Universidade do Minho
Escola de Engenharia

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Isolamento e caracterização de microrganismos vaginais e a sua associação com a Vaginose Bacteriana

RESUMO

A vaginose bacteriana (VB) é uma das condições ginecológicas mais comuns que afetam as mulheres em idade fértil em todo o mundo, tendo sido associada a graves consequências para a saúde pública. Devido à sua complexidade e à diversidade dos microorganismos presentes, a sua etiologia é desconhecida. Dados recentes associaram a presença de biofilmes anaeróbios, tanto na vagina saudável como em VB, levando à teoria de que os microorganismos que formam biofilme, como *Gardnerella vaginalis*, podem ser relevantes para a etiologia da VB. Biofilmes são estruturas complexas que protegem os microorganismos podendo conferir resistência aos antibióticos e às defesas naturais do hospedeiro. Assim, o objetivo deste trabalho foi isolar e caracterizar a população microbiana presente na vagina de mulheres portuguesas. Para isso, foram recolhidas 54 amostras de exsudado vaginal de mulheres saudáveis ou diagnosticadas com BV. Após a caracterização inicial, 15 estirpes foram isoladas e identificadas. O passo seguinte consistiu na caracterização fenotípica das estirpes, como a capacidade intrínseca para formar biofilme e a tolerância a antibióticos, através da determinação das concentrações mínimas inibitórias (CMI´s). A capacidade intrínseca de formação de biofilme de cada um dos isolados foi avaliada em condições anaeróbias durante 48 horas utilizando diferentes meios de crescimento. Foi possível observar que todas as estirpes isoladas apresentaram elevada capacidade para formar biofilme, dependendo dos meios de crescimento utilizados. As estirpes que demonstraram maior capacidade para formar biofilme foram *G. vaginalis*, *Enterococcus faecalis*, *Streptococcus* spp., *Bifidobacterium breve* e *Propionibacterium acnes*. Além disso, os resultados da susceptibilidade antimicrobiana demonstraram que a maior parte dos microrganismos apresentaram CMI´s semelhantes às previamente descritas na literatura.

Este trabalho permitiu fazer uma caracterização da flora microbiana vaginal de mulheres portuguesas e é o primeiro estudo deste tipo em Portugal. Estes resultados devem ser tidos em conta na investigação da epidemiologia e patogénese da VB.

Isolation and characterization of vaginal microorganisms and its association with Bacterial Vaginosis

ABSTRACT

Bacterial vaginosis (BV) is one of the most common gynaecological conditions affecting women in reproductive age worldwide, being linked to serious public health consequences. Due to their complexity and to the diversity of microorganisms involved in this condition, the exact aetiology is inconclusive, but recent reports referring to the presence of anaerobic biofilms led to the theory that the microorganisms which form the biofilm, like *Gardnerella vaginalis*, may be relevant for the aetiology of BV. Biofilms are complex structures that protect the microorganisms involved increasing their resistance to antibiotics and natural defences of the body. As such, the aim of this work was to isolate and characterise the microbial population present in the vagina of Portuguese women. In order to achieve this, 54 samples of vaginal exudate were collected in clinical settings from Portuguese women that were either healthy or had been diagnosed with BV. After an initial characterization, 15 unique strains were isolated and identified. The next step was the phenotypic characterization of the isolated strains, which included the determination of their intrinsic capacity to form biofilm and tolerance to some antibiotics typically prescribed by physicians around the world, by determining the minimum inhibitory concentrations (MIC). The intrinsic capacity to grow as biofilm of each one of the isolate was assessed under anaerobic conditions for 48 hours and using different media. It was observed that all isolates had the capacity to form biofilm, but this was depending on the growth media used. The strains that showed a higher capacity for biofilm formation were *G. vaginalis*, *Enterococcus faecalis*, *Streptococcus* spp., *Bifidobacterium breve* and *Propionibacterium acnes*. Furthermore, results of antimicrobial susceptibility assays showed that for the most part of microorganisms had an MIC similar to those previously reported in the literature.

This work allowed the characterization of the vaginal microbial flora in Portuguese woman and is the first study of this kind in Portugal. These results should be taken into account when researching the epidemiology and pathogenesis of BV.

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NOMENCLATURE

Symbols

P	Significance value
ºC	Temperature
min	Time (minutes)
s	Time (seconds)

ABBREVIATIONS

BAP	Biofilm-associated protein
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BV	Bacterial Vaginosis
CBA	Columbia Blood Agar
CM	Clindamycin
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular Polysaccharides
FBS	Fetal Bovine Serum
HBT	Human bilayer Tween
HIV	Human Immunodeficiency Virus
IgA	Immunoglobulin A
LAM 1	Laboratory of Applied Microbiology 1
LB	Luria Broth
LBG	Luria Broth supplemented with Glucose
MD	Metronidazole
MIC	Minimum Inhibitory Concentrations
MRS	De Man-Rogosa and Sharpe agar
MRSG	De Man-Rugosa and Sharpe agar supplemented with Glucose
NCBI	National Center for Biotechnology Information
OD	Optical density

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rDNA	ribosomal DNA
RF	Rifampin
rRNA	Ribosomal Ribonucleic acid
sBHI	Brain Heart Infusion supplemented
sBHIG	Brain Heart Infusion supplemented Glucose
SEM	Scanning electron microscope
SPSS	Statistical package for the social sciences
TAE	Tris-acetate-EDTA
tDNA-PCR	tDNA intergenic spacer PCR
TMPD	NNN'N'-tetramethyl-p-phenylenediamine
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSBG	Tryptic Soy Broth supplemented with Glucose
TZ	Tinidazole
VLY	Vaginolysin
VM	Vancomycin

LIST OF PUBLICATIONS

Abstracts and Posters

Alves P, Castro J, Sousa C, Cereija T, Henriques A, Cerca N, (2012) Biofilm formation potential of clinical isolates associated with bacterial vaginosis. In Biofilms 5, 10-12 December 2012, Paris, France.

Salgueiro D, Machado A, Alves P, Martinez J, Henriques A, Cerca N, (2011) Presence of *Gardnerella vaginalis* in healthy Portuguese women – a pilot study. In Microbiotec, 1-3 December 2011, Braga, Portugal.

I dedicate this thesis to my parents, sister and boyfriend

"*Nós nunca nos realizamos. Somos dois abismos - um poço fitando o céu.*"

Fernando Pessoa

CHAPTER 1

General Introduction

1.1. Vaginal flora microenvironment

Microbial communities have a strong influence in human health and quality of life. Therefore, the bacterial community of the human vagina can have a profound impact on women's health, since microorganisms play a critical role in determining the biochemical profile and inflammation of the vaginal environment (Srinivasan and Fredericks, 2008).

Despite decades of investigations, based on growth technologies of human vaginal flora, recent studies using culture independent methods significantly increased the knowledge of bacterial diversity in this important niche (Donachie *et al*., 2007). Diverse microorganisms can be found in healthy women that are mostly colonized with lactobacilli such as *Lactobacillus crispatus*, *Lactobacillus jensenii* and *Lactobacillus gasseri* (Zhou *et al*., 2004) though a variety of other bacteria may be present (Vásquez *et al*., 2002) (Figure 1.1).

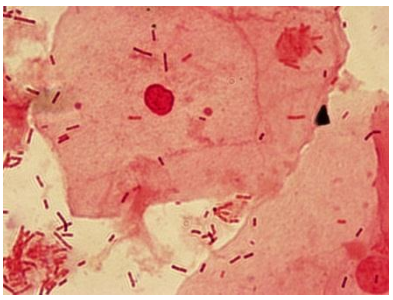


Figure 1.1. Gram staining of vaginal fluid smear from clinical sample of healthy woman that we prepared (original magnification, x1000). There is a predominance of *Lactobacillus* spp. which are responsible for suppressing the growth of other species.

The definition of a healthy vaginal environment is more complex than originally thought due to bacterial diversity observed among individuals (Kim *et al*., 2009). Thus, the identity and diversity of these populations remain largely unknown and the complex interaction between the various members of the vaginal flora is still poorly understood (Zhou *et al*., 2004). This means that it is essential to have accurate knowledge about the microbial ecosystem of the human vagina of healthy women, because there is still much to understand about how bacterial communities in this niche promote health and facilitate disease (Srinivasan and Fredericks, 2008).

1.2. Bacterial Vaginosis

Bacterial vaginosis (BV) is the main vaginal disorder of women in reproductive age worldwide (Harwich *et al.*, 2010) and, although not life threatening, leads to increased risk of preterm delivery (Hillier *et al*., 1995), and more severe gynecological infections such as pelvic inflammatory disease (Haggerty *et al*., 2004) and increase risk of Human Immunodeficiency Virus (HIV) infection acquisition (Schmid *et al*., 2000).

The etiology of this condition has been long debated, and despite the impact on women's health, little is known about its cause and pathogenesis. This microecologic disorder is characterized by not being associated with a specific etiologic agent (Aroutcheva *et al*., 2001).

Women without BV typically show Gram-positive rods, revealing a predominance of lactobacilli, particularly *L. crispatus* and *L. jensenii* (Livengood, 2009). However, it is likely that over time, microbial communities in the human vagina may be affected in a negative way due to various factors, such as woman's age, hormonal fluctuations (menses, or contraception), pregnancy, sexual activity (frequency of sex and numbers of sexual partners), health status (such as diabetes, infections) and as well as various lifestyle habits and hygiene practices (such as douching) (Srinivasan and Fredericks, 2008).

During BV, most of the beneficial bacteria (like *L. crispatus*, *L. jensenii*, *L. gasseri*) (Srinivasan and Fredericks, 2008) (Figure 1.1) are replaced, by concomitant overgrowth of anaerobic or facultative bacteria (Fredricks *et al*., 2005) generally Gram-negative or Gram-variables cocci and rods, normally associated with the gastrointestinal tract. Cultures of vaginal fluids from individuals with BV microorganisms typically present bacteria such as *Gardnerella vaginalis* and a combination of other bacteria such as, *Prevotella*, *Porphyromonas*, *Mobiluncus* spp., *Atopobium vaginae* and *Mycoplasma* spp. (Livengood, 2009).

Lactobacilli are responsible for promoting a healthy ecosystem, producing lactic acid, hydrogen peroxide (H_2O_2) and bacteriocins, which have antimicrobial properties that inhibit microorganisms that are pathogens of this niche, maintaining a healthy ecosystem (Srinivasan and Fredericks, 2008). The growth of anaerobic bacteria is associated with increased production of proteolytic enzymes, which break the vaginal epithelial peptides into a variety of amines that at, an elevated pH, becomes volatile causing bad odor. Amines are associated with an increase in transudation and exfoliation

of squamous epithelial cells, resulting in a white fluid that is expelled from the vagina, creating an imbalance in the vaginal flora (Sobel, 2000).

It is unknown if the primary event of BV is the loss of lactobacilli or the acquisition of facultative anaerobic bacterial communities found in this infection, or if these two events are simultaneous processes (Figure 1.2). It is also possible that the primary etiologic agent is another unknown factor, that changes in vaginal flora reflect a downstream event in the pathogenesis of BV (Srinivasan and Fredericks, 2008).

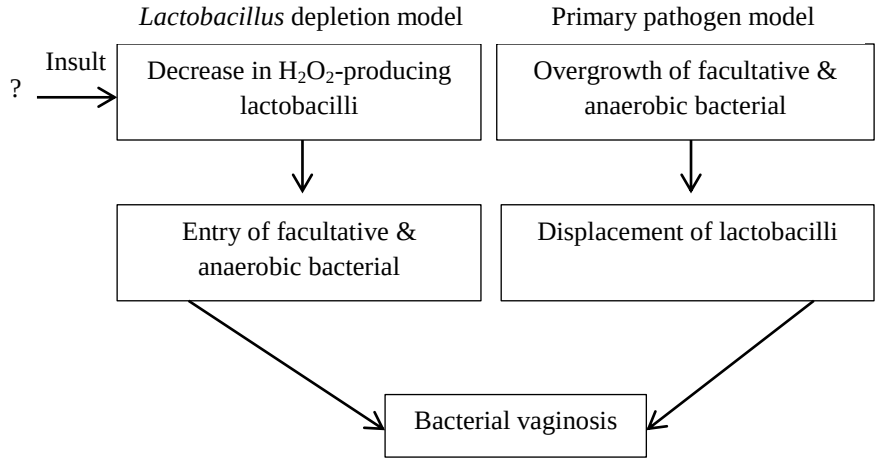


Figure 1.2. Two models explaining the pathogenesis of BV, the loss of lactobacilli model suggests that there is a decrease in the production of H_2O_2 by lactobacilli as the main event, which allows the proliferation of anaerobic bacteria, resulting in BV; while the primary pathogenic model suggests that entry of facultative anaerobic bacteria will cause loss of lactobacilli resulted in BV. Adapted from Srinivasan and Fredericks, (2008).

1.2.1. Clinical features and diagnosis of BV

The diagnosis of BV is generally done through a series of clinical criteria from pelvic examination and microscopic examination of the exudate (Amsel's criteria), or by microscopic interpretation of vaginal fluid by Gram staining technique (Nugent criteria) (Srinivasan and Fredericks, 2008). The Amsel criteria are the most frequently method used to diagnose BV in most countries, including Portugal (Henriques *et al*., 2012). At least 3 of 4 Amsel criteria must be present to establish the diagnosis of BV, including the increase of pH of vaginal fluid > 4.5; positive "wiff test" in the detection of an odor similar to rotten fish (with the addition of 10% potassium hydroxide (KOH) in a sample containing vaginal fluid); presence of “clue cells” (> 20%) in vaginal fluid where the

vaginal epithelial cells are coated with bacteria creating indistinct borders (Figure 1.3) and white vaginal discharge, with homogeneous characteristics (Srinivasan and Fredericks, 2008).

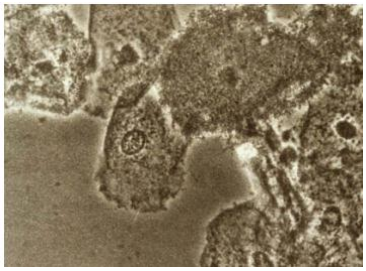
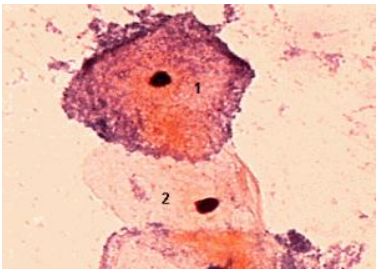
a)  **b)** 

Figure 1.3. "Clue cells" in both images (original magnification, x400). **a)** some cells have irregular edges that define the "clue cells", those that do not have this characteristic are not "clue cells"; **b)** represents the normal vaginal bacterial microscopy image bacterioscopy, in which a cell 1 is a "clue cell" in which the *G. vaginalis* bacterium is adhered to the surface of cell, while in the cell 2 does not. Adapted from Livengood, (2009).

For the clinical diagnosis of BV, Nugent's criteria form a standardized method of Gram stain interpretation of vaginal samples to detect the shift of normal vaginal flora to other microorganism allowing the scoring system of vaginal smears (Delaney and Andrew, 2001). The Nugent score is calculated by assessing the presence of some microorganisms in vaginal smears that are graded on a scale based on the presence or absence of some microorganisms, such as large Gram-positive rods - *Lactobacillus* morphotypes (decrease in *Lactobacillus* - scored as 0 to 4), small Gram-variable rods - *G. vaginalis* morphotypes (scored as 0 to 4), curved Gram-variable rods - *Mobiluncus* spp. morphotypes (scored as 0 to 2). Therefore, the score can range from 0 to 10 and score between 7 to 10 are classified as BV (Sha *et al*., 2005) (Table 1.1).

For these two evaluation criteria of vaginal flora samples, Nugent scoring allows the evaluation of a change in vaginal flora continuously. Because Amsel criteria are dependent on the accuracy of the clinician, the Nugent score was favored for diagnosing BV due to its higher sensitivity and reproducibility (Onderdonk *et al*., 1977). Nevertheless, assessment of from vaginal smear slides samples can also be subjective and thus requires the experience of a reader (Chaijareenont *et al*., 2004).

Table 1.1. Scheme for grading Gram stained vaginal contents. Adapted from Livengood, (2009).

Score	*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 +	

Total	Interpretation
0 – 3	Normal
4 – 6	Intermediate
7 - 10	Bacterial vaginosis

Morphotypes are scored as the average number seen *per* oil immersion field. Note that less weight is given to curved Gram-variable rods. Total score = lactobacilli + *G. vaginalis* and bacteroides spp. + curved rods.

Quantitation: 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.

1.2.2. Treatment of BV

Current research has revealed more detailed aspects for the treatment of BV. Aims of the treatments of BV have two main goals: to eradicate the anaerobic microorganisms, and to allow growth of lactobacilli producing H_2O_2.

Many studies have been performed in order to find antibiotics that can specifically eradicate the anaerobic microorganisms that cause the symptoms of BV, trying to overcome the resistance that these organisms sometimes show to multiple antibiotics. Results of several experiments demonstrate that the fact that in BV the vaginal flora is comprised of several Gram-variable bacteria, like *G. vaginalis*, antimicrobials specifically active against Gram-positive or Gram-negative bacteria, have little or no action against Gram-variables microorganisms, explaining the common resistance to them (Gilbert *et al.*, 1997).

In an attempt to find antimicrobial agents active against microorganisms normally associated with BV, including the bacteria *G. vaginalis* (Kharsany *et al*., 1993), various concentrations of antimicrobials were tested to find the minimum concentration capable of inhibiting the growth of pathogenic microorganisms, called a Minimum Inhibitory Concentration (MIC), as shown on table 1.2 (Kharsany *et al*., 1993).

Table 1.2. *In vitro* susceptibility of 93 strains of *G. vaginalis* to 25 antimicrobial agents. Adapted from Kharsany *et al*., (1993).

Test agent	MIC (µg/ml)		
	Range	**50%**	**90%**
Metronidazole	2.0 - 128.0	8.0	16.0
2-Hydroxy[a]	0.25-16.0	1.0	4.0
Tinidazole	1.0-128.0	8.0	8.0
Penicillin G	0.015-0.5	0.12	0.5
Ampicillin	0.03-1.0	0.5	0.5
Cefamandole	0.12-2.0	1.0	2.0
Cefoxitin	0.06-4.0	1.0	1.0
Cefuroxime	0.06-4.0	1.0	4.0
Cefotaxime	0.25-4.0	2.0	2.0
Ceftriaxone	0.06-4.0	0.5	2.0
Aztreonam	4.0-32.0	32.0	32.0
Imipenem	0.06-1.0	0.25	1.0
Tetracycline	2.0-128.0	64.0	64.0
Minocycline	0.12-16.0	2.0	16.0
Erythromycin	0.007-0.06	0.03	0.06
Clindamycin	0.007-0.03	0.01	0.03
Vancomycin	0.12-0.5	0.25	0.5
LY146032	0.5-8.0	4.0	8.0
Chloramphenicol	0.5-2.0	1.0	2.0
Amikacin	8.0-128.0	32.0	128.0
Rifampin	0.5-0.5	1.0	2.0
Ciprofloxacin	1.0-4.0	1.0	2.0
Sulfamethoxazole	128.0-128.0	>128.0	>128.0
Trimethoptim	0.5-4.0	2.0	4.0
Co-trimoxazole[b]	4.0-64.0	64.0	64.0

[a] 2-Hydroxymetabolite of metronidazole [1-(2-hydroxyethyl)-2 hydroxymethyl- 1,5-nitroimidazole]

[b] sulfamethoxazole-trimethoprim in a 19:1 ratio.

Recent investigations indicate that following intravaginal treatment with antibiotics such as metronidazole or clindamycin (Santiago *et al*., 2012) the cure rates were between 80 - 90% at the end of treatment (Livengood, 2009). However, a study suggests that three months after the end of therapy, there was a possible recurrence of BV

(Polatti, 2012). A study in Portugal on the perception of specialist doctors regarding to BV, also reported that the recurrence of these microorganisms after antimicrobial chemotherapy is high (Henriques *et al.*, 2012). This relapse can occur due to inadequate treatment of microorganisms associated with BV, due to the resistance to metronidazole and others antibiotics or due to the fact that these microorganisms have recently been recognized as biofilm-forming (described in section 1.4.3). Because of these complications, with relapse of BV, new solutions have been proposed and Nyijesy and his collaborators demonstrated that the regeneration of lactobacilli using preparations of probiotic lactobacilli, which are known to be specialized organisms which dominate the healthy vagina, is likely to happen (Nyirjesy *et al*., 2006). However, this research has been hampered by the complications involving both the application of probiotic *Lactobacillus* spp., such as dominance by these organisms, such as *L. crispatus*, *L. rhamnosus*, and *L. reuteri* (Livengood, 2009) over other existing microorganisms.

1.3. The microbiology of BV and role of recently defined BV-associated bacteria

1.3.1. The etiology of BV

BV is considered a polymicrobial condition, and recently several new fastidious bacteria have been found to be associated with BV.

An attempt to find a single etiological agent to explain the pathogenesis of BV has been inconclusive (Ferris *et al*., 2007). Particular attention has been attributed to *G. vaginalis*, as this bacterium can be found in the vaginal flora of a majority of women with BV (95% of cases); however is also present in 50% of healthy vaginal flora (Livengood, 2009). The bacteria *Mobiluncus* spp., curved bacilli highly mobile, is found only when BV is detected, but only 50% of cases of BV (Nyirjesy *et al*., 2007). *A. vaginae* is an anaerobic Gram-positive, as *G. vaginalis*, is found in more than 95% of BV, but is also present in normal flora of the vagina of healthy women (Ferris *et al*., 2007).

1.3.2. BV-associated bacteria

G. vaginalis is a Gram-variable bacterium, which was first described in 1953 by Leopold, and has been identified as the predominant BV. The name *Haemophilus*

vaginalis was originally proposed by Gardner and Dukes in 1955, due to the colony morphology of the organism and its biochemical profile. Subsequently, metabolic requirements and thorough analysis of preparations using Gram staining, it was found that the organism morphologically resembled bacilli diphtheroids and named *Corynebacterium vaginale* (Greenwood *et al*., 1979). However, due to their variable Gram staining reaction (not being positive or negative typically) was subsequently named *Gardnerella* (Figure 1.4).

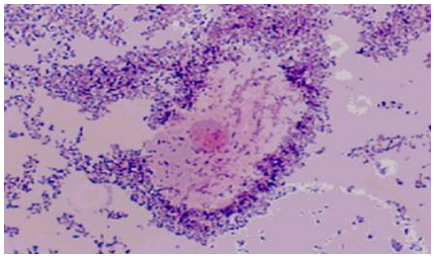
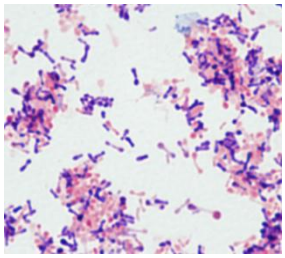
a)  **b)** 

Figure 1.4. a) Epithelial cell totally covered by Gram-positive bacteria (clue cell), *G. vaginalis*. Source: http://farm3.static.flickr.com/2056/2369779554_f4d9b48760_z.jpg, accessed August 1, (2012); **b)** Gram staining of Gram-variable *G. vaginalis* AMD bacterium that we prepared (original magnification, x1000).

Gardnerella belongs to the *Bifidobacteriaceae* family, and their cells are small, pleomorphic bacilli (Figure 1.5) in which the length and Gram stain may vary depending on the growth medium (Catlin, 1992).

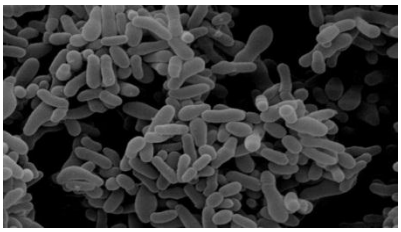


Figure 1.5. Scanning electron microscope (SEM) of *G. vaginalis* biofilm. Source: http://www.gardnerella.mic.vcu.edu/images/gv_em.jpg, accessed August 1, (2012).

G. vaginalis is considered a fastidious and anaerobic microorganism that requires a complex growth medium (Harwich *et al.*, 2010). Studies of microorganism identification using metabolic methods indicate that it is a catalase-negative microorganism, exhibits activity α-glucosidase, hydrolysis of starch and hippurate, acid phosphatase activity, salt tolerance and can use carbohydrates such as dextrins, fructose,

glucose, maltose, ribose, starch and sucrose from fermentation for growth (Catlin, 1992; Harwich *et al.*, 2010).

Researchers such as Gardner and Dukes identified this bacterium as a major etiologic agent of BV, fulfilling all the Koch's postulates (Table 1.3). However, later studies demonstrated some failures in these experiments which suggested that there were other factors in addition to *G. vaginalis* that were important in the induction of the disease, meaning that these bacteria were not the etiologic agent in BV. One of the Koch's postulates requires pathogenic microorganisms to be found in all cases of the disease and not be found in individuals who do not have the disease (Evans, 1993). In the case of *G. vaginalis*, it was found in many cases of BV, but can also detected in 50 - 60% of women who have no visible symptoms of BV, thus failing one of Koch's postulates (Fredricks and Relman, 1996).

Table 1.3. Koch´s postulates. Adapted from Evans, (1993).

The etiologic microbe should be found in every case of the 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

The role of *G. vaginalis* in BV has been extensively debated since it can be both present in the genital tract of healthy women (Hyman *et al.*, 2005) as in women with BV. However, vaginal epithelial tissue of women with BV presents a larger number of anaerobic microorganisms (Swidsinski *et al.*, 2005). Moreover, recent studies show that the biotypes of *G. vaginalis* isolates from healthy women differ from those isolated from women with BV (Numanovic *et al.*, 2008). The lack of genetic characterization of this microorganism leaves open the possibility that different pathogenic and nonpathogenic strains or subspecies exists (Harwich *et al.*, 2010).

The discovery of the presence of *A. vaginae* in the vaginal ecosystem improves the basic understanding of the pathogenesis of BV (Polatti, 2012). The bacterium *A.*

vaginae belong to the family *Coriobacteriaceae*. It is thought that this newly identified bacterium is one of the causes of complications that arise in BV (Fredricks *et al*., 2005). The genus *Atopobium* was described for the first time in 1992, and includes bacteria previously classified as lactobacilli (Rodriguez *et al*., 1999). Relatively to morphology, Gram stain shows varied morphology, as their cells appear as Gram-positive elliptical cocci or rod-shaped organisms and can be visible as single cells, in pairs or in short chains (Figure 1.6). *A. vaginae* is an anaerobic bacteria, that cannot be easily isolated by classical microbiological methods and it is rarely detected in healthy women vaginal fluid but is commonly found in the vagina of patients with BV (Verhelst *et al*., 2004).

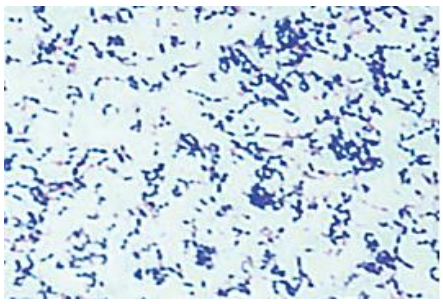


Figure 1.6. Gram staining shows Gram-positive bacteria, with *A. vaginae* visible as single cells, in pair or short chains. Adapted from Geißdörfer *et al*., (2003).

Mobiluncus spp. is another anaerobic organism most commonly associated with BV. These fastidious curved rods are only rarely cultured from vaginal smears of women without BV, yet are highly predictive of BV if found on Gram stain or wet-preparation examination of vaginal secretions (Thomason *et al*., 1984) (Figure 1.7).

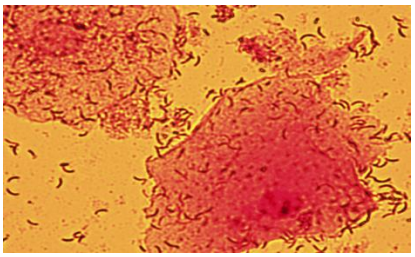


Figure 1.7. Gram staining of vaginal smear of clinical sample that we received shows curved rods, morphotypes associated with *Mobilluncus* spp. (original magnification, x1000).

The presence of *Mobiluncus* spp., specially *M. curtisii* and *M. mulieris* is highly specific, although not sensitive, for the diagnosis of BV (Roberts *et al*., 1985). The

presence and persistence of *M. curtisii*, detected by Polymerase Chain Reaction (PCR), was found to be strongly associated with recurrence of BV. Whether this organism is truly pathogenic or simply a marker for greater disturbances of vaginal flora remains unknown (Meltzer *et al*., 2008).

To better understand BV there is a need to isolate more BV-associated bacteria, such as the species detected by molecular methods (Table 1.4).

Table 1.4. Detailed composition of the vaginal microflora of 515 vaginal swab samples, as determined by culture and tDNA intergenic spacer PCR (tDNA-PCR) based identification. Adapted from Verhelst *et al.*, (2005).

Bacterial vaginosis-related anaerobe organisms	
Actinomyces neuii	*Gemella morbillorium*[b]
Aerococcus christensenii	*Mobiluncus curtisii*
Anaerococcus tetradius[b]	*Mycoplasma hominis*
Anaerococcus vaginalis[b]	*Peptoniphilus spicies.*[b]
Atopobium vaginae	*Peptostreptococcus sp.*
Bacteroides ureolyticus	*Prevotella bivia*
Dialister species	*Prevotella ruminicola*
Finegoldia magna[b]	*Prevotella species*
Gardnerella vaginalis	*Varibaculum cambriense*

[b] Formerly known as Peptostreptococcus

1.4. Vaginal microbial diversity

1.4.1. Isolation - Culture perspective

Culture studies provide critical results on the phenotypic characteristics of the microorganisms that are not easily obtained from molecular studies. Moreover, a microorganism culture allows manipulation of these organisms in experimental laboratory and testing hypotheses about their pathogenesis and virulence factors. Thus, studies using the culture of microorganisms remain an important area of research in vaginal microbiology, despite the limitations of this approach (Donachie *et al*., 2007).

Burton and Reid published a study which involved the isolation of the microorganisms in culture using selective and non-selective media and subsequent identification by phenotypic techniques. The use of a wide range of PCR primers helped to maximize the diversity of species detected and a large number of media and growth

conditions may be required for optimal isolation of diverse bacterial species (Burton and Reid, 2002). Selective media such as MacConkey agar, mannitol and Tryptic Soy Agar (TSA) with 5% sheep blood may be useful to estimate the number of aerobic and anaerobic bacteria in vaginal samples. Some of the selective media/ semi-selective agar include Rugosa, De Man-Rugosa and Sharpe (MRS) agar and Human bilayer Tween (HBT) and Columbia Blood Agar (CBA) with 5% horse blood are used to help identify microorganisms presents in vaginal swabs samples (Figure 1.8). In some of the media, such as the CBA it is possible to observe the hemolysis arising from the growth of some microorganism (eg. bacterium *G. vaginalis* shows β-hemolysis in CBA) (Totten *et al*., 1982).

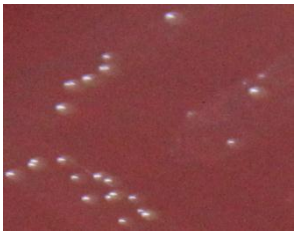


Figure 1.8. Growth of the bacterium *G. vaginalis* AMD in CBA selective medium.

Thus, using approaches based on the culture of organisms, it is possible to identify *G. vaginalis* and anaerobic bacteria such as *Neiserria gonorrhea, Streptococcus* spp., *Escherichia coli, Trichomonas vaginalis, Prevotella, Porphyromonas, Peptostreptococcus, Mobiluncus* and *Mycoplasma*, which may be largely associated with disturbances in the microbial flora in women with BV (Kalra *et al*., 2007).

1.4.2. Isolation - Molecular Perspective

Results of different research groups confirmed that the human vagina hosts numerous species of bacteria which are not yet cultured or are not easily identified by culture methods (Srinivasan and Fredericks, 2008).

With advances in technology and decreasing costs of sequencing, there is a better knowledge of the microflora of the human vagina. As the conditions of the vagina may be transient and dependent on many factors, molecular studies provide results able to demonstrate features of the vaginal microbiota under specific conditions (Hugenholtz *et al*., 1998).

Burton and Reid, were also the first researchers to examine the microbiota of the vaginal niche using a wide range of molecular methods such as PCR and Denaturing Gradient Gel Electrophoresis (DGGE) and found that different women with BV had different profiles indicating heterogeneity in bacterial composition in subjects with BV (Burton and Reid, 2002). Many researchers such as Verhelst *et al.*, (2004), Fredericks *et al.*, (2005), contributed to the current knowledge of BV, and there are many studies that have been performed in order to intensively investigate the bacterial flora of the human vagina. These studies, using methods of cultivation and molecular approaches aimed to reveal which microorganisms are the most persistent in BV.

Although progress has occurred in defining the composition of vaginal flora using Deoxyribonucleic acid (DNA) amplification, the rapid accumulation of data resulted in no therapeutic advantage. Treatment options remain limited and often have unsatisfactory results, especially in relation to the frequent relapse of symptomatic disease, leading to great frustration among patients and professionals (Srinivasan and Fredericks, 2008).

1.4.3. Virulence factors of *G. vaginalis*

The normal flora of the human vagina is a diverse set of microorganisms that live in a dynamic relationship in the colonized epithelium. Although the colonization of the lower female genital tract is usually benign, it is noted that the vast majority of infections involving female pelvic structures arise from organisms that are members of the normal flora (Larsen, 1994) due to a shift in the microbial balance caused by reasons discussed previously.

Although there is much controversy surrounding the etiology of BV, *G. vaginalis* remains the most studied organism and potential primary agent of BV. The following factors its pathogenic potential.

1.4.3.1. Initial adhesion to epithelium

Microorganisms can improve their virulence factors by producing compounds which act against host defense or in response to them. This virulence attributes include: interacting with the host defense, adhesion to host tissue, adjustment of the characteristics of virulence and production of toxins (Larsen, 1994). The organism adhesion to the epithelial surface is a significant aspect in the life of microorganisms in the vagina. The bacterium *G. vaginalis*, which is present in asymptomatic women and in abundance in

individuals with BV, is known for its ability to adhere to epithelial cells (Patterson *et al*., 2010). Boustouller *et al.* attribute the responsibility of adhering to the epithelial cells to pilli, while Scott and Smyth believe that the fibrillar matrix is responsible for foreign citoadhesion, with pilli functioning as a hemagglutinin (Scott and Smyth, 1987; Boustouller *et al*., 1989). It should be considered that although cell adhesion is important in the disease process, the fixation to the tissue does not characterize an organism as pathogenic. In fact, vaginal lactobacilli have adhesins that probably serve to promote the stable colonization of the vaginal epithelium, possibly with beneficial effects for the host (Nagy *et al*., 1992). Thus, if a microbial species is a highly virulent or only modestly virulent it is due to the interaction with the host tissue through adhesives factors.

Clearly, organisms that are usually associated with the host, described as belonging to the normal commensal flora have had little attention as producers of toxins. Although, the production of toxins with subtle effects on the physiology of the host, or his immunity, may play a role in the virulence of this microorganism (Larsen, 1994).

1.4.3.2. Cytotoxicity of *G. vaginalis*

While *G. vaginalis* can be isolated in 95% of cases of BV, studies with healthy subjects indicate that pure cultures of bacteria do not always cause BV, perpetuating the exiting doubts about it is potential as a pathogen (Catlin, 1992; Marrazzo *et al*., 2008). However, the connection between the cytolysin secreted by *G. vaginalis,* called recently vaginolysin (VLY), and BV has been made (Gelber *et al*., 2008). The production of the cytolysin by *G. vaginalis* was first reported in 1990 but was only recently named VLY. VLY, a cholesterol-dependent cytolysin recognizes the complement of the molecule CD59 on the target cell surface, which accounts for the specificity for human erythrocytes epithelial cells (Madden *et al*., 2001), leading to cell death (Gelber *et al*., 2008). Immunoglobulin A (IgA) antibodies against VLY have been linked to the mucosal immune response during BV, increasing the emphasis on the role of VLY in the pathogenesis of BV (Cauci *et al*., 2002). Studies have examined the levels of cytotoxic activity of anaerobes associated with BV, and found that only the bacteria *G. vaginalis* was able to induce lysis of vaginal epithelial cells (Patterson *et al*., 2010).

Other virulence factors produced by *G. vaginalis* include sialidase and prolidase, which are two hydrolytic enzymes that may play a role in the degradation of many

mucosa protective factors, such as mucins and contribute to the detachment and exfoliation of epithelial cells (Cauci *et al*., 2008).

1.4.3.3. Formation of biofilm

The adherence to the epithelium is the first step in the pathogenesis of a biofilm-forming pathogen as it must first adhere to host tissues in order to avoid the clearance by means of host defense mechanisms, such as urine flow and the flow of vaginal secretions, and be able to form a biofilm (Patterson *et al*., 2010). Biofilm formation is an important virulence factor because it confers tolerance to antibiotics (Figure 1.9) and increased resistance to host immune defense (Cantón *et al*., 2005).

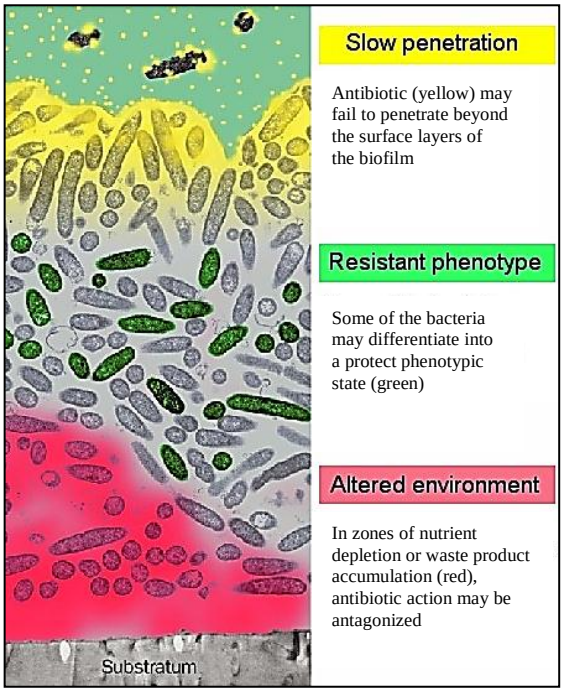


Figure 1.9. Proposed mechanisms for antibiotic resistance of biofilms. Yellow region: is characterized by low penetration, the antibiotic (yellow) may fail to penetrate the surface layers of the biofilm. Green region: some of the bacteria can differentiate themselves to protect the phenotypic state (green). Red region is in regions of lack of nutrients or accumulation of waste products (red), the action of the antibiotic can be antagonized. Adapted from Stewart and Costerton, (2001).

Microbial biofilms are communities of microorganisms that are closely associated with each other in a way that they are attached to a surface, in this case to vaginal epithelial cells, forming a porous structure where the microbial cells are involved in extracellular matrix polymers and are able to concentrate products of their own

metabolism, together with ions and nutrients that are captured from the environment that surrounds them (Costerton, 1995). This matrix consists of several components including proteins, extracellular polysaccharides (EPS), nucleic acids and other substances (Davey and O´toole, 2000) and represents approximately 85% of the biofilm (Donland and Costerton, 2002). The synthesis of polymer matrix appears to be regulated by a variety of factors in which the cell surface adhesion is very important. The exopolymers serve two main functions within the biofilm: first, the polysaccharides are produced in large amounts after initial fixation of the cell surface, being suggested to act as "cement" with which bacteria can reinforce the primary mechanisms of adhesion (Figure 1.10). Then, due to the existence of an extracellular polymer matrix (Gilbert *et al*., 1997) bacteria become protected against biocides, antibiotics and surfactants, by limiting diffusion of the agents of the surrounding medium through a combination of ionic interactions and molecular events, and are also protected from bacteriophages and other phagocytic predators such as protozoa (Dunne, 2002). Biofilms can release antigens and stimulate production of antibodies, but the bacteria living in biofilms are generally resistant to these defense mechanisms, providing the basis for persistent infections (Costerton, 1995). The microenvironment generated allows a symbiotic relationship of the species that make up the biofilm, providing necessary nutrients and allowing in some cases, due to the close proximity between different species involved, the transmission of genetic elements (Roberts *et al*., 1999).

Biofilms can be composed of populations derived from a single species or a community of diverse species but, in both cases, the development of biofilm behavior requires the multistage approach described above (Davey and O´Toole, 2000). Biofilm formation and its structure are affected by many conditions such as surface properties, nutrient availability, hydrodynamics and microbial community (Davey and O´Toole, 2000). The biofilm cells have different metabolic activities, depending on their position within the biofilm and can change over time.

Many species have been shown to use the same steps in biofilm formation, which include 1) an initial adhesion to the surface, 2) formation of the microcolonies 3) ripening microcolonies in the developing matrix thereby allowing the maturation of biofilm and 4) dispersion of planktonic bacteria (Figure 1.10). It is believed that the biofilm formation bacteria start when certain environmental factors trigger the fluctuations in cell-population density (Miller and Bassler, 2001) through a mechanism called quorum

sensing (O´Toole *et al*., 2000). Since these communities are dynamic, there is also a great exchange of information through the mechanism of quorum sensing, which allows them to better adapt their phenotype and gene expression to changes in the surrounding environment. Many bacteria use this mechanism to regulate a diverse array of physiological activities (Miller and Bassler, 2001).

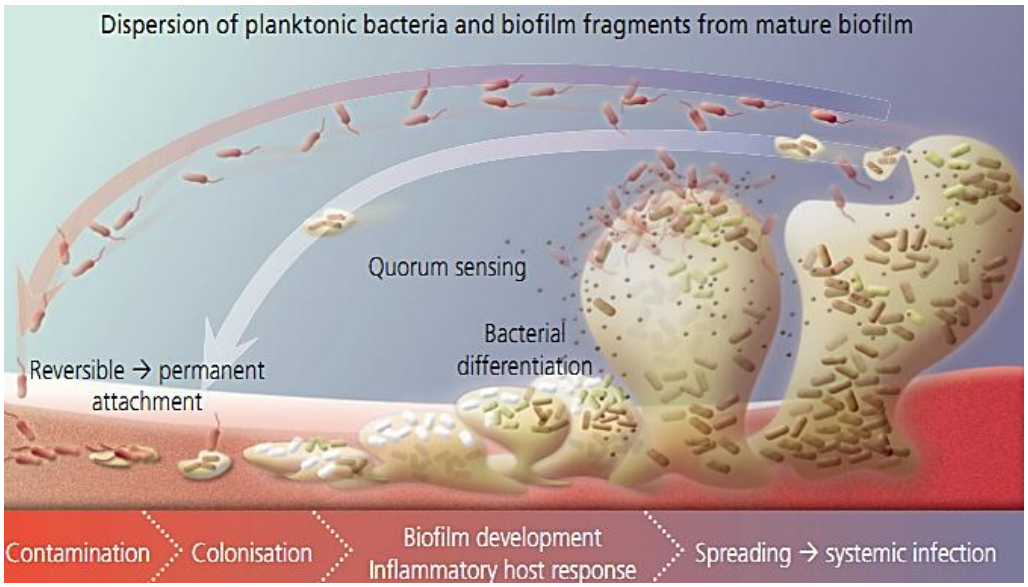


Figure 1.10. Schematic representation of the stages of microbial biofilm forming multiple bacteria development. The progression of biofilm, initiated with bacterial contamination, followed by colonization, leading to critical colonization and systemic infection. Adapted from Phillips *et al*., (2008).

In fact, direct evidence of microscopic analysis of biopsies performed on women with BV revealed the presence of bacterial biofilm on the vaginal epithelium (Swidsinski *et al*., 2005), and examining the composition and structural organization of the vaginal biofilm, has revealed that *G. vaginalis* accounted for 60 - 95% of the biofilm mass, *A. vaginae* accounted for the 1 - 40% of the biofilm mass whereas *Lactobacillus* maked up only 5% of the biofilm (Polatti, 2012). Studies using therapeutic treatments, like the antimicrobial agent metronidazole, revealed that biofilm formation is a decisive factor in the virulence of BV as it could not act in biofilm leading to relapse (Polatti, 2012). Because microorganisms in biofilms react differently to antibiotic treatment when compared with their planktonic counter-parts (Fux *et al*., 2005), antibiotic resistance is postulated as one of the reasons for persistent and recurrent BV (Polatti, 2012).

In vitro experiments show that *G. vaginalis* has a propensity to form biofilm (Figure 1.11). Patterson *et al.*, (2010) reported to presence of a protein associated with biofilm homologous to other biofilm-associated proteins (BAP) and BV. In *G. vaginalis* isolates the gene identified by Patterson *et al.*, (2010), is called by *Bapl* - Like (BapL). BAP are large proteins, which are anchored to the cell wall with the capacity to mediate adherence to host cells and intracellular adhesion, thus contributing to biofilm formation (Latasa *et al.*, 2006).

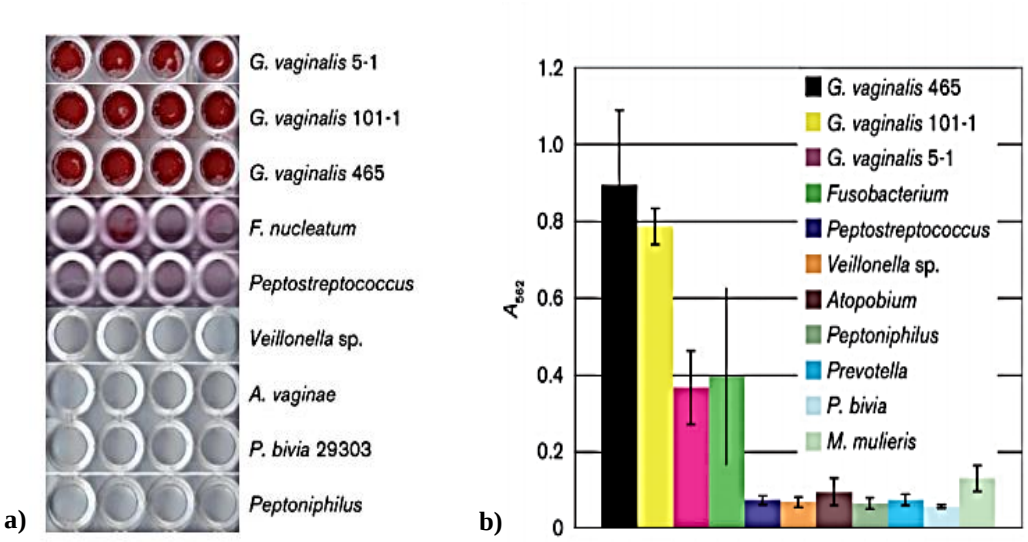


Figure 1.11. Biofilms formation of strains of *G. vaginalis* and anaerobic bacteria associated with BV. **a)** Bacteria were grown anaerobically in Brain Heart Infusion Broth supplemented with glucose (sBHIG) at 37°C for 24 h. The adhered cells were stained with safranin; **b)** Quantitative assessment of the capability of formation of the biofilm formation was made by dissolving safranin stain in 33% acetic acid and measuring optical density (OD) OD_{562}. Adapted from Patterson *et al.*, (2010).

Summarizing, there is strong evidence that *G. vaginalis* has an innate pathogenic potential compared to other BV-associated anaerobes. However there may still be many virulence factors not yet described, or maybe *G. vaginalis* has the potential of becoming more pathogenic in the presence of other species. It is also likely that the main agent of BV, if any, varies from case to case, however, several studies suggest that due to the many factors of virulence present in *G. vaginalis*, it is likely that the key agent in certain cases of BV is this bacterial species.

1.5. Outline and objectives of this thesis

In one study publish this year it was shown that BV is a prevalent gynaecological disorder in Portugal and that additional studies should be done to better characterize it in epidemiological and microbiological terms (Henriques *et al*., 2012). Thus, the characterization of vaginal microflora is a very important tool for the understanding of the vaginal flora associated with BV and can help investigate the significance of this condition in clinical pathology and to guide treatment (Srinivasan and Fredericks, 2008).

The main objective of this thesis is the study of clinical vaginal swab samples of Portuguese patients healthy or with BV. For this, this thesis is divided into 4 chapters. The first includes a general introduction to the work and includes the major implications and treatment of this condition. The methods, results and their discussion are separated into different sections, each one corresponding to a part of the experimental work. Chapter 2 concerns the studies of isolation the *G. vaginalis* and others microorganisms belonging to the vaginal flora, followed by characterization of the biofilm formation capacity and susceptibility of these strains by determining the MIC in chapter 3. The last chapter outlines the main conclusions and suggestions for future work.

Knowing more about how bacterial communities in the human vagina promoting health or facilitate disease is important, in order to optimize reproductive health.

CHAPTER 2

Isolation and identification of microorganisms from clinical vaginal samples

2.1. Introduction

The identity and diversity of populations of vaginal microflora remains largely obscure and the complex interactions of the various members of the vaginal flora are still poorly understood. However, as described in chapter 1, in recent years several new fastidious bacteria have been identified that show a high specificity for BV (Fethers *et al*., 2012). These microorganisms have been evaluated using cultivation-dependent methods of vaginal fluid samples and it was observed that most species require special conditions of culture media and incubation. Traditional methods of biochemical characterization such as oxidase, catalase reactions and Gram staining allow the identification of various microorganisms but fail to identify many vaginal microorganisms (Ledger and Witkin, 2007). On the other hand, recently, molecular biology techniques (cultivation-independent) have provided new insights regarding bacterial diversity in vaginal flora, particularly in women with BV (Zhou *et al*., 2004). What is currently accepted is that healthy women are mainly colonized by lactobacilli, though a variety of other bacteria may be present in BV (Fethers *et al*., 2012).

2.2. Objectives

The main objectives of the work described in this chapter were the isolation and identification of microorganisms from clinical vaginal samples in women with or without BV. The isolation of these microorganisms was necessary to enable phenotypic characterization of selected isolates in the next chapter of this thesis.

2.3. Materials and Methods

2.3.1. Study population and sample collection

The collection of clinical vaginal samples from patients with suspected BV and healthy vaginal flora was performed with the collaboration of some Portuguese gynecologists who performed the collections at their private clinics. All volunteers were asked to complete a questionnaire form which data was analyzed by another member of our research group (Salgueiro, 2012) and clinically examined. The doctors proceeded to the collection of the samples using an unlubricated sterile swab with Amies transport medium provided with coal (VWR) (Al-Muk and Hasony, 2001). The swabs were transported, within 48 h of collection, to the laboratory. As soon as the clinical samples

arrived at the Laboratory of Applied Microbiology 1 (LAM 1), of the Department of Biological Engineering, University of Minho, they were processed (Kharsany *et al*., 1993).

2.3.2. Treatment of the clinical samples

Each sample was used to make a smear for Gram stain (as described in section 2.3.3.2) and the evaluation of vaginal microflora of each patient and BV diagnosis were based on the method described by Nugent *et al*., (1991). After this, each sample was placed in selective culture media (as described in 2.3.3.1) and the swab was inserted into a plastic tube containing 2 ml of sterile saline solution of 0.9% sodium chloride (NaCl) (ProLAbo) which was vortexed vigorously until the solution becomes cloudy, so as to recover all the bacterial cells still present in the swab. Then, 1 mL was transferred into 2 eppendorfs, and centrifuged at 10,000 rpm for 5 min. At the end, the supernatant was discarded leaving only the pellet in eppendorfs (BIOplastics); one of them was used to prepare suspensions to be used for PCR (for selection of positive samples for *G. vaginalis*, as described in 2.3.3.3) by resuspending the pellet in ultra-pure water and the remaining pellet was used to prepare a cryovial containing the pellet resuspended in 800 µL of Brain Heart Infusion (BHI) broth (Oxoid) with 200 µL of pure sterile glycerol (Panreac) (final concentration of glycerol 20%). All samples were then stored in cryovials (VWR) properly identified and stored at -80°C. The cryopreservation of samples allows the preservation of the genotypic and phenotypic characteristics of bacteria, ensuring that the characteristics remain intact for further studies, decreasing the risk of bacterial contamination or fungal.

2.3.3. Isolation and identification methods

2.3.3.1. Culture methods

Media and growth conditions

Selected samples were cultured on Columbia Blood Agar Base (CBA) (Liofilchem) supplemented with 5% of defibrinated horse´s blood (Probiologica) with *G. vaginalis* selective supplement (Oxoid) or CBA without supplement; Man-Rogosa and Sharpe Agar (MRS) (Liofilchem) that were prepared according to the manufacturer´s instructions and Bromocresol Purple Starch Agar (Purple) which is

composed by 7.5 g/L of Peptone from Meat Peptic Digest (Merck); 7.5 g/L of Tryptone (Liofilchem); 1 g of Corn Starch from Potato (Pancreatic); 4 g/L of Dipotassium Phosphate (K_2HPO_4) (Panreac); 1 g/L of Monopotassium Phosphate (KH_2PO_4) (Panreac); 5 g/L of NaCl (ProLabo); 12 g/L of Agar (FisherScientific); 10 g/L of Starch soluble (FisherScientific); 0,0096 g/L of Bomocresol Purple (Acros-organics) and in the end of sterilization (121ºC for 15 min), after cooling, 30 µg/mL of Nalidixic Acid (Biochemica) (5 mg/mL) was added to the Purple agar. Plates (Frilabo) were incubated in 10% carbon dioxide (CO_2) for 72 h at 37ºC in anaerobic environment.

2.3.3.2. Isolation and identification of bacterial isolates

After growth of the samples on a selective culture medium growth, the colony morphologies were analyzed in order to proceed to the isolation and identification of microorganisms in each selected vaginal swabs. Once isolated strains were obtained they were characterized according to colony morphology, Gram staining, and biochemical tests such as oxidase and catalase tests (Forbes *et al*., 2007), as described below.

We analyzed the growth of each of the samples in different growth media and isolated the different colonies observed in their respective growth media, using the microbial streaking technique (Figure 2.1). Typically pure cultures were obtained by the 2^{nd} generation. If by the 2^{nd} generation we still did not have isolated strains, a suspension of the mixed cultures was used and serial decimal dilutions in 0.9% saline solution of NaCl were performed and plated in an agar plate, divided into eight quadrants and 5 µl of each dilution placed in the respective quadrants. In subsequent generations isolated colonies were obtained. To confirm the isolation Gram staining, oxidase, catalase and PCR were performed. Finally, the 16S gene was sequenced, as described below on section 2.3.3.3.

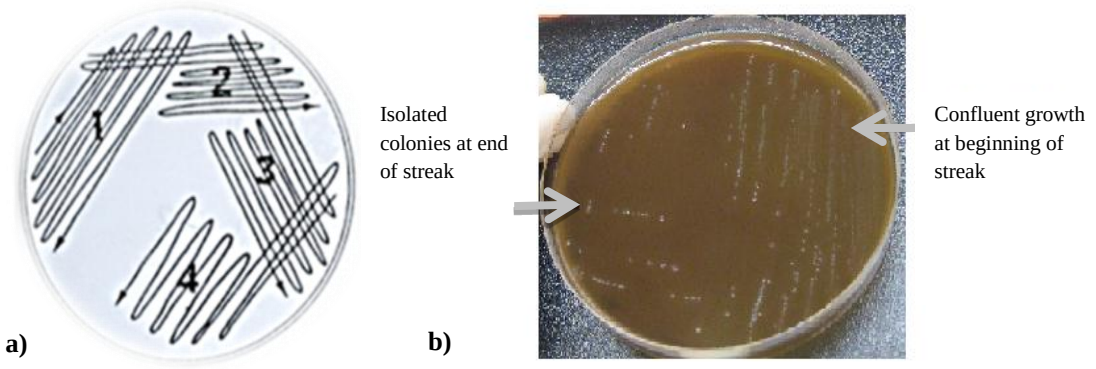


Figure 2.1. Method of making a streak plate to obtain pure cultures. **a)** Loop is flame-sterilized, and then a loopful of one colony is removed from plate. Streak is made over a sterile agar plate, spreading out the microorganisms. Following the initial streak, subsequent streaks are made at angles to it, the loop being re-sterilized with heat between streaks. Adapted from Seeley, Seeley *et al*., (1991); **b)** Colonies of the bacterium *G. vaginalis* 5-1 grown on CBA plates. This bacterium was isolated by Patterson *et al*., (2010) from women without BV as diagnosed by the Nugent Gram stain scoring system.

Gram stain

Gram staining was performed in two occasions: when the samples arrived a smear (from the original sample); and when the isolation of microorganisms was finished to make sure it was pure culture. In both cases, the fixation step was performed using heat holding the swab over a flame until all the water evaporated. Staining was done in several steps: 1) the swab was covered with crystal violet (primary dye) (AppliChem) with gentle shaking for 60 seconds; 2) sample was washed with water; 3) the smear was covered with Lugol´s solution iodine (AppliChem) leaving to react for 60 s, forming a complex with crystal violet; 4) sample was washed with water; 5) the smear was then covered with alcohol 70% (bleaching agent), stirring gently for 60 s; 6) Finally, the sample was covered with safranin solution (Liofilchem) for 30 s, and finally washed and dried with filter paper. At the end of the Gram staining, smears were observed using a light microscope, in order to characterize the Gram staining reaction (positive = purple or negative = pink) and noting their morphology. Controls were used for each individual display; negative Gram stain reaction control - *Escherichia coli* (Turovskiy *et al*., 1991) and as positive Gram stain reaction control *Staphylococcus epidermidis* (Rowlinson *et al*., 2006) (Figure 2.2).

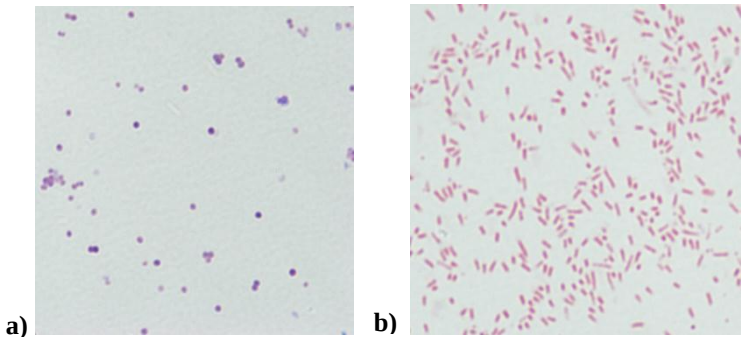


Figure 2.2. Gram staining of isolates that we prepared (original magnification, x1000). Gram stain showing **a)** Gram-positive (purple) *Staphylococcus epidermidis* bacterium; **b)** Gram-negative (pink) *Escherichia coli* bacterium.

Biochemical tests

Single colonies of all microorganisms obtained from the isolation procedure were typed, using 2 biochemical tests: oxidase and catalase test.

Oxidase test: The oxidase test is used in microbiology to determine whether a bacterium produces cytochrome c oxidase (Jurtshuk and Liu, 1983). For this, oxidase strips (Oxoid) containing NNN'N'-tetramethyl-p-phenylene-diamine dihydrochloride (TMPD) were used. This reagent turns dark blue when oxidized and colorless when reduced. When it comes in contact with oxidase-positive bacteria that have indophenol oxidase or cytochrome oxidase the indicator strip turns blue (Isenberg, 2004). On each strip a small portion of the test biomass was added and observed after 20 s. If the area of inoculation turns dark blue to brown then the result is positive. If the color does not change within the allotted time, the result is negative. The control strains used in this test were, as a positive control *Pseudomonas aeruginosa* and as negative control *E. coli* (Gaby and Hadley, 1957).

Catalase test: To perform the catalase test, a drop of H_2O_2 was added onto a microscope slide, using a sterile loop. Then, a small portion of the biomass to be analyzed is added and mixed. A positive catalase reaction was obtained when there was immediate release of oxygen (bubbles formed), which indicated the catalase activity (catalase acting on H_2O_2, water (H_2O) and releasing oxygen (O_2) according to the following reaction: $H_2O_2 \rightarrow 2\ H_2O + O_2$). Controls were used for the catalase reaction

negative control *G. vaginalis* AMD (Aroutcheva *et al*., 2001) and as positive control *S. epidermidis* (Rowlinson *et al*., 2006).

2.3.3.3. Molecular methods

Polymerase Chain Reaction (PCR)

The suspensions made for PCR from initial clinical samples, were treated with a thermal shock in order to break the cell wall and facilitating the release of DNA for the PCR reaction. To achieve this, samples were incubated for 15 minutes in a heating block at 100°C. After completing the incubation at 100°C, samples were immediately incubated on ice for at least 5 min. A master mix was prepared for the PCR reaction for n + 1 samples (where "n" is the number of samples to be tested) for each of combinations of primers to be used. Each PCR reaction (final volume of 10 µL) required 5 µL PCR 2x mix conc. (DyNazyme Master Mix: Finnzymes), 0.5 µL forward (Fw) primer, 0.5 µL reverse (Rv) primer, 0.5 µL sample (directly added to each tube and not to PCR master mix) and 3.5 µL of ultra-pure water. After the master mix was prepared, 0.5 µL of the samples were added to the respective PCR tubes and then 9.5 µL of master mix was added to each PCR tube. The specific primers used to identify *G. vaginalis*, *Lactobacillus* spp. or *A. vaginae* are described in table 2.1.The tubes were placed on a thermocycler (BioRad) and a thermocycling program was selected consisting on the following steps: 94°C for 2 min, 94°C for 30 s, 51°C for 30 s, 72°C for 40 s, repeat the step 2, 44 times, 72°C for 5 min and 4°C for ∞.

Gel Electrophoresis

PCR products were loaded on to an agarose gel which consisted of 1% (w/v) agarose in 1x Tris-acetate-EDTA (TAE) buffer. The agarose solution was heated in a microwave oven and then allowed to cool before adding 0.04 µL/mL of Midori Green DNA stain (Nippon Genetics Europe). Then, the mold was prepared with the desired comb and the agarose solution was poured into the mold. Once polymerized the gel was placed in the electrophoresis tank containing 1x TAE buffer and the comb removed, making sure that the wells were completely covered by the buffer. Before loading the samples on agarose gel, 1 µL of loading buffer was added to each PCR reaction (this turned the sample blue thus facilitating the loading of agarose gels; makes the sample settle at the bottom of the well). The samples were loaded onto the gel as well as the

DNA ladder (FastRuler Middle Range DNA Ladder - Fermentas). Finally the gel was run in the electrophoresis tank at 100 volts for 30 min and the results were visualized using the ChemiDoc (BioRad) system according to manufacturer's instructions.

The samples that tested positive for *G. vaginalis* of clinical samples (with and without BV) were selected for the isolation of the microorganisms, as described in section 2.3.3.2.

Table 2.1. Primer sequences used for the PCR assays used in this study.

Target	Primer/ Probe	PCR conditions	Primer/ Probe Sequence (5´- 3´)	References
G. vaginalis	Gard 154-454	60ºC annealing, 30 s 72ºC extension, 30 s	**Fw** CTCTTGGAAACGGGTGGTAA **Rv** TTGCTCCCAATCAAAAGCGGT	(Magalhães *et al*., 2011)
A. vaginae	Atop 167-715	60ºC annealing, 30 s 72ºC extension, 30 s	**Fw** GCGAATATGGGAAAGCTCCG **Rv** TCATGGCCCAGAAGACCGCC	(Pepin *et al*., 2011)
***Lactobacillus* spp.**	New Lacto	62ºC annealing, 30 s 72ºC extension, 30 s	**Fw** TGGAAACAGRTGCTAATACCG **Rv** GTCCATTGTGGAAGATTCCC	(Byun *et al*., 2004)

Bacterial identification by sequencing of 16S ribosomal DNA

PCR for Sequencing

The vaginal isolates presenting different characteristics such as colony morphology, and where Gram stains revealed pure cultures were selected and a single colony picked to perform PCR for production of fragments using universal primers to increase the amount of bacterial 16S ribosomal DNA (rDNA) sequences for the purposes of sequencing (Fw 5 'GTT TGA TCC AGA TGG CTC AG 3 '; Rv 5' CCA GGA ATC GGG CTA TAT TAA 3 ') (Wilson *et al*., 1990). The PCR protocol used is similar to that already described, but differed in some points. For each PCR reaction (final volume = 60 µL) was necessary to use 30 µL master mix (Phusion High-Fidelity PCR Master Mix, Finnzymes), 4 µL primers (Fw + Rv), 4 µL template (directly added to each tube and not to PCR master mix), 22 µL H_2O of ultra-pure water. The tubes were placed on a thermocycler (BioRad) with a thermocycling program which consisted of the following steps: 98°C for 2 min, 98°C for 10 s, 51°C for 30 s, 72°C for 30 s, repeat the step 2, 35 times, 72°C for 30 s and 4°C for ∞. The results of the PCR reaction

was visualized using gel electrophoresis as described before using a total volume of PCR product of 10 µL.

The PCR products amplified by the universal primers were further purified using a kit GeneJET PCR Purification Kit (Fermentas), according to manufacturer's instructions (GeneJET PCR purification Kit, 2012). The purified PCR products were sent along with the reverse primer to the company Eurofins MWG Operon (Germany) for sequencing.

Analysis of the nucleotide sequences amplified by PCR and sequenced by Eurofins was performed using the software Basic Local Alignment Search Tool (BLAST). The BLAST database, 16S ribosomal Ribonucleic acid (rRNA) gene sequences (Bacteria and Archaea) (available http://blast.ncbi.nlm.nih.gov/Blast.cgi), allows investigation of the nucleotide sequences obtained considering the homology contained in this database of the National Center for Biotechnology Information (NCBI), allowing the determination of the statistical significance of the matches.

2.4. Results and discussion

2.4.1. The importance of the vaginal microflora characterization

Molecular techniques have been shown to be very important in diagnosis of infectious diseases, promoting fast identification of clinically relevant microorganisms in BV. Over the years, many attempts have been made to improve traditional and molecular techniques in order to learn more about the human vaginal flora. Their characterization is an important step at understanding the pattern of flora associated with BV that is typically characterized by the replacement of normal vaginal microflora of lactobacilli by *G. vaginalis* and other anaerobic microorganisms. Unfortunately, much of what is known about the human vagina microbial flora is due to qualitative and descriptive studies that relied on the characterization of bacterial populations easily cultivable (Marrazzo *et al*., 2002). The technical limitations associated with classical microbiological culture techniques have unwittingly affected the ability of clinicians to understand the etiology of this clinical condition (Zhou *et al*., 2004).

The main objective of this study was the isolation and characterization of microorganisms belonging to the vaginal flora. The main problem faced in the isolation of some microorganism’s lies with the fact that they have a fastidious growth and can

lose viability very easily, as described in chapter 1. The isolation procedure was optimized, as described in section 2.3.3.2 of Materials and Methods. We were able to adjust the isolation procedure, and confirm isolates identity by doing Gram stain, biochemical tests and sending isolates for sequencing of the 16S rRNA gene in order to obtain the identity of isolates (Table 2.4). With the sequencing results it was possible to make an accurate identification of isolated microorganisms. This was important because although the biochemical tests, Gram staining, growth conditions and other tests allow us to reach some conclusions and provide information about each of the microorganisms isolated, these traditional techniques do not provide enough information to allow us to reach firm conclusions about the true identity of the microorganisms. There were organisms recovered with the same characteristics in terms of cultivability and cellular composition, which yielded similar results with the biochemical tests used. All microorganisms isolated in this study are shown in table 2.2 and 2.3.

2.4.2. Isolation and identification of vaginal isolates

We received 54 clinical samples, but only 8 samples were positive for *G. vaginalis*, as determined by PCR, and only these samples were selected to be cultured. As such, we studied the composition of the vaginal microflora of those 8 clinical vaginal samples. Some came from women with BV and others from women which presented healthy vaginal flora. In these clinical samples it was possible to isolate 16 strains. Detailed observation of these isolates characteristics such as BV situation, requirements of growth conditions, morphotypes, identity and characteristics of isolated strains are summarized in table 2.2 and 2.3.

2.4.2.1. Sequencing results

Table 2.4 shows the results of strains identification by 16S rRNA gene sequencing. The sequencing results were analyzed using bioinformatics methodology, such as the use of the BLAST database (as described in Materials and Methods) and are presented in Supplements section - S.1. The microorganism's identification was obtained by comparing the maximum percent identity and the percent query coverage obtained when comparing the isolate sequence with sequences contained in the database. The maximum percent identity reveals the highest percentage of identical

bases found within the match, while the maximum score relates to the sequence similarity percentage that corresponds to the consultation. The query coverage, however, shows the percentage of the query sequence that matches the subject sequence (GenBank Overview, 2012: available http://www.ncbi.nlm.nih.gov/genbank/).

It was not possible to identify the species for 4 microorganisms isolated, *Escherichia* spp., *Gemella* spp., *Klebsiella* spp. and *Streptococcus* spp. as BLAST results only allowed to classify these strains to genus level. For instance, in the case of the genus *Streptococcus* spp., the percent query coverage and the maximum percent identity was the same, 100% and 99% respectively, for 3 microorganisms (*Streptococcus salivarius*, *Streptococcus thermophilus* and *Streptococcus vestibularis* strains). Similar results were obtained in the mentioned strains. In the future, work should be carried out in order to sequence other genes to identify these microorganisms to species level.

Table 2.2. Microorganisms isolated from 4 clinical vaginal swabs from women with BV (UM027, UM034, UM035 and UM054).

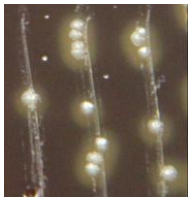
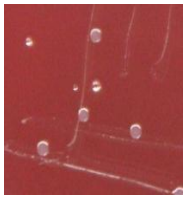
		Features of microorganisms isolated				
					Bioquimic tests	
Vaginal isolates (Results of Sequencing)	**Bacterial Vaginosis**	**Nutritional requirements of growth**	**Morphotype of colony**	**Gram staining**	**Catalase test**	**Oxidase test**
Bacillus firmus UM034	+	CBA without supplement; facultative anaerobic		Positive bacilli	+	-
Enterococcus faecalis UM035	+	CBA without supplement media; facultative anaerobic		Positive cocci	-	-
Gardnerella vaginalis UM035	+	CBA without supplement media; facultative anaerobic		Positive short rods	-	-

Table 2.2. (*Continued*).

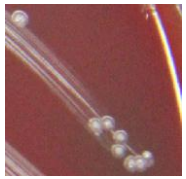
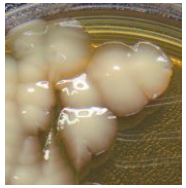
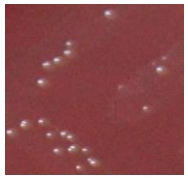
	Features of microorganisms isolated					
					Bioquimic tests	
Vaginal Isolates (Results of Sequencing)	**Bacterial Vaginosis**	**Nutritional Requirements of growth**	**Morphotype of colony**	**Gram staining**	**Catalase test**	**Oxidase test**
Gemella spp. UM034	+	CBA without supplement media; facultative anaerobic		Positive cocci	-	-
Klebsiella spp. UM034	+	MRS media; facultative anaerobic		Negative rods shaped	-	-
Mobiluncus curtisii UM027	+	CBA without supplement media; facultative anaerobic		Positive curved rods	-	-

Table 2.2. (*Continued*).

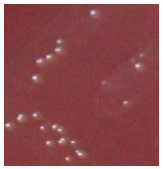
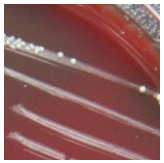
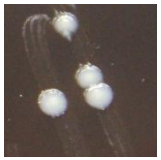
		Features of microorganisms isolated				
				Bioquimic tests		
Vaginal Isolates (Results of Sequencing)	**Bacterial Vaginosis**	**Nutritional Requirements of growth**	**Morphotype of colony**	**Gram staining**	**Catalase test**	**Oxidase test**
Mycoplasma hominis UM054	+	CBA without supplement media; facultative anaerobic		Positive short rods	-	-
Propionibacterium acnes UM034	+	CBA without supplement media; facultative anaerobic		Positive rods-shaped	+	-
Streptococcus agalactiae UM035	+	CBA without supplement media; facultative anaerobic		Positive cocci	-	-

Table 2.3. Microorganisms isolated from 4 clinical vaginal swabs from women without BV (UM016, UM022, UM031 and UM042).

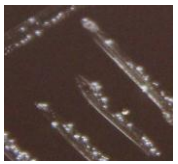
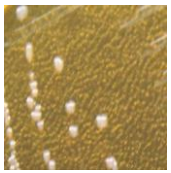
		Features of microorganisms isolated				
					Bioquimic tests	
Vaginal Isolates (Results of Sequencing)	**Bacterial Vaginosis**	**Nutritional Requirements of growth**	**Morphotype of colony**	**Gram staining**	**Catalase test**	**Oxidase test**
Alloscardovia omnicolens UM031	-	CBA without supplement media*;* facultative anaerobic		Positive short irregularly shaped rods	-	-
Bifidobacterium breve UM031	-	PURPLE media; anaerobic		Positive rod-shaped branches	-	-
Escherichia spp. UM022	-	MRS media; facultative anaerobic		Negative rods-shaped	-	-

Table 2.3. (*Continued*).

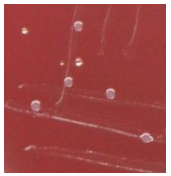
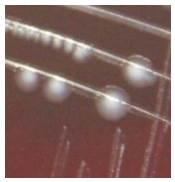
		Features of microorganisms isolated				
					Bioquimic tests	
Vaginal Isolates (Results of Sequencing)	**Bacterial Vaginosis**	**Nutritional Requirements of growth**	**Morphotype of colony**	**Gram staining**	**Catalase test**	**Oxidase test**
Gardnerella vaginalis UM016	-	CBA with supplement media; facultative anaerobic		Positive short rods	-	-
Lactobacillus gasseri UM022	-	CBA without supplement media; facultative anaerobic		Positive large rods-shaped	-	+
Staphylococcus epidermidis UM042	-	CBA without supplement media; facultative anaerobic		Positive cocci	+	-

Table 2.3. *(Continued).*

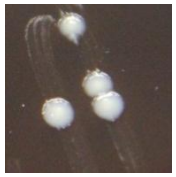
		Features of microorganisms isolated				
					Bioquimic tests	
Vaginal Isolates (Results of Sequencing)	**Bacterial Vaginosis**	**Nutritional Requirements of growth**	**Morphotype of colony**	**Gram staining**	**Catalase test**	**Oxidase test**
Streptococcus spp. UM031	-	CBA without supplement media; facultative anaerobic		Positive spherical	-	-

Table 2.4. Results of sequencing of microorganisms isolated of vaginal clinical samples using BLAST.

	BLAST Results			
Samples	**Microorganisms identified by sequencing**	**Base pairs amplified**	**Percent Query Coverage**	**Maximum Percent Identity**
UM016	*Gardnerella vaginalis*	612	100%	99%
UM054	*Mycoplasma hominis*	313	100%	99%
UM022	*Escherichia* spp.	578	100%	99%
	Lactobacillus gasseri	793	99%	99%
UM027	*Mobiluncus curtisii*	754	98%	99%
UM031	*Alloscardovia omnicolens*	623	100%	99%
	Bifidobacterium breve	753	98%	99%
	Streptococcus spp.	754	100%	99%
UM034	*Bacillus firmus*	744	100%	98%
	Gemella spp.	745	99%	99%
	Klebsiella spp.	734	100%	99%
	Propionibacterium acnes	745	99%	99%
UM035	*Enterococcus faecalis*	759	100%	99%
	Gardnerella vaginalis	458	99%	98%
	Streptococcus agalactiae	329	98%	99%
UM042	*Staphylococcus epidermidis*	206	98%	99%

2.4.3. Associations between vaginal bacterial communities in women with and without BV

The isolates of vaginal cultures obtained were very distinct despite all the isolation process was made in the same conditions (as described in 2.3.3.2. of Materials and Methods) except for the medium used, because each isolate needed a specific growth media (Table 2.2 and 2.3). Using this methodology, 15 different species related to BV conditions or related with vaginal tract were identified, *B. breve, E. faecalis, Escherichia* spp., *G. vaginalis*, *Gemella* spp., *Klebsiella* spp., *L. gasseri, M. curtisii*, *M. hominis*, *P. acnes*, *S. agalactiae*, *S. epidermidis*, *Streptococcus* spp., as described previously in literature (Verhelst *et al*., 2005) except for 2 strains, *A. omnicolens* and *B. firmus* that were not previously identified in vaginal samples.

2.4.3.1. Bacteria isolated from BV- diagnosed vaginal clinical samples

Gardnerella vaginalis

The family *Bifidobacteriaceae* consists of only two genera, named *Bifidobacterium* and *Gardnerella* (Rosenstein *et al*., 1996). Typically *Bifidobacteriaceae* family members are considered non-pathogenic microorganisms. However the bacterium *G. vaginalis* has been recognized as the main opportunistic pathogen in BV (Forsum *et al*., 2005) as described previously in chapter 1. This microorganism was isolated of one sample with BV (UM035) and another *G. vaginalis* from one healthy vaginal sample (UM016). Both of them are Gram-positive, oxidase and catalase-negative bacteria.

Enterococcus faecalis

One of the bacterium which is often isolated from vaginal samples is *E. faecalis* formerly classified as part of the group D *Streptococcus* system, is Gram-positive cocci and belongs to the family *Enterobactereaceae*. This microorganism was isolated from one clinical vaginal swab during this study, from a sample with BV, UM035. Previous studies revealed that *E. faecalis* was found in patients with signs of BV in 52.78% of cases (Jahić *et al*., 2006). The same study showed that pH change of vaginal environment was associated with an increase prevalence of *E. faecalis*, in BV cases but BV diagnosis is not predictable of the presence of *E. faecalis* in vaginal discharge.

***Gemella* spp.**

Gemella is a genus of Gram-positive cocci bacteria that thrive best at high partial pressure of carbon dioxide (CO_2). The specie of the microorganism of this genus recovered in this study was not clearly identified. However, the possible identifications obtained from the sequence were *G. haemolysans*, *G. sanquinis* and *G. morbillorum*, with *G. haemolysans* yielding a superior maximum percent identity than of the other 2 species which suggests that this strain is possibly *G. haemolysans*. On the other hand *G. haemolysans* strains have not previously been described as a species associated with BV. Previously *G. morbillorium* has been isolated from vaginal samples and is considered BV-related organism (Verhelst *et al*., 2005). *G. haemolysans* are residents of the mucous membranes of humans and of certain warm-blooded animals and are recognized opportunistic pathogens (Collins *et al*., 1999). They can occur as individual cells, short chains, or irregular clusters, as in the case of *G. morbillorum*. The members of

this specie can grow at a wide range of temperatures with optimum growth occurring between 35 - 37°C. *G. haemolysans* is facultative anaerobic and negative for catalase and oxidase tests (Ruoff, 2007).

***Klebsiella* spp.**

In our study we isolated one strain of *Klebsiella* spp. from a sample of woman which had been diagnosed with BV (UM034). Studies have revealed that these strains are a successful opportunistic pathogen associated with various ailments such as urinary tract infections, septicaemia, respiratory tract infections and diarrhea. The specie of this microorganism was not clearly identified due to the similarity detected in sequence between the sequences obtained for *Klebsiella pneumoniae* and *Enterobacter aerogenes* (Supplements section S.1 – *Klebsiella* spp.). *Klebsiella* spp. belongs to the family *Enterobactereaceae* and this is described as Gram-negative rod shaped bacterium, non-motile, encapsulated, lactose fermenting and facultative anaerobic found in the normal flora of the mouth, skin, and intestines (Carey and Klebanoff, 2001). Previous study reported that microorganisms belonging to the family *Enterobactereaceae*, including *Klebsiella* spp., were present in about 38% of samples from vaginal tract. However, these results may reflect a possible contamination of the vaginal tract by microorganisms normally associated with intestinal tract. Colonization with *Enterobacteriaceae* strains may also be a predisposing factor for urinary tract infection in women (Ovalle and Levanci, 2001). Although this microorganism has been isolated from vaginal tract previously, no study has been reported regarding its role and its influence on the vaginal tract.

Mobilluncus curtisii

This microorganism belongs to the family *Actinomycetaceae* and was isolated from a sample of a woman with BV (UM027). After isolation and identification of *M. curtisii* UM027 the strain was stored at -80ºC with glycerol, however when further experiments to further characterize this strain were attempted, we were not able to recover it from storage, perhaps due to its fastidious characteristics. Some others vaginal samples also had *Mobiluncus* spp. (as seen in the initial Gram staining), and their presence and clue cells in vaginal smears has been clinically correlated with BV (Sweet, 2000) but we did not succeed at isolating it. Gram stains of vaginal flora from women with BV, present a wide spectrum of abnormalities in the vaginal ecosystem. Gram-positive and curved

rods, felt to represent primarily *Mobiluncus* spp., may be seen in some but not all women with BV as described in some studies (Nugent *et al*., 1991). One study performed by Sweet (2000) described that some women where *Mobiluncus* spp. and clue cells were detected appeared have a higher risk of post-abortion pelvic inflammatory disease than women without presence of these microorganisms. However, whether this organism is really pathogenic or simply indicator for disorders of vaginal flora, remains unclear (Meltzer *et al*., 2008).

Mycoplasma hominis

M. hominis belongs to the family *Mycoplasmataceae* and have only been found in large numbers in the late stages of full-blown BV (Rosenstein *et al*., 1996). This microorganism was isolated from a sample with BV, UM054 and is a Gram-positive short rods bacterium. The presence of *M. hominis* may indicate additional risk factors for complications when found in women with BV. *M. hominis* infection was associated with a fishy smelling vaginal discharge, vaginal pH above 4.7, clue cells, and a positive amine test. Women infected with *M. hominis* typically had more sexually risky behavior and a greater likelihood of previous induced abortion or sexually transmitted disease than uninfected women (Mårdh *et al*., 1997).

Propionibacterium acnes

One other bacterium isolated was *P. acnes* belonging to the family *Propionibacteriaceae* that was isolated from a sample of a woman with BV (UM034). This strain is relatively slow growing, largely commensal (part of the skin microflora present on most healthy adult human skin) (Brüggemann *et al*., 2004), typically aerotolerant anaerobic Gram-positive rod bacterium. *P. acnes* are found briefly on the skin of neonates and it may also be found throughout the gastrointestinal tract in humans, and true colonization is thought to begin during the 1 - 3 years prior to sexual maturity. *P. acnes* bacterium is also suspected to be subtly involved in post-operative infections, prostheses failure, and more recently, in inflammation of lumbar nerve roots leading to sciatica (Bhatia *et al*. 2004). Strains such as *Peptostreptococcus anaerobius*, *P. acnes* and *E.coli* have been isolated significantly more frequently from vagina of women infected with *Chlamydia* and these findings confirm importance of indole-producing bacterial strains in cervicitis caused by *C. trachomatis* (Romanik *et al*., 2007).

Streptococcus agalactiae

S. agalactiae is a β-hemolytic bacterium (grown in CBA without supplement) and belongs to the streptococcus group B and to the family *Streptococcaceae*. This bacterium is facultative, fastidious, Gram-positive cocci and can be found as part of normal vaginal, intestinal, and oral flora. The virulence of the organism depends largely on the polysaccharide capsule (Cribby *et al*., 2008). This microorganism may cause various infections in adults, but the main clinical interest in these bacteria relates to their ability to cause serious neonatal illness, especially meningitis and sepsis. While neonates born by caesarian have presented with *S. agalactiae* infection, indicating ascending transmission of the microorganism from the vagina of their mothers it is considered of extreme importance in births (Regan *et al*., 1981). *S. agalactiae* was isolated from a woman that was diagnosed BV (UM035). 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.

2.4.3.2. Bacteria isolated from healthy vaginal clinical samples

***Bifidobacteriaceae* spp.**

Gardnerella vaginalis

These bacteria have been described throughout this thesis as a major species present in BV, but that can also be isolated from women without any signs or symptoms of infection. Thus, it was not surprising that we isolated one *G. vaginalis* strain from a healthy woman sample (UM016).

Bifidobacterium breve

This bacterium also belongs to the family *Bifidobacteriaceae* as *G. vaginalis,* is a Gram-positive rod-shaped branched bacterium and oxidase and catalase-negative. *B. breve* strain was isolated in this study from healthy woman (UM031). *Bifidobacterium* spp. was previously found in 12% of healthy controls, in 58% of those with intermediate microflora and in 94% of patients with BV (Hyman *et al*., 2005). *Bifidobacterium spp.* has been described as being involved in the gradual conversion from normal to BV

microflora, which in turn, may implicate a rectal-vaginal pathway in the etiology of BV (Swidsinski *et al*., 2010).

***Escherichia* spp.**

We also isolate a strain from the genus *Escherichia* from a healthy woman, UM022, but we did not identify the species of this microorganism as previously described. The options obtained in the BLAST analysis reported 4 strains belonging to the family *Enterobacteriaceae*, *Escherichia fergusonii*, *Shigella flexneri*, *Shigella dysenteriae* and *E. coli*. The genus *Shigella* is divided into four groups (or species), *S. boydii*, *S. dysenteriae*, *S. flexneri* and *S. sonnei*. Closely interrelated among them and to *E. coli*, which in reality belongs to the extremely diverse species *E. coli* (Lan and Reeves, 2002). *E. coli* strains are normally isolated from vaginal samples, but *E. fergusonii* has never been isolated from a vaginal sample. *E. fergusonii* has been associated with a wide variety of intestinal and extra intestinal infections (Mahapatra *et al*., 2005). A recent study, demonstrated that *E. fergusonii* strains have a great potential to cause human disease (Oh *et al*., 2012) and *Shigella* spp. members are widely known to cause human disease (Parsot, 2005).

Lactobacillus gasseri

In our study we isolated one *L. gasseri* strain belonging to the family *Lactobacillaceae*, from a healthy woman (UM022) these are a Gram-positive large rods-shaped, catalase-negative and oxidase-positive. In one study reported that *L. gasseri*, *L. iners*, and *L. jensenii* were cultured from 85%, 68% and 43% of healthy women; and 28%, 89% and 44% of BV women, respectively. The quantities of first 2 *Lactobacillus* species, had a significant difference between healthy women and women with BV as such the presence and quantity of *Lactobacillus* has an important guiding role in bacteria diagnostic (Dong-hui *et al*., 2009).

Staphylococcus epidermidis

S. epidermidis belongs to the family *Staphylococcaceae* and is a well-characterized, non-fastidious, anaerobic Gram-positive cocci commonly isolated in the clinical microbiology laboratory (Rowlinson *et al*., 2006). Although coagulase-negative staphylococci, including *S. epidermidis*, are often considered a contaminant in the clinical laboratory, an increasing number of reports describe their pathogenesis, in particular in infections of prosthetic devices and their involvement in nosocomial infections (Huebner

and Goldmann, 1999). This isolate was unique because it grew only under strictly anaerobic conditions. In this study, the strain of *S. epidermidis* isolated came from a woman without BV (UM042) and it is possible that this isolate is the result of contamination during the collection of the vaginal sample.

***Streptococcus* spp.**

We was also isolated *Streptococcus* spp. from healthy vaginal women (UM031), it is a Gram-positive spherical bacterium that we were not able to identify the species level through sequencing as it is very difficult to differentiate certain strains of *Streptococcus* spp. like *S. salivarius*, *S. thermophilus* and *S. vestibularis*. We obtained the same percent query coverage and maximum percent identity between the 3 potential candidates (Supplements section - S.1). Streptococci are divided into 6 phylogenetic groups: *S. anginosus*, *S. bovis*, *S. mitis*, *S. mutans*, *S. pyogenic*, and *S. salivarius*, with the group of *S. salivarius* consisting of only 3 distinct species, *S. salivarius* and *S. vestibularis*, members of the human normal microflora orally while the third, *S. thermophilus*, is found in bovine milk, all closely related (Pombert *et al*., 2009). Although, *S. agalactiae*, *S. anginosus*, *S. gallolyticus*, *S. mitis* and *S. salivarius* have been previously isolated from the sample of vaginal exudate (Verhelst *et al*., 2005). *S. salivarius* was found to account for 2% of total microorganisms in healthy vaginal flora and its presence in BV samples has been considered not to be significant for the pathology as it is present in lower numbers (Rabe *et al*., 1988).

2.4.3.3. New strains isolated in BV- diagnosed and healthy vaginal samples

There have been many recent additions to our knowledge regarding the human vaginal microbiota. Several methods, such as PCR and sequencing, have been applied to the study of the human vaginal microbial ecosystem, adding substantial data on bacterial diversity in this niche in order to understand of BV-related bacteria and their role in BV pathogenesis. However, as described before, the etiology and microbial ecology of BV remain poorly understood. Our study revealed new strains isolated from BV- diagnosed and healthy vaginal samples which will be described below. This data will contribute for improving the knowledge of human vaginal microbiota and which is critical for optimizing reproductive health.

Strain isolated from BV- diagnosed sample

Bacillus firmus

One other microorganism that was isolated from BV- diagnosed women, UM034, was *B. firmus* belonging to the family *Bacillaceae*. It is normally isolated from soil samples and is described as an extracellular amylase producing strain (Singh *et al*., 2012). It is Gram-positive and aerobic bacterium, long, spore-forming rods with a diameter of < 0.9 µm, catalase-positive and usually motile and has an optimal growth temperature of 30ºC. This bacterium is normally used in studies related with adsorption of heavy metal on the polysaccharide produced by this bacterium (Salehizadeh and Shojaosadati, 2003). Little is known about this bacterium and no reports exist regarding its relationship with vaginal ecosystem.

Strain isolated from healthy vaginal sample

Alloscardovia omnicolens

Upon its first description (Stackebrandt *et al*., 1997) the family *Bifidobacteriaceae* consisted of only two genera, namely *Bifidobacterium* and *Gardnerella*, as described previously. Although the members of this family are considered as essentially non-pathogenic, a number of exceptions have been reported. *A. omnicolens* is a member of the family *Bifidobacteriaceae* and is Gram-positive, catalase and oxidase-negative, non-motile, non-spore-forming and short irregularly shaped rods. Optimal growth occurs under anaerobic conditions after 24 h at 37ºC, but slow aerobic growth (72 h), producing pinpoint-sized colonies. In this study, this microorganism was isolated in CBA without supplement and also produced pinpoint-sized colonies (Huys *et al*., 2007). This strain was previously isolated from various human clinical samples, but no data is currently available on its potential pathogenic relevance or virulence factors (Huys *et al*., 2007). This microorganism was isolated from healthy woman, UM031. No study has yet related the association of this microorganism with BV.

CHAPTER 3

Characterization of microorganisms isolated from clinical vaginal samples

3.1. Introduction

As has been described throughout this thesis, the microbial species present in the reproductive tract have a very important role in maintaining health and preventing infections. Several studies have shown that many species commonly associated with BV are present in thick biofilms, while dominant species in the vagina such as lactobacilli may be finely distributed by epithelium (Heinemann and Reid, 2005; Oakley *et al*., 2008).

The vaginal tract colonization by bacteria, whether pathogens or commensals, is the result of adhesion of these bacteria to the vaginal mucosa and most strains possess adhesins encoded in their genome that allow it to colonize the vaginal mucosa. Besides this adhesion ability, bacteria are also capable of forming microcolonies in specific zones of the vagina, altering their surrounding environment and allowing their survival. This manipulation of the microenvironment allows microorganisms in biofilms to survive in the harshest environments (Costerton *et al*., 1999). Thus, through biofilm formation the survival of pathogenic bacteria becomes easier, possibly contributing to the establishment of BV in vaginal epithelium (Cribby *et al*., 2008).

Biofilms of *G. vaginalis* in individuals with BV were first identified, by Scanning electron microscope (SEM), as a dense tissue strongly adherent to the vaginal epithelium comprised of bacterial cells conditioned in a matrix (Scott *et al*., 1989). Later investigations showed that *G. vaginalis* formed a dense biofilm in at least half of the epithelium in 90% of biopsies from women with BV and in only 10% of healthy women (Swidsinki *et al*., 2007). The presence of the bacterial cells in biofilm allows the bacteria to reach higher concentrations than in vaginal fluid and boosts their resistance both to the host immune system and antimicrobials agents (Srinivasan and Fredericks, 2008). In fact, the antibiotics can hardly reach the bacteria residing within the film in an inactive state, leading to a reduction in antimicrobial effectiveness (Hoiby *et al*., 2010).

Typically, many women with BV initially respond to therapy (Bradshaw *et al*., 2006) with metronidazole and clindamycin, the most prescribed antibiotics throughout the world. However, there are now recent studies that demonstrate the increasing incidence of resistance associated with conventional therapy using antibiotics such as metronidazole and clindamycin for species that have been associated with BV (Austin *et al*., 2005; Bradshaw *et al*., 2006). Due to the associated high rate of resistance to antibiotic therapy and the lack of selectivity, new therapeutic approaches have emerged, such as the use of probiotics. It is necessary to understand whether the loss of protective *Lactobacillus* spp.

precedes the growth of anaerobic vaginal microorganisms or otherwise if this loss of protective agents is secondary to massive rupture of the vaginal ecosystem. Therapy should include the replacement of these microorganisms and a strategy for reducing recurrent cases (Sobel, 2005). Moreover, in recent years, therapies with probiotics *Lactobacillus* spp. have attracted the attention of the scientific community. Some studies show the interest of women and adherence to these new approaches complementary therapy and the positive effect of its implementation (Mastromarino *et al*., 2009).

3.2. Objectives

The main objective of the work described in this chapter, was the phenotypic characterization of isolates from clinical vaginal samples in Portuguese women with or without BV. The characterization of these microorganisms is necessary to better understand the pathogenicity of BV condition. For this, we used *in vitro* assays to compare biofilm-forming capacity of single-species populations from BV-associated anaerobes and microorganisms isolated from healthy women. We used a method to analyze bacterial adherence to 96-well plates and compared biofilm-producing capacities for each isolated strains chosen. We also determined the susceptibility or resistance for the clinical isolates in order to find the minimum inhibitory concentration (MIC) to some commonly used antibiotics.

3.3. Materials and Methods

3.3.1. Biofilm formation method

3.3.1.1. Media and strains used for biofilm forming assays

For the biofilm assays, 9 different growth media (Table 3.2) were prepared: Luria Broth (LB – composed by 10 g/L Tryptone (Liofilchem), 5 g/L yeast extract (Liofilchem) and 10 g/L of Sodium chloride (ProLabo); LBG [used the same composition of LB medium and supplemented with 0.25% (w/v) glucose (Liofilchem)]; Man, Rogosa and Sharpe broth (MRS, 54.3 g/L - Liofilchem), MRSG (MRS supplemented with 0.25% (w/v) of glucose); Tryptic Soy Broth (TSB, 30 g/L - Liofilchem), TSBG (TSB supplemented with 0.25% (w/v) of glucose), sBHI [BHI supplemented by 2% (w/w) gelatin (Oxoid), 0.5% (w/w) yeast extract (Liofilchem), 0.1% (w/w) starch

(FisherScientific)] , and sBHIG [supplemented with 0.25% of glucose (Liofilchem)], and finally sBHI with 10% (v/v) Fetal Bovine Serum (FBS) (Sigma-Aldrich).

3.3.1.2. Biofilm formation assays

For biofilm formation assays the first step was to prepare a pre-inoculum, in plastic tubes of 10 mL (Frilabo) for most of the isolated strains described in chapter 2. *G. vaginalis* AMD (BV isolate) and *G. vaginalis* 5-1 (non-BV isolate) were used as reference strains. All strains were cultured in 2 ml of different media described in previous section. These cultures were grown at 37ºC under anaerobic conditions; using the Anaerogen pack system (Oxoid) for 48 h. Growth was analyzed using a 96-well plates (Orange Scientific) by Enzyme-Linked Immunosorbent Assay (ELISA) reader with a 600 nm filter (Tecan Sunrise). The cultures were diluted with different media respectively an OD according to the OD of the different medium used. After this step, 200 µL of each diluted suspension were placed in 96-well plates and incubated anaerobically at 37ºC for 48 h.

Qualitative method for analysis of biofilm formation

Initially, biofilms were formed as described above and qualitatively quantified as described before by Patterson *et al*., (2010). Briefly, after cultures growth for 48 h, spent media were removed, half of 96-wells plates were washed with 200 µL 1x Phosphate Buffered Saline (PBS) [composed by 16 g/L of NaCl; 0.4 g/L of potassium chloride (KCl); 1.62 g/L of disodium phosphate ($Na_2HP.2H_2O$) and 0.4 of potassium di-hydrogen phosphate (KH_2PO_4)] to remove non-adherent bacteria, and the plates were air-dried for ± 1 h. For qualitative assessment of biofilm formation, adherent bacteria were stained with 200 µL 0.25% (w/v) safranin for 1 min. Following this, wells were washed gently to remove the safranin and the plates were air-dried. The formation of a thin or thick biofilm (qualitative classification) was determined by measuring the amount of safranin stained biofilm (by visual observation), which then had a symbol assigned to each strain: for strains not forming biofilm the symbol (-) was assigned, strains forming medium or strong biofilm (+-), and strong biofilm formation (++) in all assays.

Determination of the intrinsic ability of biofilm formation

To determine of the intrinsic capacity of biofilm formation of BV associated microorganisms independent of their growth rate we used the method described by

Harwich *et al*., (2010). This is important because a microorganism can have a greater intrinsic ability to form biofilm but form less biofilm because they might have a slower growth rate, for example. We selected 3 media in which each of the strains formed more biofilm, as determined by the qualitative analysis the pre-inoculum and biofilm forming assays were performed as described in the previous section, with the exception of the media used for each strain. After 48 h of growth, planktonic bacteria were removed from the wells and transferred to sterile eppendorfs. Part of 96-well plates were washed with 200 µL 1x PBS to removed non-adherent bacteria and the plates were air-dried for ± 1 h. For quantitative assessment of biofilm formation, were added 200 µL of the different growth media to the adherent bacteria. Afterwards, wells were scraped with a pipette tip in order to remove the biofilm attached to the well. After this step, the biofilms formed by each strain (with and without PBS washing) were placed in separate sterile eppendorfs. The OD was measured using as a blank the different media used in each case. The percentage growth as a biofilm was calculated as $OD_{600\ nm}$ biofilm/ ($OD_{600\ nm}$ biofilm + $OD_{600\ nm}$ planktonic). The data analysis was based on at least 3 independent experiments.

3.3.1.3. Statistical analysis

All the assays were compared using independent-samples T-test, using Statistical Package for Social Sciences (SPSS, version 18.0). All tests were performed with a confidence level of 95% and differences were considered significant at $P < 0.05$.

3.3.2. Antibiotic susceptibility determination

To identify the MIC range of each strain we used the method described by Harwich *et al*., (2010). Pre-inocula were prepared for each isolate including reference strains *G. vaginalis* AMD and *G. vaginalis* 5-1 (Table 3.2) in sBHI [BHI supplemented with 1% (w/w) of yeast extract, 2% (w/w) of gelatin, 0.1% (w/w) of starch], and incubated in anaerobic conditions with Anaerogen pack for ± 24 h at 37ºC. After ± 24 h growth the plates were measured in ELISA reader with a 600 nm filter, to confirm the strains growth. 5 antibiotics were diluted to a stock solution: Metronidazole (MD – Sigma), Tinidazole (TZ - Sigma), Clindamycin (CM - Sigma), Rifampicin (RF - Sigma) and Vancomycin (VM - Sigma). Antibiotics were serially diluted 2-fold in sBHI, using plastic tubes. After that, each of the 200 µL sBHI dilutions were placed in 96-well plates and 3 µL of the bacterial suspension of each strain were added to each well. The $OD_{600\ nm}$

was observed and 96-well plates were incubated in anaerobic conditions for 48 h at 37ºC with Anaerogen pack and the lowest concentrations of antibiotics that prevented visible bacterial growth were recorded.

The main characteristics of these antibiotics are described in table 3.1 and figure 3.1 presents the structure of the antibiotics used.

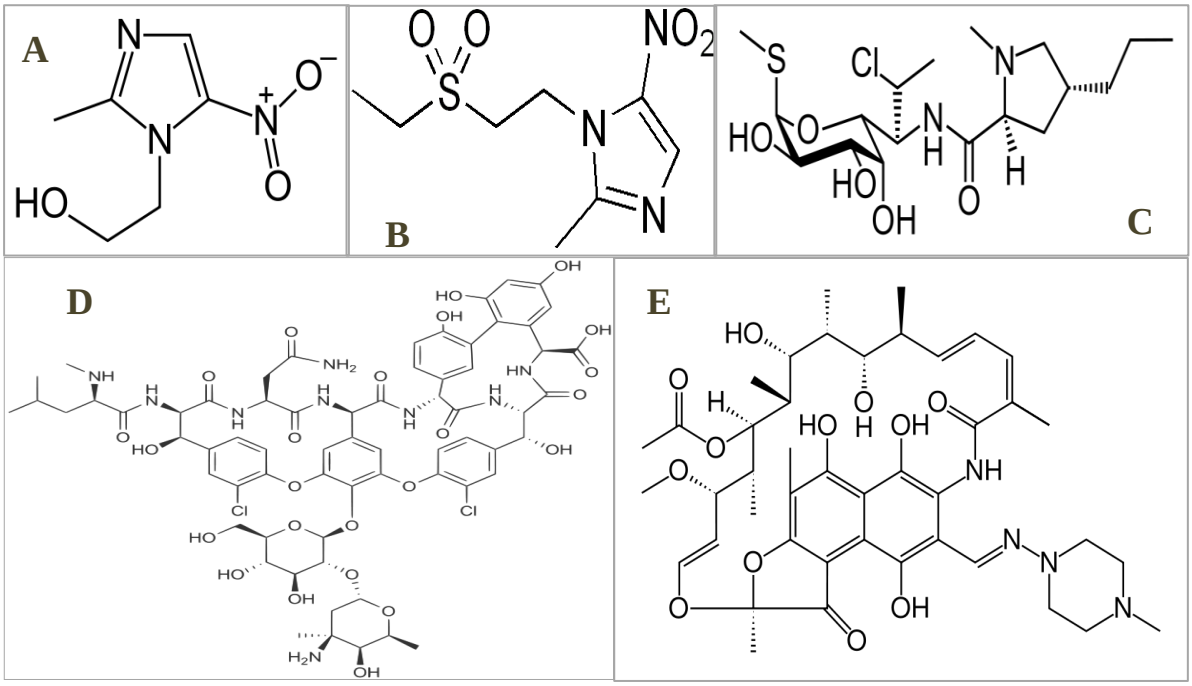


Figure 3.1. Structure of the antibiotics used in the study described in this chapter: (A) metronidazole, (B) tinidazole, (C) clindamycin, (D) vancomycin and (E) rifampicin.

Table 3.1. Characteristics of the antibiotics used in the study described in this chapter.

Antibiotic	**Mechanism of action**
Metronidazole	DNA damage
Tinidazole	DNA damage
Clindamycin	Protein synthesis inhibitor
Vancomycin	Cells wall synthesis
Rifampicin	RNA synthesis inhibitor

3.4. Results and discussion

3.4.1. Analysis of biofilm formation

Biofilms are strongly associated with human infections and up to 65% of infections treated by physicians in the developed world have been attributed to biofilms (Costerton *et al*., 2003).

The reproductive tract, as well as most of the cavities of the human body, is not a sterile environment, but a habitat for numerous species of microorganisms. Some of these microorganisms are commensal (beneficial to health), such as *Lactobacillus* spp., but other, harmless in normal situations, can lead to the onset of diseases (opportunistic pathogen) in certain conditions like has been refereed about *G. vaginalis*. Nowadays, little is known about how the different groups of bacteria involved in BV interact with the vaginal epithelial surface (Swidsinski *et al*., 2005).

As mentioned in chapter 1, the biofilm-related diseases can be multi-species or single-species. Curiously, the biofilm formed from a single-species *in vitro* and those produced in nature by various consortia species have similar structural characteristics (Davey and O´Toole, 2000). In most biofilms it is possible to observe heterogeneity, in which patches of cell aggregates, not monolayers are interspersed throughout a matrix of exopolysaccharide (described in chapter 1) that varies in density, creating open areas, which allow the passage of the necessary nutrients for the survival of these species (Davey and O´Toole, 2000). This is due to diffusion limitations, transmitted from the biofilm, resulting in local variations of nutrients, pH, and availability of oxygen and concentrations of bacterial metabolites. In addition to these complications, the study of biofilms is characterized by intrinsic limitations with regard to *in vitro* models used in the assays of biofilm formation. Despite the limitations associated with the study of biofilms, a comprehensive comparative analysis of all data can possibly enable a breakthrough in the investigation of these structures (Jefferson, 2004).

It has been described that strongly biofilm formation depends on environmental conditions, namely the growth media used. Many species have shown similar developmental steps in biofilm formation, which includes (as described in chapter 1): initial attachment to a surface, followed by the formation of microcolonies, and finally maturation of microcolonies into an EPS-encased mature biofilm. These steps are described as basic for single-species biofilm formation. It is thought that the process starts when environmental factors trigger the transition in bacteria from the planktonic growth

to a "living" surface. Biofilms can be formed on vast array of biotic and abiotic surfaces (Davey and O´Toole, 2000). In order to determine optimal conditions for *in vitro* biofilm formation, *G. vaginalis* and others microorganisms isolated in this study were cultured anaerobically in abiotic surface, 96-well plates (a non-nutritive surface, polystyrene plastic) with LB, MRS, TSB and sBHI media. Because glucose has been described to increase biofilm formation in other bacterial species (Patterson *et al*., 2007) these microorganisms were also cultured in the presence of 0.25% (w/v) of glucose (LBG, MRSG, TSBG and sBHIG). Furthermore sBHI media was supplemented with 10% (v/v) of FBS, the most widely used serum-supplement due to the very low level of antibodies present and because it contains more growth factors, allowing for versatility in many different cell culture applications. By adding these supplements to the media, bacteria (including planktonic bacteria) have access to different types of nutrients, and it may allow bacteria to adapt to different conditions and to adjust their metabolic processes to maximize the use of substrates available to protect themselves from harmful conditions and favoring biofilm production (Jefferson, 2004).

The use of these several types of growth media allowed us to analyze the behavior of the clinical isolates in relation to biofilm formation (Table 3.2). Thus, it was possible to determine that a suitable growth media is required to initiate the formation of biofilm. Initially, the biofilm formation ability was screened, qualitatively, using all 9 growth media. Table 3.2 presents the qualitatively results of biofilm formed for each isolated bacteria. As expected, it can be seen that different strains have different biofilm-forming capability depending on the medium used. Interestingly, it is clear that certain microorganisms have little or no ability to form biofilm in some media such as the *G. vaginalis* UM035 and *S. agalactiae* UM035 in LB, LBG, MRS and MRSG media; *G. vaginalis* UM016 in MRS and MRSG media; *P. acnes* UM034 in MRS, MRSG, TSB, TSBG media and *L. gasseri* UM022 in media like LB, sBHIG and sBHI supplemented with FBS. Growth and biofilm formation for all *G. vaginalis* were greatest in sBHI, sBHIG whereas when sBHI media was supplemented with FBS this biofilm was slightly less dense. This medium, sBHI, was used because it is a highly nutritious general-purpose growth medium for culturing fastidious microorganisms and non-fastidious, such as streptococci and *G. vaginalis* and because it had already been used in some studies for characterization of biofilm formation in microorganisms associated with vaginal flora.

Table 3.2. Qualitative analysis of biofilm formed for each isolated bacterium in 9 different media, based on the Patterson *et al*., (2010) method.

	Media								
Strains	**LB**	**LBG**	**MRS**	**MRSG**	**TSB**	**TSBG**	**sBHI**	**sBHIG**	**sBHI+FBS (10%)**
BV isolates									
G. vaginalis AMD	+-	+-	+-	+-	+-	+-	++	++	++
G. vaginalis UM035	-	-	-	-	+-	+-	++	++	++
Gemella spp. UM034	++	++	++	++	+-	+-	+-	++	++
E. faecalis UM035	+-	++	+-	+-	+-	++	++	+-	+-
Klebsiella spp. UM034	+-	+-	+-	+-	+-	+-	+-	+-	+-
P. acnes UM034	++	++	-	-	-	-	++	+-	++
S. agalactiae UM035	-	-	-	-	++	++	+-	++	++
non-BV isolates									
G. vaginalis 5-1	+-	+-	+-	+-	+-	+-	++	++	++
G. vaginalis UM016	+-	+-	-	-	+-	+-	++	++	++
B. breve UM031	+-	++	+-	+-	+-	++	++	++	+-
L. gasseri UM022	-	+-	+-	+-	+-	+-	++	-	-
S. epidermidis UM042	+-	++	+-	+-	+-	++	++	+-	+-
Streptococcus spp. UM031	+-	++	++	++	++	++	++	++	++

Legend: (-) no biofilm formed; (+-) formed medium or strong biofilm in all tests; (++) strong biofilm formation in all tests.

As discussed in the previous chapters, most of the vaginal microorganisms are fastidious and difficult to culture. Patterson *et al*., (2010) previously reported that the main vaginal isolates had significantly different growth rates. They argued that the most appropriate method to determine biofilm formation ability would be to determine the percentage of growth in the biofilm, as compared to total growth (biofilm + planktonic) (Harwich *et al*., 2010). Following their method, we determined the intrinsic biofilm formation ability of each isolated strain, grown in sBHI (Figure 3.2a). The sBHI medium was chosen for this analysis because it was the only medium in which all isolated strains were able to form biofilm.

Taking into account the analysis of figure 3.2a, it is possible to conclude that *G. vaginalis* BV-isolates tend to grow more in biofilms than those non-BV isolated and other anaerobes associated and not associated with BV (Figure 3.2a), as has been described in several studies (Patterson *et al*., 2010; Harwich *et al*., 2010). We also observed that quantitative measurement of biofilm growth as percentage of total growth (Figure 3.2a) indicated that *G. vaginalis* AMD had a significantly greater biofilm-

forming capacity in sBHI than the non-BV isolate *G. vaginalis* 5-1 as described in literature (Harwich MD *et al*., 2010). Through figure 3.2a, it was also found that some of the microorganisms isolated had a higher capacity to form biofilm, such as *P. acnes*, *S. epidermidis*, *E. faecalis*, *Streptococcus* spp. and *B. breve*.

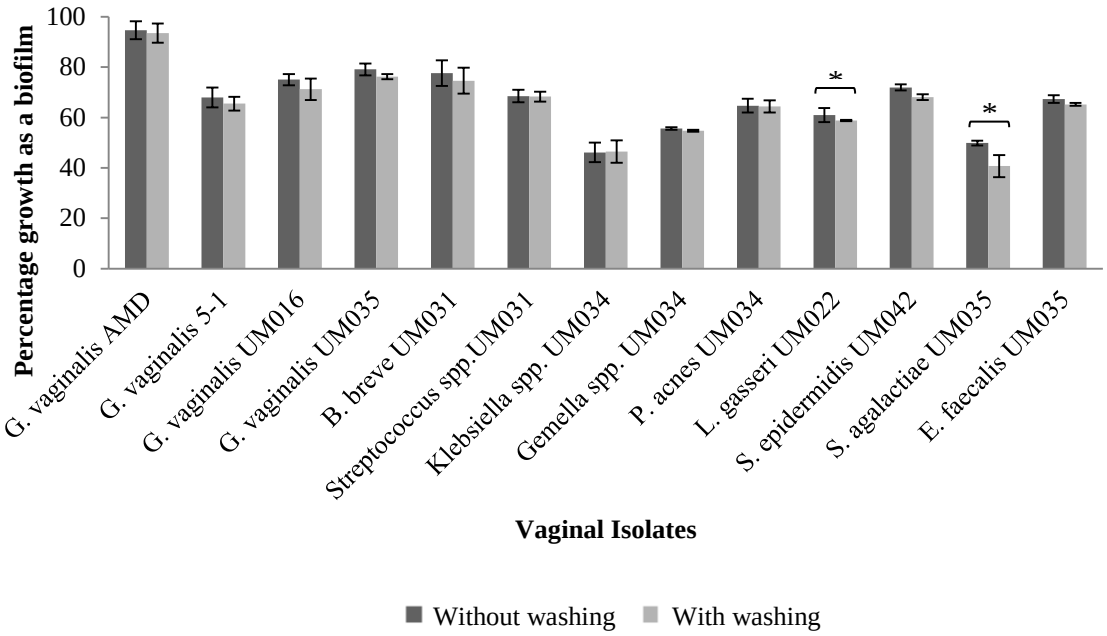


Figure 3.2a. Intrinsic ability of biofilm formation of each strain in sBHI. This assay was performed at 37ºC, during 48 h. After this time the suspension was removed, and biofilms were subjected to one wash with 1x PBS to analyze the strength of attachment. The percent growth was calculated as $OD_{600\ nm}$ biofilm/ ($OD_{600\ nm}$ biofilm +$OD_{600\ nm}$ planktonic). Error bars represent the standard deviation of 3 independent experiments. Statistical differences are marked with* ($P < 0.05$) from biofilm formation of these strains with washing or no washing of the cells with PBS buffer after removed planktonic cells were analyzed using independent-samples T-test.

To assess the strength of the association of the biofilm onto the surface to which it is attached, the biofilms were washed with PBS to remove cells that are not strongly adhered and compared to biofilms where the growth media was completely removed, but without washing, from our results it is possible to see that the PBS wash did not result in significant differences in biofilm formation (T-test, $P > 0.05$) except in the case of *S. agalactiae* UM035 and *L. gasseri* UM022 which indicates that the biofilm formed by these strains are not as strong as the others strains (T-test, $P < 0.05$).

The relationship between biofilm-forming capacity and association with BV was further investigated using the others strains isolated in this work and by quantitatively

analyzing the intrinsic capability of biofilm formation under the 3 best growth media, as determined qualitatively. We found that the BV-isolated *G. vaginalis* (*G. vaginalis* UM035), had similar biofilm formation tendency as *G. vaginalis* AMD strain and more than non-BV isolated strains, namely *G. vaginalis* 5-1 and *G. vaginalis* UM016. By observing the results from the other strains isolated in this study, it was possible to conclude that those isolated from women with BV have a stronger intrinsic capacity to grow as a biofilm than those isolated from healthy women. However, the differences detected were not statistically significant (T-test, $P > 0.05$).

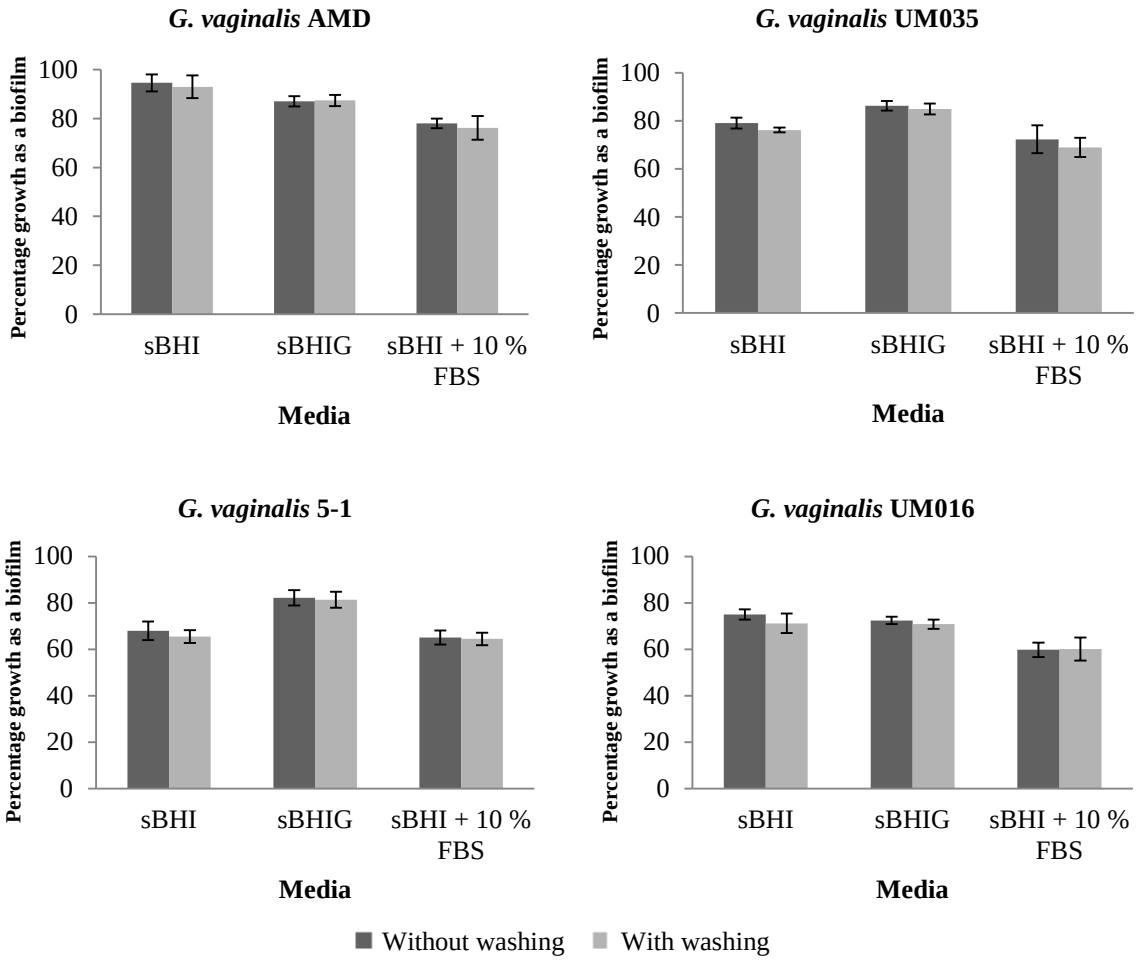


Figure 3.2b. Intrinsic ability of biofilm formation of each strain in different media. This assay was performed at 37ºC, during 48 h. After this time the suspension was removed, and biofilms were subjected to one wash with 1x PBS to analyze the resistance thereof. The percentage growth was calculated as $OD_{600\ nm}$ biofilm/ ($OD_{600\ nm}$ biofilm + $OD_{600\ nm}$ planktonic). Error bars represent the standard deviation of 3 independent experiments. Statistical differences are marked with* ($P < 0.05$) from biofilm formation of these strains with washing or no washing of the cells with PBS buffer after removed planktonic cells were analyzed using independent-samples T-test.

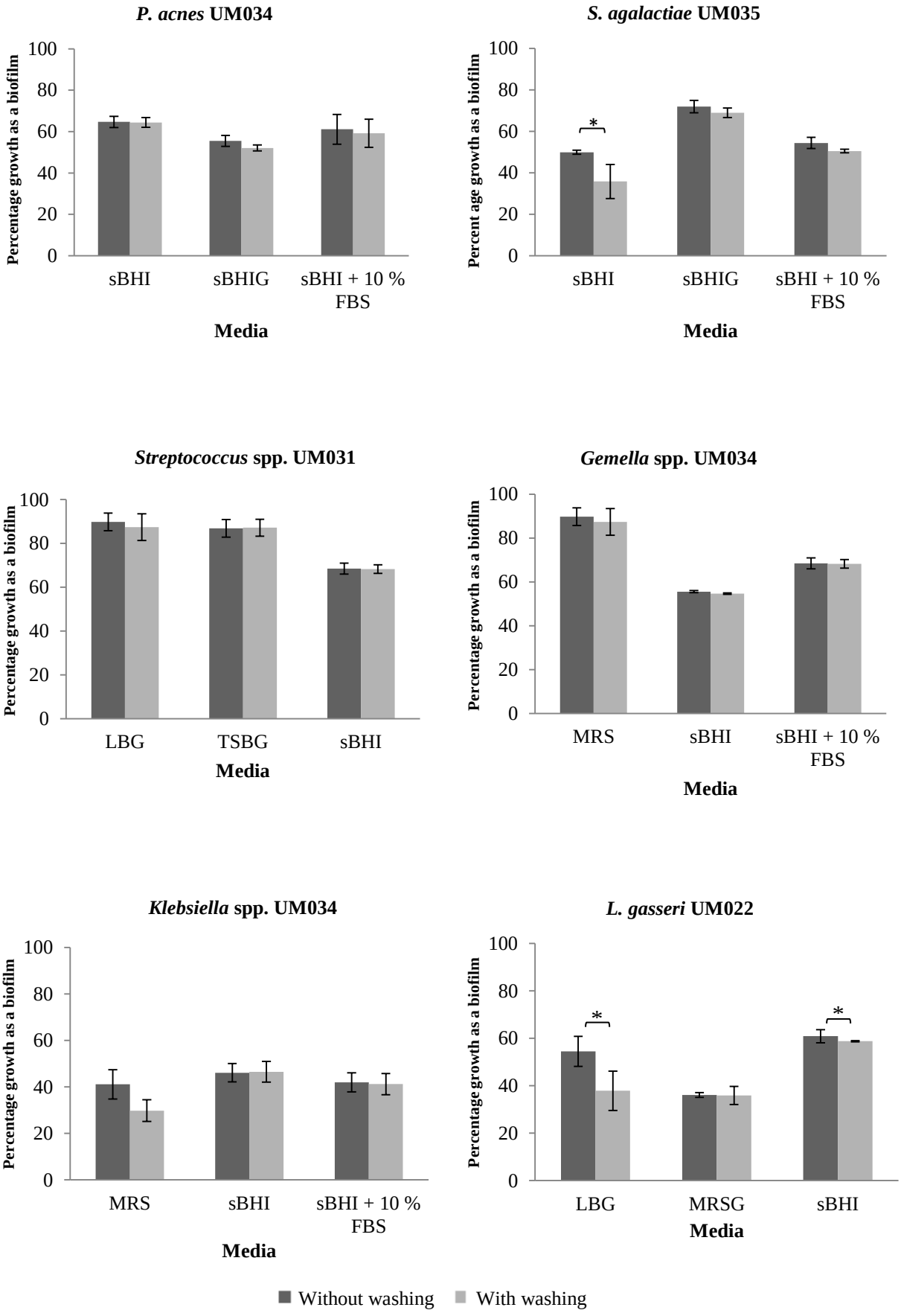


Figure 3.2b. (*Continued*).

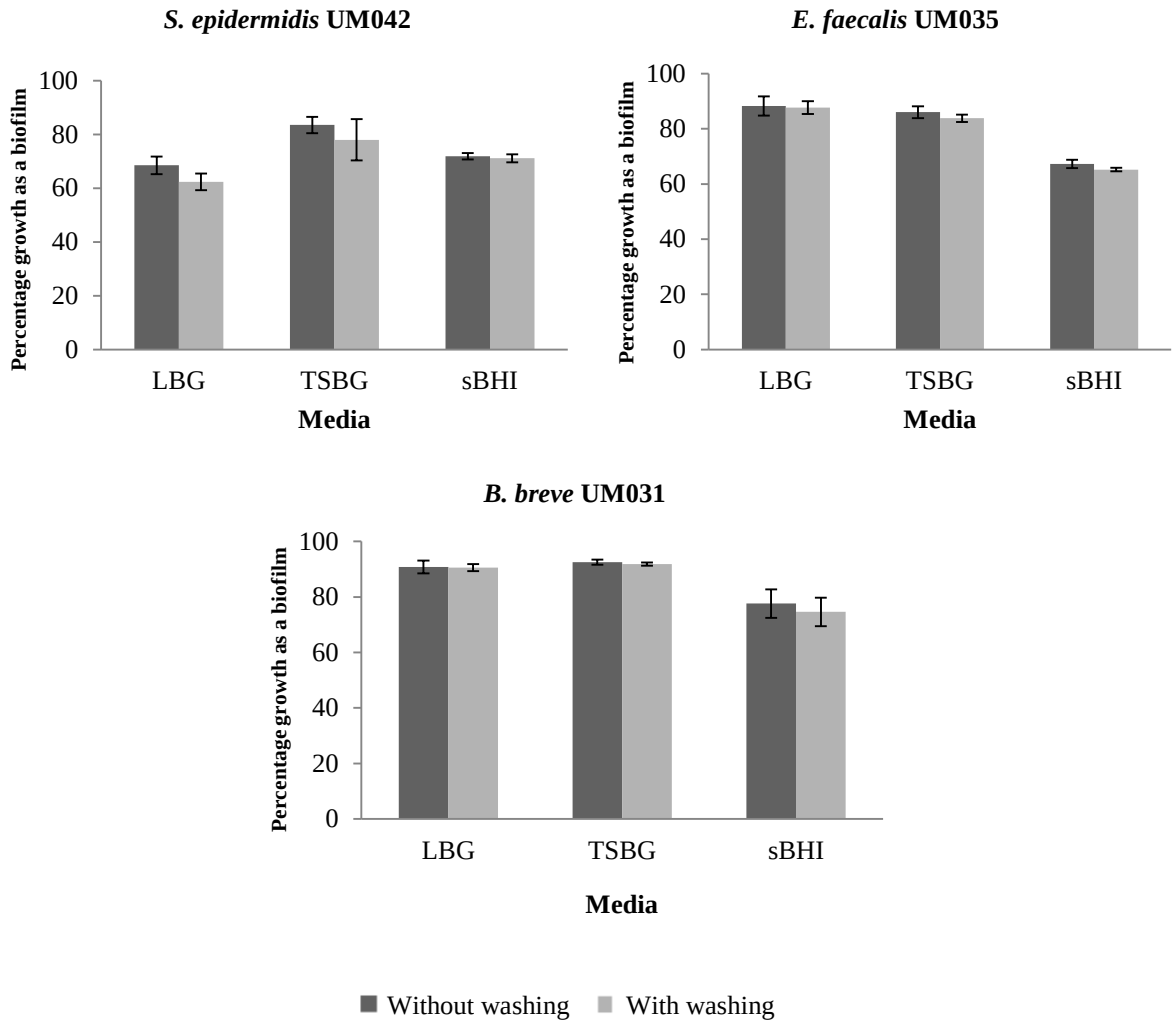


Figure 3.2b. *(Continued).*

In general it appears that *G. vaginalis* forms more biofilm when adding glucose to the medium or sBHI normal. It was found that with the use of a more complex substrate such as sBHI + 10% FBS decreases the ability to form biofilm in the 2 strains of *G. vaginalis* isolated (Figure 3.2b). The components in this type of substrate may affect somewhat the structure of the biofilm formation. This is an extremely complex mixture of many small and large biomolecules with different, physiologically balanced growth-promoting and growth-inhibiting activities. Some of the major functions of serum are to provide growth factors, hormones, attachment and spreading factors, binding proteins, lipids, and minerals (Freshney *et al*., 1994). Nonetheless, one study reported that the serum had influence in *Helicobacter pylori* bacteria in relation to their adherence and promotes their inhibition to abiotic surfaces, due the fact that these media complicate studies of bacterial metabolism and may not accurately represent the *in vivo* environment

(Williams *et al*., 2008). So our results, demonstrate the same results wherein it is found that strains of *G. vaginalis* not adhere so well in the presence of a substrate with FBS as preferred substrate glucose and normal supplement sBHI, as possible to see in figure 3.2b.

Besides the high growth tendency as biofilms of *G. vaginalis*, we also detected other organisms with similar growth trends, such as, *S. epidermidis*, *E. faecalis*, *Streptococcus* spp. and *B. breve*. *S. epidermidis* is known to possess an extracellular polyssaccaride adhesin, which is encode by *ica* operon, that represents a key virulence determinant in this bacterium and is required for biofilm formation (O´Gara and Humphreys, 2001) and *E. faecalis* is known to form dense biofilm (Kristich *et al*., 2004). In the case of *E. faecalis*, many studies reported that this bacterium has the capability to make surface pilli which can lead to the formation of a biofilm (Nallapareddy *et al*., 2006). Relatively to *P. acnes*, this bacterium has also been described to form biofilm nevertheless nothing is known about the regulation of biofilm production (Bayston *et al*., 2007).

On the other hand, the others anaerobes tested - *L. gasseri*, *Gemella* spp., *Klebsiella* spp. and *S. agalactiae* demonstrated lower biofilm formation capability, as compared to *G. vaginalis*, but it should be noted that *L. gasseri* had a higher ability to form biofilm than *S. agalactiae* and *Klebsiella* spp. This phenomenon can be explained by the fact that in the case of healthy women (as is the case of the isolated strain of *Lactobacillus*), biofilms in the vaginal tract are mainly constituted by lactobacilli, which have various mechanisms of adhesion that allow them to colonize effectively the vaginal mucosa. It is known that the adhesion of epithelial cells is accomplished through various receptors available in the vaginal mucosa and that *L. gasseri* strains bind to the vaginal epithelium through glicopeptidic receptors. Thus, the various epithelial receptors become a target for competition between bacteria in the vaginal mucosa, demonstrating the protective role of lactobacilli in the epithelium preventing connections of the epithelial receptors with other potentially pathogenic or opportunistic bacteria (Hajela *et al*., 2012). Although, it has been described that *S. agalactiae* is a common commensal organism, it may become pathogenic and endanger the life of newborns. These pathogens are characterized by presenting long filamentous structures, known as fimbriae or pilli, which are often involved in initial adhesion of bacteria to host tissues but also in bacteria-bacteria interactions, resulting in biofilm formation. Studies report that these microorganisms can form biofilms and those pilli play an important role in this process (Konto-Ghiorghi *et al*., 2009).

Results of some investigations into the ability of *G. vaginalis* and other BV-associated anaerobes, demonstrated that controversy exists as to whether *G. vaginalis* is the cause of BV or the enhancing agent BV (Patterson *et al*., 2010). Since *G. vaginalis* has been shown as the predominant species in biofilms in women presenting with BV, comparatively with others BV-associated anaerobes, it suggests the important role of biofilms in the pathogenesis of this condition (Patterson *et al*., 2010). The present study, only evaluated the biofilm formation ability of a single species and not several mixed biofilm-associated microorganisms which could reveal more information about strain interaction. One other limitation of this study is the restricted number of strains studied, as they were dependent on exudate vaginal samples received.

3.4.2. Determination of susceptibility to antibiotics

3.4.2.1. Characterization of treatment with antibiotics in BV

BV treatment has been based on empiricism, mainly due to the absence of an identified etiological agent, as discussed thought this thesis (Josey and Schwebke, 2008). The magnitude BV consequences has stimulated therapeutic research efforts towards the development more effective therapies (Menard, 2011). However, treating BV remains complicated, and antibiotic resistance is postulated as one of the reasons for persistence and recurrence of BV. Several studies report that the capacity of antibiotics to penetrate the biofilm is reduced, and considering the presence of biofilms associated with BV, antimicrobial resistance can be seriously enhanced (Hogan and Kolter, 2002). Antibiotics like nitroimidazoles (such as metronidazole and tinidazole), have been commonly used in the treatment of BV. There determining the susceptibility to metronidazole of the isolated strains of *G. vaginalis* and others vaginal anaerobes (associated or not with BV) is of the upmost importance to detect any emergence of resistance as well as to identify the appropriate therapeutic agents for use in the treatment of invasive diseases caused by *G. vaginalis* (Polatti *et al*., 2012).

At the moment there are 3 recommended therapies for BV, oral metronidazole or vaginal clindamycin (Verstraelen and Verhelst, 2009) and more recently tinidazole. So, the aim of this study was to determine the MIC of the 13 isolates obtained in this study against 5 antibiotics, 3 of them commonly used against BV (metronidazole (MD), tinidazole (TZ) and clindamycin (CM)) and also rifampicin (RF) and vancomycin (VM). Table 3.3 shows the MIC's, as determined by dilution assay in 96-well plates.

Table 3.3. *In vitro* susceptibilities of microorganisms isolated from clinical samples to 5 antimicrobial agents: Metronidazole (MD), Tinidazole (TZ), Clindamycin (CM), Rifampin (RF) and Vancomycin (VM) using method described by Harwich *et al*., (2010) in 3.3.2 section of Materials and Methods.

	Antibiotics – MIC range (µg/mL)				
Strains	**MD**	**TZ**	**CM**	**RF**	**VM**
BV isolates					
G. vaginalis AMD	16 - 32	16 - 32	8 -16	0.25 – 0.5	0.25 - 0.5
G. vaginalis UM035	16	4 - 32	> 128	0.125	0.25 - 0.5
non-BV isolates					
G. vaginalis 5-1	128	128	16 - 64	0.5 - 1	0.5 - 1
G. vaginalis UM016	32 - 64	8 - 32	64 - 128	0.25 – 0.5	0.25 – 0.5
BV isolates					
E. faecalis UM035	> 128	> 128	> 128	16	2 - 4
Gemella spp. UM034	< 16	< 4	< 0.5	< 0.03	< 0.125
Klebsiella spp. UM034	> 128	> 128	> 128	64	64 - 128
P. acnes UM034	64 - 128	> 128	0.5 - 2	0.03 - 2	0.125
S. agalactiae UM035	> 128	> 128	> 128	1 - 2	1 - 2
non-BV isolates					
B. breve UM031	16	4 - 8	> 128	0.125 - 0.25	0.125 - 0.5
L. gasseri UM022	> 128	> 128	> 128	1 - 2	0.5 - 2
S. epidermidis UM042	> 128	> 128	> 128	4 - 8	> 64
Streptococcus spp. UM031	> 128	> 128	> 128	32 - 64	2

Metronidazole and Tinidazole

MD was first used in trichomoniasis and anaerobic infections but has more recently been used in treatment of BV with good clinical results (Löfmark *et al*., 2010). In an earlier study it has observed that the majority of strains of *G. vaginalis* examined were susceptible to MD and TZ (Kharsany *et al*., 1993) but a recent study demonstrated that recent strains of *G. vaginalis* isolated are more resistant (Harwich *et al*., 2010). Generally, strains with MIC ≤ 8 µg/mL are considered susceptible, whereas those having an MIC = 16 µg/mL are considered intermediate resistant and finally with bacteria MIC ≥ 32 µg/mL are considered resistant to MD (Hecht *et al*., 2001). Having this in consideration, our MIC results were of some concern, since all strains in this study were intermediate or resistant

to MD and similar results were obtained with TZ. Several mechanisms have been proposed for MD resistance in anaerobic bacteria, although different mechanisms might be involved in different organisms, but resistance as a decrease in antibiotic absorption efficiency or altered reduction is a common explanation. Most Gram-positive anaerobic bacteria such as *Bifidobacterium, Lactobacillus* and *Propionibacterium* spp. intrinsically have reduced susceptibility to MD (Löfmark *et al*., 2010). Results similar to those obtained in this study have previously been reported where all strains tested demonstrated intermediate resistance or full resistance to MD (range for lactobacilli, including *L. gasseri* was > 256 µg/ml) (Reid *et al*., 2003). The strains that presented intermediate resistance were *B. breve, G. vaginalis AMD* and UM035 and *Gemella* spp. UM034. One other study using the same controls, *G. vaginalis* AMD and 5-1 also showed that these strains also presented intermediate resistance to MD (Harwich *et al*., 2010). Interestingly, the MIC for MD for a strain *G. vaginalis* isolated from a healthy woman, *G. vaginalis* UM016 (MICs range, 32 - 64 µg/mL) was higher than the MIC for the strain of *G. vaginalis* isolated from women with BV as *G. vaginalis* UM035 (MIC = 16 µg/mL).

Results of several experiments demonstrate that the fact that the vaginal flora in BV is comprised of several Gram-variable bacteria, like *G. vaginalis*, means that antimicrobials specifically active against Gram-positive or Gram-negative bacteria have little or no action against Gram-variables microorganisms (Gilbert *et al.*, 1997). This fact might explain the resistance observed in *G. vaginalis* strains over time. However, it is still possible to observe that the remaining strains also are very resistant to MD, as they required concentrations above 128 µg/mL.

TZ is chemically similar to MD and has a shorter treatment course. TZ may be a therapeutic alternative in the treatment of BV as it requires less than half the recommended dose of oral MD (Schwebke and Desmond, 2011), and as such it has been licensed recently in the US for the treatment of BV (Bradshaw *et al*., 2012). Although studies have shown that TZ is now the antibiotic of choice in combating BV, our results show that most microorganisms isolated in our study, including *G. vaginalis*, were resistant, except *B. breve* non-BV isolate (MIC range was 4 - 8 µg/mL) and *Gemella* spp. BV-isolate (MIC range was < 4 µg/mL) that demonstrated sensibility to this antibiotic. It should be noted that in the case of *G. vaginalis* UM035, that was BV-isolate, the MIC was variable 4 - 32 µg/mL and all of other BV-isolates were intermediate resistant or resistant (MIC range with high variability, 16 - 128 µg/mL). Results suggest that *L. gasseri* UM022 a non-BV isolate was resistant in agreement with previous studies, which

show that MD and TZ did not affect the normal microorganisms present in vaginal flora like *L. gasseri* (Polatti, 2012). Our results did not support the hypothesis that TZ is more efficacious for the treatment of BV than MD (Schewebke and Desmond, 2011) because we obtained only 2 strains, *B. breve* (MIC range, 4 - 8 µg/mL) and *Gemella* spp. (MIC range, < 4 µg/mL) were shown to be susceptible and all of others isolates present intermediate resistance (MIC range, 4 - 32 µg/mL).

Clindamycin

CM is the second antibiotic of choice used to treat BV. Belonging to the class of lincosamides, CM exhibits its action by inhibiting bacterial protein synthesis. CM has larger spectrum activity against Gram-positive aerobes and anaerobes (Bradshaw *et al*., 2012). In our results, only *Gemella* spp. (MIC range, < 0.5 µg/ mL) and *P. acnes* (MIC range, 0.5 - 2 µg/ mL), were found to be sensible to CM as previously reported (Buu-Hoi *et al*., 1982; Nishijima *et al*., 1996). Comparatively to others studies, bacteria are commonly susceptible to CM, including *G. vaginalis* strains and other vaginal aerobes and anaerobes (Backer *et al*., 2006). However, in the present study, this was not the case since they revealed intermediate resistant or resistant phenotype even in the case of *G. vaginalis* AMD and 5-1 (Patterson *et al*., 2010).

CM, while useful in combating BV is known to affect lactobacilli and thus results in a negative effect on the normal flora, increasing the potential for microbial diseases (Polatti, 2012). Other studies suggest that *L. crispatus*, for example, is more susceptible to CM than strains of *L. gasseri*. For this reason, it is possible that treatment using CM can reduce the resistance or protection conferred by lactobacilli to pathogen vaginal colonization. Moreover it is becoming apparent from clinical studies comparing CM and MD, that resistance to CM seems to develop more readily than resistance to MD (Beigi *et al*., 2004).

A study undertaken this year in Portugal, has shown that the first choice antimicrobial therapy is MD (58%) (Henriques *et al*., 2012), as has previously described in other countries (Schmid, 1999). It was also found that specialists in different geographical regions of Portugal's prescribe different antibiotic therapies. The center region of Portugal, was the only region where CM prescriptions preferred to MD, and although Portuguese doctor's do not regard recurrence as very common (in contrast to other parts of world) in the region with the highest rate of CM use perception of

recurrence rate was slightly elevated. Despite Portuguese doctor perceptions of low recurrence rates, our results indicate high levels of resistance to MD, TZ and CM from the isolates recovered in this study. However, since we were only able to isolate a few strains of each species, we can not infer to the widespread presence of resistant BV bacteria in Portugal.

Vancomycin

VM acts by inhibiting cell wall biosynthesis in Gram-positive bacteria and is not active against Gram-negative bacteria (Van Wageningen *et al*., 1998). Since the start of its use, VM has shown to be very useful and for 3 decades there were no reports of resistance to this antibiotic. However, recently reports have emerged on the isolation of *E. faecalis* resistant to high concentrations of VM (Nicas *et al*., 1989) and as such we decided to include this antibiotic in our susceptibility tests, in order to best assess overall resistance patterns in our isolates. In our results, we observed that *E. faecalis* BV-isolate was susceptible to VM in this case (MIC range, 2 - 4 µg/ mL). All strains, except *S. epidermidis* and *Klebsiella* spp., were sensitive to low concentrations of VM (MIC range, 0.125 - 2 µg/ mL). In the case of *Klebsiella* spp. it was not surprising that it was resistant as it is a Gram-negative organism. VM resistance among coagulase-negative staphylococci was first reported over 20 years (Siebert *et al*., 1979). Since that time, there have been several case reports of clinically significant coagulase-negative with decreased susceptibility to VM (Krcmery *et al*., 1996; Dunne *et al*., 2001). Further studies, have demonstrated that the incidence of these resistant organisms is very low. Possible explanations referred by Srinivasan *et al*., (2002) for this resistant phenotype include the use of high levels of VM in the months prior to the study, the fact that these isolates were all part of an outbreak, or the possibility that the researchers used a more sensitive technique to detect resistance VM. Unfortunately, none of these factors was addressed in the study.

Results obtained for *L. gasseri*, *B. breve* were similar to those obtained in other investigations (Backer *et al*., 2006).

Rifampicin

RF is a bactericidal antibiotic drug whose activity relies on the inhibition of protein synthesis (Calvori *et al*., 1965) and has been used in *in vitro* assays of several isolates related to BV for susceptibility testing (Backer *et al*., 2006; Harwich *et al*., 2010).

Our results demonstrate that with the exception of certain species of microorganisms, RF was highly active against most anaerobes tested, except for *E. faecalis* that demonstrated intermediated resistance (MIC = 16 µg/mL), *Klebsiella* spp. (MIC = 64 µg/mL) and *Streptococcus* spp. (MIC range, 32-64 µg/mL) that were resistant. Bacteria can become resistant to an antibiotic because they mutate easily and frequently. Resistance to RF arises from mutations that alter residues of the RF binding site on RNA polymerase, resulting in decreased affinity for this antibiotic (Wehrli, 1983).

Our tests showed a low MIC - value for RF (MIC range for *L. gasseri*: 1 - 2 µg/mL) that was similar with other results published (Backer *et al*., 2006). In this case, there were no significant differences regarding the effect of this antibiotic in isolates of BV and healthy samples. This result, together with the results obtained for VM suggest that lower concentrations of VM and RF are sufficient to damage the lactobacilar flora (Backer *et al*., 2006), although they were very active against *G. vaginalis*. Thus, despite this, we conclude that these antibiotics can not be used in the treatment of BV, because contribute to damaging microorganisms which are promoters of a healthy vaginal flora.

Conclusions from this study suggest that compared with previous studies, which are now some years old, there has been an increase in resistance to certain antibiotics (MD, TZ and CM) (Ayesha *et al*., 1993; Patterson *et al*., 2010). This is the first and single study in Portugal in which phenotypic characterization of the vaginal microbial strains was performed according to biofilm formation and antibiotic susceptibility. It can be said that strains of *G. vaginalis* isolated from Portuguese clinical samples appear to be more resistant to antibiotics commonly used in other studies, than other strains of *G. vaginalis*, elsewhere.

Conducting studies such as this, is important for the study of the vaginal microbiota, to better understand the preponderant factors that lead to BV becomes possible the development of new treatment regimens for BV, such as treatment with probiotics.

CHAPTER 4

Conclusion remarks and Future perspectives

It is a fact that with regards to Bacterial Vaginosis (BV), there are still many questions regarding the elucidation of its pathogenesis. Therefore, in order to find answers to some of the questions related to BV, we aimed to isolate several species from the vaginal flora of Portuguese women and study its virulence potential in the scope of BV.

Firstly, our study revealed novel strains isolated from cases of healthy or women with BV. This data will contribute to a better understanding of human vaginal microbiota which is fundamental for the optimization of reproductive health. Furthermore, besides the identification of bacterial species involved, their phenotypic characterization was considered essential in this study. Through the evaluation of the intrinsic capacity to grow as biofilm, we verified that G*ardnerella vaginalis* isolated from a case of BV showed the highest capacity for growth as biofilm. It was also found that some of the microorganisms isolated despite not having the same intrinsic capacity to form biofilm as *G. vaginalis*, also had high capacity to grow as biofilm, such as *Enterococcus faecalis*, *Streptococcus* spp., *Bifidobacterium breve* and *Propionibacterium acnes*. There are few studies related to the capacity to form biofilm of in strains isolated from vaginal flora (other than some *Lactobacillus* spp. and *G. vaginalis*) both in cases of healthy women or BV-diagnosed women.

Although, our results do not point to clear evidence that could lead to the confirmation of the etiological agent of BV, we shown than many of the organisms found on BV do not have a high virulent potential, *per se*. Furthermore, the only possible virulence factor studied was the potential of intrinsic capacity to form biofilm of these strains, so further studies must be done in this context. It is also worth to note that *G. vaginalis* and most microorganisms isolated in this study, with a high capacity to form biofilm, also demonstrate intermediate resistance or resistance to traditional antibiotics (metronidazole, tinidazole and clindamycin), and this was not surprising as resistance cases have been reported in studies conducted in other countries.

Follow up studies of interest that would improve some of the knowledge originated from this work, would be the study of mixed biofilm formation which could allow for the analysis of whether there is or not, a change in biofilm formation in the different cases of BV, and to test the hypothesis that *G. vaginalis* associated with others microorganisms can become more virulent. Clearly, isolating more different species and even different strains from the same species would be of interest, in order to achieve epidemiological significance.

Furthermore, characterization of the isolated strains at the level of expression of virulence genes (in the case of *G. vaginalis* - cytolysin secreted called vaginolysin, gene for vaginolysin, (*vly*) and biofilm-forming proteins (BAP) the gene identified (*Bapl*)) in each of the BV-associated microorganisms and non-BV isolates would allow for a better understanding of their virulence traits when in presence or absence of the other anaerobes. Testing of new therapeutic strategies in the isolated strains, such as the use of *Lactobacillus* spp. as part of the treatment of BV (probiotic treatment) and research for natural alternative treatments like using the compounds, that are naturally produced by *Lactobacillus* spp., and that could potentially be active against pathogenic bacteria isolated, would also be of great importance.

Pursuing these experiments will contribute to the better understanding of BV and its impact in women's health.

SUPPLEMENTS

S.1 – Results of sequencing and BLAST

Staphylococcus epidermidis UM042 (206 bp)

CTATACATGCAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGGACGGGT
GAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATAC
CGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGATG
GATC

Accession	Description	Query coverage	Max ident
NR_036904.1	Staphylococcus epidermidis strain Fussel 16S ribosomal RNA, partial sequence	98%	99%
NR_027519.1	Staphylococcus capitis subsp. urealyticus strain MAW 8436 16S ribosomal RNA, partial sequence	98%	97%
NR_024665.1	Staphylococcus caprae strain ATCC 35538 16S ribosomal RNA, partial sequence	98%	97%

Streptococcus agalactiae UM035 (329 bp)

CTACACATGCAGTAGAACGCTGAGGTTTGGTGTTTACACTAGACTGATGAGTTGCGAACGGGT
GAGTAACGCGTAGGTAACCTGCCTCATAGCGGGGGATAACTATTGGAAACGATAGCTAATAC
CGCATAAGAGTAATTAACACATGTTAGTTATTTAAAAGGAGCAATTGCTTCACTGTGAGATGG
ACCTGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATACATAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA
GTAG

Accession	Description	Query coverage	Max ident
NR_040821.1	Streptococcus agalactiae ATCC 13813 strain JCM 5671 16S ribosomal RNA, complete sequence	98%	99%
NR_036918.1	Streptococcus suis strain S735 16S ribosomal RNA, partial sequence	98%	94%
NR_037101.1	Streptococcus urinalis strain 2285-97 16S ribosomal RNA, partial sequence	98%	94%

Mycoplasma hominis UM054 (313 bp)

AACAAACAGCCTACGAACGCTTTACGCCCAATAATTCCGGATAACGCTTGCGACCTATGTATT
ACCGCGGCTGCTGGCACATAGTTAGCCATCGCTTTCTGACCAGGTACCGTCAGTCTGCAATCA
TTTCC

Accession	Description	Query coverage	Max ident
NR_041881.1	Mycoplasma hominis ATCC 23114 strain PG21 16S ribosomal RNA, complete sequence	100%	99%
NR_025135.1	Mycoplasma phocidae strain 105 16S ribosomal RNA, partial sequence	100%	96%
NR_024984.1	Mycoplasma falconis strain H/T1 16S ribosomal RNA, partial sequence	97%	97%

***Lactobacillus gasseri* UM022 (793 bp)**

ATCAGCGTCAGTTGCAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGC
ATTCCACCGCTACACATGGAGTTCCACTCTCCTCTTCTGCACTCAAGTTCAACAGTTTCTGATG
CAATTCTCCGGTTGAGCCGAAGGCTTTCACATCAGACTTATTGAACCGCCTGCACTCGCTTTAC
GCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAG
CCGTGACTTTCTAAGTAATTACCGTCAAATAAAGGCCAGTTACTACCTCTATCTTTCTTCACTA
CCAACAGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGC
GTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAA
TGTGGCCGATCAGTCTCTCAACTCGGCTATGCATCATTGCCTTGGTAAGCCGTTACCTTACCAA
CTAGCTAATGCACCGCAGGTCCATCCAAGAGTGATAGCAGAACCATCTTTTAAACTCTAGACA
TGCGTCTAGTGTTGTTATCCGGTATTAGCATCTGTTTCCAGGTGTTATCCCAGTCTCTTGGGCA
GGTTACCCACGTGTTACTCACCCGTCCGCCGCTCGCTTGTATCTAGTTTCATCTGGTGCAAGCA
CCAAATTCATCTAGGCAAGCTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCT
GAGCCACT

Accession	Description	Query coverage	Max ident
NR_041920.1	Lactobacillus gasseri ATCC 33323 16S ribosomal RNA, complete sequence	99%	99%
NR_044507.1	Lactobacillus taiwanensis strain BCRC 17755 16S ribosomal RNA, partial sequence	99%	99%
NR_025273.1	Lactobacillus johnsonii strain ATCC 33200 16S ribosomal RNA, partial sequence	97%	99%

***Escherichia* spp. UM022 (578 bp) of sequence**

GAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATT
TCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCTTGCCAGTATCAGATGCA
GTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTTTACG
CCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGC
CGGTGCTTCTTCTGCGGGTAACGTCAATGAGCAAAGGTATTAACTTTACTCCCTTCCTCCCCGC
TGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCG
CCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGT

GTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCCCACCTA
CTAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGT

Accession	Description	Query coverage	Max ident
NR_027549.1	Escherichia fergusonii ATCC 35469 16S ribosomal RNA, partial sequence	100%	99%
NR_026331.1	Shigella flexneri strain ATCC 29903 16S ribosomal RNA, partial sequence	100%	99%
NR_026332.1	Shigella dysenteriae strain ATCC 13313 16S ribosomal RNA, partial sequence	100%	99%

***Gardnerella vaginalis* UM016 (612 bp of sequence)**

CTTCTAGCGTCAGTAACAGCCCAGAGACCTGCCTTCGCCATTGGTGTTCTTCCCGATATCTACA
CATTCCACCGTTACACCGAGAATTCCAGTCTCCCCTACTGCACTCTAGCCCGCCCGTACCCGGC
GCAAACCCACCGTTAAGCGATGGGCTTTCACACCAGACGCGACGAACCGCCTACAAGCTCTTT
ACGCCCAATAATTCCGGATAACGCTTGCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTT
AGCCGGCGCTTATTCGAAAGGTACACTCACCCAAAAGGCTTGCTCCCAATCAAAAGCGGTTTA
CAACCCGAAGGCCTTCATCCCGCACGCGGCGTCGCTGCGTCAGGGTTTCCCCCATTGCGCAAT
ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGTCCGCC
CTCTCAGGCCGGCTACCCGTCGAAGCCTAGGTGGGCCATTACCCCGCCTACAAGCTGATAGGA
CGCGACCCCATCCCATGCCACTAAACACTTTCCCAACAAGACATGCGTCAAGTTGGAGCATCC
AGCATTACCACCCGTTTCCAAGAGCT

Accession	Description	Query coverage	Max ident
NR_044694.1	Gardnerella vaginalis 16S ribosomal RNA, complete sequence	100%	99%
NR_044692.1	Bifidobacterium minimum 16S ribosomal RNA, complete sequence	99%	93%
NR_029065.1	Bifidobacterium psychraerophilum strain T16 16S ribosomal RNA, partial sequence	99%	93%

***Mobilincus curtisii* UM027 (754 bp)**

CGCTCTCAGTGTCAGTAACGGCCCAGTGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTG
CGCATTCCACCGCTACACCAGGAATTCCAGTCACCCCTACCGCACTCAAGCCCGCCCGTACCC
ACCGCAGACCAACAGTTAAGCTGCTGGCTTTCACGACAGACGCGACGAACCACCTACGAGCTC
TTTACGCCCAATAAATCCGGACAACGCTCGCGCCCTACGTATTACCGCGGCTGCTGGCACGTA
GTTAGCCGGCGCTTCTTCCCCCACTACCATCAACACAGCCAAAACTGTGCCTTTTTCGCGAGAA
AAAGGAGTTCACAACCCGAAGGCCTACACCCTCCACGCGGCGTCGCTGCATCAAACTTTCGTC
CATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAGTGC
GACCGACCACCCTCTCAGGCCGGCTACCCGTCAAAGCCTTGGTAAGCCATCACCCCACCAACA
AGCTGATAGGCCGCGAGCCCACCCAATCCCAGAAAAAACCTTTCCAAACACCACCATGCGGT

GACGCCTGAATATCCAGTATTAGCAGCCGTTTCCAGCCGTTATCCCAAAGAAAAGGACAGGTT
ACTCACGTGTTACTCGCCCGTTCGCCACTAATCCACCACAGCAAGCTGCGGCTTCATCGTTCGA
CTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCTGAGCCAAA

Accession	Description	Query coverage	Max ident
NR_042124.1	Mobiluncus curtisii subsp. holmesii ATCC 35242 strain : CCUG 17762 16S ribosomal RNA, partial sequence	98%	99%
NR_042085.1	Mobiluncus curtisii subsp. curtisii ATCC 35241 strain CCUG 21018 16S ribosomal RNA, partial sequence	97%	99%
NR_042086.1	Mobiluncus mulieris strain CCUG 20071 16S ribosomal RNA, partial sequence	98%	98%

***Alloscardovia omnicolens* UM031 (623 of sequence)**

CTCAGCGTCAGTAATAGCCCAGAGACCTGCCTTCGCCATTGGTGTTCTTCCCGATATCTACACA
TTCCACCGTTACACCGAGAATTCCAGTCTCCCCTACTACACTCTAGCCTGCCCGTACCCAGCGC
CAATCCACCGTTAAGCGATGGACTTTCACACCAGACGCGACAAACCGCCTACGAGCTCTTTAC
GCCCAATAATTCCGGACAACGCTTGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAG
CCGGTGCTTATTCAAAAGGTACACTCACTCACGCTTGCTCCCCAATAAAAGCGGTTTACAACC
CGAAGGCCGTCATCCCGCACGCGGCGTCGCTGCATCAGGGTTCCCCCCATTGTGCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTC
AGGCCGGCTACCCGTCGAAACCTTGGTAGGCCACTACCCCACCAACAAGCTGATAGGACGCG
ATCCCATCGCATAGCACTAAAACGTTTTCCCACACACCCATGCGAGCATGTGGAACATTCGGC
ATTACCACCCGTTTCCAGGAGCTATTCCAAACTAC

Accession	Description	Query coverage	Max ident
NR_042583.1	Alloscardovia omnicolens strain : CCUG 31649 16S ribosomal RNA, complete sequence	100%	99%
NR_041347.1	Metascardovia criceti strain OMB105 16S ribosomal RNA, partial sequence	100%	95%
NR_044692.1	Bifidobacterium minimum 16S ribosomal RNA, complete sequence	98%	93%

***Bifidobacterium breve* UM031 (753 bp of sequence):**

AGATCACAGAGACCTGCCTTCGCCATTGGTGTTCTTCCCGATATCTACACATTCCACCGTTACA
CCGGGAATTCCAGTCTCCCCTACCGCACTCAAGCCCGCCCGTACCCGGCGCGGATCCACCGTT
AAGCGATGGACTTTCACACCGGACGCGACGAACCGCCTACGAGCCCTTTACGCCCAATAATTC
CGGATAACGCTTGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTC
GAAAGGTACACTCAACACAAAGTGCCTTGCTCCCTAACAAAAGAGGTTTACAACCCGAAGGC
CTCCATCCCTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCT
GCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTCAGGCCGG

CTACCCGTCGAAGCCATGGTGGGCCGTTACCCCGCCATCAAGCTGATAGGACGCGACCCCATC
CCATGCCGCAAAGGCTTTCCCAACACACCATGCGGTGTGATGGAGCATCCGGCATTACCACCC
GTTTCCAGGAGCTATTCCGGTGCATGGGGCAGGTCGGTCACGCATTACTCACCCGTTCGCCAC
TCTCACCAGGCAGCAAGCTGCCTGGATCCCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGC
GTTCATCCTGAGCCAGGATTC

Accession	Description	Query coverage	Max ident
NR_040783.1	Bifidobacterium breve DSM 20213 strain ATCC 15700 16S ribosomal RNA, partial sequence	98%	99%
NR_043442.1	Bifidobacterium pseudolongum subsp. pseudolongum strain JCM 1205 16S ribosomal RNA, partial sequence	98%	95%
NR_036854.1	Bifidobacterium boum strain RU917 16S ribosomal RNA, partial sequence	98%	95%

***Streptococcus* spp. UM031 (754 bp of sequence):**

GAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCT
ACGCATTTCACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTTGACAGTTTCC
AAAGCGAACTATGGTTGAGCCACAGCCTTTAACTTCAGACTTATCAAACCGCCTGCGCTCGCT
TTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTGCTGGCACGTAG
TTAGCCGTCCCTTTCTGGTAAGCTACCGTCACAGTGTGAACTTTCCACTCTCACACTCGTTCTT
GACTTACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGG
GTTGCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT
CCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCGTCGCCTAGGTGAGCCGTTACCTC
ACCTACTAGCTAATACAACGCAGGTCCATCTTGTAGTGGAGCAATTGCCCCTTTCAAATAAAT
GACATGTGTCATCCATTGTTATGCGGTATTAGCTATCGTTTCCAATAGTTATCCCCCGCTACAA
GGCAGGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCAAGAAGAGCAAGCTCCTCTCT
TCAGCGTTCTACTTGCATGTATTAGGCACGCCGCCAGCGATCG

Accession	Description	Query coverage	Max ident
NR_042776.1	Streptococcus salivarius strain ATCC 7073 16S ribosomal RNA, complete sequence	100%	99%
NR_042778.1	Streptococcus thermophilus strain ATCC 19258 16S ribosomal RNA, complete sequence	100%	99%
NR_042777.1	Streptococcus vestibularis ATCC 49124 strain ATCC 49124 16S ribosomal RNA, complete sequence	100%	99%

***Bacillus firmus* UM034 (744 bp of sequence):**

GTCGCCTTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCC
ACTCTTCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCT
TTCACATCAGACTTAAGGAACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACGCTT
GCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGT
CAAGGTACCGGCAGTTACTCCGGTACTTGTTCTTCCCTAACAACAGAGTTTTACGATCCGAAA
ACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTG
CTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCG
GCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCAT
CTGTAAGTGATAGCCGAAACCATCTTTCAGCTTTCCCTCATGTGAGGGAAAGAATTATCCGGT
ATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTGCCCACGTGTTACTCACCC
GTCCGCCGCTGACTTCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCAC

Accession	Description	Query coverage	Max ident
NR_025842.1	Bacillus firmus strain IAM 12464 16S ribosomal RNA, partial sequence	100%	98%
NR_043762.1	Bacillus thioparans strain BMP-1 16S ribosomal RNA, partial sequence	99%	96%
NR_044828.1	Bacillus benzoevorans strain NCIMB 12555 16S ribosomal RNA, partial sequence	100%	96%

***Gardnerella vaginalis* UM035 (456 of sequence):**

GCAGTCCAGAGACCTGCCTTCGCCATTGGTGTTCTTCCCGATATCTACACATTCCACCGTTACA
CCGGGAATTCCAGTCTCCCCTACTGCACTCTAGCCCGCCCGTACCCGGCGCAAACCCACCGTT
AAGCGATGGGCTTTCACACCAGACGCGACGAACCGCCTACAAGCTCTTTACGCCCAATAATTC
CGGATAACGCTTGCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTATT
CGAAAGGTACACTCACCCGAAAGCTTGCTCCCAATCAAAAGCGGTTTACAACCCGAAGGCCTT
CATCCCGCACGCGGCGTCGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCCACTGCTGCC
TCCCGTAAGAGTCTGGGCCGTATCTCAGTCCCAATGGGGCCGTCCG

Accession	Description	Query coverage	Max ident
NR_044694.1	Gardnerella vaginalis 16S ribosomal RNA, complete sequence	99%	98%
NR_037117.1	Bifidobacterium pseudocatenulatum strain B1279 16S ribosomal RNA, partial sequence	97%	95%
NR_041875.1	Bifidobacterium catenulatum strain ATCC27539 16S ribosomal RNA, partial sequence	97%	95%

***Gemella* spp. UM034 (745 of sequence):**

CGCGCTCAGTGTCAGTTACAGGCCAAAAAGCCGCCTTCGCCACTGGTGTTCCTCCTAATCTCTA
CGCATTTCACCGCTACACTAGGAATTCCACTTTTCTCTCCTGCACTCAAGTTTAACAGTTTCCA
ATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTATTAAACCACCTGCGCGCGCTT
TACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGTGGCTTTCTGGTTAGGTACCGTCTCTACTGTGTATAGTTACTACACAATCATTCTTCC
CTAACAACAGAGCTTTACGAACCGAAATCCTTCTTCACTCACGCGGCGTTGCTCCGTCAGGCTT
TCGCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCC
AGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTCGCCTTGGTAGGCCTTTACCCCACC
AACTAGCTAATGCACCGCAAAGCCATCTCATAGTGTTAGCAAAACCAACTTTCAAACATCAAC
CATGCAGTTAATGCTGTTATCCGGTATTAGCTATCGTTTCCAATAGTTGTCCCAGTCTATGAGG
CAGGTTCTTTACGTGTTACTCACCCGTTCGCCGCTAAGTTTTTCTAGTGCAAGCACCAGAAAAA
CTTCGCTCGACTTGCATGTATTAGGCAAGC

Accession	Description	Query coverage	Max ident
NR_025903.1	Gemella haemolysans strain ATCC 10379 16S ribosomal RNA, partial sequence	99%	99%
NR_026419.1	Gemella sanguinis strain 2045-94 16S ribosomal RNA, partial sequence	99%	98%
NR_025904.1	Gemella morbillorum strain 2917B 16S ribosomal RNA, partial sequence	99%	98%

***Klebsiella* spp. UM034 (734 of sequence):**

CGCACTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTA
CGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGA
ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCT
TTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGT
TAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGCCAAGGTTATTAACCTTAACGCCTTCCTCC
CCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCT
TGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTC
CAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCA
CCTACTAGCTAATCCCATCTGGGCACATCTGATGGCATGAGGCCCGAAGGTCCCCCACTTTGG
TCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTT
CCCAGACATTACTCACCCGTCCGCCGCTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCGCTC
GACTTGCATGTGTTAGGCCTGC

Accession	Description	Query coverage	Max ident
NR_041750.1	Klebsiella pneumoniae subsp. ozaenae strain ATCC11296 16S ribosomal RNA, partial sequence	100%	99%
NR_037084.1	Klebsiella pneumoniae subsp. rhinoscleromatis strain R-70 16S ribosomal RNA, partial sequence	100%	99%
NR_024643.1	Enterobacter aerogenes strain JCM1235 16S ribosomal RNA, partial sequence	100%	99%

***Propionibacterium acnes* UM034 (745 of sequence):**

AAATCAGCGTCAGGAAGGCCCAGAGAACCGCCTTCGCCACTGGTGTTCCTCCTGATATCTGCG
CATTCCACCGCTCCACCAGGAATTCCATTCTCCCCTACCTTCCTCAAGTCAACCCGTATCGAAA
GCACGCTCAGGGTTAAGCCCCAAGATTACACTTCCGACGCGATCAACCACCTACGAGCCCTTT
ACGCCCAATAAATCCGGACAACGCTCGCACCCTACGTATCACCGCGGCTGCTGGCACGTAGTT
AGCCGGTGCTTCTTTACCCATTACCGTCACTCACGCTTCGTCACAGGCGAAAGCGGTTTACAAC
CCGAAGGCCGTCATCCCGCACGCGGCGTTGCTGCATCAGGCTTCCGCCCATTGTGCAATATTC
CCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCACCCTCT
CAGGCCGGCTACCCGTCAAAGCCTTGGTAAGCCACTACCCCACCAACAAGCTGATAAGCCGCG
AGTCCATCCCCAACCGCCGAAACTTTCCAACCCCCACCATGCAGCAGGAGCTCCTATCCGGTA
TTAGCCCCAGTTTCCTGAAGTTATCCCAAAGTCAAGGGCAGGTTACTCACGTGTTACTCACCCG
TTCGCCACTCGAGCACCCCACAAAAGCAGGGCCTTTCCGTTCGACTTGCATGTGTTAAGCACG
CCGCCAGCGTTCGTCCTGAGCCAGTCTA

Accession	Description	Query coverage	Max ident
NR_040847.1	Propionibacterium acnes 16S ribosomal RNA, complete sequence	99%	99%
NR_025277.1	Propionibacterium propionicum strain DSM 43307 16S ribosomal RNA, partial sequence	98%	95%
NR_025274.1	Propionibacterium avidum strain DSM 4901 16S ribosomal RNA, partial sequence	98%	95%

***Enterococcus faecalis* UM035 (759 of sequence):**

GAGCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCATATATCTA
CGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCA
ATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCTCGCT
TTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAG
TTAGCCGTGGCTTTCTGGTTAGATACCGTCAGGGGACGTTCAGTTACTAACGTCCTTGTTCTTC
TCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGACT
TTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCC

CAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTGGCCTTGGTGAGCCGTTACCTCAC
CAACTAGCTAATGCACCGCGGGTCCATCCATCAGCGACACCCGAAAGCGCCTTTCACTCTTAT
GCCATGCGGCATAAACTGTTATGCGGTATTAGCACCTGTTTCCAAGTGTTATCCCCCTCTGATG
GGTAGGTTACCCACGTGTTACTCACCCGTCCGCCACTCCTCTTTCCAATTGAGTGCAAGCACTC
GGGAGGAAAGAAGCGTTCGACTTGCATGTATTAGGCACGCCGCCA

Accession	Description	Query coverage	Max ident
NR_040789.1	Enterococcus faecalis strain JCM 5803 16S ribosomal RNA, partial sequence	100%	99%
NR_042405.1	Enterococcus silesiacus strain : LMG 23085 = R-23712 16S ribosomal RNA, complete sequence	100%	96%
NR_043285.1	Enterococcus caccae strain 2215-02 16S ribosomal RNA, partial sequence	100%	96%

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