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BACTERIAL VAGINOSIS IN PORTUGAL: DIAGNOSIS OF *Gardnerella vaginalis* **AND** *Atopobium vaginae* **IN HEALTHY OR SYMPTOMATIC WOMEN**

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VAGINOSE BACTERIANA EM PORTUGAL: DIAGNÓSTICO DE *Gardnerella vaginalis* **E** *Atopobium vaginae* **EM MULHERES SAUDÁVEIS OU SINTOMÁTICAS**

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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"The most important is to never stop questioning".
Albert Einstein

Resumo

A vaginose bacteriana corresponde ao distúrbio mais comum nas mulheres, tendo um impacto importante em todo o Mundo. Estima-se que afecta cerca de 30-50% das mulheres Afro-Americanas e 10-20% das mulheres Caucasianas em idade reprodutiva. Associado ao aparecimento de vaginose bacteriana, verifica-se um decréscimo do número de *Lactobacillus* spp. no epitélio com consequente aumento do número de microrganismos anaeróbios, tais como *Gardnerella vaginalis* e *Atopobium vaginae*. Embora comumente associada à vaginose bacteriana, *G. vaginalis* foi também identificada no epitélio vaginal de mulheres saudáveis, mas em menores números. O crescimento de *G. vaginalis* pode ser identificado por beta hemólise, Gram-variável, oxidase e catalase negativa (testes microbiológicos convencionais) e ainda através de técnicas moleculares.

O principal objectivo deste projecto foi a identificação de *A. vaginae* e *G. vaginalis* na microflora vaginal de mulheres Portuguesas, saudáveis ou já diagnosticadas, à priori, como portadoras de vaginose bacteriana; através de métodos moleculares. O principal interesse no estudo destes microrganismos deveu-se ao facto de serem, nos últimos anos, os mais usualmente isolados de casos de vaginose bacteriana. *Gardnerella vaginalis* e, mais recentemente, *A. vaginae* são dois microrganismos inicialmente associados a vaginose mas actualmente identificados em mulheres saudáveis. Em Portugal, o primeiro e único estudo associado a vaginose remonta de 1998, o que justifica a importância dos dados obtidos neste estudo. Neste sentido, o estudo envolveu a recepção de amostras clínicas obtidas por auto-colheita de mulheres saudáveis, em consultório de ginecologia ou mesmo nas emergências do Hospital de Braga, e posterior tratamento das amostras. A caracterização foi levada a cabo por métodos moleculares como *Reacção em Cadeia da Polimerase* (PCR) e Microscopia Fluorescente com Hibridação *in situ* (FISH).

Os resultados demonstraram, através de métodos moleculares, que das cinquenta e sete amostras recolhidas de mulheres Portuguesas e associadas a este projecto, *G. vaginalis* foi identificada em dezasseis amostras, o que corresponde a 28% do número total de amostras. *Atopobium vaginae* foi apenas encontrado em cinco casos o que corresponde a 8% das mesmas.

Em suma, as técnicas moleculares permitiram a identificação directa de parte dos microrganismos presentes nas zaragatoas, sendo assim possível concluir que *G. vaginalis* and *A. vaginae* não estão unicamente associadas a vaginose bacteriana mas também estão presentes, em diferentes proporções, em mulheres Portuguesas saudáveis.

Palavras-chave: Vaginose bacteriana, Amostras Clínicas, *Gardnerella vaginalis, Atopobium vaginae, Lactobacillus* spp., Métodos Moleculares, Interacção, Cultura.

Abstract

Bacterial vaginosis is the leading vaginal disorder, having an important impact worldwide. It is estimated to affect 30-50% of African-American women and 10-20% of Caucasian women at reproductive age. During bacterial vaginosis, a decrease of *Lactobacillus* spp. and an increase in the number of anaerobic microorganisms, such as *Gardnerella vaginalis* and *Atopobium vaginae* in the vaginal epithelium is observed. Although commonly associated to bacterial vaginosis, *G. vaginalis* has also been associated to the vagina of healthy women, but in lower numbers. The growth *of G. vaginalis* can be identified by beta hemolysis, variable Gram staining, negative oxidase and catalase (conventional microbiological tests) and by molecular techniques.

The main goal of this study was the identification of *G. vaginalis* and *A. vaginae* in the vaginal microflora of healthy or ill women, by molecular techniques. The reason of our interest in these microorganisms was based on fact of being the mostly isolated microorganisms in cases of bacterial vaginosis. *Gardnerella vaginalis* and, most recently, *A. vaginae* were two microorganisms firstly associated to bacterial vaginosis, however more recent studies identified them on the healthy vaginal microflora. In Portugal, the unique study involving bacterial vaginosis was done in 1998, which consequently straighten up the importance of this study. By this way, our study involved the reception of swabs obtained by self-harvest, gynecological private practice or even in hospitals emergency and the posterior manipulation of the samples. The identification of *G. vaginalis* and *A. vaginae* was specially based on the analysis of the clinical samples by *Polymerase Chain Reaction* (PCR), and by Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH).

The results revealed that from fifty-seven Portuguese women samples involved in this study, the presence of *G. vaginalis* was detected in sixteen samples, which corresponds to a prevalence of 28 %. On the other hand *A. vaginae* was present in five clinical samples, which corresponds to 8 % of the samples.

The molecular techniques allowed the direct identification of part of the microorganisms present on the vaginal swabs and allowed to conclude that the *G. vaginalis* and *A. vaginae* are not only associated to bacterial vaginosis but they can also be founded, in different percentage, in a range of Portuguese healthy women.

Keywords: Bacterial vaginosis, Clinical samples, *Gardnerella vaginalis, Atopobium vaginae, Lactobacillus* spp., Molecular Methods, Interaction, Culture.

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x

Contents

List of figures

List of tables

Acronyms

BV	Bacterial vaginosis
BHI	Brain heart infusion
CBA	Columbia blood agar
DAPI	*4',6-diamidino-2-phenylindole*
DNA	*Deoxyribonucleic Acid*
ER	Hospital emergency
FISH	Fluorescence *in situ* Hybridization
FITC	Green filter
GPP	Gynecological private practice
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
PBS	Phosphate buffered saline
PCR	*Polymerase Chain Reaction*
RNA	*Ribonucleic Acid*
SH	Self-harvest
ssDNA	Stranded DNA
Ta	Temperature of annealing
TAE	Tris-Acetate EDTA
Tm	*Melting temperature*
TRITC	Red filter

1. Introduction

Bacterial vaginosis (BV) has an important impact worldwide as the leading vaginal disorder in women, affecting 30-50% of African American women and 10-20% of Caucasian women of reproductive age (Patterson *et al.,* 2010). BV is a polymicrobial syndrome characterized by causing several complications associated to the reproductive health of women (Turovskiy *et al.*, 2011), and is three to four times more common than *Trichomonas vaginalis* infections or either the vulvovaginal candidiasis (Cauci *et al.*, 1996; Aroutcheva *et al.*, 2001; Eschenbach, 2007; Patterson *et al.,* 2010).

Over the last fifty years several studies were carried out in order to understand the etiology of BV. Some risk factors were identified as associated to BV, however their correlation is still unclear because of its complexity and absence of an animal model that could be applied (Turovskiy *et al.,* 2011).

1.1 *Vaginal epithelium microflora*

The normal vagina is a complex system composed by a squamous epithelia and indigenous microbiota. The normal microflora contains lactobacilli, especially *Lactobacillus crispatus* and *L. gasseri,* present on a level of 10^7-10^8 CFUg^{-1} on fluid (Zarate and Nader-Macias, 2006) and *L. jensenii* (Pavlova *et al.*, 2002; Marrazzo, 2011). In contrast to *L. crispatus* and also *L. gasseri*, recent studies report *L. iners* as predominant in bacterial vaginosis flora with non-Lactobacillus species, what therefore contraries the first hypothesis in which it belongs to the normal vaginal flora (Menard *et al.*, 2010; Zozaya-Hinchliffe *et al.*, 2010). *Lactobacillus* spp. are Gram-positive rods, strict or facultative anaerobes, have a fastidious growth and prefer an acidic environment, by producing lactic acid (Reid, 2001; Dover *et al.*, 2008). Lactobacilli play an important role maintaining a healthy vaginal ecosystem. About 78% of vaginal lactobacilli inhibits the growth of *G. vaginalis*, acting as a barrier to infection and contributing to the control of the vaginal microbiota, by competing with other microorganisms for adherence on epithelial cells and shifting of pathogen biofilm (Aroutcheva *et al.*, 2001; Saunders *et al.*, 2007; Coudeyras *et al.*, 2008; Teixeira *et al.*, 2010). The biofilm is a functional consortium of microorganisms, in this particular case, aggregated to the vaginal epithelium (Costerton *et al.*, 1987). It is characterized by its structural heterogeneity, has an extracellular matrix of polymeric substances and the interactions between the communities are complex. Lactobacilli are also responsible for the production of byproducts such as lactic acid, which maintains a low pH, hydrogen peroxide and bacteriocins, that have antimicrobial properties thereby excluding pathogens from the niche (Mah and O'Toole, 2001; Koumans *et al.,* 2002; Larsson *et al.*, 2005).

Some factors are involved in the colonization of vaginal epithelial by lactobacilli. In fact, differences in the composition of cell wall influence the adhesion (Zarate and Nader-Macias, 2006). Hormonal changes, like estrogen, glycogen content and also the pH on vagina can also influence this adherence to epithelial cells. The particular increase of estrogen, during the menstrual cycle, increases this colonization (Cribby *et al.*, 2008). During menopause occurs the diminishing of the levels of estrogen that results in a lower quantity of lactobacilli present on vaginal tract of menopausal women (Cribby *et al.,* 2008). Postmenopausal women are more susceptible to vaginal infections being colonized by microorganisms such as *G. vaginalis* and *A. vaginae,* and the colonization by commensal

lactobacilli can restore the normal microflora (Burton *et al.*, 2004; Cribby *et al.,* 2008). Furthermore, the microbiota of a healthy premenopausal is also more susceptible to infection and is colonized, in particular, by species of lactobacilli such *as L. casei, L. crispatus, L. gasseri, L. iners, L. jenesennii, L. plantarum, L. rhamnous, L. reuteri* and *L. salivarus (Cribby et al.*, *2008).*

1.2 Monomicrobial theory

Gardner and Dukes, 50 years ago, defended that BV etiological agent was *G. vaginalis*, arguing that a pure colony of *G. vaginalis* inoculated on the vagina could cause BV (Fredricks *et al.*, 2009). However, the microorganisms associated to BV cannot fulfill the second postulate of Koch´s postulates, which defends that a pure colony of this microorganism inoculated into the vagina could cause disease. In fact, *G. vaginalis* is an example of a microorganism associated to BV and it was found in the vaginal microflora of healthy women (Gardner and Dukes, 1959; Fredricks *et al.,* 2009; Turovskiy *et al.*, 2011. More recent studies also demonstrated the revision of the potential virulence of *G. vaginalis* and the etiological idea (Patterson *et al.,* 2010).

1.3 Alteration of indigenous microflora

Recent studies demonstrated that, during BV, a microecologic imbalance takes place. There is a decrease of *Lactobacillus* spp in the vaginal microflora, such as *L. crispatus* and *L. jensenii* (Srinivasan *et al.*, 2010), which are present in the vaginal epithelium, and an increase in the number of anaerobic and Gram positive rods, such as *Pretovella* spp., *Mobilincus* spp. and *Mycoplasma hominis,* and also Gram-variable rods like *G. vaginalis* (Aroutcheva *et al.*, 2001; Sethi *et al.*, 2006; Coudeyras *et al.*, 2008) Recent studies related another important microbiological markers associated to BV: the presence of *A. vaginae* (De Backer *et al.*, 2010). The literature also reveals the association of this microorganism to *G. vaginalis. Gardnerella vaginalis* is a fastidious microorganism, presented as a Gram variable short rod in blood agar media when submitted to growth at 37 ºC; it appears as transparent/little grey colonies and sometimes β-hemolysis is visible (Lamont *et al.*, 2011; Turovskiy *et al.*, 2011). *Atopobium vaginae* is a fastidious microorganism difficult to be identified by culture. The genus *Atopobium* was proposed in 1992 by Collins and Wallbanks, but was only discovered in 1999, associated to vaginal flora. It belongs to the *Coriobacteriaceae* family and is characterized as an anaerobic Gram-positive bacteria. The characteristic cocci appear in pairs or short chains (Rodriguez *et al.*, 1999; Knoester *et al.,* 2011), and in blood agar, at 37 ºC, the growth of small colonies with grey/ white color is observed (Rodriguez *et al.*, 1999). However, only a few articles related the biochemical characterization or even the type of growth and support this identification by molecular methods, in response to its low cultibility.

During this flora alteration, an increase of the pH of the vagina also occurs, from 4.5 to 7, caused by the depletion of lactobacilli, decreasing the production of hydrogen peroxide, which is responsible for maintaining the low pH (Cauci *et al.,* 1996; Walker and Thornsberry, 1998; Witkin *et al.*, 2007; Teixeira *et al.*, 2010).

1.4 Healthy women

As referred before, the vaginal flora of healthy women is dominated by *Lactobacillus* spp. (figure 1.1 A), that are capable of producing bacteriocins, hydrogen peroxide and organic acids. *Lactobacilli,* able to produce hydrogen peroxide, are present in about 96% of healthy women and 6% of patients with BV. Non-producing *Lactobacillus* spp. are only present in 4% of healthy women and 36% of patients with BV (Livengood, 2009).

More recent studies demonstrated that *G. vaginalis* is also part of the genital tract of healthy women (figure 1.1 B). However, the numbers of *G. vaginalis* isolated from the vaginal epithelium of healthy women are lower than the numbers found in women with BV. Furthermore, it has been reported recently that the biotypes of *G. vaginalis* isolated from healthy women differ from those isolated from women with BV (Aroutcheva *et al.*, 2001; Harwich *et al.*, 2010).
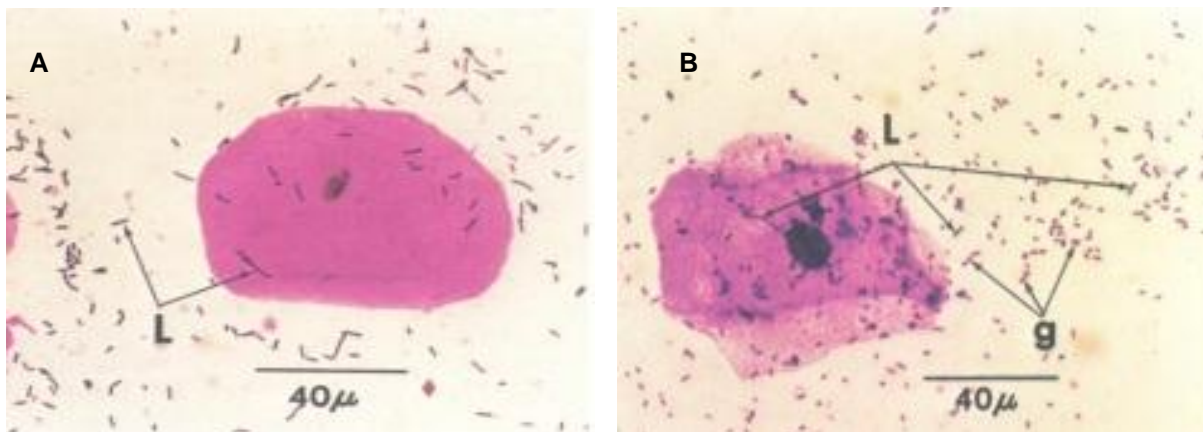
Figure 1.1: Representation of normal vaginal fluid smears. 1.1 A - clue cell with *Lactobacillus* spp. morphotype (L). 1.1 B - clue cell surrounded by *Lactobacillus* spp. (L) and also *G. vaginalis* (g) (Spiegel *et al.*, 1983).

1.5 Symptoms associated to Bacterial vaginosis

Related to the microbial change on microflora, some clinical symptoms can be associated and enumerated, such as:

1. An elevation of vaginal pH more than 4.5;

2. The change in vaginal composition is typically accompanied by an amine odor upon addiction of KOH to a drop of secretion – "positive whiff test";

3. The presence of a thin discharge;

4. The presence of bacteria coated epithelial cells termed "clue cells" (Witkin *et al.*, 2007).

Gardner and Dukes (1955), proved the vaginal clue cells value on the diagnosis of bacterial vaginosis. In fact, clue cells are squamous epithelial cells whose surfaces are heavily covered with bacteria. The desquamation of these cells results on the formation of the classic clue cells (Swidsinski *et al.,* 2005).

Otherwise, about 50 % of patients do not have symptoms and, in these cases the diagnosis can´t be done based on this clinical profile (Gillet *et al.,* 2011).

1.6 Complications associated to Bacterial Vaginosis

More serious infections in the upper genital tract have also been associated with BV, such as preterm delivery, preterm labor, post abortion endometritis, post-partum endometritis and low birth weight (Srinivasan and Fredricks, 2008; Swidsinski *et al.,* 2008; Menard *et al.*, 2012). In fact, women who give birth prematurely are more predictable to have an upper tract infection like demonstrated by analyzing the amniotic fluid, the fetal membranes or the placenta. Some studies also refer that the alteration of vaginal ecosystem can also improve the risk for the acquisition of human papiloma virus - HPV infection, which can be explained by a reduction of the levels of a specific leucocyte involved on the block of Human immunodeficiency virus - HIV infection (Gillet *et al.*, 2011). The fluid of women with BV also has an amount of enzymes, like sialidases, which are associated with the degradation of the layer of cervical epithelium causing the alteration of epithelial cells and increased predisposition to the acquisition of HPV. However this relation is still the focus of studies (Gillet *et al.*, 2011). The presence of BV also increases the risk of HIV transmission from women to men or to other women (Koumans *et al.*, 2002; Eschenbach, 2007) as well as the susceptibility to other sexually transmitted diseases (Larsson *et al.,* 2005; Srinivasan and Fredricks, 2008; Menard *et al.,* 2012).

1.7 Epidemiology

Epidemiological studies revealed that some factors increase the acquisition of BV. Factors such as environmental and ethnical characteristics can influence the predisposition to BV. Other risk factors included the ethnicity, socioeconomic status, douching and the antibiotic treatment (Gillet *et al.*, 2011; Ravel, 2011; Turovskiy *et al.,* 2011). However, according to socioeconomic aspects, a recent study identified the same profile of microorganisms associated to vaginal flora in women who lived in industrialized countries, as well as in women living in poor countries (Pepin *et al.,* 2011). The women that have a new sexual partner or even multiple partners also have an increased predisposition to BV (Livengood, 2009). Larsson *et al.* (2007) reported that smoking habits increase the predisposition of women to acquire BV, by a decline of lactobacilli that produce hydrogen peroxide. The prevalence of BV in pregnant women also differs between populations and between post and pre-menopausal women.

1.8 Diagnosis of Bacterial Vaginosis

The Papanicolaou test is the first test performed on clinical practice. On this procedure, the clinician inserts a speculum into the patient's vagina, and then removes a sample from the uterine cervix for analysis. The cytological examination improves the diagnosis of cervical infections, very common in women of reproductive age (Bukhari *et al.*, 2012). However, some studies reveal that Papanicolaou smear is not very useful and robust for the diagnosis of BV because it, typically, uses clue cells as the only criteria, excluding other clinical aspects associated to BV. The sample collected is removed from uterine cervix instead of vagina (Greene *et al.*, 2000).

The detection of BV can also be done by standardized scoring systems that allow the interpretation of Gram staining of a vaginal fluid sample and improved the detection of women with asymptomatic BV (Boskey *et al.*, 2004), such as the Amsel, Nugent and Spiegel methods.

Amsel criteria

The primary clinical diagnosis of BV is based on the presence of at least 3 of the 4 symptoms described by Amsel *et al.*, (1983), and referred on section 1.5 (Walker and Thornsberry, 1998). However, the percentage of asymptomatic women is about 50 %, which is an obstacle to this kind of diagnosis (Hay, 2010; Gillet *et al.*, 2011). For this reason, the scientific community consider microbial diagnosis, such as the Nugent score, the election method.

Nugent score

Some years after, in 1955, Gardner and Dukes described that normal vaginal fluid contains *Lactobacillus* spp. morphotypes, however smears of patients with BV demonstrated small-Gram variable organisms like *G. vaginalis* in the absence of *Lactobacilli* spp. (Gardner and Dukes, 1955; Spiegel *et al.*, 1983; Larsson *et al.*, 2005). So, the diagnosis of BV is currently supported in the analysis of Gram-stain of vaginal microflora, according to the Nugent score (table 1.1) (Aroutcheva *et al.*, 2001; Chaijareenont *et al.*, 2004). According to this test, a score of >/= 7 on a Gram stained vaginal smear indicates the presence of BV (Cauci *et al.*, 1996; Srinivasan and Fredricks, 2008; Swidsinski *et al.*, 2008).

Spiegel score

Spiegel score is another system and it is based on scoring system criteria from 0 to 10. The score of 7 indicates the presence of BV, 4-6 is an intermediate diagnosis and a score between 0 and 3 is normal. The criterion is applied for both *Lactobacillus* spp. and *G. vaginalis* morphotypes (Nugent *et al.*, 1991). Using the Spiegel score each morphotype was quantitated from 1 to 4+ based on the number of morphotypes present on the field (table 1.2). The presence of BV is revealed if lactobacilli morphotypes are fewer than five per immersion oil field and if there are five or more *G. vaginalis* morphotypes together with five or more other morphotypes (Gram-positive cocci, small Gram-negative rods, curved Gram-variable rods, or fusiform) per oil immersion field. If five or more lactobacilli and

fewer than five other morphotypes were present per oil immersion field, the Gram staining was considered to be normal by the Spiegel criteria (Nugent *et al.*, 1991).

Table 1.1: Classification criteria of BV of Nugent score´s system (In: Chaijareenont *et al.*, 2004)

Score	Number of organism morphotypes per high power field		
	Lactobacillus (parallel-sided Gram- positive rods)	*Gardnerella*/ Bacteroides (tiny, Gram-variable coccobacilli and rounded, pleomorphic, Gram negative rods with vacuoles)	*Mobiluncus* (curved, Gram-negative rods)
0	>30	0	0
1	5-30	<1	1-5
2	1-4	1-4	>5
3	<1	5-30	
4	0	>30	

Table 1.2: Classification criteria of BV of Spiegel score´s system (Nugent *et al.*, 1991)

Score	Number of organism morphotypes per field
0	No morphotypes
1+	Less than 1 morphotype
2+	1 to 4 morphotypes
3+	5 to 30 morphotypes
4+	30 or more morphotypes

1.9 Current treatment therapeutics

The BV could be controlled by the administration of antibiotics after its diagnosis; however the symbiotic relation demonstrated between the species associated can diminish the response to treatment (Pepin *et al.,* 2011). Metronidazole is an antimicrobial agent usually considered the first option to treat BV. It is cost-effective, acting against anaerobic Gram-positive or negative bacteria; and causing minor adverse effects. Metronidazole has oral, intravenous, vaginal and topical formulation (Lofmark *et al.*, 2010). The prescription of metronidazole for seven days is recommended, three times a day. Some authors defend that after the administration of metronidazole, the DNA concentration of *G. vaginalis* will decrease and the DNA concentration of *L. crispatus* will increase (Turovskiy *et al.*, 2011). However, patients with recurrent BV have shown increased levels of *G. vaginalis* DNA, even after the metronidazole treatment (Turovskiy *et al.*, 2011). Administration of clindamycin is an alternative, with safe administration for the control of BV, and currently this is the preferred approach (oral or intravaginal administration) (Koumans *et al.*, 2002). In fact, resistance to metronidazole has

been observed in some *G. vaginalis* strains and could be explained by deletion or inactivation of genes with nitroreductase activity that causes metronidazole resistance (Harwich *et al.*, 2010). The association of *A. vaginae* and *G. vaginalis* in BV cases increases the rate of recurrence of BV in comparison with *G. vaginalis*, after the treatment with metronidazole (De Backer *et al.*, 2010). However, *Lactobacillus* spp. are resistant to metronidazole and some studies demonstrated that the treatment with metronidazole improves the recolonizing of vaginal flora with lactobacilli and, consequently, increase of hydrogen peroxide. On the other hand, clindamycin has a higher activity against *G. vaginalis* and *A. vaginae*, however it can cause the remotion of lactobacillli from the normal vaginal microflora (De Backer *et al.*, 2006). However, antibiotics do not eradicate all the vaginal bacteria, being effective in about 60% of cases (Dover *et al.*, 2008). Probiotics are an alternative to the use of antibiotics on the reposition of vaginal flora (Zarate and Nader-Macias, 2006; Cribby *et al.*, 2008). The Food and Agricultural Organization of the United Nations classifies the probiotics as "live microorganisms that confer a health benefit to the host when administered in adequate amounts" (Jones and Versalovic, 2009). Some lactobacilli strains can act as probiotics, merging as a viable alternative to restore the normal flora. *Lactobacillus rhamnosus, L. reuteri* and *L. acidophilus* are being administered to the vagina of women in order to restore the antimicrobial defenses present in the epithelium, defending, some studies, that they inhibit the colonization by microorganisms such as *Escherichia coli*, *G. vaginalis*, *Candida albicans* and *Pseudomonas aeruginosa* (Reid, 2001; Zarate and Nader-Macias, 2006; Cribby *et al.*, 2008; Dover *et al.*, 2008). On the selection of the probiotics targeting genital applications, some lactobacilli hydrogen peroxide producers have been utilized, however, a recent study showed that some of them can be ineffective on the suppression of BV, because these probiotics have the capability to inhibit some lactobacilli from the normal microflora more than BV (O'Hanlon *et al.*, 2011). By this way, they defend that lactobacilli which produce lactic acid are more effective on the vagina's acidification and therefore the suppression of BV (O'Hanlon *et al.*, 2011).

1.10 *Gardnerella vaginalis* characteristics

Since *G. vaginalis* is the most relevant organism associated to BV it is worthwhile to further describe its characteristics.

Gardnerella vaginalis, initially known as *Haemophilus vaginalis*, is affiliated to the family *Bifidobacteriaceae* and, primary, was identified as the sole cause of BV. *Gardnerella vagin*alis had been detected in >98% of BV cases and has a clinically significant role in the etiology of BV (Aroutcheva *et al.*, 2001; Livengood, 2009). *Gardnerella vaginalis* cells are Gram-variable bacteria, pleomorphic short-rods, it does not possess flagella and capsules, or even produce endospores (Harper and Davis, 1982; Catlin, 1992). The cell walls of *G. vaginalis* contain alanine, lysine, glycine, galactose, glucose and glutamic acid and 6-deoxytalose. The chemical composition of the wall is typical of Gram-positive bacteria (Harper and Davis, 1982), however electron microscopy demonstrated that the peptidoglycan layer is thinner than the majority of Gram-positive organisms, which sometimes results on a negative Gram-staining. It ferments carbohydrates such as dextrin,

maltose, glucose, fructose, ribose, sucrose and starch (Harwich *et al.*, 2010). Finally, it is important to note that *G. vaginalis* is a fastidious organism and requires complex medium to grow, as well as a 10% CO_2 atmosphere, because they are facultative anaerobic bacteria (Harwich *et al.*, 2010).

In relation to virulence factors G. vaginalis is responsible for the of hemolysin and vaginolysin that can be associated to its capability for biofilm formation.

Formation of biofilm: *Gardnerella vaginalis* has the capability to form an adherent biofilm on the vaginal epithelium of women with BV. The aggregation ability of *G. vaginalis* is considered a virulence factor that enhances the bacterial attachment to epithelial surfaces. The aggregation of bacteria in monolayer prevents the access of antimicrobial agents against them, that are usually dormant; and confers resistance to the host's immune defenses (Patterson *et al.*, 2007; Swidsinski *et al.*, 2008; Patterson *et al.*, 2010). The biofilm formation increases the resistance to the bacterial byproducts such as lactic acid and hydrogen peroxide, which are normally produced by lactobacilli present in healthy vagina, and suppress the growth of anaerobes such as *G. vaginalis* (Swidsinski *et al.*, 2008; Harwich *et al*, 2010).

Hemolysin: *Gardnerella vaginalis* is able to produce hemolysin as a virulence factor. Hemolysin is the unique identified 59 kd pore-forming cytolysin, which is produced by this bacterium. It is very selective on human erythrocytes and after the formation of a pore on the target membrane, induces cell lysis through a colloid osmotic mechanism (Cauci *et al.*, 1996). *Gardnerella vaginalis* hemolysin could be associated to the alteration of epithelial cells, forming the so-called clue cells (Cauci *et al.*, 1996). Some studies also associate the elevation of immunoglobulin A levels in the vaginal fluid of many patients with acute BV with the production of a perforin-like protein (Cauci *et al.*, 1996).

Vaginolysin: This virulent factor is a cholesterol-dependent cytolisin which increases the availability of the cellular contents, like a substrate to bacterial growth (Harwich *et al.*, 2010; Patterson *et al.*, 2010). This cytolisin is a pore-forming protein and utilizes the complement regulatory molecule CD59 to activate, on human epithelial cells, the epithelial p38-mitogen-actived protein kinase, leading to the cell death. The mucosa response to that process by increasing the level of immunoglobulin A (Patterson *et al.*, 2010). Other study supports that cytolitic action also makes the vaginal epithelium more susceptible to infection by HIV virions (Gelber *et al.*, 2008).

1.11   Identification methods in Bacterial Vaginosis

Over the decades, molecular techniques have been gaining importance as methods for modern diagnosis in microbiology, overpassing the conventional characterization based on culture tests. In relation to BV, culture is still a technique that could be involved on the identification of the microorganisms. That could be explained by its simplicity, low cost, and high sensibility through biological amplification. In a clinical microbiology laboratory, the identification of positive cultures constitutes a significant part of the work and, traditionally, the identification is the result of a

combination of morphology, growth characteristics, biochemical tests and molecular techniques (Amann and Fuchs, 2008). For example, the growth of *G. vaginalis* can be identified by beta hemolysis, positive Gram staining, negative oxidase and catalase, and the formation of a biofilm in the vaginal epithelium can be visualized under fluorescence microscopy, demonstrating that the etiology of BV is heterogeneous (Swidsinski *et al.*, 2005; Srinivasan and Fredricks, 2008; Livengood, 2009). Other relevant microorganism is *A. vaginae,* which usually appears as Gram-positive elliptical cocci in pairs or short chains (Rodriguez *et al.*, 1999). In blood agar, at 37 ºC, they grow as small pinheaded colonies (Rodriguez *et al.*, 1999). Furthermore, lactobacilli were identified to the genus level by Gram staining, colony morphology and positive catalase test (Walker and Thornsberry, 1998).

1.11.1 Biochemical and culture test

Gram staining

The Gram staining has the capability to distinguish between two different types of bacterial cell wall that differ in chemical composition and molecular architecture. In the clinical microbiology laboratory, Gram staining is commonly used as the first step in classical bacteria identification. Gram staining of vaginal fluid could be utilized to distinguish between the normal vaginal flora from BV microflora and is based on the Nugent score (Fredricks *et al.*, 2005). The capability to retain the dye depends on the thickness of the peptidoglycan. In fact, Gram-positive layer is thicker than the Gram-negative layer, obtaining a dark purple color smear (Gardner and Dukes, 1955).

Relative to *G. vaginalis*, it is considered Gram-variable (Livengood, 2009), because the Gram-staining result differs between Gram-positive to Gram-negative. In fact, *G.vaginalis* has a peptidoglycan layer with several amino acids profiles, which is a common aspect in Gram-negative bacteria, but fails in the presence of ribitol teichoic acid, always common in Gram-positive (Turovskiy *et al.*, 2011). On the Gram staining of normal vaginal women, it is also possible to see clue cells surrounded by lactobacilli species that produce hydrogen peroxide, bacteriocins and organic acids involved on suppression of the growth of other microorganisms (figure 1.2) (Livengood, 2009).
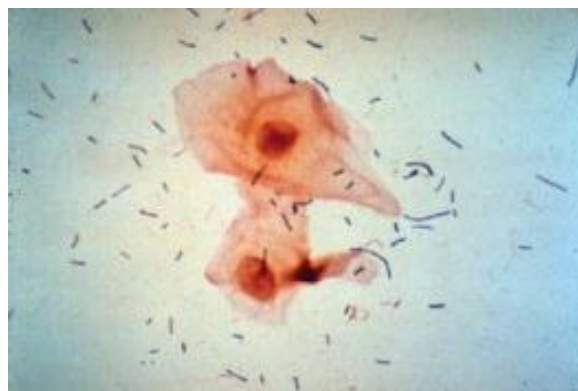
Figure 1.2: Gram staining of normal vaginal epithelium. Total magnification=400x (In: Livengood, 2009).

Oxidase and catalase test

The oxidase test is a biochemical test applied on the identification of bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. *Gardnerella vaginalis* is characterized for being oxidase negative, as well as *Lactobacilli* spp. (Harwich *et al.*, 2010).

Catalase is other biochemical test. Catalase negative bacteria are, usually, anaerobes or facultative anaerobes (Harwich *et al.*, 2010). This is the case of *G. vaginalis*. The two biochemical tests are important on the characterization of *G. vaginalis*, however, they cannot be applied directly on clinical samples, but only in isolates. *Lactobacilli* spp. is catalase positive (Harwich *et al.*, 2010)

In relation to *A. vaginae,* no study relates the result of these tests, which could be explained by its low capability of culture and posterior characterization.

Culture

Growing the bacteria in specific solid media is an usual method for the isolation of a pure culture from a mixture. So, on the agar-based surface, the inoculum is streaked, and after a few sub-cultures on fresh agar plates, isolated colonies are obtained. After the isolation, biochemical tests can be done to identify the bacteria (Arvidson, 2010). Different growth media can be used to isolate *G. vaginalis.* Medium containing 5-10% of horse blood allows the differentiation between hemolytic and non-hemolytic, and the isolation of fastidious microorganisms. Catlin (1992) also refers the value of a semi-selective medium, such as Columbia Blood Agar (CBA). *Gardnerella vaginalis* can also grow on a CBA media without antibiotic and Bromocresol purple agar medium. The incubation at 37 ºC for 48-72 hours, in a humidified atmosphere of air plus 5 to 10% of CO_2, or even the growth in a jar containing a candle or anaerobic bags, is suitable for practical purposes.

1.11.2 Molecular techniques

Besides culture tests, bacteria associated to BV can be rapidly identified by molecular methods such as *Polymerase Chain Reaction* or Peptide Nucleic Acid Fluorescence *in situ* Hybridization. These methods, besides being highly sensitive, give specific results in just a few hours (Swidsinski *et al.*, 2011).

The high level of sensibility of nucleic acid amplification tests, such as *Polymerase Chain Reaction*, allows the use of less invasive specimen types, including self-collected vaginal swabs, which are less sensitive to culture methods (McKechnie *et al.*, 2009). The Fluorescence *in situ* hybridization is as well a sensitive method that allows the analysis and discrimination of the viable bacteria present in samples. On this project, these two molecular techniques were selected in order to characterize the vaginal microflora.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is considered a direct test consisting in the amplification of nucleic acids and is commonly used in medical and biological research labs for many applications (Amann and Fuchs, 2008). The PCR technique allows the generation of thousands to millions of copies of a certain piece of DNA, starting from only a few copies, and it involves a replication process of DNA that occurs *in vitro*. The process involves repeated cycles of heating and cooling for denaturation and enzymatic replication of the DNA (figure 1.3) (Henriques, 2011). For the reaction to take place, a DNA template is required (target DNA), a set of primers, which hybridize with the 3´region of each target DNA chain, DNA polymerase, such as Taq Polymerase, which has an optimal action temperature of about 70ºC; deoxyribonucleotide triphosphate (dNTPs) that are modified nucleotides and are involved in DNA synthesis; buffer, reacquired for a high enzymatic activity and divalent cations (Mg^{2+} or Mn^{2+}) or monovalent cations (K^+), that are enzymatic co-factors. Each PCR reaction usually utilizes between 10 to 200 µl of total reaction volume (Henriques, 2011).
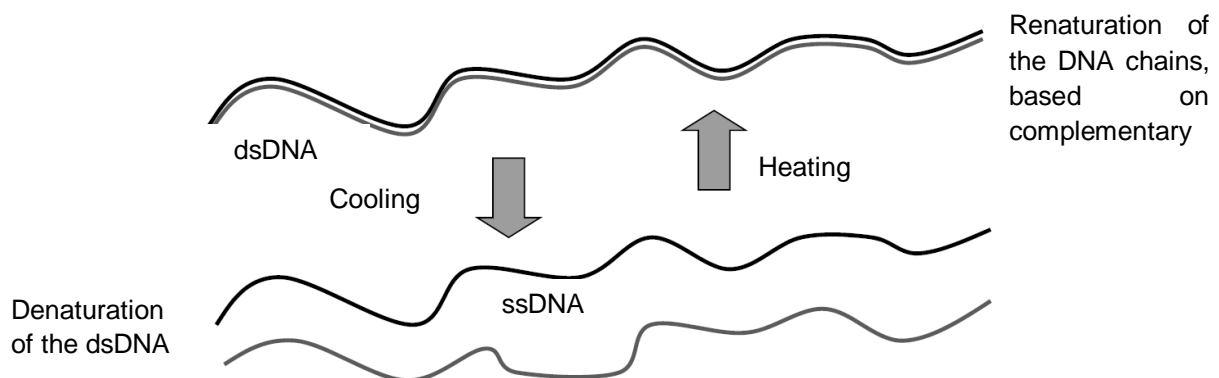
Figure 1.3: *Polymerase Chain Reaction* concept (Adapted from: Henriques, 2011).

Process of amplification

The process of DNA amplification involves three steps: denaturation, primmer annealing and elongation (figure 1.4) (Henriques, 2011). The first process is initiation, where the heating temperature differs between 94 to 96 ºC, for 1-9 minutes; a denaturation step, important for the melting of template DNA, by disruption of hydrogen bonds which results in a single stranded DNA. The next step is annealing and lasts 20-40 s, at the proper temperature for the specific primers used. The temperature of annealing (Ta) is almost lower than the melting temperature (Tm) and here, the primers hybridize partially or completely with the template and the only stable hydrogen bonds are formed. Then, there is the extension/elongation phase, where the DNA polymerases synthetize a new DNA chain, which is complementary to the template and an insertion of dNTPs occurs in 5´- 3´direction. The temperature applied in this step depends on the polymerase used (figure 1.5) (Henriques, 2011).
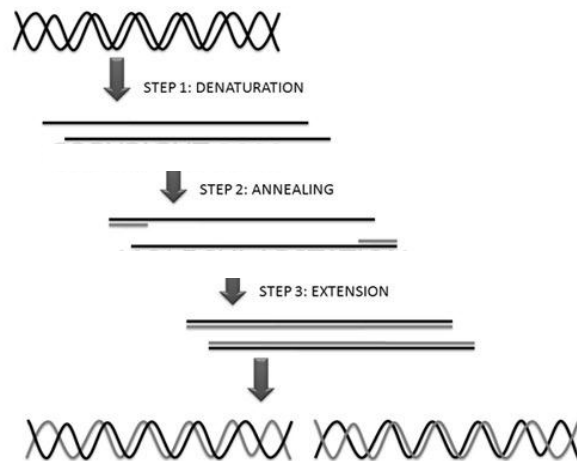
Figure 1.4: *Polymerase Chain Reaction* cycle steps (In: Molecular Station, 2011).
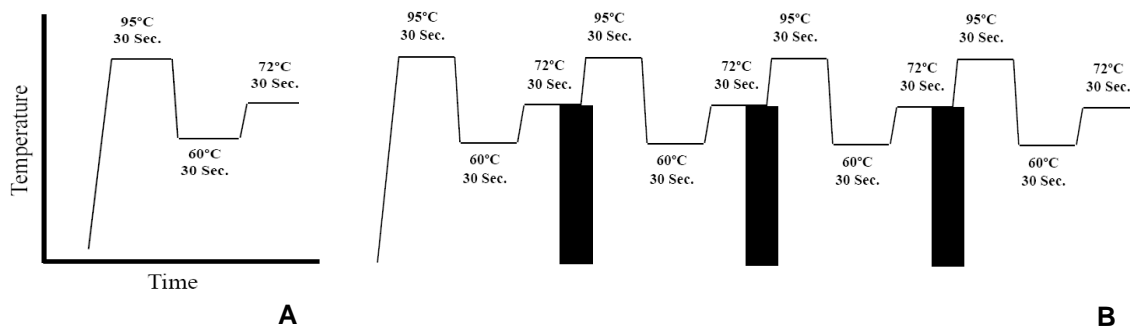
Figure 1.5: Temperatures of denaturation, annealing and elongation. Representation of one cycle (1.5 A) and four cycles (1.5 B) (In: Henriques, 2011).

Electrophoresis

After the amplification of DNA fragments, electrophoresis is needed in order to detect the amplified DNA (figure 1.6 A, B and C). Since the DNA is negatively charged, it migrates to the cathode on an agarose gel. It is necessary to add a DNA binding dye to the agarose gel, such as Midori Green or Ethidium Bromide. Midori Green consists on a nucleic acid stain that is more recently used as an alternative to the traditional ethidium bromide, because it is not toxic (Labgene Scientific Suisse, 2012). It is important to note that electrophoresis needs to be done on a running buffer, usually TAE (Tris-acetate EDTA), which facilitates the observation of the fragments migration. Negative and positive DNA ladders may also be added to comparison of results.

Figure 1.6: Representation of an agarose gel observed at naked eye   (1.6 A), a digital image of the same gel (1.6 B) and the gel transiluminated with UV radiation (1.6 C) (In: Henriques, 2011).

Polymerase Chain Reaction and clinical samples

In relation to PCR, some limitations could be associated to it. It is the case of the formation of PCR primer dimers, which corresponds to the annealing of the primers utilized with themselves, or with the other primer, resulting on a PCR product of less than 100 bp (Life Technologies, 2011). Furthermore, the result obtained on agarose gel is also based on the end-point of each reaction. This result is founded on size discrimination, which does not allow a great accuracy. So, the analysis of the end-point is non-automated, has poor precision, low sensibility and is only based on size (Labgene Scientific Suisse, 2012). The formation of a non-specific product is other case which corresponds to non-specific hybridization of the primers to the wrong sequence of DNA (Molecular station, 2011). If there are no PCR products or bands, the primers may be annealing with each other and it is necessary to design them again. Treatment with DNAses; the use of autoclaved water; the increase of DNA template amount or the annealing temperature, are some solutions for the control of this limitation (Molecular station, 2011).

On the other hand, several authors considered PCR as a sensitive method for a rapid detection of microbial pathogens in clinical samples. This alternative has an important role, especially in cases of microorganisms that are difficult to culture or if they require a long period of incubation (Life Technologies, 2011). So, despite the susceptibility of PCR to contamination, sensibility to experimental conditions or even to inhibitors, it still has significant advantages in the detection of *G. vaginalis* or other microorganisms associated to BV (Life Technologies, 2011).

More molecular techniques, such as FISH, can be used for the identification of these microorganisms and for the comprehension of the etiology of BV.

Fluorescence *in situ* hybridization (FISH)

Traditional *in situ* hybridization is based on the annealing of DNA or RNA molecules to a particular target sequence inside a cell (Cerqueira *et al.*, 2008). Fluorescence *in situ* hybridization (FISH) is a useful tool for independent detection of the target cells *in situ*, by application of oligonucleotide probes attached to a fluorescence label (Pavlekovic *et al.*, 2009). It is increasingly becoming a promising molecular biology technique. It is actually applied in pathogen detection in clinical samples, identification of biomarkers involved in cancer progression, analysis of microbial communities, evaluation of chromosomal stability in stem cell research and also the genes expression (Almeida, 2011). This technique could be applied in identification, quantification or even for phylogenetic characterization of specific microbial populations, in complex environment, and by combination with other techniques (Amann and Fuchs, 2008). The diagnosis of bacterial biofilm-related infections could also take place (Lebeer *et al.*, 2011).

Fluorescence *in situ* hybridization detects nucleic acid sequences by a fluorescence labeled probe that hybridizes, in a specific way, its complementary sequence within the intact cell. Fluorescence *in situ* hybridization is based in phylogenetic markers at 16 or 23 rRNA, which are less influenced by the growth conditions. It utilizes labeled DNA probes for the *in situ* identification of the microorganisms, by hybridization with the ribosomal RNA (Almeida *et al.*, 2011).

Peptide nucleic acids probes (PNA)

Fluorescence *in situ* hybridization methods usually involve the use of DNA oligonucleotide probes, containing about 20 base pairs. However, the use of DNA probes had some limitations associated to cell permeability, hybridization affinity and target site accessibility. This results on a lower signal and loss of sensibility and specificity (Almeida *et al.*, 2011). More recently, peptide nucleic acids (PNA) have been studied and optimized for bacterial recognition. Peptide nucleic acids probes are synthetic DNA mimics, where the negatively charged sugar-phosphate backbone of DNA is replaced by an achiral neutral polyamide backbone. This polyamide is formed by repetitive units of N-(2-aminoethyl) glycine (Guimarães *et al.*, 2007; Almeida *et* al., 2009) (figure 1.7 C). Peptide nucleic acids have the capability to hybridize with complementary nucleic acid targets, based on Watson-Crick base-pairing rules (Guimarães *et al.,* 2007). Individual nucleotide bases are attached to each of the units to offer a molecular design that enables PNA to hybridize to complementary nucleic acid targets (Amann and Fuchs, 2008). The optimization of protocol for the identification of microorganisms associated to BV was done by two members involved in this project (Carvalho, 2011; Machado *et al.*, 2011).

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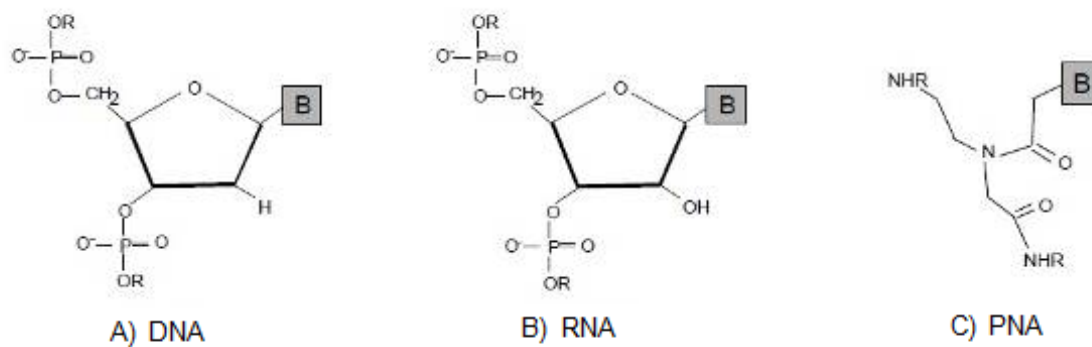
Figure 1.7: Chemical structures of DNA (1.7 A), RNA (1.7 B) and PNA (DNA mimics) (1.7 C) (In: Cerqueira *et al.*, 2008).

The use of rRNA as a target molecule

The abundance of rRNA target molecules allows the detection of individual cells and the identification by the use of fluorescent-labeled probes. The use of rRNA as a target molecule increases the sensibility of target amplification methods such as transcript-mediated amplification (Amann and Fuchs, 2008; Cerqueira *et al.*, 2008). The hybridization with PNA probes is efficient in low salt concentrations, which is ideal for nucleic acid with complex structure like rRNA (Hoshino *et al.*, 2008). These probes can also be applied on penetration on biofilms formed in BV.

PNA-FISH steps

The FISH experience can be divided in three steps: fixation, hybridization and washing. The first step is fixation which includes the application of chemical fixatives. The treatment with fixatives such as methanol, ethanol or paraformaldehyde, not only stabilizes cell morphology but also permeabilize the cell membrane for subsequent hybridization. Regarding the fixation step, formaldehyde and ethanol are still the fixatives of election, but there is still no standard permeabilization protocol for all microorganisms. Empiric optimizations often consider the specific composition of the cell wall (Amann and Fuchs, 2008). In the first step of hybridization, the probe must access the target sequence within the ribosome. To increase the effectiveness, factors such as temperature, pH, concentration of fixatives and ionic strength should be regulated. After incubation with probe, usually for a few hours, the labeled oligonucleotide diffuses to its intracellular targets and forms specific hybrids. Then, on the washing step the excess probe is washed away. The sample is then ready for single-cell identification and quantification by fluorescence microscopy (Amann and Fuchs, 2008; Cerqueira *et al.*, 2008).

1.12 Objectives

The overall goal of this project was the identification of *G. vaginalis* and *A. vaginae* in the vaginal microflora of Portuguese women, providing also additional information between normal and BV microflora. The project involved, at first, the sampling of different clinical cases and the posterior analysis using molecular analysis of the microorganisms present based on PCR and FISH. The samples were collected by self-harvest, on gynecological private practice and in hospital emergency and, after that, the treatment and analysis was done at the Center of Biological Engineering of University of Minho.

Due to the lack of proper and updated information regarding BV in Portugal, we devised the study reported here. Currently there is only one limited study to BV occurrence in the Portuguese population (Guerreiro *et al.*, 1998), and as such our study is relevant for the characterization of that pathology in Portugal. Therefore, the main objective of this study was to determine the prevalence of *G. vaginalis* in the Portugal population. We also aimed to determine if the novel described *A. vaginae* was commonly present in Portuguese women. Furthermore, a short epidemiological survey was performed.

2.0 Materials and Methods

2.1 Collection of vaginal samples

During this study fifty-seven samples were collected. Thirteen were collected by self-harvest, in healthy volunteers, thirteen in hospital emergency and thirty-one in gynecological private practice appointments. Self-harvest samples were instructed to insert the swab on the vagina. The swab was then rotated against the vaginal wall. After that, it was removed and it was put in a plastic tube containing Amies transport medium with coal. Within 48 hours after the sample collection (ideally within 24 hours) in clinical scenario or self-collection, the samples were transported to University Of Minho, Department of Biological Engineering, where the microbiological analysis were performed (Sadhu *et al.*, 1989; Boskey *et al.*, 2004; Menard *et al.*, 2012).

2.2 Treatment of samples from swabs

After the reception of the samples, the swabs were treated (Zarate and Nader-Macias, 2006; Tamrakar *et al.*, 2007). Some swabs were, initially, rotated into a microscope slide for Gram staining. After that, the swabs were streaked in Columbia Agar (Oxoid) supplemented with 5% of defibrinated horse blood, with and without *G. vaginalis* supplement containing the antibiotics Gentamicin, Nalidixic acid and Amphotericin B (Sigma – Aldrich). Plates were incubated at 37 ºC, anaerobically, using a CO_2 incubator (HERAcell 150, Thermo Electron Corporation) set with 10% CO_2 and 5% O_2. After this, the swabs were placed in a 10 ml tube with screw top, containing 2 ml of NaCl (0.9%) and the microbial cells contained in swab were suspended using vigorous vortexing. The final suspension was divided in two portions of 0.5 ml each, in sterile eppendorffs, and one other of 1 mL. The three contents were centrifuged at 10000 rpm during 5 minutes using a microcentrifuge. The pellet containing 1 ml of cell suspension was ressuspended in 800 µl of BHI and transferred to a criovial to which 200 µl of glycerol solution was added, and stored at -80 ºC. One of the pellets resulting from the centrifugation of the 0.5 ml suspension was ressuspended in 500 µL of PBS 1x and the other in 500 µL of ultra-pure water and they were stored at -20 ºC, to posterior use at FISH or PCR, respectively (Schwebke, 1999), as described on sub-section 2.4.1 and 2.5.6.

2.3 Gram-staining

During the treatment of the samples, a direct smear was done by transferring, directly, the vaginal fluid present on the swab to a glass slide. As such, the smears were heated fixed and, then, Gram staining was performed (Sadhu *et al.*, 1989; Nugent *et al.*, 1991; Boskey *et al.*, 2004). The microscope slides were visualized on the microscope OLYMPUS BX51, with a total magnification of 1000x (immersion lens). The pictures were taken using the camera OLYMPUS DP71 and the manipulation of them were done using OLYMPUS Cell B program.

2.4 *Polymerase Chain Reaction* of samples collected with a swab

2.4.1 Cell lysis and DNA extraction

The eppendorf, containing the cell suspension in ultra-pure water (sub-section), was treated with a heat shock in order to weaken the cell walls and facilitate the release of DNA during the PCR reaction. Cell suspension was incubated for 20 minutes at 95ºC in the heating block and, at the end of which, the suspension was, immediately, cooled on ice for at least 5 minutes.

2.4.2 *Polymerase Chain Reaction* conditions

The PCR Mastermix (Dynenzyme II PCR Mix, Finnenzymes) was prepared for n + 1 reactions, n being the total number of reactions necessary, and for each primer combinations that was used. For each PCR reaction, with 10 µl total volume, it was necessary to add 3.5 µl ultrapure water, 5 µl of PCR Mix 2x concentrated, 0.5 µl primer forward, 0.5 µl primer reverse and 0.5 µl template, directly, to the PCR tube. Generally, *G. vaginalis, A. vaginae* and *Lactobacillus* spp. primers targeting the conserved regions of the 16S rRNA gene were used (table 2.1). The negative control was prepared adding 4 µL of ultra-pure water, 1 µL primer Mix Control (Part of the DyNAzyme II DNA polymerase kit, Finnenzymes) and 5 µL of PCR Master Mix. For the positive control preparation it was added 0.5 µL of DNA control, 1 µL of Primer Mix Control (Part of the DyNAzyme II DNA polymerase kit, Finnenzymes), 3.5 µL of ultra-pure water and also 5 µL of Master Mix. This control corresponded to a DNA provided with the PCR kit that was amplified by the primers used. Then, the tubes were taken to the thermocycler. The thermocycler was programmed with the following PCR protocol: step one - 94 ºC for 4 minutes; step two - 94 ºC for 30 seconds; followed by 62 ºC for 30 seconds and 72 ºC for 45 seconds. Step two, was repeated for forty times. The final extension was at 72 ºC for 7 minutes and then the PCR tubes were hold at 4 ºC (Aroutcheva *et al.*, 2001).

Primer specificity was previously evaluated by another project member. The temperature of annealing tested was also referred and tested (table 2.1) (Magalhães *et al.*, 2011).

2.4.3 Agarose gel preparation

A 1% (wt/vol) agarose gel with TAE 1x buffer was prepared. The agarose was weighed, directly, to a Schott bottle and the adequate volume of TAE 1x was added. At this point the solution appeared cloudy as the agarose was still not in solution. The solution was heated in the microwave until it became clear and then, agarose solution was allowed to cool. After that, it 10 µl of Midori green (Grisp) were added and the mix was swirled gently. The desired cast was prepared with the comb and the agarose solution was poured into it. The agarose was allowed to cool and polymerise. When the gel was polymerised it was put it in the electrophoresis tank which contained TAE 1x. The comb was removed and, before loading the samples on to the agarose gel, 1 µl of loading buffer was added to each PCR reaction. The addiction of loading buffer turned the solution blue which made the gel

loading easier and samples to settle in the agasose well. The PCR samples were loaded, as well the negative and positive control and the Lambda (72- 23 130 bp, Finnenzymes) into the gel. The gel was run at 100 Volts for 30 minutes and the results were checked using ChemiDoc system.

Table 2.1: Sequence of the primers utilized on the identification of *A. vaginae*, *G.vaginalis* and *Lactobacillus* spp.; as also the temperature of annealing (ºC) and the size of amplicon (Adapted: Magalhães *et al.*, 2011)

Primers	Sequence	Temperature of annealing (ºC)	Purpose
Gv Forward	CTCTTGGAAACGGGTGGTAA		Identification of *G. vaginalis*
Gv Reverse	TTGCTCCCAATCAAAAGCGGT	62	
Av Forward	GCGAATATGGGAAAGCTCCG		Identification of *A. vaginae*
Av Reverse	TCATGGCCCAGAAGACCGCC	62	
New Lacto Forward	TGGAAACAGRTGCTAATACCG		Identification of *Lactobacillus* spp.
New Lacto Reverse	GTCCATTGTGGAAGATTCCC	62	

2.5 Fluorescence *in situ* hybridization

The FISH experiences were performed as described on some studies (Guimarães *et al.*, 2007; Almeida *et al.*, 2009; Fredricks *et al.*, 2009; Almeida *et al.*, 2011).

2.5.1 Preparation of hybridization solution

A hybridization solution of 10 ml volume was prepared by adding 1g of 10% (w/v) dextran sulfate, 0.0058 g of 10 mM NaCl, 3 ml of 30% (v/v) formamide, 0.01 g of 0.1% (W/V)(w/v) sodium pyrophosphate, 0.02 g of 0.2% (W/V) polyvinylpirroline, 0.02 g of 0.2% (W/V) FICOLL, 0.02 g of 5 mM disodium EDTA, 0.01 mL of 0.1% (V/V) Triton X-100 and 0.079 g of 50 mM Tris-HCl. After all the compounds were in solution, the final volume was adjusted with sterilized water. Then, the solution was filtered using a filter syringe (0.2 µm of porosity) and the pH adjusted to 7.5. The falcon was covered with aluminum and stored at 4 ºC.

2.5.2 Preparation of washing solution

For the preparation of 500 mL of washing solution, 0.303 g of 5 mM Tris Base, 0.438 g of 15 mM NaCl and 500 µL of 1% (V/V) Triton-x, were mixed in deionized water. After solubilization of the reagents the final volume was made up and the pH was adjusted to10. The solution was autoclaved and stored at 4 ºC. This solution was, preferably, prepared fresh for each use and never stored more than one or two weeks.

2.5.3   Preparation of 4% paraformaldehyde solution for use on fixation

For the preparation of 100 mL of paraformaldehyde solution, first, 65 mL of distilled water were heated to 60 ºC and 40 g of paraformaldehyde was added, all in the chemical hood. A few drops of NaOH (2 M) were added in order to make the white solution clear. Then, 33 mL of 3x PBS were added to the mixture, and adjusted the pH to 7.5 with HCl (1 M). After this, the solution was filtered using a syringe filter (0.2 μm of porosity). Finally, the solution was, quickly, cooled to 4 ºC.

2.5.4   Preparation of 3x phosphate buffered saline solution

For the preparation of 200 mL of phosphate buffered saline solution, it was added 4.8 g of 180 mM NaCl, 0.120 g of 3 mM KCl, 0.486 g of 9 mM $Na_2HPO_4^-$. $2H_2O$ and 0.120 g of 1.5 mM KH_2PO_4 to distilled water.

2.5.5   Preparation of PNA probes aliquots

The probes arrived as a powder and they were stored at -20 ºC. All the following procedures described for the preparation of the probes were done in the dark.

Solution used for preparation of the PNA probe original suspension: 1% TFA (trifluoroacetic acid) and 10% ACN (acetonitrile) solution - for the preparation of 10 mL of solution, 1 mL of ACN was added to 8 mL of ultra-pure water, using the chemical hood. Then, 100 μL of TFA was added and the final volume was completed with ultra-pure water. The solution was filtered with a filter syringe (0.2 μL of porosity) and stored at 4 ºC.

Preparation of original aliquot: The final concentration of this mix was of 100 μM, and it was prepared from the previous solution. The aliquot of the original probe was stored at -20 ºC and covered with aluminum.

Stock aliquots: To prepare aliquots of 4 μM, 40 μL of the original probe of 100 μM was diluted in 960 μL of ultra-pure water. The stock aliquots were stored on fridge at -20 ºC.

"In use" aliquots: The preparation of "in use" aliquot involved the addiction of 50 μL of the stock aliquot and 950 μL of the hybridization solution (200 nM). The "in use aliquots" were stored at 4 ºC.

2.5.6 Hybridization on slides

The inoculum was prepared by homogenizing a few colonies in 750 μL of PBS 1x. The hybridization took place in diagnostic slides (Thermo Scientific). It was added 45 μL of the inoculum to each well of the diagnostic slide. The least 2 slides were prepared (one for control - no probe; and another for test - with probe) and, after that, the slides were fixed using a flame. The reference strains of *G. vaginalis* were *G. vaginalis* ATCC 12457, *G. vaginalis* AMD and *G. vaginalis* 5-1 (Harwich *et al.*, 2010). For the identification of *Lactobacillus,* reference strains were used: *L. casei* CECT 5275 or *L. crispatus* ATCC 33820. The wells were covered with 45 μL of 4% paraformaldehyde solution, during 10 minutes. The slides were pressed against paper to remove the excess paraformaldehyde, and then 45 μL of 50% ethanol was added. After 15 minutes, it was removed. The slides were put in petri dishes, previously covered with aluminum and with paper towels inside. Then, was added 20 μL of the "in use solution" (200 μM) to one of the wells of the slide. In another well 20 μL of hybridization solution (negative control) was added and the slides were covered with cover-slips. The hybridization took place for 90 minutes at 60 ºC. After that, the wash solution was added to the "coplin-jar" and it was incubated along with the slides on petri dishes. The slides were removed from the petri dishes, the cover-slips were also removed, and the slides were transferred into the washing solution and incubated for 30 minutes. After this period, the slides were removed from the coplin jar and they were air-dried. Finally, coverslips were added to the slides as well as immersion oil and the slides were visualized on OLYMPUS BX51 epifluorescence microscope. The microscope contains one filter sensitive to Alexa Fluor 594, a fluorochrome that was attached to the *G. vaginalis* probe, and one filter sensitive to the fluorochrome Alexa Fluor 488 that was attached to the *Lactobacillus* spp. probe (table 2.2) (Life Technologies Corporation, 2012). Some images were also obtained using the DAPI filter which allowed the visualization of all microorganisms. The sensitivity setting in the acquisition of images was ISO 800. To visualize the presence of *Lactobacilli* spp. the green filter (FITC) was used and the red filter (TRITC) as control, because it did not have the ability to detect the signal of this probe. For the identification of *G. vaginalis* the TRITC filter was used and the FITC filter served as control. The PNA probes utilized for identification of *Lactobacillus* spp. and *G. vaginalis* on this study were, on a previous work, designed and developed by our research group (patent under submission) and the conditions of operation of probes were also optimized (Carvalho, 2011). In relation to the identification of *A. vaginae* by PNA-FISH, it was not performed because the probe was not able to be optimized.

The optimization of the protocol utilized for the visualization of specific kind of microorganisms implied the evaluation of temperature, time of hybridization, washing proprieties, definition of fixation agents, and the concentration of the probe. This step had an increased importance because it allowed the achievement of a strong fluorescence signal with specific probes and diminishes the low auto-fluorescence that can affect the other signals (Bento, 2009).

The visualization of the slides were done using a 100 x immersion objective, resulting in 1000 x of total magnification and the camera for the acquisition of color images was OLYMPUS DP71 and the *software* for the acquisition and treatment of images was OLYMPUS Cell B.

Table 2.2: Characteristics of the fluorochromes associated to the PNA probes (Adapted: Life Technologies Corporation, 2012)

Fluorochrome	Absorvation (nm)	Issuance (nm)	Colour	Filter	Microorganisms
Alexa Fluor 488	496	519	Green	FITC	*Lactobacillus* spp.
Alexa Fluor 594	590	617	Red	TRITC	*G. vaginalis*

2.5.7 Optimization of the FISH realized directly on clinical sample

In order to diminish the total coal, present in the swabs, at the moment of FISH, three approaches were tested. First, from one clinical sample obtained by self-harvest, 150 µL of media was removed and divided in 3 equal eppendorfs that were centrifuged for 5 min 10000 rpm. After that, each eppendorf were treated differently. The first treatment involved vortex cycles. The second treatment involved the rapid centrifugation at 5000 rpm only during seconds. The last treatment was the sonication of the sample at 40% intensity during ten seconds. For each treatment a portion of the sample was fixed, and then covered with violet crystal during one minute and the other portion was covered with 15 µL of DAPI (1:1200). At last all the slides were visualized on the microscope OLYMPUS BX51.

3.0 Results and discussion

This section was divided in four sub-sections and contains all the results and the respective discussion. The first one involves the description of the samples associated to this study, the second one includes the characterization of the vaginal flora by classical methods, the third one compares the molecular techniques employed in the analysis of some clinical samples and the last one relates the prevalence and also the distribution of anaerobe microorganisms in samples collected from the vaginal epithelium.

3.1 Samples characterization

This study encompassed, as already referred, the collection of vaginal samples from three different sources. The total number of patients involved on this study was fifty-seven. Thirteen were obtained by self-harvest, thirteen were collected by a specialized doctor in a hospital emergency and the last thirty-one on gynecological appointment at a private practice, which corresponds to 23% of the total samples number, for the first two cases, and 54% to the last one (figure 3.1). In relation to self-harvest of samples from vaginal flora and the respective analysis, some studies considered it as an acceptable method for the diagnosis of BV (Boskey *et al.*, 2004). The swabs were obtained with the consented agreement of all patients and the majority of these women were from the North Coast or, in some cases, Northern Interior of Portugal. The ages of the women differ between fifteen to seventy years old, and the age average is, approximately, of thirty-seven years old. The distribution by age showed that 25% corresponds to ages between fifteen and twenty-five; 29% to the ages between twenty-six and fifty-five and, finally, about 45 % of women had between fifty-six and seventy years old (figure 3.2). The table 3.1 contains additional information about the habits of the women involved in this study that could influence the final result.

Samples source
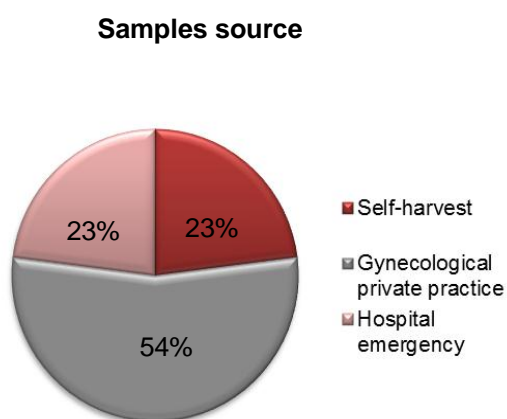
Age distribution

Figure 3.1: Graphic with the sources of the vaginal samples collected in this project.
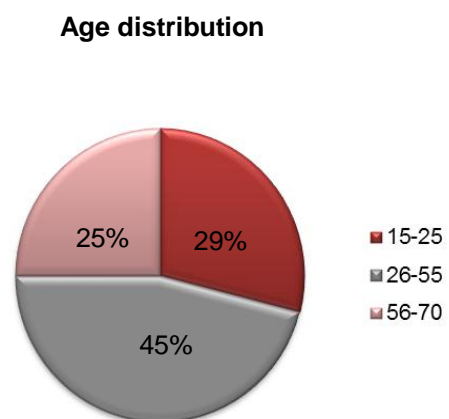
Figure 3.2: Graphic with the distribution of women´s age.

Table 3.1: Discrimination of women characteristics as well the respective number and source of the samples

Women habits/ conditions	Number and source of the samples with the respective condition	Total number of the samples
Symptoms (such as itch, pain, smell and vaginal fluid increment)	5 samples - Gynecological private practice 8 samples - Hospital emergency	
Condom use	2 samples - Self-harvest 2 samples - Gynecological private practice	
Menopause	1 sample - Self-harvest 6 samples - Gynecological private practice 1 sample - Hospital emergency	57
Pregnant	5 samples - Hospital emergency	
Pré-menopause	2 samples - Gynecological private practice	
Smoke	1 sample - Self-harvest 6 samples - Gynecological private practice 1 sample - Hospital emergency	

3.2 Characterization of vaginal flora by classical methods

- **Culture results**

From the total number of samples collected, we randomly selected some samples and proceed to the culture of them in CBA with and without *G. vaginalis* supplement, as already referred. The culture tests were only applied in a few number of samples received because the main goal of this project was the molecular identification of *G. vaginalis* and *A. vaginae* in the vaginal microflora. Four days after plates inoculation, we analyzed the morphology of colonies.

Columbia Blood Agar is a base media typically used on the culture of fastidious and pathogenic microorganisms. The nitrogen, vitamin and also the carbon source are conferred by the presence of Enzymatic Digest of Animal Tissue, Enzymatic Digest of Casein, and Yeast Enriched Peptone. For example, the corn starch present in the media enhances the hemolytic reactions of some streptococci. The sodium chloride has the capability of maintain the osmotic balance of the medium. The supplementation with blood also allows the hemolytic reactions and acts as an additional growth factor.

In Columbia Blood Agar with supplement, we observed the appearance of five of the morphotypes described on table 3.2. The analysis of the growth in Columbia Blood Agar with *G. vaginalis* supplement did not only provide the growth of *G. vaginalis*; however, analyzing the type of colonies, we can say that, the small, transparent colonies might be *G. vaginalis* (table 3.2 and 3.3). On Columbia Blood Agar without antibiotic we observed an increase in the morphologies of colonies, because in this media the absence of *G. vaginalis* supplement allows the growth of other types of microorganisms (table 3.3). On figure 3.3 it was possible to see the distribution of the number of different kind of morphologies by clinical sample. Through the analysis of the results, it was possible to note that, on the majority of samples, in CBA with antibiotic, there were only four distinct morphologies (figure 3.3 A). In 25% of the samples there was no growth. On the other, in 75% of the total samples it appeared one, two, three or five morphotypes with the same proportion (figure 3.3 A). Despite the fact that five was the maximum number of different morphologies found in each individual, we observed eight different morphologies in all plates with CBA with antibiotic. On the other hand, in CBA without *G. vaginalis* supplement, the number of different colonies increased and the presence of five different morphotypes were the most commonly case (figure 3.3 B). In CBA without antibiotic the maximum number it was also five distinct morphologies, however it was possible to visualize twelve morphotypes. Figure 3.4 is an example of growth in two media, of the same sample, where we observed distinct morphotypes.

CBA with antibiotic   **CBA without antibiotic**
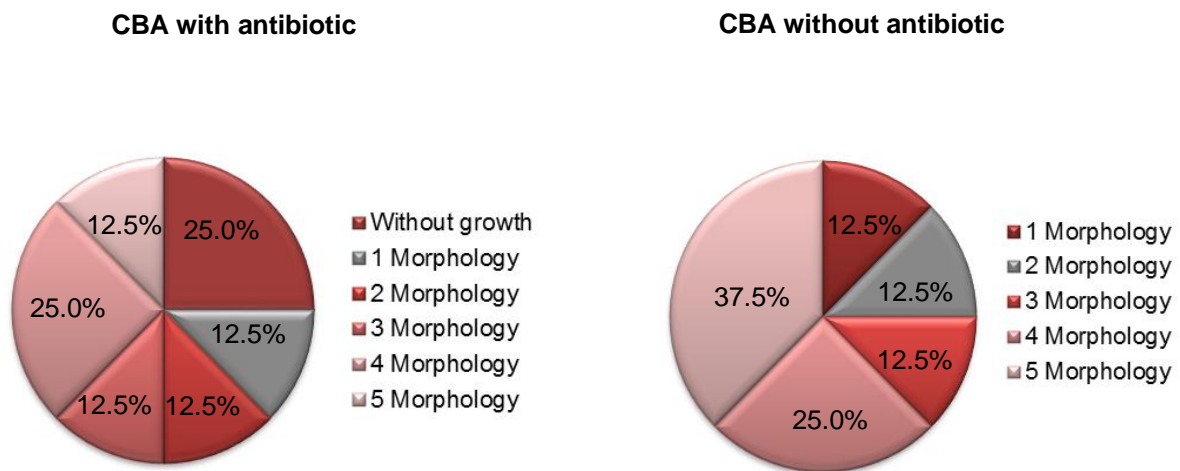
Figure 3.3: Culture results in Columbia blood agar with *G. vaginalis* supplement (3.3 A) and without supplement (3.3 B): percentile distribution of the number of morphotypes obtained in each sample tested.

Table 3.2: Description of the colonies morphotypes grown in CBA with antibiotic

Number of the colony	Description of the colonies grown in CBA with antibiotic
1	Little and transparent round colonies, regular , convex and β-hemolysis
2	Little and transparent round colonies, regular, convex, without hemolysis
3	High and white round colonies, regular, convex with hemolysis
4	Little greys and round colonies, regular, raised, without hemolysis
5	White colonies around the centre and transparent in the middle, regular, convex without hemolysis
6	Transparent colonies with irregular relief, raised and without hemolysis
7	White and round colonies with grey edges, convex and regular, without hemolysis
8	Grey round colonies with more grey biomass in the centre, umbonate, without hemolysis

Table 3.3: Description of the colonies morphotypes grown in CBA without antibiotic

Number of the colony	Description of the colonies grown in CBA without antibiotic
9	Grey and higher colonies with white center and transparent edge, without hemolysis
10	Small white round colonies, convex, without hemolysis
11	Little and transparent round colonies, regular , convex and β-hemolysis
12	Grey medium round colonies, raised, without hemolysis
13	High colonies and transparent with a white center, convex, with hemolysis
14	White colonies with small white points in the middle, convex, without hemolysis
15	Transparent and small colonies with an irregular relief, convex, without hemolysis
16	White colonies with a transparent relief and smaller colonies in the front, convex, without hemolysis
17	Grey and high colonies with a white center, convex, without hemolysis
18	Grey and high colonies with a white center, convex, with β-hemolysis
19	Transparent colonies with two white points in the middle, convex, without hemolysis
20	Small green round colonies, convex, without hemolysis
21	Higher brown round colonies, umbonate, with hemolysis

The figure 3.4 represents a plate of CBA with antibiotic (3.4 A) and without (3.4 B), where it was possible to see some of the different morphotypes related.
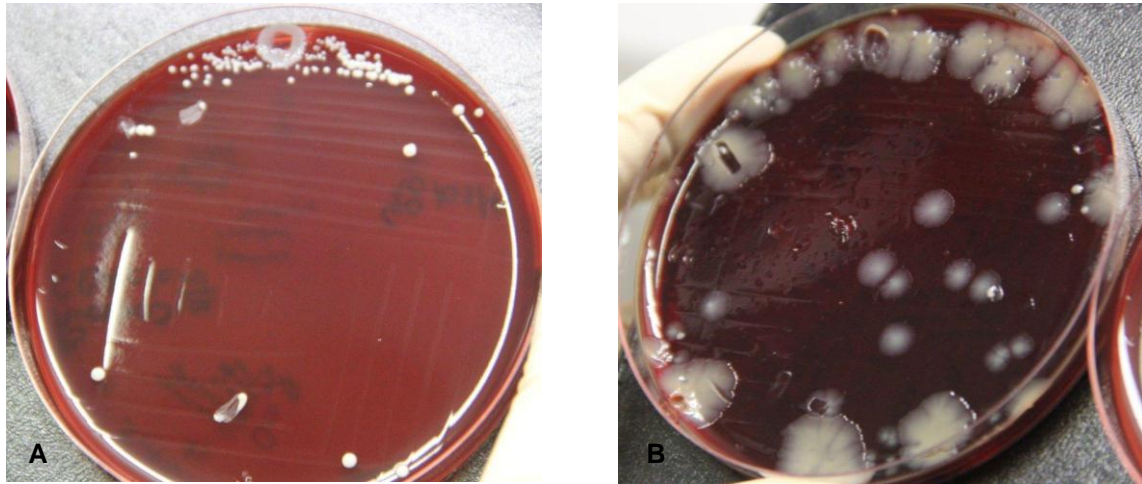
Figure 3.4: Example of the growth of one clinical sample in CBA with *G. vaginalis* supplement (3.4 A) and in CBA without antibiotic (3.4 B).

The culture of the microorganisms of the vaginal epithelium can be difficult because these microorganisms are anaerobes and require special nutritional requirements, which can interfere in the detection of a certain type of microorganisms (Burton *et al.*, 2002; Burton *et al.*, 2004; Srinivasan and Fredricks, 2008). One example of this is *A. vaginae* that is a microorganism associated to vaginal microflora that is not usually detected in culture media but can be identified by molecular techniques (section 3.3) (Burton *et* al.*,* 2004). Some studies report, as well, the failure of the identification of some species like *L. iners* (Lamont *et al.*, 2011). Microbiological culture in selective media involves hard work and is time consuming which can be an obstacle to its use.

- **Gram-staining results**

Another method used on the analysis of the diversity of microorganisms associated to vaginal flora was the optical microscopic observation using Gram-staining. We randomly selected a fraction of the total samples used to perform the Gram-staining because culture tests, as referred previous, and also Gram-staining, were not the principal technique associated to this study. According to the results obtained in Gram-staining, it was possible to represent the results in a figure, demonstrating the diversity of microorganisms presented in the clinical samples (figure 3.5). Using this technique, we found three different morphotypes described as short rods, bacilli and rods. By this way, in 39.3% of the samples tested was found two morphotypes, in 32.2% one morphotype and, at least, in 28.5%, three morphotypes. Furthermore, the figures 3.6 to 3.10 represent different Gram-staining of samples collected by self-harvest, gynecological private practice and in hospital emergency, where is possible to see some of the morphotypes described. It is also important to note that samples 3.6 - 3.8 were obtained from healthy women and 3.9 - 3.10 were collected in women with a previous diagnostic of BV.

Gram-staining results
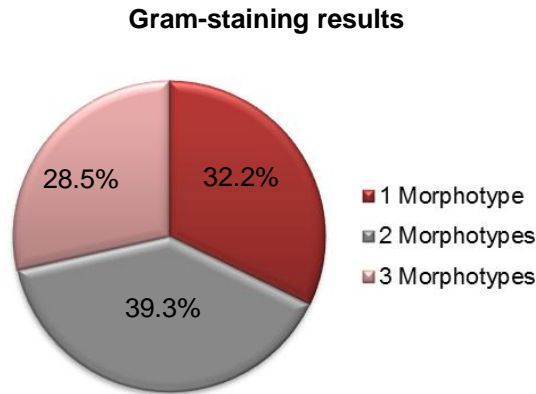
Figure 3.5: Gram-staining results: number of morphotypes identified in individual clinical samples.

On figure 3.6 it is not possible to see epithelial cells but the vaginal microflora reveals the typical morphology of *Lactobacillus* spp. and also short rods that probability could be *G. vaginalis*. The figures 3.7 and 3.8 represent a Gram-staining of a clinical sample obtained on gynecological private practice and hospital emergency, respectively, where is possible to see the presence of *Lactobacillus* spp. and also short rods, surrounding the epithelial cells. On the other hand, the picture 3.9 and 3.10 are Gram-staining of women previous identified by the doctor as unhealthy. On the first one (figure 3.9) is possible to see Gram-variable bacteria identified as *G. vaginalis.* The picture 3.10 represents a Gram-staining of a sample obtained in emergency appointment in hospital. On this picture is visible a large number of microorganisms surrounding the epithelial cells. These microorganisms might be *Mobilincus* spp. based on the curved shape. It is also visible a different shape, similar to a short-rod that could be *G. vaginalis*. It is important to note that this sample was obtained in hospital emergency and in, cases of BV, *Mobilincus* spp. could appear associated with *G. vaginalis* (Turovskiy *et al.*, 2011).
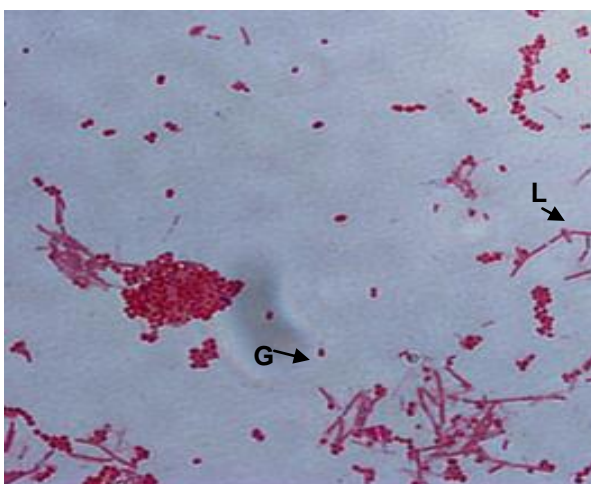
Figure 3.6: Gram staining of sample eleven, obtained by self-harvest and collected from a healthy woman. Total magnification = 1000 x. Legend: L= Possibly *Lactobacillus* spp.; G= Possibly *G. vaginalis*.
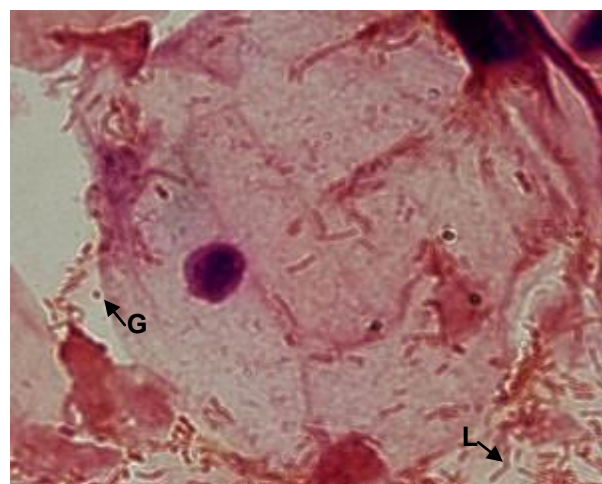
Figure 3.7: Gram staining of sample sixteen, obtained on gynecological private practice from a healthy woman. Total magnification = 1000 x.
Legend: L= Possibly *Lactobacillus* spp.; G= Possibly *G. vaginalis*.
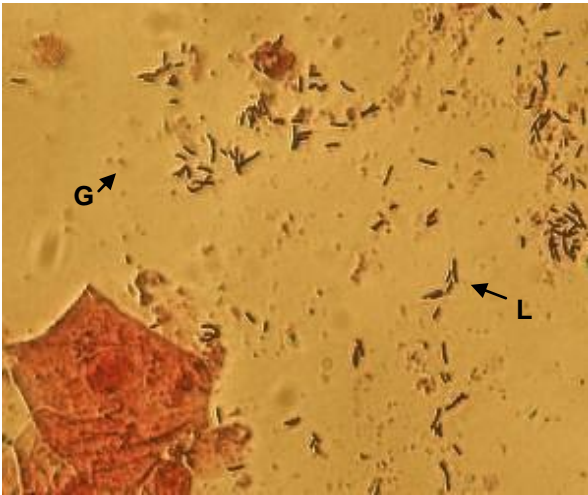
Figure 3.8: Gram staining of sample fourty-seven, obtained in hospital emergency, from a healthy woman. At= 1000 x.
Legend: L= Possibly *Lactobacillus* spp.; G= Possibly *G. vaginalis.*
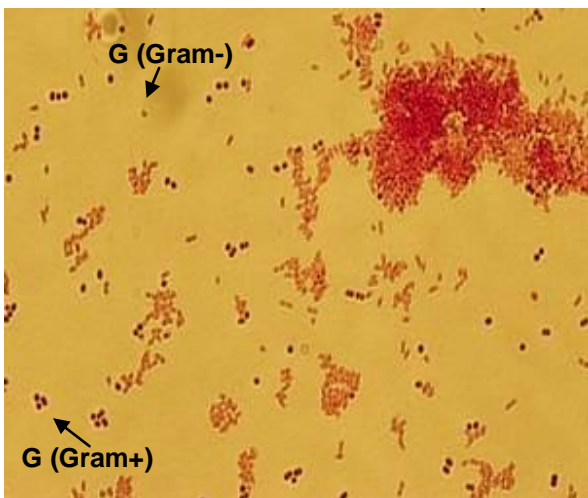
Figure 3.9: Gram staining of sample thirty-five obtained on gynecological private practice, from an unhealthy woman. At= 1000 x.
Legend: G= Possibly *G. vaginalis.*
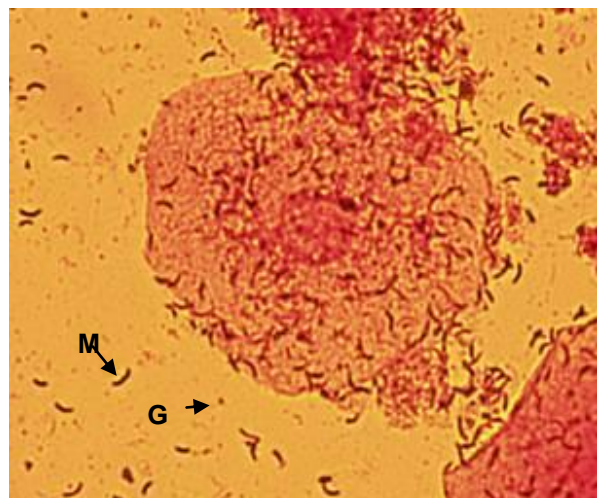
Figure 3.10: Gram staining of sample fifty-five, obtained on hospital emergency, from an unhealthy woman. At= 1000 x.
Legend: M= Possibly *Mobilincus* spp.; G= Possibly *G. vaginalis.*

The advantage of using Gram-staining of the smears of vaginal fluid is the visualization of the different morphologies of the microorganisms presented in the swabs and also the visualization of its position in the epithelial cells. However, Gram-staining has lower specificity because some microorganisms from the vaginal fluid have the similar shape. This is the case of *L. iners* that present a Gram-positive result and have similar shape to that of *G. vaginalis:* short rods (De Backer *et* al., 2007). In comparison with culture methods, Gram-staining has a few advantages, however they both

lack sensitivity. As can be seen in figure 3.5, the maximum number of different morphologies was three against five identified by culture (figure 3.3).

Furthermore, Gram-staining method can have other possible problems if during fixation excessive heat is used, which alters the cell morphology; also problems may arise by excessive cell decolorization, namely by the low concentration of crystal violet; the excessive washing between steps, the insufficient exposition to lugol and the prolonged decolorization with ethanol (Sutton, 2011). Besides this, other problem is related to the moment of specimen collection that could influence the diagnosis of BV. In fact, the microflora is, majority, colonized by lactobacilli, and, in about 80% the composition is altered day-by-day which can affect the result of Gram staining. By this way, sometimes the analysis could result on an wrong result (Schwebke, 1999).

Other biochemical tests can be used for the identification of the microorganisms present in the vaginal microflora. Namely the oxidase test and catalase that provide date that are useful in the identification of microorganisms such as *G. vaginalis* and *Lactobacillus* spp., that are present on the vaginal epithelium of healthy women. However, these biochemical tests cannot be applied on the clinical samples but only after the isolation of each colony present in the different media. Because this study did not include the purification of cultures, these steps were not performed.

3.3 Molecular characterization: PCR versus FISH

Some previous studies related the presence of a spectrum of anaerobe commensals such as *G. vaginalis*, and *Pretovella* spp. and anaerobic Gram-positive like *Mobilincus* species, *Mycoplasma hominis*, *Ureaplasma urealyticus*, by conventional culture, from vaginal fluid of women with BV. The molecular techniques, were, more recently, used in order to increase the spectrum of vaginal microorganisms associated to BV, by-pass the culture methods, and allowed the identification of *A. vaginae, L. iners, Megasphaera, Bifidiumbacterium,* among others microorganisms in this environment (Marrazzo, 2011).

3.3.1 *Polymerase Chain Reaction*

After the reception and treatment of the fifty seven samples, they were analyzed by *Polymerase Chain Reaction* (PCR). This method is very specific and allows the determination of the microorganisms present on the samples, in just a few hours. For this we used primers for the identification of *G. vaginalis A. vaginae* and *Lactobacillus spp.,* that are microorganisms usually present in the clinical samples (Magalhães *et al.*, 2011) and the microorganisms, most frequently, associated to vaginal microflora and BV. This was also the main reason for the study of these microorganisms and not microorganisms such as *Mobilincus* spp., for example. The PCR involved the amplification of 16S rRNA. That corresponds to the small ribosomal subunit of the 16S rRNA gene and it is usually applied in the majority of molecular identification because it is present in all bacteria, it has conserved regions in each species that are targeted with primers and used on the identification of the bacteria (Srinivasan and Fredricks, 2008). The table 3.4 contains the results obtained on the PCR performed when the samples were received and the table 3.5 the results obtained in FISH, for the

same samples(Salgueiro *et al.*, 2011). According to the PCR results all fourteen samples reveals the presence of *Lactobacillus* spp. while *G. vaginalis* was only identified in nine samples (sample number two, three, six, eight, nine, ten, eleven, thirteen and fourteen) (table 3.4). It is important to note that table 3.4 contains only PCR results from the first fourteen treated samples, due to FISH experiences were exclusively realized for these samples.

3.3.2 Fluorescence *in situ* hybridization

FISH is a molecular technique that involves the detection of the region based on the probe for the sequence of interest (Cerqueira *et al.*, 2008).

After the reception of the samples, FISH, besides PCR, was performed in the first fourteen samples, as already referred (table 3.5). The clinical samples were analyzed by FISH using a PNA probe for the identification of *Lactobacillus* spp., a PNA probe for identification of *G. vaginalis* and hybridization solution as a negative control. We also tested *G. vaginalis* reference strains (*G. vaginalis* AMD) and lactobacilli reference strains (*L. casei* CECT 5275 or *L.crispatus* ATCC 33820*)* (appendix - part I). According to the results obtained and summarized in table 3.5, *Lactobacillus* spp. were moderate hybridized using PNA *Lactobacillus* spp. and we had a punctual hybridization of *G. vaginalis*, on sample one. The sample two had a very high signal of *Lactobacilli* spp. fluorescence but a weak signal of *G. vaginalis*. The sample three also had a very high hybridization of *Lactobacillus* spp. as sample number five, but a weak signal of *G.vaginalis* fluorescence and absence, respectively. Sample number four and twelve, had an high signal for the detection of *Lactobacillus* spp. but no signal with PNA probe for the identification of *G. vaginalis*. Sample number six had a low hybridization with PNA *Lactobacillus* spp. but a high fluorescence of *G. vaginalis*. Then, sample number seven had moderate hybridization with PNA *Lactobacillus* spp. and also PNA *G. vaginalis*. Samples number eight; nine and thirty had an elevated signal with the two PNA tested. Sample number ten had too a weak hybridization of *Lactobacillus* spp. Otherwise, the signal with PNA *G. vaginalis* was high. At least, sample number eleven had a high number of *Lactobacillus* spp. hybridized but *G. vaginalis* had not any signal. The results for sample number fourteen, were particularly interesting as this was a sample collected from women with symptoms and diagnosed as BV by the doctor and as such it was different from the other thirteen samples collected by self-harvest. The interest in the analysis of this sample by FISH was high, and had demonstrated a very high hybridization with PNA *G. vaginalis* and low with PNA *Lactobacillus* spp.. All results were compared with the signal of a PNA *Lactobacillus* spp. and PNA *G. vaginalis* with the reference strains *L. crispatus* ATCC 33820 or even *L. casei* CECT 5275 and *G. vaginalis* AMD, respectively (appendix – part I). Hybridization solution applied to each sample instead of each probe was also tested.

Table 3.4: Results of the PCR performed for each clinical sample with *Lactobacillus* spp. and *G. vaginalis* primers and also the source of the samples collected

Table 3.5: Results of the FISH performed for the first fourteen clinical samples using PNA *Lactobacillus* spp. probe and PNA *G. vaginalis* probe

PCR Results			
Samples source	Number of clinical sample	*Lactobacillus* spp. primers	*G. vaginalis* primers
SH	1	+	-
SH	2	+	+
SH	3	+	+
SH	4	+	-
SH	5	+	-
SH	6	+	+
SH	7	+	-
SH	8	+	+
SH	9	+	+
SH	10	+	+
SH	11	+	+
SH	12	+	-
ER	13	+	+
GPP	14	+	+

FISH Results		
Number of clinical sample	PNA *Lactobacillus* spp.	PNA *G. vaginalis*
1	++	+/-
2	++++	+/-
3	++++	+
4	+++	-
5	++++	-
6	-/+	+++
7	+	+
8	+++	+++
9	+++	+++
10	+	+++
11	+++	+
12	+++	-
13	+++	+++
14	+/-	+++

Legend of the table with PCR results:

Negative result: -.
Positive result: +.
ER – Hospital emergency.
SH – Self-harvest.
GPP – Gynecological private practice.

Legend of the table with FISH results:

 - Without hybridization.
+ Weak hybridization.
++ Moderate hybridization.
+++ High hybridization.
++++ Very high hybridization.

According to the results obtained, it is possible to note that the FISH results gathered on the analysis of the clinical sample three to five and eight to thirteen, are in concordance with the PCR results using the primers specific to *G. vaginalis* and *Lactobacillus* spp. Nonetheless, analyzing the FISH result from sample one, it had a punctual hybridization of *G. vaginalis* in the sample but a negative result for PCR, using *G. vaginalis* primers. The negative result could be explained by an absence of sufficient DNA in the sample suspended for amplification. The sample number two also had a low signal of fluorescence using the *G. vaginalis* PNA; however it is a positive result even though in that aliquot, the biomass present was low. The same type of result can be observed for sample number six, although with the *Lactobacillus* spp. PNA probe. The FISH result for sample seven also reveals the same profile as the previous samples, but the positive result using PNA GARD instead the negative result, could be justified by the presence of sufficient and representative biomass in the FISH aliquot. Comparing sample seven to controls, we obtained a high fluorescence of the

reference lactobacilli with PNA GARD and of *G. vaginalis* with PNA GARD (appendix- part I). On the wells containing each clinical sample and incubated with hybridization solution (no probe) the result was, at always, an absence of fluorescence, as expected. It is also important to note that, in some FISH experiments, it was observed a high auto-fluorescence that could be explained by the high number of cells in PBS suspension, by the condition of the wash solution, or by the presence of epithelial cells in the clinical sample.

The following figures show some representative images of FISH obtained for part of the clinical samples.

Figure 3.11: Fluorescence micrographs of vaginal-fluid smears of a healthy woman (sample seven), analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *Lactobacillus* spp, with DAPI, TRITC and FITC filter (Total magnification=1000x).
   Legend: L represents the hybridized *Lactobacillus* spp.

On figure 3.11 it is possible to see some fluorescence bacilli (L), although some auto-fluorescence is seen with all the filters. Figure 3.12 represents the result of *G. vaginalis* PNA applied to clinical sample seven, collected from a healthy woman. The following figure show the presence of *G. vaginalis*, represented as G (figure 3.12).

Figure 3.12: Fluorescence micrographs of vaginal-fluid smears of a healthy woman (sample seven), analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids) of *G. va*ginalis, with DAPI, TRITC and FITC filter (Total magnification=1000x).
   Legend: EC represents the epithelial cell and G the fluorescence *G. vaginalis*.

Figure 3.13: Fluorescence micrographs of vaginal-fluid smears of a healthy woman (sample twelve), analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *Lactobacillus* spp, with DAPI, TRITC and FITC filter (Total magnification=1000x).
  Legend: EC represents the epithelial cell and L the hybridized *Lactobacillus* spp.

According to the results obtained in FISH for the sample number twelve, it is possible to see on figure 3.13 and with the FITC filter the hybridization of *Lactobacillus* spp. (reported as "L") covering the epithelial cell (EC). Analyzing the figure 3.14 it is possible to see a fluorescence point represented as G, with the TRITC filter that does not exist with the other filters. This difference in fluorescence between the filters allows us to say that *G. vaginalis* is present in this sample, although in low quantities. We also can see the presence of epithelial cell (EC) and the common fluorescence around these.
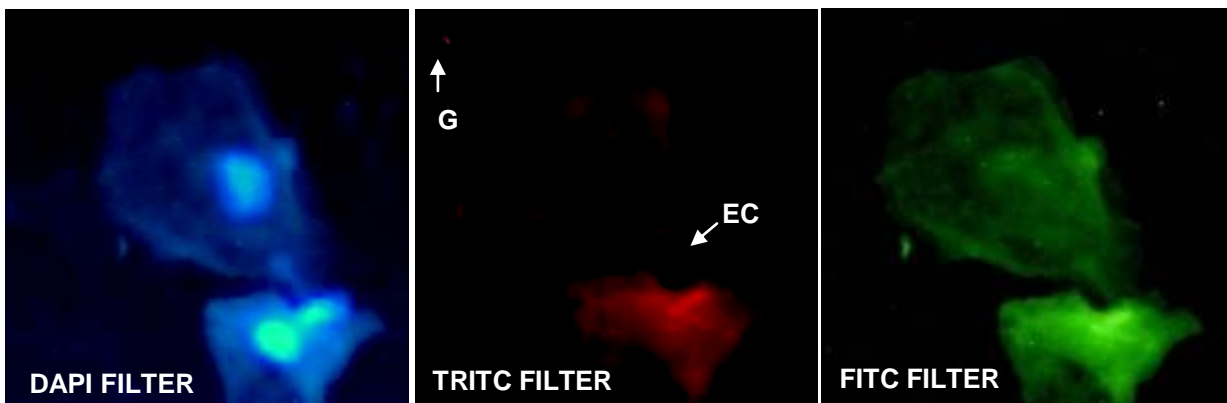
Figure 3.14: Fluorescence micrographs of vaginal-fluid smears of a healthy woman (sample twelve), analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *G. va*ginalis, with DAPI, TRITC and FITC filter (Total magnification=1000x).
  Legend: EC represents the epithelial cell and G the fluorescence *G. vaginalis.*
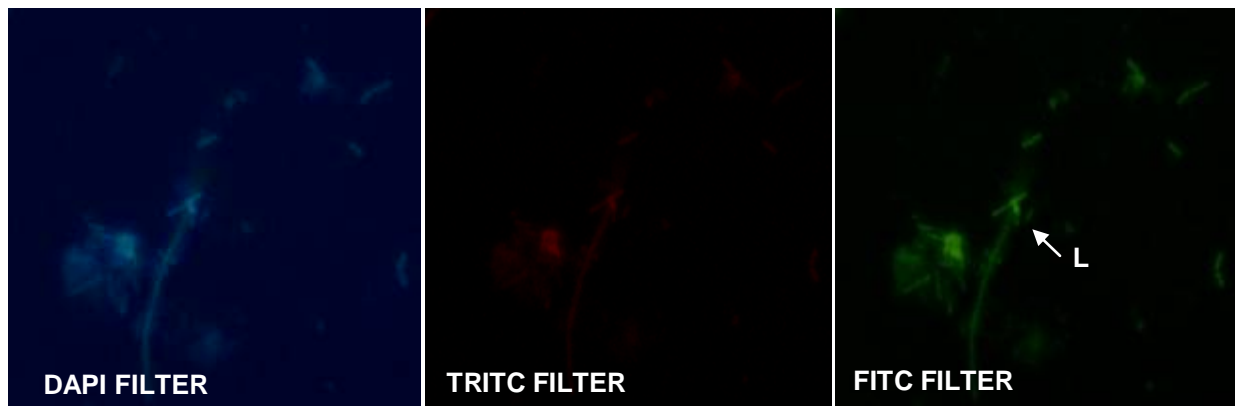
Figure 3.15: Fluorescence micrographs of vaginal-fluid smears of a healthy woman with symptoms (sample thirteen), analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *Lactobacillus* spp, with DAPI, TRITC and FITC filter (Total magnification=1000x).
Legend: L represents the hybridized *Lactobacillus* spp.

The previous images represent some FISH examples obtained in sample thirteen. Analyzing the results with FITC filter we can see hybridization of the *Lactobacillus* spp., although the signal is not string as in the other images, and this could be explained by different reasons such as low membrane permeability or even problems with the probe fluorescence and stability (figure 3.15). Using the TRITC filter is possible to see a high hybridization and the overlap of the cells (figure 3.16).
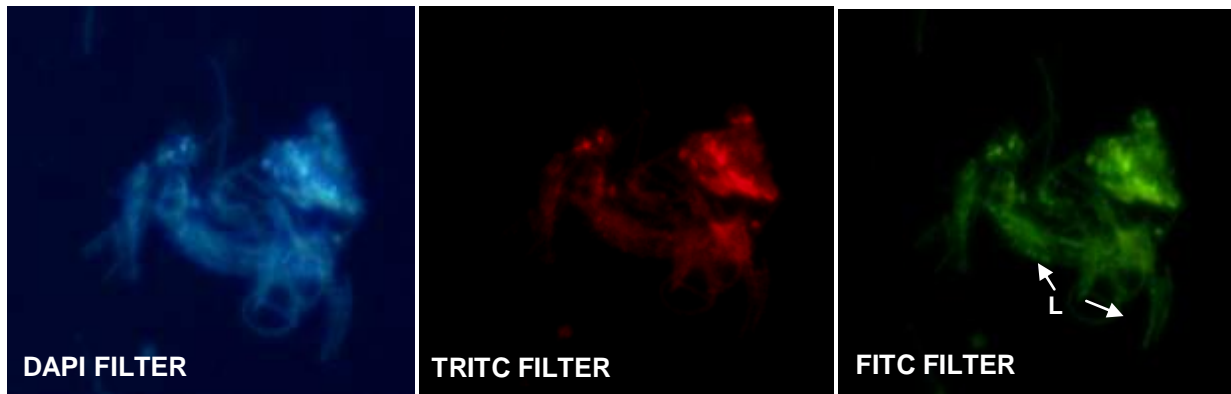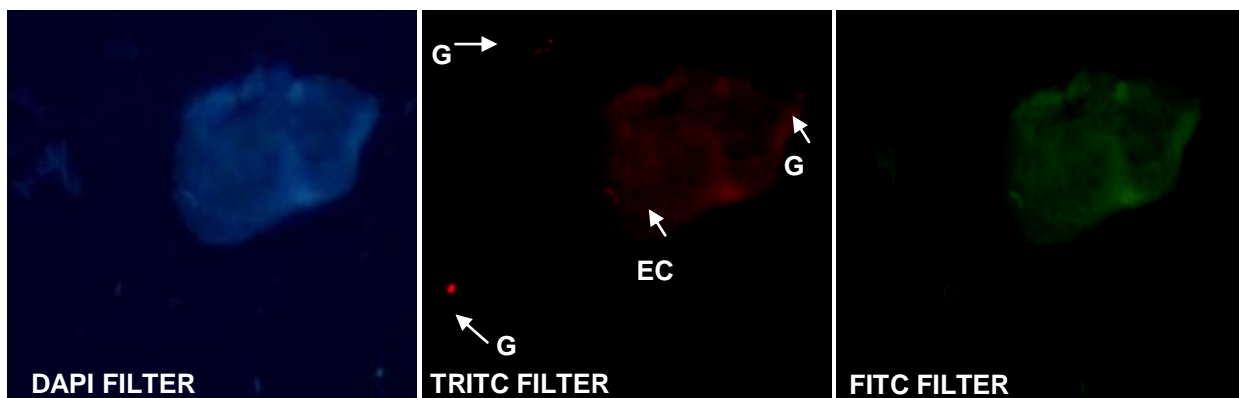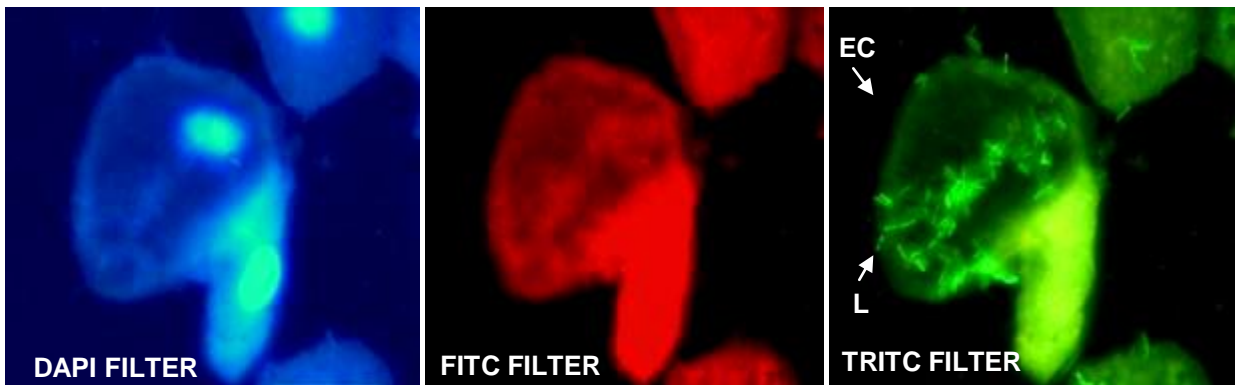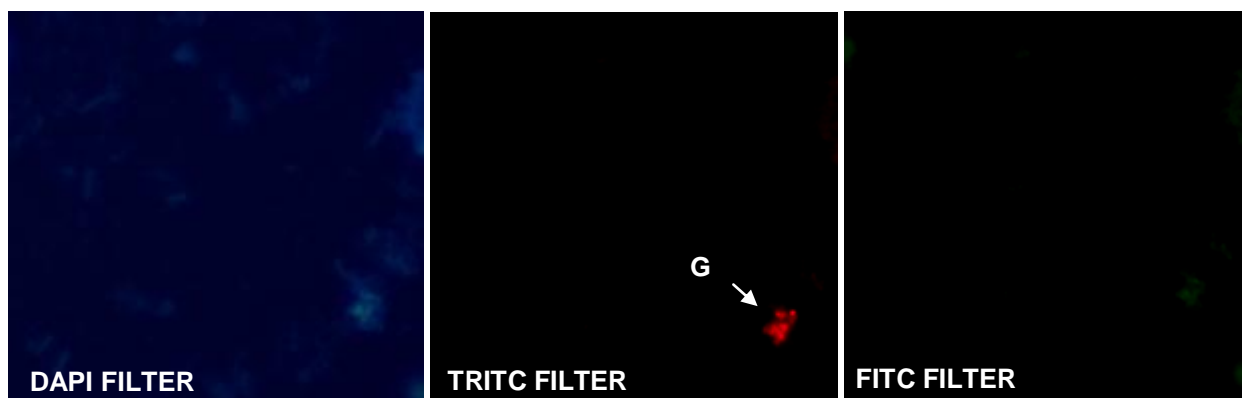

Figure 3.16: Fluorescence micrographs of vaginal-fluid smears of a healthy woman (sample thirteen), analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *G. va*ginalis, with DAPI, TRITC and FITC filter (Total magnification=1000x).
Legend: G represents the fluorescence *G. vaginalis*.

Figure 3.17: Fluorescence micrographs of clinical sample fourteen, analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *Lactobacillus* spp, with DAPI, TRITC and FITC filter (Total magnification=1000x).

Sample number fourteen was collected from women with symptoms and was diagnosed, by the doctor, as BV positive. According to the images obtained using a probe for identification of *G. vaginalis* (figure 3.17), is possible to see a high and strong signal. By the other side, with the PNA *Lactobacillus* spp., it was not possible to see hybridization on the sample. That could be explained by the replacement of normal vaginal flora, mostly *Lactobacillus* spp., by anaerobes such as *G. vaginalis* (G) (figure 3.18).

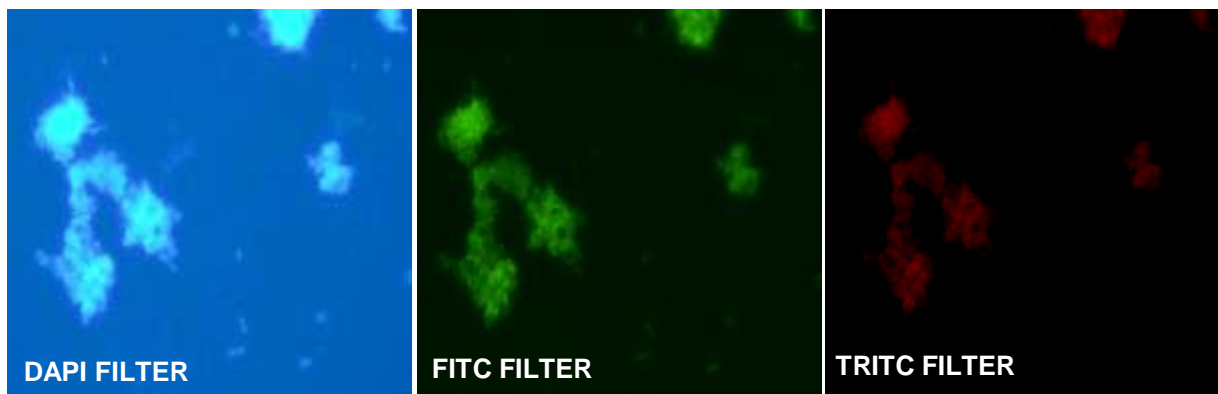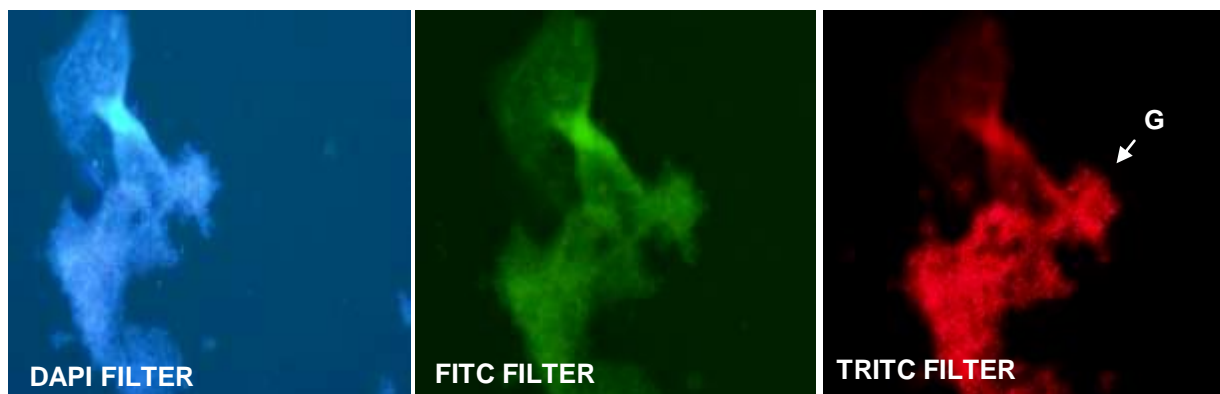**Figure 3.18:** Fluorescence micrographs of clinical sample fourteen, analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids (Total magnification=1000x) of *G. vaginalis*, with DAPI, TRITC and FITC filter.
Legend: *G* - indicates the fluorescence of *G. vaginalis.*

3.3.3 Comparison of FISH and PCR

According to literature, molecular methods, such as FISH and PCR have the capability to directly detect and identify specific microorganisms. These molecular techniques are becoming important methods in modern microbiologic diagnostic where the direct handling of the samples collected from patients is more usual and there is the need for more immediate and consistent results (Stender *et al.*, 2002; Amann and Fuchs, 2008; Srinivasan and Fredricks, 2008). Overtaking the traditional culture methods of microorganisms, these molecular techniques were, more recently, named as the gold-standard (Anukam *et al.*, 2005).

FISH is a molecular method useful for the visualization, identification and quantification of the target microorganisms present on microbial community. However, some limitations can be associated with it, such as low ribosomal content of bacterial cells, low permeability of the probe in the cellular wall and low access to the target sequence caused by the secondary or tertiary rRNA structure (Guimaraes *et al.,* 2007; Hoshino *et al.*, 2008). The alterations to the different protocols might avoid these obstacles. In fact, some factors as the hybridization step, temperature, pH and ionic strength concentrations are related to the access of the probe and hybridization with the target sequence. The washing step ensured the removal of all loosely bound or unbound labeled probes and ensured that the detection was specific. However, it does not overcome the problems related with the low target cell´s number and lack of cell permeabilization (Cerqueira *et al.*, 2008). The fluorcrome probe characteristics are the main limitation of fluorescence signal (section 2.7.4). The low signal intensity can also be associated to the low ribosomal content on bacterial cells (Guimaraes *et al.*, 2007). According to the results obtained we can say that some problems of auto-fluorescence that we obtained could be explained by this kind of problems.

In relation to PCR, some studies refer to PCR as a superior technique for detection of vaginal bacterial microflora, some of which could not be detectable and identified by standard culturing techniques (Ling *et al.*, 2010; Zozaya-Hinchliffe *et al.*, 2010; Ravel *et al.*, 2011). Because of this, a large range of studies identified vaginal species like *A. vaginae,* an uncultured microorganism*,* and also other unknown species such as *Clostridiale*s and *Megasphaera* spp. (Zozaya-Hinchliffe *et al.*, 2010). The PCR detection of microorganisms had also been described as a sensitive method for the detection of sexually transmitted infections such as *Trichomonas vaginalis*, *Neisseria gonorrhoeae* or even *Chlamydia trachomatis* (Menard *et al.*, 2012). A study involving the PCR assays also demonstrated that *G. vaginalis* presence is not a requirement for BV (Lin *et al.*, 2000).

Comparing FISH with PCR it is also possible to compare some of problems associated to the use of these techniques. In fact, PCR technique is faster than FISH, however, with FISH we have the capability to distinguish the different shapes of the microorganisms present in the clinical sample. Like PCR, FISH also involves the use of specific probes and parameters like design of the probes, temperature of hybridization, must be determined and optimized. It is also important to note that the majority of the labs have access to thermocyclers, however the access to a fluorescence microscope is not as widespread, which can limit the analysis of the samples. Other problems related to the use of a technique such as PCR is the need to perform sequence analysis after isolation of the

microorganisms which could be expensive and involves time in order to confirm result. In both methods it is important to have a representative portion of the sample involved in the analysis, because, sometimes, the positive result on the FISH is not a positive result in PCR, and this could be a limitation of FISH. The limitation of the non-visualization of the viability of the microorganisms on PCR could also be critical to further culture steps. With FISH, it is possible to see different fields of vision, which can result in a higher number of false negatives. With PCR we have to work with specific primers and the design, optimization of the temperature of annealing, for example, needs to be verified which involves time. The same occurs in FISH, but in this case, aspects such as hybridization temperature and chemical fixatives must be optimized. The moment of the treatment of the vaginal swab after the reception is also a limiting factor as ideally they should take place in about 24 to 48 hours, as to avoid the loss of viability of the microorganisms present on the swabs. In our case this was a factor that could influence the final results because the swabs collected from clinical and emergency were treated some days (4-10) days after collection. This could also result in a positive PCR and a negative FISH, since intact bacteria are required for FISH; while the presence of DNA is sufficient for PCR. Moreover, the FISH experiences involve the utilization of probes which are considerable more expensive, while the price of primers is very low which can be an advantage when using PCR. Taking in consideration the respective advantages and disadvantages of both samples, we selected PCR as the main tool to analyze the presence of *G. vaginalis* and *A. vaginae* in the subsequent thirty-three clinical samples.

3.4 Prevalence of *A. vaginae* and *G. vaginalis* in vaginal swabs

Gardnerella vaginalis plays a significant role in the aetiology of BV and the presence of this microorganism in high concentrations and the decrease of *Lactobacillus* spp. can be a microbiological indicator of the occurrence of BV. Nevertheless, it should be noted that *G. vaginalis* can be part of the normal vaginal microflora. While about 88 to 98% of women with BV are colonized with *G. vaginalis*, recent studies indicate that *G. vaginalis*, is also part of normal microflora, with a significant lower prevalence (10-40%) (Tabrizi *et al.,* 2006). To date, there is no information regarding the presence of *G. vaginalis* and *A. vaginae* in Portuguese women.

The prevalence of *A. vaginae* and *G. vaginalis* in Portuguese microflora of the women involved in this study was analyzed based on PCR results (Table 3.6).

Table 3.6: Results of the PCR realized to each fifty-seven clinical samples with *A. vaginae*; *G. vaginalis* and *Lactobacillus* spp. primers and also the source of the samples collected

PCR Results				
Source samples	Number of clinical sample	*Lactobacillus* spp. primers	*G. vaginalis* primers	*A. vaginae* primers
SH	1	+	-	-
SH	2	+	+	-
SH	3	+	+	-
SH	4	+	-	-
SH	5	+	-	-
SH	6	+	+	-
SH	7	+	-	-
SH	8	+	+	-
SH	9	+	+	-
SH	10	+	+	-
SH	11	+	+	+
SH	12	+	-	-
ER	13	+	+	+
GPP	14	+	+	+
GPP	15	+	+	-
GPP	16	+	-	-
GPP	17	+	+	-
GPP	18	+	-	-
GPP	19	+	-	-
GPP	20	+	+	-
GPP	21	+	-	-
GPP	22	+	+	-
GPP	23	+	-	-
GPP	24	+	-	-

GPP	25	+	-	-
GPP	26	+	-	-
GPP	27	+	-	-
GPP	28	+	+	+
GPP	29	-	-	-
GPP	30	+	-	-
GPP	31	+	-	-
GPP	32	+	+	-
GPP	33	+	-	-
GPP	34	+	-	-
GPP	35	+	+	-
GPP	36	+	-	-
GPP	37	+	-	-
GPP	38	-	-	-
GPP	39	+	-	-
GPP	40	+	-	-
GPP	41	+	-	-
GPP	42	+	-	-
GPP	43	+	-	-
GPP	44	+	-	-
GPP	45	+	-	-
ER	46	+	-	-
ER	47	+	-	-
ER	48	+	-	-
ER	49	+	-	-
ER	50	+	-	-
ER	51	+	-	-
ER	52	+	-	-
ER	53	+	-	-
ER	54	+	-	-
ER	55	+	-	-
ER	56	+	+	-
ER	57	+	+	+

Legend:

Negative result: -.
Positive result: +.
ER – Hospital emergency.
SH – Self-harvest.
GPP – Gynecological private practice.

According to the analysis of the table 3.6, we can conclude that all the samples were positive for the lactobacilli primers with exception of samples twenty-nine and thirty eight. These samples are also negative to the presence of *G. vaginalis* and *A. vaginae*. The negative result of *Lactobacillus* spp. (sample thirty-eight) could be justified by mitochondrial DNA degradation from the sample before the appropriate treatment. Then, looking to the results using *G. vaginalis* primers, we can say that, approximately, seventeen samples (sample two; three; six; eight; nine; ten; eleven; thirty; fifteen;

seventeen; twenty; twenty-two; twenty-eight; thirty-five; thirty-six; fifty-six and fifty-seven) were positive. From these positive samples, seven were collected by self-harvest, seven were obtained from gynecological private practice and four were collected in a hospital emergency.

Atopobium vaginae is another microorganism, found, more recently, and is defined as an interesting anaerobic and fastidious microorganism, recently associated to BV (Menard *et al.*, 2010). *Atopobium vaginae* has also been identified in the normal vaginal flora and the recurrence of BV increases in the cases where both *A. vaginae* and *G. vaginalis* have been detected in comparison to those with *G. vaginalis* only (De Backer *et al.*, 2010; Menard *et al.*, 2010). In relation to *A. vaginae* primers, only five samples were positive (samples eleven; thirteen; twenty eight; thirty six and fifty seven) and are all present in positive samples for *G. vaginalis*. It corresponds to one sample obtained from self-harvest, one from gynecological private practice and three from emergency. Our results indicate a prevalence of 28% of *G. vaginalis* in the samples collected and of 8 % of *A. vaginae* in the total number of samples*.*

4.0 Conclusions

Bacterial vaginosis is, currently, considered a polymicrobial syndrome and is very common in women at reproductive age and, sometimes, it can be asymptomatic. The real etiology of this infection remains unknown; however studies demonstrated that BV is associated with an alteration of vaginal microflora, with *Lactobacillus* spp. being replaced for some anaerobes such as *G. vaginalis*. The prevalence of this disease varies from country to country, ethnicity and age of women. Some external factors such as environmental, affects this predisposition. In Portugal there is no information about BV since 1998, which enhances the importance of this study.

Direct examination of clinical material received by microscopy is usually used by some physicians for the diagnosis of BV (Spiegel *et al.*, 1983) and it was used in this project to have a better comparison with other methods. This study involved the collection of seventy-seven samples, twelve by self-harvest, thirty on gynecological private practice and fourteen on emergency. From the total number of samples, three were from women with a previous diagnosis of BV and the rest were from healthy women. Culture tests and also Gram-staining, were performed to study the vaginal flora variability. However, a proportion of the microorganisms that inhabit the vaginal epithelium were not cultivable. Because of this, some aspects such as taxonomic composition, the structure and also the specific function of the different microorganisms identified during BV are still not understood (Ling *et al.*, 2010). Based solely on culture methods and Gram-stain results it was possible to verify the diversity and variability of microorganisms in vaginal flora.

The main conclusion of this study was that 28% of the Portuguese women involved on the study presented *G. vaginalis* as a part of their vaginal microflora. Another microorganism usually associated to the vaginal microflora is *A. vaginae* and in our study appeared in 8% of the fifty-seven clinical samples collected. The *Lactobacillus* spp. was also identified on the samples. On specific cases, FISH was used to verify the decrease of lactobacilli and its replacement by anaerobes. So, we proved that molecular techniques allowed the analysis of the bacterial diversity in the vaginal microenvironment and allowed the direct detection and identification of specific microorganisms. Because of this, molecular techniques are being considered as important methods in modern diagnostic in microbiology, where the patient can be treated rapidly and directly (Stender *et al.*, 2002; Srinivasan and Fredricks, 2008).

Despite the considerable efforts and recent studies, the microbiological cause of BV is still unclear and needs to be clarified. More studies are required to a better analysis of the vaginal microflora of the Portuguese women. The application of the molecular techniques seems like the standard methodology in the identification and characterization of the microorganisms that could be present on the vaginal swabs.

5.0 Future work

To further pursue the study of BV on Portuguese women, more samples will be required, to achieve statistical significance. It would be interesting to relate *G. vaginalis* with *A. vaginae* prevalence in the population, and try to relate this with sexual behaviors. *Gardnerella vaginalis* and *A. vaginae* were also identified in virginal women (Tabrizi *et al.*, 2006), and the analysis of the different association to the vaginal flora of these women/adolescents could be another interesting point. Other kind of epidemiological study could involve the analysis of the vaginal pH of the Portuguese women and its comparison with women from other ethnicity, because some studies reported differences among women from different environment (Ravel *et al.*, 2011).

It is also important to continue the work of culturing and isolation of the microorganisms present on the swabs. While in this work we limited our conclusion to the variability of the microflora, it would be interesting to isolate and identify the microorganism. This would mean culturing samples onto different media and also the assessment of biochemical tests, application of the molecular techniques as PCR, and the extraction and the DNA sequenciation.

In relation to the FISH it would be interesting the optimization of *A. vaginae* probes and the future application on the characterization of vaginal microflora associated to *G. vaginalis* and *Lactobacillus* spp. probes. If fresh samples of vaginal swabs could be obtained, it would be interesting to repeat this study in a more quantitative way, by advising the reduced *Lactobacillus* spp. and increasing *G. vaginalis*.

6.0. Appendix

I – Fluorescence *in situ* hybridization results of reference strains
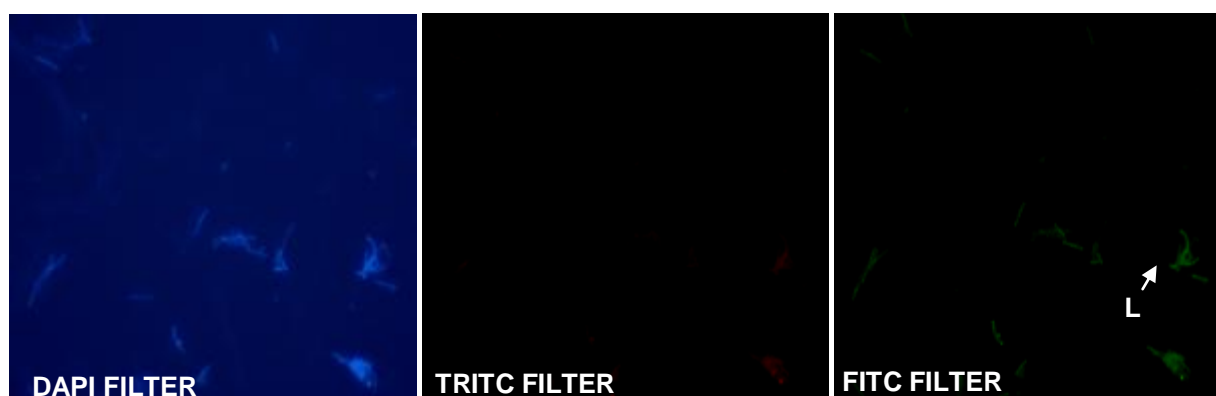
Figure i: Fluorescence micrographs of *L. crispatus* reference strain, analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *Lactobacillus* spp, with DAPI, TRITC and FITC filter (Total magnification=1000x).
    Legend: L represents the fluorescence *Lactobacillus* spp.

In all our FISH assays we always used reference strains such as *L. crispatus* ATCC 33820 using the *Lactobacillus* spp. probe (figure i). On some FISH experiments was also utilized *L. casei* CECT 5275 to control because the permeability of this microorganisms were almost higher and so, the signal was amplified. However, we always preferred to obtain signal with *L. crispatus* ATCC 33820 because it constitute part of the vaginal flora of healthy women. In relation to *G. vaginalis* reference strain we selected *G. vaginalis* AMD. The result to the AMD was as expected and a high hybridization of the cells was proved (figure ii), for the *G. vaginalis* probe.
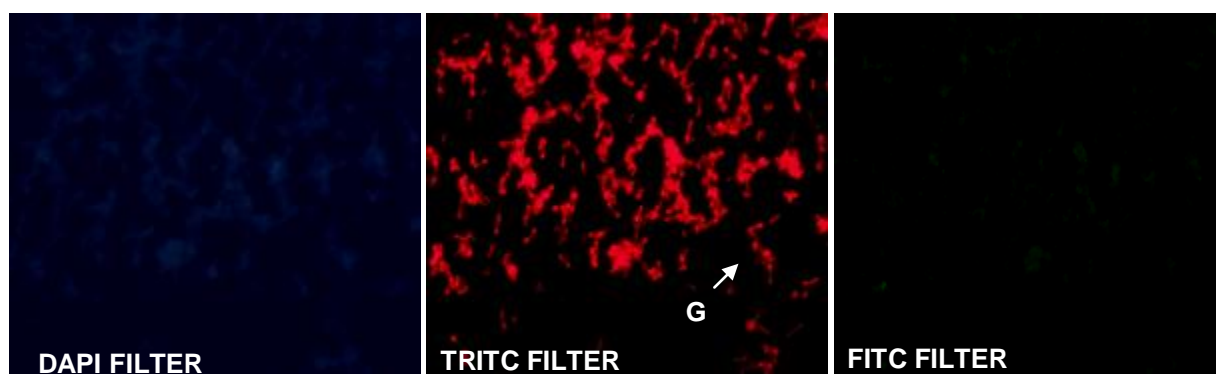
Figure ii: Fluorescence micrographs of *G. vaginalis* AMD reference strain, analyzed by FISH with labeled oligonucleotide probes targeting bacterial Peptide Nucleic Acids of *G.vaginalis*, with DAPI, TRITC and FITC filter (Total magnification=1000x)
    Legend: G represents the fluorescence G.vaginalis.

II - **Optimization of FISH protocol applied in clinical samples**

During FISH observations some problems appeared when the samples were prepared from the initial vial instead of directly from FISH aliquot. In fact, the clinical samples always have carbon residues incorporated into the swab that probably affected the quality of fluorescence. The Amies transport media contains vegetal coal, sodium chloride, dissodium phosphate, calcium chloride, magnesium chloride, monopotassium phosphate, potassium chloride, sodium tioglicolate and bacteriological agar and this could causes auto-fluorescence. The high auto-fluorescence of the samples on each well, which sometimes is observed, could be explained by an excess of biomass that made the visualization of different microorganisms harder. In relation to the last problem some measures were taken and the FISH suspension could not be opaque. However, the problem with the coal present on the swabs was optimized by submitting the same sample to three different treatments, in order to remove the coal without remove the biomass. First, the sample was centrifuged during 5 min at 10000 rpm. After that the sample was divided in three parts and each one was differently treated. The first treatment encompasses vortex cycles before proceeding to the FISH analyzes. The second treatment required the rapid centrifugation at 5000 rpm only during seconds. The last treatment was the sonication of the sample at 40% during 5 plus seconds. After that, a portion of sample was fixed and analyzed using violet crystal and DAPI. 4',6-diamidino-2-phenylindole was utilized because it allowed the visualization of the total number of bacteria and also the discrimination of the mix culture (Almeida *et al.*, 2009).

The results demonstrated similarity of the samples fixed and stained with crystal violet but the total biomass present with treatment B was superior. When DAPI was used we also observed that with the treatment B we had a slight increase in the number of bacteria compared with the other treatments. However, with all the treatments some residual coal remained. The figure iii represents an image obtained with the treatment B, and is possible to visualize different morphologies present in the clinical sample. It is also possible to see similar shapes to bacilli, represented types as B, and shapes more circular, short rods, represented as SR. After application of this treatment we repeated some FISH experiences but we still see some auto-fluorescence. Regarding these results we note that maybe the coal was not the only interference but also the residual BHI.
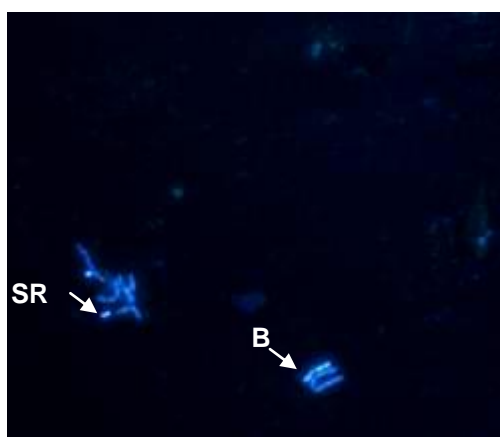

Figure iii: Image of the DAPI applied to the clinical sample number nine fixated after treatment B.

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