sourdough (Dunny and Cleary, 1991; Wood and Holzapfel, 1995; Wood and Warner, 2003). In addition, they are an important source of antimicrobial agents (Cotter *et al*, 2005). However, not all of the LAB are useful, some of them, such as several *Streptococcus* spp., are involved in food spoilage or may even be pathogen.

The definition of LAB is biological rather than taxonomical and refers to the basal metabolism of these bacteria, which primarily leads to the production of lactic acid. However, they do not comprise a monophyletic group of bacteria. Among the genera comprising the LAB, *Lactobacillus* is the largest genus (Hugenholtz, 1998), with over 100 species described. It belongs to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales* (Hammes and Hertel, 2003).

Lactobacilli are strictly fermentative and can be divided into three groups based on fermentation characteristics: obligate homo-fermentative, facultative hetero-fermentative and obligate hetero-fermentative (Hammes and Vogel, 1995; Pot *et al*, 1994). However, this phenotypic classification does not match the rRNA-based phylogeny (Vandamme *et al*, 1996). The high degree of diversity and complex phylogeny of *Lactobacillus* and related genera was acknowledged by Schleifer and Ludwig (Schleifer and Ludwig, 1995), who divided the species into three groups, based on 16S rRNA studies. The first group comprises *Lactobacillus delbrueckii* and many other obligate homo-fermentative lactobacilli (*L. acidophilus*, *L. gasseri*, and *Lactobacillus johnsonii*). The second group, the so-called *Lactobacillus casei-Pediococcus* group, is the largest of the three groups and contains both homo-fermenters and hetero-fermenters species (*Lactobacillus plantarum*, *L. casei*, *Pediococcus pentosaceus*, and *Lactobacillus brevis*). The *Leuconostoc* group is composed of all members of the genus *Leuconostoc* and some obligate hetero-fermentative lactobacilli (*Leuconostoc mesenteroides* and *Oenococcus oeni*). Streptococci (*Streptococcus thermophilus*) and lactococci (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) form a separate branch (Siezen *et al*, 2004). Reclassifications have been frequent following the identification of new species and classification techniques, and the taxonomy is generally considered unsatisfactory (Felis and Dellaglio, 2007; Schleifer and Ludwig, 1995).

1.3.1. Vaginal lactobacilli

The genus *Lactobacillus* comprises a phenotypically heterogeneous group of facultatively anaerobic, catalase-negative, rod-shaped LAB (Kandler and Weiss, 1986). Over 120 species of *Lactobacillus* have been identified, and more than 20 species have been detected in the vagina, where lactobacilli are recognized as markers of “normal” vaginal microflora (Donders, 2007). Some of the species initially identified using phenotypic assays include *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. jensenii*, *L. casei*, *L. delbrueckii*, *L. vaginalis*, and *L. salivarius* (Levison *et al*, 1977; Nagy *et al*, 1991; Rogosa and Sharpe, 1960). The most recent studies on the vaginal *Lactobacillus* microbiota report that the most frequently occurring species are *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, with the vaginal ecosystem of most healthy women dominated by a single *Lactobacillus* species (Ravel *et al*, 2011; Shi *et al*, 2009; Thies *et al*, 2007; Vásquez *et al*, 2002; Vitali *et al*, 2007; Yamamoto *et al*, 2009; Zhou *et al*, 2004).

Recently, a new classification of the undisturbed vaginal microflora was suggested, based on the Ison and Hay criteria (Ison and Hay, 2002), that classified the vaginal microflora through an estimation of the ratios of the observed cellular types: 0, no bacteria; I, normal microflora; II intermediate microflora; III, bacterial vaginosis; and IV, streptococci-associated microflora.

Verhelst *et al* splitted up grade I "normal" microflora into four categories, designated grade Ia, Ib, Iab and I-like (Verhelst *et al*, 2005). Grade Ia was shown to contain predominantly *L. crispatus*, grade Ib predominantly *L. gasseri* and *L. iners*, grade Iab a mixture of these three species and grade I-like *Bifidobacterium* spp. rather than *Lactobacillus* spp. Grade I-like microflora was considered as not representative for normal vaginal microflora, but as probably an unrecognized type of disturbed vaginal ecosystem (Verstraelen *et al*, 2007) that had previously not been distinguished from a healthy vaginal microflora.

The existence of *L. iners* was unknown prior to 1999, but is now known to play a significant role in the vaginal microbial ecosystem. Phylogenetically, the new species clusters with the *L. delbrueckii* group of species and shows a specific association with *L. gasseri* and *L. johnsonii* (Falsen *et al*, 1999). The first report of the isolation of *L. iners* in a woman with a "normal" vaginal microflora was in 2002 (Burton and Reid, 2002). *L. iners* only grows on blood agar (Falsen *et al*, 1999) and previous works using cultivation-based techniques failed to isolate *L. iners* because Rogosa or MRS agar were used (Antonio *et al*, 1999; Giorgi *et al*, 1987; Pavlova *et al*, 2002; Song *et al*, 1999; Wilks *et al*, 2004). Hereafter, cultivation-independent methods have detected *L. iners* at high levels in most subjects with and without BV, and in some studies it was the only *Lactobacillus* species detected in BV-positive women (Burton and Reid, 2002; Fredricks *et al*, 2005; Thies *et al*, 2007; Verhelst *et al*, 2004; Wertz *et al*, 2008). It has been postulated that this might be because *L. iners* may be better adapted to the conditions associated with BV, such as the polymicrobial state of the vaginal microflora and elevated pH (Wertz *et al*, 2008). Alternatively, *L. iners* could be resistant to unknown factors that lead to the demise of other *Lactobacillus* spp. during the onset of BV. In a real-time PCR-based study, De Backer *et al* reported a significant negative correlation between *L. iners* and *L. gasseri*, and a positive correlation between *L. iners* and *G. vaginalis* (De Backer *et al*, 2007). They categorized women using the Ison and Hay criteria, as refined by Verhelst *et al* (Verhelst *et al*, 2005), and observed that *L. iners* was abundantly present in most grades, but predominantly in grade III, as it was *G. vaginalis*; while it was virtually absent in grades I and II, where *L. gasseri* was more prevalent. It has also been suggested that *L. iners* may become a dominant part of the vaginal microbiota during the transitional stage between abnormal and normal, following the resolution of bacterial vaginosis (Ferris *et al*, 2007; Jakobsson and Forsum, 2007; Kalra *et al*, 2007). When analyzing the stability of the normal vaginal ecosystem in pregnancy (Verstraelen *et al*, 2009), it appeared that the presence of *L. gasseri* or *L. iners* is associated with a weakly stable microflora. They possibly offer poorer colonization resistance thereby allowing the overgrowth of other bacteria and predisposing to the occurrence of "abnormal" vaginal microbiota.

L. crispatus and *L. jensenii* are the predominant species of lactobacilli that colonize the vagina of women of reproductive age. As H_2O_2-producing lactobacilli, they may protect a woman from developing BV by inhibiting the growth of BV-related microorganisms, such as *G. vaginalis*, anaerobic Gram-negative rods, and *Mycoplasma hominis* (Antonio *et al*, 1999). They are found in samples of all grades, even if the concentration of *L. crispatus* is much higher in grade I, particularly grade Ia (De Backer *et al*, 2007). The presence of *L. jensenii* is associated with normal microflora, but this species elicits both poor colonization strength and poor colonization resistance, conferring only intermediate stability. On the contrary, *L. crispatus* is associated with a particularly stable vaginal ecosystem (Verstraelen *et al*, 2009). Vaginal bacterial communities dominated by *L. crispatus* also show the lowest median pH (~ 4.0), while communities dominated by other species of *Lactobacillus* have slightly higher pH, ranging from 4.4 to 5.0 (Figure 2), indicating that these communities as a whole might not produce as much lactic acid as those dominated by *L. crispatus* or might have different buffering capabilities (Ravel *et al*, 2011).

The production of H_2O_2, the maintenance of vaginal pH ~ 4.0 and the capability to colonize the vaginal ecosystem with high colonization strength and high colonization resistance, make *L. crispatus* the main marker of a healthy and stable vaginal ecosystem, which is less likely to shift away to an abnormal microbiota.

1.3.2. Defense factors of vaginal lactobacilli

The ability of lactobacilli to maintain the normal vaginal ecosystem has been attributed to the secretion of lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins (Hillier, 1998). The production of organic acids maintains the vaginal pH at < 4.5, thereby creating an inhospitable environment for the growth of most endogenous pathogenic bacteria. In addition, H_2O_2 inhibits the growth of vaginal microorganisms either directly or through the enhancement of the enzyme peroxidase-halide (Eschenbach *et al*, 1989; Hillier *et al*, 1992). Hydrogen peroxide was found to be lethal to *G. vaginalis*, *Prevotella bivia*, and *Escherichia coli* (Cantoni *et al*, 1989; Hillier *et al*, 1993-a; Klebanoff *et al*, 1991), and vaginal colonization with H_2O_2-producing lactobacilli was associated with a decreased occurrence of BV (Hawes *et al*, 1996). The production of bacteriocin or lactocin with a broad range of antibacterial activity further suppresses the endogenous pathogenic bacteria, to maintain a healthy vaginal ecosystem (Klaenhammer *et al*, 1988; McGroarty and Reid, 1988; Skarin and Syl, 1986). Finally, the growth of *Lactobacillus* species maintains a higher oxidation-reduction potential in the vaginal environment, inhibiting the growth of the obligate anaerobic bacteria.

It is highly probable that all these factors act synergistically to suppress the growth of pathogens and other opportunistic organisms (Aroutcheva *et al*, 2001-a). Several investigators have demonstrated that the activity of the *Lactobacillus*-produced bacteriocins is influenced by the hydrogen ion concentration in the environment, and is higher at a low pH (Dembélé *et al*, 1998; Skarin and Syl, 1986). The activity of H_2O_2 was also found to be pH-dependent. Hydrogen peroxide was stable in an acid environment and degraded as the hydrogen ion concentration decreased (Fontaine and Taylor-Robinson, 1990). Therefore, as the pH of the vagina increases, bacteriocin loses its effectiveness, hydrogen peroxide is degraded, and lactobacilli cannot compete against the other bacteria. Lactic acid appears to be the primary vaginal acidifier, but the concentration of lactic acid in the medium is not solely responsible for the pH change, it depends also on the production and secretion of other organic acids, including acetic acid (Radler and Bröhl, 1984). Lactic acid and acetic acid have the ability to inhibit bacterial growth and act synergistically, indeed the activity of acetic acid is potentiated by the lower pH produced by lactic acid. Furthermore, the biocell mass or numbers of bacteria in the vaginal environment is responsible for pH changes, thus the active growth of bacteria present is also important (Aroutcheva *et al*, 2001-a). It is possible that a reduction in growth of lactobacilli results in an elevation in the pH of the vagina and therefore in an overgrowth of other bacteria that leads to an imbalance within the endogenous vaginal microflora.

1.3.3. Other lactic acid-producing bacteria

As previously reported, three lactic acid-producing taxa, namely *A. vaginae*, *Megasphaera* spp. and *Leptotrichia* spp., were found to be constituents of the normal microflora of some women (Ravel *et al*, 2011; Rodriguez *et al*, 1999; Zhou *et al*, 2004; Zhou *et al*, 2007). Possibly, when lactobacilli are unable to predominate in a particular vagina for whatever reason, another lactic-acid producing species fills this niche, in order to maintain the acidification activity which is essential to promote a healthy vaginal environment.

A. vaginae belongs to the genus *Atopobium*, which lies within the family *Coriobacteriaceae*, and forms a distinct branch within the phylum *Actinomycetes* (Stackebrandt and Ludwig, 1994). Three species formally designated *Lactobacillus minutus*, *Lactobacillus rimae*, and *Streptococcus parvulus*, within the lactic acid-producing group of bacteria (Collins and Wallbanks, 1992), have been reclassified as the genus *Atopobium*. In 1999, an organism similar to these three species was isolated from the vagina of a healthy woman in Sweden, and the organism was named *Atopobium vaginae* (Rodriguez *et al*, 1999). In addition to producing lactic acid (Dewhirst *et al*, 2001; Downes *et al*, 2001), some species of *Atopobium* exhibit peptidyl peptidase activity, and produce significant quantities of ammonia in environments where sugars are a scarce source of energy (Eschenlauer *et*

al, 2002; Kazor *et al*, 2003; Kumar *et al*, 2003). This may be why *A. vaginae* is found more often in the vagina of postmenopausal women who are not on hormone replacement therapy (Burton *et al*, 2004). Moreover, ammonia is known to act as a substrate to promote the growth of *G. vaginalis* (Pybus and Onderdonk, 1997), and *A. vaginae* is found much more commonly in women with BV than in those with normal flora (Biagi *et al*, 2009; Bradshaw *et al*, 2006-b; Ferris *et al*, 2004; Fredricks *et al*, 2005; Menard *et al*, 2008; Oakley *et al*, 2008; Verhelst *et al*, 2004; Verstraelen *et al*, 2004; Vitali *et al*, 2007; Zhou *et al*, 2007). The renamed *Atopobium parvulum*, *Atopobium minitum*, and *Atopobium rimae* have been associated with dental abscesses and oral infections (Downes *et al*, 2001; Kumar *et al*, 2003; Olsen *et al*, 1991; Paster *et al*, 2001), tubo-ovarian abscesses (Geissdörfer *et al*, 2003), and abdominal wound infection, supporting the view that these organisms may be pathogenic to the host.

Leptotrichia, an anaerobic Gram-negative rod, is reportedly part of the normal oral flora and has rarely been isolated from clinical material (Könönen *et al*, 1994; Kroes *et al*, 1999; Tee *et al*, 2001). However, *Leptotrichia sanguinegens/amnionii* has been reported in association with postpartum endometritis, adnexal masses, and fetal death (Gundi *et al*, 2004; Hanff *et al*, 1995; Shukla *et al*, 2002), and has been detected in the amniotic fluid of women with preterm labor, premature prelabor rupture of membranes (PPROM), and pre-eclampsia (DiGiulio *et al*, 2008; DiGiulio *et al*, 2010). Little is known about the ecology of *Leptotrichia* spp., but they do produce lactic acid as the primary fermentation product from glucose (Tee *et al*, 2001) and may represent opportunistic pathogens. *Megasphaera* spp. isolated from the vagina had modest similarity (89–95%) to *Megasphaera cerevisiae*, a Gram-negative, obligate anaerobe that is associated with beer spoilage by causing turbidity, off-flavors and off-odors (Doyle *et al*, 1995; Ziola *et al*, 2000). *Megasphaera* and *Leptotrichia* are also capable of producing malodorous metabolites (Kazor *et al*, 2003; Ziola *et al*, 2000). This renders detection of a vaginal odor in women lacking a *Lactobacillus*-dominated vaginal microflora as not indicative of BV or any other disease condition, especially in asymptomatic women.

1.4 Abnormal and intermediate vaginal microflora

Lactobacillary grade III, and to a lesser extent Lactobacillary grade IIb, are more likely to be linked with pathological conditions, and are said to be “abnormal vaginal microflora”.

Up to the 1950s, symptomatic women with Lactobacillary grade III were diagnosed with “non-specific vaginitis”, as the microbial etiology of lactobacillary deficiency was still uncertain at that time. This was resolved by Gardner and Dukes, who discovered a new genus of bacteria held

responsible for the condition we now know as bacterial vaginosis (BV) (Gardner and Dukes, 1955). The use of the wet mount was usually preferred to the Gram stain for the diagnosis of such abnormal lactobacillary grades (Donders *et al*, 2000-b), but BV is characterized by the overgrowth of different microorganisms, and most of them cannot be visualized on wet-mount microscopy. For this reason many attempts were made to cast the diagnosis in microbiological terms; quantitative bacteriology was used to try and explain symptoms in terms of numbers of different bacteria (Lindner *et al*, 1978). The diagnosis of BV based on Gram-stained specimens was first done by Spiegel *et al* (Spiegel *et al*, 1983-a) and later refined and quantified by Nugent *et al* (Nugent *et al*, 1991), thereby progressively moving the diagnosis of a common clinical condition into the laboratory. By counting the different cell types (*Lactobacillus* spp., *G. vaginalis*/*Bacteroides* spp., *Mobiluncus* spp.) a score between 0 and 10 is obtained (Table 1). A score of 1–4 of lactobacillary morphotypes, a score of 1–4 of *G. vaginalis*/*Bacteroides* morphotypes, and a score of 1 or 2 for *Mobiluncus* morphotypes has to be added to obtain the global Nugent score (Nugent *et al*, 1991); whereby a score of ≥ 7 corresponds to BV, a score of ≤ 3 corresponds to normal microbiota, and a score of 4–6 is considered "intermediate microflora". Even if Nugent score is nowadays accepted as the gold standard for the diagnosis of BV in most clinical trials, it is not an ideal scoring system, as on a continuous scale of 1–10 there is no consensus on what the intermediate group with a score of 4–6 stands for. Ideally the "intermediate microflora" state represents a turning point from a normal state into BV, or from BV to normal. In reality, however, most of the women with so-called "intermediate microflora" according to Nugent will have neither BV nor a normal ecosystem. Different studies reported that the intermediate group was linked to a different and usually more

Table 1. Nugent criteria for the diagnosis of bacterial vaginosis (BV).

	***Lactobacillus* morphotypes**	***G. vaginalis/Bacteroides* morphotypes**	***Mobiluncus* morphotypes**
0	4 +	0	0
1	3 +	1 +	1-2 +
2	2 +	2 +	3-4 +
3	1 +	3 +	
4	0	4 +	

BV: score ≥ 7; normal microflora: score ≤ 3; "intermediate microflora": score 4–6.

serious range of complications, including mid-trimester pregnancy loss, than the full-blown BV (Donders *et al*, 1998; Hay *et al*, 1994; McDonald *et al*, 1994). Therefore, in the intermediate group, "partial BV" as well as other "abnormal" conditions, such as aerobic vaginitis (AV), may be present (Donders, 2007). AV is a newly recognized disorder of the vaginal ecosystem, and should not be confused with BV. Hence, although BV is a condition of abnormal vaginal flora, abnormal vaginal flora is not always BV.

The "abnormal" microflora can be disturbed by anaerobic overgrowth (BV) (Figure 1E) or by aerobic microorganisms, such as *E. coli*, group B streptococci, and enterococci, (AV) (Figure 1D), or can be a mixture of both (mixed abnormal microflora). Concomitant infectious conditions, such as candidiasis, trichomoniasis, BV, AV or cervicitis may occur (Donders *et al*, 1993). Appropriate diagnosis and distinction between these infectious conditions is crucial as their treatments are different; for example, AV does not respond well to metronidazole, the treatment of choice for trichomoniasis and BV. In order to get to such a diagnosis, the use of Lactobacillary grades to decide whether or not a bacterial ecosystem is "normal" (Lactobacillary grade I or IIa) is essential (Donders, 1999). In the case of an "abnormal" microbiota (Lactobacillary grade IIb or III), extra criteria based on microscopy are added to distinguish between BV and AV, and a further scrutiny is required to find other pathogens such as *Trichomonas* or *Candida*. As abnormal Lactobacillary grades are also associated with *Chlamydia trachomatis*, gonorrhoea and syphilis, a screen for sexually transmitted pathogens should also be considered (Donders *et al*, 1993).

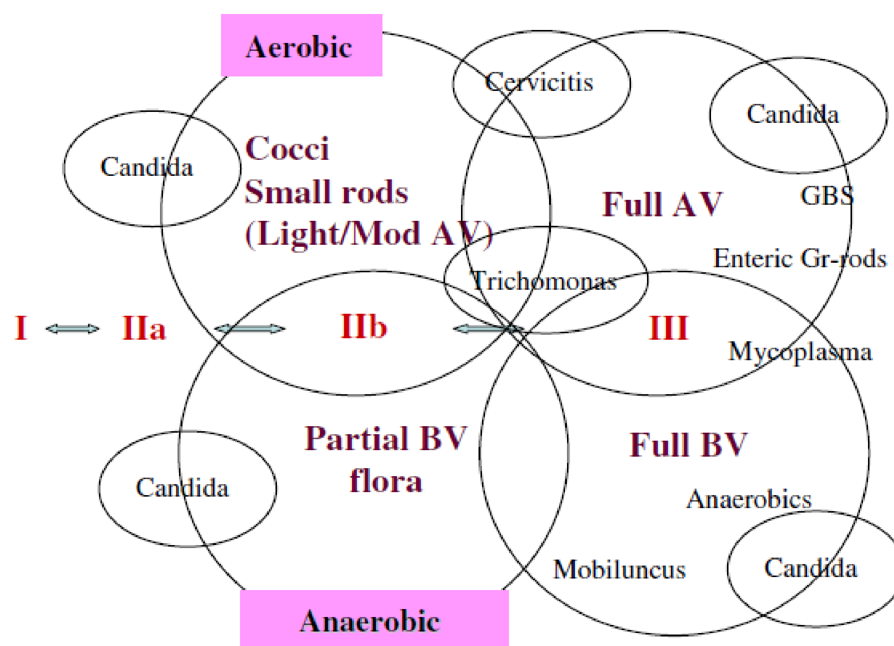

Figure 3. Vaginal infectious disease in different types of microflora (Donders, 2007).

Figure 3 represents the different pathogenic conditions of vaginal microbiota disturbance; the progressive loss of lactobacilli is seen as one dimension (x axis) and the gradual redox potential (aerobic–anaerobic) as the other (y axis). On the anaerobic side, normal microflora may gradually become partial BV, which contains some anaerobic microorganisms but not enough to cause the appearance of clue cells, and further down the line full-blown BV. On the aerobic side normal microflora may be progressively altered into moderate AV and further deteriorates to severe AV or "desquamative inflammatory vaginitis" (DIV). *Trichomonas vaginalis* will usually be found in cases with "abnormal" Lactobacillary microflora, but can also be accompanied by anaerobic-dominant or with aerobic-dominant microflora, or both. *Candida* will be more frequently found in the aerobic rather than the normal Lactobacillary microflora (lower left in the diagram), but it can also be encountered in all other compartments of the diagram. On the aerobic side, *Candida* may often grow together with group B streptococci (Monif, 1999).

1.4.1. Bacterial vaginosis (BV)

Bacterial vaginosis is an ecological disorder of the vaginal microbiota in which the normal *Lactobacillus*-dominant microflora is replaced by a 100–1000-fold increase in the numbers of anaerobic bacteria (Eschenbach, 1993-a). Thus, BV is usually a polymicrobial infection, and organisms responsible for infection include: *G. vaginalis*, *M. hominis*, *Bacteroides* spp., *Peptostreptococcus* spp., *Fusobacterium* spp., *Prevotella* spp. (Forsum *et al*, 2005; Spiegel, 1991). Symptoms are few, and most women do not realize they have the condition. When present, the most common symptom is a thin, gray, non-pruritic discharge with a fishy odor, while the vagina is not red or inflamed and there are no prominent symptoms of burning, pain or dyspareunia. These together with a pH > 4.5 and typical clue cells on microscopy suggest the clinical diagnosis according to Amsel *et al* (Amsel *et al*, 1983). A typical feature of bacterial vaginosis is the absence of inflammation. In BV there is only a slight increase in IL-1β and an unexpectedly low production of IL-8, preventing the attraction of inflammatory cells such as macrophages and neutrophils (Cauci, 2004; Sodhani *et al*, 2005). In non-pregnant women, the presence of BV is associated with an increased risk of upper genital tract and sexually transmitted infections (Plitt *et al*, 2005; Schwebke, 2003; Soper *et al*, 1990) and with the acquisition of HIV (Cohen *et al*, 1995; Martin *et al*, 1999; Taha *et al*, 1998). In pregnancy, BV increases the risk of post-abortal sepsis (Larsson *et al*, 2000), early miscarriage (Ralph *et al*, 1999), recurrent abortion (Llahi-Camp *et al*, 1996), late miscarriage (Hay *et al*, 1994; Llahi-Camp *et al*, 1996), preterm prelabor rupture of membranes (PPROM) (Azargoon and Darvishzadeh, 2006), spontaneous preterm labor (SPTL) and preterm birth (PTB) (Hay *et al*, 1994; Hillier *et al*, 1995; Kurki *et al*, 1992; Lamont, 2004), histological

chorioamnionitis (Gibbs, 1993; Martius and Eschenbach, 1990), and postpartum endometritis (Jacobsson *et al*, 2002; Watts *et al*, 1990).

1.4.2. Aerobic vaginitis (AV)

Aerobic vaginitis (AV) is defined as a disruption of the lactobacillary population, accompanied by signs of inflammation and the presence of a rather scarce, predominantly aerobic microflora, composed of enteric commensals or pathogens (Donders *et al*, 2011).

Table 2. Criteria for the microscopic diagnosis of aerobic vaginitis (AV) (×400 magnification, phase contrast microscope).

AV score	Lactobacillary grades	Number of leukocytes	Proportion of toxic leukocytes	Background microflora	Proportion of parabasal epitheliocytes
0	I and IIa	≤10/hpf[1]	None or sporadic	Unremarkable or cytolysis	None or <1%
1	IIb	>10/hpf and ≤10/epithelial cell	≤50% of leukocytes	Small coliform bacilli	≤10%
2	III	>10/epithelial cell	>50% of leukocytes	Cocci or chains	>10%

"Severe AV" or "desquamative inflammatory vaginitis": score > 6; "moderate AV": score 5–6; "light AV": score; "no signs of AV": score < 3.

[1]hpf: high-power field (×400 magnification).

The diagnosis of AV is based on five microscopic criteria, each of which can be absent (0 points), moderate (1 point) or severe (2 points) (Table 2). The first criterion is Lactobacillary grading (LBG): LBG I and LBG IIa are considered normal (score 0), LBG IIb scores 1 point, LBG III scores 2 points, and is characterized by the complete replacement of lactobacilli by other bacterial morphotypes, such as cocci, anaerobic coccobacilli, or small bacilli. The second criterion

is the proportional number of leukocytes: if there are more than 10 leucocytes present per high-power field (1 point), the mean number surrounding each epithelial cell is counted, and a score of 2 is assigned when there is a mean of > 10 leukocytes per epithelial cell. The third criterion is the presence of toxic leukocytes, that is round and bloated leukocytes, containing swirling lysosomes: if the proportion of toxic leukocytes *vs* the total number of leukocytes is < 50% of the total leukocytes, 1 point is given; if there are > 50%, 2 points are given. The fourth criterion is the type of background flora: if it is unremarkable, or shows debris and bare nuclei from lysed epithelial cells (cytolysis) a score of 0 is assigned; a score of 1 is given if the lactobacillary morphotypes are very coarse or resemble small bacilli (other than lactobacilli); and a score of 2 is given if prominent single or chained cocci are visible. Finally, the presence of parabasal epithelial cells, that are small, rounded cells with large nucleus and dark cytoplasm, is evaluated: superficial and intermediate epitheliocytes are normal (score 0), but the presence of parabasal epithelial cells are abnormal. If between 1 and 10% of epitheliocytes are parabasal cells 1 point is given, but if more than 10% are parabasal cells, 2 points are assigned (Donders *et al*, 2002). The sum of the points establishes the composite AV score, with a maximum score of 10. A composite score of 1–2 represents normality, a score of 3–4 corresponds to slight AV, a score of 5–6 to moderate vaginitis, and a score > 6 to severe AV. In practice, a score of 8–10 matches the definition of "desquamative inflammatory vaginitis", so that such a diagnosis can be seen as the most extreme form of AV (Gardner, 1968; Sobel, 1994).

AV is recognized to be a separate vaginal microbiota disorder, and should not be confused with BV. Clinical features associated with the two entities and the host response are specific and clearly different for each condition. First of all, the diagnosis of AV is best accomplished on wet mounts, in order to better identify the presence of the signs of inflammation, such as the presence of toxic leukocytes and parabasal cells (Figure 4). The microscopic evaluation also permits to distinguish between the presence of aerobic enteric commensals, such as *E. coli*, *Staphylococcus aureus*, and group B streptococci, which is typical of AV, and the overgrowth of anaerobic bacteria (*G. vaginalis*, *Mobiluncus* spp., *Bacteroides* spp., *Prevotella* spp., *Peptostreptococcus* spp.), representative of BV. Not only are the microscopic details different between AV and BV, the local immune response elicited in the host is unique in each condition. The concentrations of pro-inflammatory cytokines, IL-1β, IL-6 and IL-8, are clearly linked to an abnormal vaginal microflora, and are inversely proportional to the numbers of lactobacilli (Donders *et al*, 2000-a; Donders *et al*, 2003). BV is associated with a moderate elevation of the pro-inflammatory cytokine IL-1β, but does not result in the subsequent induction of IL-6 and IL-8 (Cauci *et al*, 2003). Whereas in AV, IL-1β is produced in the vagina to a much greater extent, and even more dramatic is the elevated

concentration of IL-6 (Donders *et al*, 2002). IL-6 is a well-known marker for bacterial amnionitis and imminent term and preterm delivery, and, together with IL-8, is a chemo-attractant directly linked to increased prostaglandin production and delivery (Imseis *et al*, 1997; Romero *et al*, 1993).

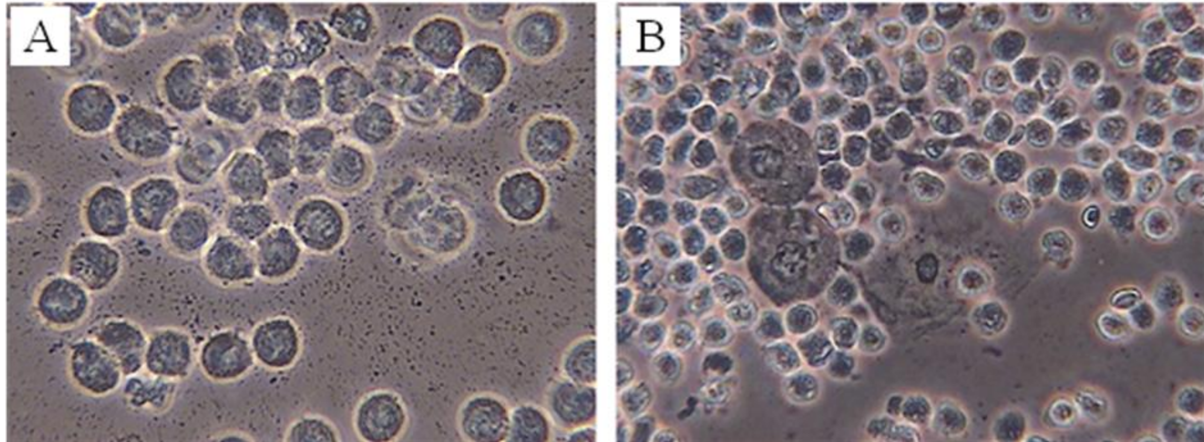

Figure 4. Images of phase-contrast microscopy (×400) of vaginal fluid from patients with aerobic vaginitis (AV): (A) The toxic leukocytes, full of lysozymic granules. (B) The typical AV flora, also illustrating the parabasal cells. (Donders, 2007).

Many women have microscopic signs of AV with the apparent absence of any symptoms. Probably the severity of the abnormalities found by microscopy (AV score) is linked to the severity of symptoms. As a consequence of the increased immune response in AV, symptoms are related to the thinned vaginal mucosa and increased inflammatory reaction. There is almost invariably an increased vaginal discharge, which is abundant and yellowish, but devoid of fishy amine odor. Also, severe burning and stinging is often prominent, and is commonly linked to dyspareunia. Because of this thinning of the vagina and the possible ulcerations, AV may predispose women to the acquisition of HIV or other sexually transmitted infections (Donders *et al*, 1991; Donders *et al*, 1993).

The best approach for treating AV in both pregnant and non-pregnant women is unknown. The inflammatory component of most patients with AV suggests that antibiotics may not be sufficient. Clindamycin may be a better choice than metronidazole for pregnant women with an abnormal vaginal flora. Some local, non-absorbable antibiotics may hold special promise because they induce a favorable anti-inflammatory environment (Brown *et al*, 2010), as these treatments have led to high cure rates in inflammatory bowel diseases such as Crohn's disease, diverticulitis and colitis ulcerosa (Guslandi, 2010; Latella and Scarpignato, 2009; Shafran and Burgunder, 2010).

1.4.3. Candida vaginitis (CA)

Candida vaginitis is one of the most common causes of vaginal infections (Anderson *et al*, 2004). *Candida albicans* is responsible for the 70% to 90% of cases, the remaining cases being caused by *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* (Nyirjesy, 2008; Richter *et al*, 2005; Sobel, 2007). In the absence of immunosuppression or damaged mucosa, *Candida* spp., mostly *C. albicans*, can be isolated in the vaginal tracts of 20% to 30% of healthy asymptomatic non-pregnant women (Beigi *et al*, 2004-b); it is usually not associated with any signs of disease and is thus referred to as colonization (Figure 5). Vaginal colonization typically begins following menarche and stems from increased vaginal glycogen stores induced by the rise in endogenous estrogen levels. If the balance between colonization and the host is temporarily disturbed, *Candida* can cause vaginitis, which is associated with clinical signs of inflammation. Risk factors that can precipitate infection include conditions with broad-spectrum antibiotic use, inadequately controlled diabetes mellitus, infection, and genetic predispositions (Goswami *et al*, 2000; Sobel, 2007). Episodes of CA occur mostly during childbearing years and an increased frequency has been reported during the premenstrual week (Eckert *et al*, 1998) and during pregnancy (Cotch *et al*, 1998).

In most women, the hallmark symptoms of CA are vulvar pruritus and burning, frequently accompanied by soreness and irritation leading to dyspareunia and dysuria (Anderson *et al*, 2004). On physical exam, vulvar and vaginal erythema, edema, fissures, and a thick curdy vaginal discharge are commonly found (Eckert *et al*, 1998). The clinical symptoms are nonspecific and can be associated with a variety of other vaginal diseases and infections. However, the vaginal pH is normal (4.0 to 4.5), and previous studies have failed to find an altered or abnormal bacterial vaginal ecosystem in patients affected by CA, as a predominant presence of lactobacilli was observed (Sobel and Chaim, 1996; Vitali *et al*, 2007; Zhou *et al*, 2009). However, a PCR-DGGE study by

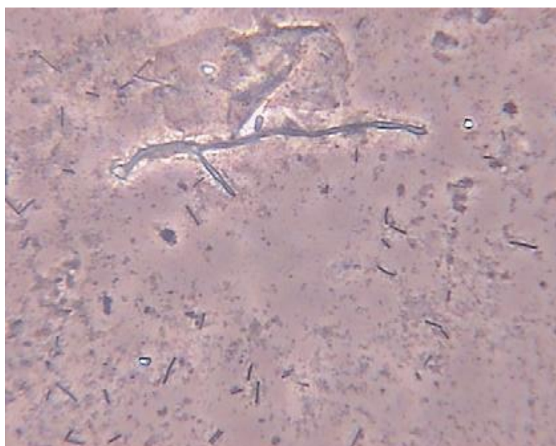

Figure 5. Images of phase-contrast microscopy (×400) of vaginal fluid from women with *Candida* in a normal bacterial microflora (Lactobacillary grade I) (Donders,

2007).

Vitali *et al*, showed a different frequency of particular *Lactobacillus* species in respect to the frequency detected under healthy conditions, with a decrease in H_2O_2-producing species (*L. acidophilus*, *L. gasseri*, and *L. vaginalis*) and an increase of non-H_2O_2-producing *L. iners* (Vitali *et al*, 2007). Thus, hydrogen peroxide rather than lactic acid, could be responsible for the control of *Candida* spp. overgrowth in the vaginal environment (Sobel and Chaim, 1996).

The 2006 STD Treatment Guidelines from the Centers for Disease Control and Prevention (CDC) classifies *Candida* vaginitis as either uncomplicated or complicated (Centers for Disease Control and Prevention, 2006). Uncomplicated infections are easily treated and defined as sporadic or infrequent infections of mild-to-moderate severity caused by *C. albicans* in immunocompetent women. Complicated infections include cases of severe vaginitis, CA caused by non-*C. albicans* species, CA associated with pregnancy or other concurrent conditions, such as uncontrolled diabetes or immunosuppression, and recurrent *Candida* vaginitis. Recurrent CA is defined as at least four episodes of *Candida* vaginitis during 1 year (Sobel *et al*, 1998).

Successful treatment of uncomplicated CA is achieved with single-dose or short-course therapy in over 90% of cases. Several topical and oral drugs are available, without evidence for superiority of any agent or route of administration (Pappas *et al*, 2009), although among the topically applied drugs, azoles are more effective than nystatin (Centers for Disease Control and Prevention, 2006). Non-*C. albicans*-related disease is less likely to respond to azole therapy (Nyirjesy *et al*, 1995). Vaginal boric acid, administered in a gelatin capsule, AmB suppositories, or topical 17% flucytosine cream alone or in combination with 3% AmB cream are all rather good treatment options for non-*C. albicans* CA (Pappas *et al*, 2009; Phillips, 2005; Sobel *et al*, 2003). In cases of recurrent CA, long-term suppressive therapy with oral fluconazole is the most convenient and well-tolerated regimen among other options and was shown to be effective in over 90% of patients. Against expectations, these patients have shown little evidence of developing fluconazole resistance in *C. albicans* isolates or superinfection with non-*C. albicans* species (Shahid and Sobel, 2009; Sobel *et al*, 2004). Many women with recurrent CA turn to probiotics as adjunct or sole therapy to control their recurrences. Most probiotics, in oral or topical formulation, contain lactobacilli, which are felt to inhibit or reduce the growth of *Candida* spp. in the vaginal tract, but results from different clinical trials are in disagreement (Falagas *et al*, 2006).

1.4.4. Cytolytic vaginosis

Cytolytic vaginosis is a non-inflammatory condition in which H_2O_2-producing lactobacilli cause an extreme vaginal acidity (pH < 4), leading to epitheliolysis (Cibley and Cibley, 1991). Usually the abundant presence of coarse, equal-sized lactobacilli is evident, together with bare nuclei, patches of cytoplasmic debris of lysed epithelial cells, and paucity of leukocytes (Figure 6). The condition can typically cause a burning sensation and increased vaginal discharge, and is often confused with *Candida* vaginitis (Demirezen, 2003).

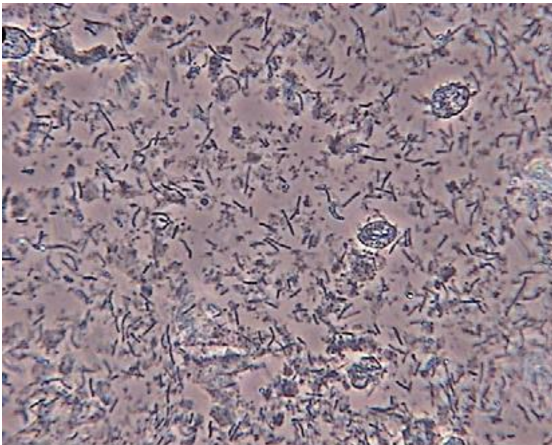

Figure 6. Images of phase-contrast microscopy (×400) of vaginal fluid from patients with cytolysis of epithelial cells, with numerous bare epithelial nuclei and cytolyic debris clearly visible (cytolytic vaginosis) (Donders, 2007).

1.4.5. Trichomonial vaginitis (TV)

Trichomonas vaginalis is one of the most frequent sexually transmitted pathogens worldwide (Kingston *et al*, 2003). It is a unicellular, flagellated protozoan and is responsible for 15% to 20% of patients with vaginitis (Anderson *et al*, 2004). Up to 50% of patients may be asymptomatic (Sobel, 2005), while a frothy, malodorous, yellow-green discharge is a common complaint of the symptomatic women affected by TV. Like BV, studies have demonstrated a link between trichomonas infection and a higher risk for premature rupture of membranes, chorioamnionitis, and preterm birth during pregnancy. In non-pregnant women, infection is felt to be a risk factor for the transmission of HIV, cervical neoplasia, atypical pelvic inflammatory disease, tubal infertility, and post-hysterectomy infection (Soper, 2004).

1.4.6. Cervicitis

Cervicitis is an inflammation of the uterine cervix, frequently asymptomatic. It is generally considered to result from infection with a sexually acquired microorganism, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* or *Mycoplasma genitalium* (Cohen *et al*, 2002; Gaydos *et al*,

2009; Marrazzo *et al*, 1997; Miller and Zenilman, 2005). In many women with cervicitis these microorganisms are not always detected, even when highly sensitive diagnostic tests are performed (Marrazzo *et al*, 2002-a; Nyirjesy, 2001). Cervicities should be suspected in women with a yellow endocervical discharge, easily induced cervical bleeding, edema of the cervical ectropion, or leucorrhea (> 10 white blood cells per high-power field on microscopic examination of vaginal fluid) (Marrazzo and Martin, 2007). Because cervicitis increases the risk of poor pregnancy outcome, predicts upper genital tract disease, and is associated with increased shedding of HIV-1 from cervix, determining the etiology of this condition should be a priority.

2. Bacterial vaginosis

2.1 Overview

Bacterial vaginosis (BV) is the most common vaginal tract syndrome in women of child-bearing age (Holst *et al*, 1987), and occurs at a rate between 5% and 50% (Sobel, 2000). It is characterized by dramatic shifts in the types and relative proportions of a diverse community of bacteria as the vaginal ecosystem changes from a healthy to a diseased state (Eschenbach, 1993-b; Fredricks *et al*, 2005). Because BV is fundamentally a result of changes to the vaginal microbial community, several studies focused on the knowledge of how the healthy community is altered in its taxonomic composition, community structure and function, in order to understand the etiology of BV and develop a successful treatment.

Up to the 1950s, symptomatic women with Lactobacillary grade III were diagnosed with "non-specific vaginitis", as the microbial etiology of lactobacillary deficiency was still uncertain at that time. In 1923, Curtis had described a vaginal discharge syndrome in women and called it the "white-discharge" syndrome (Curtis, 1914), then renamed "non-specific vaginitis". Using culture techniques, he associated this syndrome with black-pigmented anaerobes, curved anaerobic motile rods, anaerobic cocci and Gram-variable diphtheroidal rods. He also noted a relative death of the "Döderlein's bacillus" in women with this syndrome. Thus, over 80 years ago, it was known that significant shifts in the vaginal microbiota were associated with a symptomatic vaginal discharge syndrome in women, but many investigators believed that this syndrome was caused by a single microorganism. In 1955, Gardner and Dukes isolated a small, pleomorphic Gram-variable coccobacillus from women with "non-specific vaginitis", the condition now known as BV, and postulated that this was the primary etiological agent (Gardner and Dukes, 1955). This organism, first called *Haemophilus vaginalis* and then repeatedly renamed, is now classified as *Gardnerella vaginalis*, the sole member of the genus *Gardnerella* (Greenwood and Pickett, 1979; Piot *et al*, 1980). Phylogenetic analysis based on 16S rRNA places *Gardnerella* in the Gram-positive family *Bifidobacteriales*. In their paper on *Haemophilus vaginalis* vaginitis, Gardner and Dukes provided retrospective evidence that more than one organism is required to establish BV (Gardner and Dukes, 1955). They reported that when 15 clinically healthy volunteers were inoculated with vaginal material from BV patients, 11 developed BV. However, when 13 volunteers were inoculated with pure cultures of *G. vaginalis*, only one developed BV, suggesting that *G. vaginalis* alone was not sufficient to induce disease and that additional factors/organisms were necessary.

Since that time, numerous BV-associated bacteria have been identified using both standard culture and cultivation-independent techniques and BV is generally regarded as a polymicrobial disease.

2.2 Etiology and risk factors

BV represents an imbalance in the ecology of the normal vaginal microflora (McDonald *et al*, 2003), characterized by a reduction in the prevalence and concentration of H_2O_2-producing lactobacilli (Eschenbach *et al*, 1989; Hawes *et al*, 1996) and an increase in the prevalence and concentration of other bacteria, including *G. vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Mobiluncus* spp., anaerobic Gram-negative rods belonging to the genera *Prevotella*, *Porphyromonas* and *Bacteroides*, and anaerobic *Peptostreptococcus* spp. (Hillier *et al*, 2008; Larsson and Forsum, 2005; Livengood, 2009; Marrazzo, 2003; Srinivasan and Fredricks, 2008; Verhelst *et al*, 2004; Verstraelen *et al*, 2004). All these organisms are of relatively low virulence. Therefore, BV is not caused by the mere presence of the potential pathogens but rather by their unrestrained increase in number, reaching cell counts that are 100- to 1000-fold above the normal bacterial levels of the vagina (Eschenbach, 1993-a; Forsum *et al*, 2005; St John *et al*, 2007). The majority of bacterial species (> 99%) have not been cultivated in the laboratory (Hugenholtz *et al*, 1998), but molecular approaches now enable their detection.

Recent studies using cloning and sequencing of the 16S rRNA gene for the identification of genital microbiota, have demonstrated a high diversity of organisms in women with BV compared with women with normal microflora. Collectively, these studies showed that bacteria that were previously unidentified represent a substantial fraction of the BV microbiota in many women (Ferris *et al*, 2004; Fredricks *et al*, 2005; Hyman *et al*, 2005; Oakley *et al*, 2008; Verhelst *et al*, 2004; Zozaya-Hinchliffe *et al*, 2008). *Atopobium vaginae*, *Leptotrichia amnionii*, *Megasphaera* spp., *Sneathia* spp., *Eggerthella*-like uncultured bacteria, and three newly described members of the *Clostridiales* order (BVAB1, BVAB2 and BVAB3) have been found to have high specificity for BV (Ferris *et al*, 2004; Fredricks *et al*, 2005); although *A. vaginae* has also been detected in subjects without bacterial vaginosis (Zhou *et al*, 2004). Moreover, the number of phylotypes found in association with BV was statistically significantly greater than the number detected in the presence of a normal microbiota (Biagi *et al*, 2009; Burton and Reid, 2002; Diao *et al*, 2011; Fredricks *et al*, 2005; Vitali *et al*, 2007). These studies have demonstrated that different subjects with BV have different microbial profiles, indicating heterogeneity in the composition of bacterial taxa in women with BV. Unlike healthy women, whose bacterial communities are dominated by *Lactobacillus*

spp., women with BV does not possess a single dominant phylotype, but instead had a diverse array of vaginal bacteria, often at relatively low abundances.

The etiology of bacterial vaginosis remains enigmatic. BV does not follow Koch's postulate that a single pathogen is responsible for a specific disease, and many cultivated bacteria linked to this condition, especially *G. vaginalis*, frequently colonize the vagina of healthy women (Sobel, 1989). Thus, even if *G. vaginalis* remains one of the most frequently isolated organisms in women with BV (Menard *et al*, 2008), its presence ''per se'' is not sufficient for the development of BV and it probably acts synergistically with other microorganisms to cause the syndrome (Aroutcheva *et al*, 2001-b; Totten *et al*, 1982). It has never been resolved which microorganisms within the mixture associated with BV are essential for its development and maintenance (Hill, 1993), and the factors which lead to the decline of the lactobacilli and the overgrowth of other bacteria remain unknown (Eschenbach, 1989; Sobel, 1989). Microbial interactions can exert their effect either by modifying the environment, so it becomes suitable for another organism, or by releasing particular metabolites which influence the growth of other organisms (Atlas and Bartha, 1993). These may, in turn, contribute to the pathogenesis of BV.

In 1999, Pybus and Onderdonk reviewed the positive interactions among three microorganisms that were recognized as being significantly associated to BV: *G. vaginalis*, anaerobes, and *M. hominis* (Pybus and Onderdonk, 1999). They highlighted that both *M. hominis* and *G. vaginalis* could be either dependent on nutrients produced by the BV-associated microflora which precede them in colonizing the vagina or are a consequence of alterations to the vaginal environment as a result of BV. Several studies reported that when *M. hominis* and *G. vaginalis* were isolated from the female genital tract they were invariably associated with anaerobic organisms, suggesting that their presence in the vagina is dependent on other BV-associated bacteria (Blanco *et al*, 1983; Hill *et al*, 1985; Holst *et al*, 1987; Rosenstein *et al*, 1996). Further analysis regarded the identification of specific nutritional pathways involving BV-related organisms, to support their interactions. Chen *et al* hypothesized a commensal relationship between *G. vaginalis* and anaerobes, such that amino and keto acids, especially pyruvate, produced by *G. vaginalis* are utilized by anaerobes (Chen *et al*, 1979). Furthermore, they demonstrated the production of the volatile amines putrescine and cadaverine by metronidazole-resistant organisms. Pybus and Onderdonk suggested the existence of a commensal relationship between *Prevotella bivia* and both *G. vaginalis* (Pybus and Onderdonk, 1997) and *Peptostreptococcus anaerobius* (Pybus and Onderdonk, 1998). They reported that the growth of the two microorganisms was stimulated in *P. bivia*-conditioned supernatants, as *G. vaginalis* utilizes ammonia, and *P. anaerobius* utilizes aminoacids, both produced by *P. bivia*. In addition, it was postulated that anaerobes, via the

production of the alkaline metabolites amines and ammonia, could raise the pH of the vaginal environment and thus encourage the growth of *G. vaginalis* (Chen *et al*, 1979; Spiegel *et al*, 1980). Therefore, 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 (trimethylamine, putrescine, and cadaverine), which, in high pH, become volatile and malodorous (Figure 7). The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating the typical discharge. In conditions of elevated pH, *G. vaginalis* more efficiently adheres to the exfoliating epithelial cells, creating clue cells. Amines further provide a suitable substrate for *M. hominis* growth. What remains unknown is whether the loss of lactobacilli precedes or follows this massive disturbance in the microflora (Sobel, 2000).

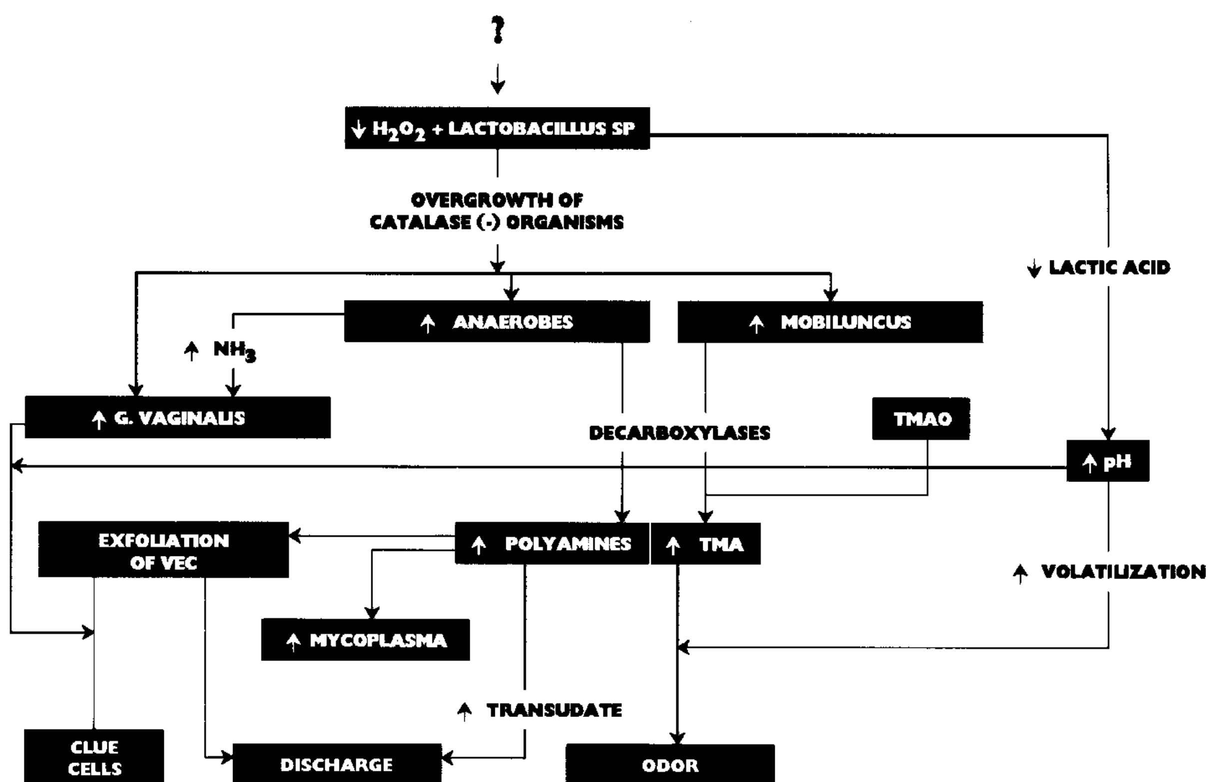

Figure 7. Pathophysiology of bacterial vaginosis (Sobel, 2000).

2.2.1. Risk factors

Numerous epidemiologic investigations have been done to identify factors that increase a woman's risk of BV. The risk of BV is increased in women of African ethnicity (Cherpes *et al*, 2008; Klatt *et al*, 2010; Simhan *et al*, 2008), and in other women who have no or low amounts of

H_2O_2-producing lactobacilli in the vagina (Hawes *et al*, 1996). Other risk factors include low socioeconomic status, cigarette smoking, douching, menses, use of intrauterine devices, antibiotic treatment for another condition, lack of condom use, and acquisition of a new sex partner. In general, women with BV have more sex partners and an earlier age of sexual debut than women without BV (Cherpes *et al*, 2008; Fethers *et al*, 2009; Merchant *et al*, 1999; Schwebke and Desmond, 2005; Schwebke *et al*, 1999; Verstraelen, 2008; Verstraelen *et al*, 2010; Yen *et al*, 2003).

The fact that many of the high-risk behaviors are also well-established risk factors for acquisition of common sexually transmitted infections (STIs) suggests that BV could be transmitted sexually (Gardner and Dukes, 1955; Verstraelen, 2008). Additionally, the concordance of BV status in monogamous lesbian couples, ranging up to 95% (Berger *et al*, 1995; Marrazzo *et al*, 2002-b), strongly suggests that BV may be sexually associated, although an infectious correlate has not been identified (Schwebke, 2009-b). Since the original transmissible nature of BV was demonstrated by Gardner and Dukes in 1955, several studies have confirmed that *G. vaginalis* and other BV-associated organisms may be transferred sexually; however, transmission alone does not seem enough to cause disease because most of the microorganisms are normally found in low numbers in the healthy vagina. The fact that there is no evidence for a decrease in the rates of BV recurrence following antibiotic treatment of men sexually involved with affected women is another distinction between BV and the common STIs (Verstraelen *et al*, 2010). In fact, many researchers prefer to view BV not as an infection but as a complex microbial imbalance, with a significant role played by the indigenous vaginal lactobacilli (Guise *et al,* 2001; Hay, 2005; Schwiertz *et al*, 2006).

Five decades of intense research established many risk factors for acquisition of BV; however, because of the complexity of BV and lack of a reliable animal model for this condition, its exact etiology remains enigmatic. Afterwards the major theories on the etiology of BV are described, but none of them on their own can reliably explain the epidemiological data. Instead, BV is caused by a complex interaction of multiple factors, which include the numerous components of the vaginal microbial ecosystem and their human host, and many of them are yet to be characterized (Turovskiy *et al*, 2011).

2.2.2. The "Gardnerella vaginalis" theory

BV was initially thought to be a STI propagated by a bacterium that is now known as *G. vaginalis*. As the polymicrobial nature of BV became evident, the role of this microorganism in the

etiology of this condition became less clear. As a result, general interest in *G. vaginalis* declined in the late 1980s, only to re-emerge in recent years as its relationship to BV was re-evaluated.

The original discovery of *G. vaginalis* was made by Leopold in 1953 (Leopold, 1953), while Gardner and Dukes were the first to describe the microorganism in relation to BV (Gardner and Dukes, 1955). The bacterial cell's morphology, apparent negative reaction to Gram staining, and inability to grow on agar media lacking blood convinced these researchers that they were dealing with a new *Haemophilus* species, which they named *Haemophilus vaginalis*. Afterwards, because of its unique cell wall structure and nutritional requirements, the microorganism has been renamed several times (Catlin, 1992; Deane *et al*, 1972; Dunkelberg *et al*, 1970). Finally, two large taxonomic studies evaluating multiple criteria (Greenwood and Pickett, 1980; Piot *et al*, 1980) proposed a new genus named *Gardnerella*, with *G. vaginalis* being the only species in it. Due to the uncertainties in the taxonomic status of *G. vaginalis*, its cell wall has been investigated for decades. *Gardnerella vaginalis* is commonly described as a Gram-variable or Gram-uncertain microorganism, because its reaction to Gram staining can vary from negative to positive (Catlin, 1992). It is likely that the age of the culture and the growth conditions may influence the reaction to Gram staining, determining fluctuations in thickness of the peptidoglycan layer, which becomes thinner as the culture ages (Catlin, 1992; Sadhu *et al*, 1989). Overall, the cells of *G. vaginalis* are small, pleomorphic rods having average dimensions of 0.4 by 1.0–1.5 μm (Catlin, 1992). The bacterium is immotile, with the cells frequently occurring in clumps in vaginal smears and when grown in liquid media (Greenwood and Pickett 1980; Taylor-Robinson, 1984). Strands of exopolysaccharide produced by cells are presumed to be responsible for the cell clumping effect (Catlin, 1992). Recently, the genomes of several *G. vaginalis* strains were sequenced, providing new information about the microorganism. These studies estimated the genome of *G. vaginalis* to be 1.62–1.67 Mb with a low GC content (41–42%) (Yeoman *et al*, 2010); thus, the bacterium's genome size is relatively small. This, together with its deficiencies in important biochemical pathways, are consistent with the parasitic lifestyle of *G. vaginalis*. Among the other biochemical properties, *G. vaginalis* has a hemolytic activity, which is thought to be mainly due to secretion of vaginolysin (VLY). VLY is a cytolysin with specific activity against human erythrocytes, which, *in vivo*, is thought to increase nutrient availability for its producer strain (Yeoman *et al*, 2010).

Gardner and Dukes were the first to report that *G. vaginalis* was isolated from the lower genital tract of BV-affected women in 92% of cases, compared to a 0% isolation rate from healthy women (Gardner and Dukes, 1955). Consequently, they hypothesized that the microorganism was the only etiological cause of BV (Eschenbach *et al*, 1989). Subsequent studies found *G. vaginalis* in the vaginas of 14–69% of BV-free women, an incidence that is considerably higher than the 0%

originally reported (Cristiano *et al*, 1989; Gardner and Dukes 1955; Masfari *et al*, 1986; Mikamo *et al*, 2000; Totten *et al*, 1982). To explain epidemiological data showing the common occurrence of *G. vaginalis* in healthy women, numerous studies were directed to identify specific virulent subtypes of this organism responsible for BV (Aroutcheva *et al*, 2001-b; Benito *et al*, 1986; Briselden and Hillier, 1990; Piot *et al*, 1984), but it is still unclear whether any of the biochemical characteristics selected for biotyping *G. vaginalis* are linked to the virulence of the microorganism. Genetic subtyping proved to be challenging because of the great variability in the DNA sequence of different *G. vaginalis* isolates. Different strains isolated from women with or without BV showed genomic differences regarding adherence, aggregation, and cytolysin productions (Harwich *et al*, 2010; Yeoman *et al*, 2010)

The most interesting factor that might be of importance in the pathogenesis of BV is the ability to adhere to the vaginal epithelia and to form biofilms (Harwich *et al*, 2010). Swidsinski *et al* showed by FISH with specific probes that a characteristic dense biofilm in confluent or patchy layers was attached to the epithelial surface in 90% of the vaginal biopsies of patients with BV (Swidsinski *et al*, 2005; Swidsinski *et al*, 2008). Bacteria within the biofilm were calculated to reach concentrations of nearly 10^{11} per mL; *Gardnerella* were the predominant bacteria, while *Atopobium* were present in 80% and contributed up to 40% of the biofilm mass. It was shown that the classical clue cells were desquamated cells from the biofilms (Swidsinski *et al*, 2005). The adherent *G. vaginalis* biofilm typically resist antibiotic treatment (Swidsinski *et al*, 2008; Swidsinski *et al*, 2010), which might explain the high recurrence rates of BV. The metabolic activities of *Gardnerella* and other microbes stop during treatment but slowly start again after treatment cessation, eventually leading to vaginal discharge and odor that are products of enzymatic activities (Hillier *et al*, 2008).

In a recent review, Turovskiy *et al* proposed that, under favorable conditions, BV infection can be seeded by *G. vaginalis*, as well as a group of other microorganisms (including *A. vaginae*) that are relatively tolerant to the healthy vaginal environment, thus allowing the more fastidious opportunists to take advantage of the created niche (Turovskiy *et al*, 2011).

2.2.3. The "incompetent vaginal lactobacilli" theory

The role of vaginal lactobacilli as a primary line of defense against various vaginal pathogens has been recognized for decades. Accordingly, researchers hypothesized that women affected by recurring BV are colonized by *Lactobacillus* strains that are not particularly competent as "defenders". In 1989, Eschenbach *et al* were the first to postulate that H_2O_2 production by vaginal lactobacilli is critical for sustainment of healthy vaginal microbiota (Eschenbach *et al*, 1989).

Afterwards, several studies recognized an inverse association between BV and the occurrence of vaginal H_2O_2-producing *Lactobacillus* spp. (Dimitonova et al, 2007; Hawes *et al*, 1996; Hillier *et al*, 1992; Hillier *et al*, 1993-a; Martinez et al, 2008; Nagy *et al*, 1991). Additionally, the presence of many BV-related pathogens including *G. vaginalis*, *P. bivia*, *Bacteroides* spp. and *Mobiluncus* spp. was inversely related to the presence of H_2O_2-producing *Lactobacillus* spp. (Hillier *et al*, 1993-a). Therefore, a protective role for the production of H_2O_2 by vaginal lactobacilli against BV has been suggested.

Multiple studies attempted to model the antagonism between vaginal H_2O_2-producing *Lactobacillus* spp. and BV-related pathogens *in vitro*. The great majority of these studies demonstrated the inhibitory and/or bactericidal properties of vaginal H_2O_2-producing lactobacilli against pathogens (Atassi *et al*, 2006; Atassi and Servin, 2010); however, the relative contribution of the H_2O_2 produced by the *Lactobacillus* spp. to the overall antimicrobial effect is still a matter of debate. Some *in vitro* studies tried to quantify the levels of H_2O_2 produced by the vaginal *Lactobacillus* spp., but the concentration of H_2O_2 largely depended on oxygen availability, thus multiple studies reported undetectable levels of H_2O_2 in vaginal *Lactobacillus* cultures grown under anaerobic conditions (McLean and McGroarty, 1996; O'Hanlon *et al*, 2010; Strus *et al*, 2006). In hypoxic conditions, the metabolism of H_2O_2-producing *Lactobacillus* strains shifts away from production of H_2O_2 and towards the production of lactic acid, because the enzymes involved in these two metabolic pathways compete for NADH (McLean and McGroarty, 1996).

Some clinical trials also evaluated the efficacy of topical application of H_2O_2 in restoring the microbial balance in BV-affected individuals (Cardone *et al*, 2003; Chaithongwongwatthana *et al*, 2003; Winceslaus and Calver, 1996). It has been reported that intravaginal administration of hydrogen peroxide may represent a promising treatment for BV; however, additional clinical trials are needed to evaluate the efficacy of this approach.

It has been demonstrated that H_2O_2 is an integral part of the natural defenses produced by vaginal lactobacilli (Atassi and Servin, 2010). Conversely, the expected levels of H_2O_2 production in the vaginal environment, which is hypoxic, are very low. Therefore, the role of this antimicrobial *in vivo* is still unclear, and the causal relationship between vaginal H_2O_2-producing *Lactobacillus* spp. and BV is yet to be established.

2.2.4. The "phage" theory

Some theories state that the overgrowth of pathogens characteristic to BV has to be preceded by a major disturbance within the *Lactobacillus* population (Blackwell, 1999; Pavlova *et al*, 1997). The resultant decline of lactobacilli allows for a shift in the vaginal microbiota composition.

Pavlova *et al* proposed that bacteriophages could cause a decline in vaginal lactobacilli (Pavlova *et al*, 1997). The involvement of bacteriophages in the etiology of BV would explain why this condition is epidemiologically similar to STIs, and yet the rate of its recurrence in women is unaffected by an antibiotic treatment of their male partners (Blackwell, 1999). *In vivo* and *in vitro* studies identified *Lactobacillus* strains that were phage carriers (lysogens), especially from BV-affected women (Kilic *et al*, 2001; Pavlova *et al*, 1997). Some of these phages were able to infect a broad range of *Lactobacillus* species originating from different women. All the phages identified were temperate, but, once released from a bacterial cell, some of these phages would undergo a lytic cycle in a different bacterial strain (Kilic *et al*, 2001; Pavlova *et al*, 1997). It has been proposed that, *in vivo*, some external factors can induce these normally temperate phages into a lytic cycle (Blackwell, 1999; Kilic *et al*, 2001). For example, a study revealed that the lysogenic *Lactobacillus* strains of vaginal origin can be induced to release phages by cigarette smoke chemicals (Pavlova and Tao, 2000). The phage theory of the lactobacilli decline, along with this finding, may explain why cigarette smoking is a significant risk factor for BV.

If bacteriophages truly play a significant role in the etiology of BV, then intravaginal installation of phage-resistant probiotic strains could be effective for treatment and prophylaxis of this condition (Blackwell, 1999).

2.2.5. The "altered immunity" theory

BV-associated pathogens can frequently be detected in the lower genital tract of asymptomatic women, and it remains unclear why certain women are unaffected by these pathogens while others develop BV. Furthermore, the specific factors determining the severe BV-related complications in some women but not in others are also poorly understood (Witkin, *et al*, 2007-b). Currently, many researchers are leaning towards the idea that host immunity is a decisive factor in the equation determining the initial development and later course of BV (Forsum *et al*, 2005; St John *et al*, 2007; Witkin, *et al*, 2007-b). The main purpose of these studies is to relate the etiology of both BV and the associated complications to the differences in the immune response of diverse hosts. So far, research primarily targeted components of the innate immune system, because of their prominent role in other infectious conditions (Genc and Schantz-Dunn, 2007; Misch and Hawn, 2008). The innate immune system recognizes pathogen-associated molecular patterns that are non-variant components of many microbial invaders. Recognition of microorganisms by the innate immune system is the initial trigger for a successful antimicrobial immune defense.

Witkin *et al* proposed that BV develops as a result of inhibition of Toll-like receptors (TLR) activation and that the negative consequences of BV are facilitated by a decrease in 70-kDa heat shock protein (hsp70) production and release and/or inadequate mannose-binding lectin (MBL) function (Witkin, *et al*, 2007-b). TLRs are transmembrane proteins and represent the major determinants for the recognition of microbial pathogens and the initiation of immune system activation (Janssens and Beyaert, 2003; O'Connell *et al*, 2005). The binding of an invariant microbial component to a specific TLR triggers a sequence of events leading to the selective activation of genes coding for pro-inflammatory cytokines (O'Connell *et al*, 2005). These cytokines, in turn, initiate a microbial pathogen-specific immune response. TLRs have been identified on a variety of cell types, including epithelial cells in the female genital tract (Abrahams, 2005; Fazeli *et al*, 2005). The inducible hsp70 is a newly recognized component of innate immunity. Its synthesis is greatly up-regulated under non-physiological conditions, such as infection, inflammation, ischemia, and exposure to toxic chemicals. Intracellularly, hsp70 binds to other proteins, preventing their denaturation and degradation, but it can also be released from cells into the extracellular milieu in response to stress. This extracellular hsp70 functions as an early warning signal. By binding to TLRs, hsp70 initiates a nonspecific pro-inflammatory immune response to fight any microbial pathogens that might be present (Campisi *et al*, 2003). MBL possesses antimicrobial activity against fungi, bacteria, and viruses. It recognizes and binds to specific carbohydrate patterns that are present on the surface of many microorganisms (Babovic-Vuksanovic *et al*, 1999). Subsequent to MBL binding to a microbial surface, serine proteases associated with MBL are activated and initiate complement system activation (Fujita *et al*, 2001). In addition to being present in serum, MBL has also been identified in vaginal secretions (Babula *et al*, 2003), suggesting a role in antimicrobial defense at this site.

It has been proposed that the bacteria present in the vaginas of healthy women maintain vaginal epithelial cell TLR activation at a steady level, resulting in sufficient cytokine production to inhibit the proliferation of abnormal BV-associated bacteria. Examination of vaginal secretions of women with *Lactobacillus*-dominant microflora has identified the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in low concentrations (Donders *et al*, 2003). However, various effector molecules, such as bacterial proteases and toxins produced by BV-related bacteria, might inactivate TLRs on cervicovaginal epithelia. These compounds can inactivate local immune response through a direct degradation of TLRs, by interference with TLR-ligand recognition, or by inducing anti-inflammatory cytokines, such as IL-10 (Witkin *et al*, 2007-b). The inactivation of the innate immune response would allow unrestrained multiplication of pathogens, manifested as BV. Most importantly, it has been suggested that certain polymorphisms in genes coding for innate immune

system components, such as TLRs, would make women susceptible to these bacterial molecules and therefore vulnerable to BV (Witkin *et al*, 2007-b).

The role of intrinsic host factors in the etiology of BV is still unclear, requiring more research to be conducted. Moreover, predisposition to BV is probably determined not by a single allele but by a specific allelic combination known as a haplotype (Cauci *et al*, 2007). Nevertheless, it is also important to take into account that although heredity may predispose a woman towards BV, the condition itself is caused by interaction of intrinsic host factors with the environment.

2.2.6. The "coitus-induced" theory

In a recent review, Leppäluoto wrote about the thesis of the "coitus-induced dynamics of vaginal bacteriology", in which the etiology BV is considered in relation to sexual behaviors and BV is seen as a physiological phenomenon instead of a sexually transmitted infectious disease (Leppäluoto, 2011). He hypothesized that the dual concept of BV as a monobacterial and polymicrobial entity may derive from a common physiological etiology.

The impetus for the physiological concept derived from an observation that spermatozoa were frequently seen in the cocci dominant (Leppäluoto, 1971-a) conventional cytologic smear (Wied and Bahr, 1959), which also provided a clue to the presence of *G. vaginalis* (Bergman *et al*, 1965; Gardner and Kaufman, 1969). Studies revealed that in most of the women, after free intravaginal deposition of an ejaculate, the dominant *Lactobacillus* morphotype microflora seen in pre-coital smears was replaced by *G. vaginalis* morphotype dominant microflora in post-coital smears. An hypothesis was suggested, that coitus-connected factors would effect a physiological change of the vaginal *Lactobacillus* microflora to a *Gardnerella* microflora (Leppäluoto, 1971-a). *In vivo* observations on vaginal acidity in relation to intravaginal deposition of an ejaculate indicated that the alkalinity of the ejaculate might neutralize the pre-coital high vaginal acidity (Masters and Johnson, 1966), which is associated to the *Lactobacillus* microflora of healthy women. This could lead to replacement of the *Lactobacillus* microflora with *Gardnerella* microflora, which instead is associated with a relatively low acidity (Gardner and Dukes 1955). The pre-coital highly acidic environment would then be restored along with a decreasing neutralizing effect of an ejaculate that lasted from six to seven up to 16 hours (Masters and Johnson, 1966), with a resultant replacement of *Gardnerella* by *Lactobacillus* microflora, seen in the pre-coital smears (Leppäluoto, 1971-a; Leppäluoto, 1971-b). The biological purpose of the elevated pH of post-coital vaginal acidity associated with *G. vaginalis* microflora, together with immobilization of *G. vaginalis* bacteria in a thin film on the vaginal wall (Gardner and Dukes 1955) and the paucity of leukocytes, could be necessary to help protect ejaculated spermatozoa (Leppäluoto, 1989). Accordingly, as a proposed

physiological phenomenon, the coitus-induced change of vaginal acidity and microbial ecosystem would thus reflect a conditional and reversible event (Leppäluoto, 1971-b).

The occurrences of BV in virginal adolescent girls (Bump and Buesching, 1988; Vaca *et al*, 2010) and in women who have sex with women (Marrazzo *et al*, 2002-b) could be a result of a psychic and/or physical sexual stimulation (Masters and Johnson, 1966). This practice could induce and maintain vaginal "lubrication", a transudation of neutrally acidic vaginal fluid with initial pH 7.3 (Moghissi, 1979) that would neutralize vaginal acidity to a degree that would favor *G. vaginalis* microflora and BV.

Leppäluoto also suggested that the dual occurrence of BV as a monobacterial physiological entity and as a polymicrobial disease would basically be due to alterations in the vaginal milieu attributable to changing sexual habits in the era of the contraceptive pills, rather than to improved diagnosis of vaginal microbiology (Leppäluoto, 2011). Oral contraception and the increasingly liberal sexual attitudes will have made free deposition of an ejaculate in the vagina much more common. Coital acts repeated at short intervals might first have resulted in an incidental post-coital *Gardnerella* microflora and then in a prolonged duration of or repeated change to lower vaginal acidity, which could have predisposed to transfer and colonization of predominantly anaerobic bacteria, presumably from the perianal region (Holst, 1990).

The connotation that BV, a common genital condition, is in origin a normal physiological state, and not a suspected transmitted sexual disease, would be immensely important to women, in terms not just of physical health but also self-esteem and the image of women as individuals in a social setting.

2.3 Clinical features and diagnosis

Clinical presentation of BV is typical. Women have an unpleasant, "fishy-smelling" discharge that is more noticeable after intercourse. The discharge is off-white, thin, and homogeneous. Erythema and inflammation are usually absent, and most patients are asymptomatic (Sobel, 1997).

The diagnosis of BV (Centers for Disease Control and Prevention, 2006) is based on clinical criteria or Gram stain, which are both fairly subjective and thus complicate research and clinical practice. Simple diagnostic criteria established by Amsel *et al* have proved useful in clinical practice (Amsel *et al*, 1983). At least 3 of the following 4 elements must be present for diagnosis of BV: 1) the presence of "clue cells" on microscopic examination, that are vaginal epithelial cells heavily coated with bacteria; 2) a thin, homogeneous, milky discharge that smoothly coats the

vaginal walls; 3) pH of vaginal fluid > 4.5; 4). a positive whiff test, that is the production of a fishy odor when 10% KOH is added to a slide containing vaginal fluid (Amsel *et al*, 1983). Amsel's criteria, although widely accepted as the best available means to diagnose BV in the clinical setting, may fail to identify women with asymptomatic BV.

Alternatively, BV maybe diagnosed using the Nugent scoring system (Nugent *et al*, 1991) for interpretation of Gram-stained vaginal smears (see paragraph 1.4). This method assesses the number of lactobacilli relative to BV-associated bacterial morphotypes in order to characterize vaginal microbiota as normal, intermediate or abnormal (BV) (Table 1), and is generally preferred in the scientific community (Schwiertz *et al,* 2006).

The diagnostic criteria used are a critical issue in studies on the etiology of BV. While numerous studies have shown that women with high numbers of *Lactobacillus* spp. generally do not have BV, it is misleading to conclude that women whose vaginal communities contain few or no *Lactobacillus* spp. have BV. Unfortunately, this erroneous belief is the premise of the Nugent criteria wherein the degree of "healthiness" is largely influenced by scoring the relative abundance of *Lactobacillus* spp. with typical cell morphology. It can be stated that while high numbers of lactobacilli corresponds to a "normal and healthy" microbiota, the converse is not necessarily true, that is low numbers of or no lactobacilli does not always correspond to an "unhealthy" ecosystem (Forney *et al*, 2006). Thus, BV is often over-diagnosed by Gram's staining. This could partly account for the reported high incidence of so-called asymptomatic BV in reproductive-age women (Eschenbach et al, 1988; Sobel 2000) and could also explain a proportion of BV treatment failures and apparent recurrences of BV in women (Hillier and Holmes, 1990; Schwebke and Desmond, 2007).

2.4 Complications and sequelae

BV may lead to potentially severe complications and sequelae. A large body of literature confirms that BV is associated with considerable gynecologic and obstetric disease (Table 3). Several studies have demonstrated an increased risk of abortion in the first trimester and late miscarriage, premature ruptures of the membranes, chorioamnionitis, and preterm birth in women with BV (Azargoon and Darvishzadeh, 2006; Gibbs, 1993; Hay *et al*, 1994; Hillier *et al*, 1995; Jacobsson *et al*, 2002; Llahi-Camp *et al*, 1996; Ralph *et al*, 1999). A causal relationship has been established also between BV and pelvic inflammatory disease (PID), plasma cell endometritis, postpartum fever, post-hysterectomy vaginal-cuff cellulitis, and post-abortion infection (MacDermott, 1995; Ness *et al*, 2005; Oleen-Burket and Hillier, 1995). Moreover, epidemiologic

studies have demonstrated that BV is associated with a markedly increased risk for acquisition (Cherpes *et al*, 2003; Martin *et al*, 1999; Peters *et al*, 2000; Wiesenfeld *et al*, 2003) and transmission (Cherpes *et al*, 2005; Cohn *et al*, 2005; Coleman *et al*, 2007; Cu-Uvin *et al*, 2001; Sha *et al*, 2005) of sexually transmitted infections, including HIV (Atashili *et al*, 2008; Cu-Uvin *et al*, 2001; Martin *et al*, 1999).

2.4.1. Preterm birth and chorioamnionitis

The association between BV and preterm birth has been confirmed repeatedly and consistently, even if the role of BV itself in the pathogenesis of preterm labor and delivery is not well understood (McGregor and French, 2000). A recent meta-analysis study concluded that BV more than doubled the risk of preterm delivery in asymptomatic patients and of preterm labor in patients with symptoms, and also significantly increased the risk of late miscarriages and maternal infection (Leitich and Kiss, 2007).

A possible explanation to this association involves alterations in the host defense mechanism that leads to chorioamnionitis. Chorioamnionitis refers to inflammation of the fetal membranes and placental chorion most typically due to an ascending bacterial infection. It is believed that vaginal organisms first invade the choriodecidual space (between the maternal tissues and the fetal membranes) and then infect the amniotic fluid by crossing intact chorioamniotic membranes (Goldenberg *et al*, 2000). The presence of microbes in the chorioamnion generates a maternal and, in some cases, a fetal inflammatory response characterized by the release of pro-inflammatory cytokines and chemokines (Hillier *et al*, 1993-b) that may lead to cervical ripening, membrane injury, labor at term or premature birth at earlier gestational ages (Tita and Andrews, 2010). Intrauterine infection may result from a chronic infection early in pregnancy that eventually becomes symptomatic with contractions and leads to spontaneous abortion or preterm birth (Goldenberg *et al*, 2000). This hypothesis is supported by the results of two studies, which confirmed that BV in early pregnancy may be a stronger risk factor for preterm delivery (Hay *et al*, 1994; Kurki *et al*, 1992).

In most cases of preterm labor and delivery, intrauterine infection is not clinically apparent. This might be because many hospital laboratories still use the traditional culturing methods for detecting bacterial infections (Han *et al*, 2009). When molecular methods has been used, then bacteria has been found in the membranes of up to 70% of women undergoing elective caesarean section at term (Steel *et al*, 2005). Since these were not cases of preterm birth, these findings

suggest that the presence of bacteria in the chorioamnion alone is not always sufficient to cause an inflammatory response. It seems that after entering the amniotic cavity, the bacterial numbers must

Table 3. Bacterial vaginosis and adverse pregnancy outcome: patients, methods and results of individual studies (Donati *et al*, 2010). Modified from Leitich and Kiss, 2007.

Study	Patient inclusion Criteria	Diagnosis of BV	Mean gestational age at BV screening (weeks)	Outcome	Patients with BV	Patients without BV	OR (95%CI)
Andrews et al. [71] (RCT)	Singleton pregnancy at 21–25 weeks and positive fetal fibronectin test	Gram stain (Nungent score ≥7)	23.7	Delivery <37 weeks	15/99	23/217	1.51 (0.75–3.03)
Daskalakis et al. [72]	Singleton pregnancy at 22–25 weeks and no previous preterm delivery	Gram stain (Nungent score ≥7) and vaginal pH >4.5	23.5	Delivery <37 weeks	16/95	88/1102	2.33 (1.31–4.17)
De Seta et al. [73]	Singleton pregnancy at 13–18 weeks	Gram stain (Nungent score ≥7)	15.5	Delivery <37 weeks	14/95	35/503	2.31 (1.19–4.49)
Edwards et al. [67]	Singleton pregnancy at 23–32 weeks and preterm labor	Gram stain (Nungent score ≥7)	28.3	Delivery <37 weeks	9/23	25/105	2.06 (0.80–5.32)
Genc et al. [74]	Singleton pregnancy at 18–22 weeks and no previous preterm delivery <24 weeks	Gram stain (Nungent score ≥7)	20.0	Delivery <37 weeks	4/30	19/177	1.28 (0.40–4.06)
Goffinet et al. [68]	Pregnancy at 24–34 weeks and preterm labor	Gram stain (Nungent score ≥ 7)	29.0	Delivery <35 weeks	6/24	71/330	1.22 (0.47–3.18)
				Delivery <33 weeks	6/24	41/330	2.35 (0.88–6.26)
				Chorioamnionitis	1/24	7/330	2.01 (0.24–17.01)
				Neonatal infection	2/24	19/330	1.49 (0.33–6.80)
Goyal et al. [75]	Pregnancy at 23–36 weeks and preterm labor	Gram stain (Nungent score ≥7)	30.2	Delivery <37 weeks	18/19	30/41	6.60 (0.79–55.48)
Guerra et al. [69]	Singleton pregnancy at <10 weeks and ≥1 previous preterm delivery	Gram stain (Nungent score ≥7)	8.5	Delivery <37 weeks	33/72	45/138	1.75 (0.97–3.14)
			25.0	Late miscarriage ≤25 weeks	23/95	9/147	4.90 (2.15–11.14)
				Delivery <37 weeks	12/36	60/168	0.90 (0.42–1.93)
Kalinka et al. [31]	Singleton pregnancy at 8–16 weeks	Gram stain (Nungent score ≥7)	12.3	Delivery <37 weeks	9/55	14/141	1.77 (0.72–4.38)
Kalinka et al. [76]	Singleton pregnancy at 22–34 weeks	Gram stain (Nungent score ≥7)	29.0	Delivery <37 weeks	5/31	10/83	1.40 (0.44–4.49)
Kiss et al. [77] (RCT)	Singleton pregnancy at 15–19 weeks	Gram stain (Nungent score ≥7)	17.0	Delivery <37 weeks	10/179	116/1918	0.92 (0.47–1.79)
Oakeshott et al. [70]	Singleton pregnancy at <10 weeks	Gram stain (Nungent score ≥7)	7.0	Delivery <37 weeks	7/112	47/785	1.05 (0.46–2.38)
				Late miscarriage at 13–23 weeks	5/117	10/795	3.50 (1.18–10.44)
Purwar et al. [78]	Singleton pregnancy at 12–28 weeks	Gram stain (Nungent score ≥7)	25.5	Delivery <37 weeks	32/115	40/823	7.55 (4.50–12.66)
Thorsen et al. [79]	Pregnancy at <24 weeks	Gram stain (Nungent score ≥7)	17.0	Delivery <37 weeks	13/401	99/2526	0.82 (0.46–1.48)
				Delivery <34 weeks	7/401	39/2526	1.13 (0.50–2.55)
				Delivery <32 weeks	2/401	22/2526	0.57 (0.13–2.44)

breach some threshold to trigger an intra-amniotic inflammatory response, which in turn induces preterm labor (Romero *et al*, 2002). *U. urealyticum*, *Fusobacterium* spp., and *M. hominis* are the bacterial species most commonly isolated from the amniotic cavity of women with preterm labor (Gerber *et al*, 2003; Perni *et al*, 2004). Other microorganisms found in the amniotic fluid include *Streptococcus agalactiae*, *Peptostreptococcus* spp., *Staphylococcus aureus*, *G. vaginalis*, *Streptococcus viridians*, and *Bacteroides* spp. (Gardella *et al*, 2004; Hitti *et al*, 1997; Oyarzun *et al*, 1998). While some of these bacteria have been associated with skin, fecal, and gut microbiota, most are related to those found in the human "abnormal" vagina, which can be a potential source of infecting organisms (Goldenberg *et al*, 2008).

2.4.2. Other gynecologic complications

Another important complication of BV is spontaneous abortion. Three studies analyzed the risk of spontaneous abortion in women with BV and reported that the risk was significant and was nearly 10-fold in women with BV. However, more evidence is needed to study this association.

Vaginal anaerobic microflora has also been incriminated in pelvic inflammatory disease (PID), especially in the absence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Hillier and Holmes, 1999). The relationship of pelvic inflammatory disease to BV has to be established, but it has been postulated an association with endometritis, which, in turn, is associated with PID (Hillier *et al*, 1996; Korn *et al*, 1998; Wiesenfeld *et al*, 2002). Moreover, vaginal BV-associated anaerobes, such as *Prevotella* spp. and *Peptostreptococcus* spp. were found in fallopian tube tissue or on endometrial biopsy of patients with acute PID (Soper *et al*, 1994).

Numerous studies have shown an association of BV with mucopurulent endocervicitis (Moi, 1990; Paavonen *et al*, 1986), and with inflammatory changes noted on cervical cytology (Eltabbakh *et al*, 1995). Postoperative infections, including post-abortion PID, post-hysterectomy cuff cellulitis, and post-Caesarian endomyometritis, have been shown to be associated with asymptomatic BV (Soper *et al*, 1990). Moreover, preoperative antibiotic prophylaxis that covers BV-associated microflora can reduce these complications (Penney *et al*, 1998).

2.4.3. Acquisition of sexually transmitted infections

Numerous longitudinal studies have demonstrated that BV-associated vaginal microbiota is related with increased incidence of STIs (Brotman *et al*, 2010; Cherpes *et al*, 2003; Martin *et al*, 1999; Peipert *et al*, 2008). BV diagnosed by Nugent score increases the risk of infections by *Chlamydia trachomatis, Neisseria gonorrhoeae*, *Trichomonas vaginalis*, HIV, HSV, and Human

papillomavirus (Brotman *et al*, 2010; Cherpes *et al*, 2003; Watts *et al*, 2005). Both ulcerative (Syphilis, Cancroid, and herpes) and non-ulcerative (Gonorrhea and Chlamydia) STIs have been associated with higher rates of transmission and acquisition of HIV (Laga *et al*, 1993; Wasserheit, 1992). In the largest longitudinal cohort study to evaluate vaginal microbiota as a risk factor for incidence of STIs, the NIH Longitudinal Study of Vaginal Flora (LSVF) study team found that intermediate and high Nugent scores were associated with a 1.5- to 2-fold increased risk for incident trichomonal, gonococcal, and/or chlamydial infection (Brotman *et al*, 2010). The LSVF study is consistent with other longitudinal studies of vaginal microbiota and STI risk (Cherpes *et al*, 2003; Martin *et al*, 1999).

A large randomized trial of home screening of BV to prevent acquisition of STIs is ongoing and seeks to determine whether restoring the vaginal microbiota to a stable, low pH, high lactic acid and/or *Lactobacillus*-dominated state may help prevent acquisition of STIs upon exposure (NIAID, 2011). Only a few molecular studies and, to date, no longitudinal molecular studies have investigated the interaction between vaginal microbiota and STIs (Spear *et al*, 2008). The strong interaction between vaginal microbiota and acquisition of STIs has already been highlighted, while the details of how the vaginal ecosystem provides protection against STI is still an active area of research. The lack of lactic acid–producing bacteria in vaginal microbiota of women with BV in addition to the high pH and local cytokine production that accompany BV appear to be the major factors affecting STI risk (Hillier, 1998; Redondo-Lopez *et al*, 1990; Yudin *et al*, 2003). Information obtained from future longitudinal molecular studies may form the basis for new intervention strategies to prevent STIs based on the protective features of the vaginal microbiome.

2.4.4. BV and HIV

Various evidences suggest that BV may increase the risk of human immunodeficiency virus (HIV) acquisition and transmission. It is feasible that absence of H_2O_2-producing lactobacilli increases susceptibility to HIV as well as BV. Alternatively, BV per se may facilitate HIV transmission.

The magnitude of the association between BV and HIV has varied in epidemiological studies. Analyses of HIV incidence studies indicate that BV increases the risk of HIV acquisition by approximately 60%. In several populations of women studied, BV prevalence was higher, reaching rates as high as 70% (Atashili *et al*, 2008). Studies of HIV prevalence tended to find higher HIV prevalence in women with BV, but recent studies have also associated an increased risk of HIV acquisition in women with intermediate grades of vaginal microflora (van de Wijgert *et al*, 2009).

A disturbed vaginal ecology caused by BV creates a more permissive environment for acquiring HIV (Schmid *et al*, 2000; Schwebke, 2003; Sha *et al*, 2005; Watts *et al*, 2005). The depletion of H_2O_2-producing lactobacilli may reduce vaginal defense against microorganisms, including HIV (Hillier, 1998; Schmid *et al*, 2000), and increases vaginal pH, which makes the vaginal environment more favorable for HIV proliferation (Taha *et al*, 1998). High vaginal pH may increase the availability of vaginal HIV target cells (Hill and Anderson, 1992), the adherence and survival of HIV (Taha *et al*, 1998), and intravaginal levels of IL-10, making macrophages more susceptible to HIV (Cohen *et al*, 1999); on the contrary, the levels of secretory leukocyte protease inhibitor (SLPI) (Hillier *et al*, 2004), which has been shown to block HIV infection *in vitro* (Draper *et al*, 1998), are reduced in BV vaginal fluid. Furthermore, major BV-associated organisms, such as *G. vaginalis*, *P. bivia* and *Peptostreptococcus asaccharolyticus* directly upregulate HIV replication (Al-Harthi *et al*, 1999; Hashemi *et al*, 2000; Simoes *et al*, 2001; Zariffard *et al*, 2005). These changes, combined with the difficulties of successfully eradicating BV (Wilson, 2004), may explain the increased risk observed in most epidemiology studies.

Moreover, it has been hypothesized that some intravaginal practices, such as the use of cloth or paper to wipe out the vagina, the application of products intended to dry or tighten the vagina, or the use of soap to clean the vagina, could increase the risk of HIV infection by causing physical abrasions or by disrupting the vaginal epithelium and increasing the occurrence of BV (Hilber *et al*, 2007; Myer *et al*, 2005; Shattock and Moore, 2003).

If these associations are true, then treatment of BV has the potential to decrease the incidence of STIs significantly and could have a major impact in the HIV epidemic, especially in Africa and other locations where prevalent rates of BV are very high.

2.5 Antibiotic treatment

The currently recommended treatment regimes for BV are oral or vaginal metronidazole or vaginal clindamycin (ACOG Practice Bulletin, 2006). Treatment efficacy is supposed to be high (Joesoef and Schmid G, 2004; Kane and Pierce R, 2001), but many women remained colonized by BV-associated anaerobes after antibiotic therapy (Boris *et al*, 1997; Ferris *et al*, 1995). In clinical practice the efficacy is not more than 60% after 4 weeks (Larsson and Forsum, 2005), with a recurrence rate of 30–40% (Bannatyne and Smith, 1998; Colli *et al*, 1997; Eriksson *et al*, 2005; Paavonen *et al*, 2000). Reasons for recurrence are unclear and include the possibility of reinfection, but it more likely reflects vaginal relapse with failure to eradicate the offending organisms and/or

failure of the normal protective *Lactobacillus*-dominant vaginal microflora to reestablish itself (Sobel, 2000).

Lots of studies focused on the evaluation of the success of different antibiotic treatment, but often the results were not comparable because the approaches have varied widely from study to study (Joesoef and Schmid G, 1995; Sobel, 1997). The major differences among diverse studies regarded the diagnostic criteria for BV before or after treatment, the inclusion and exclusion criteria, the time of follow-up evaluation after treatment, the design of the study, the dose and duration of antibiotic therapy, and the study populations.

2.5.1. Metronidazole and clindamycin

The most established method of treating BV is with oral metronidazole. It is employed in a number of different therapy regimens most commonly with 400 or 500 mg twice daily for 7 days. Alternatively, topical therapy with 2% clindamycin once daily for 7 days or 0.75% metronidazole gel once daily for 5 days has been shown to be as effective as oral metronidazole (Ferris *et al*, 1995). Two studies compared metronidazole vaginal gel and clindamycin efficacy (Beigi *et al*, 2004-a; Ferris *et al*, 1995). One of them considered a follow-up time of 35–45 days after start of treatment and reported a resolution of BV of only 49% *vs* 48% for treatment with metronidazole vaginal gel and clindamycin ovules, respectively (Beigi *et al*, 2004-a). Management of acute BV symptoms during relapse includes oral or vaginal metronidazole or clindamycin, usually prescribed for a longer treatment period (10–14 days). However, maintenance antibiotic regimens have had largely disappointing results, and new approaches include exogenous *Lactobacillus* re-colonization using suppositories that contain selected bacteria (Sobel, 2000).

Although both antibiotics have been recommended for the treatment of symptomatic BV, their antimicrobial spectrum is not identical. Clindamycin and other macrolides have a broader antimicrobial activity against organisms involved in BV, including *Mobiluncus* spp. and *Mycoplasma* spp. (Barry *et al*, 1987; Spiegel, 1987; Spiegel *et al*, 1983-b). Importantly, these antibiotics have anti-inflammatory properties (Esterly *et al*, 1978; Konno *et al*, 1994; Mikasa *et al*, 1992). A study, reported that patients with the highest Nugent score (7-10), in which *Mobiluncus* spp. is frequently found, respond better to clindamycin than those who had intermediate flora (Nugent score 4-6) (Lamont *et al*, 2003; Rosenstein *et al*, 1996). *In vitro* studies have demonstrated that metronidazole and other nitroimidazoles are largely inactive against *G. vaginalis*, *M. hominis*, *U. urealyticum*, and *A. vaginae* (Ferris *et al*, 2004; Goldstein *et al*, 2002; Xiao *et al*, 2006). Yet, metronidazole administration to women with symptomatic BV is associated with a treatment success rate similar to clindamycin (Centers for Disease Control and Prevention, 2007). This has

been attributed to the activity of the hydroxy metabolite of the drug *in vivo*, which is effective against the organisms involved in BV. Alternatively, metronidazole may change the microbial ecosystem by eradicating bacteria susceptible to it; this elicits the lack of nutritional and metabolic interactions typical of the BV-associated bacteria, and favors cure of BV (Bradshaw *et al*, 2006-a; Pybus and Onderdonk, 1999). A recent study found that treatment of BV with clindamycin ovules is associated with significant development of antimicrobial resistance of anaerobic isolates. The anaerobic isolates had 17% baseline resistance to clindamycin, which increased to 53% after treatment, while less than 1% of the anaerobic isolates had resistance to metronidazole (Beigi *et al*, 2004-a). Thus, clindamycin treatment is questionable from a bacterial ecology point of view and metronidazole remains the preferred treatment.

2.5.2. BV treatment during pregnancy

BV is associated with late miscarriage and preterm delivery, but the mechanisms involved are not yet fully understood. Clinical trials of antibiotic therapy to reduce these complications have yielded conflicting results. However, these trials were conducted in mixed populations of pregnant women with variable risk profiles for preterm delivery, and investigators used different criteria for diagnosis, treated with different antibiotics at different doses and via different routes, and initiated treatment at different gestational ages. Due to the heterogeneity of the studies, the results of various systematic reviews were limited.

In general, the results of trials to treat BV in pregnancy have not proved to be effective in reducing the incidence of preterm birth; for example, treatment with intravaginal therapy with 2% clindamycin increased the incidence of preterm delivery, possibly because of an anti-*Lactobacillus* effect (Koumans *et al*, 2002). In an review of antibiotics for treating BV in pregnancy it is reported that 13 high-quality trials involving 5300 women found that antibiotic treatment was effective in eradicating BV in pregnancy but it was not significant in reducing the risk of preterm birth, reducing the risk of pPROM, or decreasing the risk of subsequent preterm birth in women with a previous preterm birth. Only in women with a previous preterm birth the use of antibiotics was associated with decreased risk of pPROM and low birth weight (McDonald *et al*, 2005).

BV is present in as many as 15 to 20% of pregnant women; however, most of them do not experience preterm birth or postpartum infectious complications. A higher relative risk for preterm delivery is found among women with BV early in pregnancy and it is associated with early miscarriage in the first trimester; thus it has been proposed that priority should be given to studies that examine screening and treatment strategies before pregnancy or in early pregnancy (Koumans and Kendrick, 2001). The importance of the timing of antibiotic administration has recently become

more apparent because there is evidence that exposure to either bacterial products or bacteria itself, may predispose to a subsequent viral infection, and this, in turn, leads to both preterm labor and fetal damage (Cardenas *et al*, 2010). Antimicrobials must be used early enough so that eradication of the microorganisms would be followed by resolution of any inflammatory response (Cauci *et al*, 2003; Donders *et al*, 2002; Donders *et al*, 2003) and its unintended consequences (Gomez *et al*, 1998; Gotsch *et al*, 2007; Kim *et al*, 2009).

2.6 Probiotics in the treatment of BV

2.6.1. Definition and general features

Probiotics are dietary supplements containing potentially beneficial microorganisms. According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). The most common type of microorganisms used are usually lactic acid producers, in particular strains of the genera *Lactobacillus* and *Bifidobacterium* (Tannock, 2005).

The rationale for the use of probiotics is based on the gastrointestinal and genitourinary regulatory role played by the commensal microflora and the need for restoration of this microbial ecosystem after insult. In particular, probiotics were initially developed as dietary supplements to assist the gut microbiota to re-establish themselves after such detrimental events.

An effective probiotic should have the following features:

- exert a beneficial effect on the host;
- be non-pathogenic and non-toxic;
- contain a large number of viable cells;
- be capable of surviving and metabolizing in the gut;
- remain viable during storage and use;
- have good sensory properties;
- be isolated from the same species as its intended host.

Health advantages associated with probiotic intake are the following:

- alleviation of symptoms of lactose intolerance;
- increase in natural resistance to infectious and inflammatory diseases of the gastrointestinal tract, such as *Helicobacter pylori* infections, acute gastroenteritis, antibiotic-associated

diarrhea, travelers' diarrhea, irritable bowel syndrome, inflammatory bowel disease, pouchitis, diverticular disease;

- suppression of cancer;
- reduction in serum cholesterol concentrations;
- improved digestion;
- stimulation of gastrointestinal immunity (Fuller, 1992; Gibson and Roberfroid, 1995; Gonzalez *et al*, 1995; Saavedra, 1995).

The mechanisms involved may include the following:

- a reduced gut pH through stimulation of the lactic acid–producing microflora (Langhendries *et al*, 1995);
- direct antagonistic effects on pathogens (De Vuyst and Vandamme, 1994; Gibson and Wang, 1994);
- competition for binding and receptor sites that pathogens may occupy (Fujiwara *et al*, 1997);
- improved immune function and stimulation of appropriate immunemodulatory cells (Isolauri *et al*, 1995);
- competition for available nutrients and other growth factors.

There is no published evidence that probiotic supplements are able to replace the body's natural microflora when these have been killed off; indeed bacterial levels in feces disappear within days when supplementation ceases. It is hoped, however, that probiotics do form beneficial temporary colonies which may assist the body in the same functions as the natural microbiota, while allowing the natural microflora time to recover from depletion. The probiotic strains are then thought to be progressively replaced by a naturally developed gut microbiota.

2.6.2. Probiotic lactobacilli in the vaginal ecosystem

The well-known physiological role played by a normal and stable *Lactobacillus*-dominated microbiota in preventing vaginal infections have raised interest for the potential of bacteriotherapy with probiotic lactic bacteria in the setting of urogenital infections. The rationale for urogenital probiotics comes from the belief that replenishing the normal microbes will counteract pathogens and lead to a return to the *Lactobacillus*-dominated state found in healthy women.

In the development of a probiotic product, several properties of the candidate *Lactobacillus* strains should be considered, in order to assure the maintenance of a pathogen-free vaginal environment. The characteristics needed for a *Lactobacillus* strain to serve effectively as a probiotic

include avid adherence to epithelial cells, interference with the adhesion of pathogens, and production of antimicrobial molecules capable of inhibiting the growth of pathogens (Mastromarino *et al*, 2002). The adherence in an essential factor for the antimicrobial activity of lactobacilli (Reid and Sobel, 1987), as it establishes the ability of lactobacilli to colonize the vaginal mucosa (McLean and Rosenstein, 2000). It has been suggested to be the result of specific and non-specific binding mechanisms, with the latter involving electrostatic or hydrophobic interactions. Thus, cell surface charge and hydrophobicity influence the strength of adhesion. In particular, the reduction in surface negative electric charge promotes the attachment of several lactobacilli to the cell membrane. *In vitro* evidence indicates that different *Lactobacillus* strains have varying degrees of adhesion to vaginal epithelial cells, with the greatest adhesion among specific strains of *L. gasseri*, *L. brevis* and *L. acidophilus* (Mastromarino *et al*, 2002; McLean and Rosenstein, 2000). Another important feature can be the coaggregation activity, that is the capability of a probiotic strain to bind a pathogen to block adhesion and/or displace previously adherent pathogens on vaginal epithelial cells. The coaggregation could be an important factor in establishing and maintaining a healthy ecosystem because of the production of a microenvironment around the pathogen where the concentration of inhibiting substances produced by lactobacilli is exacerbated. Finally, for use as probiotics in genitourinary tract, lactobacilli must exhibit adequate antibacterial activity. The most relevant property in this context is the ability to maintain a vaginal pH ≤ 4.5. This ability depends particularly on the number of lactobacilli present to produce lactic acid, re-emphasizing the importance of colonization. An acidic vaginal environment is conducive to replication of lactobacilli and subsequent production of additional antibacterial substances, including bacteriocin and hydrogen peroxide. However, different strains of lactobacilli produce varying amounts of these substances (Aroutcheva *et al*, 2001-a). In addition, lactobacilli may offer protection against infections through production of biosurfactants. These substances discourage the growth of uropathogens by inhibiting adhesion of microorganisms along uroepithelial cells (Velraeds *et al*, 1996). The contribution of these individual antibacterial properties to clinical efficacy is unclear, as the properties vary by *Lactobacillus* strain and by the susceptibility of specific pathogens. Only randomized controlled trials can establish the role of lactobacilli in the treatment and prevention of bacterial urogenital infections.

2.6.3. Probiotic lactobacilli against BV

Probiotic preparations containing lactobacilli represent the most common alternative therapy used by women with BV (Nyirjesy *et al*, 1997).

The first example of vaginal probiotics use was that of Stanley Thomas in 1928 following his observation that lactobacilli were absent in the presence of gonococci. He reported on two experiments, one *in vitro* and the other *in vivo*, in which the addition of a thin layer of whey broth from a culture of *L. acidophilus* demonstrated the eradication of *Neisseria gonorrhoeae* (Thomas, 1928). Exogenous strains of lactobacilli have then been suggested as a means of establishing or re-establishing normal vaginal microflora. Of the strains that showed the most promise, a combination of two, *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 were developed in oral and intravaginal formulations. Numerous studies have reported the different attributes of these two organisms, with several recent studies further illustrating clinical effectiveness and potential application to prevent preterm labor (Reid *et al*, 2009). Use of oral formulations of these strains has been associated with normalization of vaginal lactobacilli within 28 to 60 days after the initiation of treatment (Reid and Burton, 2002; Reid *et al*, 2003). Evidence of fecal and vaginal colonization by these strains was observed after 14 days of oral administration (McLean and Rosenstein, 2000). With vaginal formulations, local colonization by these strains was noted after 3 days and continued to be evident at 12 days (Gardiner *et al*, 2002; Reid *et al*, 2001). Other clinical trials presented significant variations in the study design and reported different outcomes. Three randomized controlled trials reported enhanced cure rates or a reduced recurrence of BV among premenopausal women treated with oral (capsules or yogurt) lactobacilli (Anukam *et al*, 2006-a; Neri *et al*, 1993; Shalev *et al*, 1996). On the contrary, other four randomized controlled trials found no beneficial effect of probiotics in the treatment of BV (Anukam *et al*, 2006-c; Eriksson *et al*, 2005; Fredricsson *et al*, 1989; Hallén *et al*, 1992). The results do not provide sufficient evidence for or against recommending probiotics for the treatment of BV. Further clinical trials, including larger samples of women with BV, in which lactobacilli are compared either with a placebo or metronidazole, need to be conducted before it will be possible to reach definitive conclusions as to whether probiotics represent an effective and safe method for treating women with BV.

Alternatively research might be focused on the predominant lactobacilli strains inhabiting the vagina, *L. crispatus* and *L. jensenii*. These species intrinsically meet two requirements for use in successful probiotic therapy: they are endogenous vaginal lactobacilli, and they adhere to vaginal epithelial cells. In addition, they are known to be good producer of H_2O_2. Strains of probiotic *L. crispatus* CTV-05 have been demonstrated to have high mean adherence to vaginal epithelial cells *in vitro*, and have established vaginal colonization in seven out of nine women when administered vaginally. Combined vaginal and rectal colonization by H_2O_2-producing lactobacilli is associated with a four-fold decrease in the incidence of BV (Antonio *et al*, 2005; Antonio and Hillier, 2003).

Vaginal colonization of women with endogenous species may be advantageous in the maintenance of a normal microflora and the prevention of sexually transmitted diseases.

2.6.4. VSL#3

VSL#3 is a probiotic preparation characterized by a very high bacterial concentration (300 billion/g), consisting of eight strains of viable lyophilized probiotic bacteria: four strains of *Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*B. longum*, *B. breve*, *B. infantis*) and one strain of *Streptococcus salivarius* subsp. *thermophilus*. Rationale for the use of a cocktail containing large numbers of different strains has been the concept of high efficacy through a synergistic action of the different strains in the mixture.

Numerous experimental studies have been exerted in the attempt to unravel the precise mechanisms by which probiotic bacteria in VSL#3 and their metabolic products (short-chain fatty acids, vitamins) exert their beneficial effects. Data obtained from these studies indicate that the VSL#3 bacterial strains have immunomodulatory effects. Attenuation of severity of disease activity by means of improvement of histologic grading has been described in various animal models (Madsen *et al*, 2001). A decrease of neutrophil tissue influx and activity has also been shown (Shibolet *et al*, 2002). Further, a diminished pro-inflammatory IL-1β, TNF-α, IFN-γ, IL-12, and IL-8 cytokine production and an enhanced production of the anti-inflammatory cytokine IL-10 have been reported (Hart *et al*, 2004; Jijon *et al*, 2004; Lammers *et al*, 2002; Lammers *et al*, 2003; Madsen *et al*, 2001; Otte and Podolsky, 2004; Ulisse *et al*, 2001). Coincubation of probiotic bacteria with pro-inflammatory stimuli or pathogenic bacteria revealed that probiotic bacteria inhibited the pro-inflammatory cytokine production induced by these inflammatory stimuli, and further, that probiotics prevented *Salmonella*-induced damage of the intestinal barrier integrity (Lammers *et al*, 2002; Otte and Podolsky, 2004). Maintenance of the epithelial barrier function is necessary for preserving mucosal integrity. Impaired barrier function and increased permeability are observed in gastrointestinal diseases (Schmitz *et al*, 1999). Therefore, reinforcement of the barrier function, together with immunomodulatory and metabolic properties, may be central in the mechanism of action of probiotic bacteria. The exact mechanisms by which probiotics can influence barrier function remain to be elucidated. It is known that certain lactobacilli adhere to mucosal surfaces, inhibit the attachment of pathogenic bacteria, and enhance the secretion of mucins, improving the mucosal barrier function and decreasing the permeability to macromolecules and toxins (Bernet *et al*, 1994; Mack *et al*, 1999; Willemsen *et al*, 2003). Recently it was shown that

VSL#3 probiotic bacteria reinforce the barrier function by secreting soluble factors that enhance the barrier integrity and by regulating tight junctions (Madsen *et al*, 2001; Otte and Podolsky, 2004).

VSL#3 has been widely investigated for inflammatory and functional bowel disorders. In particular, clinical trials have been developed to assess the efficacy of VSL#3 in the maintenance treatment of patients with ulcerative colitis in remission (Venturi *et al*, 1999), in the maintenance treatment of patients with chronic pouchitis (Gionchetti *et al*, 2000), in the maintenance of antibiotic-induced remission in patients with refractory or recurrent pouchitis (Mimura *et al*, 2004), in the prevention of pouchitis onset in patients operated (Lammers *et al*, 2005), and in the prevention of post-operative recurrence of Crohn's disease (Gionchetti *et al*, 2003). In general, encouraging results have been obtained from these studies. More than one study reported that fecal concentrations of lactobacilli, bifidobacteria and *S. thermophilus* increased significantly in all patients and persisted through the treatment period. Within a few days after the end of the treatment, bacterial concentrations of lactobacilli, bifidobacteria and *S. thermophilus* turned to basal levels, whereas no modification of fecal concentrations of *Bacteroides*, enterococci, coliforms, clostridia and total anaerobes and aerobes was observed (Gionchetti *et al*, 2000; Venturi *et al*, 1999). These data suggest that the effect was not mediated by suppression of endogenous luminal bacteria.

The ability of several bacterial species to colonize both the gastrointestinal tract and the reproductive tract has been demonstrated in several studies (Antonio *et al*, 2005; El Aila *et al*, 2009; Morelli *et al*, 2004). Thus, it has been suggested that a healthy gut microbiota contributes to protecting against female genitourinary tract infections and that imbalance in the intestinal microbiota favors the suppression of lactobacilli, which in turn leads to overgrowth of anaerobes in the vagina (Petricevic *et al*, 2012). In the light of such assumption, oral probiotic products primarily designed for the treatment of gastrointestinal disorders, such as VSL#3, could be used for the prevention of vaginal tract imbalances and their sequelae.

3. Microbial ecology: molecular culture-independent approaches

3.1 From cultivation to molecular techniques

The characterization of the community composition is the first step in the study of a complex bacterial ecosystem. The microbial inhabitants of the female genital tract and the contribution of these organisms to health and disease have been investigated for well over a century, yet they remain incompletely understood.

The earliest studies focused on the microbiology of the lower genital tract and relied upon growth of bacteria in rich medium and identification based on observable characteristics, such as morphology, Gram stain, and arrangement of cells. These investigations led to important insights including the identification of lactobacilli as the predominant members of the vaginal ecosystem in most women, and to the hypothesis that these organisms might serve protective functions at the vaginal mucosal surface. However, only a fraction of microbes can be cultured in the laboratory even with the most modern bacteriological techniques, which resulted inadequate to describe the true complexity of the vaginal microbiota accurately. In fact, it is now recognized that < 20% of the organisms comprising the human microbiome may be cultivatable (Dethlefsen *et al*, 2007). Additionally, culture-based methods are laborious, time-consuming, and prone to statistical and methodological errors. Thus, in the last decade many molecular tools have been developed, allowing faster and more accurate investigations of complex microbial ecosystems.

Cultivation-independent techniques bypass the need to grow microbes in culture and rely instead upon isolation of DNA from a given sample followed by techniques to identify individual microbial community members. The most efficient culture-independent strategies for exploring microbial biodiversity are based on the 16S ribosomal RNA (rRNA) gene sequence. The 16S rRNA gene (Figure 8) consists of about 1,500 nucleotides and contains regions highly conserved

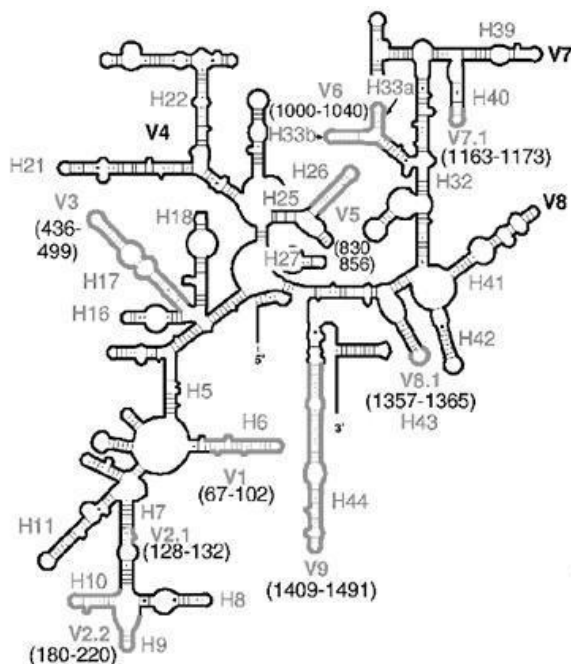

Figure 8. *E. coli* 16S rRNA secondary structure. Position of the 9 hypervariable regions (V1-V9) is indicated (Tannock, 1999).

among all the bacteria, interspersed with 9 hypervariable regions (V1-V9), that can allow identification of different bacterial phylotypes to the genus or species level (Tannock, 1999). Sequencing of the 16S rRNA genes has resulted in more than one million small subunit rRNA entries, which are available through databases, such as GeneBank, EMBL, Ribosomal Database Project (RDP).

Molecular studies of microbial ecosystems are based on nucleic acid extraction, amplification and sequencing of variable regions of the 16S rRNA gene using oligonucleotide primer sequences that complement the conserved regions of this gene. The polymerase chain reaction (PCR) amplicons represent a sample of all the bacteria present in a specimen, and they can be analyzed in several ways to assess community structure. Cloning and sequencing of 16S rRNA genes yields the most detailed data and gives significant information about the identity of uncultured bacteria, but is laborious, expensive and hardly quantitative. The so-called “fingerprinting techniques” are more appropriate and economical to study a complex bacterial community, although they are only semi-quantitative. Denaturing/temperature gradient gel electrophoresis (D/TGGE) and terminal-restriction fragment length polymorphism (T-RFLP) are the most important; they are used for monitoring community shifts, for example in response to a treatment, or comparing individuals. D/TGGE separates the PCR product on the base of sequence differences by subjecting them to the activity of a gradient of denaturing chemicals (DGGE) or to an increasing temperature (TGGE). T-RFLP digests the ampolicons with restriction enzymes, yielding patterns of fragments used for identification. Even if limited to genus-level identification of microbes, these studies provides a glimpse of the vast, unappreciated diversity of uncultivated bacteria present at the vaginal mucosal surface. Other culture-independent approaches frequently applied to characterize environmental samples are the real time-PCR and the Fluorescent In-Situ Hybridization (FISH). These are quantitative techniques that use specific 16S rRNA-targeted primers and probes, respectively. The disadvantage of these quantitative techniques resides in the fact that primers and probes can be designed and validated only for bacterial groups which are known, and whose 16S rRNA has been sequenced. Moreover, the study consists in one experiment for each probe or primer set.

16S-based studies have largely confirmed the broad conclusion of Döderlein, that lactobacilli are the dominant organisms in the vaginal tract of most healthy premenopausal women, but they have also refined that view significantly. The microbiota of the vagina has been revealed as considerably more dynamic and complex than previously suspected, with important implications for the health of women.

More recently, the microarrays technology has been applied to the study of the diversity of complex ecosystems. Diversity microarrays allow identification of bacterial species in unknown

samples. In the majority of the cases they are based on the 16S rRNA gene, but microarrays based on other functional genes (rpoB, recA, gyrB, groEL, and atpD) can be used to distinguish between closely related bacteria, having a resolution below the species level. Lots of efforts have been made to develop a diversity microarray specific for the intestinal tract (Paliy *et al*, 2009; Palmer *et al*, 2007; Rajilic-Stojanovic *et al*, 2009; Wang *et al*, 2002). As regards the analysis of vaginal microbiota, Dols *et al* recently developed a PCR-based microarray containing probes that represent BV-associated genera, to provide a more insightful diagnosis of this condition (Dols *et al*, 2011). The microarray technology, with its ability to detect and measure thousands of distinct sequences simultaneously, has been recognized as a valuable tool to explore and systematically characterize complex microbial communities. However, further efforts should be made to develop a microarray able to cover the complete diversity of the human vaginal microbiota in health and diseased states, in order to fully characterize this complex ecosystem.

3.1.1. PCR-DGGE

Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community; one of the most used is DGGE (Denaturing Gradient Gel Electrophoresis) (Muyzer and Smalla, 1998).

In DGGE DNA fragments of the same length but with different sequences can be separated (Fischer and Lerman, 1983). Separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). The melting of DNA fragments proceeds in discrete so-called “melting domains”, that is stretches of base-pairs with an identical melting temperature. Once a domain with the lowest melting temperature reaches its melting temperature (Tm) at a particular position in the denaturing gradient gel, a transition of a helical to a partially melted molecule occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel.

By using DGGE, 50% of the sequence variants can be detected in DNA fragments up to 500 bp (Myers *et al*, 1985). This percentage can be increased to nearly 100% by the attachment of a GC-rich sequence, a so-called GC-clamp, to one side of the DNA fragment (Myers *et al*, 1985; Sheffield *et al*, 1989). A sequence of G and C is added to the 5’end of one of the PCR primers, co-amplified and thus introduced into the amplified DNA fragments (Sheffield *et al*, 1989). The GC-rich sequence acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands. DNA bands in DGGE profiles can be visualized using ethidium

bromide. Alternatively, a more sensitive detection method is silver staining (Bassam *et al*, 1991), which permits to identify community members by sequencing of DNA eluted from excised DGGE bands.

Prior to DGGE analysis of DNA fragments it is necessary to determine the melting behavior of the DNA fragments. Furthermore, to obtain the best separation of different DNA fragments, it is necessary to optimize the gradient and the duration of electrophoresis. The melting behavior of DNA fragments, as well as the optimal gradient, can be determined experimentally with perpendicular gradient gels. These gels have an increasing gradient of denaturants from left to right, perpendicular to the direction of electrophoresis. The electrophoretic pattern will appear as a sigmoid-shaped curve: DNA molecules at the left side of the gel, where the concentration of denaturants is low, will migrate as double-stranded DNA; at the other side of the gel, where the concentration of denaturants is high, the molecules melt into branched molecules as soon as they enter the gel and therefore halt; at intermediate concentrations of denaturants, the molecules have different degrees of melting, and concomitantly different mobility. The optimal time of electrophoresis is determined by parallel gradient electrophoresis. Parallel gradient gels have an increasing gradient of denaturants from top to bottom, parallel to the direction of electrophoresis and they are used for analyzing multiple samples on the same gel. By using parallel DGGE many samples taken at different time intervals during the study can be simultaneously analyzed.

Limitations of the technique are represented by the possibility to separate only relatively small fragments, up to 500 bp (Myers *et al*, 1985) and the limited number of different DNA fragments which can be separated by DGGE. In general, this electrophoretic technique will only display the rDNA fragments obtained from the predominant species present in the community. Furthermore, co-migration of DNA fragments can be a problem for retrieving clean sequences from individual bands. Another problem is the presence in some bacteria of multiple rrn operons with sequence micro-heterogeneity, which might lead to an overestimation of the number of bacteria within natural communities. Nevertheless, substantial information about the species composition can be obtained from very complex microbial communities by DGGE analysis. It can give a direct display of the predominant constituents in microbial communities and are remarkably suited to investigate the temporal and spatial distribution of bacterial populations. This aspect is certainly the most important reason for the popularity of this technique in microbial ecological studies. Other aspects are its easiness, reproducibility, reliability, and speed (Muyzer and Smalla, 1998).

The use of DGGE might be especially interesting to answer questions on the behavior of microbial communities or certain “indicator microorganisms” after environmental perturbations in the vaginal ecosystem, such as the onset of a vaginal infection or a probiotic/antibiotic treatment.

3.1.2. Real-time PCR

Real-time PCR is a quantitative technique in which the amount of product formed during the amplification is monitored during the course of the reaction. By monitoring the fluorescence of dyes or probes introduced into the reaction, that is proportional to the amount of product formed, it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample. Typical uses of real-time PCR include pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, and phylogenetic analysis.

Differently from a traditional PCR reaction, where end-point measurements only distinguish a positive from a negative sample but tell nothing about the initial amounts of target molecules, real-time PCR allows the monitoring of the growth phase of the reaction, thanks to a fluorescent reporter that generates a fluorescence signal proportional to the amount of product formed. During the growth phase of the reaction response curves are separated on the basis of the difference in their initial amounts of template molecules. The difference is quantified by comparing the number of amplification cycles required for the samples' response curves to reach a particular threshold fluorescence signal level (CT). The amplification response curves are expected to be parallel in the growth phase of the reaction, and the setting of the threshold level is usually made manually. This does not affect significantly the differences between CT values, though it affects the values of the individual CTs. Thus, comparing individual CT values between experiments should be avoided, and one reference per run to which all the other response curves can be related should be included. Typically, a standard curve based on serial dilution of a standard, which can be a purified PCR product or a purified plasmid that contains the target sequence, is included. The CT values of the diluted standards are read out, and plotted versus the logarithm of the samples' concentrations, number of template copies or dilution factor.

Today fluorescence is exclusively used as the detection method in real-time PCR. Both sequence specific probes and non-specific labels are available as reporters. One of the most popular dyes is the intercalator SYBR Green I (Zipper *et al*, 2004). This dye has virtually no fluorescence when it is free in solution, but it becomes brightly fluorescent when it binds to double-stranded DNA, presumably to the minor groove. In real-time PCR the fluorescence of SYBR Green I increases with the amount of double-stranded product formed, hence, the dye is excellent for quantitative PCR when samples are compared at the same level of fluorescence in absence of interfering DNA. Although minor groove binding dyes show preference for runs of AT base-pairs, SYBR Green I is considered sequence non-specific reporters in real-time PCR. It gives rise to fluorescence signal in the presence of any double stranded DNA including undesired primer–dimer

and non-specific products. It is therefore good practice to control for primer–dimer formation or non-specific amplification. This can be done by melting curve analysis after completing the PCR. The temperature is gradually increased and the fluorescence is measured as function of temperature. The fluorescence decreases gradually with increasing temperature and, when the temperature is reached at which the double-stranded DNA strands separate, the dye comes off and the fluorescence drops abruptly (Ririe *et al*, 1997).

Other kinds of reporter are labeled primers and probes based on nucleic acids or some of their synthetic analogues (Costa *et al*, 2004; Egholm *et al*, 1992). The dye labels are of two kinds: fluorophores with intrinsically strong fluorescence, such as fluorescein and rhodamine derivatives, which through structural design are brought into contact with a quencher molecule, and fluorophores that change their fluorescence properties upon binding nucleic acids. Examples of probes with two dyes are the hydrolysis probes, popularly called Taqman probes (Holland *et al*, 1991). The dyes form a donor–acceptor pair, where the donor dye is excited and transfers its energy to the acceptor molecule if it is in proximity. Energy transfer and quenching are distance-dependent and structural rearrangement of the probe, or, in the case of hydrolysis probes, degradation, change the distance between the donor and acceptor and, hence, the fluorescence of the system.

Real-time PCR is a very useful technique, as it is rapid and reliable, and allows the specific quantification of targeted groups of bacteria. However, primers and probes can be designed only for bacterial groups which are covered by the reference sequences. Differently from DGGE and the other fingerprinting techniques, that can characterize complex bacterial communities, real-time PCR yields more complete information on a limited number of selected bacteria.

3.2 Pyrosequencing: a deep sequencing approach

The molecular techniques used to characterize complex ecosystems were limited in that the number of sequences that could be reasonably analyzed by traditional Sanger sequencing is far too low to enable a complete understanding of a microbial community structure. Even if diversity microarrays are very straightforward techniques to use for comparative community profiling, faster and cheaper than high-coverage sequencing, they can only detect taxa that are covered by the reference sequences. What microbiologists needed was a “high-throughput” sequencing technique that could provide thousands of sequences per specimen at a manageable cost.

In recent years, next generation sequencing technologies have been developed, allowing the massive sequencing of a vast numbers of (partial) 16S rRNA genes from many complex bacterial ecosystems, at much lower cost than Sanger's capillary electrophoresis method (Claesson *et al*,

2009). Pyrosequencing is a sequencing-by-synthesis method, based on the detection of the pyrophosphate release occurring at the nucleotide incorporation during the sample amplification. This technique produces a shorter sequence length than the amplification and cloning approaches described earlier, but these shorter sequences are sufficient for identifying bacteria at least at the genus level in most cases. The number of reads available enables characterization of individual patient's microbiota to the extent that there is reasonable confidence that the true structure of each individual community can be analyzed and compared with the microbiota of other individuals.

The method first adapted to this purpose was pyrosequencing using the 454 FLX Sequencing System, in which hundreds of thousands of beads, each carrying millions of copies of a unique single strand DNA molecules, are sequenced in parallel (Spear *et al*, 2008). The availability of computational analysis programs to analyze large numbers of sequences and the drop in the cost of high-throughput sequencing, allowed the application of pyrosequencing to many life science fields. A key innovation, currently used in comparative studies of microbial communities, is multiplexing. In the so-called "barcoded pyrosequencing" each sample is tagged with a unique molecular barcode (a short key sequence added during PCR) and can be sequenced together with other barcoded samples in the same run (Hamady and Knight, 2009). Using this approach, Ravel *et al* recently reported the results of a pyrosequencing analysis of vaginal specimens from a sample of 400 healthy women (Ravel *et al*, 2011).

3.3 The "metagenomic" era

Despite the advances in understanding the composition of microbial ecosystems brought about by pyrosequencing analyses of 16S rRNA sequences, the focus of research in this field may be shifting, as experts are suggesting the need for moving away from simply describing the microbiota to determining what the organisms are actually doing. One way to gain insight into potential functions and activities of microbes without the need of cultivation is the metagenomic approach.

The term metagenomics was first used in the late 1990s, and was defined as the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms without the necessity for culturing. The availability of "next-generation" sequencing technologies have made it such that a cloning step is no longer essential for metagenomic projects and now the total microbial community or microbiome (the number and relative abundance of microbial species present) and their metagenomes (the microbial functional content) can be rapidly defined. Metagenomic studies using shotgun sequencing approaches are

being used to identify most of the bacterial genes present in the microbiota at a particular site at a specific time. Such data permit the determination of metabolic pathways most common in a chosen environment. Moreover, direct measurement of metabolites in samples is possible. An additional approach to discovering what the microbiota is doing is transcriptomics, which measures the specific mRNAs being produced by the microbiota. Ultimately, proteomic approaches can be used to identify and measure the gene products actually being produced by the microbiota (Figure 9). The application of all these meta-"omics" approaches will provide an understanding of the overall composition and physiology of the vaginal microbiome, as well as the other human microbiomes (Zoetendal *et al*, 2008).

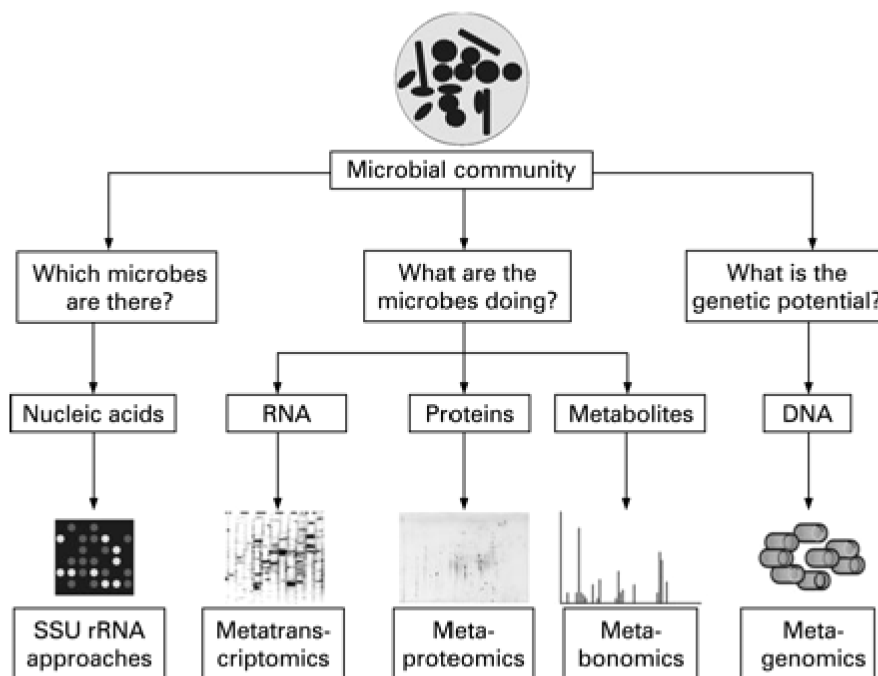

Figure 9. Schematic representation of the metagenomic and other community-based "omics" approaches (Zoetendal *et al*, 2008).

Metagenomics holds significant promise for increasing our understanding of many microbial diseases associated with the human body, especially those considered to be polymicrobial in origin, such as BV. Indeed, the landscape of microbial ecology as it relates to human disease has led to the National Institutes of Health (NIH) Human Microbiome Project (HMP). The HMP has the overall goal of demonstrating the role the human microbiome plays in human health and disease by promoting a combined assessment of microbial population structure and of community function. This will establish at each body site if there is a "core microbiome" indicative of a healthy status and will provide the baseline community structure, which will enable application of Koch's

postulates to previously intractable human diseases. That is, the correlation of microbial community structure or microbiome, rather than of a single microorganism, to healthy and diseased states (White *et al*, 2011).

4. Rifaximin

Rifaximin ($C_{43}H_{51}N_3O_{11}$, molecular weight 785.9 daltons) is derived from rifamycin, and is a structural analogue of rifampin (Figure 10). The unique properties of this medication, including its broad spectrum of antimicrobial activity, high fecal concentrations, and low systemic absorption, make it an ideal agent for the treatment of gastrointestinal diseases.

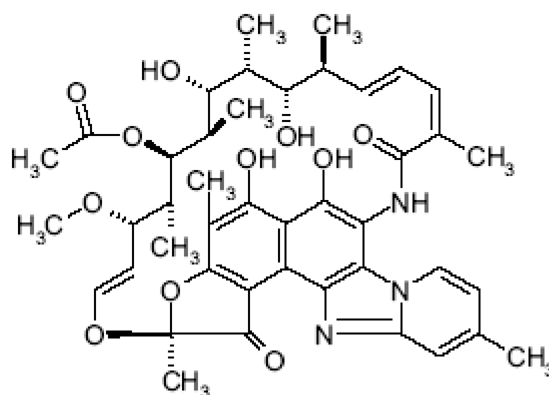

Figure 10. Chemical structure of Rifaximin.

4.1 Mechanism of action

Rifaximin is a highly potent inhibitor of bacterial RNA polymerase. It forms a complex with the β-subunit of the enzyme (RpoB), resulting in the blockage of the traslocation step that would ordinarily follow formation of the first phosphodiester bond during the transcription process (McClure and Cech, 1978).

4.2 Metabolism and pharmacokinetics

Rifaximin is a non-absorbable oral antibiotic that acts locally in the gastrointestinal tract. This enhances fecal concentrations of the antibiotic and limit its systemic toxicity, which is comparable to placebo (Descombe *et al*, 1994; Scarpignato and Pelosini, 2006). According to an FDA drug advisory briefing, absorption of rifaximin following oral dose is minimal, due to low intestinal permeability and poor water solubility; in addition, absorption is decreased by P-glycoprotein efflux

pump (FDA, 2010). Consistent with this finding, studies with radio-labeled rifaximin had demonstrated < 0.4% of detectable rifaximin dose in blood and urine, undetectable levels in bile and breast milk, and 97% recovered unchanged in the stool after oral ingestion (Descombe *et al*, 1994).

As a virtually non-absorbed antibiotic, drug interactions with rifaximin are uncommon. Despite the fact that rifaximin is related to rifamycin and rifampin, it is not known to have major drug interactions through cytochrome P450 (CYP450) enzyme systems. According to the drug manufacturer, *in vitro* data show that rifaximin can induce CYP3A4; however, clinical studies have shown no significant effect on drug metabolism by cytochrome P450 isoenzymes (Pentikis *et al*, 2007; Trapnell *et al*, 2007).

With its lack of systemic absorption, rifaximin is relatively well tolerated drug and is associated with a low incidence of adverse events. Clinical trials evaluating rifaximin for several gastrointestinal diseases support the safety and tolerance of this medication (Leevy and Phillips, 2007; Pimentel *et al,* 2006; Prantera *et al*, 2006).

4.3 Antimicrobial activity and resistance

Rifaximin is active against a variety of aerobic and anaerobic Gram-positive and Gram-negative organisms, as well as protozoa infections (Table I) (Amenta *et al*, 1999; Gillis *et al*, 1995; Megraud *et al*, 1994; Ripa *et al*, 1987). In *in vitro* and *in vivo* studies, rifaximin levels in the stool were 160 to 250 times higher than MIC_{90} (4 to 64 µg/ml) for most intestinal pathogens (Jiang and DuPont, 2005; Jiang *et al*, 2000).

The primary mechanism for developing resistance to rifaximin is chromosomal alteration of the drug target, the DNA-dependent RNA polymerase (Scarpignato and Pelosini, 2006; Spratt, 1994). *In vitro* data showed that most common organisms to develop resistance to rifaximin are aerobic Gram-positive cocci. However, intestinal lumen is predominantly an anaerobic environment, theoretically limiting selection of isolates resistant to rifaximin (Scarpignato and Pelosini, 2006). In addition, because rifaximin lacks systemic absorption, drug concentrations stay well above the MIC of pathogens to disallow for subtherapeutic drug concentrations in the intestine. Despite these advantages, and potentially due to rifaximin's structural relationship to other rifamycins, resistance rates for *Enterococcus*, *Bacteroides*, *Clostridium*, and *Enterobacteriaceae* range between 30% to 90% after 5 days of treatment. After rifaximin is stopped, these resistant strains tend to disappear within 1 to 12 weeks (De Leo *et al*, 1986).

Culture-dependent analysis of fecal samples showed that the antibiotic did not alter the concentration of some major bacterial groups, while it induced an increase in bifidobacteria (Jiang and DuPont, 2009). A recent study on the use of rifaximin in the treatment of travelers' diarrhea further confirmed that clinical effectiveness of the antibiotic is not associated with evident alterations of the colonic microbiota (Jiang and DuPont, 2009).

4.4 Therapeutic applications

Since its approval in Italy in 1987, rifaximin has been licensed in over 30 countries for the treatment of a variety of gastrointestinal diseases. Rifaximin was approved by the US Food and Drug Administration (FDA) in 2004 for the management of travelers' diarrhea caused by non-invasive strains of *Escherichia coli* (Xifaxan, 2010). In 2010, rifaximin received an additional labeling for reduction in the risk of the recurrence of overt hepatic encephalopathy (HE) in patients with advanced liver disease (NDA, 2010).

4.4.1. Treatment and prevention of travelers' diarrhea

The effectiveness of rifaximin as a therapeutic agent for travelers' diarrhea has been demonstrated in several pivotal randomized, double-blind clinical trials. The primary endpoint of these trials was the time to last unformed stool (TLUS), defined as the time from the first dose of medication to the passage of the last unformed stool, after which patients are declared well. Rifaximin has been shown to be more effective than placebo (Steffen *et al*, 2003) and similar in efficacy to traditionally used antibiotics, trimethoprim–sulfamethoxazole (TMP-SMX) (DuPont *et al*, 1998) and ciprofloxacin (DuPont *et al*, 2001; Taylor *et al*, 2006), in shortening the duration of travelers' diarrhea. The recommended dosage of rifaximin for the treatment of travelers' diarrhea caused by non-invasive strains of *E. coli*, including ETEC and EAEC, in patients more than 12 years of age is 200 mg three times per day for 3 days (DuPont *et al*, 2009-b).

Some experts consider antibiotic chemoprophylaxis for travelers' diarrhea to be unnecessary and excessive because of their fears of antimicrobial resistance promotion and potential risk of adverse events for a selflimited disease in the majority of travelers' diarrhea cases. As a gut-selective antibiotic with minimal systemic toxicity and a lack of drug interactions, rifaximin appears to be an ideal prophylactic drug for travelers' diarrhea (DuPont *et al*, 2009-a).

Rifaximin's bactericidal activity appears to be limited to the lumen of the gastrointestinal tract. As a result, rifaximin is less effective in treating invasive bacterial pathogens, such as *Shigella*

spp., *Campylobacter jejuni*, and *Salmonella* spp. (Taylor *et al*, 2006). In contrast, rifaximin's protective effect may extend to even travelers' diarrhea cases associated with invasive enteric pathogens. It has been hypothesized that rifaximin may eradicate invasive diarrheagenic pathogens in the gastrointestinal tract prior to mucosal infiltration (DuPont, 2008).

4.4.2. Clostridium difficile infection (CDI)

According to the 2010 update on the Clinical Practice Guidelines for CDI in Adults by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), rifaximin may be a treatment option for the management of patients with multiple recurrent episodes of *Clostridium difficile* infections (Cohen *et al*, 2010).

Rifamycins have been considered as a treatment option for CDI on the basis of *in vitro* susceptibility data (Hecht *et al*, 2007). The high levels of rifaximin that can be achieved in the gut are ideal for treatment of CDI. The efficacy of rifaximin has been reported for the treatment of refractory or recurrent CDI in several studies (Basu *et al*, 2010; Garey *et al*, 2009; Johnson *et al*, 2009; Neff *et al*, 2010), but optimal dosing, duration, and use of rifaximin for CDI is unclear.

Caution is recommended with use of rifaximin because of the potential for isolates to develop resistance. *In vitro* studies suggest that rifampin resistance predicts rifaximin resistance in *C. difficile* (O'Connor *et al*, 2008). Prevalence of rifampin resistance in *C. difficile* may suggest limited utility with rifaximin; therefore, the SHEA-IDSA guidelines do not provide an official recommendation on its use.

4.4.3. Irritable bowel syndrome (IBS)

Irritable bowel syndrome is a chronic gastrointestinal illness estimated to affect between 1% and 20% of the general population. IBS can be characterized by constipation and/or diarrhea, change in bowel habits, abdominal pain, and bloating. IBS diagnosis is usually a diagnosis of exclusion; organic causes of the abdominal symptoms have to be ruled out before the diagnosis of IBS is made. Various clinical criteria could be used to diagnose IBS (Brandt *et al*, 2009). The etiology of IBS remains unknown. However, many causes have been proposed, such as changes in central and peripheral sensorium, altered intestinal motility, and exaggerated response to stress. Additionally, some data suggest that alterations in small intestinal flora and bacterial overgrowth may contribute to the pathophysiology of IBS, although available evidence is conflicting (Brandt *et al*, 2009).

Symptomatic improvement in IBS symptoms has been demonstrated with tetracyclines and fluoroquinolones, with high relapse rates following antibiotic discontinuation (Attar *et al*, 1999). There are concerns about the development of antibiotic resistance with the widespread use of antimicrobial agents. Two randomized, double-blind, placebo-controlled trials evaluated the efficacy of rifaximin treatment for IBS. Rifaximin patients experienced significantly greater symptomatic relief than the placebo group (Sharara *et al*, 2006). Though, long-term efficacy and tolerability data are currently lacking to support use beyond what was studied in short-term trials (Brandt *et al*, 2009).

4.4.4. Inflammatory bowel disease (IBD) and pouchitis

An abnormal host immune response associated with a loss of tolerance to the commensal intestinal microbiome is believed to play an important role in the pathogenesis of inflammatory bowel diseases (IBDs), including Crohn's disease, ulcerative colitis, and pouchitis (Sartor, 2008). As a result, antibiotics such as metronidazole and ciprofloxacin have been used for the medical management of IBD. However, despite the potential beneficial effect of prolonged antimicrobial therapy in IBD, side effects of these antibiotics limit their extended use.

As a non-absorbable antibiotic, rifaximin is an attractive alternative, but a lack of well designed clinical trials with sufficient power contribute to the difficulty in assessing the efficacy of rifaximin for IBD (Guslandi, 2010; Isaacs *et al*, 2007; Prantera *et al*, 2006; Shafran and Burgunder, 2010; Shen *et al*, 2008).

4.4.5. Colonic diverticular disease

Although the majority of individuals with diverticular disease are asymptomatic, approximately 20% of patients experience clinical illness and are at risk for complications, such as diverticulitis and hemorrhage (Almy and Howel, 1980). Dietary fiber supplementation is considered the standard therapy for symptomatic diverticular disease and may prevent complications (Painter and Burkitt, 1975). Multiple randomized clinical studies have demonstrated that the addition of monthly rifaximin to fiber supplementation may further improve symptoms and prevent complications in patients with symptomatic, uncomplicated diverticular disease (Colecchia *et al*, 2007; D'Inca *et al*, 2007; Latella *et al*, 2003; Papi *et al*, 1995).

4.4.6. Hepatic encephalopathy (HE)

Hepatic encephalopathy is one of the complications associated with portal hypertension in patients with chronic liver disease and cirrhosis. Usually, HE presents as impaired thinking, confusion, and behavioral and motor changes. The etiology of HE is poorly understood. One of the proposed mechanisms is poor liver ability to remove nitrogenous waste produced by the gastrointestinal bacteria. Ammonia is a byproduct of protein catabolism, which is metabolized to urea and excreted renally in patients with normal liver function. Patients with HE are not able to convert NH_3 to urea and have elevated ammonia concentrations in the blood and NH_3 accumulation in the central nervous system, resulting in impaired neurotransmission (Ryan, 2010).

In today's clinical practice, the most frequently administered agents for both treatment of overt HE and prevention of recurrent HE episodes are lactulose and rifaximin (Bajaj, 2010). Lactulose exerts its effect by catharsis and by increasing conversion of NH_3 to the ionized form, thereby decreasing its absorption into systemic circulation, leading to increased removal of nitrogenous waste via fecal route (Ryan, 2010). An alternative is the use of oral antibiotics to decrease the load of ammonia-producing bacteria in the gastrointestinal tract. However, use of antibiotics long term is limited due to adverse effects (Blei and Córdoba, 2001). Rifaximin showed a limited risk to select resistant bacteria. Multiple small trials investigated efficacy of rifaximin for this indication compared with other active control medications or placebo. They reported safety and effectivess of rifaximin for the prevention of recurrent HE in patients who also receiving lactulose.

Rifaximin is a broad-spectrum non-absorbed rifamycin antibiotic with an excellent safety profile, a lack of drug interactions, and minimal effect on the intestinal microbiome. Thanks to these features and to its ability to they induce a favorable anti-inflammatory environment (Brown *et al*, 2010), rifaximin appears promising as a therapeutic agent for infections and/or imbalances involving other human ecosystems, such as the vaginal microbiota.

PROJECT OUTLINE

Project outline

Bacterial vaginosis (BV) represents a very common imbalance in the ecology of the normal vaginal microflora, affecting women of childbearing age (McDonald *et al*, 2003). It is a polymicrobial disorder associated with an increase of the taxonomic richness and diversity of the vaginal microbiota (Ling *et al*, 2010; Oakley *et al*, 2008), characterized by a reduction in the prevalence and concentration of H_2O_2-producing lactobacilli (Eschenbach *et al*, 1989; Hawes *et al*, 1996) and an increase in the prevalence and concentration of other bacteria, including *Gardnerella vaginalis*, anaerobic Gram-negative rods, anaerobic Gram-positive cocci, *Mycoplasma hominis*, and *Mobiluncus* spp. (Ling *et al*, 2011; Turovskiy *et al*, 2011). 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 (putrescine, cadaverine and trimethylamine) which, at high pH conditions, become volatile and malodorous. The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating the typical discharge (Chen *et al*, 1979, Sobel, 2000). The clinical consequences of BV could be important. Indeed, 40% of the cases of spontaneous preterm labor and preterm birth are thought to be associated with BV or other types of "abnormal" vaginal microflora (Donders *et al*, 2011). A large body of literature confirms that BV is associated with these and other considerable gynecologic and obstetric diseases. Moreover, epidemiologic studies have demonstrated that BV is associated with a markedly increased risk for acquisition and transmission of HIV and other sexually transmitted infections, including *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and Herpes simplex virus type-2 infection (Cherpes *et al*, 2003; Martin *et al*, 1999; Wiesenfeld *et al*, 2003).

The association of lower genital tract infection, such as BV, with an increased risk of preterm delivery and preterm rupture of the fetal membranes has recently attracted great interest in the pathogenesis of such infection-related mechanisms (Goldenberg *et al*, 2008; Mattison *et al*, 2001). The mechanisms linking BV with preterm delivery have not been fully identified, but local immune response is hypothesized to be crucial (Beigi *et al*, 2007). Parturition is characterized by cervical ripening and myometrial maturation with subsequent uterine contractions leading to cervical dilatation and birth (Norwitz *et al*, 1999). The process of labor displays many of the hallmarks of inflammation. Acute inflammatory features, such as increased influx of leucocytes and elevated expression of pro-inflammatory cytokines, have been observed in cervical tissues and fetal membranes during both term and preterm labor (Challis *et al*, 2009; Dubicke *et al*, 2010; Houben *et al*, 2009).

BV is considered as a complex microbial imbalance, and is caused by the interaction of multiple factors, which include the numerous components of the vaginal microbial ecosystem and their human host, and many of them are yet to be characterized (Turovskiy *et al*, 2011). Due to the unknown etiology of BV, it is difficult to find an efficient cure for this condition. The currently recommended treatment regimes are oral or vaginal metronidazole or vaginal clindamycin (ACOG Practice Bulletin, 2006), but long-term follow-up suggests recurrence rates as high as 58% one year after treatment (Bradshaw *et al*, 2006-a). The reasons for recurrence may include the failure to eradicate the offending organisms, due to the formation of a prolific bacterial biofilm adherent to the vaginal epithelium (Swidsinski *et al*, 2005). The main components of this polymicrobial biofilm are *G. vaginalis* and *Atopobium vaginae*. Other hypothesis for the etiology and recurrence of BV may include the incapability of H_2O_2-producing *Lactobacillus* strains, which are considered competent as "defenders" (Atassi and Servin, 2010), to efficiently recolonize the vaginal environment after the antibiotic therapy; the depletion of these lactobacilli strains due to the activation of bacteriophages (Pavlova *et al*, 1997); or an incompetent immune response to BV-associated pathogens by the host (Witkin, *et al*, 2007-b).

The role of a healthy vaginal microbiota is fundamental to protect women from genital tract infections and to maintain the natural balance of the vaginal microbial ecosystem, especially during pregnancy (Donati *et al*, 2010). Thus, the high recurrence rates resulting in repeated exposure to antibiotics and the emergence of drug-resistant strains suggest a need for alternative therapeutic tools.

A potentially novel way to prevent and to protect against infection-mediated preterm birth is to use probiotic bacteria. Probiotics, defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002), are being studied for their ability to replenish vaginal lactobacilli and modulate immunity (Reid *et al*, 2003; Reid *et al*, 2005). The ability of several bacterial species to colonize both the gastrointestinal tract and the reproductive tract has suggested that a healthy gut microbiota contributes to protecting against female genitourinary tract infections and depletion of lactobacilli (Antonio *et al*, 2005; El Aila *et al*, 2009).

Rifaximin is a semisynthetic rifamycin derivative, with a broad antimicrobial spectrum (Rivkin and Gim, 2011; Scarpignato and Pelosini, 2005) and a good safety profile because of its negligible grade of systemic absorption. Thanks to its antibacterial activity covering *G. vaginalis* and other pathogens responsible for urogenital infections (Hoover *et al*, 1993), rifaximin could be a suitable alternative for the local treatment of BV.

In the present study the impact of a dietary supplementation with the probiotic product VSL#3, a mixture of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains, on the vaginal microbial ecology and immunological profiles of asymptomatic healthy women during late pregnancy, was evaluated. The dynamics of the vaginal bacterial communities prior and after the probiotic ingestion were assessed by PCR-DGGE and real-time PCR, while the modulation of the cytokine secretion in vaginal fluids was measured by Luminex® Immunoassay. Although previous studies demonstrated the therapeutic efficacy of VSL#3 in the management of gastrointestinal disorders, this is the first study that investigates the indirect effects of this probiotic formula on the vaginal ecosystem.

Afterward, the impact of two doses of rifaximin vaginal tablets (100 mg and 25 mg) administered for different periods (2 days and 5 days), on the vaginal microbiota of 102 European patients with BV enrolled in a multicentre, double-blind, randomized, placebo-controlled study was assessed. Bacterial DNA extracted from standardized vaginal rinsings was analyzed using the culture-independent techniques PCR-DGGE and real-time PCR.

CHAPTER 1

DIETARY SUPPLEMENTATION WITH PROBIOTICS DURING LATE PREGNANCY: OUTCOME ON VAGINAL MICROBIOTA AND CYTOKINE SECRETION

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Paper submitted to BMC Microbiology

Background

The vaginal microbiota of healthy women consists of a wide variety of anaerobic and aerobic bacterial genera and species dominated by the facultative, microaerophilic anaerobic genus *Lactobacillus* (Lidbeck and Nord, 1993). The activity of lactobacilli is essential to protect women from genital tract infections and to maintain the natural healthy balance of the vaginal microbial ecosystem. This role is particularly important during pregnancy because vaginal infection has been claimed as one of the most important mechanisms responsible for preterm birth and perinatal complications (Donati *et al*, 2010).

The association of lower genital tract infection with an increased risk of preterm delivery and preterm rupture of the fetal membranes has recently attracted great interest in the pathogenesis of such infection-related mechanisms (Goldenberg *et al*, 2008; Mattison *et al*, 2001). Earlier studies showed an increased rate of prematurity in women with bacterial vaginosis (BV). The mechanisms linking BV with preterm delivery have not been fully identified, but local immune response is hypothesized to be crucial (Beigi *et al*, 2007). Parturition is characterized by cervical ripening and myometrial maturation with subsequent uterine contractions leading to cervical dilatation and birth (Norwitz *et al*, 1999). The process of labor displays many of the hallmarks of inflammation. Acute inflammatory features, such as increased influx of leucocytes and elevated expression of pro-inflammatory cytokines, have been observed in cervical tissues and fetal membranes during both term and preterm labor (Challis *et al*, 2009; Dubicke *et al*, 2010; Houben *et al*, 2009).

A potentially novel way to protect against infection-mediated preterm birth is to use probiotic bacteria, especially lactobacilli. In addition, administration of probiotics to the mother during pregnancy and breast-feeding appears to be a safe and effective mode of enhancing the immunoprotective potential of the breast milk and preventing atopic eczema in the infant (Huurre *et al*, 2008; Rautava *et al*, 2002).

In the present study the impact of a dietary supplementation with the probiotic product VSL#3, a mixture of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains, on the vaginal microbial ecology and immunological profiles of asymptomatic healthy women during late pregnancy was evaluated. The dynamics of the vaginal bacterial communities prior and after the probiotic ingestion were assessed by PCR-DGGE and real-time PCR, while the modulation of the cytokine secretion in vaginal fluids was measured by Luminex® Immunoassay. This is the first study that investigates the indirect effects of this probiotic formula on the vaginal ecosystem.

Materials and methods

Patients, study medication and sample collection

A pilot, not randomized, controlled and perspective study was conducted. The study protocol was approved by the ethical committee of the University of Bari, Italy. Written informed consent was obtained from all the participants in the study. A total of 27 healthy pregnant women (21 to 42 years of age; mean = 32) who had no symptoms of vaginal or urinary tract infection were included in the present study (Table 1.1). None of the subjects had received oral or local antimicrobial therapy within the previous 2 weeks. The recruited patients were divided into 2 groups: probiotic group [P (n=15)], and control group [C (n=12)] on the basis of their availability to consume the probiotic product. Patients of the P group consumed 1 sachet once/day of VSL#3 (VSL Pharmaceuticals, Fort Lauderdale, FL) for 4 weeks from the 33rd (W33) to the 37th (W37) week of gestation. Patients of the C group did not receive any dietary supplementation. VSL#3 sachet contains 900 billion viable lyophilized bacteria consisting of 4 strains of *Lactobacillus* (*L. paracasei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*), 3 strains of *Bifidobacterium* (*B. longum*, *B. breve*, *B. infantis*) and 1 strain of *Streptococcus thermophilus*. Mid-vaginal swabs were collected from the women of both P and C groups at the time points W33 and W37. Samples were placed in 1 ml of sterile saline and stored immediately at -80°C until use.

The individual characteristics (age, type of delivery and gestational age at birth) of the women enrolled in the present study are reported in Table 1.1. Gestational age was determined by utilizing the last menstrual period and earliest ultrasound.

DNA extraction from vaginal samples

Frozen vaginal swabs were thawed, mixed by vortex shaker for 1 min and then removed from the liquid. The liquid was centrifuged at 10,000 × *g* for 15 min, and the pellet was washed 3 times in saline at 40°C. The pellet was resuspended in 180 µl of enzymatic lysis buffer (20 mM Tris-HCl, pH 8, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) and incubated at 37°C for 30 min. Glass beads (200 mg) were added and the sample was mixed by vortexing for 1 min. Total DNA was extracted by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol "Pretreatment for Gram-positive bacteria". A slight modification was introduced: a centrifugation step (8000 × *g* for 5 min) was carried out after the incubation with proteinase K to remove glass beads. DNA amounts were quantified by using NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

Table 1.1. Characterization of the subjects included in the study groups.

Woman N.	Age	Type of delivery1	Gestational age at birth
Probiotic (n = 15)			
1	31	SD	39 week + 6 days
2	32	CD	40 week + 3 days
3	39	SD	40 week + 1 day
4	31	SD	40 week + 2 days
5	33	SD	40 week + 3 days
6	30	SD	39 week
7	33	SD	41 week + 3 days
8	34	CD	39 week
9	36	CD	38 week + 4 days
10	38	SD	38 week + 5 days
11	42	SD	39 week + 4 days
12	30	SD	39 week
13	29	SD	40 week + 2 days
14	33	CD	39 week + 2 days
15	25	SD	40 week + 1 day
Control (n = 12)			
16	28	SD	40 week + 6 days
17	33	SD	39 week + 3 days
18	33	CD	37 week + 4 days
19	32	CD	41 week + 3 days
20	34	SD	40 week
21	21	SD	39 week + 5 days
22	30	SD	38 week + 6 days
23	30	SD	40 week + 2 days
24	34	CD	39 week + 6 days
25	38	CD	41 week + 1 days
26	38	CD	38 week + 5 days
27	30	SD	40 week + 2 days

[1] SD: spontaneous delivery; CD: caesarean delivery.

PCR-DGGE and cluster analysis

The amplification reactions were performed in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany). GoTaq Flexi DNA Polymerase (Promega, Madison, WI) was used

as thermostable DNA polymerase. The reaction mixture contained 0.5 μM of each primer, 200 μM of each dNTP, 2 mM $MgCl_2$ solution, 1.25 U of GoTaq Flexi DNA Polymerase, 5 μl of Green GoTaq Flexi buffer 5×, and 2 μl of the bacterial DNA template (30-40 ng) in a final volume of 25 μl. The universal primers HDA1-GCclamp and HDA2 for bacteria (Walter *et al*, 2000) were used to amplify a conserved region within the 16S rRNA gene. The thermocycle program consisted of the following time and temperature profile: 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 60 s; and 72°C for 8 min. The *Lactobacillus* genus-specific primers Lac1 and Lac2-GCclamp (Walter *et al*, 2001) were used to amplify a specific region of the 16S rRNA gene of lactobacilli. The amplification program was 95°C for 5 min; 35 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 60 s; and 72°C for 8 min. A volume of 8 μl of PCR samples was loaded on DGGE gels, containing 30-50% and 25-55% gradients of urea and formamide for universal bacteria and lactobacilli amplifications, respectively. DGGE analysis was performed by using the D-Code Universal Mutation System Apparatus (Bio-Rad, Los Angeles, CA), as previously described (Vitali *et al*, 2007). Following electrophoresis, the gel was silver stained (Bassam *et al*, 1991) and scanned using a Molecular Imager Gel Doc XR System (Bio-Rad). DGGE gel images were analyzed using the FPQuest software version 4.5 (Bio-Rad). In order to compensate for gel-to-gel differences and external distortion to electrophoresis, the DGGE patterns were aligned and normalized using an external reference marker. The marker for the DGGE analysis with the universal primers for bacteria contained PCR amplicons from *Bacteroides*, *Coriobacterium*, *Enterococcus faecalis*, *Bifidobacterium bifidum*, *Lactobacillus casei*, *Acidaminococcus fermentas* and *Atopobium*. The marker for the DGGE analysis with *Lactobacillus*-specific primers contained PCR amplicons from *L. plantarum*, *L. paracasei*, *L. brevis*, *L. gasseri*, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*. After normalization, bands were defined for each sample using the appropriate densitometric curve. The similarity in the profiles was calculated on the basis of the Pearson correlation coefficient with the Ward clustering algorithm. Cluster analysis of the DGGE patterns was performed using the FPQuest software.

Sequencing of DGGE fragment

The DNA fragment of interest was excised from the denaturing gel with a sterile scalpel, washed once in 1× PCR buffer, and incubated in 20 μl of the same buffer overnight at 4°C. Two μl of the buffer solution were used as template for PCR reaction. Reamplification of the 16S rRNA region was conducted as described above by employing the primers Lac1 and Lac2 (without the GC-clamp). The re-amplified fragment was purified using the Wizard SV Gel and PCR Clean-up system (Promega), and then subjected to automated sequence analysis of both DNA strands with

Lac1 and Lac2. BigDye terminators (ABI-PerkinElmer, Foster City, CA) were used with a 377 sequencer (ABI). The sequence identity was determined by comparison with the rRNA gene sequences deposited in GenBank database using BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

Quantitative real-time PCR

Quantitative PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Each DNA sample was amplified with different genus- or species-specific primer sets targeted to 16S rRNA gene or 16S-23S rRNA spacer region: Bact-0011f/Lab-0677r (Heilig *et al*, 2002) for *Lactobacillus*, Bif164/Bif662 (Kok *et al*, 1996) for *Bifidobacterium*, Th1/Th2 (Tilsala-Timisjärvi and Alatossava, 1997) for *Streptococcus thermophilus*, F-GV1/R-GV3 (Zariffard *et al*, 2002) for *Gardnerella vaginalis*, c-Atopo-f/c-Atopo-r (Matsuki *et al*, 2004) for *Atopobium*, g-Prevo-f/g-Prevo-r (Matsuki *et al*, 2002) for *Prevotella*, VeilloF/VeilloR (Rinttilä *et al*, 2004) for *Veillonella*. Amplifications were carried out in a final volume of 20 µl containing 0.5 µM of each primer, 4 µl of LightCycler-FastStart DNA Master SYBR Green I (Roche) and either 2 µl of template or water (no-template control).

The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min followed by 30 (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*), 35 (*Prevotella*) or 40 (*Bifidobacterium*, *S. thermophilus*) cycles of denaturation at 95°C for 15 s; primer annealing at 63°C (*Lactobacillus*, *S. thermophilus*), 62°C (*Veillonella*), or 60°C (*Bifidobacterium*, *Atopobium*, *Prevotella*, *G. vaginalis*) for 20 s; extension at 72°C for 45 s (*Lactobacillus*, *Atopobium*, *Prevotella*, *G. vaginalis*, *Veillonella*), 30 s (*Bifidobacterium*), or 15 s (*S. thermophilus*) and a fluorescence acquisition step at 85°C (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*, *S. thermophilus*), 87°C (*Prevotella*) or 90°C (*Bifidobacterium*) for 5 s. DNAs extracted from *L. acidophilus* NCFM, *B. longum* NCC2705, *G. vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, *Veillonella parvula* ATCC 10790, *Atopobium vaginae* ATCC BAA-55 and *S. thermophilus* ATCC 19258 were used as standards for PCR quantification. DNAs extracted from vaginal samples were amplified in triplicate for each primer set and the mean value was used for statistical analysis. Data were expressed as ng of DNA of the targeted genus or species per µg of total DNA extracted from the vaginal sample.

Bioplex immunoassay

Cytokine levels were determined using a multiplexed bead immunoassay. Prior to assay, vaginal samples were concentrated 10 times with Microcon spin devices (YM3, Millipore Corporation, Billerica, MA) and subsequently resuspended in Bio-Plex Assay Buffer. The levels of 27 immune-mediators, 15 cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IFN-β, TNF-α), 7 chemokines (MCP-1, MIP-1α, MIP-1β, RANTES, Eotaxin, IL-8, IP-10) and 5 growth factors (PDGF-BB, FGF basic, G-CSF, GM-CSF, VEGF), were measured using the human ultrasensitive cytokine 27-plex antibody bead kit (Bio-Rad). The assays were performed in 96-well filter plates, as previously described (Vignali, 2000). Briefly, the filter plate was prewetted with washing buffer (Bio-Rad) and the solution was aspirated from the wells using a vacuum manifold (Millipore Corporation). Microsphere beads coated with monoclonal antibodies against the different target analytes were added to the wells. Samples and standards were pipetted into the wells and incubated for 30 min with the beads. The wells were washed using a vacuum manifold (Millipore Corporation) and biotinylated secondary antibodies were added. After incubation for 30 min, beads were washed then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein, R-phycoerythrin (streptavidin/R-phycoerythrin). After washing to remove the unbound streptavidin/R-phycoerythrin, the beads (a minimum of 100 per analyte) were analyzed in the Luminex 200 instrument (MiraiBio, Alameda, CA). The Luminex 200 monitors the spectral properties of the beads to distinguish the different analytes, while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. The concentration of the samples was estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor (Bio-Plex Manager software version 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve. The intra-assay CV including ultrafiltration and immunoassay averaged 19%. Concentrations of cytokines, chemokines and growth factors were then converted in pg of the target molecule per μg of total proteins present in the vaginal sample.

Statistical analysis

Statistical analysis was performed using SigmaStat (Systat Software, Point Richmond, CA). For each subject, variations of the DGGE profiles related to the time points W33 and W37 were analyzed by Pearson correlation. Significant differences in the intensity of each DGGE band among all vaginal samples and in the amounts of the bacterial genera and species determined by qPCR

were searched by using Wilcoxon Signed Rank Test. This test was also used to analyze differences in cytokines, chemokines and growth factors. A *P* value below 0.05 was considered statistically significant.

Results

Bacterial population profiling with PCR-DGGE

PCR-DGGE analysis with universal primers for bacteria (HDA1-GC/HDA2) was used to investigate the stability of the predominant vaginal bacterial communities over a period of 4 weeks in the last trimester of pregnancy, from the 33rd (W33) to the 37th (W37) week of gestation, and the influence of the oral consumption of the probiotic VSL#3 from W33 to W37 on the predominant vaginal microbiota (Figure 1.1). DGGE band profiles displayed a relatively low complexity for both probiotic (P) and control (C) groups, as assessed by the richness index. Mean values of the richness index were 6.6 at both W33 and W37 for C group and shifted from 8.4 (W33) to 7.4 (W37) for P group without significant variations between W33 and W37. Pearson correlation was used to calculate the similarity index (SI) between DGGE patterns related to the time points W33 and W37 for each pregnant woman (Table 1.2). The SI median values of P group and C group were 73% and 79%, respectively. In particular, 3 women belonging to P group (N. 2, 9 and 10) and only one woman belonging to C group (N. 24) showed SI values lower that 50%. For each woman, significant differences between DGGE profiles related to W33 and W37 were searched by Wilcoxon Signed Rank Test. No significant variations were detected between W33 and W37 in control women. Significant differences ($P < 0.05$) were found for 5/15 (33%) women belonging to P group (N. 4, 5, 9, 10, 11). Interestingly, women N. 9 and 10 were the same presenting SIs < 50%. These data suggested a potential role of the probiotic formula in modulating the vaginal bacterial communities. The peak heights of the DGGE densitometric curves were analyzed using the Wilcoxon Signed Rank Test in order to search for significant differences in single species abundances between W33 and W37. No significant changes in species abundance were found for both P and C groups, even in women N. 4, 5, 9, 10, 11, suggesting that the modulation of the vaginal microbiota exerted by the probiotic VSL#3 was not related to a particular bacterial species. Cluster analysis confirmed the stability of the vaginal microbiota in the last trimester of pregnancy since the DGGE profiles related to the time points W33 and W37 clustered together for all the control women, except for the woman N. 24 (Figure 1.1). Four treated women (N. 2, 9, 10 and 15)

Table 1.2. Similarity index (SI) of DGGE profiles related to W33 and W37 obtained with universal (HDA1/HDA2) and *Lactobacillus*-specific (Lac1/Lac2) primers.

Woman N.	HDA1-GC/HDA2 SI (%)	Lac1/Lac2-GC SI (%)
Probiotic (P)		
1	55.2	21.6
2	28.4	62.0
3	84.0	84.0
4	87.7	84.1
5	78.0	87.8
6	64.5	68.1
7	77.2	85.6
8	88.5	95.5
9	37.5	86.2
10	41.3	91.9
11	95.3	96.6
12	94.5	93.3
13	84.7	96.9
14	94.3	94.3
15	81.1	44.5
Control (C)		
16	91.2	90.9
17	87.8	93.7
18	81.6	76.9
19	83.7	91.5
20	67.7	81.3
21	87.1	94.3
22	94.6	74.4
23	85.3	74.1
24	25.4	46.0
25	84.7	84.2
26	78.3	68.1
27	84.5	86.3

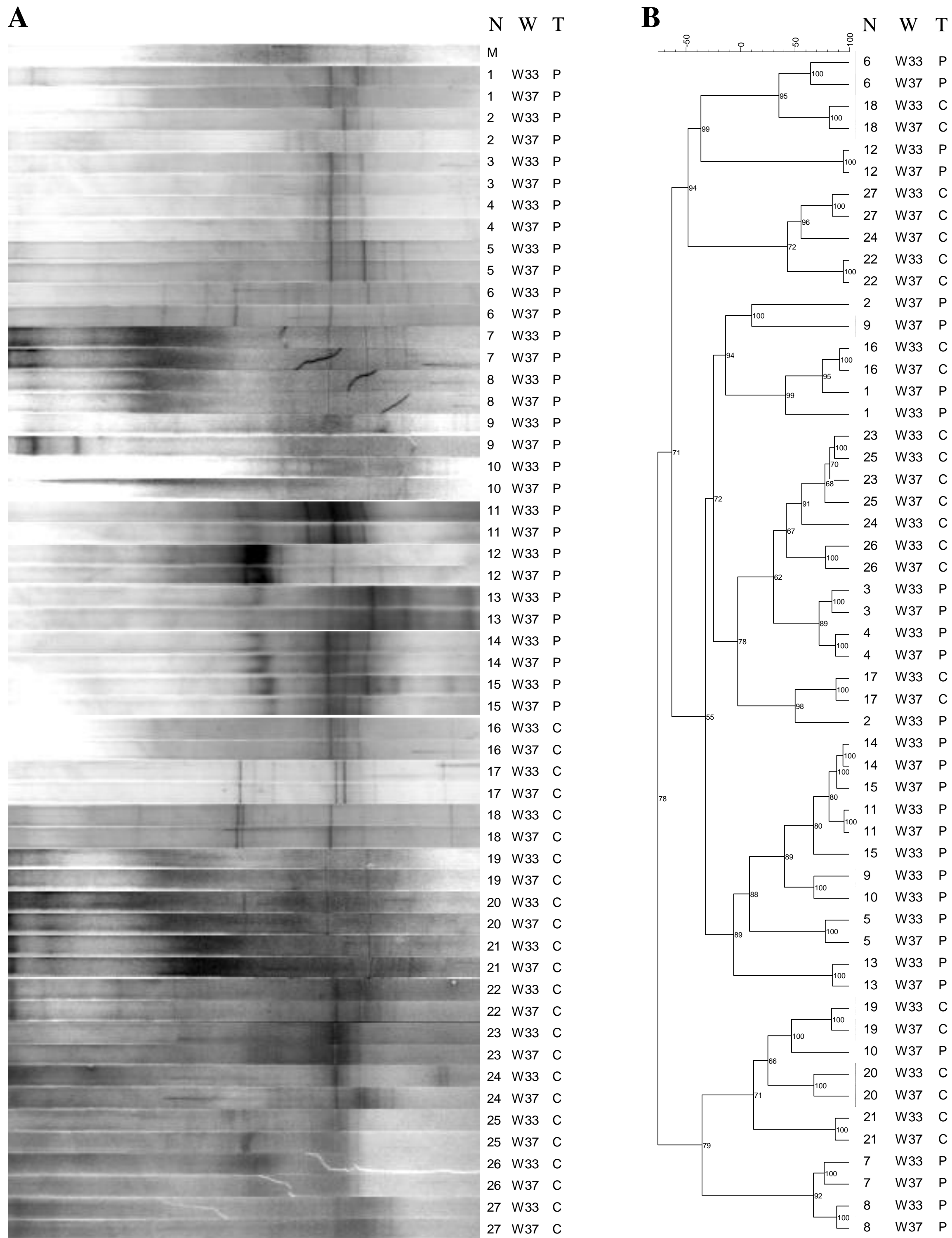

Figure 1.1. PCR-DGGE analysis with universal primers for bacteria. Analysis was conducted on the vaginal samples collected at 33rd (W33) and 37th (W37) week of gestation from 15 women administered with the probiotic VSL#3 [(P) N. 1-15] and 12 control women [(C) N. 16-27]. N: woman number; W: week of gestation; T: type of treatment. (A) PCR-DGGE fingerprints. M, external reference marker. (B) Dendrogram of the DGGE profiles shown in panel A. Pearson correlation was used to calculate the similarity in DGGE profiles.

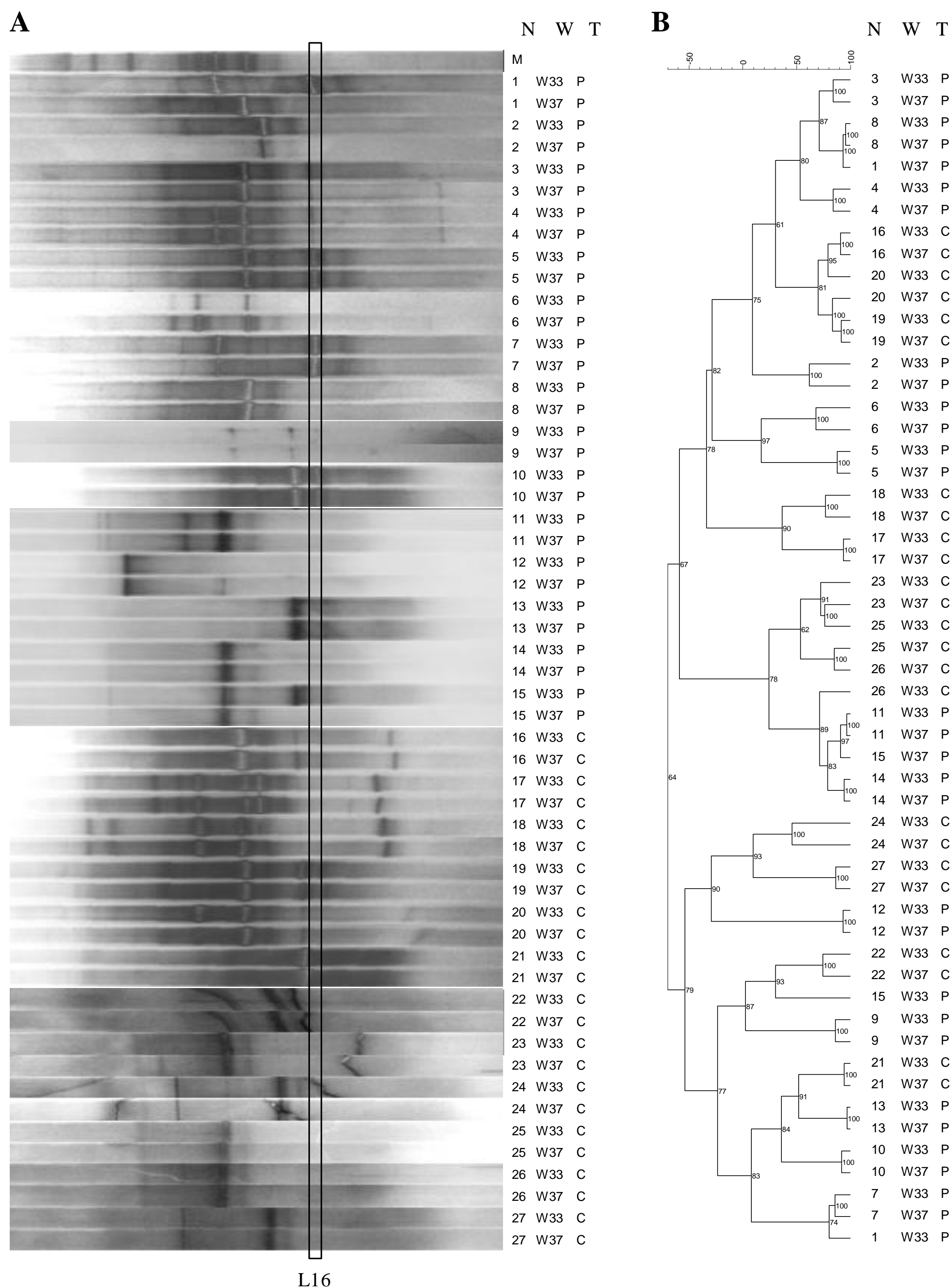

Figure 1.2. PCR-DGGE analysis with *Lactobacillus*-specific primers. Analysis was conducted on the vaginal samples collected at 33rd (W33) and 37th (W37) week of gestation from 15 women administered with the probiotic VSL#3 [(P) N. 1-15] and 12 control women [(C) N. 16-27]. N: woman number; W: week of gestation; T: type of treatment. (A) PCR-DGGE fingerprints. M, external reference marker. Band L16 corresponds to *L. helveticus* (GenBank accession number: AB571603) (B) Dendrogram of the DGGE profiles shown in panel A. Pearson correlation was used to calculate the similarity in DGGE profiles.

showed W33 and W37 DGGE profiles not closely related. However, the DGGE patterns of the majority of the women administered with VSL#3 grouped according to the subject and not to the time point, revealing that the inter-individual variability was higher than the variability induced by the probiotic supplementation.

Because of the importance of lactobacilli in the establishment of a healthy vaginal environment (Donati *et al*, 2010), DGGE analysis with *Lactobacillus*-specific primer set (Lac1/Lac2-GC) was also carried out. This analysis allowed to investigate the variations in lactobacilli population occurring physiologically from W33 and W37 and potentially associated with the VSL#3 intake (Figure 1.2). Richness indexes ranged from 5.7 (W33) to 5.4 (W37) for P group and from 6.3 (W33) to 6.8 (W37) for C group. Mean values of SI were 79% and 80% for P and C groups, respectively (Table 1.2). Only 2 women included in P group showed SIs < 50% (N. 1 and 15). Wilcoxon Signed Rank Test highlighted significant differences between DGGE profiles related to W33 and W37 for women N. 7 and 10, accounting for 13% of the women included in P group. Comparing this percentage with the 33% obtained by DGGE analysis with HDA1-GC/HDA2 primer set, the probiotic intake seemed to have a more extended impact on total bacteria than lactobacilli. Notably, only for woman N. 10, significant differences were found between W33- and W37-related DGGE patterns for both HDA1-GC/HDA2 and Lac1/Lac2-GC primer sets. The peak height analysis by Wilcoxon Signed Rank Test allowed to identify a band, denominated L16 (Figure 1.2), which significantly changed after the probiotic treatment. The sequencing of the DNA extracted from this band revealed 100% homology with *L. helveticus* strains. The nucleotide sequence of this DGGE fragment has been deposited in DDBJ Nucleotide Sequence Database under the accession number AB571603. *L. helveticus* was found to be a representative species within lactobacilli population since it was detected in 9 women treated with VSL#3 and 2 control women, corresponding to a frequency of occurrence of 40.7%. Notably, a general decrease in the intensity of *L. helveticus* band was observed in P group while no variations were appreciable in C group. Cluster analysis showed that *Lactobacillus*-specific DGGE profiles related to the time points W33 and W37 were closely related for all control women and for the majority of the women administered with VSL#3, except for the subjects N. 1 and 15 (Figure 1.2).

Quantitative variations of vaginal bacterial populations

Quantitative real-time PCR (qPCR) was performed to analyze changes in concentration of *Lactobacillus*, *Bifidobacterium* and *Streptococcus thermophilus*, that were included in the probiotic VSL#3, and *Gardnerella vaginalis*, *Atopobium*, *Prevotella* and *Veillonella*, that are important BV-associated genera and species (Biagi *et al*, 2009; Vitali *et al*, 2007). qPCR efficiency for all assays

was between 90% and 110% and correlation coefficients for genomic DNA standards were > 0.99. The sensitivity of qPCR assays was 9.1×10^{-3}, 1.5×10^{-4}, 3.7×10^{-4}, 1.7×10^{-1}, 1.4×10^{-2}, 4.9×10^{-4}, 3.3×10^{-1} ng of target DNA for *Lactobacillus*, *Bifidobacterium*, *S. thermophilus*, *G. vaginalis*, *Atopobium*, *Prevotella* and *Veillonella*, respectively. All patients naturally harbored strains belonging to *Lactobacillus*, *Bifidobacterium*, *Atopobium* and *Prevotella*, as demonstrated by the presence of these genera in the vaginal samples collected at W33. Woman N. 9 (P group) was the only exception lacking lactobacilli at both the baseline and after one-month intake of VSL#3 (Table 1.3). *G. vaginalis* was found in two women belonging to C group (N. 18 and 20) at both time points at the concentration of $5.5 \times 10^1 \pm 3.8$ (N. 18: W33), $7.5 \times 10^1 \pm 4.6$ (N. 18: W37), $2.2 \times 10^2 \pm 1.8 \times 10^1$ (N. 20: W33) and $1.9 \times 10^2 \pm 3.2 \times 10^1$ (N. 20: W37). *S. thermophilus* and *Veillonella* were not detected in any pregnant woman enrolled in this study. Statistical elaboration of qPCR data related to *Lactobacillus*, *Bifidobacterium*, *Atopobium* and *Prevotella* was performed to search for significant variations of these genera associated with the going on of pregnancy or the probiotic treatment (Figure 1.3). No significant changes in the amounts of these bacteria were found

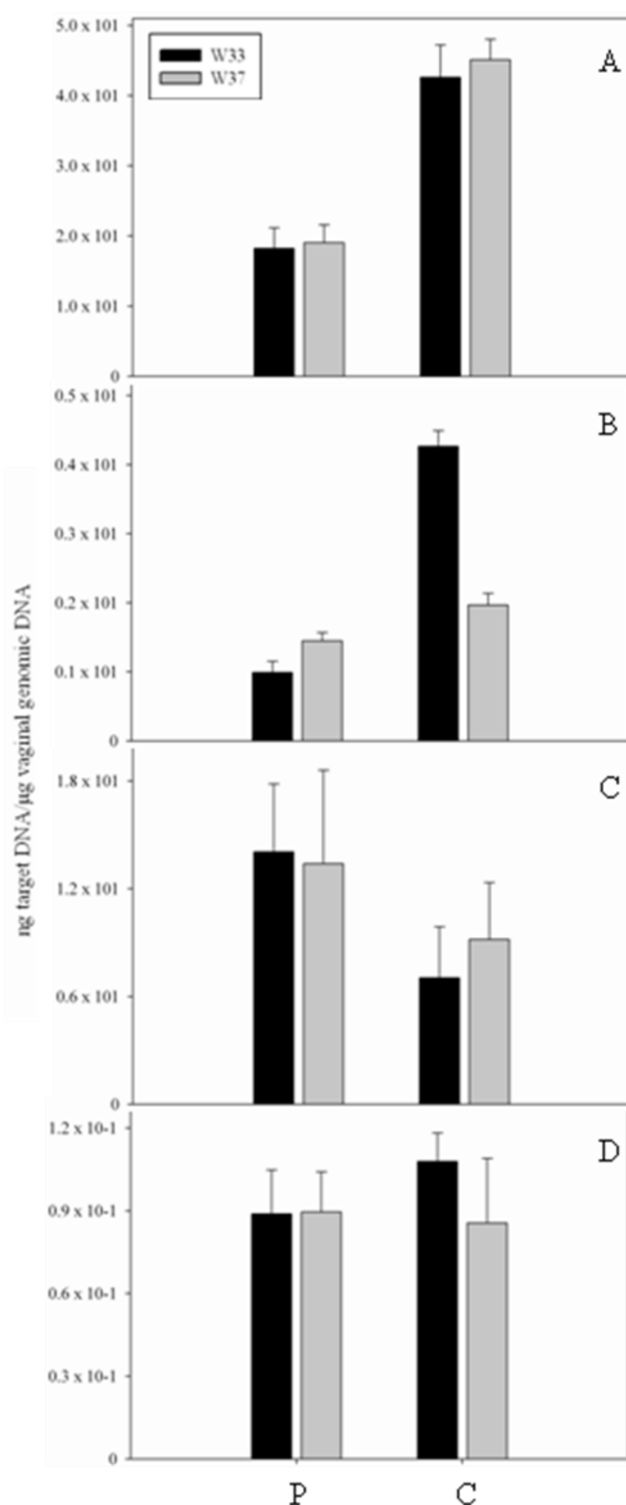

Figure 1.3. qPCR evaluation of *Lactobacillus* (A), *Bifidobacterium* (B), *Atopobium* (C) and *Prevotella* (D). Analysis was performed on vaginal samples collected at 33rd (W33) and 37th (W37) week of gestation from pregnant women treated (P) and not treated (C) with VSL#3. Data are expressed as ng of DNA of the target genus per µg of total bacterial DNA extracted from the vaginal sample. The diagrams show the mean values with the error bars representing the standard deviations.

Table 1.3. qPCR data of *Lactobacillus*, *Bifidobacterium*, *Atopobium* and *Prevotella*.

		ng of target DNA/µg vaginal genomic DNA (mean ± SD)			
Woman N.	Time point	*Lactobacillus*	*Bifidobacterium*	*Atopobium*	*Prevotella*
Probiotic (P)					
1	W33	$2.4 \times 10^{1} \pm 1.1$	$1.9 \times 10^{-2} \pm 7.4 \times 10^{-3}$	3.6 ± 1.5	$2.1 \times 10^{-2} \pm 1.0 \times 10^{-2}$
	W37	$3.0 \times 10^{1} \pm 3.1$	$3.1 \times 10^{-2} \pm 2.7 \times 10^{-4}$	$1.3 \times 10^{1} \pm 6.8$	$9.1 \times 10^{-2} \pm 1.6 \times 10^{-2}$
2	W33	$9.6 \pm 8.7 \times 10^{-1}$	$3.1 \times 10^{-2} \pm 8.8 \times 10^{-3}$	$5.4 \times 10^{1} \pm 7.4$	$1.4 \times 10^{-1} \pm 4.8 \times 10^{-2}$
	W37	$5.9 \times 10^{-1} \pm 4.9 \times 10^{-2}$	$2.4 \times 10^{-2} \pm 1.2 \times 10^{-2}$	$2.4 \times 10^{1} \pm 1.9 \times 10^{1}$	$1.1 \times 10^{-1} \pm 1.1 \times 10^{-2}$
3	W33	$2.4 \times 10^{1} \pm 2.9$	$2.4 \times 10^{-2} \pm 4.2 \times 10^{-3}$	$1.1 \times 10^{1} \pm 6.0$	$1.1 \times 10^{-1} \pm 7.7 \times 10^{-3}$
	W37	$2.2 \times 10^{1} \pm 2.4$	$3.0 \times 10^{-2} \pm 2.4 \times 10^{-3}$	4.0 ± 2.3	$5.2 \times 10^{-2} \pm 8.2 \times 10^{-3}$
4	W33	$2.2 \times 10^{1} \pm 2.0$	$6.8 \times 10^{-2} \pm 8.3 \times 10^{-3}$	4.7 ± 1.9	$7.3 \times 10^{-2} \pm 2.9 \times 10^{-2}$
	W37	$1.5 \times 10^{1} \pm 1.4$	$2.1 \times 10^{-2} \pm 7.2 \times 10^{-3}$	5.2 ± 2.0	$4.6 \times 10^{-2} \pm 9.5 \times 10^{-3}$
5	W33	$2.5 \times 10^{1} \pm 4.5$	$2.1 \times 10^{-2} \pm 3.4 \times 10^{-3}$	$1.2 \times 10^{1} \pm 3.0$	$9.3 \times 10^{-2} \pm 8.3 \times 10^{-3}$
	W37	$2.2 \times 10^{1} \pm 4.5$	$1.4 \times 10^{-2} \pm 3.2 \times 10^{-3}$	$1.5 \times 10^{1} \pm 1.9$	$3.0 \times 10^{-2} \pm 1.1 \times 10^{-2}$
6	W33	$1.1 \times 10^{-1} \pm 3.4 \times 10^{-3}$	$7.1 \times 10^{-2} \pm 7.1 \times 10^{-3}$	$1.0 \times 10^{1} \pm 4.1$	$1.2 \times 10^{-1} \pm 1.3 \times 10^{-2}$
	W37	$2.2 \pm 6.0 \times 10^{-1}$	$2.1 \pm 1.7 \times 10^{-1}$	$2.4 \times 10^{1} \pm 1.0 \times 10^{1}$	$1.5 \times 10^{-1} \pm 1.2 \times 10^{-2}$
7	W33	$4.1 \times 10^{1} \pm 8.5$	$3.7 \times 10^{-2} \pm 5.4 \times 10^{-3}$	$2.9 \times 10^{1} \pm 9.2$	$1.2 \times 10^{-1} \pm 2.1 \times 10^{-2}$
	W37	$2.0 \times 10^{1} \pm 2.6$	$1.7 \times 10^{-2} \pm 4.4 \times 10^{-3}$	$2.6 \times 10^{1} \pm 7.7$	$1.1 \times 10^{-1} \pm 1.1 \times 10^{-3}$
8	W33	$1.0 \times 10^{1} \pm 1.7 \times 10^{-1}$	$1.3 \times 10^{-2} \pm 1.9 \times 10^{-3}$	5.5 ± 1.2	$4.2 \times 10^{-2} \pm 1.9 \times 10^{-2}$
	W37	$2.1 \times 10^{1} \pm 2.0$	$1.5 \times 10^{-2} \pm 2.6 \times 10^{-3}$	$1.6 \times 10^{1} \pm 6.6$	$5.1 \times 10^{-2} \pm 3.3 \times 10^{-3}$
9	W33	0.0 ± 0.0	$7.1 \times 10^{-3} \pm 2.8 \times 10^{-5}$	$1.8 \times 10^{1} \pm 7.1$	$6.7 \times 10^{-2} \pm 1.5 \times 10^{-2}$
	W37	0.0 ± 0.0	$1.1 \times 10^{1} \pm 1.0$	$1.5 \times 10^{1} \pm 6.8$	$2.3 \times 10^{-1} \pm 8.0 \times 10^{-2}$
10	W33	$6.7 \pm 6.1 \times 10^{-1}$	$2.0 \times 10^{-2} \pm 4.8 \times 10^{-3}$	$1.4 \times 10^{1} \pm 4.3$	$8.6 \times 10^{-2} \pm 2.0 \times 10^{-2}$
	W37	$1.1 \times 10^{1} \pm 1.4$	$2.3 \times 10^{-2} \pm 1.5 \times 10^{-2}$	$1.7 \times 10^{1} \pm 9.7$	$8.0 \times 10^{-2} \pm 2.9 \times 10^{-2}$
11	W33	$2.7 \times 10^{1} \pm 1.7$	$2.9 \times 10^{-3} \pm 1.7 \times 10^{-3}$	2.3 ± 1.8	$3.2 \times 10^{-2} \pm 3.3 \times 10^{-3}$
	W37	$3.0 \times 10^{1} \pm 5.6$	$1.3 \times 10^{-2} \pm 8.5 \times 10^{-3}$	$1.3 \pm 7.5 \times 10^{-1}$	$3.6 \times 10^{-2} \pm 1.3 \times 10^{-2}$
12	W33	$2.2 \pm 5.6 \times 10^{-1}$	$1.5 \times 10^{1} \pm 2.3$	$1.4 \times 10^{1} \pm 2.9$	$2.2 \times 10^{-1} \pm 2.1 \times 10^{-2}$

	W37	$2.0 \pm 3.1 \times 10^{-1}$	$8.7 \pm 5.6 \times 10^{-1}$	$1.2 \times 10^{1} \pm 2.3$	$1.0 \times 10^{-1} \pm 1.8 \times 10^{-2}$
13	W33	$3.7 \times 10^{1} \pm 5.4$	$3.0 \times 10^{-2} \pm 4.5 \times 10^{-3}$	$7.0 \pm 2.6 \times 10^{-1}$	$2.7 \times 10^{-2} \pm 5.0 \times 10^{-4}$
	W37	$6.6 \times 10^{1} \pm 5.9$	$1.1 \times 10^{-2} \pm 2.2 \times 10^{-3}$	$6.8 \pm 6.6 \times 10^{-1}$	$5.7 \times 10^{-2} \pm 2.0 \times 10^{-3}$
14	W33	$2.2 \times 10^{1} \pm 8.5$	$1.7 \times 10^{-2} \pm 4.9 \times 10^{-3}$	$9.0 \pm 4.4 \times 10^{-1}$	$6.7 \times 10^{-2} \pm 6.6 \times 10^{-3}$
	W37	$1.6 \times 10^{1} \pm 4.9$	$2.8 \times 10^{-2} \pm 4.7 \times 10^{-3}$	$1.1 \times 10^{1} \pm 1.1$	$1.1 \times 10^{-1} \pm 1.8 \times 10^{-3}$
15	W33	$2.2 \times 10^{1} \pm 7.1$	$1.4 \times 10^{-2} \pm 7.1 \times 10^{-3}$	$1.8 \times 10^{1} \pm 5.6$	$1.1 \times 10^{-1} \pm 1.4 \times 10^{-2}$
	W37	$2.8 \times 10^{1} \pm 3.4$	$4.7 \times 10^{-3} \pm 2.3 \times 10^{-3}$	$1.1 \times 10^{1} \pm 2.4 \times 10^{-1}$	$7.4 \times 10^{-2} \pm 2.4 \times 10^{-3}$
Control (C)					
16	W33	$5.4 \times 10^{1} \pm 4.0$	$2.1 \times 10^{-2} \pm 5.6 \times 10^{-3}$	$1.1 \times 10^{1} \pm 4.6$	$6.8 \times 10^{-2} \pm 1.1 \times 10^{-2}$
	W37	$2.0 \times 10^{1} \pm 1.7$	$2.0 \times 10^{-2} \pm 7.4 \times 10^{-3}$	$1.4 \times 10^{1} \pm 5.0$	$5.6 \times 10^{-2} \pm 5.4 \times 10^{-3}$
17	W33	$5.5 \pm 5.3 \times 10^{-1}$	$6.0 \pm 1.6 \times 10^{-1}$	$1.2 \times 10^{1} \pm 4.3$	$5.9 \times 10^{-2} \pm 2.3 \times 10^{-2}$
	W37	$1.5 \times 10^{1} \pm 2.9$	$9.3 \pm 5.3 \times 10^{-1}$	$1.9 \times 10^{1} \pm 8.7$	$5.4 \times 10^{-2} \pm 1.0 \times 10^{-2}$
18	W33	$2.6 \pm 1.6 \times 10^{-1}$	$1.8 \pm 3.5 \times 10^{-2}$	$1.3 \times 10^{1} \pm 5.5$	$8.8 \times 10^{-2} \pm 1.7 \times 10^{-2}$
	W37	$1.2 \times 10^{1} \pm 2.0$	$2.9 \pm 7.5 \times 10^{-2}$	$3.3 \times 10^{1} \pm 4.4$	$4.5 \times 10^{-2} \pm 2.8 \times 10^{-3}$
19	W33	$7.6 \times 10^{1} \pm 3.3 \times 10^{-1}$	$1.2 \pm 7.9 \times 10^{-3}$	$1.3 \times 10^{1} \pm 3.6$	$1.9 \times 10^{-1} \pm 3.2 \times 10^{-3}$
	W37	$2.7 \times 10^{1} \pm 3.8$	$2.7 \times 10^{-2} \pm 4.7 \times 10^{-3}$	8.2 ± 4.6	$1.1 \times 10^{-1} \pm 2.6 \times 10^{-2}$
20	W33	$1.6 \times 10^{1} \pm 1.4$	$1.1 \times 10^{1} \pm 1.2$	$1.2 \times 10^{1} \pm 5.5$	$8.6 \times 10^{-2} \pm 1.5 \times 10^{-2}$
	W37	$1.0 \times 10^{1} \pm 6.4 \times 10^{-2}$	$1.1 \times 10^{1} \pm 1.4$	$1.2 \times 10^{1} \pm 4.7$	$1.1 \times 10^{-1} \pm 3.1 \times 10^{-2}$
21	W33	$5.6 \times 10^{1} \pm 8.3$	$1.7 \times 10^{-2} \pm 1.7 \times 10^{-3}$	$2.1 \times 10^{1} \pm 1.0 \times 10^{1}$	$1.3 \times 10^{-1} \pm 2.0 \times 10^{-2}$
	W37	$6.4 \times 10^{1} \pm 1.5$	$3.3 \times 10^{-2} \pm 8.7 \times 10^{-3}$	$2.2 \times 10^{1} \pm 1.0 \times 10^{1}$	$1.2 \times 10^{-1} \pm 2.4 \times 10^{-2}$
22	W33	$4.3 \times 10^{1} \pm 2.0$	$1.2 \times 10^{-1} \pm 2.8 \times 10^{-2}$	$2.3 \times 10^{-1} \pm 1.5 \times 10^{-2}$	0.0 ± 0.0
	W37	$6.8 \times 10^{1} \pm 5.1$	$2.7 \times 10^{-2} \pm 6.6 \times 10^{-3}$	$1.9 \times 10^{-1} \pm 2.0 \times 10^{-2}$	0.0 ± 0.0
23	W33	$2.6 \times 10^{1} \pm 5.6$	$2.3 \times 10^{-1} \pm 3.6 \times 10^{-2}$	0.0 ± 0.0	0.0 ± 0.0
	W37	$6.3 \times 10^{1} \pm 2.0$	$8.2 \times 10^{-3} \pm 1.9 \times 10^{-3}$	$1.6 \times 10^{-1} \pm 2.9 \times 10^{-2}$	$5.3 \times 10^{-1} \pm 1.8 \times 10^{-1}$
24	W33	$1.2 \times 10^{1} \pm 1.0$	$2.7 \times 10^{1} \pm 2.1 \times 10^{-1}$	$1.8 \pm 1.5 \times 10^{-1}$	$6.8 \times 10^{-1} \pm 3.4 \times 10^{-2}$
	W37	$7.5 \times 10^{1} \pm 3.8$	$9.7 \times 10^{-3} \pm 3.7 \times 10^{-3}$	$3.7 \times 10^{-1} \pm 3.4 \times 10^{-2}$	0.0 ± 0.0
25	W33	$6.5 \times 10^{1} \pm 1.0 \times 10^{1}$	$3.0 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$7.5 \times 10^{-2} \pm 7.5 \times 10^{-3}$	0.0 ± 0.0
	W37	$6.6 \times 10^{1} \pm 7.1$	$9.1 \times 10^{-3} \pm 5.1 \times 10^{-4}$	$2.5 \times 10^{-1} \pm 2.7 \times 10^{-2}$	0.0 ± 0.0
26	W33	$8.5 \times 10^{1} \pm 6.3$	$4.4 \pm 9.3 \times 10^{-1}$	$3.2 \times 10^{-1} \pm 3.9 \times 10^{-2}$	0.0 ± 0.0
	W37	$5.4 \times 10^{1} \pm 4.5$	$2.0 \times 10^{-2} \pm 6.1 \times 10^{-4}$	$3.6 \times 10^{-1} \pm 4.2 \times 10^{-2}$	0.0 ± 0.0
27	W33	$7.0 \times 10^{1} \pm 1.5 \times 10^{1}$	$3.3 \times 10^{-2} \pm 4.7 \times 10^{-3}$	$2.8 \times 10^{-1} \pm 2.6 \times 10^{-2}$	0.0 ± 0.0
	W37	$6.6 \times 10^{1} \pm 3.6 \times 10^{-1}$	$2.1 \times 10^{-2} \pm 1.6 \times 10^{-2}$	$4.0 \times 10^{-1} \pm 3.8 \times 10^{-2}$	0.0 ± 0.0

between W33 and W37 in both P and C groups. However, in spite of the lack of statistical relevance, a weak modulation was observed for *Bifidobacterium* and *Atopobium*. Regarding bifidobacteria (Figure 1.3B), a physiological tendency to decrease was observed in vaginal samples of control women at the end of the study period (mean value, W33: $4.3 \pm 2.2 \times 10^{-1}$; W37: $2.0 \pm 1.7 \times 10^{-1}$). This trend seemed to be counterbalanced in the women consuming VSL#3 since bifidobacteria amount slightly increased during the treatment period (mean value, W33: $9.9 \times 10^{-1} \pm 1.6 \times 10^{-1}$; W37: $1.4 \pm 1.2 \times 10^{-1}$). An opposite trend was observed for *Atopobium* (Figure 1.3C). This genus increased at W37 (mean value, 9.2 ± 3.2) compared to W33 (mean value, 7.0 ± 2.8) in C group, while it remained constant after the VSL#3 treatment (mean value, W33: $1.4 \times 10^{1} \pm 3.8$; W37: $1.3 \times 10^{1} \pm 5.2$).

Immunological profiles

The effect of the probiotic intake on the vaginal immune response was evaluated by measuring the levels of 27 cytokines, chemokines and growth factors in the vaginal samples of the pregnant women belonging to P and C groups.

Figure 1.4 shows the cytokines and chemokines whose concentration significantly changed in P and C groups during the study period ($P < 0.05$). In group C, significant reductions at W37 were found for 5 mediators, 4 cytokines [IL-4 (mean value, W33: $2.8 \times 10^{-2} \pm 1.5 \times 10^{-2}$; W37: $1.3 \times 10^{-2} \pm 6.9 \times 10^{-3}$), IL-7 (mean value, W33: $1.2 \times 10^{-1} \pm 8.6 \times 10^{-2}$; W37: $6.1 \times 10^{-2} \pm 3.5 \times 10^{-2}$), IL-9 (mean value, W33: $1.1 \pm 5.6 \times 10^{-1}$; W37: $3.7 \times 10^{-1} \pm 1.5 \times 10^{-1}$) and IL-10 (mean value, W33: $1.5 \times 10^{-1} \pm 1.1 \times 10^{-1}$; W37: $9.4 \times 10^{-2} \pm 5.4 \times 10^{-2}$)] and 1 chemokine [RANTES (mean value, W33: 4.3 ± 2.9; W37: $1.3 \pm 3.9 \times 10^{-1}$)]. Both IL-4 and IL-10 are produced by Th_2 cells

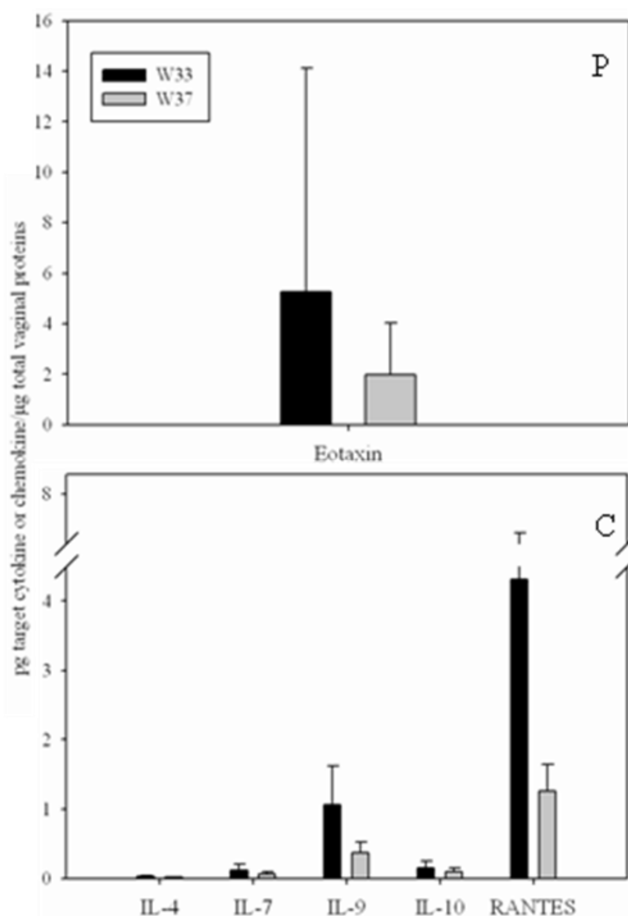

Figure 1.4. Cytokines and chemokines whose concentration significantly changed during the study period ($P < 0.05$). P, probiotic group; C, control group; W33, 33rd gestational week; W37, 37th gestational week. Cytokine or chemokine names are reported in x-axis.

and exert a regulatory role in the immune response. IL-7 and IL-9 are hematopoietic growth factors that control proliferation and homeostasis of a variety of hematopoietic cells. RANTES is a pro-inflammatory chemokine which attracts monocytes, lymphocytes, basophils and eosinophils in the inflammatory response. In P group a significant variation was registered only for the chemokine Eotaxin, which decreased after the probiotic treatment (mean value, W33: 5.3 ± 8.8; W37: 2.0 ± 2.1). Eotaxin exerts a pro-inflammatory activity by recruiting eosinophils during allergic responses.

Figure 1.5 shows the women, belonging to P and C groups, who registered significant variations in total levels of immune-mediators during the study period ($P < 0.05$). Significant changes were found for women N. 18, 19, 20, 21, 23, 24, 25 and 27 (8/12; 67%) of C group and women N. 1, 2, 3, 10, 11 (5/15; 33%) of P group.

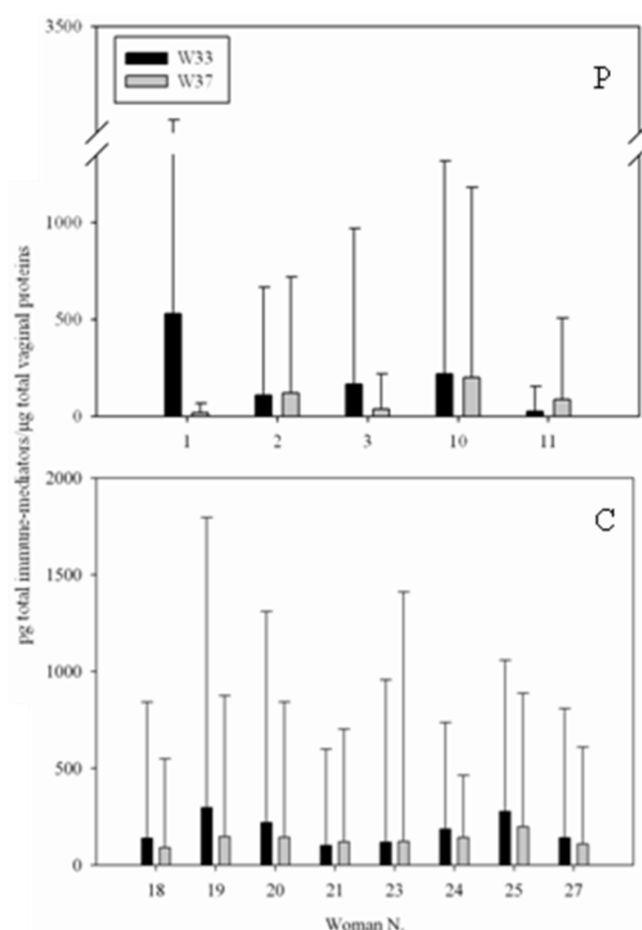

Figure 1.5. Women registering significant variations in total levels of immune-mediators. P, probiotic group; C, control group; W33, 33rd gestational week; W37, 37th gestational week. The identification numbers of the women registering significant variations are reported in x-axis.

Discussion

To our knowledge, this is the first study describing the effect of a probiotic mixture, orally consumed during the last trimester of pregnancy, on the vaginal microbiota and immune response. Although several health-promoting activities of probiotics have been described in relation to the gut homeostasis (Hart *et al*, 2004; Reid *et al*, 2005), less information is available regarding the interactions between orally administered probiotic bacteria and the vaginal microbial habitat.

The first step in ascertaining the influence of the dietary supplementation with the probiotic VSL#3 on the vaginal ecosystem of pregnant women was the characterization of vaginal bacterial communities by using an integrated approach based on PCR-DGGE and qPCR.

DGGE population profiling, conducted with universal primers for bacteria and *Lactobacillus*-specific primers, allowed to investigate the variations of the predominant vaginal bacterial communities and lactobacilli species occurring both physiologically in the last trimester of pregnancy and potentially associated with VSL#3 intake. The influence of the probiotic intake in modulating the predominant bacterial populations and lactobacilli species could be hypothesized since significant differences between DGGE profiles at W33 and W37 were found only in women belonging to P group. Notably, the lower percentage of women belonging to P group who displayed significant differences in *Lactobacillus*-specific DGGE profiles between W33 and W37, compared to the universal bacterial DGGE patterns, suggested a major stability of lactobacilli population and a more extended impact of the probiotic VSL#3 on total bacteria than lactobacilli. However, no significant changes in single species abundances were found between W33- and W37-related universal DGGE profiles. Differently, the statistical analysis of the peak heights of the *Lactobacillus*-specific DGGE densitometric curves allowed to identify a band, corresponding to *L. helveticus*, which significantly decreased after the probiotic treatment. Strains belonging to *L. helveticus* are used as starter cultures in the manufacturing of a variety of fermented dairy products, to modulate flavor. The presence of *L. helveticus* in vagina, likely due to the migration from the gut, can be related to a diet rich in yogurt and cheese. This work is not the first describing *L. helveticus* in vaginal samples. Stoyancheva *et al* identified this species among several *Lactobacillus* isolates from vaginal fluids of healthy Bulgarian women in childbearing age by using three different molecular techniques, amplified ribosomal DNA restriction analysis, ribotyping and PCR with species-specific primers (Stoyancheva *et al*, 2006). The decrease of *L. helveticus* observed in our study could be due to a competition between the *Lactobacillus* strains present in VSL#3 formula and dairy *L. helveticus* strains in colonizing vaginal environment.

Cluster analysis of universal and *Lactobacillus*-specific DGGE profiles confirmed the stability of the vaginal microbiota in the last trimester of pregnancy since the DGGE profiles related to the time points W33 and W37 of the control women were closely related. Also the DGGE patterns of the majority of the women administered with VSL#3 grouped according to the subject and not to the time point, revealing that the inter-individual variability was higher than variability induced by the probiotic supplementation. Thus, the DGGE population profiling suggested the ability of the probiotic VSL#3 to modulate the vaginal microbiota, without compromising the inter-individual species variability.

The hypothesis of a positive action of VSL#3 on the vaginal microbiota of pregnant women was further supported by qPCR results, which suggested a role of the probiotic product in counteracting the decrease of the health-promoting *Bifidobacterium* genus and the increase of the BV-related *Atopobium* genus, that physiologically occur in late pregnancy.

The second step of the present research was the investigation of the vaginal immunological profiles of the pregnant women in order to search for correlations between the VSL#3 intake and changes in vaginal immune response. Pregnancy has been referred to as a state of relative immune compromise. This notion has been related to both demonstration of depression of certain aspects of cell-mediated immunity and clinical observations of an increased severity of numerous infectious conditions in pregnant women (Beigi *et al*, 2007). On the other hand, preterm cervical ripening can be likened to an inflammatory process with cytokines as important mediators (Törnblom *et al*, 2005).

Bioplex immunoassay was used in the present work to measure levels of 27 cytokines, chemokines and growth factors in the vaginal samples of the pregnant women belonging to P and C groups. In group C a significant reduction at W37 was found for IL-4, IL-7, IL-9, IL-10 and RANTES. IL-4 is a key regulator in humoral and adaptive immunity. It has many biological roles, including the stimulation of activated B-cells and T-cell proliferation, and the differentiation of CD4+ T-cells into Th_2 cells. A regulatory role is also exerted by IL-10. In relation to pregnancy, IL-10 decreases the production of pro-inflammatory cytokines, such as IL-8, IL-6, TNFα, IL-1β and prostaglandin E2 in lipopolysaccharide-stimulated fetal membranes (Brown *et al*, 2000; Fortunato *et al*, 1997). Both IL-4 and IL-10 are produced by Th_2 cells. IL-7 and IL-9 are hematopoietic growth factors that act as regulators of cell survival, proliferation and homeostasis of a variety of hematopoietic cells. RANTES is a potent and versatile chemokine, capable of attracting monocytes, lymphocytes, basophils and eosinophils. This cytokine has been implicated in the regulation of the inflammatory response and recruitment of macrophages to the implantation site in early pregnancy (Athayde *et al*, 1999). However, no variations in RANTES levels have been associated with preterm cervical ripening and labor (Törnblom *et al*, 2005). Immunological profiles related to the women belonging to C group indicated that some fluctuations in vaginal immune-modulators occurred physiologically during the last trimester of pregnancy. In particular, it is noteworthy the decrease of IL-10 and IL-4, important regulatory cytokines controlling the inflammatory reaction responsible for uterine contractions and cervical ripening at the labor time (Dubicke *et al*, 2010). In P group a significant variation was registered only for the chemokine Eotaxin, which decreased after the probiotic treatment. Eotaxin selectively recruits eosinophils, and for this reason is implicated in allergic responses (Garcia-Zepeda *et al*, 1996). By comparing the data related to the

two study groups, the following hypotheses could be formulated regarding the possible impact of the probiotic intake on the cytokine secretion during late pregnancy: probiotics counteracted the decrease of anti-inflammatory cytokine levels occurring in C group; probiotics induced the decrease of a pro-inflammatory cytokine in P group, showing a global anti-inflammatory effect on the vaginal immunity. In addition, a stabilization effect on the vaginal immunity during late pregnancy could be attributed to the probiotic intake, since the percentage of women with modified amounts of immune-mediators decreased from 67% to 31% in relation to the dietary supplementation.

The impact of the oral intake of the probiotic VSL#3 on the vaginal microbiota and immune response of pregnant women was investigated by molecular fingerprinting techniques (PCR-DGGE and qPCR) and Luminex® immunoassay. The major findings of this study are the following: the VSL#3 intake seems to be associated with a modulation of the predominant vaginal bacterial communities, without compromising the inter-individual species variability; VSL#3 modulation of lactobacilli population appears to be related to variations of *L. helveticus* species; a potential role of the probiotic product in counteracting the physiological decrease of *Bifidobacterium* and increase of *Atopobium* could be hypothesized; the probiotic treatment can be associated with a global anti-inflammatory effect on the vaginal immunity, with potential implications in preventing preterm birth.

CHAPTER 2

EFFICACY OF RIFAXIMIN VAGINAL TABLETS IN THE TREATMENT OF BACTERIAL VAGINOSIS: A MOLECULAR CHARACTERIZATION OF THE VAGINAL MICROBIOTA

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Paper submitted to Antimicrobial Agents and Chemotherapy

Background

Bacterial vaginosis (BV), a common vaginal syndrome affecting pre- and post-menopausal, non-pregnant as well as pregnant women, is a complex, polymicrobial disorder associated with an increase of the taxonomic richness and diversity of the vaginal microbiota (Ling *et al*, 2010; Oakley *et al*, 2008). It is characterized by an overgrowth of *Gardnerella vaginalis*, anaerobic Gram-negative rods, anaerobic Gram-positive cocci, *Mycoplasma hominis*, and *Mobiluncus* spp., and a reduction in lactobacilli, particularly those producing hydrogen peroxide (Ling *et al*, 2010; Ling *et al*, 2011; Turovskiy *et al*, 2011). The clinical consequences of BV could be important. Indeed, 40% of the cases of spontaneous preterm labor and preterm birth are thought to be associated with BV or other types of lactobacilli devoid flora (Donders *et al*, 2011; Pretorius *et al*, 2007). Furthermore, in this state of diminished colonization resistance, BV renders women particularly vulnerable to the acquisition of HIV and other sexually transmitted diseases, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and Herpes simplex virus type-2 infection (Cherpes *et al*, 2003; Martin *et al*, 1999; Wiesenfeld *et al*, 2003). The diagnosis of BV is based on Amsel's criteria (Amsel *et al*, 1983) or Nugent score (Nugent *et al*, 1991) determinations. Current therapy of BV involves effective oral or intra-vaginal administration of metronidazole or clindamycin, but long-term follow-up suggests recurrence rates as high as 58% one year after treatment (Bradshaw *et al*, 2006-a). These high recurrence rates resulting in repeated exposure to antibiotics and the emergence of drug-resistant strains suggest a need for alternative therapeutic tools.

Rifaximin is a semisynthetic rifamycin derivative, with a broad antimicrobial spectrum (Rivkin and Gim, 2011; Scarpignato and Pelosini, 2005) and a good safety profile because of its negligible grade of systemic absorption. Thanks to its antibacterial activity covering *G. vaginalis* and other pathogens responsible for urogenital infections (Hoover *et al*, 1993), rifaximin could be a suitable alternative for the local treatment of BV.

The aim of the study was to evaluate the impact of two doses of rifaximin vaginal tablets (100 mg and 25 mg) administered for different periods (2 days and 5 days) on the vaginal microbiota of women affected by BV. Bacterial DNA extracted from standardized vaginal rinsings was analyzed using the culture-independent techniques PCR-DGGE and real-time quantitative PCR (qPCR).

Material and methods

Study population

The present molecular study was performed on the vaginal fluids collected from 102 patients enrolled in a multicentre, double-blind, randomized, placebo-controlled study (EudraCT: 2009-011826-32). Diagnosis of BV was made using both Amsel's criteria and Nugent scoring at the screening visit (V1). Patients with Nugent score > 3 and positive for at least 3 of 4 Amsel's criteria were included. At the randomization visit (V2), the patients were distributed into 4 treatment groups: group A received rifaximin 100 mg vaginal tablet once daily for 5 days, group B received rifaximin 25 mg vaginal tablet once daily for 5 days, group C received rifaximin 100 mg vaginal tablet once daily for the first 2 days and placebo vaginal tablet for the remaining 3 days, group D received placebo vaginal tablet once daily for 5 days. Study medication was administered intra-vaginally at bedtime. At 7 days after the end of the therapy the first follow-up visit (V3) was performed. Patients showing remission according to Amsel's criteria and Gram stain Nugent score attended the second follow-up visit (V4) 28 days after the end of the treatment. Table 2.1 shows the distribution of the selected 102 patients into the four treatment groups according to the geographic origin and the rate of remission. Rifaximin and placebo vaginal tablets were purchased by Alfa Wassermann S.p.A., Bologna, Italy.

Standardized vaginal rinsings with 2 ml of saline were collected for molecular studies at V1, V3 and V4, by flushing and re-aspirating the fluid through a 22 Gauge needle in the left, central and right upper vaginal vaults as described elsewhere (Donders *et al*, 2009). The vaginal rinsings were subsequently stored at -80°C until use.

Extraction of bacterial DNA from vaginal rinsings.

One ml of vaginal rinsings was centrifuged at 9,500 × *g* for 15 min, and the pellets washed three times in saline at 40°C. Each pellet was resuspended in 180 µl of Enzymatic Lysis Buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) and incubated at 37°C for 30 min. Glass beads (200 mg) were added and the sample was mixed by vortexing for 1 min. Total DNA was extracted by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol "Pretreatment for Gram-positive bacteria". A slight modification was introduced: a centrifugation step (6,000 × *g* for 5 min) was carried out after the incubation with proteinase K to remove glass beads. DNA amounts were quantified by using NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

Table 2.1. Distribution of the patients into the treatment groups (A, B, C and D) according to the geographic origin and the rate of remission

	Treatment groups			
	A (100 mg/5d)	B (25 mg/5d)	C (100 mg/2d)	D (PBO)
Total (N:)	27	25	25	25
Italy (N:)	7	2	1	2
Germany (N:)	9	11	13	12
Belgium (N:)	11	12	11	11
Remission at V3 (N:)	6	12	9	4
Remission at V3 (%)	22	48	36	16

Real-time quantitative PCR

Quantitative PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Each DNA sample was amplified with different genus- or species-specific primer sets targeted to 16S rRNA gene or 16S-23S rRNA spacer region: Bact-0011f/Lab-0677r (Heilig *et al*, 2002) for *Lactobacillus*, F-GV1/R-GV3 (Zariffard *et al*, 2002) for *G. vaginalis*, c-Atopo-f/c-Atopo-r (Matsuki *et al*, 2004) for Atopobium, g-Prevo-f/g-Prevo-r (Matsuki *et al*, 2002) for *Prevotella*, VeilloF/VeilloR (Rinttilä *et al*, 2004) for *Veillonella*, MycF/MycR (Wiesenfeld *et al*, 2003) for *M. hominis* and Mob-s/Mob-as (Tiveljung *et al*, 1996) for *Mobiluncus*. Amplifications were carried out in a final volume of 20 µl containing 0.5 µM of each primer, 4 µl of LightCycler-

FastStart DNA Master SYBR Green I (Roche) and either 2 µl of template or water (no-template control). The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min followed by 30 (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*, *Mobiluncus*), 35 (*Prevotella*) or 40 (*M. hominis*) cycles of denaturation at 95°C for 15 s; primer annealing at 63°C (*Lactobacillus*, *Mobiluncus*), 62°C (*Veillonella*, *M. hominis*), or 60°C (*Atopobium*, *Prevotella*, *G. vaginalis*) for 20 s; extension at 72°C for 45 s (*Lactobacillus*, *Atopobium*, *Prevotella*, *G. vaginalis*, *Veillonella*) or 30 s (*M. hominis*, *Mobiluncus*) and a fluorescence acquisition step at 82°C (*M. hominis*), 85°C (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*), 87°C (*Prevotella*) or 88°C (*Mobiluncus*) for 5 s. DNAs extracted from *Lactobacillus acidophilus* NCFM, *Gardnerella vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, *Veillonella parvula* ATCC 10790, *Atopobium vaginae* ATCC BAA-55, *Mycoplasma hominis* DSM 19104 and *Mobiluncus curtisii* ATCC 43063 were used as standards for PCR quantification. DNAs extracted from vaginal samples were amplified in triplicate for each primer set. Data were expressed as ng of DNA of the targeted genus or species per µg of total DNA extracted from the vaginal sample.

PCR-DGGE analysis

Amplification of the V2-V3 region of the bacterial 16S rRNA gene was carried out using the universal eubacterial primers GCclamp-HDA1 and HDA2 (Walter *et al*, 2000), supplied by M-Medical, Milan, Italy. The amplification reactions were performed in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany). GoTaq Flexi DNA Polymerase (Promega, Madison, WI) was used as thermostable DNA polymerase. The reaction mixture contained 0.5 µM of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$ solution, 1.25 U of GoTaq Flexi DNA Polymerase, 5 µl of Green GoTaq Flexi buffer 5×, and 30 ng of the bacterial DNA template in a final volume of 25 µl. The thermocycle program consisted of the following time and temperature profile: 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 60 s; and 72°C for 8 min. Samples (5 µl) of the amplified products (200 bp) were subjected to gel electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

DGGE analysis was performed using the D-Code Universal Mutation System Apparatus (Bio-Rad, Hercules, CA) with 20-cm by 20-cm by 0.75-mm gels. The sequence-specific separation of the PCR fragments was obtained in 8% (w/v) polyacrylamide (acrylamide-N,N'bisacrylamide, 40:3 w/v) gels in 0.5× TAE buffer (20 mM Tris, 10 mM glacial acetic acid and 0.5 mM EDTA pH 8). The denaturing gels contained a 30% to 50% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (v/v) formamide and 7 M urea. A stacking gel containing 8% (w/v) polyacrylamide was applied onto the denaturing gel. A

volume of 8 µl of PCR samples was loaded onto the stacking gel. Electrophoresis was conducted at an initial voltage of 150 V for 5 minutes and then at a constant voltage of 90 V and temperature of 60°C for 16 h. Following electrophoresis, the gel was silver stained (Bassam *et al*, 1991) and scanned using a Molecular Imager Gel Doc XR System (Bio-Rad). Cluster analysis of the DGGE patterns was performed using the FPQuest software version 4.5 (Bio-Rad) with the Ward clustering algorithm. The Similarity Indexes (SIs) of profiles were calculated on the basis of the Pearson correlation coefficient. SI indicates the % of similarity among 2 or more DGGE profiles belonging to the same woman, taking into account the presence/absence and intensity of the bands. The Richness Indexes (RIs), of the profiles were also calculated from the densitometric curves, using FPQuest. RI indicates the number of bands of each DGGE profile.

Statistical analysis

Statistical analysis was performed using SigmaStat (Systat Software, Point Richmond, CA). Differences in the amounts of the bacterial genera and species determined by qPCR were analyzed using Wilcoxon Signed Rank Test. Kluskal-Wallis One Way Analysis of Variance (ANOVA) on Ranks test was applied to compare the SIs of the DGGE profiles related to V1-V3 for all women, and V1-V3 and V3-V4 for women who were in remission at V3. Subsequently, Dunn's test was used to make all pairwise comparisons. Differences in the SI values for different dosages and duration of treatment were evaluated by a Two Way ANOVA test. Wilcoxon Signed Rank Test was used to compare the RIs of the DGGE profiles related to V1-V3 for all women, and V1-V3 and V3-V4 for women who were in remission at V3. Finally, a Friedman Repeated Measures ANOVA on Ranks test was used to compare the RIs of the DGGE profiles related to V1, V3 and V4 for women who were in remission at V3, and Tukey's test was then used to make all pairwise comparisons. A P value below 0.05 was considered significant in all statistical tests.

Results

Variations of lactobacilli and BV-related bacterial populations

qPCR was performed to quantify *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis* and *Mobiluncus*, that are the principal bacterial groups which change in presence of BV (Biagi *et al*, 2009; Lamont *et al*, 2011; Vitali *et al*, 2007). Efficiency of qPCR for

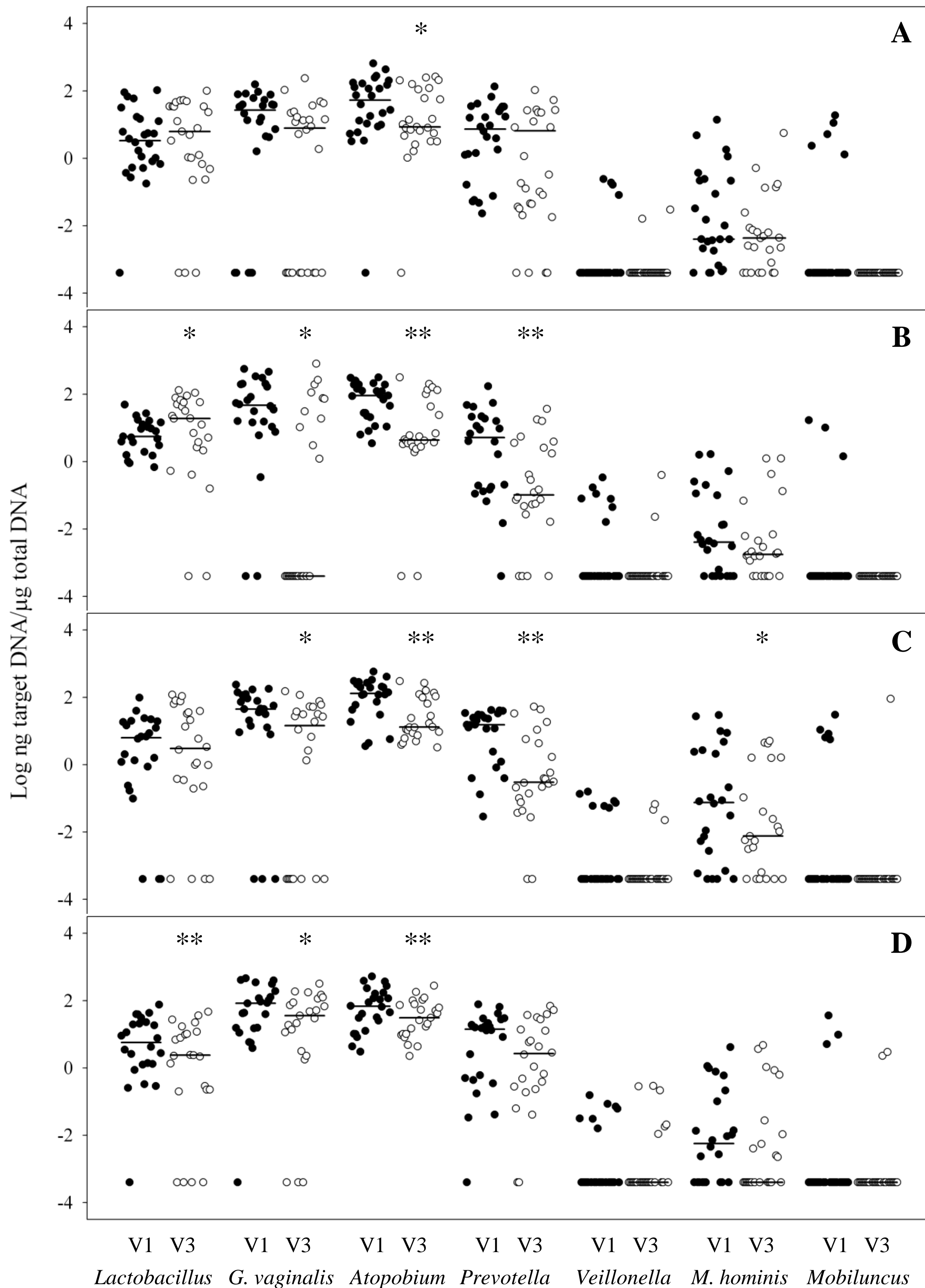


Figure 2.1. qPCR evaluation of *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis* and *Mobiluncus* at time points V1 and V3 for the treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo). Data are related to the total number of women analyzed (N: 102). The scatter plots show the distributions of quantification data, with the horizontal bars representing the median values. *$P < 0.05$; **$P < 0.01$.

all assays was between 90% and 110% and correlation coefficients for genomic DNA standards were > 0.99.

At the baseline (V1) the percentages of women harboring *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis* and *Mobiluncus* were 95%, 89%, 99%, 98%, 30%, 78% and 20%, respectively. These data show the high frequency of occurrence of *G. vaginalis*, *Atopobium*, *Prevotella* and *M. hominis* in the vaginal ecosystem of women affected by BV, differently from *Veillonella* and *Mobiluncus*, which were detected in less than 50% of the patients.

Quantitative variations of lactobacilli and BV-related bacterial groups were visualized in scatter plots showing the distributions of qPCR data expressed as log of ng of DNA of the target genus or species per μg of total bacterial DNA extracted from the vaginal sample, with the horizontal bars representing the log median values (Figure 2.1 and 2.2).

Figure 2.1 shows the qPCR results at V1 and V3 related to all the women analyzed (N: 102), randomized into the four treatment groups (A, B, C, D). Treatment with rifaximin 100 mg/day for 5 days (group A), induced a slight increase in the amount of *Lactobacillus* and a decrease in *G. vaginalis* and *Atopobium*, with a significant reduction only for *Atopobium* genus [V1: 1.73 log ng/μg; V3: 0.93 log ng/μg; ($P = 0.02$)]. At V3 the log median value of this genus was similar to that of lactobacilli. Concentrations of the other BV-related groups did not change after the treatment. In response to the administration with rifaximin 25 mg/day for 5 days (group B), *Lactobacillus* significantly increased [V1: 0.75 log ng/μg; V3: 1.28 log ng/μg; ($P = 0.03$)] and *G. vaginalis* [V1: 1.67 log ng/μg; V3: -3.40 log ng/μg; ($P = 0.01$)], *Atopobium* [V1: 1.96 log ng/μg; V3: 0.64 log ng/μg; ($P = 0.005$)] and *Prevotella* [V1: 0.72 log ng/μg; V3: -0.99 log ng/μg; ($P = 0.004$)] significantly decreased at V3. A slight decrease at V3 was also observed for *M. hominis*. Comparing all the bacterial concentrations at V3, *Lactobacillus* log median value was the highest one. These data indicate the efficacy of the treatment with rifaximin 25 mg/day for 5 days. Treatment with rifaximin 100 mg/day for 2 days (group C) resulted in a significant reduction of *G. vaginalis* [V1: 1.65 log ng/μg; V3: 1.16 log ng/μg; ($P = 0.01$)], *Atopobium* [V1: 2.12 log ng/μg; V3: 1.11 log ng/μg; ($P = 0.003$)], *Prevotella* [V1: 1.18 log ng/μg; V3: -0.52 log ng/μg; ($P = 0.003$)] and *M. hominis* [V1: -1.13 log ng/μg; V3: -2.12 log ng/μg; ($P = 0.01$)], together with a slight reduction of *Lactobacillus* genus. Differently from the group B, in the group C the log median values of *G. vaginalis* and *Atopobium* at V3 were higher than that of lactobacilli, suggesting that rifaximin 100 mg/day for 2 days was less effective against BV than rifaximin 25 mg/day for 5 days. In the placebo group (group D), a reduction in the amounts of all the bacterial groups was observed, with statistical significance for *Lactobacillus* [V1: 0.76 log ng/μg; V3: 0.38 log ng/μg; ($P = 0.007$)], *G. vaginalis* [V1: 1.92 log ng/μg; V3: 1.55 log ng/μg; ($P = 0.04$)] and *Atopobium* [V1: 1.83 log ng/μg; V3: 1.49

log ng/µg; (*P* = 0.005)]. However, the log median values of *G. vaginalis* and *Atopobium* remained higher than that of lactobacilli at V3. In general, no variations were observed for *Veillonella* and *Mobiluncus*, being these genera found in a low percentage of women, as described above, and at low concentrations (< -0.47 log ng/µg and < 1.56 log ng/µg, respectively). However, a decrease in the percentage of women who harbored *Veillonella* and *Mobiluncus* was observed in all the treatment groups after the antibiotic administration [*Veillonella* (A, V1: 11.1% *vs* V3: 7.4%; B, V1: 24.0% *vs* V3: 8.0%; C, V1: 28.0% *vs* V3: 12.0%; D, V1: 28.0% *vs* V3: 24.0%); *Mobiluncus* (A, V1: 4.0% *vs* V3: 0%; B, V1: 8.0% *vs* V3: 0%; C, V1: 20.0% *vs* V3: 4.0%; D, V1: 16.0% *vs* V3: 12.0%)].

Figure 2.2 shows the qPCR results at V1, V3 and V4 related to the women who were in remission at V3 and who performed the last study visit V4 (N: 31), divided into the four treatment groups. Treatment with rifaximin 100 mg/day for 5 days (group A) did not significantly affect any bacterial group, even though a reduction trend was observed for the BV-related bacteria, and *Lactobacillus* log median concentration after the treatment was higher than that of the other genera and species. Treatment with rifaximin 25 mg/day for 5 days (group B) caused an increase of *Lactobacillus* at V3 and V4, which was significant at V4 [V1: 0.72 log ng/µg; V4: 1.57 log ng/µg; (*P* = 0.04)]. A significant decrease at V3 and V4 compared to V1 was observed for *G. vaginalis* [V1: 1.73 log ng/µg; V3: -3.40 log ng/µg; V4: -3.40 log ng/µg; (*P* = 0.004, V3 *vs* V1), (*P* = 0.004, V4 *vs* V1)], *Atopobium* [V1: 2.02 log ng/µg; V3: 0.52 log ng/µg; V4: 0.56 log ng/µg; (*P* = 0.004, V3 *vs* V1), (*P* = 0.02, V4 *vs* V1)] and *M. hominis* [V1: -2.32 log ng/µg; V3: -2.81 log ng/µg; V4: -3.40 log ng/µg; (*P* = 0.02, V3 *vs* V1), (*P* = 0.04, V4 *vs* V1)]. *Prevotella* decreased at V3 and V4, with a significant variation at V3 only [V1: 0.95 log ng/µg; V3: -1.27 log ng/µg; (*P* = 0.002)]. Treatment with rifaximin 100 mg/day for 2 days (group C) showed a relapse at V4, with *Lactobacillus* concentration increasing at V3 and significantly decreasing at V4 compared to V3 [V3: 1.80 log ng/µg; V4: 0.33 log ng/µg; (*P* = 0.03)], and BV-related bacterial groups decreasing at V3 and increasing again at V4, with significance for *G. vaginalis* [V1: 1.91 log ng/µg; V3: -3.40 log ng/µg; V4: 1.96 log ng/µg; (*P* = 0.005, V3 *vs* V1), (*P* = 0.05, V4 *vs* V3)], *Atopobium* [V1: 2.09 log ng/µg; V3: 0.98 log ng/µg; V4: 2.17 log ng/µg; (*P* = 0.003, V3 *vs* V1), (*P* = 0.005, V4 *vs* V3)] and *Prevotella* [V1: 1.18 log ng/µg; V3: -1.06 log ng/µg; V4: 1.79 log ng/µg; (*P* = 0.008, V3 *vs* V1), (*P* = 0.03, V4 *vs* V3)]. Despite of the absence of significant variations in the placebo group (group D), *Lactobacillus* gradually decreased at V3 and V4 while *Prevotella* decreased at V3 and increased again at V4. Notably, *Lactobacillus* log median concentration was lower than that of *G. vaginalis* and *Atopobium* after the treatment. Similarly to the analysis of V1 and V3 for the total number of women, no variations were observed for *Veillonella* and *Mobiluncus*, due to the low concentrations

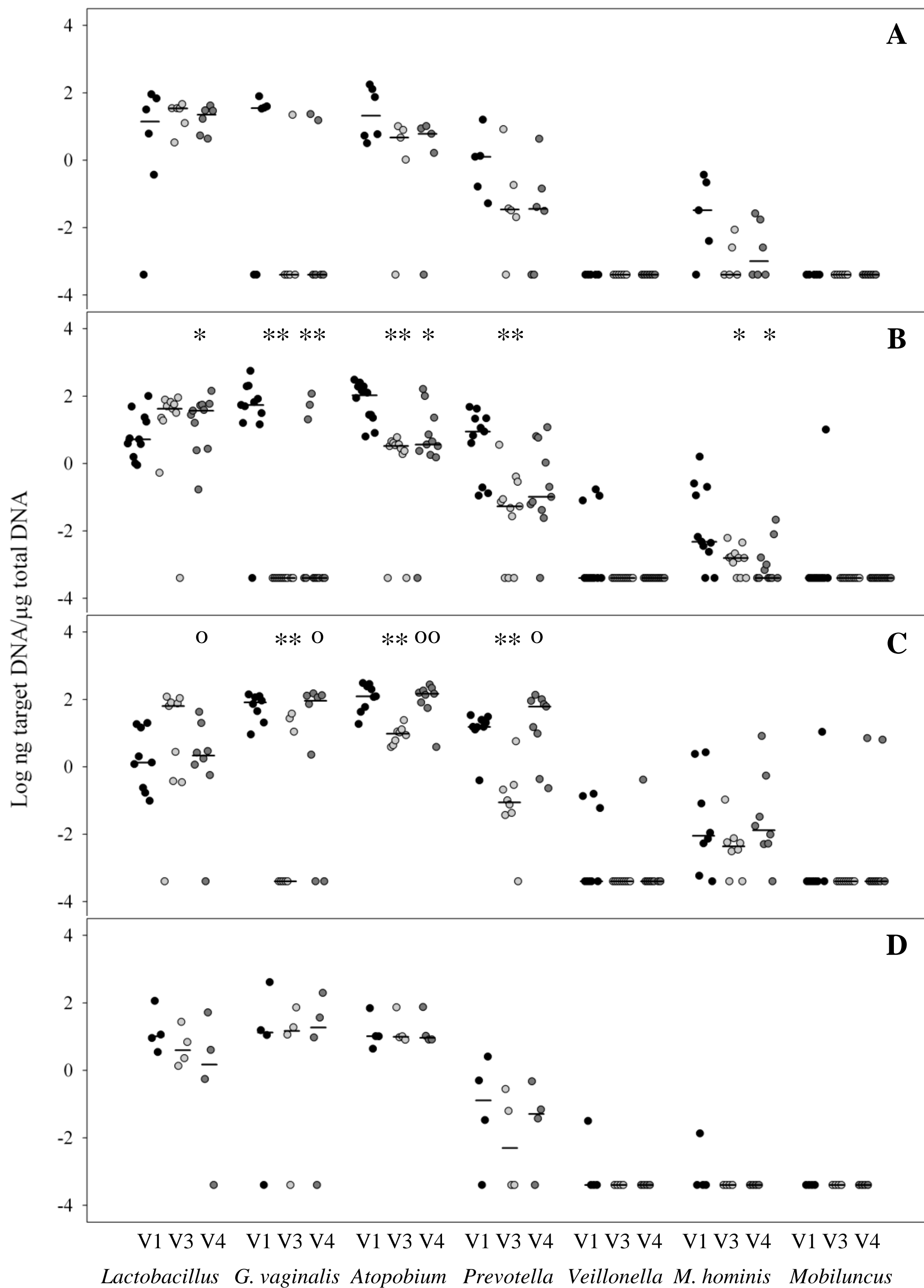


Figure 2.2. qPCR analysis of *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis* and *Mobiluncus* at time points V1, V3 and V4 for the treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo). Data are related to the women who were in remission at V3 and performed the last study visit V4 (N: 31). The scatter plots show the distributions of quantification data, with the horizontal bars representing the median values. *$P < 0.05$; **$P < 0.01$, related to V1; °$P < 0.05$; °°$P < 0.01$ related to V3.

(< -0.77 log ng/µg and < 1.01 log ng/µg, respectively) and the low frequency of occurrence of these genera.

Bacterial population profiling with PCR-DGGE

PCR-DGGE analysis with universal primers for bacteria (HDA1-GC/HDA2) was carried out on the vaginal samples collected at V1, V3 and V4 to evaluate the impact of rifaximin on the composition of BV microbiota.

Cluster analyses were performed on DGGE profiles grouped on the basis of the treatment. Figure 2.3 represent the cluster for the treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo). The percentages of women with V1 and V3 profiles clustering together were 37%, 24%, 28% and 56% for the treatment groups A, B, C and D, respectively. This result highlights the ability of rifaximin to modulate the vaginal microbiota

Table 2.2. Mean values of Similarity Indexes [SIs (%)] of DGGE profiles related to the treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo).

Treatment group	Mean SI (%)		
	Total (N: 102)[1]	Remission (N: 31)[2]	
	V1-V3	V1-V3	V3-V4
Group A	61.6	53.3	66.8
Group B	48.4	45.3	62.1
Group C	54.0	40.4	42.8
Group D	75.4	83.9	58.7

[1] SIs for V1-V3 were calculated for the total number of women (N: 102).

[2] SIs for V1-V3 and V3-V4 were calculated for the women who were in remission at V3 and performed the last study visit V4 (N: 31).

composition exerting the strongest impact at the dosage of 25 mg/day for 5 days. By considering the women who were in remission at V3 and performed the last study visit (V4), the percentages of V1, V3 and V4 DGGE profiles clustering together were 0% for A and B treatment groups, 11% for C group and 50% for D group, further confirming the impact of rifaximin on the vaginal communities.

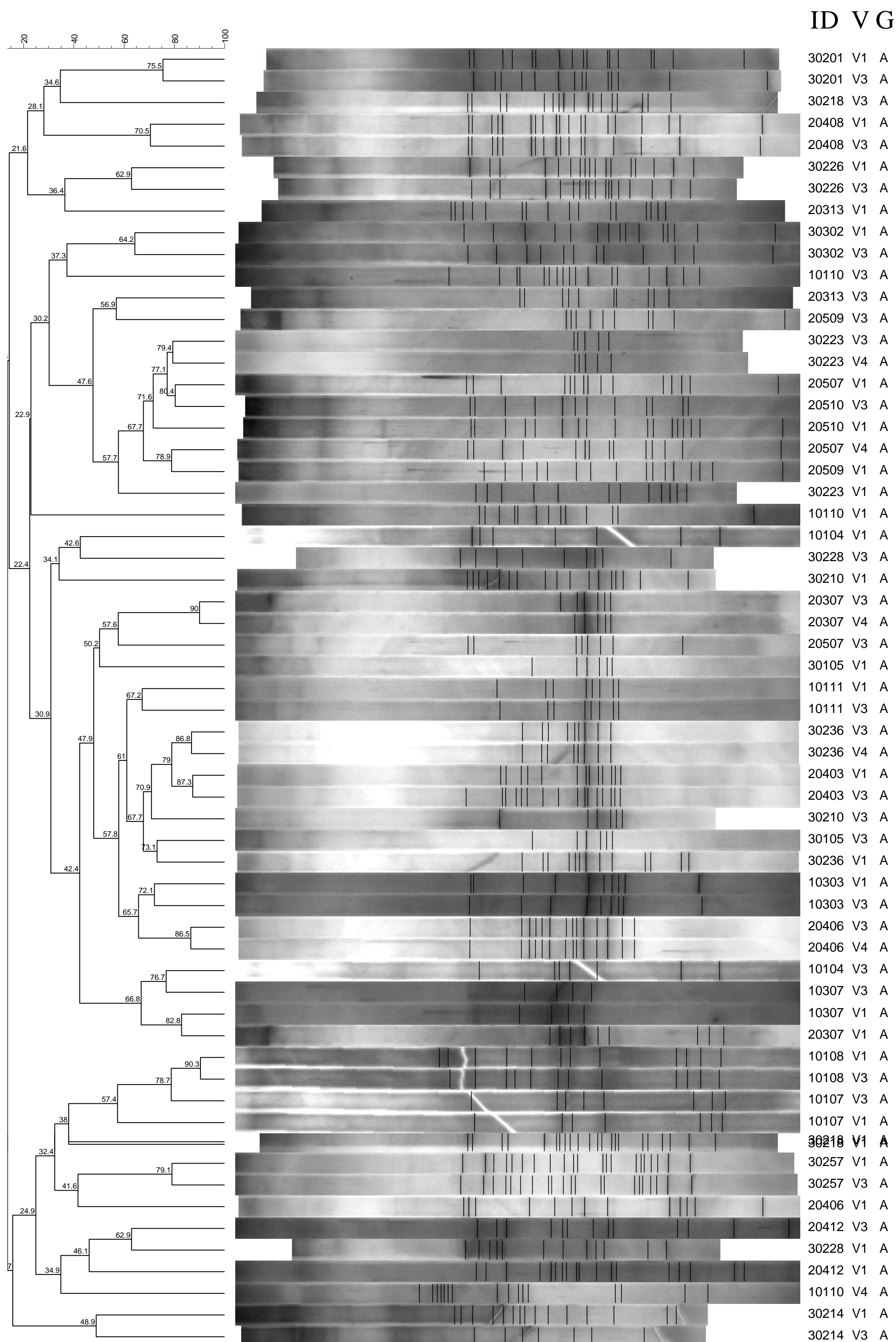
Table 2.2 reports the mean values of Similarity Indexes (SIs) of the DGGE profiles related to V1-V3 for total women analyzed, and V1-V3 and V3-V4 for the women in remission, according to the different treatment groups. Considering the total number of women, the highest SI mean value of V1-V3 was found for the group D, administered with placebo (75.4%). Among the groups administered with rifaximin, the highest SI mean value was found for the treatment group A (61.6%), followed by C (54.0%) and B (48.4%). Considering the women who were in remission at V3, a similar trend was observed for SI mean values of V1-V3. The placebo group presented the highest value (83.9%), followed by the treatment groups A (53.3%), B (45.3%) and C (40.4%). The lower SI mean values of V1-V3 for the groups administered with rifaximin compared to the placebo group support the previously formulated hypothesis that the antibiotic treatment induces changes in the structure of BV microbiota. By considering the SI mean values of V3-V4 for the women in remission, maintenance of the antibiotic effect on the vaginal communities was deduced for the treatment groups A and B, on the basis of their high SIs (66.8% and 62.1%, respectively). On the contrary, the SI mean value for group C was the lowest one (42.8%), indicating that the changes induced at V3 by rifaximin 100 mg/day for 2 days were not preserved until the last time point V4.

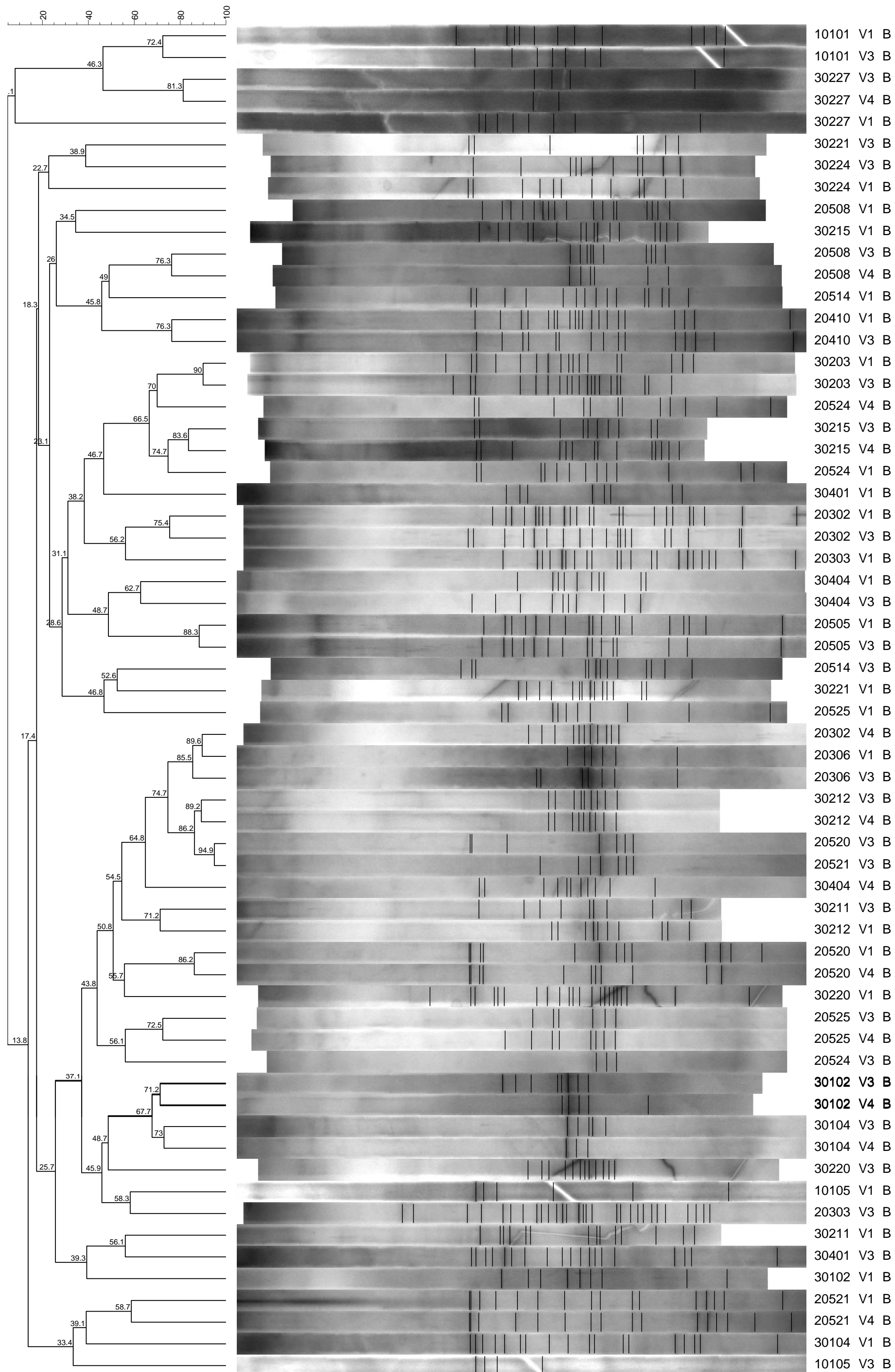
SI values were compared using the Kluskall-Wallis One Way ANOVA on Ranks test. SIs of V1-V3 related to the total number of women were significantly different by comparing all the treatment groups ($P < 0.001$). In particular, Dunn's test revealed significant differences for A *vs* D, B *vs* D, and C *vs* D ($P < 0.05$). Also for the women in remission at V3, SIs of V1-V3 resulted significantly different by comparing all the treatment groups ($P < 0.05$); a pairwise comparison by Dunn's test revealed significant differences for B *vs* D and C *vs* D ($P < 0.05$).

A Two Way ANOVA test was used to compare the influence of the two variables, dosage (100 mg *vs* 25 mg) and duration (5 days *vs* 2 days) of treatment, on SIs of DGGE profiles related to V1-V3. A significant difference was found for women who received different doses of antibiotic ($P < 0.05$), while no difference was observed for women treated for a different number of days ($P > 0.05$). This result indicates that the dosage exerts the major influence on the vaginal microbiota composition.

Table 2.3 shows the mean values of Richness Indexes (RIs) for DGGE profiles related to V1 and V3 for total women, and to V1, V3 and V4 for the women who were in remission at V3,

Figure 2.3. Cluster of DGGE profiles of vaginal samples collected from women belonging to treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo). ID indicates the patient identification number, V indicates the visit number, G indicates the group of treatment. Similarity values are indicated at each node.

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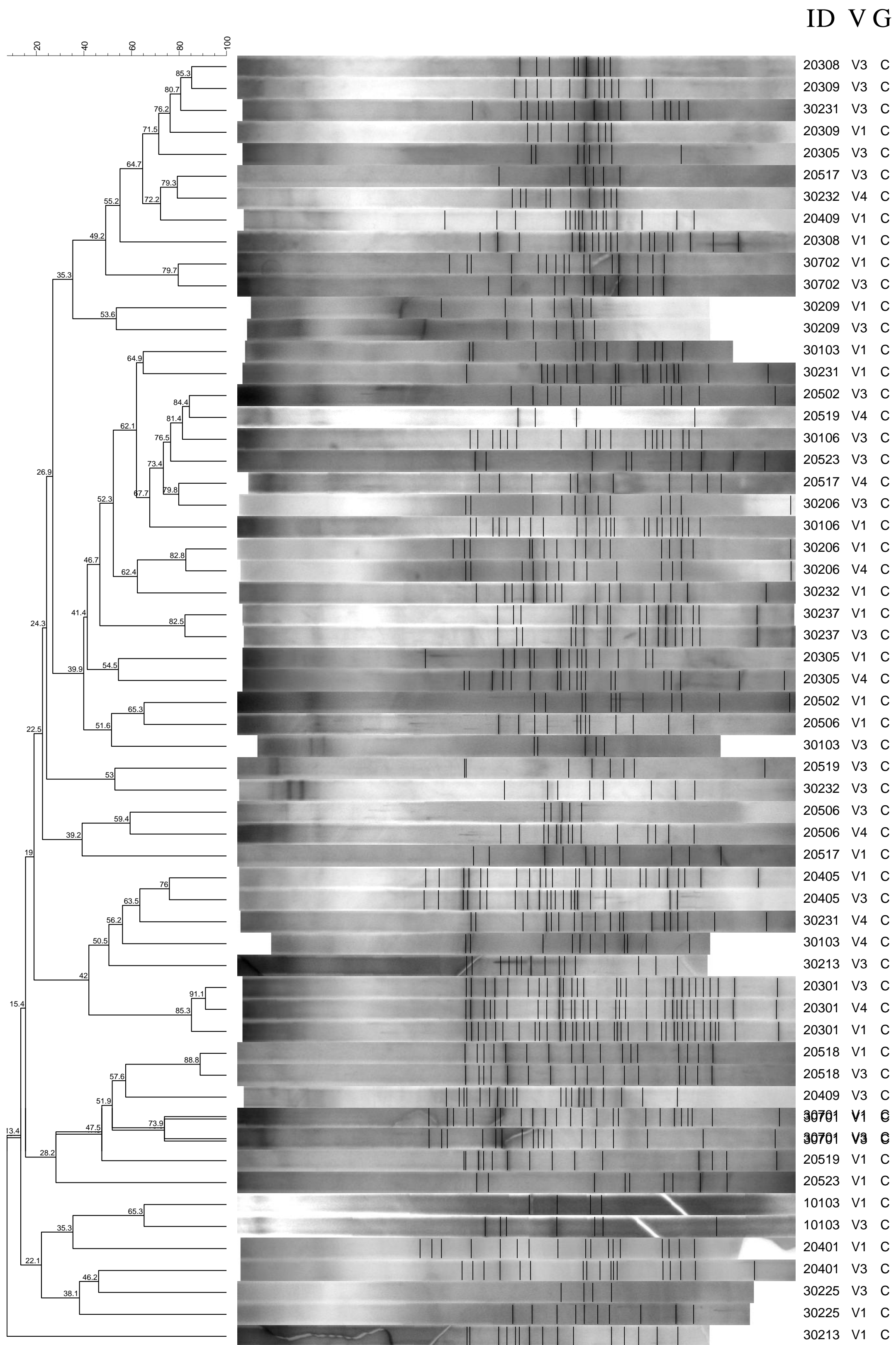

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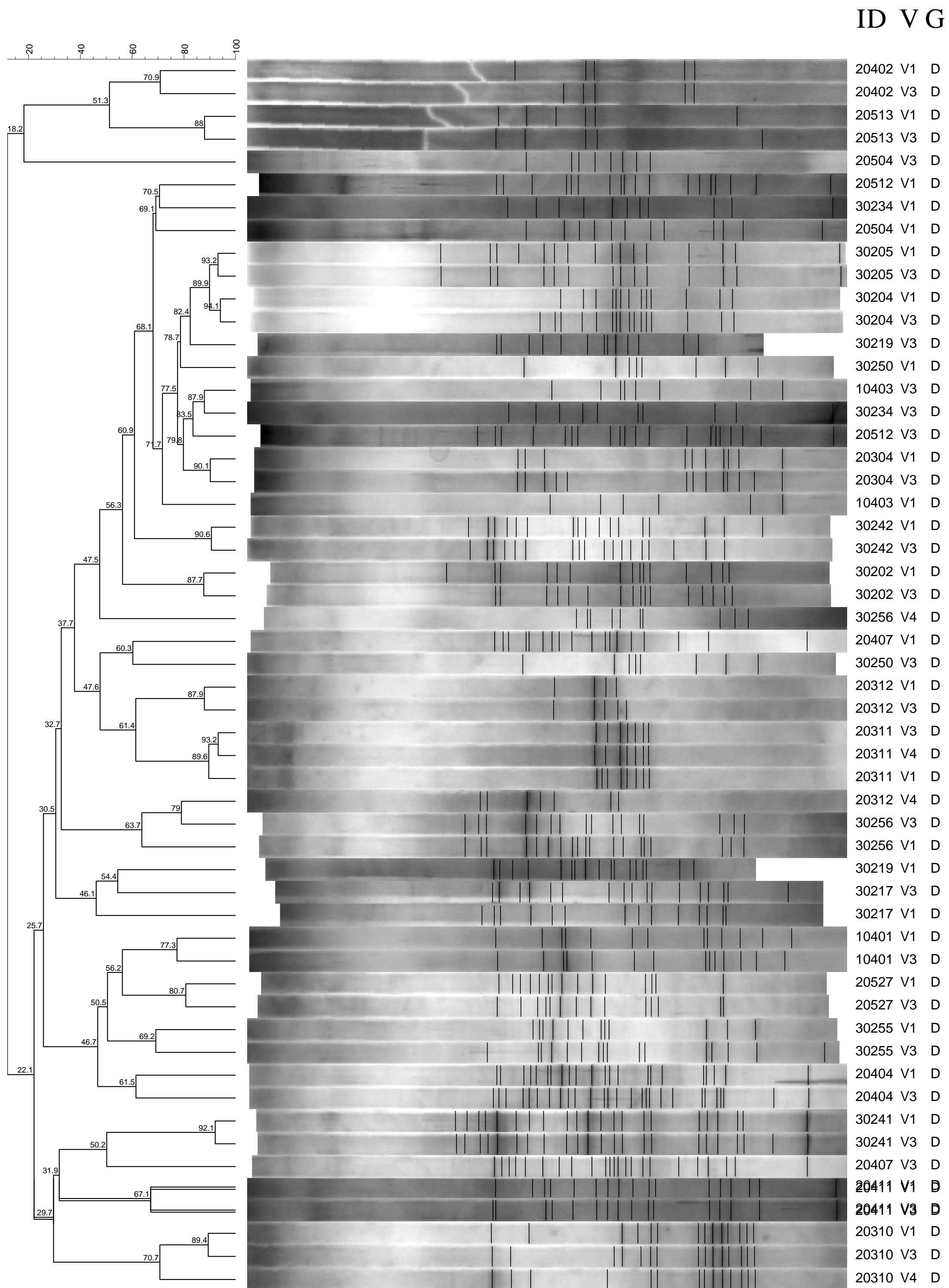

according to each group of treatment. Considering the total number of women, a reduction of the RI mean values at V3 was observed in the groups administered with rifaximin, A (V1: 13.5 *vs* V3: 10.9), B (V1: 14.0 *vs*V3: 10.7) and C (V1: 15.4 *vs* V3: 12.2). Similar RI mean values at V1 and V3 were found for the placebo group (V1: 13.3 *vs* V3: 13.9). The RI mean values for the women in remission decreased at V3 compared to V1 in all the groups treated with the antibiotic (A, V1: 12.5

vs V3: 9.8; B, V1: 13.6 *vs* V3: 8.2; C, V1: 16.2 *vs* V3: 10.4). The general decrease of RI at V3 indicates the capability of the antibiotic to reduce the complexity of the vaginal microbiota, complexity that is a typical feature of BV. RI mean values at V4 were similar to those calculated at V3 for the groups A (10.2) and B (9.0), indicating a maintenance of the antibiotic effect. Differently, the RI mean value for the group C (14.6), after the decrease at V3, approximated the baseline value at V4, reflecting a restoration of the complexity of the BV microbiota.

Table 2.3. Mean values of Richness Indexes (RIs) for the treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo).

Treatment group	Mean RI				
	Total (N: 102)[1]		Remission (N: 31)[2]		
	V1	V3	V1	V3	V4
Group A	13.5	10.9	12.5	9.8	10.2
Group B	14.0	10.7	13.6	8.2	9.0
Group C	15.4	12.2	16.2	10.4	14.6
Group D	13.3	13.9	11.3	10.8	9.5

[1] RIs for V1 and V3 were calculated for the total number of women (N: 102).

[2] RIs for V1, V3 and V4 were calculated for the women who were in remission at V3 and performed the last study visit V4 (N: 31).

Wilcoxon Signed Rank test revealed significant differences of RIs between V1 and V3 for the groups A ($P < 0.05$), B ($P < 0.05$) and C ($P < 0.001$), considering the total number of women, and for the groups B ($P < 0.001$) and C ($P < 0.001$), considering the women in remission. Friedman Repeated Measures ANOVA on Ranks test showed a significant difference of RIs among V1, V3 and V4 in the groups B ($P < 0.05$) and C ($P < 0.001$); a further pairwise analysis with the Tukey's test revealed significant differences for V1 *vs* V3 and V1 *vs* V4 in the group B, V1 *vs* V3 and V3 *vs* V4 in the group C ($P < 0.05$). These results stress again the efficacy of rifaximin 25 mg/day for 5

days in the follow-up period, and the risk of relapse associated to the treatment with rifaximin 100 mg/day for 2 days.

In conclusion, DGGE results are in strong agreement with qPCR data, confirming and reinforcing them. This integrated molecular analysis allowed us to identify the best treatment regimen for the cure of BV, which is rifaximin 25 mg/day for 5 days.

Discussion

Symbiosis between the vaginal microbiota and the human host is crucial for the reproductive tract health. The importance of this relationship is reflected by the fact that BV has serious gynecological and obstetric complications, and it creates a permissive environment for acquiring sexually transmitted infections (Turovskiy *et al*, 2011). Conventional treatment of BV with metronidazole and clindamycin does not eradicate all BV-associated bacteria, as evidenced by a BV recurrence rate of 58% (Bradshaw *et al*, 2006-a). Moreover, these treatments play a significant role in the expansion of drug resistance in *G. vaginalis* and *Prevotella* spp. (Bryskier, 2001; Liebetrau *et al*, 2003). The failure of conventional antibiotics in BV cure and eradication raises the question for alternative therapeutic tools.

Rifaximin is a non-absorbable broad spectrum antibiotic that is associated with a low risk of bacterial resistance. While several studies report the efficacy of rifaximin in the treatment of gastrointestinal disorders (Rivkin and Gim, 2011; Scarpignato and Pelosini, 2005), the use of this antibiotic in genital tract infections has not been explored until now. The purpose of the present study was to evaluate the efficacy of different doses of rifaximin vaginal tablets (100 mg/day for 5 days, 25 mg/day for 5 days, 100 mg/day for 2 days) in BV cure and remission maintenance of 102 European patients. A combination of two molecular techniques, PCR-DGGE and qPCR, was used to achieve a detailed overview of the bacterial composition of the vaginal environment related to the BV condition and to the effects of the antibiotic intake.

Consistent with data from previous studies (Fredricks *et al*, 2009; Ling *et al*, 2010), our qPCR results showed that BV-associated microbiota is dominated by several anaerobic bacterial species which coexist with lactobacilli. In particular, at the screening visit (V1) *Atopobium* and *G. vaginalis* were the predominant bacteria, found at higher amounts than lactobacilli. *Prevotella* showed a baseline concentration similar to that of *Lactobacillus*, while *Veillonella* and *Mobiluncus* genera, which have been previously associated to BV infection (Biagi *et al*, 2009; Holst *et al*, 1987; Vitali *et al*, 2009), were detected only in few women and in low amounts. Remarkably, *M. hominis* was recovered in high amounts and was present in 78% of patients, while not more than 35% of patients have been found infected with *M. hominis* by using culture-based methods, highlighting the power

of the molecular techniques for ecological studies. *Mobiluncus*, on the other hand, was only recovered in 20% of BV positive women. Still, according to the morphology as recognized in Gram stains, *Mobiluncus* was suspected in 26% of patients, as their Nugent score was 9 or 10, indicating a mismatch between microscopic findings and the molecular data. In a previous study (Marconi *et al*, in press), the concentration of *A. vaginae* was found to correlate extremely well with the Nugent scores, with the highest *A. vaginae* concentration in patients with Nugent scores 9 and 10. This leads us to the suggestion that *Atopobium* should be seen as one of the most important markers of severity of the microbial disturbance encountered in BV. Furthermore, as *A. vaginae* was also recovered in less amounts in vaginal fluid expressing intermediate Nugent scores 4-6, and *Mobiluncus* is only represented in score 9 or 10, the role of the latter seems to have been overrated in the past and its importance in the diagnosis of BV should be re-appraised.

By comparing the qPCR data related to the follow-up visits (V3 and V4) and the screening visit (V1), we sought to determine whether changes in quantities of vaginal bacteria, as reflected by bacterial DNA concentrations, are associated with the cure of BV after rifaximin therapy. In particular, we aimed at identifying the most efficient treatment able to reduce the concentrations of BV-related bacteria and to allow *Lactobacillus* spp. to recolonize the vaginal environment, reaching the highest abundance in the community. We demonstrated that the treatment with the highest dosage of rifaximin (100 mg/day for 5 days, group A) was less effective, as only *Atopobium* genus significantly decreased after the antibiotic intake and lactobacilli did not succeed in recolonizing the vaginal mucosa. The intake of rifaximin 100 mg/day for 2 days (group C) gave a better response at the first follow-up visit (V3). All the BV-associated bacteria significantly decreased in terms of concentration (*Atopobium*, *G. vaginalis*, *Prevotella* and *M. hominis*) or percentage of presence (*Veillonella* and *Mobiluncus*), while *Lactobacillus* significantly increased up to a higher amount than all the other groups in the women who were in remission at V3. However, bacterial concentrations at the second follow-up visit (V4) were similar to the baseline, indicating a relapse that was probably due to the short duration of the antibiotic intake. Rifaximin 25 mg/day for 5 days (group B) was found to be the most effective treatment, giving a good response at V3 and maintaining the effect until V4. Indeed, a significant increase of *Lactobacillus* genus together with a significant decline of bacteria associated with BV was observed at both the time points V3 and V4. The lack of *in vivo* rifaximin susceptibility of lactobacilli is not surprising. This peculiar feature had already been pointed by previous studies reporting that rifaximin did not affect the intestinal amounts of several health-promoting microbial groups, such as *Lactobacillus* spp., *Bifidobacterium* spp., *Atopobium/Collinsella* spp. (Maccaferri *et al*, 2010). Our hypothesis that rifaximin 25 mg/day for 5 days (group B) represents the most effective treatment against BV is in accordance with the

rates of remission at V3 assessed by both Amsel and Nugent criteria. The highest percentage of remission was found in group B (48%), while lower percentages were calculated for the other treated groups (A: 22% and C: 36%). The lowest rate of remission was registered in the placebo group (16%), demonstrating that spontaneous remission could be possible at low rates, as reported in the literature (Schwebke, 2000; Voorspoels *et al*, 2002).

Information derived from qPCR assays related to specific targeted bacteria were integrated with the PCR-DGGE data, which provided an overall picture of the diversity and richness of the global bacterial microbiota. Cluster analyses of DGGE profiles revealed a higher intra-individual variability in the women treated with the antibiotic compared to the women administered with placebo, highlighting the ability of rifaximin to modulate the vaginal microbiota composition. This assumption was made by considering that the treatment groups A, B and C presented lower percentages of women with V1 and V3 profiles clustering together and lower similarity indexes (SIs) for V1-V3 profiles, compared to the placebo group. In particular, the lowest SI values were found for V1-V3 profiles of women belonging to the groups B and C, when considering the total number of women or the women who were in remission at V3, respectively. A maintenance of the antibiotic effect on the vaginal bacterial communities was demonstrated for the treatment groups A and B on the basis of their highest SI values of V3-V4 profiles related to the women who were in remission at V3. The lowest SI value of the group C suggested that the treatment with rifaximin 100 mg/day for 2 days did not avoid relapse. In general, these results confirm the hypothesis suggested by qPCR data that the treatment with rifaximin 25 mg/day for 5 days is the most effective. In fact, the low similarity of V1-V3 DGGE profiles of the group B demonstrates that the antibiotic intake induced a variation in the overall composition of the vaginal microbiota, while the high similarity of V3-V4 DGGE profiles for the women in remission indicates the maintenance of the effect. The identification of rifaximin 25 mg/day for 5 days as the best treatment for the cure of BV was further supported by the finding that the variable “dosage” exerted a major influence on the diversity of the vaginal microbiota compared to the variable “duration” of the treatment.

Previous PCR-DGGE studies indicated that the level of complexity of the vaginal microbiota was higher in BV infection than in healthy status (Ling *et al*, 2011; Vitali *et al*, 2007). Here, the complexity of the vaginal communities was evaluated by calculating the richness indexes (RIs) of DGGE profiles. The significant decrease of RI values after the antibiotic intake in all the treated groups indicates the rifaximin effectiveness in reducing the complexity of the vaginal ecology, and restoring a community structure similar to a healthy microbiota. Considering the RI values at V4, the maintenance of remission in women treated with rifaximin 25 mg/day for 5 days and the relapse occurring in women treated with rifaximin 100 mg/day for 2 days were further confirmed.

In conclusion, our results suggest that rifaximin is a potential good candidate for the treatment of BV. In particular, the treatment with rifaximin 25 mg/day for 5 days is active against the pool of bacteria recognized to cause BV, while it does not affect the normal population of lactobacilli. Based on these encouraging results, further clinical studies will be undertaken in order to confirm the real advantage of this antibiotic in comparison to standard BV treatments.

CONCLUDING REMARKS

Concluding remarks

In the present study the impact of two different therapeutic approaches on the composition of the vaginal ecosystem has been studied by using an integrated molecular fingerprinting approach based on PCR-DGGE and real-time PCR.

The oral administration of the probiotic mixture VSL#3 in the last trimester of pregnancy of healthy women was analyzed to evaluate the beneficial effects on the vaginal microbiota composition and immune response. The microbiological analyses showed only a blind modulation of the bacterial population composition in women who consumed the probiotic mixture, without significant differences induced by the intake. The DGGE population profiling suggested the ability of the probiotic VSL#3 to modulate the vaginal microbiota, without compromising the inter-individual species variability. Real-time PCR results suggested a role of the probiotic product in counteracting the decrease of the health-promoting *Bifidobacterium* genus and the increase of the BV-related *Atopobium* genus, that physiologically occur in late pregnancy.

As regards the investigation of the vaginal immunological profiles of the pregnant women, the probiotic intake counteracted the decrease of anti-inflammatory cytokine levels occurring in the control group, and induced the decrease of a pro-inflammatory cytokine in the group treated with VSL#3, showing a global anti-inflammatory effect on the vaginal immunity, with potential implications in preventing preterm birth.

A combination of the two molecular techniques, PCR-DGGE and qPCR, was also used to achieve a detailed overview of the bacterial composition of the vaginal environment related to the BV condition and to the effects of the antibiotic therapy with rifaximin. In particular, the aim was to identify the most efficient treatment able to reduce the concentrations of BV-related bacteria and to allow *Lactobacillus* spp. to recolonize the vaginal environment, reaching the highest abundance in the community. The results suggested that rifaximin is a potential good candidate for the treatment of BV. In particular, the treatment with rifaximin 25 mg/day for 5 days is active against the pool of bacteria recognized to cause BV, while it does not affect the normal population of lactobacilli. A modulation in the global composition of the bacterial community is demonstrated by the low similarity among DGGE profiles of the same woman before and after antibiotic intake. Patients treated with this antibiotic regimen (rifaximin 25 mg/day for 5 days) showed the highest rate of remission at follow-up visit after one month from the end of the treatment.

Based on these encouraging results, an oral probiotic therapy can be suggested for the preventive modulation of the vaginal microbial community composition and immune response of

the host, in order to maintain the right balance among the vaginal microorganisms and to elicit the right physiological immune response, especially during pregnancy.

Rifaximin has been demonstrated to represent a valid alternative candidate for the cure of BV. Further clinical studies will be undertaken in order to confirm the real advantage of this antibiotic in comparison to standard BV treatments.

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