Joana Isabel Reis Castro **Adhesion of vaginal microorganisms to epithelial cells and its association with Bacterial Vaginosis**

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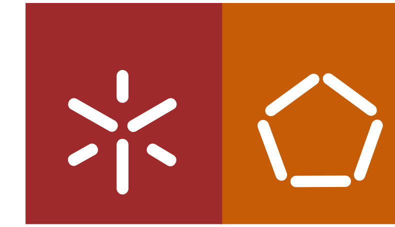

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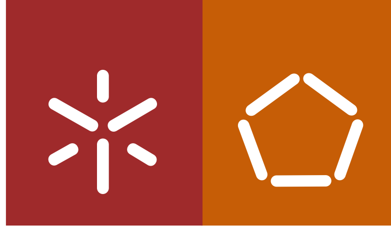

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Adhesion of vaginal microorganisms to epithelial cells and its association with Bacterial Vaginosis

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Trabalho efetuado sob a orientação do
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e co-orientação da
Professora Doutora Mariana Henriques

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TÍTULO DA TESE DE MESTRADO:
Adhesion of vaginal microorganisms to epithelial cells and its association with Bacterial Vaginosis

A adesão de microrganismos vaginais a células epiteliais e a sua associação com a Vaginose Bacteriana

ORIENTADOR: Doutor Nuno Cerca

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

Universidade do Minho, ___/___/______

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A adesão de microrganismos vaginais a células epiteliais e a sua associação com a Vaginose Bacteriana

RESUMO:

A vaginose bacteriana (VB) é um distúrbio da flora vaginal normal e um importante problema de saúde pública em mulheres de idade reprodutiva. A VB é caracterizada pela substituição de lactobacilos vaginais por microrganismos, predominantemente, anaeróbios. Desconhecendo-se a etiologia da VB, duas hipóteses tentam explicar esta condição: a hipótese polimicrobiana, que infere que a VB é causada por uma mistura de bactérias patogénicas, principalmente anaeróbias; e outra hipótese que aponta para a *Gardnerella vaginalis* como o verdadeiro agente causador da VB. No entanto o isolamento frequente desta espécie em mulheres aparentemente saudáveis lançou dúvidas sobre essa afirmação. Assim, num esforço para se compreender a etiologia desta doença, foram realizados ensaios de adesão *in vitro* para comparar a capacidade de adesão de vários isolados vaginais provenientes de exsudados vaginais de mulheres que foram diagnosticadas como tendo VB e de mulheres saudáveis. No total, foram caracterizados 15 isolados vaginais quanto à sua capacidade de adesão inicial numa monocamada de células HeLa. Estes ensaios revelaram que os isolados de *G. vaginalis* apresentaram uma capacidade de adesão inicial mais forte do que os outros isolados analisados. Além disso, estirpes de *G. vaginalis* isoladas de pacientes com VB apresentaram uma maior capacidade de adesão inicial do que as estirpes de *G. vaginalis* que foram isoladas de mulheres saudáveis. Assim, a fim de compreender as diferenças verificadas, foi estudada a competição entre lactobacilos (*Lactobacillus iners*, *Lactobacillus crispatus* e *Lactobacillus casei*) e estirpes de *G. vaginalis* (não-patogénicas e patogénicas). Todos os ensaios de competição foram quantificados por microscopia de fluorescência, usando DAPI para contar as células totais e uma sonda de PNA-FISH para quantificar *G. vaginalis*. Os resultados mostraram que a adesão de *L. iners* não diminuiu na presença de estirpes patogénicas de *G. vaginalis*. Pelo contrário, o *L. crispatus* mostrou uma diminuição na capacidade de adesão às células epiteliais na presença de estirpes patogénicas de *G. vaginalis*. O *L. crispatus* mostrou, também, que tem uma grande capacidade de inibir a adesão de isolados patogénicos de *G. vaginalis*. Por sua vez, o *L. casei* foi o lactobacilos menos aderente de todos os utilizados no presente estudo. Como resultado, estes estudos de adesão ajudam a fornecer informações sobre a situação clínica na qual os lactobacilos vaginais indígenas podem interferir com a presença de *G. vaginalis* na microflora vaginal.

Adhesion of vaginal microorganisms to epithelial cells and its association with Bacterial Vaginosis

ABSTRACT:
Bacterial vaginosis (BV) is an unhealthy disturbance of the normal vaginal flora and an important public health problem in women in reproductive age. BV is characterized by the replacement of vaginal lactobacilli by predominantly anaerobic microorganisms. The lack of basic information about the etiology of BV has lead to the postulation of two hypotheses. The first is the polymicrobial hypothesis, which infers that BV is caused by a mixture of pathogenic bacteria, mainly anaerobes. The second is that a single pathogenic species, in many cases *Gardnerella vaginalis* is the causative agent of BV, but frequent isolation of this species from seemingly healthy women has cast doubt on this claim. So, in an effort to tease apart the aetiology of this disorder, *in vitro* adherence assays were performed to compare the initial adhesion, the first step of biofilm formation, of *G. vaginalis* relative to other microorganisms isolated from vaginal swabs from patients with BV and healthy women. In total, 15 unique vaginal isolates were characterized for their initial adhesion ability to a monolayer of the HeLa cells. These assays revealed that *G. vaginalis* isolates had a stronger initial adhesion capability than the other isolates recovered. Furthermore, *G. vaginalis* strains isolated from BV patients had stronger initial adhesion ability than *G. vaginalis* isolated from healthy women. In order to understand these differences, the competition between lactobacilli (*Lactobacillus iners*, *Lactobacillus crispatus* and *Lactobacillus casei*) and *G. vaginalis* strains (non-pathogenic and pathogenic) was studied. All competition assays were quantified by fluorescence microscopy, using DAPI for total cell count and PNA-FISH probe for *G. vaginalis* quantification. The results showed that *L. iners* did not decrease in presence of pathogenic *G. vaginalis* strains. In contrast, *L. crispatus* showed a decreased adherence capacity to epithelial cells in the presence of pathogenic *G. vaginalis* strains. Furthermore, the results showed that *L. crispatus* could be important for antagonizing the pathogenic strains of *G. vaginalis*. In turn, *L. casei* was the least adherent of the all lactobacilli used in this study. As a result, adherence studies help to provide insight into the clinical situation in which indigenous vaginal lactobacilli can interfere with *G. vaginalis* presence in vaginal microflora.

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NOMENCLATURE

Symbols

P – significant value

R^2 – correlation factor

t – time (min)

T – temperature (ºC)

Abbreviations

Abs – Absorbance

ANOVA – Analysis of variance

ATCC – American type culture collection

BME – Basal medium Eagle's

BV – Bacterial Vaginosis

CBA – Columbia Blood Agar

CDC – Cholesterol-dependent cytolysin

CECT – Colección Española de Cultivos Tipo

CFU – Colony forming unit

CoNS – Coagulase-negative staphylococci

DC – Dendritic cells

DMEM – Dulbecco's modified Eagle's medium

DMSO – Dimethly sulfoxide

DNA – Desoxiribonucleic acid

ELISA – Enzyme-linked immunesorbent assay

EMEM – Eagle's minimal essential medium

EPS – Extracellular polymeric substances

FBS – Fetal bovine serum

FISH– Fluorescence in situ Hybridization

HBT – Human Blood-Bilayer-Tween

HIV – Human immunodeficiency virus

IgA – Immunoglobulin class A

MRS – De Man-Rogosa-Sharpe

PBS – Phosphate-buffer saline

PCR – Polymerase chain reaction

PNA – Peptide nucleic acid

RPMI – Roswell Park Memorial Institute medium

sBHI – supplemented Brain heart infusion

SD – Standard deviation

SEM – Scanning electron micrograph

SPSS – Statistical package for the social sciences

TEM – Transmission electron microscopy

TLR – Toll-like receptors

UTIs – Urinary tract infections

VCU – Virginia Commonwealth University

VEC – Vaginal epithelial cells

Vs – Versus

VYL – Vaginolysin

LIST OF PUBLICATIONS

Abstracts and Posters

Castro J, Machado A, Henriques A, Henriques M, Jefferson K and Cerca N (2012). Competitive initial adhesion between *Lactobacillus* spp. and *Gardnerella vaginalis* strains against vaginal epithelium, in *Biofilms 5*, 10-12 December 2012, Paris, France

Alves P, Castro J, Sousa C, Cereija T, Henriques A and Cerca N (2012). Characterization of biofilm-forming microorganisms isolated from vaginal exudate in Portugal, in *Biofilms 5*, 10-12 December 2012, Paris, France

Magalhães A, Castro J, Henriques A and Cerca N (2011). Multiplex PCR for the discrimination of vaginal bacterial population. *Microbiotec*, 1-3 December 2011, Braga, Portugal

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“The important thing is not to stop questioning.

Curiosity has its own reason for existing.”

Albert Einstein

CHAPTER 1

Introduction

1.1 Healthy vaginal microbiota

The healthy vaginal ecosystem harbors a microbiota that is being increasingly recognized as protecting it from invading pathogens, including those that cause urinary tract infections and sexually transmitted diseases (Boris, 1998). The healthy vaginal flora is composed by *Lactobacillus* spp., particularly *L. rhamnosus, L. crispatus, L. gasseri, L. jensenii, L. fermentum, L. plantarum, L. brevis, L. vaginalis, L. salivarius, L. delbrueckii, L. reuteri* and *L. iners* (Figure 1.1) (Cribby *et al*., 2008; Dover *et al*., 2008; Srinivasan *et al.,* 2008). A diverse array of other microorganisms such as *Staphylococcus, Ureaplasma, Corynebacterium, Streptococcus, Peptostreptococcus, Gardnerella, Bacteroides, Mycoplasma, Enterococcus, Escherichia, Veillonella, Bifidobacterium* and *Candida* can also be present, but in much lower numbers (Turovskiy *et al*., 2011; Zhou *et al*., 2004).

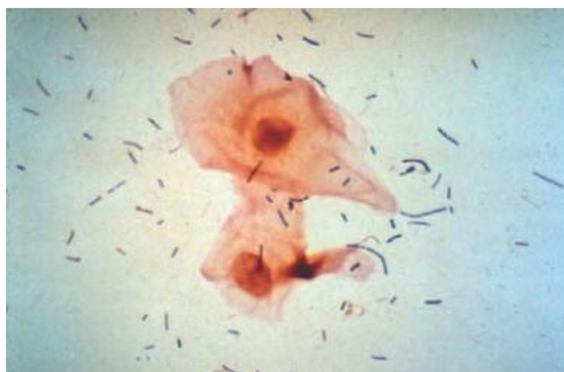


Figure 1.1 – Gram stain of normal vaginal contents (original magnification, x400). Note predominance of *Lactobacillus* species (Livengood, 2009).

The composition of vaginal floral is influenced by symbiotic relationship between vaginal lactobacilli and their human host, and it is modulated by the hormones (particularly estrogen) circulating in female's body, which stimulate the vaginal epithelium to produce glycogen (Hay, 2005). Changes in the vaginal microbiota occur during each menstrual cycle, with the high concentrations of estrogen increasing adherence of lactobacilli to vaginal epithelial cells (VEC). At menopause, with decrease in estrogen levels, there is a decrease in lactobacilli present in the vaginal tract (Cribby *et al*., 2008). Thus, these hormonal changes can affect the ability of lactobacilli to adhere to epithelial cells and colonize the vaginal tract (Cribby *et al*., 2008). The glycogen is metabolized to glucose and then to lactic acid by vaginal lactobacilli. Lactic acid is largely responsible for the normal vaginal pH being acidic (< 4,5) (Donati *et al*., 2010; Turovskiy *et al*., 2011). The acidic environment of a healthy vagina is not permissive

for over-growth of many potential pathogens and protects the vagina against Human immunodeficiency virus (HIV) (Aroutcheva *et al.*, 2001a; Donati *et al.*, 2010; Dover *et al.*, 2008; Valore *et al.*, 2006). An increase of the vaginal pH leads to the decrease of the *Lactobacillus* spp. that are associated with antimicrobial activity (Aroutcheva *et al.*, 2001 b; Donati *et al.*, 2010).

It has been postulated that *Lactobacillus* spp. are responsible to maintain the vaginal ecosystem in healthy condition by preventing overgrowth by pathogens and other opportunistic organisms by producing antimicrobials such as weak organic acids like lactic acid, hydrogen peroxide and bacteriocins (Dover *et al.*, 2008).

Eschenbach *et al.* (1989) were the first to postulate that hydrogen peroxide (H_2O_2) production by vaginal lactobacilli is critical for maintenance of healthy vaginal microbiota, because it creates an unacceptable environment for growth of anaerobes and a more difficult environment for HIV transmission. This study reported that H_2O_2 producing lactobacilli were found in 96 % of healthy women and in only 6 % of patients with BV; non-hydrogen peroxide–producing lactobacilli were found in only 4 % of the normal vaginas and in 36 % of those with BV. The amount of H_2O_2 produced in the vaginal fluid of women with a healthy vaginal microflora was estimated to 1 – 15,5g.mL^{-1} (Strus *et al.*, 2006). H_2O_2 seems to add to the antimicrobial defense of the vaginal environment, but is probably not a crucial factor (Strus *et al.*, 2006). Host factors can also work synergistically with the lactobacilli to inhibit growth of other bacteria (Zhou *et al.*, 2004). For example, myeloperoxidase and chloride ions enhance the toxicity of H_2O_2 (Zhou *et al.*, 2004).

Bacteriocins are low-molecular-weight proteins or peptides, that inhibit a wide range of Gram-positive and, under certain conditions, Gram-negative bacteria (Aroutcheva *et al.*, 2001 a). These antimicrobial compounds promote cell membrane permeabilization and ion efflux, thereby depleting the transmembrane potential within the bacteria cell (Machado, 2011). These bacteriocins can inhibit the growth of pathogens, such as *G. vaginalis*. However, one species of *Lactobacillus* can produce a bacteriocin that is able to inhibit the growth of other lactobacilli (Aroutcheva *et al.*, 2001 a).

1.2 Bacterial vaginosis

Bacterial vaginosis (BV) is characterized by the replacement of vaginal lactobacilli, such as, *L. crispatus* and *L. jensenii* by predominantly anaerobic microorganisms (Patterson *et al*., 2007; Sobel, 2000; Turovskiy *et al*., 2011). The exception is *L. iners*, a non-hydrogen peroxide producer, which is found commonly in the BV flora (Livengood, 2009).

The most common symptom of BV is a vaginal discharge. It may look grayish white. Other sign of BV can be an odor that is usually described as “fishy”. This is caused by the production of amines (including putrescine, trimethylamine, and cadaverine) by the anaerobic bacteria (Spear *et al*., 2007; Zariffard *et al*., 2005). These amines volatilize increasingly with pH raise, so that patients often note a worsening of this symptom when vaginal alkalinity is enhanced, such as after sex (due to the presence of semen) and during menses (due to the presence of blood). Other symptom of BV is irritation (itching, burning and pain) (Livengood, 2009; Srinivasan *et al.,* 2008). All patients with vaginal symptoms should be examined to confirm the diagnosis. Researches that have used routine screening to identify patients with BV have found that more than 50 % of affected individuals are asymptomatic (Livengood, 2009).

BV poses a significant health risk because it predisposes women to serious disorders, such as pelvic inflammatory disease, low birth weight, chorioamnionitis, post-partum endometritis and preterm delivery, which is a leading cause of infant death in the United States (Dover *et al.*, 2008; Spear *et al*., 2007). It is thought that these problems arise when microorganisms associated with BV ascend from the lower reproductive tract (Zariffard *et al*., 2005). BV has also been associated with decrease success of *in vitro* fertilization procedures, and increasing risk of cystitis (Livengood, 2009).

The epidemiology of BV suggests a sexually transmissible agent, but this does not explain the high prevalence of BV in sexually inactive women (Zariffard *et al*., 2005). Several studies have treated the male partners of women with BV with clindamycin and nitroimidazole agents typically used for the treatment of BV (Aroutcheva *et al*., 2001 b; Kharsany *et al.*, 1993). These studies all failed to demonstrate a decrease in recurrent BV among the women whose partners were treated. Thus, if BV is caused by a transmissible agent, it is unlikely to be a clindamycin or nitroimidazole susceptible anaerobe (Kharsany *et al.*, 1993). In this sense, BV may also increase risk of acquiring sexually transmitted

diseases, such as HIV infection (Verstraelen *et al.*, 2010). Studies show that genital tract secretions from women with BV induce HIV expression in infected cells (Spear *et al.*, 2007). The increased HIV expression appears to be due, at least in part, to activation through Toll-like receptors (TLR), specifically TLR2, that are express by dendritic cells (DC) (Zariffard *et al.*, 2005). DC are suggested to be one of the first cells that take up HIV during sexual transmission. DC are important for antigen processing and presentation to the immune system. Studies reported that BV may substantially affect local DC antigen presenting function in women (Zariffard *et al.*, 2005; Spear *et al.*, 2007). However, further research is needed to elucidate how BV contributes to HIV acquisition and transmission (Zariffard *et al.*, 2005; Spear *et al.*, 2007).

The lack of basic information about pathogenesis of BV has lead to the postulation of two competition models. It is not known whether the primary event initiating BV is the loss of key lactobacilli or acquisition of the complex bacterial communities found in this syndrome; these may be simultaneous processes (Figure 1.2) (Oakley *et al.*, 2008; Srinivasan *et al.*, 2008; Turovskiy *et al.*, 2011). It is also possible that some other factor is the primary etiological agent, and that the changes in vaginal microbiota reflect downstream event in pathogenesis of BV. These doubts still remain due of the complexity of BV and the lack of reliable animal model for this condition (Srinivasan *et al.*, 2008; Turovskiy *et al.*, 2011).

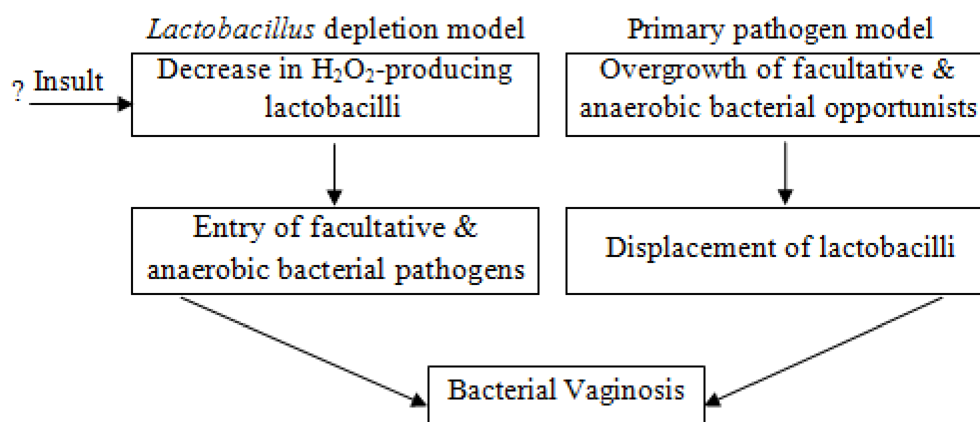


Figure 1.2 – Competition models for the pathogenesis of BV. At least two models exist to explain the pathogenesis of BV. The first model is the *Lactobacillus* depletion and suggests that there is a decrease in H_2O_2 producing lactobacilli as the primary event that allows for the overgrowth of facultative anaerobes resulting in BV. The second model is primary pathogen and suggests that the entry of facultative anaerobes causes the displacement of lactobacilli thereby resulting in BV (Srinivasan *et al.* 2008).

1.2.1 The diagnosis of BV

Gardner and Dukes (1955) described the clinical findings of BV in 1181 patients as (i) vaginal squamous cells with a granular appearance and indistinct borders, (ii) "disagreeable" odor, (iii) an elevated pH of 5 to 5,5 and (iv) a thin, gray, adherent discharge. These findings were adjusted later and are now known as the Amsel criteria (Livengood, 2009; Turovskiy *et al.*, 2011). The diagnosis of BV in clinical settings is usually based on the fulfilment of three of four clinical criteria described by Amsel and colleagues (1983). Amsel's criteria include (i) elevated vaginal pH (> 4,5), (ii) presence of white adherent discharge and (iii) numerous exfoliated epithelial cells with bacteria (Gram-variable polymorphic rods) attached to their surface (clue cells) and (iv) has a characteristic fishy odor of the discharge especially when 10 % potassium hydroxide (KOH) is added (whiff test) (Amsel *et al.,* 1983).

Amsel criteria do not require a greater than normal volume of vaginal discharge; only a thinning of the consistency. A normal squamous cell has sharp, clear, linear edges, whereas a clue cell has granular, cloudy, rough edges (Figure 1.3) (Amsel *et al.*, 1983; Livengood, 2009; Simoes *et al.*, 2006).

Later, investigators revealed that accuracy of the criteria could be enhanced if a vaginal pH ≥ 4,7 were used in place of a vaginal pH > 4,5 and if > 20 % of the vaginal squamous cells were clue cells (Eschenbach *et al.,* 1988).

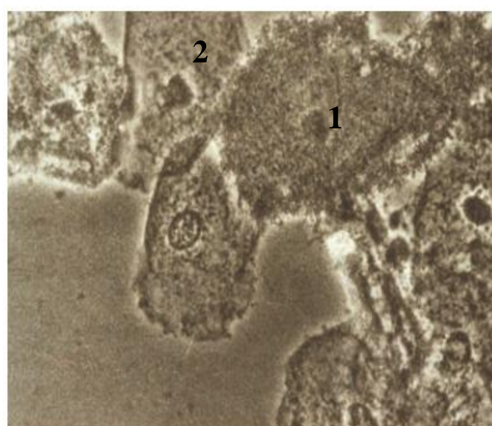


Figure 1.3 – Clue cells in saline (original magnification, x400). Note the rough, cloudy, irregular borders that define the clue cell in cell 1. The cell 2 has stippling over the cytoplasm, but edges are sharp and linear, this is not a clue cell (Livengood, 2009).

Due to the fact that BV can be asymptomatic in about 30 – 50 % of women, microbiological diagnostic methods, such as Nugent's scoring system (Nugent *et al.*, 1991) are preferred in the scientific community (Schwiertz *et al.*, 2006; Turovskiy *et al.*, 2011). In the Gram stain scoring system a greater density of *Lactobacillus* morphotypes lowers the score, while a greater density of *Gardnerella vaginalis, Bacteroides* morphotypes and other curved rods bacteria increases the score. Scores of 0 to 3 are considered normal, 4 to 6 are intermediate, and 7 to 10 are BV (Table 1.1) (Nugent *et al.*, 1991). The agreement of Gram stain score and diagnosis by the clinical criteria is imperfect. Gram stain is more sensitive, whereas the Amsel criteria are more specific. Overall the concordance between them is of 80 % to 90 % (Livengood, 2009; Nugent *el al.*, 1991)

Table 1.1 – Scheme for Grading Gram-stained Vaginal Contents (Nugent *el al.*, 1991) [a]

Score[b]	*Lactobacillus* Morphotypes	*Gardnerella* and *Bacteroides* spp. Morphotypes	Curved Gram-Variable Rods
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

[a] Morthotypes are scored as the average number seen per oil immersion field. Total score = lactobacilli + *G. vaginalis* and *Bacteroides* spp. + curved rods.
[b] Quantification: 0, No morphotypes present; 1+, <1 morphotype present; 2+, 1 to 4 morphotypes present; 3+, 5 to 30 morphotypes present; 4+, 30 or more morphotypes present.

Gram stain scoring may be the most accurate approach, but requires a delay of 1 to 2 days to confirm the diagnosis. On the other hand, it is relatively easy to determine the dominant bacterial morphotype while examining vaginal swabbing (*wet prep*) (Livengood, 2009; Nugent *el al.*, 1991). Finally, the criteria used for the diagnosis of BV in routine clinical practice are often a matter of the user preference (Livengood, 2009).

1.2.2 The treatment of BV

The treatment of BV has the goal to eradicate anaerobic microorganisms and providing the re-growth of *Lactobacillus* spp. producing H_2O_2 (Livengood, 2009). Nyirjesy and coworkers (2006) have revealed that re-growth of lactobacilli producing hydrogen is likely to occur. Healthy vaginal lactobacilli are active against several microorganisms including *G. vaginalis* (Dover *et al.,* 2008). Aroutcheva and their coworkers (2001 a) reported that in 22 vaginal *Lactobacillus* strains, isolated from healthy women, 73 % showed to be active against *G. vaginalis*.

The most common treatment for BV is the use of antibiotics, namely, with oral or vaginal metronidazole or with vaginal clindamycin (Swidsinski *et al.*, 2008). Recent studies indicate a new antimicrobial agent for the treatment of BV, called tinidazole. It has a twice-longer serum half-life than metronidazole, and their side effects have been reported at half the frequency when compared with of metronidazole. Thus, tinidazole offers a well-tolerated, and it is highly competitive new option for treatment of BV, while requiring less than half as many doses as the currently recommended oral metronidazole regimen (Dover *et al*., 2008; Eriksson *et al*., 2005).

However, antibiotics do not eradicate all vaginal pathogenic bacteria. Treatment of BV is only effective in 60 % cases, with a common recurrence rate of 30 – 40 % within 6–12 months (Eriksson *et al*., 2005). Moreover, a recent trial reported that all *G. vaginalis* strains develop resistance to metronidazole in recurrent BV cases. In this sense, there is an interest in developing alternative therapies against BV that might be safer and more efficient than antibiotics. Such as more selective antimicrobials, probiotics and acidification procedure that will inhibit BV pathogenic bacteria without killing healthy lactobacilli. Some of these alternative therapies would also reduce the risk of infection's reoccurrence by promoting healthy *Lactobacillus* spp. growth (Dover *et al.,* 2008; Falagas *et al.*, 2007; Wang *et al*., 2010).

Probiotic *Lactobacillus* preparations are known to contain the specialized organisms that dominate the healthy vagina. Investigations are being conducted to isolate, store, and deliver them to patients in an effort to enhance the success of therapy. These efforts have been hindered by nuance involving both re-growth and establishment of dominance by these organisms (such as *L. crispatus*, *L. rhamnosus*, and *L. reuteri*) (Livengood, 2009).

Recently vaginal probiotic capsules were developed and these are particularly appealing, because of their ease of use and high satisfaction rates when are compared with creams, gels, and oral yogurt consumption (Wang *et al.,* 2010). Researchers showed that probiotic prophylaxis with vaginal capsules (that contain 8 billion colony-forming units of *L. rhamnosus*, *L. acidophilus*, and *Streptococcus thermophilus*) is well tolerated and yields dramatic reductions in BV recurrence and *G. vaginalis* risk through eleven months after treatment in women with history of recurrent BV (Wang *et al.,* 2010).

1.2.3 The epidemiology of BV

Epidemiological studies show that the risk of BV is higher in black and non-Hispanic woman (Figure 1.4) (Allsworth and Peipert, 2007). Other risk factors include: low socioeconomic status, antibiotic treatment of another condition, douching, young age of coitarche, new partner, a recent history of multiple partners and cigarette smoking (Turovskiy *et al.,* 2011).

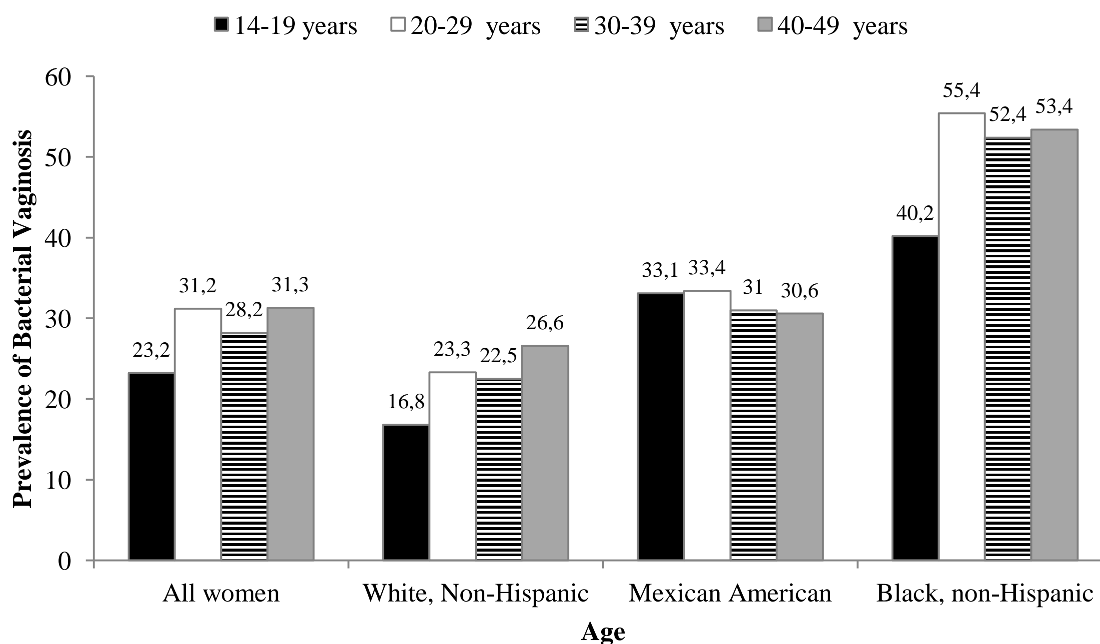


Figure 1.4 – Prevalence of bacterial vaginosis in the United States of America by age stratified by race or ethnicity (Allsworth and Peipert, 2007).

In Portugal, most doctors agree that BV is frequent (74 %) with a lower prevalence during pregnancy (55 %). In this country BV is mainly diagnosed by using

Amsel's scoring system (75 %), which is mainly based on the clinical observation of the aspect, odour and pH of the vaginal discharge. Although the first choice antimicrobial therapy is metronidazole (58 %), Henriques *et al.* (2012) found that doctors from different geographical regions do prescribe different antibiotic therapies. The centre region of Portugal was the only region where clindamycin prescriptions (49 %) were preferred to metronidazole (45 %). Finally, most Portuguese doctors involved in the study feel that BV relapses are not very frequent (62 %), in contrast with the results of studies reported from other parts of the world. In this study, the first covering all regions of Portugal, investigators showed that there are diverse perceptions regarding the prevalence of BV, as well as different diagnostic approaches and antimicrobial treatments used (Henriques *et al.,* 2012).

1.3 *Gardnerella vaginalis*

In 1953, Leopold described *G. vaginalis* as a novel *Haemophilus* – like species associated with prostatitis and cervicitis. Two years later, Gardner and Dukes were the first to associate this microorganism with BV. Through the morphology of the bacterial cell, of the apparent negative reaction to Gram staining, and of the inability to grow on agar media in the lack of blood, researchers concluded that they were in the presence of a new species of *Haemophilus*, what they called a *Haemophilus vaginalis*, based on its origin (Greenwood and Pickett, 1980; Piot and Dyck, 1983). In 1980, taxonomic studies evaluating multiple criteria revealed the lack of genetic relationship between *H. vaginalis* and other established morphology and physiologically similar genera (Greenwood and Pickett, 1980). The main difference between the two types of bacterial cells was shown through the results of Gram staining. As a result, Greenwood and Pickett (1980) proposed the name of *G. vaginalis* (Figure 1.5), and this proposal was supported by Piot and Dyck (1983).

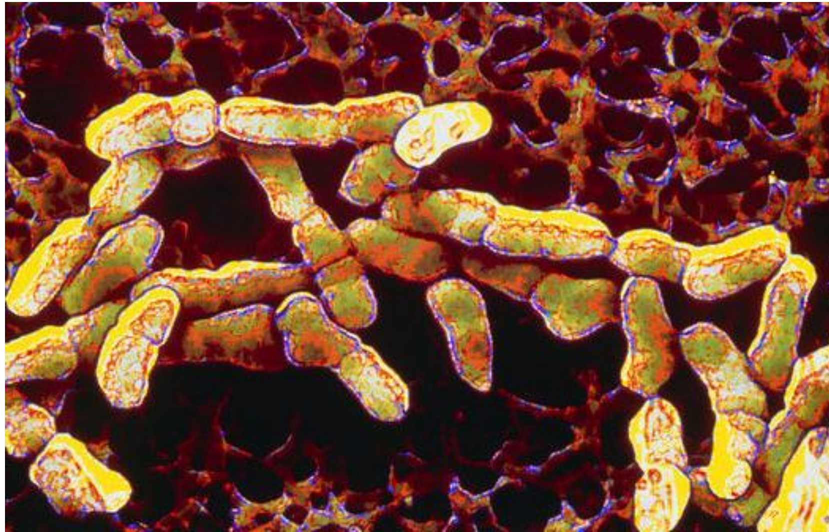


Figure 1.5 – *G. vaginalis*. Coloured Scanning Electron Micrograph (SEM) of a colony of *G. vaginalis*. Magnification: x30'000. Adapted from http://www.sciencephoto.com/media/11256/enlarge (2012).

1.3.1 Structure and physiology of *G. vaginalis*

G. vaginalis is a facultative anaerobic, but some rare anaerobic strains exist. Their growth is the best at 35 ^{o}C and is enhanced by carbon dioxide (CO_2) (Spiegel, 1991). This bacterium is nonencapsulated, nonspore-forming, nonmotile, pleomorphic, and has also revealed fimbriae (pili) on their surface (Spiegel, 1991). Studies reported that the outer fibrillar coat was responsible for the attachment of *G. vaginalis* to exfoliated VEC (clue cells); on the order hand, pili were involved in the attachment of the pathogen to human red blood cells (Scott *et al*., 1989; Catlin 1992).

G. vaginalis belongs to the Bifidobacteriaceae family, and is commonly described as a Gram-variable or Gram-uncertain microorganism. This means that reaction to Gram staining can vary from negative to positive, depending of the growth conditions (Catlin, 1992; Turovskiy, *et al.,* 2011). Thus, results of the antimicrobial susceptibility studies are consistent with the notion that *G. vaginalis* is neither typically Gram-positive nor typically Gram-negative, since antimicrobial agents regarded as specifically active against Gram-positive or Gram-negative organisms showed relatively poor activity (Kharsany *et al.,* 1993).

G. vaginalis is considering a fastidious microorganism and requires complex medium for growth. Some different selective and semi-selective media have been used to

isolate *G. vaginalis* from clinical specimens, such as medium Columbia Blood Agar (CBA) and agar medium Human Blood-Bilayer-Tween (HBT) (Catlin, 1992).

Biochemical tests revealed that *G. vaginalis* is catalase-negative, oxidase-negative and β-glucosidade-negative. *G. vaginalis* exhibits α-glucosidase activity and β-haemolysis on human blood (Catlin, 1992; Turovskiy, *et al.,* 2011).

1.3.2 The role of *G. vaginalis* in BV

As briefly discussed in section 1.2 there are two main theories try to answer the BV riddle. In 1955, Gardner and Dukes related *G. vaginalis* with the syndrome nonspecific vaginits, currently known as BV. These investigators believed that *G. vaginalis* was the sole cause of BV, and began to fulfill Koch's postulates (see Appendix A) for disease causation in a series of clinical experiments. Later studies demonstrated pitfalls in these experiments (Srinivasan *et al.,* 2008). As *G. vaginalis* can be detected in 30 – 50 % of women without BV (Fredricks *et al.,* 2007), suggesting that *G. vaginalis* is not the sole etiological agent in BV. This is because Koch's postulates demand that the etiological microbe should be found in every case of disease but should not be detected in subjects without disease that did not happen (Srinivasan *et al.* 2008).

After more than half a century of research, the scientific community is still debating about the origin of this pathology. Through several studies it is thought that the *G. vaginalis* likely plays an important role in the pathogenesis of BV (Livengood, 2009; Patterson *et al.,* 2010). Recently it has been described that some strains of *G. vaginalis* form thick biofilms (Patterson *et al.,* 2010).

1.3.2.1 Biofilm formation

Microbial adhesion to host surfaces is a prerequisite for infection, as a potential pathogen must first adhere in order to avoid clearance by host defense mechanisms, such as the flow of vaginal secretions, the mucociliary escalator and the urine flow (Sobel *et al.,* 1982; Harwich, *et al.,* 2010). Adhesion to urogenital epithelial cells allows microorganisms to colonize, thereby minimizing contact of the bacteria with potentially deleterious extracellular enzymes and local antibodies and reducing their chances of

being flushed away in vaginal fluid or urine (Catlin, 1992). Several strains of *G. vaginalis* are known to form biofilms, after adhering to the vaginal epithelium (Harwich, *et al.,* 2010; Patterson *et al.,* 2010).

Biofilms are described as a community of bacteria that do not live as pure culture of dispersed single cells but instead accumulate at interfaces to form polymicrobial aggregates (Flemming and Wingender, 2010; O'Toole, *et al.,* 2000). Biofilm formation and structure is affected by several conditions like: surface properties, nutrient availability, composition of the microbial community and hydrodynamics. Biofilms are dynamic and heterogeneous communities in constant evolution. Cells in a biofilm have different metabolic activities, depending on the special position inside the biofilm, and this can change over time (Sutherland, 2001; Trevors, 2011).

Several bacterial pathogens form biofilms having complex interactions with components of the innate host defense system (O'Toole, *et al.* 2000). There is evidence that BV is associated to biofilm formation and it has been suggested that this biofilm may be critical in pathogenesis (Catlin, 1992; Patterson *et al.,* 2007).

Many species have shown distinct steps in biofilm formation, which include (Figure 1.6): (a) the adhesion of planktonic bacteria cells to surface, in this case, to VEC, (b) binary division of attached cells, that is formation microcolonies, and finally (c) aggregation of single cell or cell flocs from the bulk fluid to the developing biofilm, resulting in extracellular polysaccharide matrix, that accounts for the majority (~85 %) of the biofilm volume. Compounds such as extracellular polymeric substances (EPS), fimbriae, mating pili, can all function as extracelular matrix components (Leid, 2009).

Initially, planktonic bacteria form a reversible attachment on the surface. After the initial attachment, other chemical and physical interactions transform the reversible attachment to enduring irreversible adsorption. After the irreversible attachment, bacteria produce EPS mainly composed of polysaccharides (Flemming and Wingender, 2010; O'Toole, *et al.* 2000; Leid, 2009). EPS can be considered as the primary component of biofilms. EPS immobilize biofilm cells and keep them in close proximity, thus allowing for intense interactions, including cell-cell communication. Cell-to-cell signaling, termed *quorum sensing*, has been shown to play an important role in virulence factors, biofilm differentiation, cell attachment and detachment (Leid, 2009). EPS may also have

functions like: adhesion (because allows the initial steps in the colonization of surfaces by planktonic cells and the long-term attachment of whole biofilms to surfaces); sorption of inorganic ions and organic compounds; retention of water; nutrient source; protective barrier (because it confers resistance to host defences during infection) and antibiotic resistance (by slowing down the diffusion of antibiotics into the biofilm) (Flemming and Wingender, 2010; O'Hanlon *et al.*, 2011; O'Toole, *et al.* 2000).

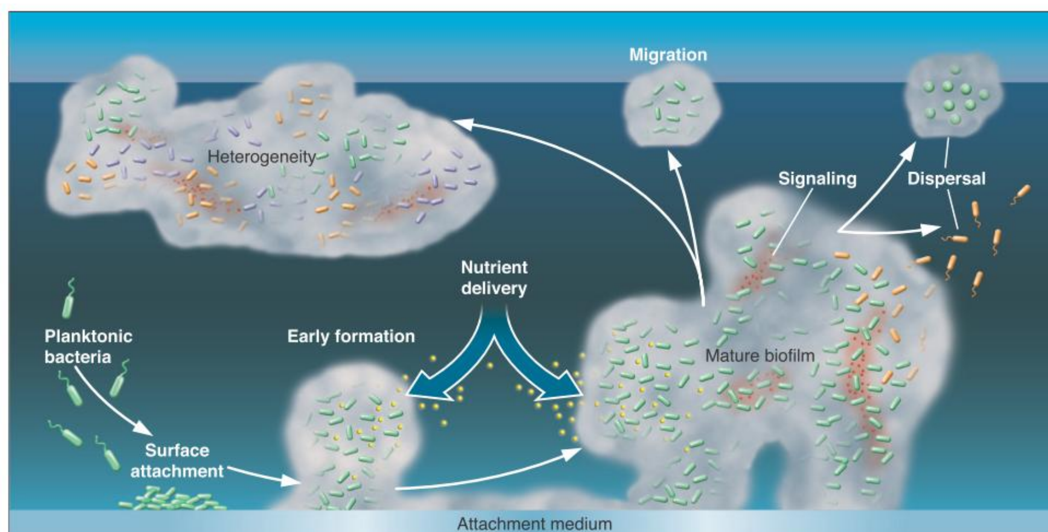


Figure 1.6 – Depiction of the dynamic nature steps of a biofilm community. The community starts to form when single cells called planktonic bacteria attach to surface. As those individual cells strongly adhere, and expand in number, they are surrounded by an extracellular matrix. As the community matures, partly by cell to cell communication (signaling), parts of the biofilm can disperse, migrate, or the community can continue to develop into a heterogeneous population of cells that are metabolically, physiologically and genetically distinct from one another. If appropriate attachment reservoirs are available downstream from the dispersion event, the entire cycle can start over again (Leid, 2009).

G. vaginalis adherence to VEC increased with increasing acidity of the test medium, being greatest at pH 4 in phosphate-buffered saline (PBS) than at pH 5 to 6 in citrate-acetate-phosphate buffer. It is known that both VEC and bacteria carry net negative charges that create an electrostatic repulsive force. This is reduced at a lower pH, with the result that binding is increased. So, adherence in the vaginal microenvironment is also influenced by pH (Catlin, 1992).

The ability for a strain to grow as a biofilm would likely confer resistance to mucosal immune defenses and antibiotic resistance (Figure 1.7), which could contribute to initial and recurrent colonization. Furthermore, lactobacilli normally associated with

the healthy vagina produce byproducts such as lactic acid and hydrogen peroxide that normally suppress the growth of anaerobes such as *G. vaginalis*, but biofilm formation leads to increased resistance to these byproducts. Therefore, biofilm formation may enable proliferation of *G. vaginalis* even in the presence of lactobacilli. Thus, biofilm formation is associated with increased antibiotic resistance (Flemming and Wingender, 2010) and appears to play a role in treatment failure and recurrence in cases of BV (Patterson *et al.,* 2010).

Due to the fact that bacterial biofilm are not effectively cleared by the immune system, or completely killed by antibiotics, biofilm-related infections tend to be chronic and/or relapsing. BV tends to be a smoldering infection with a high rate of relapse or recurrence (Swidsinski *et al.,* 2005).

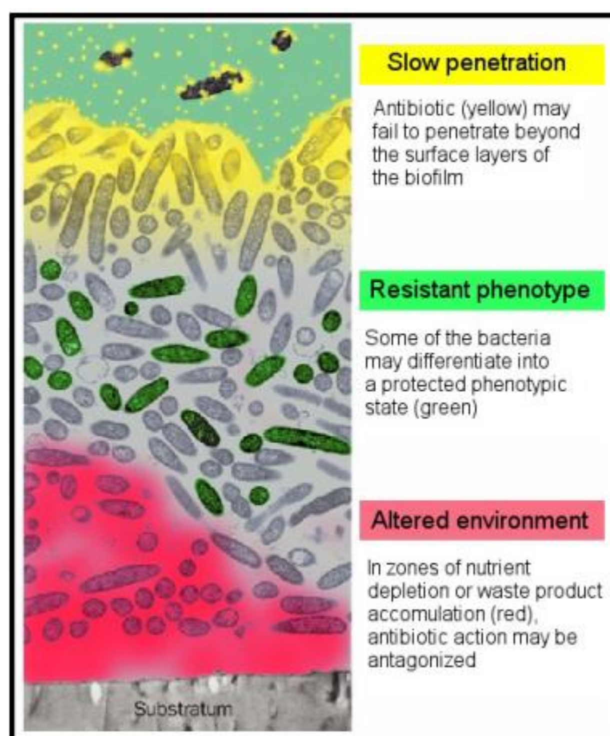


Figure 1.7 – Some of the most discussed hypothesis for biofilm resistance to antibiotics. Adapted from Lancet (2001), 358, 135–138.

1.3.2.2 Biotypes of G. vaginalis

Recent studies led by Harwich and their coworkers (2010) demonstrated that *G. vaginalis* strain isolated from a healthy patient had fundamental genomic differences, as compared with the genome of a *G. vaginalis* isolated from a patient with BV. Back in

1990, a research demonstrated that the lipase-positive isolates of *G. vaginalis* exhibited a stronger correlation with BV and women who acquired a new biotype were more likely to develop BV (Briselden and Hillier, 1990).

In addition to lipase, the production of a cytolysin appears to be an important virulence factor of *G. vaginalis*. It was named vaginolysin (VYL), since it is a member of the cholesterol-dependent cytolysin (CDC) family (Zvirbliene *et al.*, 2010). Similar cytolysins appear to contribute to virulence by making cellular contents more available as a substrate for bacterial growth. VYL is characterized as a pore-forming cytotoxin that utilizes the complement regulatory molecule CD59 to activate the epithelial p38-mitogen-activated protein kinase pathway in human epithelial cells, leading to cell death (Gelber *et al.*, 2008; Patterson *et al.,* 2010). IgA antibodies against VYL have been linked to the mucosal immune response during BV, further supporting the role of VYL in BV pathogenesis (Patterson *et al.,* 2010). VLY from *G. vaginalis* 5-1 (non-pathogenic strain) and *G. vaginalis* AMD (pathogenic strain) were 99 % identical at the amino acid level, differing at a single amino acid (T35A), this difference could be responsible for virulence of *G. vaginalis* AMD (Patterson *et al.,* 2010; Zvirbliene *et al.*, 2010).

The research group of Patterson *et al.* (2010) has also been involved in determining other differences between isolates of *G. vaginalis* from healthy subjects and BV patients that may explain the outcome of colonization. They investigate whether the disparity in the sequences and expression of biofilm-associated protein (BAP) could translate into differences in biofilm forming activity. BAP are cell wall-anchored adhesions that can mediate both adherence to host cells, how intracellular adherence, which confers the biofilm formation capacity (Harwich *et al.*, 2010; Patterson *et al.,* 2010). These investigators showed that gene sequences for AMD and 5-1 BAP were quite disparate. This was particularly noticeable in the repeat regions, the region of BAP proteins that generally mediates adherence. Researches also noted that *G. vaginalis* from a healthy individual isolate appeared to be coated in capsular structure, whereas the *G. vaginalis* isolated from a BV patient did not appear to express this structure (Figure 1.8) (Harwich *et al.*, 2010). This may negatively impact the adherence to VEC, or biofilm formation (Harwich *et al.*, 2010).

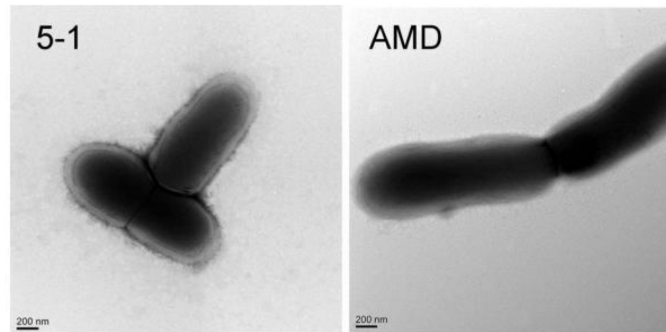


Figure 1.8 – Transmission Electron Microscopy (TEM) of *G. vaginal*is strains. The left panel is *G. vaginalis* 5-1 (non-pathogenic strain) while the panel on the right is *G. vaginalis* AMD (pathogenic strain). When the strains were grown in the presence of 10 % human serum, a capsule-like material was present on *G. vaginalis* 5-1, but undetectable on *G. vaginalis* AMD (Harwich *et al.*, 2010).

Harwich *et al.* (2010) analyzed the ability of the two *G. vaginalis* strains to adhere to vaginal epithelial cell (ME-180 cells). While equal amounts of the two strains were added to epithelial cells monolayers, adherence of strain AMD from a BV isolated was much more pronounced relatively to that of the non-BV isolated strain 5-1. It was also observed that strain AMD was more aggregative than was strain 5-1 (Figure 1.9). This suggests that the capability of BV isolates to bind to and adhere to vaginal epithelium may be higher than non-BV isolates, and that BV-isolates tend to produce more biofilm growth than do non-BV isolates of *G. vaginalis*, which would reasonably be consistent with greater virulence (Harwich *et al.*, 2010).

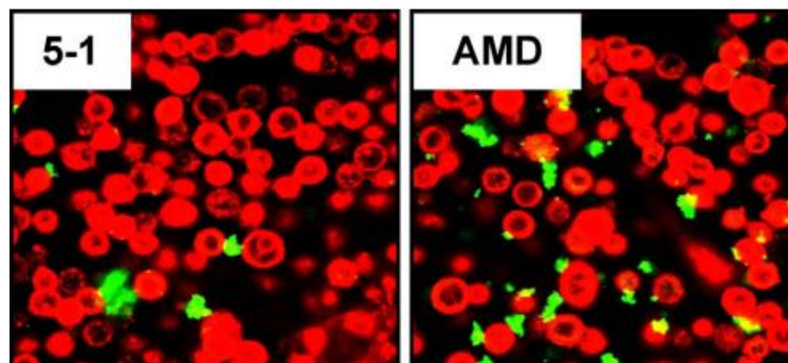


Figure 1.9 – Adherence of *G. vaginalis* to cultured vaginal epithelium. Equal amounts of the indicated strains of *G. vaginalis* (green) were added to epithelial cells (red). The cells were stained with BacLight green and Vybrant Red stains respectively. Adherence was analyzed by confocal microscopy following incubation and extensive washing with 1x PBS (Harwich *et al.* 2010).

1.3.2.3 Virulence of G. vaginalis and Host defense

The dynamic relationship between host defense and microbial virulence in infectious diseases indicates that it is insufficient to view virulence attributes as a collection of substances that do some harm to host cells. Microorganisms may enhance their virulence by producing factors that act against host defense factors or in response to them (Larsen, 1994; Witkin *et al.,* 2007).

The presence of an extensive resident microflora represents another measure of effectively protecting the host's mucosal surfaces. Thus, colonization by pathogens in the presence of a resident flora requires successful strategies that enable invading microbes to successfully compete for nutritional and spatial resources and displace commensal organisms from the microbial niche. In addition to their ability to attract professional immune cells, the epithelial body surfaces themselves provide effective innate antimicrobial defense (Turovskiy *et al.,* 2011; Witkin *et al.,* 2007).

Lactobacillus spp. that are part of the resident microflora, are believed to interfere with pathogens by different mechanisms. The first is the competitive exclusion of genitourinary pathogens from receptors present on the surface of the genitourinary epithelium. Second, lactobacilli coaggregate with some uropathogenic bacteria, a process that, when linked to the production of antimicrobial compounds, such as lactic acid, hydrogen peroxide and bacteriocin-like substances would result in inhibition of the growth of the uropathogenic bacteria, as *G. vaginalis* (Harwich *et al.,* 2010). Clearly, microorganisms that normally associate with the host as normal commensal flora have been given little attention as toxin producers. But the production of toxic substances with subtle effects on the host physiology or immunity will play a role in the virulence of these microorganisms (Larsen, 1994).

1.3.3.4 Additional virulence factors of G. vaginalis

Additional virulence factors produced by *G. vaginalis* include sialidase and prolidase (Pleckaityte *et al.*, 2012; Santiago *et al*., 2011), which are two hydrolytic enzymes that may have a role in degrading several key mucosal protective factors, such as mucins, as well as contributing to exfoliation and detachment of vaginal epithelial cells (Cauci *et al.,* 2008).

In sum, there is a strong evidence that *G. vaginalis* does possess innate pathogenic potential (Eren *et al*., 2011; Yeoman *et al*., 2010). Other BV-associated anaerobes might have virulence factors that have not yet been described, or they may be more pathogenic in the presence of other species. It is also likely that the primary aetiological agent of BV, if there is one, varies on a case by-case basis. However, Patterson *et al.,* (2010) suggests that, due to its collection of virulence factors, it is likely that *G. vaginalis* has a pathogenic capacity, and could be the key agent in certain cases of BV.

1.4 The role of other anaerobes in BV

Recent studies demonstrate the occurrence other anaerobe microorganisms associated with BV (Table 1.2). *Mobiluncus* spp. and *Atopobium vagiane* have been also reported as microorganisms associated with BV, although they are not found in every cases of BV (Livengood, 2009; Srinivasan *et al*., 2008; Turovskiy *et al.,* 2011).

Table 1.2 – Detailed composition of the anaerobe microorganisms associated with BV in vaginal microflora of 515 vaginal swab samples (Verhelst, *et al*. 2005)

Species associated with BV
Gardnerella vaginalis
Peptoniphilus sp. [a]
Aerococcus christensenii
Atopobium vaginae
Dialister sp.
Actinomyces neuii
Bacteroides ureolyticus
Finegoldia magna [a]
Prevotella bivia
Varibaculum cambriense
Mycoplasma hominis
Anaerococcus tetradius [a]
Anaerococcus vaginalis [a]
Gemella morbillorium [a]
Mobiluncus curtisii
Prevotella ruminicola
Prevotella sp.

[a] Formerly known as *Peptostreptococcus*.

Mobiluncus spp., a highly motile curved bacillus, is found only when BV is present, but in only 50 % of cases of BV (Hillier *et al.,* 1991). *A. vaginae* is a Gram-positive anaerobe which is found in the flora of over 95 % of BV cases, but also occurs in the vagina of healthy women (Ferris *et al.,* 2007; Livengood, 2009). Thus, BV flora can include mixtures of multiple genera of Gram-positive and Gram-negative microorganisms (Figure 1.10).

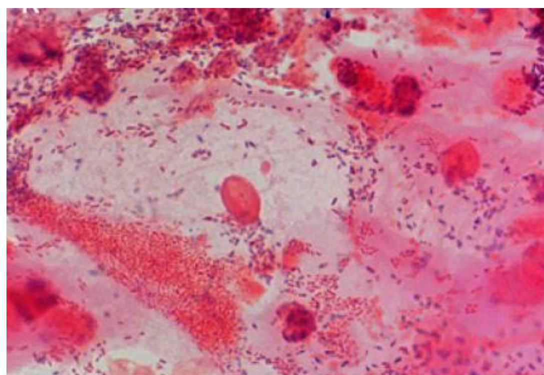


Figure 1.10 – Microscopic image (1000 ×) of Gram-stained vaginal smears illustrating the case of BV (Verhelst *et al.,* 2005).

1.4.1 *Mobiluncus* spp.

Mobiluncus spp. are anaerobic bacteria. These anaerobic curved rods have been given the genus name *Mobiluncus,* and two species, *M. curtisii* and *M. mulier.* The two species can be differentiated by cell morphology and biochemical reactions. The most useful aspects that are used for differentiation are: β-galactosidase activity; hippurate hydrolysis and metronidazole susceptibility (Hallén, *et al.,* 1988).

M. curtisi microorganisms are referred to as short form Gram-variable, comma-shaped, resistant to metronidazole, and have positive results in all these differential biochemical tests. In contrast, *M. mulieris* microorganisms are referred to as long forms, Gram-negative, curved, sensitive to metronidazole, and have negative results in biochemical reactions. Additional problem is the fastidious nature of the *Mobiluncus* spp. which complicates their isolation and subsequent cultivation (Hillier *et al.,* 1991). *Mobiluncus* spp. is detected by traditional methods, as: Nugent's scoring system (Nugent *et al.,* 1991).

Many questions remain concerning the ecology of *Mobiluncus* spp., including the factors responsible for vaginal colonization and the clinical manifestations of vaginal colonization with these microorganisms (Hillier *et al.,* 1991). There seems to be an association between *Mobiluncus* with black race, unemployment, and low income suggests the need to assess further the role of behavior or risk factors among low socioeconomic status women as risk factors for colonization by this bacterium (Hillier *et al.*, 1991). Hallén and their coworkers (1988) have reported that *Mobiluncus* can be recovered from up to 53 % of the rectal specimens from women with BV (Hallén, *et al.,* 1988; Hillier *et al.,* 1991).

1.4.2 *Atopobium vaginae*

A. vagianae has been increasingly identified with the advent of molecular technical. The genus *Atopobium* was proposed in 1992 to accommodate bacterial isolates previously classified as *Lactobacillus minitus*, *Lactobacillus imae* and *Streptococcus parvulus* (Polatti, 2012; Santiago *et al.*, 2012; Rodriguez *et al.,* 1999). Gram stain appearance is that of a small Gram-positive cocco-bacillus. Members of the genus are known to produce large amounts of lactic acid. On this basis, some species belonging to the genus *Atopobium* were originally identified as *Lactobacillus* spp. (Rodriguez *et al.,* 1999). Differences between bacteriologic methods could explain this discrepancy. It may be that some identification systems do not correctly separate anaerobic lactobacilli and streptococci from *A. vaginae*. This bacterium, when detected with molecular techniques, are present in 86,4 % of BV samples (Verhelst *et al*., 2004; Verhelst *et al*., 2005).

A potentially important observation is that *A. vaginae* appears to be highly metronidazole resistant (Ferris *et al.*, 2004). Ferris and their coworkers (2004) showed that *A. vaginae* strains were susceptible to clindamycin, cephalosporins carbapenems, ampicillin and linezolid and were moderately susceptible to the quinolones. It is interesting to speculate that this microorganism could possibly contribute to BV treatment failures or relapses which are common (Backer *et al.*, 2006; Santiago *et al.*, 2012).

The bacterium *A. vaginae* is a strict anaerobe. Its clinical significance is unknown but it has been isolated from a tubovarium abcess. Investigators suggest that *A. vaginae*

may be an important component of the complex bacterial ecology that constitutes abnormal vaginal flora.

Prospective treatment studies are needed to determine whether or not *A. vaginae* and other metronidazole resistant microorganisms such as *M. curtisii* might play such a role in BV (Backer *et al.,* 2006; Polatti, 2012).

CHAPTER 2

Gardnerella vaginalis and others microorganisms collected in vaginal samples

2.1 Introduction

The vaginal microflora constitutes a complex environment, composed of several microbiological species in variable quantities and proportions (Donders *et al.,* 2005). Studies reported that *Gardnerella vaginalis, Streptococcus agalactiae*, *Peptostreptococcus* spp., *Bacteroides* spp., *Prevotella* spp., *Mobiluncus* spp., *Escherichia coli* and opportunistic pathogens such as *Staphylococcus epidermidis* and *Enterococcus faecalis* can be present in vaginal microflora of women with BV. However, some of these microorganisms can also be present in women with asymptomatic vaginal infections (Allsworth and Peipert, 2011; White *et al.,* 2011).

In order to better understand the etiology of BV, Alves *et al*. (2012), Harwich *et al.* (2010) and Patterson *et al.* (2010) isolated microorganisms from vaginal swabs from healthy women and BV patients (Table 2.1).

In United States, Patterson *et al.* (2010) isolated *G. vaginalis* strain 5-1 from a woman without BV and strain 101 from a woman with BV as diagnosed by the Nugent Gram stain scoring system. Both *G. vaginalis* strains were collected from swabs specimens at Brigham and Women´s Hospital, Boston, MA. Strain AMD was isolated from a woman diagnosed with BV on Amsel criteria at Virginia Commonwealth University (VCU) Women´s Health Clinic, Richmond by Harwich *et al.* (2010). They were interested in determining if different strains of *G. vaginalis* had different virulence potential. To expand this study, Alves *et al.* (2012) isolated more strains from different microorganisms (Table 2.1). In all vaginal specimens taken from women who were diagnosed with BV or were otherwise healthy, BV status was determined using the Amsel criteria and were collected in a gynecology private practice clinic from the Oporto region. Portuguese isolates were obtained by using selective growth media; testing for catalase and oxidase reactions; polymerase chain reaction (PCR) for BV-specific pathogens, Gram stain and, finally, by DNA sequencing technique performed by Eurofins MWG Operon, Germany.

Table 2.1 – Composition of the vaginal microflora isolate from vaginal swabs from healthy women and from women with BV (Alves *et al*., 2012; Harwich *et al.*, 2010; Patterson *et al.*, 2010)

Species	Strain *	BV diagnosis	Reference
Bifidobacterium breve	UM031	No	Alves *et al.*, 2012
Enterococcus faecalis	UM035	Yes	Alves *et al.*, 2012
Gardnerella vaginalis	5-1	No	Patterson *et al.*, 2010
	UM016	No	Alves *et al.*, 2012
	AMD	Yes	Harwich *et al.*, 2010
	101	Yes	Patterson *et al.*, 2010
	UM034	Yes	Alves *et al.*, 2012
	UM035	Yes	Alves *et al.*, 2012
Gemella haemolysans	UM034	Yes	Alves *et al.*, 2012
Klebsiella pneumoniae	UM034	Yes	Alves *et al.*, 2012
Lactobacillus gasseri	UM022	No	Alves *et al.*, 2012
Propionibacterium acnes	UM034	Yes	Alves *et al.*, 2012
Staphylococcus epidermidis	UM016	No	Alves *et al.*, 2012
Streptococcus agalactiae	UM035	Yes	Alves *et al.*, 2012
Streptococcus salivarius	UM031	No	Alves *et al.*, 2012

* According to Alves *et al.* (2012) the name attributed to the strain corresponds to the code of vaginal swabs, which means that the same vaginal swab yielded more than one isolate.

G. vaginalis characteristics have already been discussed in the section 1.3 of Chapter 1. Thus, the next sections present the main characteristics of microorganisms isolated from vaginal samples of Portuguese women.

2.1.1 Bacteria isolated from women without BV

Bifidobacterium breve

Bifidobacteria are generally characterized as Gram-positive, non-spore forming, non-motile, catalase-negative anaerobes (Charteris *et al.*, 1997). The possible contribution of the potentially beneficial *Bifidobacterium* genus to the vaginal bacterial microbiota may have been underestimated due to confusion with the more commonly detected *G. vaginalis*. Such confusion may be the result of similar phenotypic and molecular traits used for identification (Burton *et al.,* 2003). *B. breve* is probably the most common bifidobacterium in infants but remains in the gut throughout adulthood. It is a lactic acid-producing bacterium found in the small and large intestines (Thorsen *et al.,* 1998).

Lactobacillus gasseri

Lactobacillus species are the most often found inhabitants of vaginal ecosystem of fertile women. *Lactobacillus gasseri* is an anaerobic, Gram-positive bacterium that falls into the category of lactic acid producer bacteria (Boris and Barbés, 2000). It is also a rod shaped and of the non-spore-forming type and typically found in the gastrointestinal tracts of humans due to its largely fermentative function. This bacterium is very important, because provides fewer complications in the digestive system, it is the best choice to ferment meat. *L. gasseri* decrease the ability of pathogens like *Staphylococcus aureus* to grow in meat (Arihara *et al*., 1998). Studies observed that the lactic acid and the hydrogen peroxide produced by *L. gasseri* had a very strong effect on the pathogen *S. aureus* (Otero and Nader-Macías, 2006). Recently, its function as a probiotic has been the area of major interest (Petricevic *et al.*, 2012).

Staphylococcus epidermidis

Although *Staphylococcus epidermidis* normally colonizes the skin and nose, some studies reported that it can also be present in vaginal flora (John *et al.,* 2003; Verhelst *et al*., 2005). These bacteria are common opportunistic pathogens and typically, cause infections and diseases when illness or injury occurs (John *et al.,* 2003; Wang *et al.,* 2007). *S. epidermidis* belongs to the coagulase-negative staphylococci (CoNS), which are distinguished from coagulase-positive staphylococci, such as *S. aureus*, by their lack of the enzyme coagulase. As part of the human epithelial microflora, *S. epidermidis* usually

has a benign relationship with its host. Furthermore, it has been proposed that *S. epidermidis* may have a probiotic function by preventing colonization of the host by more severe pathogens (John *et al.,* 2003; Wang *et al.,* 2007). Despite that, *S. epidermidis* is often associated with nosocomial infections (Otto, 2009).

Streptococcus salivarius

Streptococcus salivarius is a facultative Gram-positive cocci. *S. salivarius* is a member of the viridians group of streptococci, commensals of the human upper respiratory, gastrointestinal and female genitourinary tract, but most prevalent in the oral cavity and possessing an excellent potential for use as a probiotic targeting the oral cavity (Conte *et al.,* 2006). *S. salivarius* may exert their protective effect through *in situ* production of the antibiotic enocin (Sanders and Sanders, 1982). It is reported to have very low pathogenic potential (Sanders and Sanders, 1982).

2.1.2 Bacteria isolated from women with BV

Enterococcus faecalis

Enterococcus faecalis is a Gram-positive, facultative anaerobic, coccus which occurs isolated, in pairs or short chains. It is a normal inhabitant of the intestinal tract and female genital tract. *E. faecalis* is an opportunistic bacterium that has become one of the most troublesome hospital pathogens. *E. faecalis* is also catalase-negative, non-spore forming, and is usually nonmotile (Thorsen *et al.,* 1998; Pál *et al.,* 2005). In most cases, they cause no infection, however, in some people, enterococci can cause serious infections, especially those found in the urinary tract (UTIs), wounds, and blood. Vancomycin is often the antibiotic used to treat enterococcal infections, but even this is becoming ineffective as new resistant strains are found (Thorsen *et al.*, 1998; Pál *et al.,* 2005).

Gemella haemolysans

Gemella haemolysans was first described in 1938 and it had been classified for a long time in the *Streptococcus* or *Nesseria* group due to its molecular resemblance, so its identification is difficult (Gatibelza *et al.,* 2009; Malik *et al.,* 2010). *G. haemolysans* is a

facultative, anaerobic, Gram-positive coccus with a low virulence, which is catalase and oxidase negative. *G. haemolysans* is commensal organism of the human genitourinary, gastrointestinal and upper respiratory tracts, and oral cavity (Gatibeleza *et al.,* 2009). However, as an opportunistic pathogen, *G. haemolysans* is able to cause severe systemic infections including endocarditis, spondylodiscitis, meningitis, endophthamitis, thoracic empyema, and septicemia (Anil *et al.*, 2007; Gatibelza *et al.,* 2009). *Gemella* spp. is generally resistant to vancomycin, teicoplanin, erythromycin and tetracycline (Gatibelza *et al.,* 2009; Malik *et al.,* 2010).

Klebsiella pneumonia

Klebsiella pneumoniae is the most frequent cause of nosocomial respiratory tract infections and the second most common cause of Gram-negative bacteraemia and UTIs (Ahmad *et al.,* 2012). *K. pneumoniae* is nonmotile and rod-shaped and is able to overcome innate host immunity through several means. *K. pneumoniae* possess a polysaccharide capsule, which is the main determinant of their pathogenicity. The capsule is composed of complex acidic polysaccharides. Its massive layer protects the bacterium from phagocytosis by polymorph nuclear granulocytes. In addition, the capsule prevents bacterial death caused by bactericidal serum factors (Thorsen *et al.,* 1998). *K. pneumoniae* is naturally resistant to ampicillin, amoxicillin and carbenicillin (Ahmad *et al.,* 2012).

Propionibacterium acnes

Propionibacterium acnes is a slow growing Gram-positive anaerobe, usually pleomorphic, bacillus, which has been considered as a commensal bacterium with low pathogenic potential. However, it has the ability to act as an opportunistic pathogen. *P. acnes* is part of the microflora of the skin, the oral, and the intestinal mucous membranes and can be also present in vaginal flora (Csukás *et al*., 2004; Verhelst *et al*., 2005). They may cause several infections, as acne vulgaris, orbital or dental abscess, sinusitis, hepatitis, endocarditis, osteomyelitis and sepsis. Furthermore, several reports indicate that *P. acnes* may also be the etiological agent of ophthalmic infections (Csukás *et al.*, 2004). *P. acnes* has also been involved in postoperative disorders and opportunistic infections in immunosuppressed hosts (Nakamura *et al.*, 2003). *P. acnes* is usually susceptible to a

wide range of common antibiotics, although some, particularly in acne patients, have been found to be resistant to clindamycin, erythromycin and tetracyclines. Despite their susceptibility, they are sometimes remarkably difficult to eradicate, and long courses of antimicrobial treatment are often recommended (Bayston *et al.,* 2007).

Streptococcus agalactiae

Streptococcus agalactiae is known to cause various infections in adults, but clinical interest in these bacteria mainly relates to their ability to cause serious neonatal illness, especially meningitis and sepsis. Although neonates born by caesarian section have presented with *S. agalactiae* infection, indicating ascending transmission of the microorganism from the vagina of their mothers, in the majority of cases the neonate acquires the infection during labour through contact with the vaginal secretions of the mother colonized by *S. agalactiae* (Pasnik *et al.*, 2006). It is well known that *S. agalactiae* colonizes the female genital tract but it is unclear if this bacterium can cause true infection of the vagina (Brimil *et al.,* 2006). Studies have concluded that invasive infections caused by *S. agalactiae* are not uncommon and that they pose a major problem not only in pregnant women and neonates but also in non-pregnant adults, especially the elderly and patients with chronic diseases (Brimil *et al.,* 2006; Konto-Ghiorghi et *al.*, 2009). Despite adequate antibiotic therapy for this invasive disease often results in high mortality even in recent years (Brimil *et al.,* 2006). The capsule of *S. agalactiae* has long been recognized as one of the most important virulence factors. *S. agalactiae* is resistant to tetracycline, gentamicin, amikacin and erythromycin and it is susceptible to penicillin (Gao et *al.,* 2012; Thorsen *et al*., 1998; Pál *et al.,* 2005).

2.2 Aim

The aim of the work described in this chapter is to characterize the various microorganisms isolated from the genital tract in women with or without the clinical diagnosis of BV and to get the relationship between the absorbance and the number of viable cells for each isolate. With the data obtained from the calibration curves of the various vaginal isolates, it is possible to know which cell concentration correspondent the absorbance of the bacteria inocula. This data is crucial for the experiments described in the following chapters.

2.3 Materials and Methods

2. 3.1 Strains and culture conditions

Bacterial strains used in this study were isolated from vagina flora of the healthy women and BV-diagnosed women, and the source of the strains, is listed in Table 2.1. In addition to the microorganisms isolated from vaginal swab samples, we also included some reference *Lactobacillus* spp. strains. The *Lactobacillus* species studied were *Lactobacillus casei* reference strain CECT 5275 (obtained from the Colección Española de Cultivos Tipo), *Lactobacillus iners* reference strain ATCC 55195 (obtained from American Type of Culture Collection) and *Lactobacillus crispatus* reference strain VCUVAHMP00053 (obtained d from VCU Women´s Health Clinic, Richmond, USA). Once in the laboratory, all clinical isolates were properly stored at -80 ºC (stock collection) and at -20 ºC. All bacteria except *B. breve* and *L. casei* were maintained in Columbia blood agar (CBA – Liofilchem) with 5 % defibrinated horse blood added; this medium was prepared according to the manufacturer's instructions. *B. breve* was maintained in Bromocresol Purple Starch Agar medium which is composed by Peptone from Meat Peptic Digest 7,5 g.L^{-1} (Merck), Tryptone 7,5 g.L^{-1} (Liofilchem), Corn Starch from Potato 1 g.L^{-1} (Pancreatic), Dipotassium Phosphate 4 g.L^{-1} (Pancreatic), Monopotassium Phosphate 1 g.L^{-1} (Pancreatic), Sodium chloride 5 g.L^{-1} (ProLabo), Agar 12 g.L^{-1}, Starch soluble 10 g.L^{-1} (FisherScientific) and Bromocresol Purple 0,0096 g.L^{-1} (Acros-organics). After preparing the medium, it was sterilized by autoclaving at 121 °C for 15 minutes. Finally, after cooling, Nalidixic Acid 30 µg.mL^{-1} (Applichem) was added. *L. casei* was maintained in De Man-Rogosa-Sharpe (MRS – Liofilchem) medium plus the addition of agar (Liofilchem) and it was also prepared according to the manufacturer's

instructions. All isolates were cultured in supplemented Brain heart infusion broth [sBHI; brain heart infusion broth (Oxoid) containing 2 % (w/w) gelatin (Oxoid), 0,5 % yeast extract (Liofilchem), 0,1 % starch (FisherScientific), and 0,1 % glucose (Liofilchem)] (Patterson *et al.*, 2010). All cultures were grown at 37 ºC under anaerobic conditions, using the Anaerogen pack system (Oxoid).

2.3.2 Correlation between viable cells and optical density

Bacteria inoculums were incubated for 48 h at 37 ºC, under anaerobic conditions. Growth was analyzed using a 96-well plate (Orange Scientific) by ELISA 96-well plate reader with a 600 nm filter (Tecan Sunrise). Dilutions were performed with the same culture medium, to cover the range of absorption measurements of interest. Dilutions (usually up to 10^{-8}) of the inoculum were performed in NaCl (0,9 %) and plated, for colony forming unit (CFU) count. CFUs were determined using the micro drop technique, in which droplets (20 μL) of each dilution were placed on agar plates and allowed to dry. Subsequently, the plates were incubated at 37 °C in anaerobic conditions for a suitable period of time (typically 48 hours) to enable the counting of colonies.

2.4 Results and discussion

Calibration curves reflect the relationship between absorbance and the number of viable cells and allow the quantification of the number of cells present in a cell suspension by reading the respective value of absorbance. The graphs relating the calibration curves obtained of the microorganisms used in this study are presented in Figure 2.1.

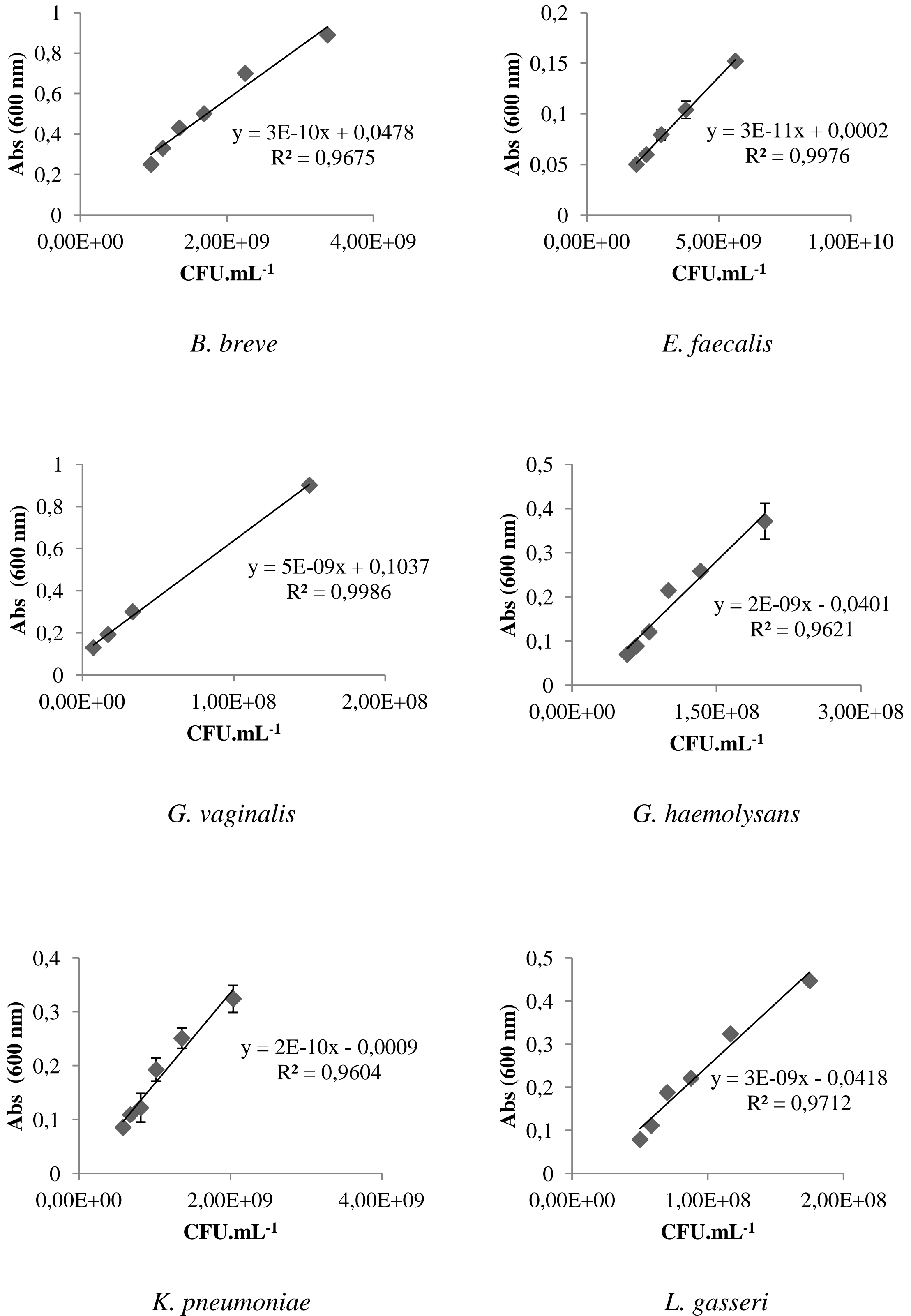
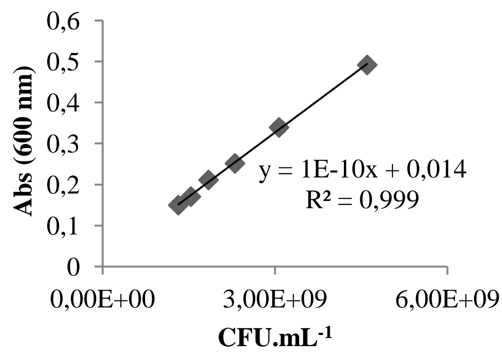
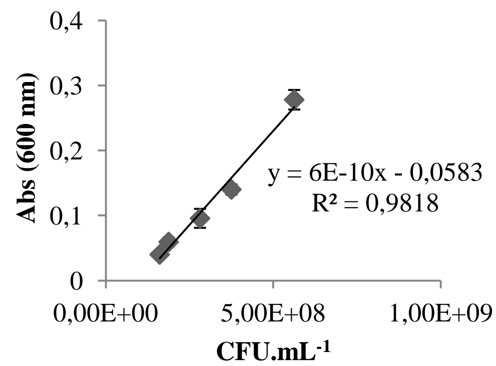


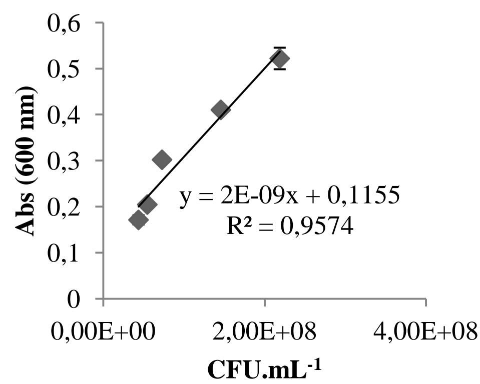
Figure 2.1 – Calibration curves of all bacteria isolates.



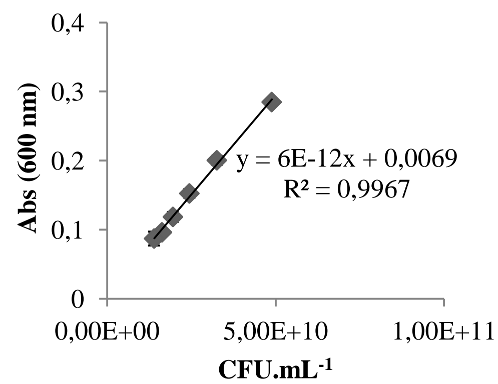
P. acnes



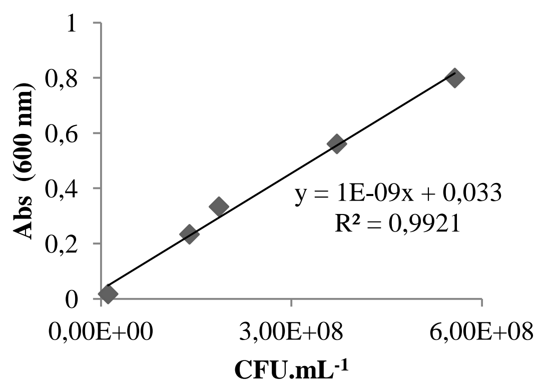
S. epidermidis



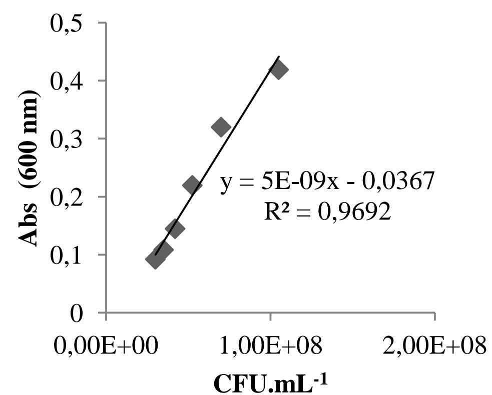
S. agalactiae



S. salivarius

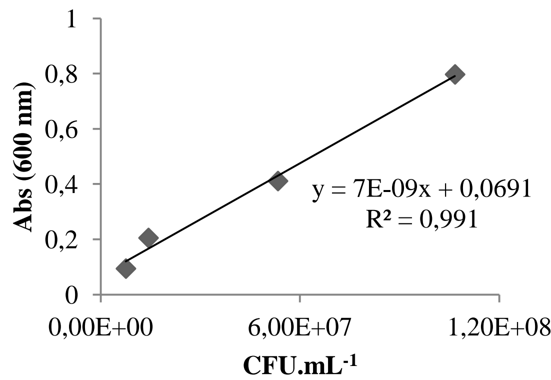


L. casei



L. iners

Figure 2.1 – Calibration curves of all bacteria isolates (continued).



L. crispatus

Figure 2.1 – Calibration curves of all bacteria isolates (continued).

The relationship between absorbance and CFU is different for each species of bacteria studied, which can be due to the size and number of cell varies with phase of growth. Therefore it is important to have the cells in known physiological state of growth, in this case in stationary phase. So, growth curves of microorganisms allow the identification of the growth phase in which the microorganism is in function the incubation time.

The size of the organism, the preparation of the suspension, and other factors all influence the absorbance readings. There are several factors that can affect this curve (e.g., quality of lamp output, size of slit, condition of filter, condition of detector, microorganism characteristics). Despite the inherent inaccuracy of the method, if the procedure is adequately controlled and calibrated, the estimation of microbial numbers by optical density is sufficiently accurate for use in preparing inocula for further studies. The method offers the overwhelming advantages of being rapid, low cost and non-destructive (Sutton, 2011).

Although six different strains of *G. vaginalis* were studied (see Table 2.1), only one calibration curve was performed, because the size of cells is identical. However, there were calibration curves of the different *Lactobacillus* species. This is because, the various *Lactobacillus* species used in this study differed in phenotypic characteristics, namely in structure (*L. casei, L. crispatus* and *L. gasseri* are rod shaped and *L. iners* is curved shaped) and size of bacteria.

So, the determination of calibration curves was necessary for all species of microorganisms, because data about the relationship between CFU and absorbance were necessary to be able to study the initial adhesion of each microorganism to epithelial cells, which will be described in next chapter.

CHAPTER 3

Adherence of vaginal microorganisms to epithelial cells

3.1 Introduction

The interaction between vaginal species and mucosal cells is believed to be one of the critical events in the initial development of BV. Thus, studies on the bacterial adhesion to epithelial cells are important to understand the phenomena involved in the interactions between microorganisms and human tissue (Dunne, 2002; Patterson *et al.*, 2010). However, little is known on how different species of bacteria implicated in BV interact with the vaginal epithelial surface (Swidsinski *et al.,* 2005).

3.1.1 Role of bacterial adhesion in BV

Bacterial adhesion to epithelial cells is one of the characteristics that allow microorganisms to colonize specific tracts of the host. The specific attachment of bacteria to epithelial cells surface (Figure 3.1) is well documented (Catlin, 1992; Swidsinski *et al*. 2005), and it is known that it can lead to the biofilm formation in BV. Initial adhesion is studied both in pathogens and in commensal bacteria that are usually present in vaginal flora (Zárate and Nader-Macias, 2007). Some studies have shown that the high adhesion capacity of certain microorganisms indicates that they can compete for nutrients with a higher efficiency than the non-adherent bacteria (Atassi *et al.*, 2006; Otero and Nader-Macías, 2007).

The healthy vaginal flora is mainly composed by *Lactobacillus* strains, which possess high adherence ability (Atassi *et al.*, 2006). This capacity to adhere to vaginal epithelium is an advantage for prevention of adherence and colonization of pathogenic bacteria (Duary *et al.*, 2011). Despite this, adhesion to vaginal epithelial cells also allows pathogens, as *G. vaginalis* to colonize, thereby minimizing contact of the bacteria with potentially deleterious extracellular enzymes and local antibodies and reducing their chances of being flushed away in vaginal fluid or urine (Atassi *et al.*, 2006; Catlin, 1992).

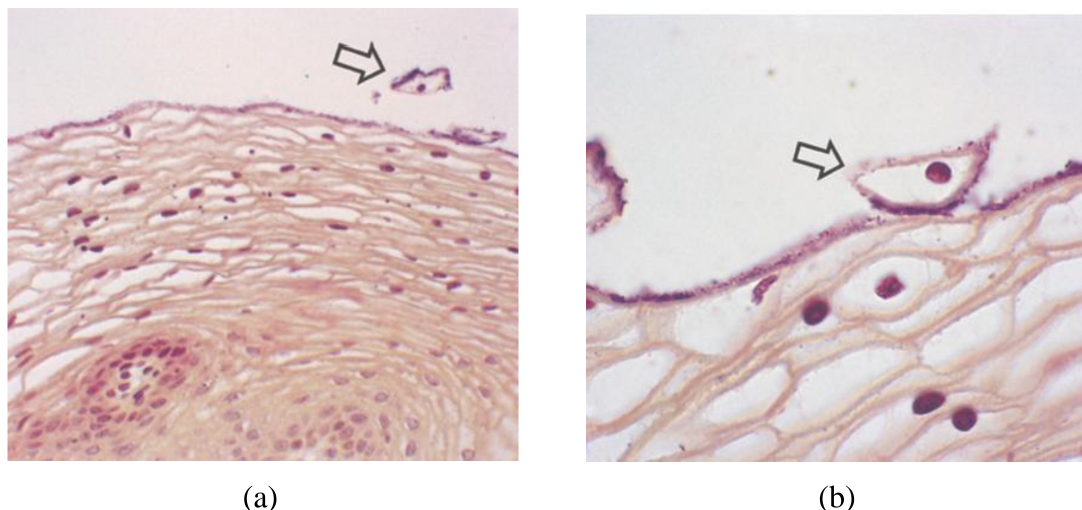


Figure 3.1 – A biofilm can be histologically detected on the vaginal epithelial surface in patients with BV. Original magnifications: left panel, x100 (a); right panel, x250 (b). Note the desquamation of surface epithelial cells containing the biofilm that can be detected as ''clue cells'' in the vaginal smear (arrows). Adapted from Swidsinski *et al.,* 2005.

Researchers are now interested in the determination of the possible interactions of adhesion of various vaginal bacteria to vaginal epithelium (Marrs *et al.,* 2012). Characteristics of cellular interaction differ for a given microorganism or host tissue. The molecular basis of this specificity varies widely, and a microorganism may possess more than one type of adhesion (Bibel *et al.*, 1987; Marrs *et al.,* 2012).

3.1.2 Bacterium–host cell interaction

As stated above, microbial adherence to vaginal cells is the initial step in the development of BV. Specific interaction between microbial surface ligands or adhesins and host receptors influence the distribution of microbes in the sites of infection. These interactions include the mechanisms of adhesion of bacteria to tissue cultured cells, and the events that occur after the bacteria associate with the host cell, such as the capacity to invade these cells (Albert *et al.,* 2000; Dunne, 2002).

Pathogenic microorganisms and those that are part of the normal microbiota have shown to posses macromolecules in their surface that participate in their adhesion to epithelial cells (Otero and Nader-Macías, 2007).

The process of bacterial attachment to an available epithelial surface and the subsequent development of a biofilm is dictated by a number of variables, including the

bacteria species, surface composition, environmental factors, and essential gene products. Adhesion to living or devitalized tissue is accomplished through specific molecular (lectin, ligand, or adhesion) docking mechanism. In the most basic form, bacterial adhesion (as a process distinct from, but integral in biofilm formation) can be divided into two stages: the primary or docking stage and the secondary or locking phase (Dunne, 2002).

Docking: Primary Bacterial adhesion

Primary adhesion constitutes the serendipitous meeting between epithelial surface and a planktonic microorganism. This stage is reversible and is dictated by a number of physiochemical variables that defines the interaction between the bacterial cell surface and the conditioned surface of interest (An *et al*., 2000; Dunne, 2002). First, the microorganism must be brought into close approximation of the vaginal epithelium, propelled either randomly (for example, by a stream of fluid flowing over a vaginal epithelium) or in a directed fashion via hemotaxis and motility. Once the microorganism reaches critical proximity to a surface (usually 1 nm), the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces. These interaction forces, involving a bacterium and epithelial cells, can be non-specific and include electrostatic forces, van der Waals forces, hydrodynamic and hydrophobic forces (Dunne, 2002). The hydrophobic effect is said to be non-specific because any hydrophobin adhesion will interact any hydrophobic receptor. However, the specificity determinants of the hydrophobic effect are poorly understood (An *et al.,* 2002).

Locking: Secondary Bacterial adhesion

The second stage of adhesion is the anchoring or locking phase and employs molecularly mediated binding between specific adhesins and the epithelial surface (An *et al*., 2000; Boland *et al*., 2000; Dunne, 2002). At this point, loosely bound microorganisms consolidate the adhesion process by producing exopolysaccharides that complex with receptor-specific ligands located on pili, fimbriae, and fibrillae, or both. At the end of the second stage, adhesion becomes irreversible in the absence of physical or chemical

intervention, and the microorganism is attached firmly to the surface. Interestingly, the presence of one species of microorganism on a surface can promote of the adhesion of another. All bacteria produce multiple adhesins, and some are regulated at the transcriptional level, permitting organisms to switch from sessile to planktonic forms under different environmental influences (An *et al*., 2000; Dunne, 2002).

One of the critical steps in these adherence studies is the culture of epithelial cells from woman, which requires some specific techniques, since these cells are very difficult to obtain and maintain.

3.1.3 Epithelial cells

In order to understand the phenomena of adherence of vaginal microorganisms to epithelial cells it is necessary to use *in vitro* models. The use of vaginal epithelial cell lines is a key factor to understand BV, due to the lack of a tractable animal model for BV, which has thwarted efforts to understand the etiology of this common and important health concern of women.

The ability to manipulate and maintain cells *in vitro* is a critical phase of cells culture. This process includes several steps as: cryopreservation, culture maintenance and prevention and detection of external contamination (Henriques, 2005).

Cryopreservation

Cryopreservation is a determinant step, since it is necessary to keep and maintain cells stored so that they can repeatedly be used from the same “starting” state. The cell response to the cryopreservation depends on the method of freezing, mainly the cooling rate. If extracellular ice is formed it rises to a chemical potential difference across cell membranes, driving water out of the cell by osmosis (Freshney, 2005; Henriques, 2005). However, the plasma membrane has a finite permeability to water, the magnitude of which determines the rate of water efflux and the corresponding time scale of cell dehydration. Consequently, if the rate of cooling is sufficiently slow to allow the intracellular solution to equilibrate with its external environment by expelling water through the cell membrane, the cell will dehydrate extensively with decreasing

temperature. On the other hand, if the cooling rate is fast compared with the rate of water efflux, low temperatures are reached before significant dehydration can occur. In this situation, the cell remains largely deformed, but there is a very high probability of ice formation in the cell, as the intracellular solution is in a super cooled non-equilibrium state (Freshney, 2005; Henriques, 2005; Karlsson and Toner, 2000). The effect of cooling rate on the cell survival is presented schematically in Figure 3.2.

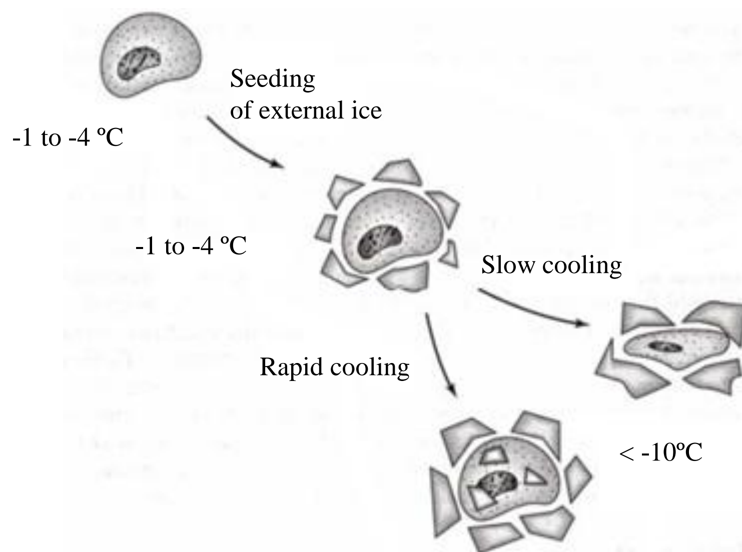


Figure 3.2 – Effect of cooling rate on cell survival. Adapted from Palsson and Bhatia (2004).

So, it can be established an optimum cooling rate at which the two mechanisms of damage are balanced, and the probability of cell survival reaches a maximum (Karlsson and Toner, 2000). Usually, during the freezing process a permeant cryoprotective additive such as dimethyl sulfoxide (DMSO) is used. The freezing process includes several steps, as keeping the cells at -20 ºC, decreasing the temperature to - 80ºC and storing at - 136 ºC in liquid nitrogen.

Cell maintenance

The *in vitro* cell maintenance depends on the medium in which cells are grown, which provides inorganic salts and other nutrients. The tissue culture media can include: basal medium components such as sodium chloride (adjusts osmotic pressure), inorganic

salts (provide electrolyte balance similar to blood), sodium bicarbonate (provides buffering capacity), D-glucose (as source of energy, carbon), amino acids (as source of nitrogen for protein synthesis), vitamins (cofactors in various intracellular biochemical reactions) and phenol red (as a visual pH indicator); serum (that provides cell growth, attachment factors, hormones and carrier proteins); growth factors, hormones (that stimulate growth function); and antibiotics (used to prevent contamination by microorganisms) (Freshney, 2005; Henriques, 2005).

The most common commercially available media are: BME (basal medium Eagle's), EMEM (minimum essential medium with Earl's salts), DMEM (Dulbecco's modified Eagle's medium) and RPMI 1640 (created by Roswell Park Memorial Institute). The selection of the optimal medium to be used depends on the culture type (Henriques, 2005).

The serum used in medium preparation is a fraction of whole blood. Plasma is the non cellular fraction of the blood, whereas serum is the liquid that remains after plasma is allowed to clot. Serum is typically added to culture medium in a proportion of approximately 1 to 20 % by volume. Despite of the advantages, the use of serum has some disadvantages, that is: the chemically constituents that are note defined or may vary; the extensive testing necessary before use; the lack of reproducibility; the difficulty of standardization of experimental and production protocols; the risk of contamination; the availability and costs; and may contain growth and metabolism inhibitors (Freshney, 2005; Henriques, 2005).

If cells grow adhered to the bottom of a T-flask it will be necessary to detach them. For that, chemical or physical processes can be used. Among the latter, scrapping is commonly used when the adhesion is not tight. Sometimes chemical detachment is needed and it can be done with digestion enzymes. The enzyme digestion can be performed using different enzymes depending on the tissue type. Examples of enzymes are: trypsin, collagenase, elastase and papain, but the most common is trypsin (Doyle and Griffiths, 2000).

Contamination

Maintenance of cells in physiological, nutrient-rich environment provides an ideal medium for the growth of microorganisms that are contaminants. Sterile culture techniques and the use of antibiotics are designed to prevent contaminations; nevertheless, microbial contaminants are routinely encountered. Included in these microbial contaminants are: bacteria, fungi, yeasts (that can be detected by an increase in the medium turbidity and a changing in the color of the medium) and mycoplasma (that is the most difficult contaminant to detect). The detection of mycoplasm includes techniques as staining, culture, DNA probes and co-cultivation (Doyle and Griffiths, 2000).

3.1.4 Aim

The aim of the work described in this chapter is to analyze the adherence, to epithelial cells, of microorganisms isolated from the genital tract of women with or without clinical diagnosis of BV, as well as, of *Lactobacillus* strains previously selected.

Most studies of bacterial adhesion to host cells have been done mostly with *G. vaginalis* (Scott *et al*., 1989; Catlin, 1992; Harwich *et al*., 2010; Patterson *et al*., 2010). However, there are few studies of adherence of *G. vaginalis* isolated from a BV patient and from a healthy woman to epithelium (Harwich *et al*., 2010; Patterson *et al*., 2010), and there are not studies of adherence of Portuguese vaginal isolates, such as: *B. breve*; *E. faecalis*; *G. haemolysans*; *K. pneumonia*; *L. gasseri*, *P. acnes*; *S. epidermidis*; *S. agalactiae* and *S. salivarius*.

3.2 Materials and Methods

3.2.1 Strains and culture conditions

The 15 vaginal microorganisms, used in this study, were isolated from vagina flora of the healthy women and BV-diagnosed women. Microorganisms isolated were: *B. breve; E. faecalis; G. vaginalis*; *G. haemolysans; K. pneumonia; L. gasseri, P. acnes; S. epidermidis; S. agalactiae* and *S. salivarius* and the source of the strains, is listed in Table 2.1. In addition to the microorganisms isolated from vaginal swabs, some reference *Lactobacillus* spp. strains were also included, namely *L. casei* CECT 5275, *L. iners*

ATCC 55195 and *L. crispatus* VCUVAHMP00053. All isolates except *L. casei* were cultured in sBHI broth (see section 2.3.1). *L. casei* was cultured in MRS broth (see section 2.3.1). All cultures were grown at 37 ºC, during 48 h under anaerobic conditions, using the Anaerogen pack system (Oxoid). All isolates were grown in appropriate media and were collected by centrifugation at 7 000 rpm for 10 min. The culture medium was discarded, and the bacteria were washed once with PBS (composed by 16 g.L^{-1} of NaCl; 0,4 g.L^{-1} of KCl; 1,62 g.L^{-1} of $Na_2HPO_4^-.2H_2O$ and 0,4 g.L^{-1} of KH_2PO_4). After this, bacterial suspension was calibrated for appropriate optical density, using a 96-well tissue culture plates (Orange Scientific) by ELISA 96-well plate reader with a 600 nm filter (Tecan Sunrise). So, the absorbance value was adjusted in accordance with the calibration curve (Figure 2.1) of each microorganism. Thereafter, bacterial suspension was centrifuged again and the absorbance value was again confirmed. The PBS was discarded, and the bacteria were resuspended in D-MEM medium. Finally, bacterial suspensions were prepared for epithelial cells' infection.

3.2.2 Cell line and culture conditions

Human cervical HeLa cells, kindly provided by the Instituto Gulbenkian de Ciência, were cultured at 37 ºC, in 5 % CO_2 (Shel Lab CO_2 series incubator), in D-MEM (Sigma-Aldrich) supplemented with 15 % Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1 IU penicillin/streptomycin mL^{-1} (Sigma-Aldrich).

After being slowly defrosted, cells were added to a falcon tube containing 5 mL of medium and were centrifuged for 6 min at 1000 rpm. The pellet was resuspended in 2 mL of medium and then the suspension was added to a T-flask (25 cm^2) containing 3 mL of fresh medium. The flask was maintained in a CO_2 incubator (Shel Lab CO_2 series incubator) at 37 ºC until 80 % of confluence was obtained. At this point, the medium was removed and the cells were washed once with 2 mL of PBS (Sigma-Aldrich). After discarding the PBS, 1 mL of trypsin (Sigma-Aldrich) was added and the cells were kept for 10 minutes at 37 ºC until they were detached from the flask. To stop the trypsin activity, 3 mL of D-MEM medium were added to the flask. Cells were enumerated in a Neubauer chamber and were then diluted in 5 mL of medium to 1×10^6 cells.mL^{-1}. The new flasks were incubated in 5 % of CO_2 and 37 ºC. The trypsinization was repeated to prepare new flasks – to maintain the cells, or to prepare cells for adhesion assays. In the

latter, after detachment, 2×10^5 cells.mL^{-1} were added to a 24-well tissue culture plates (Orange Scientific containing circular glass lamellas (12 mm Ø, thickness 0,13 – 0,16 mm, Marienfeld, Germany) in the bottom. The well plate was incubated for 48 h or until a monolayer of cells has been formed. After reaching confluence, the growth medium of epithelial cells was discharged and circular glass lamellas were washed one time with 500 μL of PBS (Sigma-Aldrich) and were ready to be used in the adhesion assays.

3.2.3 Adhesion assay

To perform the adhesion assay, 250 μL of the microbial cells suspension (10^8 CFU.mL^{-1} of *G. vaginalis* strain 5-1, AMD and 101; and 10^9 CFU.mL^{-1} to the reference strains: *L. casei*, *L. iners* and *L. crispatus*) were added to each well of 24-well plate, which had a circular glass lamella with a monolayer of HeLa cells in the bottom. After 10, 15, 20 or 30 minutes of incubation of bacteria (at 37 ºC, in 5 % CO_2), each well of 24-well plate was washed once with 500 μL of PBS (Sigma-Aldrich). In order to assess the influence of washing the cells in bacterial adhesion quantification a replicate well was used, in the same conditions as described above, without the washing step. After this, each circular glass lamella was removed from the 24-well plate, and transferred to a surface coated with absorbent paper.

Fixing bacteria and epithelial cells

For fixing the bacteria and epithelial cells to the circular glass lamellas, 45 μL of methanol 100 % were added and lamellas were left at room temperature for 30 minutes. After this, 45 μL of paraformaldehyde 4 % was added and lamellas were left at room temperature for 10 minutes. Then, the excess of paraformaldehyde 4 % was removed with the help of tweezers. Finally, 45 μL of ethanol 50 % was added to each lamella and these were left at room temperature overnight or until dry.

Cell enumeration

The circular glass lamellas were taken to the microscope room, and 20 μL of blue-fluorescent DAPI nucleic acid stain (referring to a concentration of 2,5 $\mu g.mL^{-1}$) was added immediately before adherence visualization using a fluorescence microscope (Olympus BX51), using DAPI filter ($\lambda_{excitation}$ = 365-370 nm). The images were captured with a video camera that was coupled to the microscope and connected to a computer, using *Cell – Imaging Software for Life Sciences Microscopy*. Twenty fields were randomly counted in each sample. Thereafter, it was counted the number of bacteria adhered to epithelial cells and also eukaryotic cells per image, using the *ImageJ Software*. Results were expressed as bacteria per HeLa cells. Adherence assays were repeated three times on separate days, with three fields of view assessed each time.

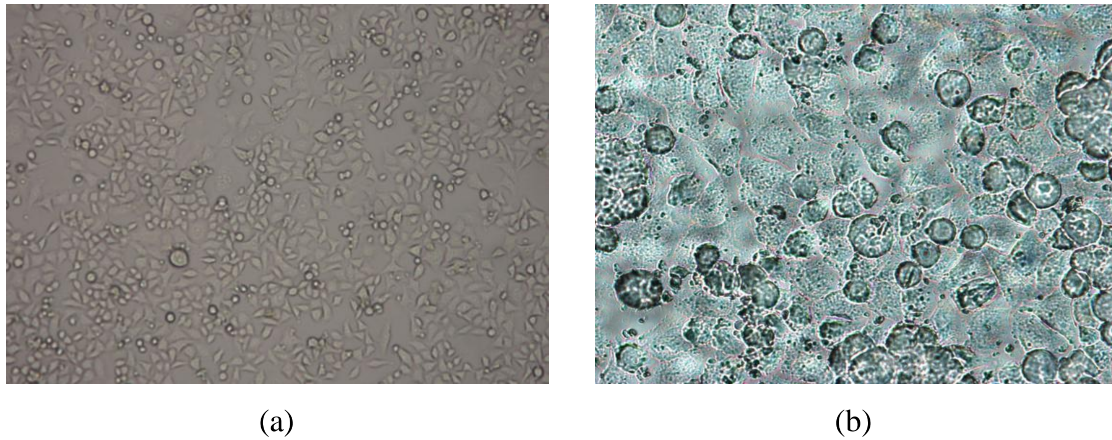
3.2.4 Statistical analysis

The data were statistically analyzed using SPSS (Statistical Package for the Social Sciences – version 18). Results are expressed as the mean ± standard error of the mean (SD). Student's t-test was used for statistical comparison. In some cases, Tukey's test was also used, after analysis of variance (ANOVA). In both tests, *P*-value of 0.05 was considered statistically significant.

3.3 Results and discussion

3.3.1 HeLa cells

Some of the commonly used non-polarized cell-lines for studying the bacteria – host interaction are HeLa cells derived from human cervical epithelial carcinoma (Figure 3.3). The continuous or immortal cell lines can arise spontaneously or by transformation either by treatment with carcinogenic chemicals or as a result of exposure to DNA tumor viruses (Doyle and Griffiths, 2000).



(a) (b)

Figure 3.3 – HeLa cells. Original magnifications: left panel, x100 (a); right panel, x400 (b). HeLa cells attachment in T-flask surface (a); HeLa cells fixed in circular glass lamella (b).

Cultured cells, which represent a single cell type, can be grown in defined media under reproducible conditions. Nevertheless, there are certain limitations which may affect interpretation of experimental data. During the process of immortalization, cell lines lose many traits of the original tissue from which they were derived (An *et al.*, 2000). One feature that can be lost in this process is tissue-specific surface molecules that normally function as receptors for bacterial adhesions (Freshney, 2005). This may explain the fact that many bacterial pathogens that are highly specific for a particular tissue of the host are frequently able to adhere to cultured cells derived from tissue that they do not normally infect (An *et al.*, 2000). Another problem is that most cultured cells exhibit changes in their normal morphology. A further limitation of cultured cells as representatives of human mucosal surfaces is that mucosal surfaces, *in vivo*, are coated by mucus and bathed in solutions that are difficult to mimic in an *in vitro* system (Doyle and Griffiths, 2000; Freshney, 2005). Furthermore, real tissues consist of multiple cell types, not of a single cell type as seen in most tissue culture models. In spite of the numerous limitations of existing cell lines, these have been extremely useful when investigating bacterium – host cell interaction, and, if their limitations are kept in mind, cultured cells will continue to be invaluable models (An *et al.*, 2000; Doyle and Griffiths, 2000).

3.3.2 Kinetics of initial adhesion of *Lactobacillus* spp. to epithelial cells

As the ability of bacteria to adhere to host cells is an important factor for colonization in different host (Kaewsrichan *et al.,* 2006), the adhesiveness of lactobacilli to host epithelial cells was investigated. At present, little is known about the mechanisms by which lactobacilli from healthy vaginas adhere to vaginal epithelial cells (Atassi *et al.,* 2006; Boris *et al.*, 1998). In recent years, the role of lactobacilli has been recognized in maintenance of homeostasis within dynamic ecosystems such as the vagina. Loss of vaginal lactobacilli may predispose women to the acquisition of genitourinary infections (Srinivasan and Fredricks, 2008). For this reason, the prophylactic use of *Lactobacillus* may be an effective means of restoring the normal microbiota in the vagina, and thus preventing infections. The characteristics needed for a *Lactobacillus* strain to serve effectively as a prophylactic agent include avid adherence to vaginal epithelial cells, interference with the adhesion of other pathogens and the production of molecules capable of inhibiting the growth of pathogens (Srinivasan and Fredricks, 2008).

In this study, several critical parameters that could affect the outcome of adherence assays were evaluated in order to provide insights into which steps on this process are affected by variations in experimental conditions. In order to understand the kinetics of the adhesion of *Lactobacillus* to epithelial cells, four time points (10, 15, 20 and 30 minutes) were analyzed. The results of the adherence of *L. iners, L. crispatus* and *L. casei* to HeLa cells are showed in Figure 3.4. The values of adhesion of lactobacilli to epithelial cells are showed in two conditions: with and without washing with PBS.

Since bacterial binding to epithelial cell surfaces is not only mediated by bacterial fimbriae but also by hydrophobic interactions between bacteria and various host cells (Bos *et al.,* 1999; Brauner *et al.,* 1990), the washing step can influence this process. It has been suggested that, since the adhesion method would always be affected by the air-liquid interface in the washing and drying processes, this method evaluates the retention of cells rather than adhesion, i.e. the ability to adhere to a surface and to resist shear forces that exist in natural environments (Albert *et al.,* 2000; Bos *et al.,* 1999). However, the effect of the air-liquid interface varies and is dependent on the substratum properties, cell surface properties and the velocity of the passing air-bubble. It has been demonstrated that the passage of an air-liquid interface through a lawn of adherent bacteria can detach some of the cells, although this effect is attenuated in the presence of a more hydrophilic substrate or with more rapid washing of adherent cells (Bos *et al.,* 1999; Brauner *et al.,*

1990). In this chapter, the washing procedure, when applied, was performed carefully in order to minimize formation air-liquid interface.

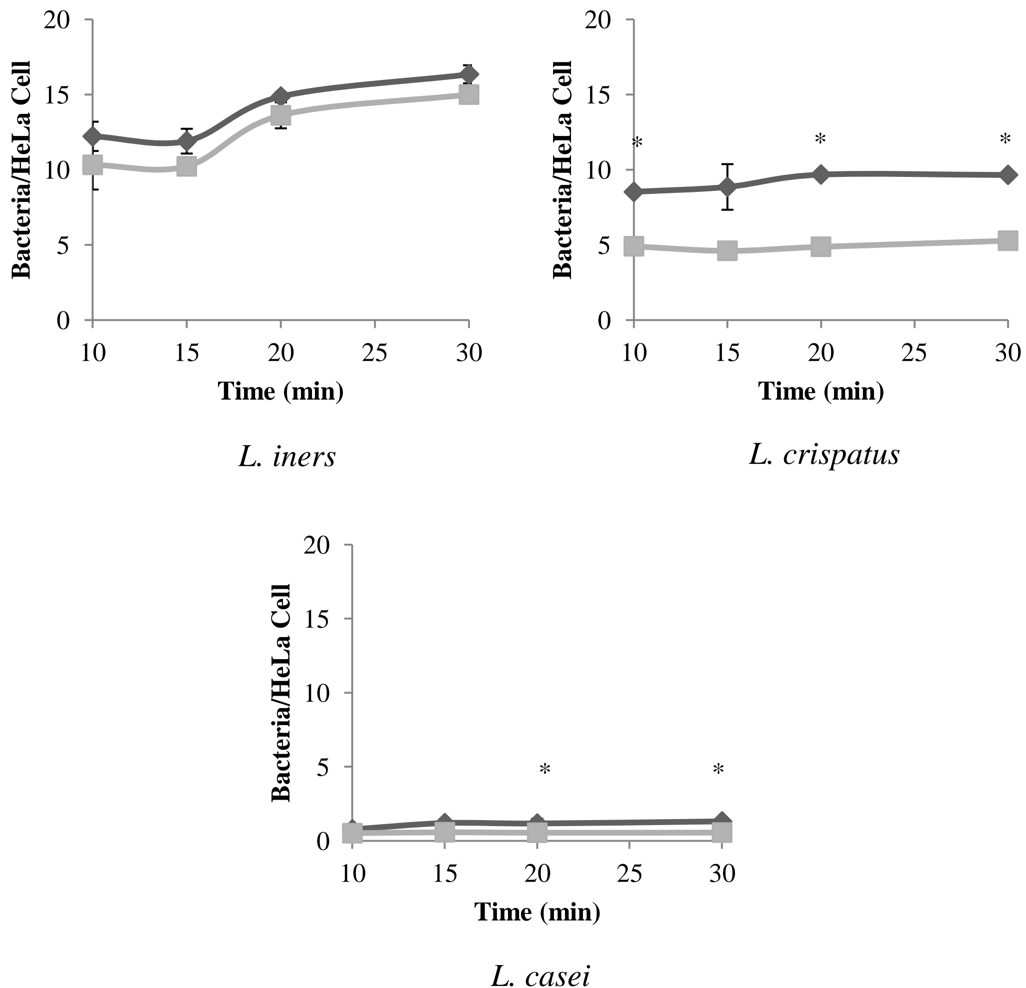


Figure 3.4 – Quantification of initial adhesion of *L. iners*, *L. crispatus*, and *L. casei* to epithelial cells: under the conditions of no washing with PBS (darker line) after 10, 15, 20 and 30 minutes of incubation with bacteria; and washing with PBS (lighter line) after 10, 15, 20 and 30 minutes of incubation with bacteria. *Significantly different values between washing and no washing of the cells with PBS after incubation with bacteria ($P<0.05$).

Lactobacillus colonization and adhesion within the vaginal tract have remained poorly characterized, in part due to our poor knowledge of the adhesive surface components expressed by lactobacilli (Duary *et al*., 2011). Chauviere and their coworkers (1992) reported previously that not all strains of *Lactobacillus* developed adhesiveness to Caco2 cells, thereby, indicating that this property is highly strain specific (Duary *et al*., 2011). The involvement of carbohydrates and lipoteichoic acids in the adherence of lactobacilli to genital epithelia has also been reported (Boris *et al*., 1998; Granato *et al*.,

1999; Neeser et *al.*, 2000). Furthermore, regular structures on the outside of lactobacilli are known as S-layer and they have been associated on the adhesion events. S-layer proteins of lactobacilli have a molecular mass between 40 and 55 KDa, and are, in general, non-glycosylated. Nevertheless, the functions of S-layers in *Lactobacillus* spp. have remained poorly characterized (Frece *et al.*, 2005). Overall, our various results suggest that lactobacilli adhere to host tissues via mechanisms that could vary in different species.

Our results demonstrated that certain strains of lactobacilli, including *L. iners* and *L. crispatus* adhered in large numbers to the epithelial cells, whereas *L. casei* bound significantly less. In this present study, *L. casei* was used as negative control, because it is not a common colonizer bacterium in the vagina epithelium (Ingrassia *et al.*, 2005). Nevertheless, *L. casei* is major members of the indigenous bacterial flora in the gastrointestinal tract of humans and animals. Thus, our results showed that *L. casei* did not have statistical differences (p>0.05) between levels of adherent cells regardless of the exposure times to the epithelial cells.

L. iners is widely present in healthy women as well as those suffering from BV, suggesting that it is an important indigenous species of the vagina. Nevertheless, this microorganism is fastidious and not easy to work *in vitro* (Saunders *et al.*, 2007). Females with BV have loss of many *Lactobacillus* spp., except *L. iners* (Srinivasan and Fredricks, 2008). It has been suggested that a factor that may contribute to *L. iners* to host cell adhesion is a highly expressed and secreted CDC proteins (Macklaim *et al.*, 2011). The CDC proteins use cholesterol in host cell membranes as a receptor for binding. On binding, a conformational change in the protein causes cell lysis by forming large pores in membrane. Thus, *L. iners* may exhibit specialized adaptation mechanism to be vaginal environment (Macklaim *et al.*, 2011). Therefore, a recent study showed the ability of *L. iners* to adhere to VEC (Macklaim *et al.*, 2011). Nonetheless, the present study is the first to examine the adherence of *L. iners* to HeLa cells. In the present case, when no washing step was used, a great number of adherent bacterial cells were generally obtained. Furthermore, without washing, there was no difference (p>0.05) between levels of adherent cells regardless of the exposure times to the epithelial cells, except to t=15 and t=20 min (p<0.05). In other hand, using washing, there were some statistical differences between levels of adherent bacteria to epithelial cells, *L. iners* had differences statistical

($p<0.05$) between t=10 and t=30 min and t=15 and t=30 min. Thus, significant differences were not found between t=20 and t=30 min. In this sense, the adherence of *L. iners* to epithelial cells remains constantly after 20 min of exposure. However, quantification studies of adherence of *L. iners* have not been reported, thereby impeding a comparison with the present study.

L. crispatus was chosen in this study because this specie has been reported as prevalent among women with *Lactobacillu*s-predominant healthy vaginal flora in North America, Europe and Asia. *L. crispatus* is one of the predominant H_2O_2 producing species, is under development as a probiotic for the treatment of BV (Antonio and Hillier, 2003). Studies showed that *L. crispatus* have a collagen-binding surface component, identified as the S-layer protein CbsA (Antikainen *et al.*, 2002; Toba *et al.*, 1995). CbsA of *L. crispatus* exhibits affinity for collagens of the epithelial cells (Antikainen *et al.*, 2002). Our results showed that *L. crispatus* had differences statistical ($p<0.05$) in bacterial adherence to epithelial cells between t=10 and t=30 min; t=15 and t= 30 min and t=20 and t=30 min, using washing process. Zárate and Nader-Macías (2006) reported that *L. crispatus* showed the capability to adhere to VEC, when this bacterium was exposure for 60 min. However, these researchers (Zárate and Nader-Macías, 2006) used a filter an 8-µm pore size membrane to remove all non-adherent bacteria, instead of the washing process. Finally, the results suggest that *L. crispatus* has adhesive properties as well as a quickly initial adhesion to epithelial cells.

3.3.3 Kinetics of initial adhesion of *G. vaginalis* to epithelial cells

Studies showed that *G. vaginalis* have strong adherence to vaginal epithelial cells and a propensity to form a dense biofilm; these findings suggest a key role for *G. vaginalis* in BV pathogenesis (Patterson *et al.,* 2010). Additional virulence factors produced by *G. vaginalis* include sialidase and prolidase, which are two hydrolytic enzymes that may have a role in degrading several key mucosal protective factors, such as mucins, as well as contributing to exfoliation and detachment of vaginal epithelial cells. In sum, there is strong evidence that *G. vaginalis* possess innate pathogenic potential (Scott *et al.,* 1989). In order to understand the ability of *G. vaginalis* to adhere to epithelial cells, several strains were used: *G. vaginalis* AMD (pathogenic isolate), *G.*

vaginalis 101 (pathogenic isolate) and *G. vaginalis* 5-1 (non-pathogenic isolate). Adherence was assayed by fluorescence microscopy, and results are showed in Figure 3.5.

In a time-course assay, Patterson *et al.* (2010) showed that adherence of *G. vaginalis* to epithelial cells did not increase after 15 minutes of contact. Furthermore, after 30 minutes of incubation with the bacteria, the cell lines began to exhibit cytopathogenic changes. Therefore, in this study, bacteria were incubated for up to 30 minutes, in a 24-well plate with circular glass lamellas in the bottom, in order to understand the kinetics of the adhesion of *G. vaginalis* to epithelial cells.

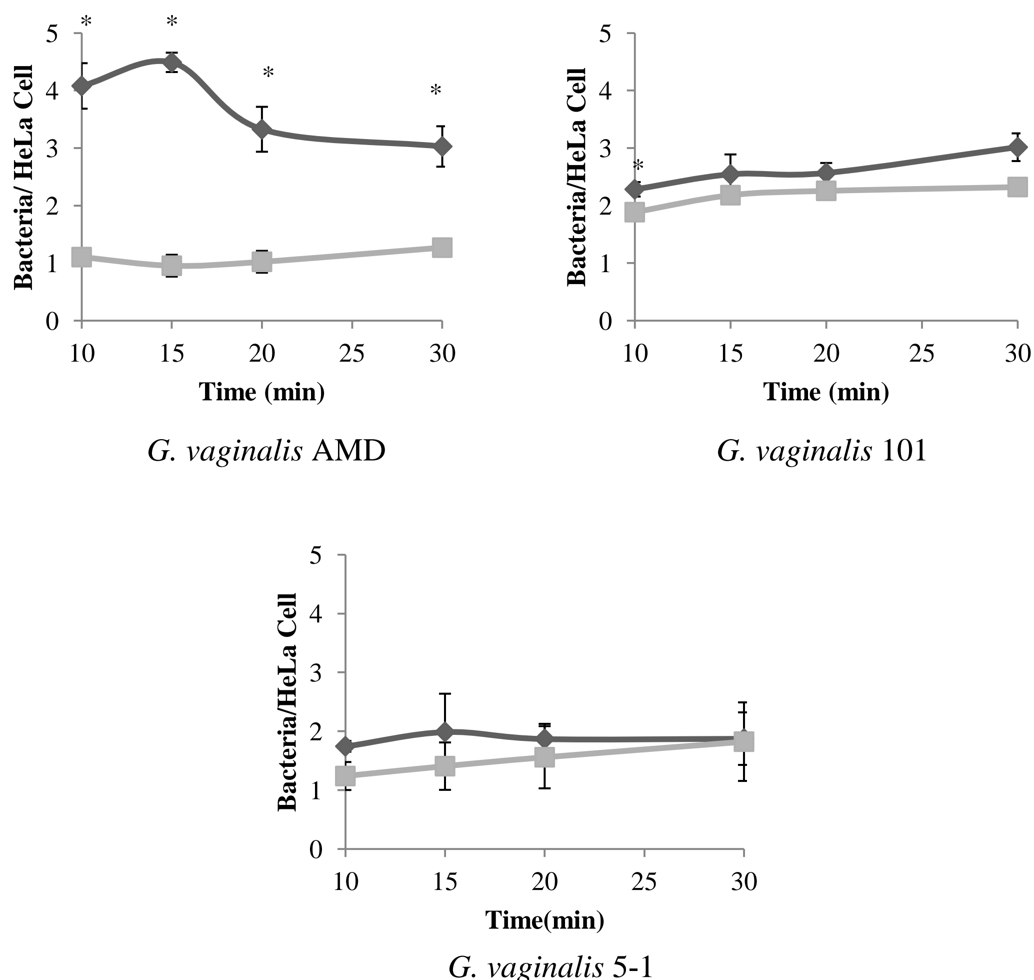


Figure 3.5 – Quantification of initial adhesion of *G. vaginalis* AMD (BV isolate), *G. vaginalis* 101 (BV isolate), and *G. vaginalis* 5-1 (non-BV isolate) to epithelial cells: under the conditions of no washing with PBS (darker line) after 10, 15, 20 and 30 minutes of incubation with bacteria; and washing with PBS (lighter line) after 10, 15, 20 and 30 minutes of incubation with bacteria. *Significantly different values between washing and no washing of the cells with PBS after incubation with bacteria ($P<0.05$).

Overall, the results of bacterial adhesion after the washing step showed a lower standard deviation than there was not any washing step. So, the washing step must occur to discriminate effectively adherent from merely deposited cells, in order to accurately quantify and compare among different experimental conditions the number of adherent cells to epithelial surface. When comparing washing and no washing, it was found that adhesion levels determinate by both procedures were different for each time of exposure evaluated. So, the difference is most noted for *G. vaginalis* strain AMD, that showed significantly different values ($p<0.05$) between washing and no washing for each time.

While equal amounts of the three strains were added to HeLa cells monolayer, adherence of strain AMD and 101 (pathogenic isolates) was more pronounced relative to that of non-BV isolate strain 5-1. However, there was an exception, with washing procedure after incubation of bacteria, being the number of *G. vaginalis* AMD per HeLa cells was approximate the number obtained with *G. vaginalis* 5-1, under the same conditions. Nevertheless, it was observed that strain AMD and 101 were more aggregative than strain 5-1. This confirms that the capability of BV isolates to bind to and adhere to vaginal epithelium may be higher than non-BV isolates, as previous suggested (Patterson *et al.*, 2010). Furthermore, without washing, there was no difference between levels of adherent cells, regardless of the exposure times to the epithelial cells, except to *G. vaginalis* AMD that had differences statistical ($p<0.05$) between t=10 and t=30 min; between t=15 and t=20 min and between t=15 and t=30 min. In other hand, using washing, there were some statistical differences between levels of adherent bacteria to the epithelial cells, this is because *G. vaginalis* 101 had statistical differences ($p<0.05$) between t=10 and t=15 min and t=10 and t=20 min and between t=10 and t=30 min. In this case, *G. vaginalis* AMD and 5-1 did not have statistical differences ($p>0.05$) between levels of adherent cells regardless of the exposure times to the epithelial cells.

In general, it was concluded that there were no significant changes in adhesion of bacteria (*Lactobacillus* spp. and *G. vaginalis*) to the epithelial cells up to 30 minutes.

3.3.4 Adhesion of Portuguese vaginal isolates to epithelial cells

Other BV-associated anaerobes might have virulence factors that have not yet been described, or they may be more pathogenic in the presence of other species. Therefore the ability of BV-associated anaerobes to adhere to epithelial cells was

analyzed. This study did not include all the bacterial species that have been found to be associated with cases of BV. BV is a polymicrobial disorder, and the diversity of species associated with this disorder continues to increase as detection methods (Patterson *et al*., 2010). Because of the large number of candidates, and because many bacteria found to be associated with BV are unculturable, this study focused instead on a relatively small number isolated Portuguese species (Alves *et al*., 2012).

Portuguese vaginal isolates were added to each circular glass lamella at 10^8 CFU.mL^{-1} for only time, that was 30 minutes, in conditions and procedure previously optimized (see section 3.2). This time of incubation of bacteria to epithelial cells was also previously optimized (see section 3.3.2 and 3.3.3). Adherence of bacteria to epithelial cells was assayed by fluorescence microscopy, and results are showed in Table 3.1.

When comparing washing and no washing, it was found that the level of adherence reached using both procedures was significantly different ($p<0.05$) for *L. gasseri* UM022 and *G. vaginalis* UM016. So, as presented in Table 3.1, the adhesion to epithelial cells was major when the step of washing was not performed. In this sense, the ability of *L. gasseri* UM022 and *G. vaginalis* UM016 to resist to shear forces (air-liquid interface), that exist in natural environments (mimic through the washing step), was lower than other Portuguese vaginalis isolates analyzed in this studied.

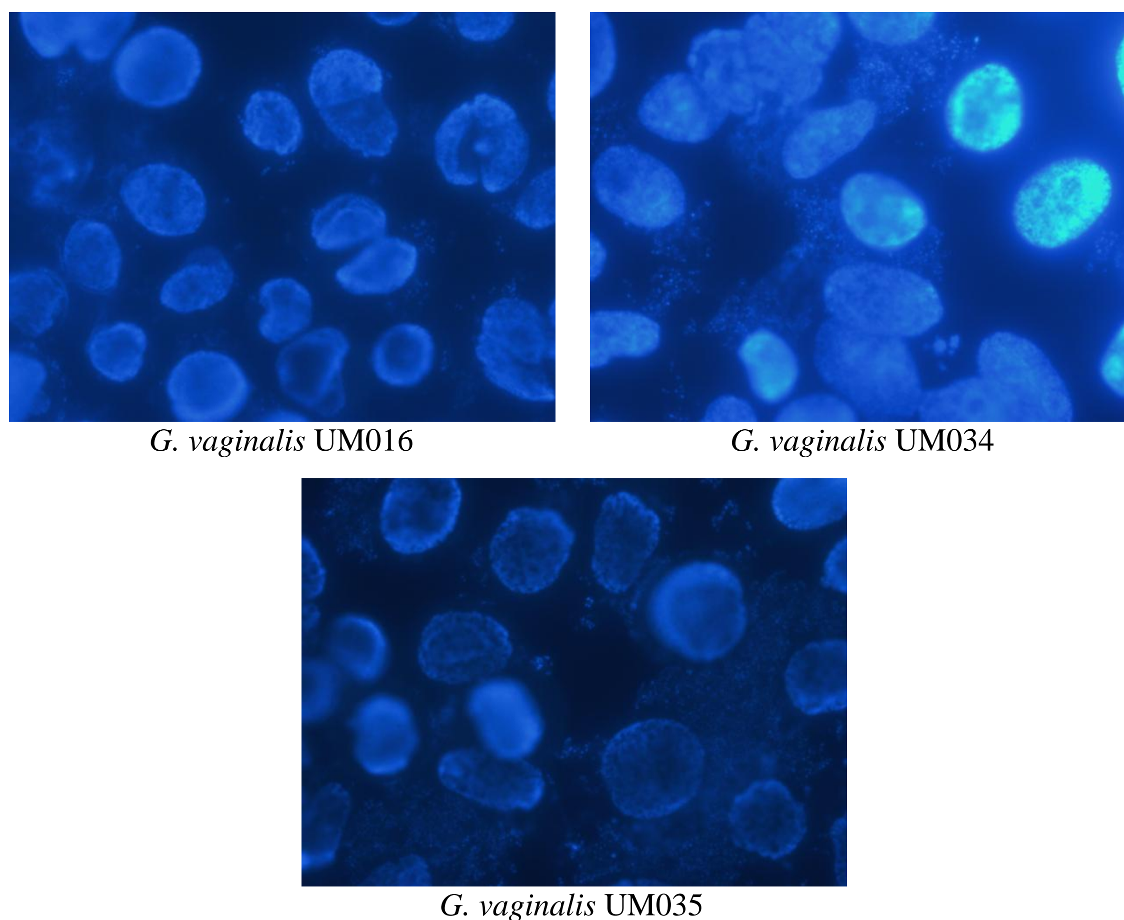
Table 3.1 – Quantification of initial adhesion of vaginal isolates of Portuguese women to epithelial cells (HeLa cells): under the conditions of no washing with PBS and washing with PBS after 30 minutes of incubation of bacteria

Bacteria	**Bacteria/HeLa Cell**	
	Without washing with PBS	**With washing with PBS**
B. breve UM031	0,91 ± 0,27	0,63 ± 0,06
E. faecalis UM035	0,55 ± 0,09	0,42 ± 0,06
G. vaginalis UM016	3,45 ± 0,11*	2,76 ± 0,37*
G. vaginalis UM034	9,23 ± 2,53	7,74 ± 1,37
G. vaginalis UM035	18,28 ± 5,57	15,72 ± 1,13
G. haemolysans UM034	1,19 ± 0,43	0,80 ± 0,43
K. pnemoniae UM034	0,40 ± 0,08	0,32 ± 0,10
L. gasseri UM022	1,61 ± 0,67*	0,44 ± 0,18*
P. acnes UM034	0,32 ± 0,12	0,15 ± 0,08
S. epidermidis UM016	0,50 ± 0,09	0,34 ± 0,09
S. agalactiae UM035	2,76 ± 0,42	2,01 ± 0,49
S. salivarius UM031	0,46 ± 0,25	0,20 ± 0,09

Each value shown is the mean ± SD from three experiments. * Significantly different values between washing and no washing of the cells with PBS after incubation with bacteria.

The results obtained (Table 3.1) showed that *G. vaginalis* isolates had a higher adherence to epithelial cells when compared with other bacteria isolated from a vaginal swab. Because, *G. vaginalis* strains were able to adhere to, and form a biofilm on (Swidsinski *et al.,* 2005) women epithelium, it is plausible that it is an initial colonizer that paves the way for additional species with low innate pathogenic potential to become established in the vagina. *G. vaginalis* strains UM034 and UM035 isolated from patients diagnosed with BV showed a greater adherence than *G. vaginalis* UM016 isolated from a healthy patient (Figure 3.6). *G. vaginalis* tended to form clumps of bacteria adhered to cells. This data confirm results of previous studies that have shown that *G. vaginalis* adheres to McCoy cells and human red blood cells (Scott *et al.*, 1989). The adherence of *G. vaginalis* to McCoy cells appeared to be mediated by an outer fibrillar coat while adherence to red cells appeared to be mediated by fimbriae (Scott *et al.*, 1989). Moreover, our results showed that there are differences on the number of bacteria per HeLa cell between each *G. vaginalis* strain isolate, which can be due to the fact that these bacteria were isolated from different Portuguese women. Thus, to understand these differences in

the number of bacteria adhered to epithelial cells it would be necessary to study the genes and proteins expressed in these bacteria, using transcriptomics and proteomics analysis.



G. vaginalis UM016 *G. vaginalis* UM034

G. vaginalis UM035

Figure 3.6 – Adherence of *G. vaginalis* to epithelial cells. Original magnifications: x1000. Bacteria were grown anaerobically in sBHI at 37 ºC for 48 h. Bacteria cultures were standardized to ensure equal numbers, and add to vaginal epithelial cells. After incubation for 30 min, non-adherent bacteria were removed by washing with PBS.

E. faecalis, *G. haemolysans*, *K. pnemoniae*, *P. acnes* and *S. agalactiae* were isolated from women diagnosed with BV. These bacteria showed a lower adhesion to epithelial cells than *G. vaginalis* strains (Table 3.1). However, *S. agalactiae* was the bacteria that had a greater ability to adhere to epithelial cells after the *G. vaginalis.* Studies have concluded that invasive infections caused by *S. agalactiae* are not uncommon and that they pose a major problem not only in pregnant women and neonates but also in non-pregnant adults, especially the elderly and patients with chronic diseases (Brimil *et al.,* 2006; Konto-Ghiorghi *et al.,* 2009). In this case, this *S. agalactiae* strain was isolated from a non-pregnant woman, but with a chronic disease (not disclosed).

Most bacterial pathogens have long filamentous structures known as pili or fimbriae, which are often involved in the initial adhesion of bacteria to host tissues but also in bacteria – bacteria interactions, resulting in biofilm formation. *S. agalactiae* showed to encode a pilin. One of the accessory pilins is responsible for the adhesive property of the pilus (Konto-Ghiorghi *et al.,* 2009). As a result, studies reported that *S. agalactiae* has ability to adhere to intestinal human epithelial cells (Konto-Ghiorghi *et al.,* 2009). Also, other researches showed that *K. pnemoniae* has capability to adhere to VEC (Osset et *al.,* 2001) and that *E. faecalis* has ability to adhere to Caco-2 and to urinary tract epithelial cells (Pruzzo *et al.,* 2002). Nevertheless, we were not found studies of adherence ability of the *G. haemolysans* and *P. acnes* to VEC, but these bacteria were recently isolated from vaginal samples (Verhelst *et al.*, 2005).

B. breve, *L. gasseri*, *S. epidermidis* and *S. salivarius* were isolated from healthy Portuguese women. These bacteria showed a lower adhesion to epithelial cells (Table 3.1). Studies reported that *B. breve* has ability to adhere to human enterocyte-like Caco-2 cells (Bernet *et al.*, 1993). Also, Atassi and their coworkers (2006) showed the ability of *L. gasseri* isolated from vaginal samples to adhere to HeLa cells. Although *S. epidermidis* adhere to skin epithelial cells (Vuong *et al*., 2004), some studies reported that it can also be present in vaginal microflora (John *et al.*, 2003; Verhelst *et al*., 2005). So, *S. epidermidis* usually has a benign relationship with its host (Wang *et al*., 2007). Finally, Weerkamp and McBride (1980) showed that *S. salivarius* have ability to adhere to human buccal epithelial cells, but this bacterium is also present in female genitourinary tract (Conte *et al*., 2006).

Summarizing, these assays revealed that *G. vaginalis* isolates had a stronger initial adhesion capability than the other isolates recovered, the weakest initial adhesion being observed with *P. acnes.* The majority of the infective microorganisms involved in the pathogenesis of urinary infection have their origin in the intestinal tract (Osset et *al.*, 2001). In women, these fecal microorganisms successively colonize the perineum, the vagina, and the periurethral and ascend until they reach the bladder (Cribby *et al*., 2008; Osset et *al.*, 2001). In this sense, over 50 microbial species have been recovered from the vaginal tract (Cribby *et al*., 2008; Oakley *et al.,* 2008). Thus, the microbiota of the lower female genital tract is a dynamic, complex example of microbial colonization, the regulation of which is not fully understood.

CHAPTER 4

Assessment of adhesion competition of vaginal microorganisms to epithelial cells

4.1 Introduction

Lactobacillus adhesion to vaginal epithelium is a critical step in the colonization and is suggested as one of the mechanisms by which they could protect the vagina from pathogens (Atassi *et al*., 2006). This can occur by their competition for the surface receptors of the genitourinary epithelium (Boris and Barbes, 2000). Bacterial adhesion to mucosal surfaces includes interactions that take place between specific adhesins of the bacterial surface and receptors of host tissues (Otero and Nader-Macías, 2007).

Recent studies have shown that there are limitations in the use of antibiotic in BV treatment, especially during pregnancy and in their inability to remove biofilms (Saunders *et al*., 2007). In this sense, it is important that alternative therapies, as probiotic applications can be considered in BV treatment. The term ‘probiotic’ is defined as a viable mono – or – mixed culture of microorganisms, applied to animals or humans, which beneficially affects the host by improving the properties of the indigenous microbial communities (Kaewsrichan *et al.*, 2006).

The regulatory roles attributed to lactobacilli in the vaginal microbiota have attracted interest because of potential therapeutic applications. The ability of lactobacilli to produce antimicrobial compounds, such as lactic acid, H_2O_2 and bacteriocin-like substances, can result in inhibition of growth of the pathogens (Atassi *et al.*, 2006; Botis *et al.*, 1998). Antonio *et al*. (2005) reported that *Lactobacillus* strains isolated from the vaginas of healthy women develop antagonistic activities against vaginosis-associated *G. vaginalis*. Therefore, it is important to study the ability of lactobacilli to inhibit the adhesion of uropathogens to epithelial cells, in order to obtain probiotics for BV treatment. However, little is known about the mechanisms by which lactobacilli adhere to VEC, although the variety of surface structures in these bacteria implies that a spectrum of adherence mechanisms may exist (Boris *et al*., 1998; Boris and Barbes, 2000) as described in chapter 3.

4.1.1 Aim

The aim of the work described in this chapter is to assess the ability of three distinct *Lactobacillus* strains to compete against *G. vaginalis* adhering to human cervix

epithelial cells. The purpose of this study is to investigate the differences of virulence of commensal and pathogenic *G. vaginalis* in the presence of lactobacilli. In this sense, competition adhesion assays will be performed in order to determine the inhibitory effect of different *Lactobacillus* strains on the adhesion of *G. vaginalis* strains to HeLa cells.

4.2 Materials and Methods

4.2.1 Strains and culture conditions

The 4 vaginal microorganisms, used in this study, were isolated from the vagina flora of healthy women and BV-diagnosed women. Microorganisms isolated were: *G. vaginalis* strains 5-1 and 101 (American isolates) and UM016 and UM035 (Portuguese isolates); the source of the strains is listed in Table 2.1. In addition to the microorganisms isolated from vaginal swab, some reference *Lactobacillus* spp. strains were also included, namely *L. casei* CECT 5275, *L. iners* ATCC 55195 and *L. crispatus* VCUVAHMP00053. All isolates except *L. casei* were cultured in sBHI broth (see section 2.3.1). *L. casei* was cultured in MRS broth (see section 2.3.1). All cultures were grown at 37 ºC, during 48 h under anaerobic conditions, using the Anaerogen pack system (Oxoid). All isolates were grown in appropriate media and were collected by centrifugation at 7 000 rpm for 10 min. The culture medium was discarded, and the bacteria were washed once with PBS, composed by 16 $g.L^{-1}$ of NaCl; 0,4 $g.L^{-1}$ of KCl; 1,62 $g.L^{-1}$ of $Na_2HPO_4^-.2H_2O$ and 0,4 $g.L^{-1}$ of KH_2PO_4). After this, bacterial suspension was calibrated for appropriate optical density, using a 96-well tissue culture plates (Orange Scientific) by ELISA 96-well plate reader (Tecan Sunrise) with a 600 nm filter. The absorbance value was adjusted in accordance with the calibration curve (Figure 2.1) of each microorganism. Thereafter, bacterial suspension was centrifuged again and the absorbance value was again confirmed. The PBS was discarded, and the bacteria were resuspended in D-MEM medium. Finally, bacterial suspensions were prepared for epithelial cells' infection.

4.2.2 Cell line and culture conditions

Human cervical HeLa cells were cultured at 37ºC, in 5% CO_2 (Shel Lab CO_2 series incubator) in D-MEM (Sigma-Aldrich) supplemented with 15 % FBS (Sigma-Aldrich) and 1 IU penicillin/streptomycin mL^{-1} (Sigma-Aldrich). After being slowly defrosted, cells were added to a falcon tube containing 5 mL of medium and were

centrifuged for 6 min at 1000 rpm. The pellet was resuspended in 2 mL of medium and then the suspension was added to a T-flask (25 cm^2) containing 3 mL of fresh medium. The flask was maintained in a CO_2 incubator (Shel Lab CO_2 series incubator) at 37 ºC until 80 % of confluence was obtained. At this point, the medium was removed and the cells were washed once with 2 mL of PBS (Sigma-Aldrich). After discarding the PBS, 1 mL of trypsin (Sigma-Aldrich) was added and the cells were kept for 10 minutes at 37 ºC until they were detached from the flask. To stop the trypsin activity 3 mL of D-MEM medium were added to the flask. Cells were enumerated in a Neubauer chamber and were then diluted in 5 mL of medium to 1×10^6 cells.mL^{-1}. The new flasks were incubated in 5 % of CO_2 and 37 ºC. The trypsinization was repeated to prepare new flasks to maintain the cells, or to prepare cells for adhesion assays. In the latter, after detachment, 2×10^5 cells.mL^{-1} were added to a 24-well tissue culture plates (Orange Scientific) containing circular glass lamellas (12 mm Ø, thickness 0,13 – 0,16 mm, Marienfeld, Germany) in the bottom. The well plate was incubated for 48 h or until a monolayer of cells has been formed. After reaching confluence, the growth medium of epithelial cells was discharged and circular glass were washed one time with 500 µL of PBS (Sigma-Aldrich) and were ready to be used in the adhesion assays.

4.2.3 Adhesion assay

To perform the competition adhesion assay, 300 µL of the microbial cells with 2 different concentrations: high level of bacteria - 10^9 CFU.mL^{-1} and low level of bacteria - 10^3 CFU.mL^{-1} and their controls (Figure 4.1) were added together in a ratio 1:1 (i.e., 150 µL of lactobacilli and 150 µL of *G. vaginalis*) to each well of 24-well plate, which had a circular glass lamella with a monolayer of HeLa cells in the bottom. After 30 minutes of incubation of bacteria (at 37 ºC, in 5 % CO_2), each well of 24-well plate was washed once with 500 µL of PBS (Sigma-Aldrich). This adhesion time was previously optimized (see chapter 3). After this, the circular glass lamellas were removed from the 24-well plate, and transferred to a surface coated with absorbent paper.

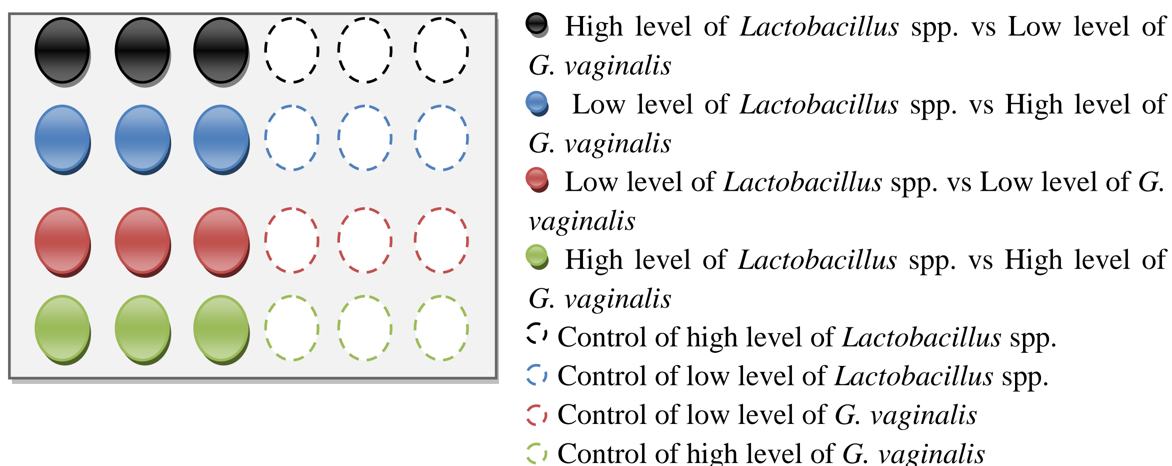


Figure 4.1 – Representative diagram of a competition adhesion assay. In the diagram are showed various combinations of concentrations of lactobacilli and *G. vaginalis* incubated in a 24-well plate. A low level of concentration of microbial cells suspension corresponds to 10^3 CFU.mL^{-1} and a high level of concentration of microbial cells suspension corresponds to 10^9 CFU.mL^{-1}.

Fixing bacteria and epithelial cells

For fixing the bacteria and epithelial cells to the circular glass lamellas, 45 µL of methanol 100 % were added and lamellas were left at room temperature for 30 minutes. After this, 45 µL of paraformaldehyde 4 % was added and lamellas were left at room temperature for 10 minutes. Then, the excess of paraformaldehyde 4 % was removed with the help of tweezers. Finally, 45 µL of ethanol 50 % was added to each lamella and these were left at room temperature overnight or until dry. After this, the circular glass lamellas were placed in Petri dishes previously covered with aluminum and with absorbent paper inside (Cerqueira *et al.,* 2008).

PNA FISH (Peptide nucleic acid fluorescence in situ hybridization)

After performing the fixing of bacteria and epithelial cells to circular glass lamella, 10 µL of the PNA probe (Gard162 Probe) was added (Machado *et al.*, submitted), in order to label *G. vaginalis.* Then, another slide (22×22 mm, JMGS) was placed over the circular glass lamella and both were placed in Petri dishes, previously covered with aluminum and with absorbent paper inside. Hybridization was performed in the oven at 60 °C (temperature more suitable for the probe used) for 90 minutes. At the same time, 5 mL of a washing solution (Table 4.1) were added to each well of the 6-well

plate. The washing solution was prepared the day before the experience. After the incubation period, the slide that was placed over the circular glass lamella was removed from de Petri dishe. After this, the circular glass lamellas were placed in the washing solution in a 6-well plate and left at 60 ºC for 30 minutes. Then, the circular glass lamellas were removed from 6-well plate, and were left to air-dry, in the dark-inside the incubator for 10 minutes. This procedure was performed in the dark.

Table 4.1 – Composition of the washing solution

Compounds of washing solution	Quantity for 500 mL
5 mM Tris Base	0,303 g
15 mM NaCl	0,438 g
1% (V/V) Triton-x	500 µL

Cell enumeration

The circular glass lamellas were taken to the microscope room, and 20 µL, referring to a concentration of 2,5 $\mu g.mL^{-1}$, of blue-fluorescent DAPI nucleic acid stain was added immediately before adherence visualization, using fluorescence microscope (Olympus BX51), using DAPI filter ($\lambda_{excitation}$ = 365-370 nm) and a magnification of 1000 times. The images were captured with a video camera that was coupled to the microscope and connected to a computer, using *Cell – Imaging Software for Life, Sciences Microscopy*. Twenty fields were randomly counted in each sample. Thereafter, it was counted the number of bacteria adhered to epithelial cells, using the *ImageJ Software*. Eukaryotic cells were also counted per field of view. All adhesion assays were quantified by fluorescence microscopy, using DAPI for total cell count and PNA-FISH probe for *G. vaginalis* quantification (using TRICT filter, $\lambda_{excitation}$ = 530-550 nm). Results for the four conditions of concentrations were expressed as the average number of *G. vaginalis* and *Lactobacillus* spp. per HeLa cell. The number of *G. vaginalis* was compared with respective control values (i.e. microbial cells without lactobacilli) and the number of *Lactobacillus* spp. was also compared with control values (microbial cells without *G. vaginalis*). The control values were taken as 100 % of adhesion and the inhibition of *G. vaginalis* and *Lactobacillus* spp. adherence was calculated by subtracting each adhesion

percentage from their correspondent control value. Adherence assays were repeated three times on separate days, with three fields of view assessed each time.

4.2.4 Statistical analysis

The results are expressed as the mean of three independent experiments. Significant differences between mean values were determined by Tukey's test after analysis of variance (ANOVA) with SPSS (Statistical Package for the Social Sciences – version 18). A *P*-value of 0.05 was considered statistically significant.

4.3 Results and discussion

The vaginal ecosystem harbors a microbiota that is being increasingly recognized as protecting it from invading pathogens. Lactobacilli play a major role in maintaining the urogenital health by preventing the overgrowth and invasion of pathogenic bacteria by competing with other microorganisms for nutrients (Atassi *et al*., 2006; Boris and Barbes, 2000). *G. vaginalis* was used in this study, because it has been described as the most prevalent pathogenic bacteria in patients diagnosed with BV (Harwich, 2010; Patterson *et al.*, 2010). Nevertheless, frequent isolation of this species from seemingly healthy women has cast doubt on this claim. Recent studies of the biofilm-forming potential and cytotoxic activity of *G. vaginalis* have renewed interest in the virulence potential of this microorganism (Patterson *et al.*, 2007; Patterson *et al.*, 2010). In this sense, we designed an adhesion assay to analyze the competition between lactobacilli and *G. vaginalis* (commensal and pathogenic isolates) for initial adhesion to epithelial cells.

4.3.1 Competition between lactobacilli and American isolates of *G. vaginalis* for initial adhesion to epithelial cells

Our study aims to understand the differences between *G. vaginalis* present in healthy women and in women with BV, based on the analyzing of the effect of lactobacilli on the attachment of *G. vaginalis* to HeLa cells, under conditions of competition. For this, four different concentration of *Lactobacillus* spp. mixed with *G. vaginalis* were used to study the ability of lactobacilli to block the adherence of *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) to HeLa cells by

competition, i.e. lactobacilli and *G. vaginalis* were incubated together in a monolayer of HeLa cells.

In order to understand the interaction between lactobacilli and *G. vaginalis* under competition conditions we tested the situation that would mimic a healthy vaginal microflora. In this sense, a high concentration of lactobacilli (10^9 CFU.mL^{-1}) and a low concentration of *G. vaginalis* (10^3 CFU.mL^{-1}) were used. The concentration of lactobacilli used, in this case, represents a normal situation in a vaginal flora constituted by 10^7-10^8 CFU of lactobacilli per gram of fluid (Osset *et al*., 2001). Moreover, in a healthy vaginal microflora there is a low concentration of anaerobes, as *G. vaginalis*, so the low level of *G. vaginalis* was used for mimic this situation. Results of adherence of high level of *Lactobacillus* spp. and low level of *G. vaginalis* strains to epithelial cells are showed in Figure 4.2.

According to Fredricks and their coworkers (2005), *L. iners* was the only lactobacilli in patients with BV according to the Amsel criteria. The fastidious *L. iners* may exhibit specialized adaptation mechanisms to the vaginal environment (Macklaim *et al.,* 2011). The detection of *L. iners* in women with and recovering from BV has led to the suggestion that is not protective against disease. However, unlike other microorganisms they seem to be easily displaced by pathogens and infectious conditions, the ability of *L. iners* to persist may prove it to be important in recovery of the microbiota, post-disease resolution (Macklaim *et al.,* 2011). Nevertheless, our results showed that there were no significant differences ($p>0.05$) in adherence of *G. vaginalis* strains, comparing with the control value in the presence of *L. iners* (Figure 4.2). Thus, it was necessary to do more experiments in order to understand the competition of between *L. iners* and *G. vaginalis* in adhesion to epithelial cells.

Women with BV have lost many *Lactobacillus* species (except *L. iners*) and acquired a variety of anaerobic and facultative bacteria (Swidsinski *et al*., 2005). The depletion of the normal genital microbial communities, such as *L. crispatus* can be caused by the competition with uropathogens (Antonio and Hillier, 2003). In this sense, it could be expected that *L. crispatus* in the presence of a pathogenic strain would have a greater decrease of adhesion to epithelial cell than in the presence of a non-pathogenic *G. vaginalis*. Nonetheless, this fact was not observed (Figure 4.2). When *L. crispatus* was mixed with *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate)

we observed a greater adherence of *L. crispatus* in presence of *G. vaginalis* 101 in comparison with *G. vaginalis* 5-1 ($p<0.05$). Conversely, no statistical differences were found in the adhesion levels of *L. iners*, when competing with both *G. vaginalis* strains ($p>0.05$).

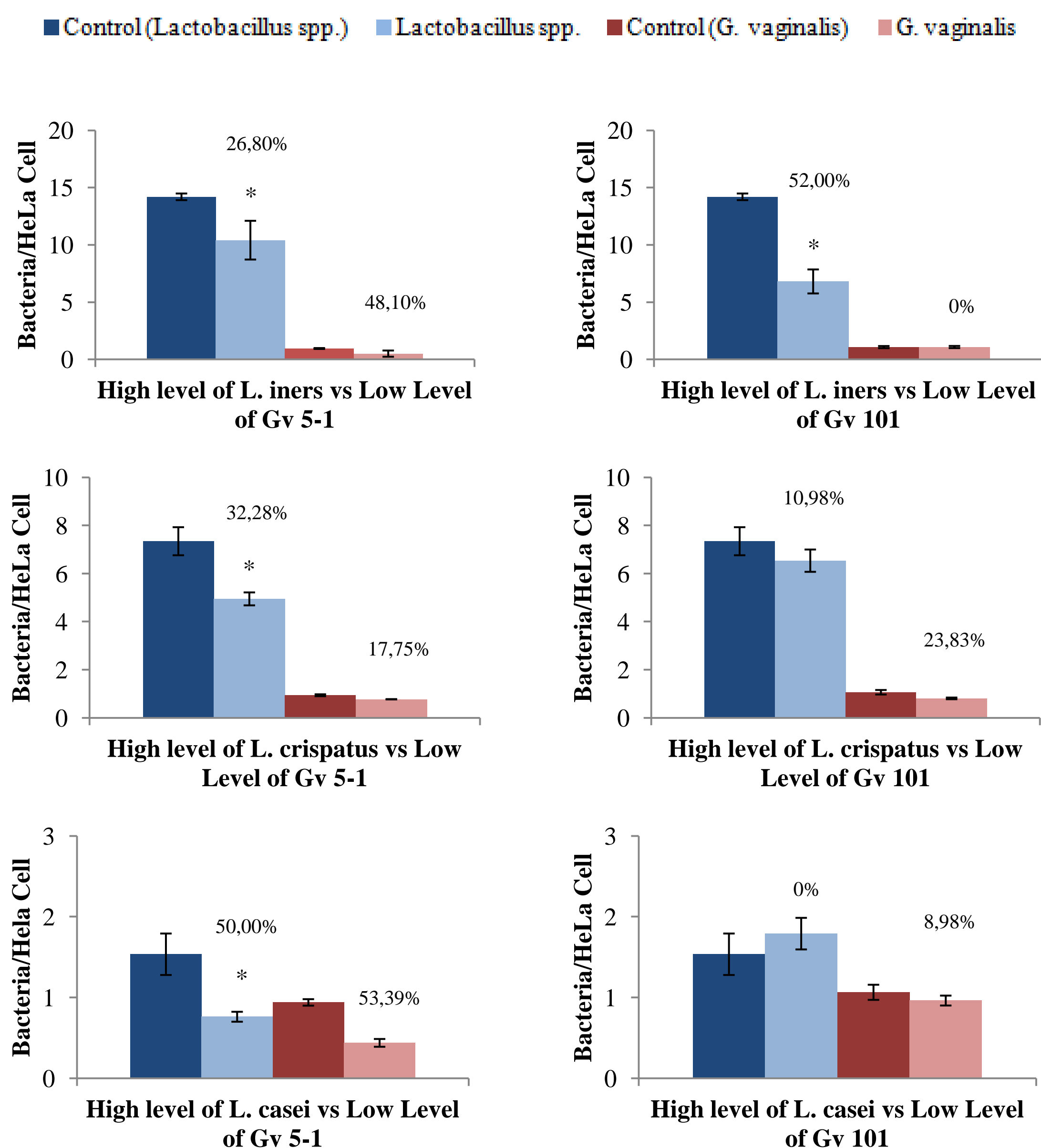


Figure 4.2 – Adhesion average of *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) to epithelial cells at high level of concentration of *Lactobacillus* spp. and low level of concentration of *G. vaginalis* strains, under the condition of competition. The control values were taken as 100 % of adhesion. The inhibition % of bacteria adherence was calculated by subtracting each adhesion percentage from their correspondent control value. *Values that are significantly different from the control value ($P< 0.05$).

To determine if the competition with *G. vaginalis* was specific for vaginal lactobacilli, we also included *L. casei* in all these studies, as a non-sense control, since this lactobacilli strain is not a common colonizer of the vagina epithelium (Ingrassia *et al*., 2005). However, *L. casei* was still able to inhibit adherence of *G. vaginalis* strains to epithelial cells, as represented in Figure 4.2. This was somewhat surprising as there have been some published literature indicating that not all lactobacilli can antagonistically act against *G. vaginalis* (Atassi *et al.*, 2006; Osset *et al.*, 2001).

In order to further test our experimental design, we performed a similar experiment, were we used high concentrations of *G. vaginalis* against low concentrations of lactobacilli, in order to mimic BV. Results of adherence of low level of *Lactobacillus* spp. and high level of *G. vaginalis* strains are showed in Figure 4.3.

In this situation, our results showed that there were significant differences ($p<0.05$) in the adhesion of *G. vaginalis* 101 (pathogenic isolate) to HeLa cells when compared with adhesion of *G. vaginalis* 5-1 (healthy isolate), in the presence of a low concentration of *L. iners* (Figure 4.3). So, the adherence of *G. vaginalis* 101 was greater to epithelial cells than the adherence of *G. vaginalis* 5-1. Researchers reported that *L. iners* is widely present in healthy females as well as those suffering from BV, suggesting that it is an important indigenous species of the vagina (Macklaim *et al.,* 2011; Zozaya-Hinchliffe *et al.,* 2010). Recent quantification of bacterial numbers in these different stages of vaginal health has shown that the abundance of *L. iners* remains relatively constant, despite the fluctuating environmental conditions (Macklaim *et al.,* 2011; Zozaya-Hinchliffe *et al.,* 2010). This remarkable ability to survive under a range of conditions suggests that, rather than *L. iners* being somehow associated with an aberrant microbiota, it may be an important member of the host's defenses by being a persistent lactobacilli involved in restoration and maintenance of the normal microbiota. However, there were no significant differences ($p>0.05$) in the adherence of *L. iners* to epithelial cells, under conditions of competition with *G. vaginalis* strains (non-pathogenic and pathogenic), probably due to the use of a low concentration of this lactobacilli in this experience. Nevertheless, significant differences ($p<0.05$) were found in adherence of *L. iners* in comparison to the control, as represented in Figure 4.3.

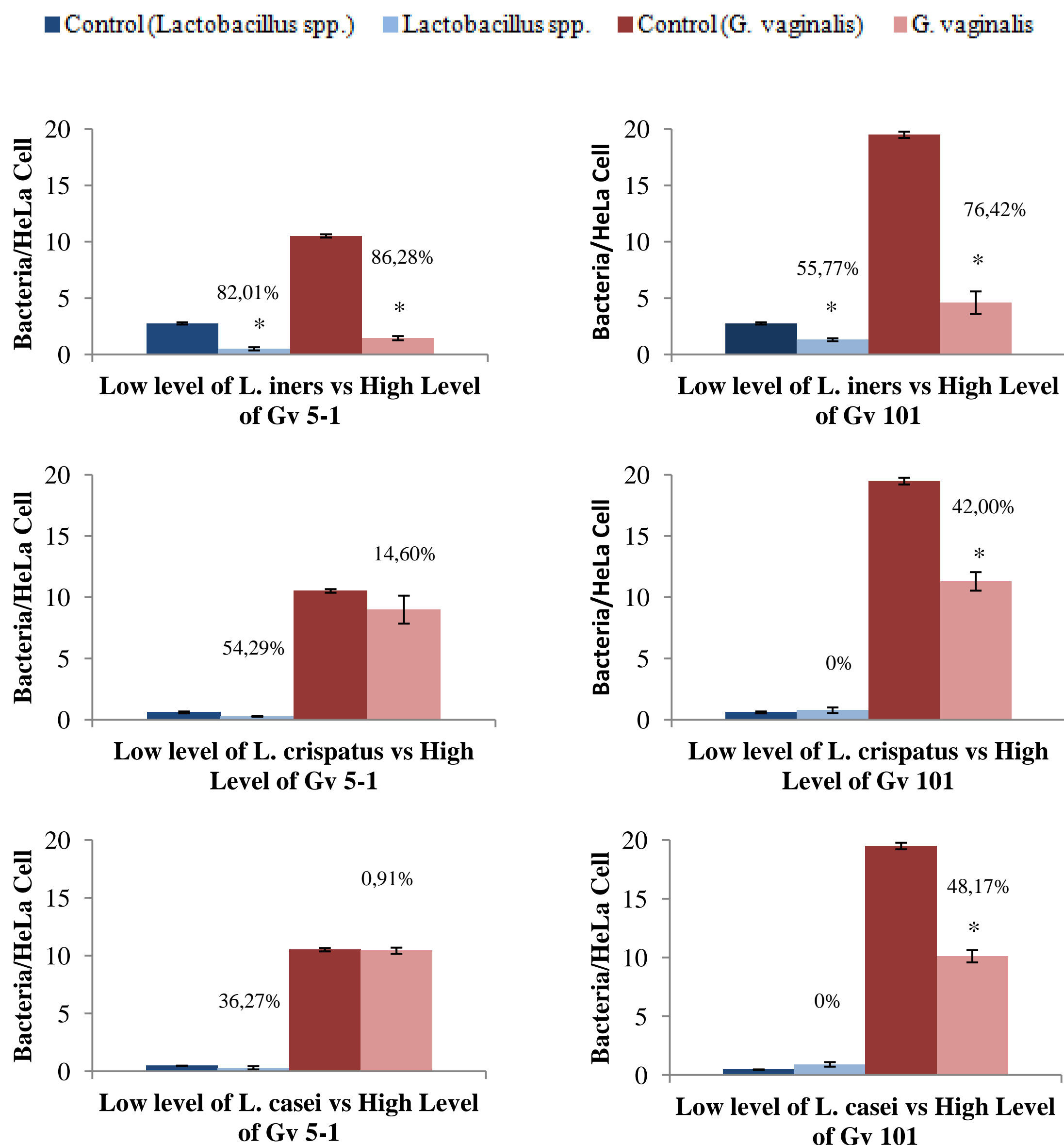


Figure 4.3 – Adhesion average of *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) to epithelial cells at low level of concentration of *Lactobacillus* spp. and high level of concentration of *G. vaginalis* strains, under the condition of competition. The control values were taken as 100 % of adhesion. The inhibition % of bacteria adherence was calculated by subtracting each adhesion percentage from their correspondent control value. *Values that are significantly different from the control value ($P<$ 0.05).

In the case of competition of *L. crispatus* against *G. vaginalis* it was observed that a low concentration of *L. crispatus* cause a greater decrease of adhesion in a pathogenic isolate of *G. vaginalis* 101 than *G. vaginalis* 5-1. *L. crispatus* is under development as a probiotic for the treatment of BV (Antonio and Hillier, 2003). Therefore, these results were not surprising because *L. crispatus* would have a greater decrease of adherence in the presence of a pathogenic strain of *G. vaginalis* (Antonio and Hillier, 2003).

Nevertheless, there were no significant differences ($p>0.05$) in adherence of *L. crispatus* to epithelial cells, under conditions of competition with *G. vaginalis* strains, as represented in Figure 4.3.

A difference experimental setup was performed, in order to simulate the interactions that occur in the woman vaginal epithelium after antimicrobial therapy. The most common treatment for BV is the use of antibiotics, specifically, with oral or vaginal metronidazole or with vaginal clindamycin. However, antibiotics do not eradicate all vaginal pathogenic bacteria. Furthermore, antibiotics can also kill healthy *Lactobacillus* spp. in vaginal flora. This is a problem because healthy vaginal lactobacilli are active against several pathogens including *G. vaginalis* (Dover *et al.,* 2008). After antibiotic therapy, the bacterial load on the vaginal epithelium can be drastically reduced. To simulate this situation, we tested adhesion with low concentration of bacteria.

Our results showed that there were significant differences ($p<0.05$) in the adherence of *L. iners* to epithelial cells in the presence of *G. vaginalis* strains, in comparison to the control (Figure 4.4). In this situation, we found a lower number of *L. iners* adhered to epithelial cells. However, significant differences ($p>0.05$) were not found in adherence of *G. vaginalis* strains in the presence of *L. iners,* compared to the control.

When *L. crispatus* was mixed with *G. vaginalis* 101 (pathogenic isolate), the results showed that there were significant differences ($p<0.05$) in the adhesion of *L. crispatus* and *G. vaginalis* isolated from a BV patient, comparing with the respective control value, as represented in Figure 4.4. Conversely, no differences ($p>0.05$) were found in adherence of *G. vaginalis* 101 in the presence of *L. casei*, compared to the control. Nevertheless, as the number of bacteria adhered to epithelial cells was very low, it is still necessary to do more experiences in order to understand the competitive initial adhesion of lactobacilli against *G. vaginalis* to epithelial cells.

Thus, our results (Figure 4.4) showed that some significant differences ($p<0.05$) were only found in adherence of lactobacilli and of *G. vaginalis* to epithelial cells, comparing to the control value, as described above. So, significant differences ($p>0.05$) were not found in the adherence of lactobacilli under competition conditions, in this

experimental set-up. The same was found between *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) when these bacteria were mixed with lactobacilli. Therefore, as the number of bacteria adhered to the epithelial cells was very low, the method that was used might not be sensitive enough to detect the possible antagonistic effect between *Lactobacilli* spp. and *G. vaginalis*.

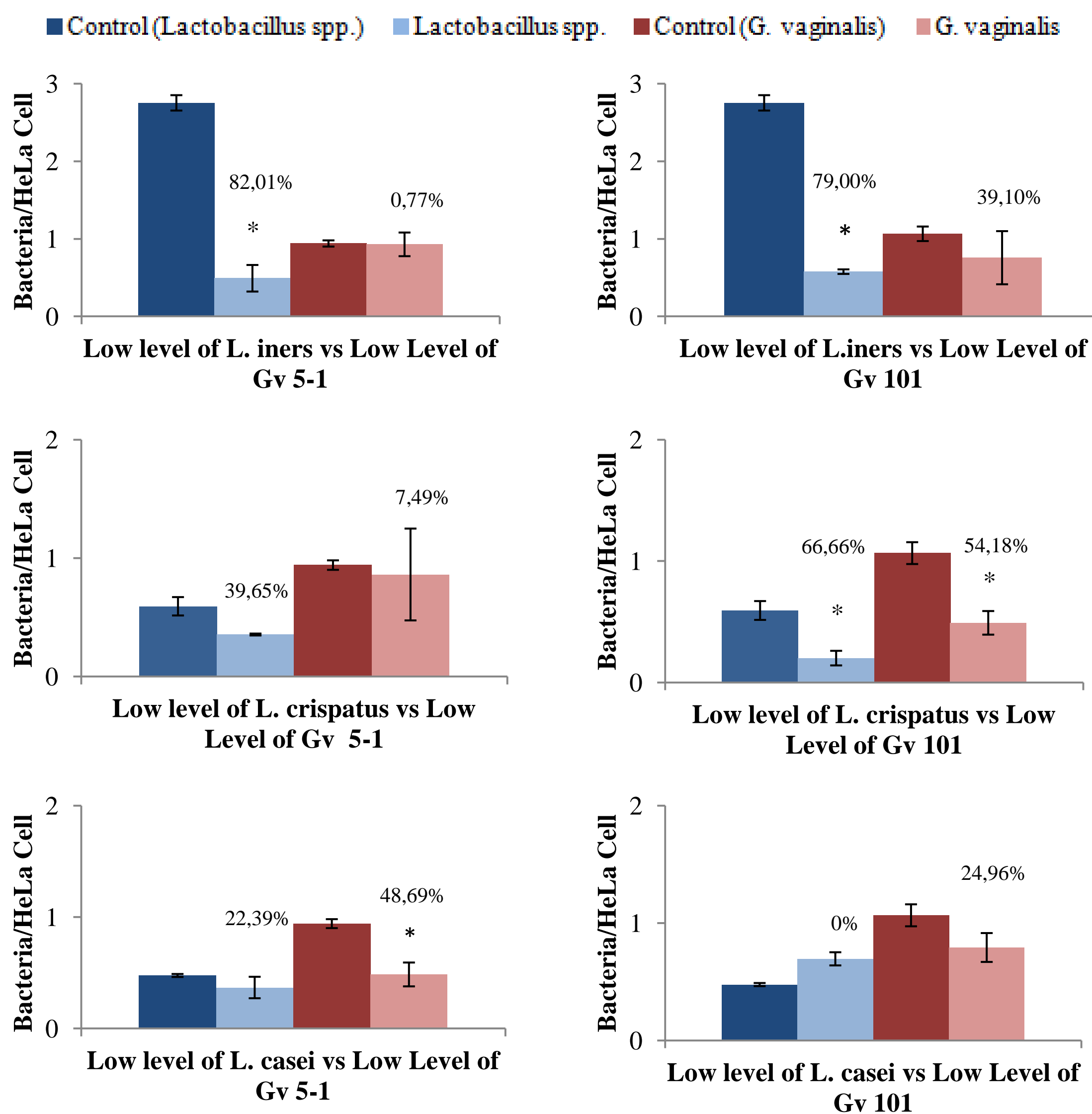


Figure 4.4 – Adhesion average of *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) to epithelial cells at low level of concentration of *Lactobacillus* spp. and low level of concentration of *G. vaginalis* strains, under the condition of competition. The control values were taken as 100 % of adhesion. The inhibition % of bacteria adherence was calculated by subtracting each adhesion percentage from their correspondent control value. *Values that are significantly different from the control value ($P<0.05$).

To overcome the limitation of the experimental technique used, we decided to saturate the system the use of low concentrations of bacteria. So, we performed a final experiment, where we used a high concentration of both species (Figure 4.5).

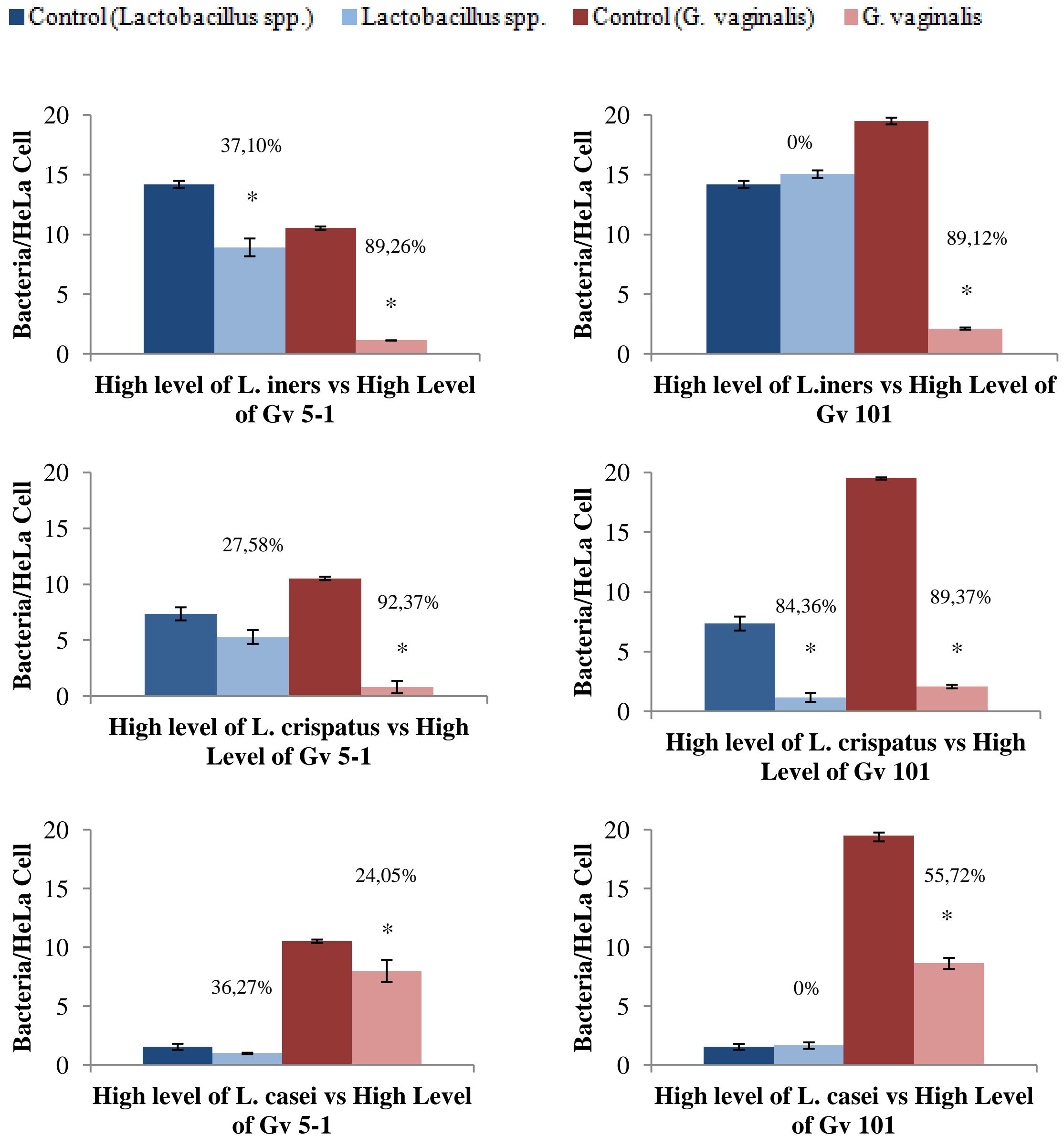


Figure 4.5 – Adhesion average of *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) to epithelial cells at high level of concentration of *Lactobacillus* spp. and high level of concentration of *G. vaginalis* strains, under the condition of competition. The control values were taken as 100 % of adhesion. The inhibition % of bacteria adherence was calculated by subtracting each adhesion percentage from their correspondent control value. *Values that are significantly different from the control value (P<0.05).

The results from this last experimental set-up showed that there were significant differences (p<0.05) in the adherence of *L. iners* to epithelial cells against *G. vaginalis* 5-

1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate) (Figure 4.5). While there was no inhibition of *L. iners* in the presence of *G. vaginalis* 101, in the presence of *G. vaginalis* 5-1 *L. iners* had 37,1 % lower adhesion levels, as compared to the control. Conversely, when *G. vaginalis* 101 was used with *L. iners*, it was found that *G. vaginalis* 101 did not have ability to decrease the adherence of *L. iners* to epithelial cells. In the other hand, *G. vaginalis* is greatly affected by the adherence of *L. iners* to epithelial cells. The inhibition of *G. vaginalis* strains was about 89 % compared to the respective control in the presence of *L. iners.* These results validate the studies of Saunders and their coworkers (2007) that showed that *L. iners* was able to reduce the viability of *G. vaginalis*. *L. iners* may exhibit specialized adaptation mechanisms to the vaginal environment. The cause of a greater adherence of *L. iners* to epithelial cells can be due to *L. iners* genome that encodes an open reading frame with significant primary sequence similarity to VLY (68,4 % similarity), the CDC from *G. vaginalis* (Macklaim *et al*., 2011; Rampersaud *et al.,* 2011). These proteins are typically found in pathogenic bacteria but are not found in other lactobacilli. However, further analysis of the functional characteristics associated with the *L. iners* genome would uncover desirable characteristics of a microbe that could contribute to the maintenance, and potentially restoration, of a healthy and stable vaginal microbiota (Macklaim *et al*., 2011; Rampersaud *et al.,* 2011).

In addition, our results showed that there were significant differences ($p<0.05$) in adherence of *L. crispatus* to HeLa cells against *G. vaginalis* 5-1 (healthy isolate) and *G. vaginalis* 101 (pathogenic isolate). While the inhibition of *L. crispatus* was 27,58 % compared to control in the presence of *G. vaginalis* 5-1, the inhibition of *L. crispatus* was 84,36 % compared to control in the presence of *G. vaginalis* 101, as represented in Figure 4.5. This result validates that a depletion of the normal genital microbial communities, especially of lactobacilli can be caused by the competition with uropathogens (Antonio and Hillier, 2003). Therefore, the results revealed that a large reduction in adherence of *G. vaginalis* was observed when these bacteria were added together with *L. crispatus* to epithelial cells. When indigenous lactobacilli compete against pathogenic bacteria, it could be possibly that lactobacilli exclude the colonization of pathogenic bacteria by occupying (by steric hindrance) their potential binding sites in the mucosa (Kaewsrichan *et al.,* 2006; Zárate and Nader-Macias). Our findings are consistent with previous reports of Atassi and their coworkers (2006) that reported that *Lactobacillus* strains isolated from

the vaginas of healthy women develop antagonistic activities against vaginosis-associated *G. vaginalis.* Moreover, mechanism by which *Lactobacillus* spp. antagonizes *G. vaginalis*, showing that the killing activity results from antimicrobial compounds producing (H_2O_2, lactic acid and bacteriocins) by these lactobacilli. However, not all lactobacilli express these properties with the same intensity; on the contrary, there are important differences among strains (Osset *et al.,* 2001). In this sense, studies of production of antimicrobial substances by lactobacilli could be necessary in order to assess the capacity of *Lactobacillus* spp. to inhibit the growth of uropathogens and block their adhesion to VEC (Osset *et al.,* 2001; Saunders *et al.,* 2007).

This work clearly demonstrates differences in competitive initial adhesion between lactobacilli and *G. vaginalis* strains against epithelial cells that could impact the ability of *G. vaginalis* to cause disease. However, the limitation of this present study is the restricted number of *G. vaginalis* strains studied. In this sense, the precise role for *G. vaginalis* in BV pathogenesis is still unclear. Further studies of other virulence properties, such as cytotoxicity of *G. vaginalis* strains and their ability to form biofilm are required to understand their role in BV.

4.3.2 Competitive between lactobacilli and Portuguese isolates of *G. vaginalis* for initial adhesion to epithelial cells

To see the impact of *G. vaginalis* strains recently isolated from Portuguese women, the competitive adhesion assays were performed in order to confirm the results described in the previous section. In this sense, studies were performed using a high concentration of lactobacilli and a high concentration of *G. vaginalis*, for Portuguese isolates of *G. vaginalis*.

Our results showed that there were significant differences ($p<0.05$) in adherence of *L. iners* to epithelial cells against *G. vaginalis* UM016 (healthy isolate) and *G. vaginalis* UM035 (pathogenic isolate) (Figure 4.6). While the inhibition of *L. iners* was 1,02 % compared to control in the presence of *G. vaginalis* UM035, the inhibition of *L. iners* was 58,34 % compared to control in the presence of *G. vaginalis* UM016, as represented in Figure 4.6. Furthermore, our results showed that the adherence of *G.*

vaginalis strains to epithelial cells was greatly affected when we used of *L. iners.* Nevertheless, the adherence of *G. vaginalis* UM035 was greater comparing with *G. vaginalis* UM016.

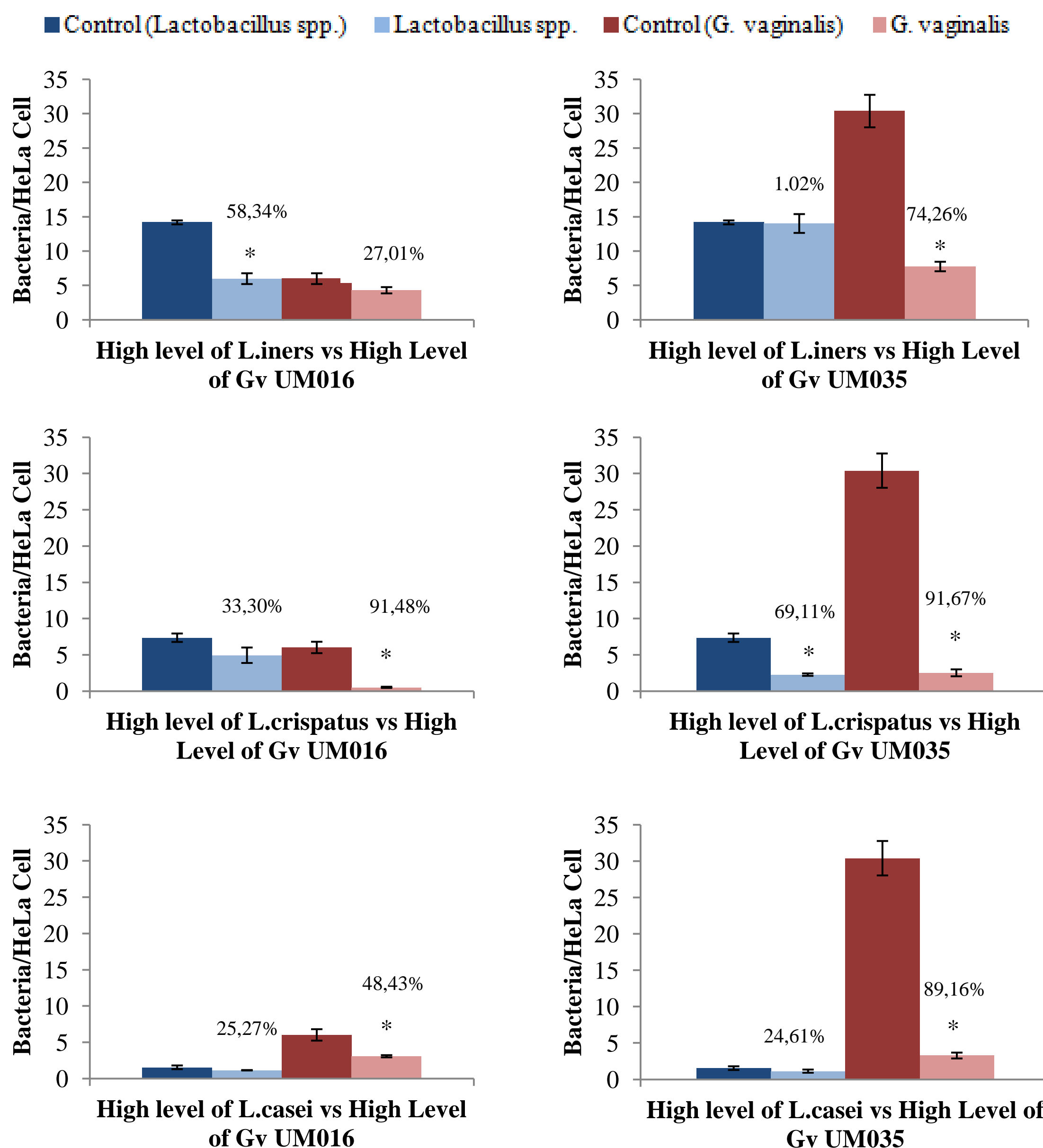


Figure 4.6 – Adhesion average of *G. vaginalis* UM016 (healthy isolate) and *G. vaginalis* UM035 (pathogenic isolate) to epithelial cells at high level of concentration of *Lactobacillus* spp. and high level of concentration of *G. vaginalis* strains, under the condition of competition. The control values were taken as 100 % of adhesion. The inhibition % of bacteria adherence was calculated by subtracting each adhesion percentage from their correspondent control value. *Values that are significantly different from the control value ($P < 0.05$).

Significant differences ($p<0.05$) were found in adherence of *L. crispatus* to HeLa cells against *G. vaginalis* UM016 (healthy isolate) and *G. vaginalis* UM035 (pathogenic

isolate). While the inhibition of *L. crispatus* was 33,3 %, compared to the control, in the presence of *G. vaginalis* UM016, the inhibition of *L. crispatus* was 69,11 %, compared to the control, in the presence of *G. vaginalis* UM035, as represented in Figure 4.6. Therefore, the results revealed that a large reduction in adherence of *G. vaginalis* was observed when these bacteria were added together with *L. crispatus* to epithelial cells. The degree of competition is determined by the affinity of adhesion on the respective bacterial surfaces for the specific receptors that they are competing for; or their relative positions in the case of steric hindrance (Lee *et al.*, 2003). The results suggest that *L. crispatus* used in the present study could prevent colonization of the urogenital tract by relevant pathogens such as *G. vaginalis* through interference mechanisms (competition).

These results support the results obtained previously (see Figure 4.5) with American isolates of *G. vaginalis* strains. So, the vaginal lactobacilli interfered to different extents with the adherence of genitourinary pathogens.

The evaluation of a greater number of uropathogenic strains should be needed in order to confirm the higher virulence potential of *G. vaginalis* isolated from BV. Finally, the data suggest a probiotic potential of these lactobacilli, mainly *L. crispatus*, as anti-infective agents in the vagina and encourage further *in vivo* studies, such as clinical trials designed to test their capacity to prevent and manage urogenital tract infections in females (Zárate and Nader-Macias, 2006).

CHAPTER 5

Conclusions and suggestions for future work

The main goal of this dissertation was to understand the role of *G. vaginalis* in the aetiology of Bacterial Vaginosis (BV). To achieve this, we compared the ability of vaginal isolates from patients with BV and healthy women to adhere to epithelial HeLa cells. The role of lactobacilli in the inhibition of adhesion to epithelial cells was also tested using commensal or pathogenic *Gardnerella vaginalis*.

The main conclusions drawn from the results are that *G. vaginalis* isolates had a stronger initial adhesion capability than the other isolates recovered, the weakest initial adhesion being observed with *P. acnes.* Moreover, *G. vaginalis* strains isolated from BV patients had stronger initial adhesion ability than the *G. vaginalis* isolated from healthy women. It was concluded that adherence of the *G. vaginalis* to epithelial cells is a critical step during the stage of vaginal colonization.

After analyzing the influence of lactobacilli in the adhesion of commensal and pathogenic *G. vaginalis* to epithelial cells, we concluded that the lactobacilli used in this study, mainly *L. crispatus*, might protect the vaginal epithelium through interference mechanisms, specially in the presence of the pathogenic *G. vaginalis* strains. Furthermore, *L. iners* did not decrease the ability to adhere to epithelial cells in the presence of *G. vaginalis* isolated from BV patients. Thus, *L. iners* seems to be somehow associated with an aberrant microbiota.

The first consideration about future work is the suggestion to use more clinical strains of *G. vaginalis* isolated from a healthy patients and women with BV. A larger array of samples would help confirming the results and understanding strain variations. Another point to consider is the use of cell lines related with vaginal epithelium, as ME-180 epithelial cells, or primary explants, in order to mimic more closely the *in vivo* conditions. Other consideration about future work is the proposal to study the ability of vaginal lactobacilli, from Portuguese vaginal flora, to adhere to vaginal epitelial cells (VEC) in competition with *G. vaginalis* strains (non-pathogenic and pathogenic). Also, the selection of anaerobic lactobacilli which produce inhibitory compounds against vaginal pathogens could be interesting to obtain probiotics, in order to restore the normal microbial communities in the vaginal ecosystem. Thus, studies of production of antimicrobial substances by lactobacilli could be necessary in order to assess the capacity of *Lactobacillus* spp. to inhibit the growth of uropathogens and block their adhesion to

VEC. Also very interesting would be to characterize the differences in gene and protein expression between isolates of *G. vaginalis* from healthy women *vs* women with BV. This characterization could be based on transcriptomic and proteomic analysis of these isolates and virulence studies. The genomic and proteomic characterization of *G. vaginalis* strains would clarify the presence of certain virulence factors. Moreover, further studies of other virulence properties, such as cytotoxicity of *G. vaginalis* strains and their ability to form biofilms should be performed to understand their role in BV. Possibly, from the results obtained it would be possible to design new therapies for this infection, so common in women of childbearing age.

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APPENDIX

Appendix A – Koch's postulate

Table A.1 – Koch's postulates (Srinivasan and Fredricks, 2008)

- The etiologic microbe should be found in every case of disease
- The etiologic microbe should not be found in subjects without disease (specificity)
- The etiologic microbe should be isolated in pure culture on lifeless media and be capable of causing the characteristic disease anew upon inoculation in a susceptible host
- The etiologic microbe should be reisolated from the experimentally inoculated host.

Appendix B – Kinetics of HeLa cells growth

As a growth curve of cells allows the identification of the growth phase in which the cells are in the function of the incubation time, the growth curve of the HeLa cells was performed as represented in Figure B.1

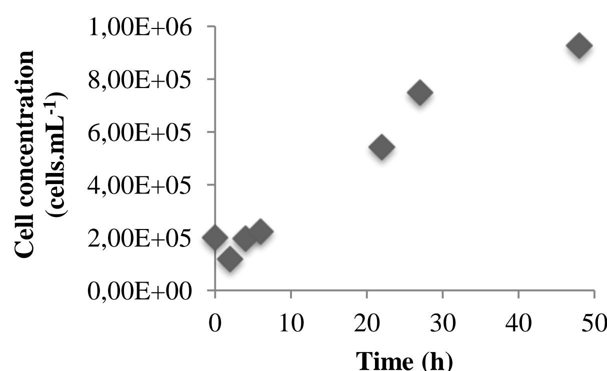


Figure B.1 – Growth curve of Hela cells.

Through the data of growth curve (Figure B.1) were calculated the specific growth rate, the doubling time and the number of the generations of HeLa cells. These data are showed in Table B.1.

Table B.1 – Values of the specific growth rate, of the doubling time and the number of generations of the HeLa cells.

Specific growth rate (h^{-1})	Doubling time (h)	Number of cell generations
0,0576	12,03	3,99

Appendix C – Competition between lactobacilli and American isolates of *G. vaginalis* (5-1 and 101) for initial adhesion to epithelial cells

While figures obtained in the adhesion assay are not to representative, since they represent only a field of view of circular glass lamella, figures of competition between lactobacilli and *G. vaginalis* strains 5-1 (non-pathogenic) and 101 (pathogenic), both with high levels of concentration (10^9 CFU. mL) are showed below.

In Figure C.1 is showed the adherence of high level of *L. iners vs* high level of *G. vaginalis* strains to epithelial HeLa cells. It is noted a greater adherence of *L. iners* in presence of *G. vaginalis* 101.

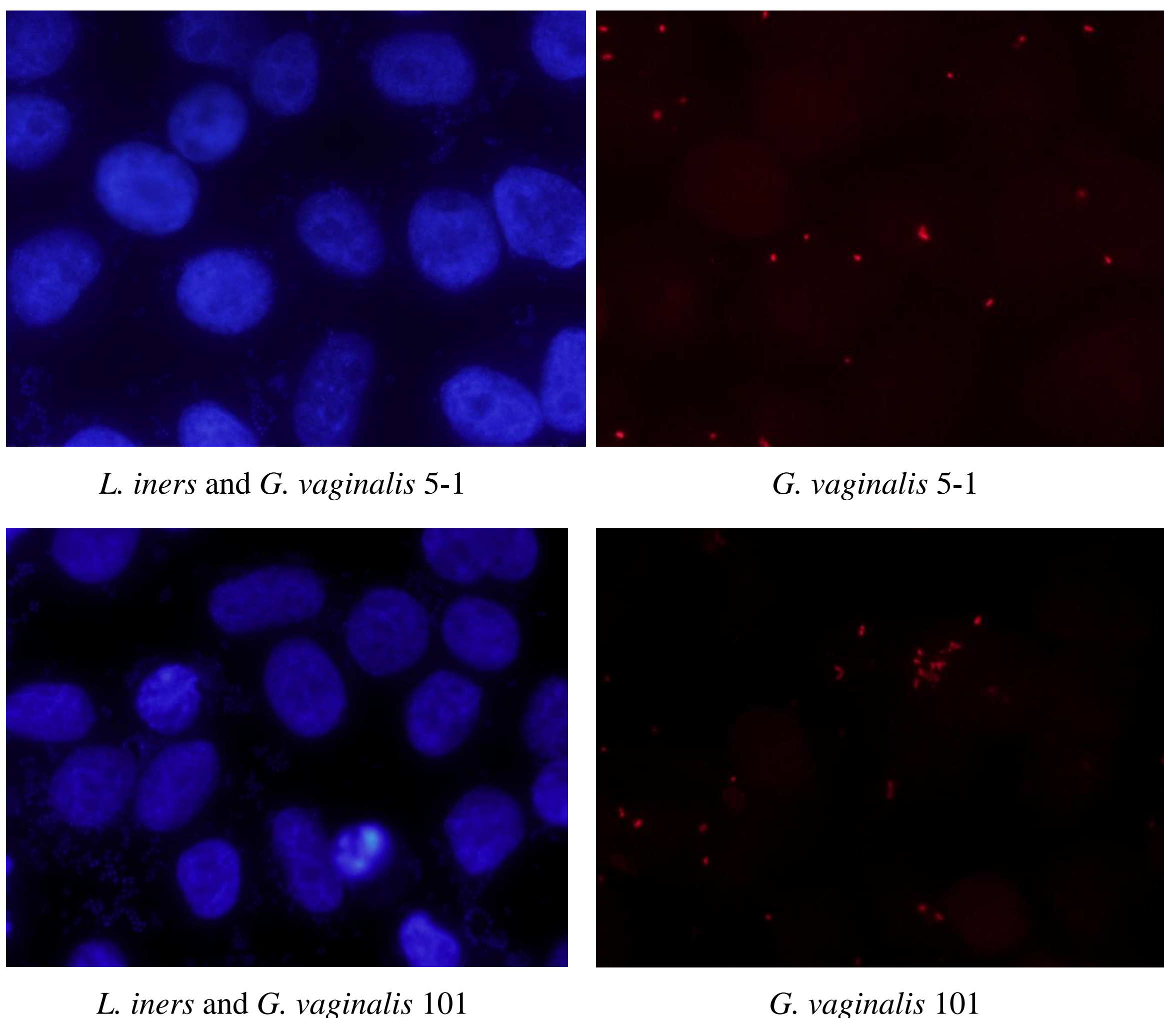
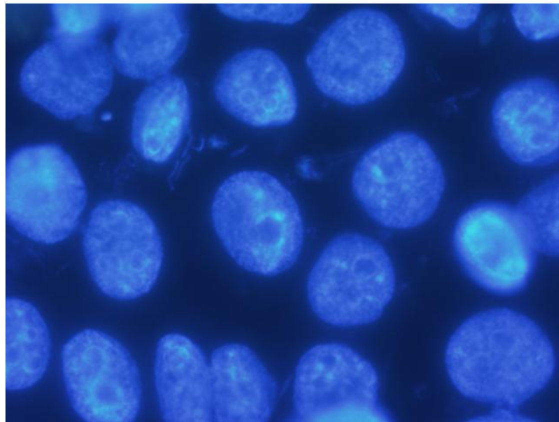
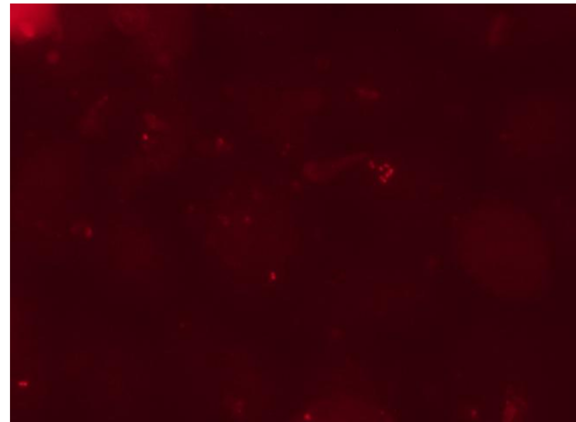


Figure C.1 – Adherence of high level of *L. iners* vs high level of *G. vaginalis* strains to epithelial HeLa cells. Original magnifications: x1000. Bacteria and HeLa cells were quantified by fluorescence microscopy, using DAPI (blue) for total cell count and PNA-FISH probe (Gard162 Probe) (red) for *G. vaginalis* quantification.

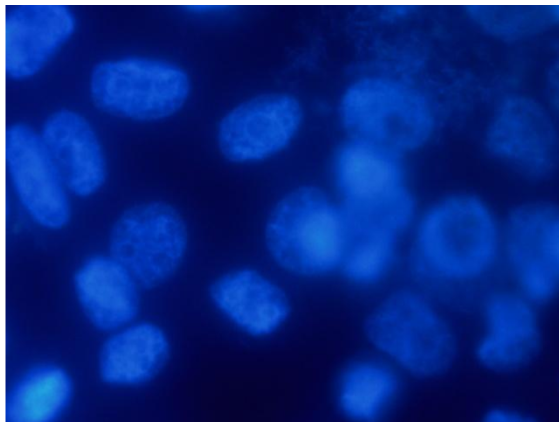
In Figure C.2 is showed the adherence of high level of *L. crispatus* versus high level of *G. vaginalis* strains to epithelial HeLa cells.



L. crispatus and *G. vaginalis* 5-1



G. vaginalis 5-1

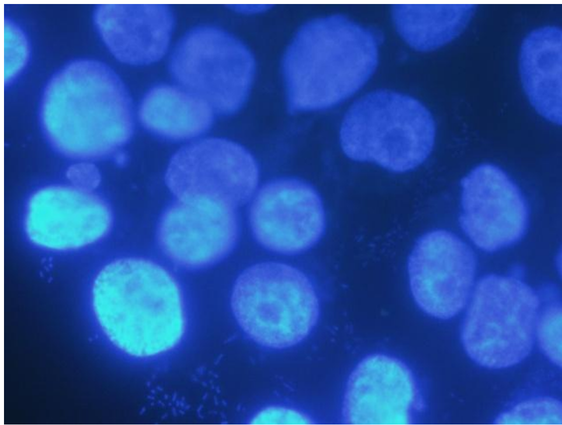


L. crispatus and *G. vaginalis* 101

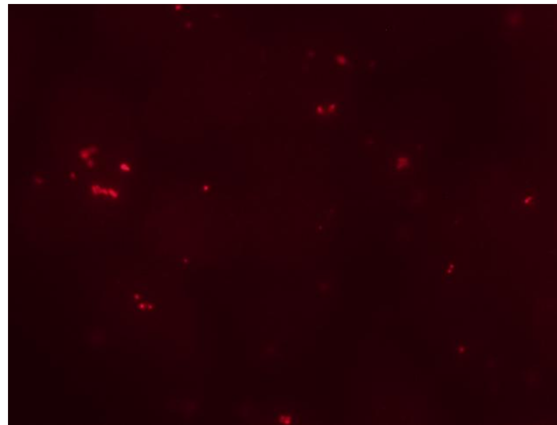
G. vaginalis 101

Figure C.2 – Adherence of high level of *L. crispatus* vs high level of *G. vaginalis* strains to epithelial HeLa cells. Original magnifications: x1000. Bacteria and HeLa cells were quantified by fluorescence microscopy, using DAPI (blue) for total cell count and PNA-FISH probe (Gard162 Probe) (red) for *G. vaginalis* quantification.

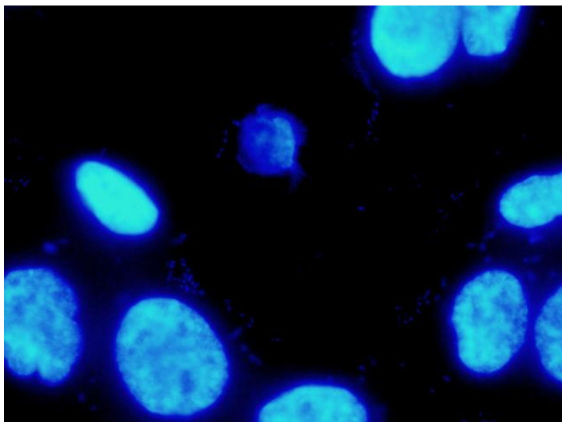
Finally, in Figure C.3 is showed the adherence of high level of *L. casei* versus high level of *G. vaginalis* strains to epithelial HeLa cells.



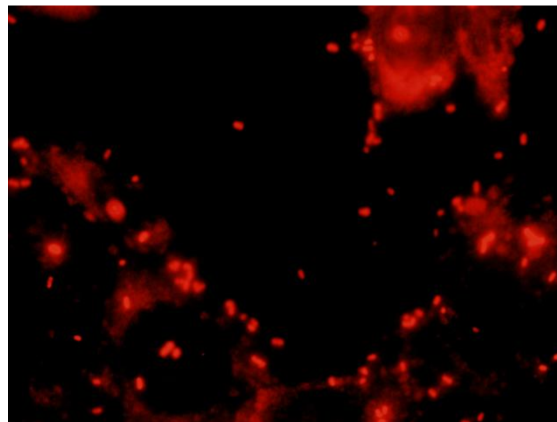
L. casei and *G. vaginalis* 5-1



G. vaginalis 5-1



L. casei and *G. vaginalis* 101



L. casei and *G. vaginalis* 101

Figure C.3 – Adherence of high level of *L. casei* vs high level of *G. vaginalis* strains to epithelial HeLa cells. Original magnifications: x1000. Bacteria and HeLa cells were quantified by fluorescence microscopy, using DAPI (blue) for total cell count and PNA-FISH probe (Gard162 Probe) (red) for *G. vaginalis* quantification.