U.PORTO

DOUTORAMENTO

CIÊNCIAS BIOMÉDICAS

Determining the Pathogenic Potential of Commensal and Clinical *Gardnerella vaginalis* Isolates

Joana Isabel Castro

D

2018

U.PORTO

Joana Isabel Castro. Determining the Pathogenic Potential of Commensal and Clinical *Gardnerella vaginalis* Isolates

Determining the Pathogenic Potential of Commensal and Clinical *Gardnerella vaginalis* Isolates

Joana Isabel Reis Castro

D.ICBAS **2018**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

JOANA ISABEL REIS CASTRO

DETERMINING THE PATHOGENIC POTENTIAL OF COMMENSAL AND CLINICAL *GARDNERELLA VAGINALIS* ISOLATES

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutor Nuno Cerca

Categoria – Investigador Principal

Afiliação – Centro de Engenharia Biológica, LIBRO – Laboratório de Investigação em Biofilmes Rosário Oliveira, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Coorientador – Doutora Kimberly Kay Jefferson

Categoria – Professora Associada

Afiliação – Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA, 23298-0678c, USA

"The important thing is not to stop questioning.
Curiosity has its own reason for existing."
Albert Einstein

The work presented in this thesis was funded by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684), the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte. JC acknowledges the FCT individual grant with reference SFRH/BD/93963/2013.

Acknowledgments

Um guerreiro da luz nunca esquece a gratidão. Durante a luta, foi ajudado pelos anjos; as forças celestiais colocaram cada coisa em seu lugar, e permitiram que ele pudesse dar o melhor de si. (…) Sua gratidão, porém, não se limita ao mundo espiritual; ele jamais esquece os amigos, porque o sangue deles se misturou ao seu no campo de batalha.

Paulo Coelho, em *O Manual do Guerreiro da Luz*

Ao meu orientador, **Doutor Nuno Cerca**, pela orientação, partilha de conhecimentos, por todas as oportunidades, pela confiança e amizade ao longo destes anos.

I would like to acknowledge my co-supervisor, **Doctor Kimberly Jefferson,** who kindly welcomed me in her lab at the Virginia Commonwealth University and by the guidance, knowledge transfer and assistance in writing reports, papers and also in the thesis.

A **todos os meus colegas e amigos do "grupo NC"**, pelo bom ambiente de trabalho, pelo apoio e amizade. Um especial agradecimento para a **Ângela França**, pela colaboração no estudo do transcriptoma da *Gardnerella vaginalis* (capítulo 5).

Gostaria também de agradecer aos **antigos membros do "grupo NC"**, em especial à **Tatiana Cereja**, **Patrícia Alves** e **Cármen Sousa**, pelo suporte técnico na caracterização fenotípica dos vários isolados de *G. vaginalis* (capítulo 3) e à **Daniela Machado** pela colaboração no trabalho dos biofilmes mistos (capítulo 7).

Não posso deixar de referir o Centro de Engenharia Biológica (que tem sido uma segunda casa nos últimos anos) e todos os que dele fazem parte.

A todos os outros meus amigos e em especial à **Célia Fortuna, Lília Marques, Liliana Maia, Madalena Maia, Marino Maciel, Natália Martins, Paulo Silva,** e **Sara Pimenta** pela amizade sincera. **Terrie Scherer,** I will never forget your kindness and friendship.

Não poderia de deixar de fazer um agradecimento especial aos meus **Pais, Irmão e Avós** por serem o meu porto de abrigo, pelos valores transmitidos, pelos meios que sempre me proporcionaram, pela força e pela coragem que sempre me incutiram para seguir em frente e realizar este sonho.

E por fim, a ti, **Pedro Silva** quero agradecer-te por todos os teus gestos de amor, que se refletiram na infinita paciência, compreensão e apoio moral que sempre me mostraste em todos os momentos.

A todos, enfim, reitero o meu apreço e a minha eterna gratidão.

Scientific outputs

Under the terms of the Decree of Law 74/2006, article 34º, point 1, published in Diário da República, 1ª série, nº60, in 24 of March 2006, altereded by the Decree of Law 11/2013, published in Diário da República, 1ª série, nº151, in 7 of August 2013, that proceeds the 3rd alteration of Decree of Law 74/2006, the author hereby declared that has actively participated in the design and technical execution of the work, interpretation of the results and manuscript preparation of the original articles included in this thesis. Under the terms of the referred Decreto-Lei, the author hereby declared that the following original articles/communications were prepared in the scope of this thesis.

Papers in peer reviewed journals

Castro J, França A, Bradwell KR, Serrano MG, Jefferson KK, Cerca N **(2017)**. Comparative transcriptomic analysis of *Gardnerella vaginalis* biofilms *versus* planktonic cultures using RNA-seq. *NPJ Biofilms Microbiomes* 3:3 (doi: 10.1038/s41522-017-0012-7).

Castro J, Machado D, Cerca N **(2016)**. *Escherichia coli* and *Enterococcus faecalis* are able to incorporate and enhance a pre-formed *Gardnerella vaginalis* biofilm. *Pathogens and Disease* 74:3 (doi: 10.1093/femspd/ftw007).

Castro J, Cerca N **(2015)**. BV and non-BV associated *Gardnerella vaginalis* establish similar synergistic interactions with other BV-associated microorganisms in dual-species biofilms. *Anaerobe* 36:56-9 (doi: 10.1016/j.anaerobe.2015.10.008).

Castro J, Alves P, Sousa C, Cereija TB, França A, Jefferson KK, Cerca N **(2015)**. Using an *in-vitro* biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis *Gardnerella vaginalis* isolates*. Scientific Reports* 5:11640 (doi: 10.1038/srep11640).

Book chapter

Castro J, Machado D, Cerca N **(2015)**, "*Gardnerella vaginalis* gene expression in biofilms" in Impact of biofilms in health: a transcriptomics perspective (Ed. Cerca N) 227-243, Universidade do Minho – DEB, Braga, Portugal, ISBN: 978-989-97478-6-9.

Oral presentations

Castro J, Cerca N* **(2018)**. The microbiome during bacterial vaginosis: who is cooperating with *Gardnerella vaginalis* in enhancing crucial gene expression in a dual-species

biofilm model? *IUSTI, 2018 Word + European Congress*, 27th – 30th June, Dublin, Ireland (*presenting author).

Castro J*, Cerca N **(2018)**. Bacterial cooperation in Bacterial Vaginosis: are social interactions relevant to enhance *Gardnerella vaginalis* virulence? *First Meeting of the PhD in Biomedical Sciences*, Instituto de Ciências Biomédicas Abel Salazar, 7th May, Porto, Portugal (*presenting author).

Castro J, Machado D, Cerca N* **(2017)**. Can cooperation within the vaginal microbiome lead to the development of bacterial vaginosis? *NZ Microbiological Society (NZMS) Conference*, 20th – 23rd November, Auckland, New Zealand (*presenting author).

Castro J*, França A, Bradwell KR, Serrano MG, Jefferson KK, Cerca N **(2016)**. What happens to *Gardnerella vaginalis* when growing as a biofilm: a comparative transcriptomic analysis by RNA-seq. *Biofilms 7 - Microbial Works of Art*. No. SPM: 3, ISBN: 978-989-97478-7-6, 26th – 28th June, Porto, Portugal (*presenting author).

Poster presentations

Castro J, Cerca N* **(2018)**. Can the differential response to innate immune components by commensal and clinical *Gardnerella vaginalis* isolates be the key leading to the development of bacterial vaginosis? *IUSTI, 2018 Word + European Congress*, 27th – 30th June, Dublin, Ireland (*presenting author).

Castro J*, França A, Bradwell KR, Serrano MG, Jefferson KK, Cerca N **(2016)**. The bacterial vaginosis riddle: how transcriptomics can highlight key physiological adaptations of *G. vaginalis* when growing as biofilms? *The New Microbiology - EMBO|FEBS Lecture course*, 24th August – 1st September, Spetses, Greece (*presenting author).

Castro J*, Machado D, Cerca N **(2015)**. Assessing synergistic interaction between *Gardnerella vaginalis* and other urogenital pathogens. *Eurobiofilms 2015: Fourth European Congress on Microbial Biofilms*, 23rd – 26th June, Brno, Czech Republic (*presenting author).

Determinação do potencial patogénico de isolados clínicos e comensais de *Gardnerella vaginalis*

Sumário

No último meio século, a vaginose bacteriana (VB) tem sido um tema controverso na microbiologia médica. Curiosamente, apesar de todo interesse e investigação no campo da VB, o agente etiológico ainda não foi definitivamente identificado. Os primeiros estudos realizados nesta área sugeriram que o agente infecioso causador da VB era a *Gardnerella vaginalis*. No entanto, de acordo com dados posteriores descobriu-se que *G. vaginalis* também estava presente em mulheres saudáveis. Tais descobertas levantaram dúvidas acerca do papel desta bactéria como agente etiológico na VB. Além disso, existem evidências que *G. vaginalis* não é capaz de causar VB de forma consistente. É de salientar, que outras espécies bacterianas também têm sido, comumente, associadas à VB. Tal facto, levou à postulação da teoria polimicrobiana para o desenvolvimento desta infeção. No entanto, dados epidemiológicos subsequentes também revelaram inconsistências com esta última teoria. Recentemente, surgiram as primeiras descrições dos biofilmes polimicrobianos de VB. Interessantemente, a *G. vaginalis* parece constituir a maior parte da biomassa desses biofilmes, que apresentam maior tolerância a estímulos externos. Estas descobertas levaram à formulação de uma nova hipótese, que sugere que variantes de estirpes de *G. vaginalis* com capacidade de induzir um biofilme podem, de facto, ser o agente causador de VB.

Assim, numa tentativa de compreender as diferenças entre estirpes de *G. vaginalis* isoladas de mulheres com vaginose bacteriana (VB) *versus* mulheres com flora normal saudável (não-VB), comparou-se o potencial de virulência de 7 estirpes de *G. vaginalis* VB e 7 estirpes não-VB. Para esse efeito foram analisadas várias características fenotípicas, nomeadamente: capacidade de formação de biofilme, adesão inicial a células humanas, interações ecológicas com bactérias endógenas com potencial benéfico, atividade citotóxica e adaptação fisiológica ao ambiente vaginal que contém fatores de proteção solúveis, como moléculas responsáveis pela imunidade inata. Notavelmente, os nossos resultados revelaram que as estirpes isoladas de mulheres com VB foram mais virulentas do que as estirpes que colonizaram as mulheres saudáveis. É de notar que apenas as estirpes de *G. vaginalis* associadas a VB foram capazes de destacar, em grande número, os lactobacilos endógenos previamente aderidos a uma monocamada de células epiteliais. Tais evidências sugerem que este parece ser o fator responsável pelo início do desenvolvimento da VB. No entanto, apesar de todas as diferenças entre os dois grupos de *G. vaginalis*, os nossos resultados demonstraram que o fator chave

na diferenciação de estirpes isoladas de mulheres com VB e de mulheres saudáveis não está relacionado com uma melhor adaptação destas aos componentes imunes do hospedeiro.

Posteriormente, numa tentativa de estudar outros fatores de virulência da *G. vaginalis*, realizou-se uma análise do transcriptoma, por sequenciação do ácido ribonucleico, de uma estirpe proveniente de uma mulher com VB. Com base nesta análise, constatou-se que *G. vaginalis* altera o seu perfil transcriptómico quando se apresenta sob a forma de biofilme. Este fenótipo corresponde a um estado fisiológico que pode promover a natureza crónica e recorrente da VB. É de salientar, que essas alterações no perfil transcriptómico da *G. vaginalis* são, provavelmente, importantes para a persistência do biofilme e, consequentemente, para a virulência dessa bactéria.

Por último, analisou-se de que forma outras espécies associadas à VB poderiam influenciar o desenvolvimento do biofilme de *G. vaginalis*. Para isso, inicialmente, determinou-se se as estirpes de *G. vaginalis* isoladas de mulheres com VB apresentariam alguma vantagem sobre os isolados não-VB, quando outras espécies bacterianas são associadas a um biofilme pré-estabelecido de *G. vaginalis*. Os nossos resultados apontaram que a principal diferença no potencial de virulência entre os dois grupos de *G. vaginalis* parece não estar relacionada com a maturação do biofilme. Posteriormente, foram investigadas as interações ecológicas entre uma estirpe de *G. vaginalis* isolada de uma mulher com VB e outras espécies bacterianas, também associadas à VB, e que tinham apresentado previamente um sinergismo com um biofilme pré-estabelecido de *G. vaginalis*. Curiosamente, este estudo revelou que as interações ecológicas foram muito específicas para cada consórcio bacteriano, confirmando que nem todos os colonizadores secundários contribuíram para o aumento da patogénese da VB, com base nos níveis de transcrição de genes de virulência da *G. vaginalis*. Em suma, este estudo lançou uma nova luz relativamente ao papel de várias espécies bacterianas associadas à VB no desenvolvimento do biofilme de VB, podendo estas modular de forma diferente os fatores de virulência da *G. vaginalis*. O trabalho desenvolvido nesta tese permitiu retirar novas ilações acerca da virulência de *G. vaginalis* e da etiologia da VB e pode, em última instância, ajudar a delinear novas estratégias de prevenção da VB, bem como reduzir as taxas de recorrência que lhe estão associadas.

Determining the pathogenic potential of commensal and clinical *Gardnerella vaginalis* isolates

Abstract

In the past half century, bacterial vaginosis (BV) has been a controversial topic in medical microbiology because, despite interest and investigation, the etiological agent has not yet been definitively identified. Earlier advances suggested *Gardnerella vaginalis* as the infectious causative agent of BV but soon after it was found that *G. vaginalis* was also present in healthy women, and this cast doubts of its role as the etiological agent in BV. Furthermore, *G. vaginalis* was not able to cause BV consistently. Importantly, other bacterial species started to be commonly associated with BV, and this raises the theory of the multi-species infection. However, subsequent epidemiological data also revealed inconsistencies with this latter theory. Recently, the first descriptions of multi-species biofilm communities were described in BV. Interestingly, *G. vaginalis* appears to account for most of the biomass of BV biofilms. Further studies demonstrated that *G. vaginalis* biofilm cells presented higher tolerance to external stresses. These findings derived a new hypothesis, where strain variants of *G. vaginalis* strains could induce a biofilm and be, in fact, the causative agent of BV, owing to its higher virulence potential.

In an effort to better understand the differences between *G. vaginalis* isolated from women with bacterial vaginosis (BV) *versus* normal healthy flora (non-BV), we compared the virulence potential of 7 non-BV and 7 BV associated *G. vaginalis* isolates by scrutinizing its phenotypic features, namely: biofilm forming-capacity, initial adhesion to human cells, ecological interactions with endogenous beneficial bacteria, cytotoxic activity, and the physiological adaptation to vaginal niche which contains soluble innate immune molecules. Remarkably, our results revealed that strains from BV women were more virulent than strains colonizing healthy women. Notably, we demonstrated that only BV associated *G. vaginalis* strains were able to dramatically displace pre-coated vaginal protective lactobacilli and we hypothesize this to be a trigger for BV development. However, despite all the differences between both *G. vaginalis* groups, our results suggested that a better adaptation to the host immune components is not a key factor differentiating between isolates from women with BV and from healthy women.

We also conducted a transcriptomic analysis by RNA-sequencing, in which we showed that a BV associated *G. vaginalis* changes its transcriptomic profile when growing as a biofilm, resulted in a distinct physiologic status that may promote the chronic and recurrent nature of BV. These changes are likely important for biofilm persistence and, consequently, for the

virulence of this bacterium, suggesting that biofilms indeed play a key role in BV development. This phenotype may contribute towards the chronic and recurrent nature of BV.

We also addressed how other BV associated species could be contributing to the development of multi-species biofilms. For that, we first determined whether BV associated *G. vaginalis* presented any advantage over non-BV isolates in biofilm enhancement by other BV associated bacterial species, using an *in vitro* dual-species biofilm formation model. However, our findings pointed out that the key difference in virulence potential between the two *G. vaginalis* groups seems not be related with biofilm maturation. Furthermore, we also investigated the ecological interactions between a BV associated *G. vaginalis* strain with other BV associated bacteria that had previously indicated synergism with a pre-formed *G. vaginalis* biofilm. Interestingly, this study revealed that ecological interactions were very specific to each consortium, confirming that not all BV-secondary bacteria are able to enhance the BV pathogenesis by influencing the transcriptomic profile of key virulence genes of *G. vaginalis*. Finally, our study casts a new light on how BV associated species can modulate the virulence aspects of *G. vaginalis,* contributing to a better understanding of the development of BV associated biofilms. Together, these new findings about the virulence traits of *G. vaginalis* and the etiology of BV could ultimately help to shape new strategies for BV prevention and reduction of BV rates.

Aos meus pais. Ao meu irmão.

E a ti, Pedro.

Table of content

Abbreviation list

AMPs – antimicrobial peptides

ARDRA – amplified ribosomal DNA restriction analysis

BFI – biofilm formation index

BHI – brain heart infusion

bp – base pair

BV – bacterial vaginosis

BVAB – BV associated bacteria

cDNA – complementary DNA

CFU – colony-forming units

CLSM – confocal laser scanning microscopy

CV – crystal violet

DAPI – 4'-6-Diamidino-2-phenylindole

DNA – deoxyribonucleic acid

FDR – false discovery rate

FISH – fluorescence *in situ* hybridization

FRT – female reproductive tract

FW – forward

GO – gene ontology

Gv – *Gardnerella vaginalis*

HBD – human β-defenin

HBD2 – human β-defenin 2

HD – human defensin

HNP – human neutrophil peptide

KEEG – Kyoto Encyclopedia of Genes and Genomes

LB – luria broth

LF – lactoferrin

LYS – lysozyme

mGTS – medium simulating genital tract secretions

MIC – minimum inhibitory concentration

MRS – de man-rogosa and sharpe agar

OD – optical density

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PFGE – Pulsed-field Gel Electrophoresis

PNA – peptide nucleic acid

qPCR – quantitative PCR

RAPD – random amplification of polymorphic DNA

RNA – ribonucleic acid

RNA-seq – RNA-sequecing

RPKM – reads per kilobase per million

RQI – RNA quality indicator

rRNA – ribosomal RNA

Rv – reverse

sBHI – supplemented brain heart infusion

SD – standard deviation

SEM – standard error of mean

SLPI – secretory leukocyte protease inhibitor

STRING – Search Tool to the Retrieval of Interacting Genes/proteins

TLRs – Toll-like receptors

tRNA – transfer RNA

TSB – tryptic soy broth

Index of Figures

CHAPTER 6

CHAPTER 7

CHAPTER 8

Index of Tables

CHAPTER 6

CHAPTER 7

CHAPTER 1

Introduction

Summary

This chapter provides a brief outline of the thesis. The background, research questions, hypothesis, aims, and significance are presented here.

1.1 Background

Bacterial vaginosis (BV), characterized by a shift of the vaginal microbiota from a *Lactobacillus*-dominated community to a dense biofilm containing a complex mixture of microorganisms, is an important risk factor in poor reproductive health. The high prevalence, high relapse rate, and associated complications make this disorder of paramount global importance [1,2]. Therefore, control of BV has been advocated for decreasing the prevalence of these complications, but the precise etiology remains unknown [3]. As a result, current treatment regimens and prevention strategies are inadequate. Such a lack of understanding not only inhibits our ability to effectively manage BV but also severely affects our ability to prevent its associated complications [4].

Microbiological analysis of BV has shown *Gardnerella vaginalis* to be the most frequent microorganism in BV, being isolated in more than 95% of cases [2]. However, there has been much debate in the literature concerning the contribution of *G. vaginalis* to the etiology of BV, since it is also present in a considerable proportion of healthy women [5,6]. The research group of Dr. Nuno Cerca has been involved in determining the differences between *G. vaginalis* and other vaginal isolates in order to explain the outcome of colonization [7-9]. A recent study [7] clearly demonstrated that *G. vaginalis* may be more suited as an early colonizer relative to the others BV associated anaerobes tested in the initial adhesion and that it may play a key role in the early establishment of BV biofilms. Of high importance, a study led by Dr. Kimberly Jefferson demonstrated that a non-BV isolate had fundamental genomic differences, as compared with the genome of a BV isolate of *G. vaginalis* [10]. This lead to the hypothesis that non-virulent *G. vaginalis* strains could occur in healthy women, while virulent strains could cause BV.

This study was designed to determine the presence of putative virulence markers in *G. vaginalis* strains isolated from Portuguese women with BV (n = 7) or without BV (n = 7). Comparison of the two sets of isolates is expected to reveal factors that may assist in the diagnosis of BV. Furthermore, we also set out to study the ecological interactions of *G. vaginalis* and other BV associated bacteria to understand the impact of the bacterial cooperation on *G. vaginalis* virulence. Together, this thesis attempts to advance our understanding of the mystery of BV pathogenesis, since this is essential to make progress in the control and prevention of this common, important condition.

1.2 Research questions

The following questions will be addressed in this thesis:

1. Can BV associated *G. vaginalis* isolates exhibit more virulence factors than non-BV isolates?

2. Can the differential response to innate immune components by non-BV and BV associated *G. vaginalis* isolates be key in BV development?

3. What happens to the *G. vaginalis* virulence profile when it is growing as a biofilm?

4. Do other BV associated species cooperate with *G. vaginalis* and enhance its virulence?

Answers to these research questions will provide new knowledge regarding the etiology of BV and might contribute to the design of improved treatment strategies.

1.3 Hypothesis and aims

1.3.1 Hypothesis

G. vaginalis can colonize the vaginal epithelium of both women with and without BV. This investigation tested the following hypothesis:

Clinical *G. vaginalis* strains are able to cause BV owing to phenotypic and genotypic adaptations that provide an ecological niche advantage over non-BV *G. vaginalis*.

1.3.2 Aims

In an effort to better understand the differences between commensal and clinical *G. vaginalis* isolates, *in vitro* assays will be performed in order to compare virulence properties of *G. vaginalis* strains isolated from Portuguese women with and without BV. This will be approached using the following sub-aims.

Aim 1: To assess the possible differences in the phenotype and genotype of non-BV and BV associated *G. vaginalis* strains.

a) To analyse the initial adhesion of non-BV and BV associated *G. vaginalis* strains to a monolayer of epithelial cells and to analyse their cytotoxic effects.

b) To compare the biofilm-forming capacity between both *G. vaginalis* groups.

c) To investigate the antimicrobial susceptibility profile of both *G. vaginalis* groups and their ability to displace beneficial endogenous bacteria from the epithelial cells.

d) To analyse the expression of virulence-related genes.

e) To identify the subgroups of non-BV and BV associated *G. vaginalis* strains according to a clade-specific genotyping system.

Aim 2: To identify the possible differences in the physiological adaptation of non-BV and BV associated *G. vaginalis* strains to the innate immune system. To achieve this, we will perform a series of *in vitro* assays to evaluate the antimicrobial susceptibility of both *G. vaginalis* groups to innate molecules (lysozyme, lactoferrin, human β defensin-2); and its initial adhesion ability, the biofilm-forming capacity, as well as the planktonic growth in the presence of physiological vaginal concentrations of the innate molecules.

Aim 3: To gain insight into the role of *G. vaginalis* biofilms in the pathogenesis of BV, we will carry out a comparative transcriptomic analysis between planktonic and biofilm cultures, using RNA-sequencing.

Aim 4: To investigate the ecological interactions between non-BV or BV associated *G. vaginalis* strains with other BV associated bacterial species, using a dual-species biofilm assembly consisting of *G. vaginalis* and secondary BV associated species.

a) To assemble pairwise combinations between non-BV or BV associated *G. vaginalis* isolates and 24 other BV associated bacteria, and compare the synergistic, neutral or antagonistic interactions between the two bacteria through the quantification of total biofilm biomass.

b) To discriminate the bacterial populations of dual-species biofilms using a validated peptide nucleic acid fluorescence *in situ* hybridization approach. Herein and in the following points, only BV associated bacteria which present a synergistic interaction with a pre-formed *G. vaginalis* biofilm will be analysed.

c) To analyse the dual-species biofilms structures by confocal laser scanning microscopy.

d) To investigate the impact of the second BV associated species on *G. vaginalis* pathogenicity, by analyzing the expression of genes related to cytotoxicity, biofilm formation, antimicrobial resistance and evasion of the immune system in cells from mono- and dual-species biofilms.

e) To analyse the bacterial coaggregation ability between *G. vaginalis* and other BV associated species.

1.4 Significance

The research question to be addressed by this thesis was the dilemma of *G. vaginalis* vaginal colonization in both healthy and BV women. It is noteworthy that a hallmark feature of BV is the presence of a highly structured polymicrobial biofilm primarily consisting of *G. vaginalis*, strongly adhered to vaginal epithelium, and a variety of other bacteria. Thus, it is essential unveiling whether non-BV and BV *G. vaginalis* strains interact differently with both the host, and with other BV associated bacteria to shed a new light on the development of BV. This could represent a significant advancement towards the characterization of ecological interactions and virulence factors that contribute to symptoms of BV. Furthermore, this thesis could lead to new insights into the interaction between both *G. vaginalis* groups and beneficial endogenous bacteria. Together, the findings on *G. vaginalis* virulence traits and BV etiology could lead to new strategies to BV prevention and consequently reduction of BV rates.

1.5 Thesis outline

Chapter 2 presents a literature review, providing a general outline of major aspects of BV, carefully emphasizing the composition of healthy and BV associated microflora. Furthermore, the BV associated biofilm development will be focused as the key step on BV establishment. Lastly, special emphasis will be also given to the dilemma of vaginal colonization of *G. vaginalis* in healthy women.

Chapters 3, 4, 5, 6, and 7 are the experiment chapters and address the four aims of this thesis. Each of the experiment chapters stands alone, providing a summary, brief introduction, materials and methods, results, and discussion.

Chapter 3 is focused on the examination of phenotypic and genotypic characteristics of 14 *G. vaginalis* strains isolated from women with and without BV (7 of each group). *G. vaginalis* bacteria were isolated from Portuguese women and identified by partial sequencing of 16S rRNA coding gene and screened for a small panel of putative virulence factors. The isolates underwent analysis for initial adhesion, biofilm formation, antimicrobial susceptibility profile, presence and expression of potential virulence related-genes and the capacity of *G. vaginalis* to displace beneficial lactobacilli.

Chapter 4 shows how commensal and clinical isolates are adapted to the innate immune system, evaluating the initial adhesion, the biofilm-forming capacity and the bacterial fitness in presence of physiological vaginal concentrations of the innate molecules. Furthermore, this chapter provides genotyping information of the 14 *G. vaginalis* strains, based on a recent clade-specific system.

Chapter 5 addresses the third aim of this thesis, comparing the transcriptomic profile of *G. vaginalis* cultured under planktonic and biofilm conditions by RNA-sequencing. This chapter provides data regarding the upregulation of the transcription of potential virulence genes in *G. vaginalis* biofilms.

Chapter 6 presents the ecological interactions between non-BV or BV associated *G. vaginalis* strains and 24 other BV associated isolates, using an *in vitro* dual-species biofilm model.

Chapter 7 is focused on deciphering the impact of other BV associated species on BV associated *G. vaginalis* virulence profile. After the first screening of ecological interactions (chapter 6), we set out to better analyse the cases of synergistic interactions between *G. vaginalis* and other BV associated bacterial species using the same *in vitro* dual-species biofilm model.

Finally, **Chapter 8** presents a summary of the thesis findings, major outcomes, the significance of the findings, limitations and future directions.

A schematic diagram which shows the general layout of this monograph and relationships of the chapters to the thesis aims is presented below in Figure 1.1.

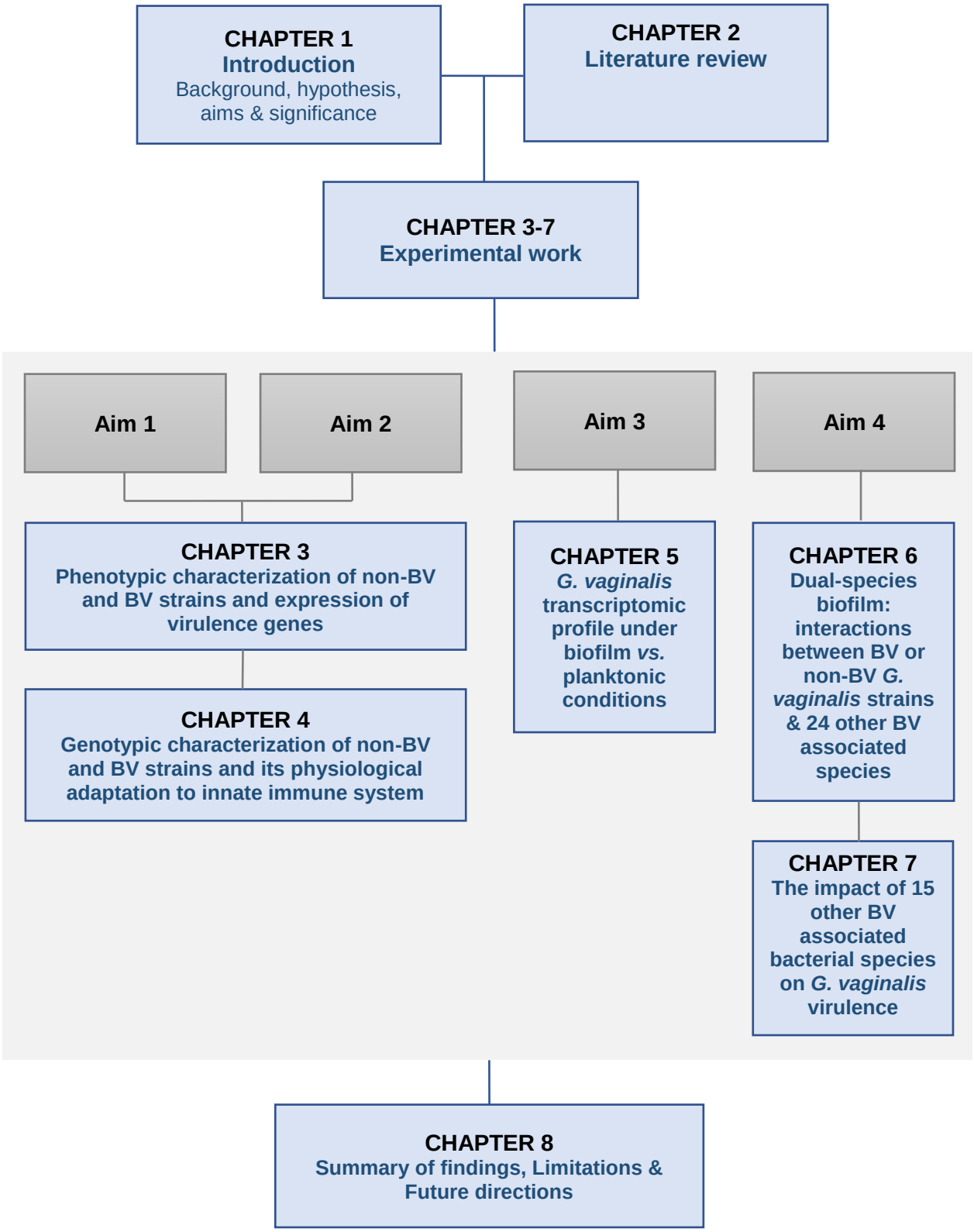


Figure 1.1. Thesis outline

1.6 References

[1] Bradshaw, C.S. et al. **(2006)**. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* 193, 1478-86.

[2] Bagnall, P. and Rizzolo, D. **(2017)**. Bacterial vaginosis: A practical review. *Jaapa* 30, 15-21.

[3] Pirotta, M., Fethers, K.A. and Bradshaw, C.S. **(2009)**. Bacterial vaginosis - More questions than answers. *Aust Fam Physician* 38, 394-7.

[4] Schwebke, J.R., Muzny, C.A. and Josey, W.E. **(2014)**. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis* 210, 338-43.

[5] Aroutcheva, A., Simoes, J., Behbakht, K. and Faro, S. **(2001)**. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin Infect Dis* 33, 1022-27.

[6] Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A. and Pleckaityte, M. **(2017)**. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infect Dis* 17, 394.

[7] Machado, A., Jefferson, K.K. and Cerca, N. **(2013)**. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 14, 12004-12.

[8] Alves, P., Castro, J., Sousa, C., Cereija, T.B. and Cerca, N. **(2014)**. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *J Infect Dis* 210, 593-6.

[9] Machado, A., Salgueiro, D., Harwich, M., Jefferson, K.K. and Cerca, N. **(2013)**. Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. *Anaerobe* 23,1-4.

[10] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

CHAPTER 2

Literature review

Summary

This chapter provides a general outline of major aspects of BV, carefully emphasizing the current hypothesis of the pathogenesis of BV. Furthermore, a special importance will be also given to the dilemma of vaginal colonization of *G. vaginalis* in healthy women.

2.1 The vaginal ecosystem

The female vaginal environment is a complex and dynamic nutrient-rich milieu for microorganisms resulting in a unique microbiome [1]. The composition of the vaginal ecosystem is not static: fluctuations in relative and absolute amounts of microbial species can occur over time due to several factors including, hormonal changes [2], sexual activity [3], hygienic practices [4], and underlying health conditions [5].

Since the first microbiological study of the human vagina, published by Döderlein in 1892 [6], the vaginal microflora of healthy premenopausal women has been described as constituted predominantly by Gram-positive bacilli of the genus *Lactobacillus*. Traditionally, lactobacilli colonization is believed to be beneficial since it prevents other microorganisms from colonizing the vaginal tract, using several protective mechanisms [7,8]. Firstly, the majority of *Lactobacillus* species produce lactic acid, which contributes to the maintenance of the vaginal pH below 4.5 [9,10]. This acidic environment constitutes an efficient mechanism of protection of the vaginal epithelium since it makes the environment inhospitable to other bacteria, including pathogens [11,12]. Secondly, *Lactobacillus* species are also known to produce other antimicrobial compounds, including hydrogen peroxide [13,14] and target-specific bacteriocins [15,16]. Despite some studies have demonstrated that hydrogen peroxide could inhibit the colonization of pathogenic bacteria [17,18], it was shown that under normal physiological concentration no detectable effect was observed in 17 vaginal pathogens under anaerobic growth conditions [19]. The vagina is virtually an anaerobic environment wherein dissolved oxygen levels are low. Therefore, it is unlikely that significant amounts of hydrogen peroxide are produced and accumulate to a toxic level to preventing the colonization of bacteria [19]. Regarding bacteriocins, their antimicrobial activity is usually based on the permeabilization of the target membrane [20]. Thus, in the vagina, bacteriocins could play a significant role in fending off non-indigenous bacteria or pathogenic microorganisms [21,22]. In addition, vaginal lactobacilli competitively block the adhesion of pathogenic bacteria to vaginal epithelial cells [23,24].

Remarkably, advances in culture-independent approaches, such as high-throughput 16S rRNA gene sequencing, have generated renewed knowledge in the composition and abundance of vaginal bacterial species in asymptomatic reproductive-age women, showing at least five major types of vaginal microflora, known as community state types. Four of these community state types are dominated by *L. crispatus, L. iners, L. gasseri* and *L. jensenii,* and one does not contain a significant number of lactobacilli (20 – 30% of the cases) but is composed of a diverse array of facultative and strictly anaerobic microorganisms, including *Atopobium, Corynebacterium, Anaerococcus, Peptoniphilus, Prevotella, Gardnerella,*

Sneathia, Eggerthella, Mobiluncus and *Finegoldia* among others [1,25]. Interestingly, these differences between community state types appear to be driven by a combination of cultural, behavioural, genetic and other uncharacterized underlying factors [1,25,26]. Overall, these findings challenged the wisdom that the occurrence of the high number of lactobacilli is synonymous with “normal” or “healthy”.

While knowledge accumulated over the past few decades has provided some insights into the vaginal ecosystem, there remains a need to define and better understand factors that affect the composition and dynamics of vaginal microbiota in both health and diseases. This knowledge will facilitate the development of new strategies for disease diagnosis and personalized treatments to promote health and improve the quality of women’s lives [25].

2.2 Vaginal innate immunity

In addition to the protective effects of the beneficial endogenous vaginal microflora, the colonization of pathogenic microorganisms in the female reproductive tract (FRT) is prevented by local components of the innate and adaptive immune systems. The innate immune system constitutes the first line of response to infection and, for this reason, it has a pivotal role in the host. In the FRT, the innate immune system consists of mechanical, chemical, and cellular components. The mucus lining and epithelial cells act as a mechanical barrier. The chemical barrier can be divided into natural antimicrobial peptides (AMPs) and pattern recognition receptors, especially Toll-like receptors (TLRs) [27,28]. TLRs recognize conserved pathogen-associated molecular patterns synthesized by microorganisms including bacteria, fungi, parasites, and viruses as well as endogenous ligands associated with cell damage. Specifically, the vaginal epithelium expresses TLR2 and its partners TLR1 and TLR6, which in combination (TLR1/2 and TLR2/6), recognize lipopeptides present on both Gram-positive and Gram-negative bacteria; TLR4, which recognizes lipopolysaccharide of Gram-negative bacteria; and TLR5, which recognizes flagellin, a component of the flagellum responsible for bacterial motility. Therefore, it has been thought that the expression of TLRs on the epithelium plays an important role in antigen detection, initiation of the immune response and in the connection between innate and adaptive immunity [29].

Importantly, the synthesis of AMPs has commonly emerged as the most ancient primary mechanism of the immune system [30]. AMPs possess additional functions apart from microbicidal activity, including cell proliferation, cytokine induction, chemotaxis, and modulation of innate and adaptive immunity [27]. Major AMPs with different structural and

functional characteristics include defensin, elafin, cathelicidin, secretory leukocyte protease inhibitor (SLPI), lysozyme, and lactoferrin. These factors are briefly described below:

2.2.1 Defensin

Defensins are small cationic peptides consisting of 30 – 42 amino acids and have molecular weights between 3.5 – 4.5 kDa. They are subdivided into α and β-defensins. Six α-defensins have been recognized in humans: human neutrophil peptide (HNP) 1 – 4 and human defensin 5 and 6 and are produced by neutrophil granulocytes [27]. Also, six human β-defensins, HBD1 to 6 have been identified, which are structurally similar to α-defensins. Four of them are expressed by mucosa and epithelial cells of the FRT [31-33]. Importantly, the permeabilization of target membranes is the crucial step in defensin-mediated antimicrobial activity and cytotoxicity. In bacteria, permeabilization leads to inhibition of RNA, DNA, and protein synthesis ultimately, bacterial cell death [33].

2.2.2 Elafin and secretory leukocyte protease inhibitor

Elafin and SLPI are two low-molecular-mass elastases inhibitors that are mainly synthesized by macrophages and epithelial cells [34]. It is thought that their physiological properties allow them to efficiently inhibit target enzymes, such as neutrophil elastase [35,36]. This proteolytic enzyme is capable of degrading elastin, which provides elasticity and resilience to tissues [37,38]. So, the main function of SLPI is to protect local tissue against the detrimental consequences of inflammation [37]. In addition to their antiprotease activity, both elafin and SLPI have a broad range of antibacterial activity against Gram-positive and Gram-negative species [39-41]. The antimicrobial activity is mediated via their cationic charge, which, like many cationic antimicrobial proteins, allows them to destabilize bacterial membranes [42]. It is also important to note that these proteases inhibitors are found in vaginal secretions [43,44].

2.2.3 Cathelicidin LL37, lactoferrin, and lysozyme

Another component of the FRT secretions is cathelicidin, which was named according to its ability to inhibit the protease cathepsin-L, a lysosomal endoprotease [45]. In humans, LL37 is the only cathelicidin, and it is produced by neutrophils and epithelial cells of the lower FRT [46]. It is found in vaginal fluid and cervical mucus [30,31]. Similarly, lactoferrin, an iron-binding cationic glycoprotein, is also produced by neutrophils and epithelial secretions. Lactoferrin is both anti-viral and anti-bacterial, and it effects can occur by sequestration of iron essential for microbes under acidic conditions, such as lower part of the FRT [47,48]. Furthermore, lactoferrin can also prevent the entry of bacteria or virus into the host cells in the early phase

of infection, either by blocking cellular receptors or by direct binding to the bacterial adhesins or virus particles [49,50]. Regarding lysozyme, it is synthesized by neutrophils, monocytes and macrophages [51]. In addition to the enzymatic lysis of peptidoglycan present on bacterial cell walls leading to the rapid killing of Gram-positive bacteria [52,53], lysozyme can also kill bacteria by a non-enzymatic mechanism, owing to its highly cationic nature, through the formation of pores on the bacterial cell membrane [54,55]. Furthermore, it blocks the human immunodeficiency virus-1 viral entry and its replication [56]. Interestingly, lysozyme displays synergism with lactoferrin, which promotes innate immune protection in the FRT [57]. Notably, endogenous AMPs can act synergistically, resulting in enhancement of their antimicrobial properties [58].

2.3 Bacterial vaginosis

Worldwide, BV is the most common gynaecological disorder among women of childbearing age, affecting ~ 29% of women in the general population and 50% of African American women [59]. Microbiologically, BV is characterized by a dramatic shift in the vaginal microflora from the dominant lactic acid and H_2O_2-producing lactobacilli to a polymicrobial flora, consisting of strictly and facultatively anaerobic bacteria, where *G. vaginalis* plays a pivotal role [60]. Importantly, the loss of lactobacilli may be a consequence of the changes in vaginal microflora rather than to be a cause of BV, as the anaerobic vaginal environment of BV is not conducive to the lactobacilli dominance [60,61]. The hypothesis of the depletion of lactobacilli as the cause of BV has not been supported by the fact that some women maintain a “healthy” vaginal environment without lactobacilli [62]. Curiously, some strains of *Atopobium* spp., *Leptotrichia* spp. and *Megasphaera* spp. are reportedly capable of producing lactic acid. Therefore, the presence of non-lactobacilli vaginal microbiota and the lack of beneficial lactobacilli may not necessarily be sufficient to cause BV [3,63].

In the last years, BV has emerged as a global issue of concern due to its association with a wide array of adverse outcomes. It has been reported that BV significantly increases the risk of development of gynaecological postoperative infections [64], pelvic inflammatory disease [65], urinary tract infections [66] and infertility [67]. Moreover, BV has been also associated with adverse pregnancy outcomes such as miscarriage and recurrent pregnancy losses [68]; preterm delivery and low birth weight [69]; and increased neonatal morbidity [70]. Furthermore, BV facilitates the transmission of sexually transmitted agents including the human immunodeficiency virus [71], human papillomavirus [72], *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [73].

2.3.1 Clinical features and diagnosis

Over the last three decades, there has been a fascinating evolution in our understanding of BV. Causing profuse vaginal discharge and fishy vaginal odour in symptomatic women, BV has been also recognized as being asymptomatic in approximately one-half of the women who experience it [74-76]. The abnormal vaginal discharge results in part from degradation of the protective vaginal mucin gel, which is performed by mucin-degrading enzymes produced by BV associated bacteria [77]. The fishy odour is due to the volatilization of amines produced as the result of the metabolism of anaerobic bacteria [78]. In clinical settings, BV is commonly diagnosed using the Amsel criteria, which include the presence of at least three of the following precepts: (*i*) thin and homogenous discharge, (*ii*) vaginal pH over 4.5, (*iii*) positive "whiff test" (detection of fishy odour through the addition of 10% potassium hydroxide to vaginal fluid), (*iv*) and presence of clue cells on microscopic examination of vaginal fluid [79]. However, these clinical signs are not always present, making Amsel criteria somewhat subjective [80].

In an attempt to improve the accuracy in BV diagnosis, Nugent and colleagues proposed a Gram stain scoring system for examining vaginal smears [81]. This method derived from the modification of the Gram-stained protocol proposed by Spiegel *et al*. [82] and currently it is regarded as the gold standard for BV diagnosis. According to Nugent criteria, Gram-stained smears are used for identification, classification, and quantification of the following bacterial morphotypes: large Gram-positive bacilli (*Lactobacillus* spp.); small Gram-variable rods (*Gardnerella* and *Bacteroides* spp.); and curved Gram-variable rods (*Mobiluncus* spp.), as presented in Table 2.1.

Table 2.1 Scheme for grading Gram-stained vaginal contents

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+	
Vaginal microflora diagnosis by Nugent score system			
Total score [a]		**Interpretation**	
0 – 3		Normal vaginal microflora	
4 – 6		Intermediate vaginal microflora	
7 – 10		Bacterial vaginosis in vaginal microflora	

[a] Morphotypes are scored as the average number see per oil immersion field. Quantification of each individual score: 0 for no morphotype present; 1+ for 1 morphotype present; 2+, 1 to 4 morphotypes present; 3+, 5 to 30 morphotypes present; 4+, 30 or more morphotypes present. The total score is the sum of the average classification of *Lactobacillus*, *Gardnerella* and *Bacteroides*, and finally *Mobiluncus* spp. Adapted from Nugent and colleagues [81].

Each morphotype is scored from 0 to 4+, regarding the number of morphotypes observed per oil immersion field. The total score is obtained by adding each individual morphotype score, ranging between 0 – 10. Thus, a score of 0 – 3 is considered normal vaginal microflora, 4 – 6 as intermediate microflora and 7 – 10 as BV (Figure 2.1). Nevertheless, Nugent score system has also some disadvantages, especially related to the inter-observer variability and it requires skilled personnel to perform it.

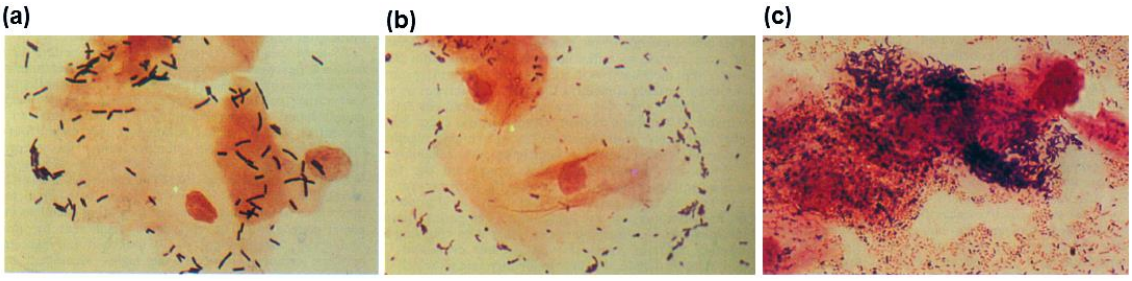


Figure 2.1 Gram-staining vaginal smears illustrate the vaginal microflora. (a) Normal vaginal epithelial cells. **(b)** Intermediate vaginal microflora. **(c)** BV associated microflora, showing a vaginal clue cell, which corresponds to vaginal squamous epithelial cells coated with the *G. vaginalis* and other anaerobic bacteria. Adapted from Nugent and colleagues [81].

Importantly, the relationship between Gram stain score and diagnosis by the clinical criteria is imperfect. Gram stain is more sensitive, whereas the Amsel criteria can be more specific. Overall the concordance between them is of ~80% to 90% [83]. These shortcomings of standard methods make BV diagnosis a challenging task, and, therefore, alternative methods for BV diagnosis have been investigated. The molecular methodologies, such as polymerase chain reaction (PCR) [84], quantitative PCR (qPCR) [85] or fluorescence *in situ* hybridization (FISH) [86], have allowed the detection or even quantification of the main BV associated bacteria. In fact, they have improved our knowledge of how microbial species interact among themselves and with the human host. However, most of these alternative methods are expensive and many of them still require validation. Until then, the Amsel and Nugent criteria remain the most commonly used methods for BV diagnosis [87].

2.3.2 Epidemiology

BV status has been referred to as “one of the most prevalent enigmas in the field of medicine” [88]. Despite the high clinical importance of BV, its real prevalence is unknown since it varies according to the characteristics of the studied population [59,89]. Epidemiological studies indicated that the risk factors of BV include *(i)* the concurrent use of medications [76], *(ii)* low socioeconomic status [90], *(iii)* increasing age [91], *(iv)* cigarette smoking [92], *(v)* young age

at coitarche [93], *(vi)* daily habits (such as vaginal douching or use of tight jeans/trousers) [94-96], *(vii)* the use of intrauterine devices [97], *(viii)* a new sexual partner [98], and *(ix)* multiple sexual partners [99]. Importantly, there has also been considerable debate in the literature as to whether BV is a sexually enhanced disease or a sexually transmitted disease [59,93]. The balance of evidence suggests that sexual transmission of the organisms associated with BV is at least an important aspect of its epidemiology [100].

A 2013 systematic review reported that BV prevalence varies between and within countries worldwide [59]. In Portugal, the epidemiological data about prevalence and risk factors of BV are limited to five studies. The first epidemiological study about BV was developed by Guerreiro *et al.* that reported a prevalence rate of 7% among contraceptive users and established a positive association between of intra-uterine device and BV occurrence [101]. In 2012, Henriques and colleagues assessed Portuguese doctors' perception of BV prevalence through 197 anonymous questionnaires [102]. They verified that most doctors considered BV a frequent condition in Portugal (74%) with a lower prevalence during pregnancy (55%). However, effective epidemiological data were not obtained at that point. These researchers also verified that most doctors use the Amsel criteria to diagnose BV disorder (75%). The main symptoms observed were the increase of vaginal exudate (54%) and malodour (43%). Finally, most Portuguese doctors involved in the study considered that BV relapses are not very frequent (62%), in contrast with the results of studies reported from other parts of the world [103]. Later, Silva and coworkers performed a cross-sectional epidemiological study to determine the prevalence of BV, *G. vaginalis* and *A. vaginae* in 260 Portuguese women and correlate the presence of these bacteria with BV risk factors. These researchers pointed out that *G. vaginalis* and *A. vaginae* were detected in 36.9% and 11.9% of vaginal samples, respectively. Furthermore, women that had been previously diagnosed with BV accounted for 20% of the samples and had a 1.26-fold higher risk of harboring *G. vaginalis* [104]. Afterward, Machado and colleagues performed a prospective epidemiological study to evaluate the accuracy of a multiplex peptide nucleic acid (PNA) FISH as a diagnostic tool for BV in comparison with the Nugent scoring method. These researchers, when analysing 200 vaginal samples, attributed BV-positive scores to 13% of the samples according to Nugent score. Furthermore, they demonstrated that PNA FISH methodology had a high sensitivity (84.6%) and specificity (97.6%) in BV diagnosis, proposing this molecular technique as a valuable alternative to diagnose this disorder [86]. In 2017, a cross-sectional epidemiological study revealed a low prevalence of BV (3.88%) and high *G. vaginalis* colonization (67.48%) among 206 Portuguese pregnant women. Despite the lower number of women with BV, prevalence ratios and association with risk factors were similar to recent European studies [105,106]. However, the percentage of healthy women colonized by *G. vaginalis* was significantly higher

than many previous studies, confirming that *G. vaginalis* colonization does not always lead to BV development [107].

2.3.3 Etiology

In spite of over 50 years of medical research, the etiology of BV still remains controversial. The lack of basic information about etiopathogenesis of BV led to the postulation of two hypotheses. The first is the primary pathogen hypothesis, which infers that a single pathogenic species, *G. vaginalis*, is the etiological agent of BV, usually transmitted by sexual contact [108]. In contrast, the second is the polymicrobial hypothesis, which argues that *G. vaginalis* acts in concert with other bacteria, principally anaerobes, to cause the disorder [109].

Historically, in 1955, Gardner and Dukes identified *G. vaginalis* as a major etiological agent of BV, fulfilling all the Koch's postulates described in Table 2.2 [110]. However, later, a study pointed out some failures in these experiments, since they showed that the artificial infection with a pure culture of *G. vaginalis* did not always cause BV [111]. The assumption was then made that *G. vaginalis* is not the specific causative agent of BV, failing one of Koch's postulates [112].

Table 2.2 Koch's postulates

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 re-isolated from the experimentally inoculated host

These first events led to the hypothesis that BV has a polymicrobial etiology. Thenceforward, the role of anaerobic bacteria in the clinical manifestations of BV started to have a special focus [113,114]. A study carried out by Chen and colleagues showed that the characteristic vaginal odour can be attributed to amine production as a byproduct of anaerobic metabolism, suggesting that anaerobic activity is instrumental in producing the symptoms of BV [115]. However, the presence of a bacterium in BV has been rarely supported by microbiological functional studies, demonstrating, thus, a lack of virulence profile characterization of such species [116]. Notwithstanding all these findings, the polymicrobial hypothesis is still

incongruent with the epidemiological profile of BV since multiple studies have been revealing that BV reflects the behaviour of a sexually transmitted or enhanced disease [93,98,117].

2.3.4 Anaerobes involved in BV

Even though the current knowledge about BV etiology remains scarce, the common consensus is that BV is always associated with the overgrowth of numerous bacterial species, including *G. vaginalis, A. vaginae*, *Fusobacterium nucleatum, Mobiluncus mulieris, Mycoplasma hominis, Prevotella bivia,* and *Ureaplasma urealyticum* [83].

With the advance in culture-independent methods, the spectrum of anaerobes detected in women with BV was greatly expanded with the addition of *Bifidobacterium, Dialister, Eggerthella, Leptotrichia, Megasphaera,* and *Slackia* organisms, as well as other bacteria related to *Arthrobacter, Caulobacter*, and *Butyrivibrio* organisms [118,119]. Furthermore, the Vaginal Human Microbiome Project has detected several newly described bacteria in the *Clostridiales* order, which were initially designated BV associated bacteria (BVAB): BVAB1, BVAB2, or BVAB3 [120,121]. To date, only BVAB3 has been cultured and biochemically characterized and the remaining two BVAB (BVAB1, BVAB2) have not yet been isolated by culture [122]. The species name of BVAB3 was proposed as *Mageeibacillus indolicus* [122]. Interestingly, differences in the BV vaginal microbiome between American women and women of European ancestry were found, with American women more likely to be colonized by *Anaerococcus tetradius*, BVAB1, BVAB3, and *Coriobacteriaceae, Sneathia, Parvimonas, Dialister, Megasphaera, Bulleidia, Prevotella,* and *Atopobium* species, while women of European ancestry were more likely to be colonized by *M. hominis, Dialister micraerophilus*, and *Gemella* species [120]. Thus, the diversity of anaerobic colonizers can vary according to women with different ancestries and from different geographical locations.

Unfortunately, despite the development of a more comprehensive picture of the vaginal microflora during BV through the use of high-throughput 16S rRNA sequencing, the significance of these findings remains unclear, since it is not known whether these microorganisms are pathogens that cause BV or if they simply are opportunistic microorganisms that take advantage of the temporary higher pH environment and thus increase in numerical dominance [25].

2.3.5 BV associated biofilm as the key step on BV establishment

Biofilm formation is a dynamic and complex process that involves multiple interactions between single or multiple bacterial species and the host cells [123,124]. To date, the exact process of the development of a biofilm in BV remains unknown [62,116]. However, it is known that microbial adhesion to host surfaces is a prerequisite for infection, since any potential pathogen must first adhere in order to avoid clearance by host defense mechanisms, such as the flow of vaginal secretions, the mucociliary escalator, or the urine flow [125]. Remarkably, the ability of *G. vaginalis* to colonize vaginal cells was already established in the eighties [126,127]. Such coating of epithelial cells with multiple layers of bacteria is exactly what one expects to see in case of biofilm formation, thus the name “clue cell” [124]. Indeed, clue cells were recognized for decades, without the knowledge that they were a marker for biofilm formation. The biofilm life cycle generally includes 3 main stages: initial adhesion, accumulation, and dispersal (Figure 2.2).

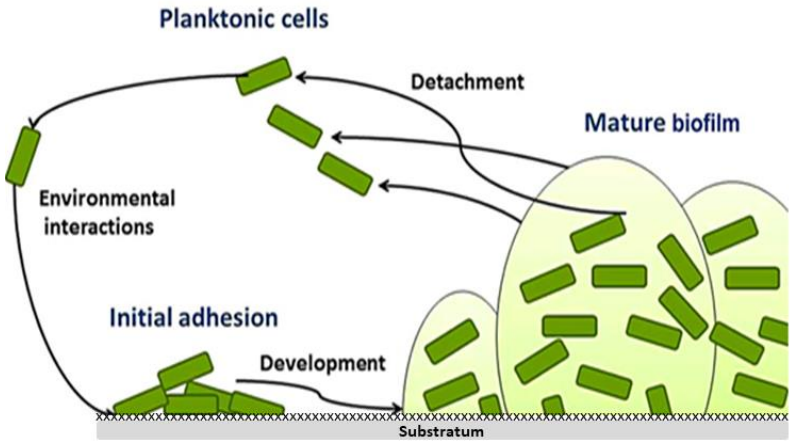


Figure 2.2 Conceptual mono-species model of the biofilm formation. According to the conceptual mono-species biofilm life cycle, when environmental conditions are appropriate, planktonic cells will adhere to the epithelial cells and grow into a multilayer structure composed of bacteria and extracellular components. Eventually, bacteria will detach from the biofilm, and the resulting cells will be able to restart the biofilm cycle in a different location. Adapted from Machado and Cerca [108].

Earlier studies conducted by Mardh and colleagues tested the ability of inoculation with *G. vaginalis* and *Mobiluncus* species to cause BV in monkeys. They showed that increasing the anaerobic bacteria concentrations on the monkeys’ vaginal epithelium was not sufficient to induce BV development since the vaginal discharges produced in the monkeys did not contain any clue cells [128]. However, at that time, the association of biofilms with BV was unknown, and these investigators were not able to explore the biofilm phenotype during BV development. Two decades later, a new light on the development of a BV associated biofilm was possible

using FISH approach to analyse vaginal biopsy specimens from women with BV [129]. These findings provide further evidence that a distinctive feature of BV is the presence of a bacterial biofilm adherent to the vaginal epithelium [129]. The discovery of a biofilm in BV explains the appearance of clue cells in the vaginal fluid, which are biofilm-coated epithelial cells desquamated from the epithelial surface. Although the biofilm was shown to contain high concentrations of a variety of bacterial groups, *G. vaginalis* was found to be the predominant constituent [130-132]. Many follow up studies validated these findings and it is currently accepted that BV associated biofilms are strongly associated with *G. vaginalis* [130,133,134].

Remarkably, Machado and colleagues showed that *G. vaginalis* was able to adhere to vaginal epithelium and displace pre-coated protective lactobacilli, while other BV associated anaerobes, such as *A. vaginae*, *F. nucleatum*, *M. mulieris* and *P. bivia*, were easily outcompeted by *L. crispatus* [135]. A subsequent study confirmed that *G. vaginalis* has a higher virulence potential than 29 other BV associated bacteria [136]. Nevertheless, an enduring enigma is whether *G. vaginalis* alone is capable of causing BV or whether *G. vaginalis* must interact with other bacterial species to cause BV. Some studies have been addressed this issue, postulating the plausive role of *G. vaginalis* in the early adhesion stages that could lead to the formation of clue cells [132,137]. As result, the more recent hypothesis suggests *G. vaginalis* is the BV initial colonizer that enables other BV associated bacteria to colonize the vagina after the initial biofilm development, as represented in Figure 2.3 [116,125].

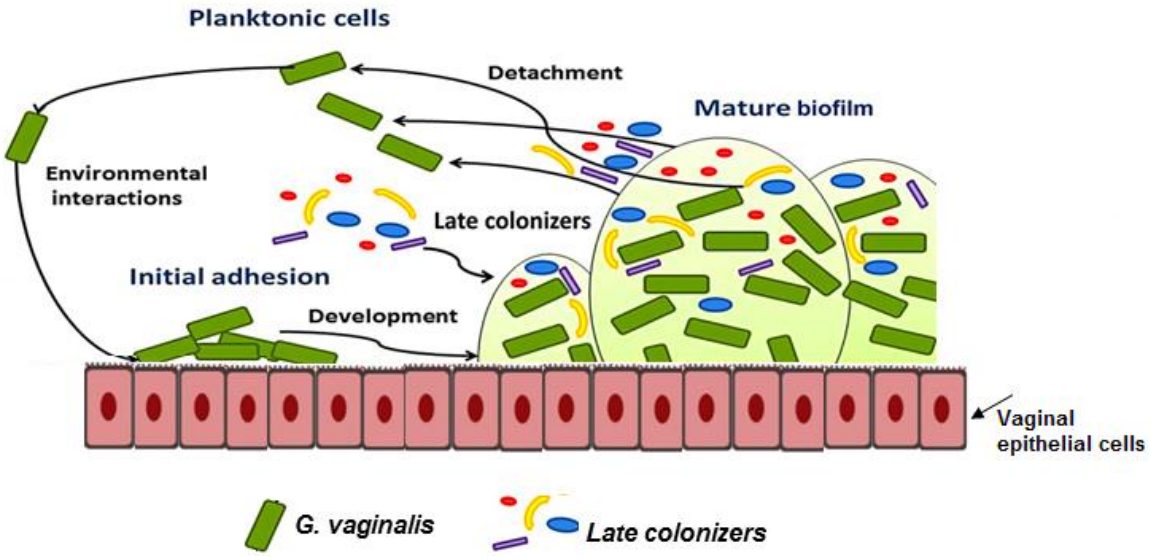


Figure 2.3 Conceptual multi-species model of the BV associated biofilm formation. In polymicrobial biofilms, such as those in BV, secondary bacteria will incorporate the biofilm after the initial colonizer species, *G. vaginalis*, has already adhered to the vaginal epithelial cells. A synergetic relationship can then be formed, allowing the biofilm to prosper. Adapted from Machado and Cerca [116].

G. vaginalis can be found in healthy women, however, pointing to its important role in BV, it is typically found at significantly lower abundance during health [132]. Nevertheless, understanding why *G. vaginalis* is also present in healthy women is a question that remains to be answered.

2.3.6 Treatment of BV

The Centers for Disease Control and Prevention [138] and the International Union against Sexual Transmitted Infections [139] recommend that all symptomatic women should be treated, since they recognize numerous benefits of therapy including the relief of the symptoms and signs of infection and reduction in the risk of acquiring sexually transmitted diseases and BV associated complications, mainly in pregnancy [138]. However, there is insufficient evidence to recommend routine treatment of asymptomatic women [75,140,141]. Conventionally, BV is treated with antibiotics, namely metronidazole, clindamycin or tinidazole as described in Table 2.3 [138,139].

Table 2.3 Regimens for BV treatment

Regimen	Antibiotic	Dose
Recommended	Metronidazole[a]	500 mg orally twice a day for 7 days
	Metronidazole	gel 0.75% one full applicator (5 g) intravaginally daily for 5 days
	Clindamycin[a]	cream 2%, one full application (5 g) intravaginally at bedtime for 7 days
Alternative	Tinidazole[a]	2 g orally once daily for 2 days
	Tinidazole[a]	1 g orally once daily for 5 days
	Clindamycin[a]	300 mg orally twice daily for 7 days
	Clindamycin	ovules 100 mg intravaginally once at bedtime for 3 days

[a] Regimens for BV treatment according to guidelines of *Sociedade Portuguesa de Ginecologia* [142]. In addition, metronidazole ovules 500 mg intravaginally for 5 days and dequalinium chloride vaginal tablets (10 mg) for 6 days are also suggested as possible regimens for BV treatment in Portugal.

Currently, metronidazole, the most widely known of nitroimidazole drug class, represents the first line therapy for BV and trichomonas vaginitis [143]. However, several side effects are associated with metronidazole therapy, such as nausea, vomiting and gastrointestinal complaints [143,144].

Clindamycin is the second recommended antimicrobial agent for the treatment of BV, with similar efficacy as metronidazole [145,146]. This lincosamide antibiotic has various formulations including vaginal dosage forms (ovule and cream) and oral (systemic) pills [146]. However, topical clindamycin tented to cause a lower rate of adverse side effects (metallic taste in the mouth, nausea, vomiting) than oral metronidazole. Nonetheless, topical clindamycin has been associated with *Clostridium difficile* colitis [147]. Furthermore, because both clindamycin ovules and cream are oil-based, their use might interfere with the safety of latex condoms and diaphragms [138]. Curiously, in Portugal, although the first choice antimicrobial therapy is metronidazole (58%), the doctors from different geographical regions do prescribe different antibiotic therapies. The Centre region of Portugal was the only region where clindamycin prescriptions (49%) were preferred to metronidazole (45 %) [102].

Tinidazole is currently considered an alternative antimicrobial agent for BV treatment particularly whenever metronidazole and clindamycin are not tolerated [138]. Being a second generation nitroimidazole, tinidazole requires lower dosages and is administered less frequently than metronidazole due to its longer half-life [148]. Other antibiotics like azithromycin [149], ornidazole [150] and secnidazole [150-152] have been tested as alternatives to treat BV.

Despite some studies reported short-term high clinical cure rates of antibiotic therapy [145,150], high recurrence rates have been demonstrated within 3 –12 months [103,153]. The ability for a strain to grow as a biofilm would likely confer resistance to both mucosal immune defenses and antibiotics [154-156] (Figure 2.4), which could contribute to initial and recurrent colonization [157]. Therefore, the low efficacy of antibiotics in preventing recurrences is thought to be due to their inability to fully eradicate BV associated biofilms [158]. In fact, Swidsinski *et al.* investigated the influence of oral metronidazole therapy on *G. vaginalis* biofilms and pointed out that biofilms were only temporarily suppressed, and that in most cases rapidly regained activity following treatment cessation [159].

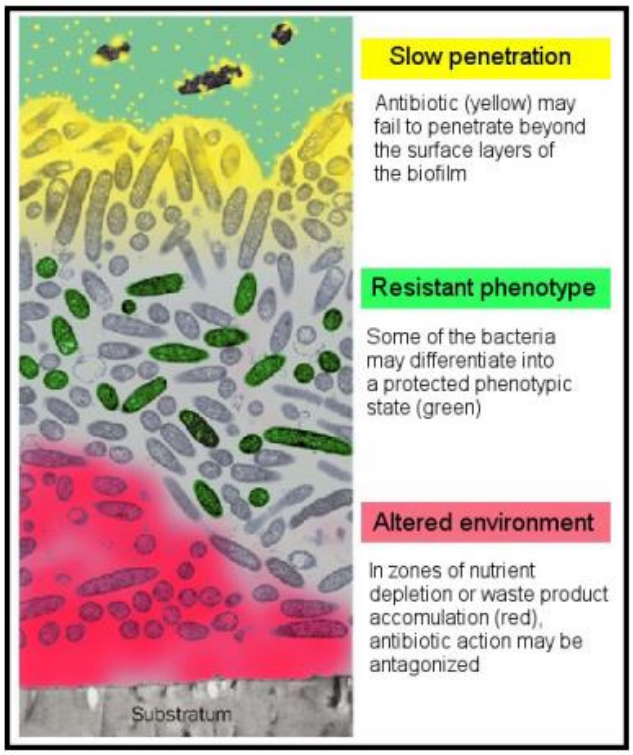


Figure 2.4 Some of the most discussed hypothesis for biofilm resistance to antibiotics. Adapted from Stewart & Costerton [160].

The increasing evidence that BV is a biofilm-mediated infection sparked the interest of the scientific community in exploring agents aimed to disrupt biofilms. Thus, in recent years, studies of anti-BV agents started to include biofilm disruptor candidates, namely DNases [161], retrocyclins [162], probiotics [163], antiseptics [164], plant-derived compounds [165] and natural antimicrobials [166]. In order to achieve the sustained cure, it is possible, even likely, that we may need an approach that combines a number of these strategies such as the use of antibiotics with biofilm-disrupting agents and partner treatment [167].

2.4 *Gardnerella vaginalis*

G. vaginalis, more than any other species in the vaginal ecosystem, has been studied because it is recovered from the vaginal samples of almost all women with BV [84,168-170].

Historically, the original discovery of *G. vaginalis* dates back to 1953, when Leopold described this microorganism as a novel "*Haemophilus*-like" species associated with prostatitis and cervicitis [171]. Two years later, Gardner and Dukes described this microorganism in relation to nonspecific vaginitis (a historical name for BV), renaming this bacterium to *Haemophilus vaginalis* [110]. Afterward, this bacterium was reclassified within in the genus *Corynebacterium* [172]. However, two large taxonomic studies demonstrated the lack

of similarity between of this bacterium and other established genera, which resulted in the emergence of the new genus named *Gardnerella* [173,174]. Currently, *G. vaginalis* remains the only recognized species in its genus, with its closest relatives found in the genus *Bifidobacterium.* Regarding its description, *G. vaginalis* cells are non-encapsulated, non-spore forming, pleomorphic rods with an average size of 0.5 to 1.5 µm (Figure 2.5) [169]. Upon Gram staining, *G. vaginalis* envelope architecture is Gram-positive [175]. Furthermore, the cellular surface of *G. vaginalis* is covered with fimbriae, which are responsible for its attachment to vaginal epithelial cells [127]. *G. vaginalis* is a fastidious and commonly known as a facultative anaerobic microorganism, that grows better at 37ºC in complex media in an atmosphere with 5 to 10% of carbon dioxide (CO_2) or in a candle flame extinction jar [169,176]. However, it was demonstrated that certain *G. vaginalis* strains are strict anaerobes [177,178]. Finally, biochemical tests revealed that *G. vaginalis* is catalase, oxidase, and β-glucosidase negative [169].

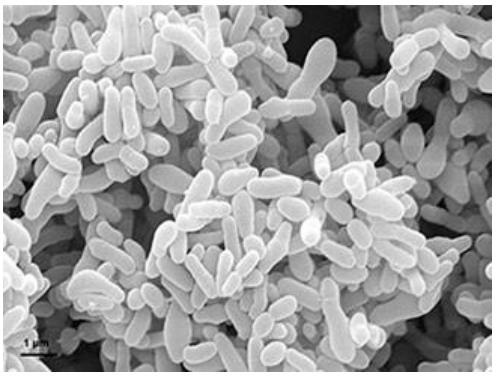


Figure 2.5 Scanning electron micrograph of *G. vaginalis*. Credit: K.K. Jefferson/Virginia Commonwealth University.

2.4.1 The dilemma of vaginal colonization by *G. vaginalis* in healthy women

The strong correlation between BV and *G. vaginalis* has sometimes been taken as direct evidence of causation of BV [179]. Nevertheless, *G. vaginalis* vaginal colonization does not always lead to BV [180]. In fact, *G. vaginalis* is often a major constituent of the vaginal microbiota of healthy, asymptomatic women of all ages [1,121,181], including girls prior to the onset of menarche [182] and postmenopausal women [183]. Therefore, the common occurrence of a putative pathogen in healthy asymptomatic women is a paradox that needs resolution so that the role of *G. vaginalis* in BV pathogenesis can be properly understood. One possibility is that only certain lineages of *G. vaginalis* are pathogenic and others are natural commensals. Another possible hypothesis is that *G. vaginalis* is an opportunistic pathogen present in vaginal microflora, that under specific conditions might turn into in a more virulent state [177]. To examine these hypothesis, many efforts have been made to decipher the

features associated with strain virulence using a variety of techniques that characterize the diversity within *G. vaginalis* strains, analyzing the phenotypic properties, genotyping differences, ecotypes and *in vitro* functional properties.

2.4.1.1 Biotyping, genotyping and ecotyping of *G. vaginalis* strains

Over more than 30 years, the scientific community has been conducting a wide of bacterial typing assays, so as to find the possible factors which might lead to different virulence traits among *G. vaginalis* strains isolated from healthy and BV women, as described in Table 2.4.

The phenotypic diversity of *G. vaginalis* isolates is well-established and has been used as the basis for classification system whereby isolates were divided into biotypes based on the biochemical properties, namely: production of β-galactosidase, lipase, and hippurate hydrolysis [184]. Attempts have been made to correlate these biotypes with BV, with one study finding that lipase-positive isolates were more frequently isolated from women with BV than those without BV [185]. However, other studies found no association between any specific biotype and BV [186-189]. Likewise, observations of genotypic differentiation of *G. vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA) and their association with biotype or specific virulence factors are also variable [190-192]. Furthermore, there has been little success in reconciling the genotypic and phenotypic characteristics with each other, or in identifying patterns of association of any genotype or phenotype with demographic or clinical characteristics [186,190,192].

Table 2.4 Studies of *G. vaginalis* differentiation using biotyping, genotyping or ecotyping approaches

Technique	Number (*n*) of strains or vaginal samples	Main conclusion	Reference
Biotyping			
Detection of hippurate hydrolysis, β-galactosidase and lipase; fermentation of arabinose, galactose and xylose	*n* = 359 strains	8 biotypes were found; No significant differences in biotypes distribution	[184]
	n = 197 strains	17 biotypes were found; No significant differences in biotypes distribution	[193]
	n = 140 strains	33 biotypes were found; Significant differences in biotypes distribution, suggesting that some biotypes were associated with BV	[187]

Table 2.4 Continued

Technique	Number (*n*) of strains or vaginal samples	Main conclusion	Reference
Detection of hippurate hydrolysis, β-galactosidase activity; lipase activity with oleate as a substrate	*n* = 261 strains	Significant differences in biotypes distribution, with the lipase-positive biotypes (biotypes 1, 2, 3, and 4) being more predominant in women with BV	[185]
Detection of hippurate hydrolysis, lipase and β-galactosidase activity	*n* = 408 vaginal specimens	No statistically significant difference between the biotype distribution	[189]
Genotyping			
Whole-sequencing genome	*n* = 3 strains	BV isolates from symptomatic BV encode numerous proteins with role in mucin degradation	[194]
	n = 2 strains	Cytolysin proteins encoded by the two strains were nearly identical, differing at a single amino acid, and were transcribed at similar levels; The BV associated strain encoded a different variant of a biofilm associated BAP protein gene	[195]
	n = 17 strains	*G. vaginalis* isolates were divided in 4 different subgroups: A, B, C and D; Each of the 4 groups has its own characteristic genome size, GC ratio, and greatly expanded core gene content	[196]
Random amplified polymorphic deoxyribonucleic acid (RAPD) and with amplified ribosomal deoxyribonucleic acid restriction analysis (ARDRA) Sialidase presence and its activity	*n* = 134 strains	3 genotypes were distinguished by both RAPD and ARDRA; Only 2 genotypes encoded and produced sialidase	[197]

Table 2.4 Continued

Technique	Number (*n*) of strains or vaginal samples	Main conclusion	Reference
Targeting clade-specific genes	*n* = 60 vaginal specimens	Clade 1 – positive association with BV Clade 2 – positively associated with intermediate vaginal flora Clade 3 – positive association with BV Clade 4 – no correlation with BV	[198]
	n = 149 vaginal specimens	All 4 clades are nearly ubiquitous in clinical specimens from women with normal, intermediate or abnormal vaginal flora, but differ significantly in their concentration, being found a higher abundance in women with abnormal vaginal flora	[199]
	n = 184 vaginal specimens	Clade 1 – no association with BV Clade 2 – no association with BV Clade 3 – no association with BV Clade 4 – association with BV Multi-clade *G. vaginalis* communities showed a positive association with BV	[200]
Targeting clade-specific genes and presence of sialidase gene	*n* = 109 vaginal specimens	Clade 1 – strongly associated with BV Clade 2 – strongly associated with BV Clade 3 – no association with BV Clade 4 – no association with BV Clade 4 was most frequently detected (79.4%) followed by clade 1 (63.7%), clade 2 (42.2%), and clade 3 (15.7%); The gene coding for sialidase was detected in all isolates of clade 1 and clade 2, but not in clade 4 isolates	[201]

Table 2.4 Continued

Technique	Number (*n*) of strains or vaginal samples	Main conclusion	Reference
Reconcile *cpn60* UT-based molecular subgroups A-D with previously published clades Define *cpn60* UT subgroups and compare to ARDRA genotyping and clade-specific Detection the presence/activity of sialidase	*n* = 112 strains	*cpn60* subgroups A – clade 4 (absence of sialidase gene) *cpn60* subgroups B – clade 2 (presence of sialidase activity in all isolates) *cpn60* subgroups C – clade 1 (presence of sialidase gene, with 9% of activity) *cpn60* subgroups D – clade 3 (presence of sialidase gene but no activity) *cpn60* subgroup A – ARDRA genotype 1 *cpn60* subgroup C – ARDRA genotype 2 *cpn60* subgroups B and D – ARDRA genotype 1 or 2	[191]
Presence of vaginolysin gene	*n* = 179 strains	Vaginolysin gene was detected in all *G. vaginalis* isolates from women without BV and in 98.3% isolates from women with BV	[202]
Presence and amount of sialidase	*n* = 120 vaginal specimens	It was found a strong association between the positive diagnosis of BV and the detection of high loads of the sialidase gene	[203]
Biotyping vs. genotyping			
Detection of hippurate hydrolysis, lipase, and β-galactosidase activity DNA typing by pulsed-field gel electrophoresis (PFGE)	*n* = 43 strains	No specific phenotype or genotype of *G. vaginalis* causes BV	[186]
Detection of hippurate hydrolysis, β-galactosidase, and lipase enzymatic activities *Cpn60* sequencing, ARDRA genotyping, sialidase gene presence	*n* = 44 vaginal specimens	*Cpn60* sequence groups of *G. vaginalis* comprises 4 distinct subgroups: A, B, C and D; They did not correspond with the Piot biotyping scheme, but showed consistency with ARDRA genotyping and sialidase gene presence	[190]

Table 2.4 Continued

Technique	Number (*n*) of strains or vaginal samples	Main conclusion	Reference
Detection of hippurate hydrolysis, β-galactosidase, and lipase enzymatic activities ARDRA genotyping and expression of vaginolysin and sialidase	*n* = 17 strains	ARDRA revealed 2 genotypes, being genotype 2 more complex than genotype 1; We did not find any correlation between vaginolysin production level and *G. vaginalis* genotype/biotype; A link between *G. vaginalis* genotype 2 and sialidase production was established	[192]
Ecotyping			
Combined analysis of core genome and accessory genome	n = 35 strains	Functional gene enrichment analysis suggests 3 lineages of *G. vaginalis* with differences in pathogenic capacities, including genes involved in mucus degradation like sialidases	[204]

More recently, the advent of culture-independent methods for determining the composition of the vaginal microbiome based on whole-genome sequencing has provided an unprecedented opportunity to investigate *G. vaginalis* diversity [205,206]. Efforts to exploit whole genome sequencing of *G. vaginalis* isolates showed disparities in virulence potential among isolates [194,195]. Although the results of these comparative genomics studies revealed some evidence regarding the distribution of genes responsible for virulence-associated traits such as adhesion [195] and degradation of mucus [194], conclusions were limited by the small number of strains studied.

Later, in a cohort study, which analyzed the vaginal microbiome of Kenyan women, based on PCR amplification and sequencing of the “universal target” region of the gene encoding the 60 kDa chaperonin (*cpn60*), researchers described four different subgroups of *G. vaginalis* [190]. Interestingly, whole genome average nucleotide identity values between *cpn60*-defined subgroups were less than 95% [190]. Confirmation that *cpn60*-based subdivisions of *G. vaginalis* were not the result of PCR artifact was supported by a whole genome sequencing study of 17 *G. vaginalis* strains [196]. This study revealed that *G. vaginalis* is highly diverse, with only of 52% of the genome of each isolate consisting of conserved genes [196]. Furthermore, this study also grouped *G. vaginalis* isolates into four subgroups based on their genome sequence. Notably, the reconciliation of the *cpn60* based on subgroups and whole genome sequence based on “clades” proposed by Ahmed *et al.* [196] was further achieved in

a recent study by Schellenberg and colleagues [191] where *cpn60* subgroups A, B, C and D [190] were shown to correspond to clades 4, 2, 1 and 3 [196], respectively (Figure 2.6). However, Janulaitiene and colleagues showed that some strains of *G. vaginalis* did not belong to any clade detectable by clade-specific PCR [201]. Furthermore, the establishment of phenotypic properties that differentiate the four subgroups is so far limited to the observation that all subgroup B isolates (and only some subgroup C isolates) are sialidase-activity positive [191,207], and lipase activity may characterize subgroup C [190]. The development of clade-specific PCR assays allowed investigation of the prevalence of each subgroup in vaginal specimens. In this sense, a study employing multi-target quantitative PCR with subgroup-specific primers detected multiple subgroups in 70% of the 60 vaginal samples examined [198]. This issue is particularly problematic given that multi-clade *G. vaginalis* communities showed a positive association with BV, suggesting that women with BV were colonized with multiple strains of *G. vaginalis* [200,201].

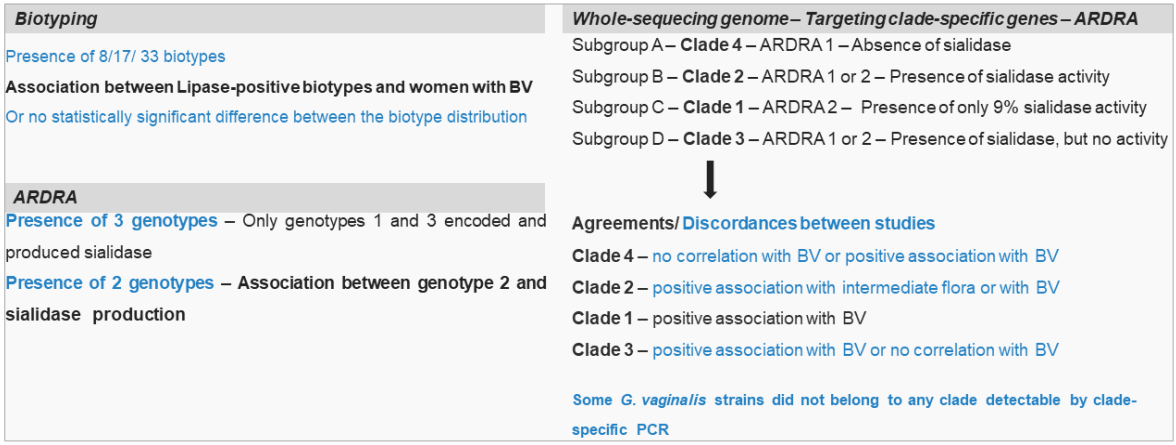


Figure 2.6 Association between data provided by the whole-sequencing genome, clade-specific system and ARDRA genotyping. In blue are highlighted the discordances found among *G. vaginalis* typing studies.

A new light shed on the diversity of *G. vaginalis* was introduced by a ecotypes study [204]. An ecotype is defined as a set of strains that are genetically similar to one another but ecologically distinct from others [208]. Genetic similarity is characterized using a phylogenetic approach to identify sequence clusters that reflect shared the evolutionary history, while the ecological distinctness can be inferred by determining sets of shared genes or similarities in gene expression patterns under the same environmental conditions. Ecotypes thus represent lineages within species that possess unique adaptations and ecological capacities [204,208]. Noticeably, Cornejo and colleagues pointed out the existence of three major ecotypes based

on the phylogenetic structure of their core and accessory genes and the cohesiveness in functional gene composition with ecotypes. They found that genomes of isolates in ecotype 1 uniquely encode several glycosidases (e. g., galactosidases, glucosidases, and fucosidases) and have expanded capabilities for galactoses and pentose sugar metabolism. The most notable feature of isolates in ecotype 2 is the possession of at least of two distinct genes encoding sialidase (also a type of glycosidase). Interestingly, the majority of the genomes in ecotype 3 lack genes for any of these enzymes [204].

Together, there remains a great deal of work to be done in elucidating the basic biology and metabolism of *G. vaginalis* subgroups. It has been suggested that the functional role played by *G. vaginalis* within the vaginal microflora could differ significantly depending on the subgroup (s) dominating the BV-type microflora [177]. However, the lack of a good animal model for the human vaginal microbiome remains a significant obstacle to investigating interactions of the *G. vaginalis* subgroups with the vaginal epithelium. In addition, the isolation of *G. vaginalis* strains of an unknown clade-specific subgroup underscores the high complexity of the genus *Gardnerella*. Thus, future studies are required to elucidate the clinical significance of genotypic differences of the various *G. vaginalis* strains.

2.4.2.2 Functional analysis of virulence potential of *G. vaginalis* strains based on in vitro assays

Despite these recent findings provided by employing higher-resolution approaches, a considerable number of culture-based studies have showed the wide variety of functional features observed for *G. vaginalis* isolates in terms of cytotoxicity, adhesion to epithelial cells, biofilm formation and antibiotic susceptibility, as demonstrated in Table 2.5.

Remarkably, a hallmark of BV is a presence of thick biofilm on vaginal epithelial cells [129,190,195,209]. The association of the biofilm phenotype to *G. vaginalis*–mediated BV development raised an important question: do all *G. vaginalis* strains possess the capability to develop a biofilm? In other biofilm associated diseases, it has been widely demonstrated that not all strains within the same species have the ability to grow as biofilms [210]. In order to answer this question, the biofilm-forming ability for *G. vaginalis* strains isolated from women with or without BV was evaluated by Harwich and colleagues [195]. They showed that the biofilm-forming ability was significantly higher in a BV strain than in a non-BV isolate. Furthermore, BV associated *G. vaginalis* strains revealed a high ability to cause in cytotoxic effects on epithelial cells, what seemed to be related to the adherence function, in which a BV associated isolate adhered more noticeably to the epithelial cells than non-BV associated isolates [195,211].

Table 2.5 *In vitro* studies of functional virulence properties of *G. vaginalis* strains isolated from women with BV versus women without BV

Type of assay	Number of strains (*n*)	Main conclusion	Reference
Antimicrobial resistance			
Minimal inhibitory concentration (MIC) assays	*n* = 43	Strains isolated from healthy women were also resistance to metronidazole	[186]
	n = 204	High resistance was observed for ampicillin (54.4%), metronidazole (59.8%), tinidazole (60.3%) and secnidazole (71.6%)	[202]
Biofilm formation			
Biofilm formation in 96 well-plates	*n* = 9	Isolates from all four subgroups produced biofilm	[190]
Cytotoxicity			
Cytotoxicity to HeLa epithelial cell	*n* = 6	BV-positive *G. vaginalis* strains were able to induce more extensive damages on the HeLa monolayer than BV-negative strains	[211]
Adherence, cytotoxicity, biofilm formation and antimicrobial resistance			
Adherence/cytotoxicity to ME-180 epithelial cells	*n* = 2	BV strain was able to adhere to larger numbers and cause more cytotoxic effects to ME-180 epithelial cells;	[195]
Biofilm formation in 96 well-plates		Both strains exhibited similar antimicrobial tolerance to tested 12 antibiotics;	
MIC assays		BV strains showed a high biofilm-forming capacity	

Another important insight providing evidence that not all *G. vaginalis* strains have the same virulence potential was derived from a work carried out by Swidsinski and colleagues. They highlighted the importance of *G. vaginalis* biofilms when they observed that only biofilm-forming *G. vaginalis* were present in the sex partners of women with BV [209]. These findings led them to propose that the mere presence of loosely adherent *G. vaginalis* on the vaginal

epithelium had a lesser clinical significance and that BV was sexually transmissible only in the presence of high-density clusters of *G. vaginalis* [209]. Later, the biofilm-forming ability of strains belong to four *G. vaginalis* subgroups was also tested. Unexpectedly, these findings showed that all isolates, belong to four different groups, were able to produce biofilm *in vitro* [190]. Similarly, it has been also demonstrated high antimicrobial resistance percentages for both *G. vaginalis* strains isolated from both health and BV women [186,195,202].

In sum, all of these data support the hypothesis that certain *G. vaginalis* subspecies are unable to induce BV, whereas other strains are suited to establishing *G. vaginalis* biofilms and, eventually, to eliciting BV, but this clearly needs further study. So, genomic sequencing and bacterial interaction studies of initial adhesion during BV and biofilm development are essential to clarify the etiology of BV.

2.5 References

[1] Ravel, J. et al. **(2011)**. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA* 108

[2] Farage, M.A., Miller, K.W. and Sobel, J.D. **(2010)**. Dynamics of the vaginal ecosystem—hormonal influences. *Infectious Diseases: Research and Treatment* 3, IDRT. S3903.

[3] Gajer, P. et al. **(2012)**. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 4, 132ra52.

[4] Crann, S.E., Cunningham, S., Albert, A., Money, D.M. and O'Doherty, K.C. **(2018)**. Vaginal health and hygiene practices and product use in Canada: a national cross-sectional survey. *BMC Women Health* 18, 52.

[5] Zheng, N.N., Guo, X.C., Lv, W., Chen, X.X. and Feng, G.F. **(2013)**. Characterization of the vaginal fungal flora in pregnant diabetic women by 18S rRNA sequencing. *Eur J Clin Microbiol Infect Dis* 32, 1031-40.

[6] Döderlein, A. **(1982)**. Das Scheidensekret und seine Bedeutung für das Puerperalfieber, *Zbl Bakteriol* 11, 699.

[7] Kovachev, S. **(2018)**. Defence factors of vaginal lactobacilli. *Crit Rev Microbiol* 44, 31-9.

[8] Vaneechoutte, M. **(2017)**. The human vaginal microbial community. *Res Microbiol* 168, 811-25.

[9] Godha, K., Tucker, K.M., Biehl, C., Archer, D.F. and Mirkin, S. **(2017)**. Human vaginal pH and microbiota: an update. *Gynecol Endocrinol*, 1-5.

[10] Tachedjian, G., Aldunate, M., Bradshaw, C.S. and Cone, R.A. **(2017)**. The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Res Microbiol* 68, 782-92.

[11] Prabhurajeshwar, C. and Chandrakanth, R.K. **(2017)**. Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An *in vitro* validation for the production of inhibitory substances. *Biomed J* 40, 270-83.

[12] O'Hanlon, D.E., Moench, T.R. and Cone, R.A. **(2013)**. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One* 8, e80074.

[13] Eschenbach, D.A., Davick, P.R., Williams, B.L., Klebanoff, S.J., Young-Smith, K., Critchlow, C.M. and Holmes, K.K. **(1989)**. Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *J Clin Microbiol* 27, 251-6.

[14] Hawes, S.E., Hillier, S.L., Benedetti, J., Stevens, C.E., Koutsky, L.A., Wolner-Hanssen, P. and Holmes, K.K. **(1996)**. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. *J Infect Dis* 174, 1058-63.

[15] Karaoğlu, Ş.A., Aydin, F., KILIÇ, S.S. and Kilic, A.O. **(2003)**. Antimicrobial activity and characteristics of bacteriocins produced by vaginal lactobacilli. *Turk J Med Sci* 33, 7-13.

[16] Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J.A., Gurguis, A. and Faro, S. **(2001)**. Defense factors of vaginal lactobacilli. *Am J Obstet Gynecol* 185, 375-9.

[17] Atassi, F. and Servin, A.L. **(2010)**. Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain *Lactobacillus johnsonii* NCC933 and vaginal strain Lactobacillus gasseri KS120.1 against enteric, uropathogenic and vaginosis-associated pathogens. *FEMS Microbiol Lett* 304, 29-38.

[18] Sgibnev, A.V. and Kremleva, E.A. **(2015)**. Vaginal protection by H_2O_2-producing Lactobacilli. *Jundishapur J Microbiol* 8, e22913.

[19] O'Hanlon, D.E., Moench, T.R. and Cone, R.A. **(2011)**. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BMC Infect Dis* 11, 200.

[20] Oscariz, J.C. and Pisabarro, A.G. **(2001)**. Classification and mode of action of membrane-active bacteriocins produced by Gram-positive bacteria. *Int Microbiol* 4, 13-9.

[21] Stoyancheva, G., Marzotto, M., Dellaglio, F. and Torriani, S. **(2014)**. Bacteriocin production and gene sequencing analysis from vaginal *Lactobacillus* strains. *Arch Microbiol* 196, 645-53.

[22] Dover, S.E., Aroutcheva, A.A., Faro, S. and Chikindas, M.L. **(2008)**. Natural antimicrobials and their role in vaginal health: a short review. *Int J Probiotics Prebiotics* 3, 219-30.

[23] Castro, J., Henriques, A., Machado, A., Henriques, M., Jefferson, K.K. and Cerca, N. **(2013)**. Reciprocal Interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on initial adherence to epithelial cells. *Int J Med Sci* 10, 1193.

[24] Leccese Terraf, M.C., Juarez Tomas, M.S., Rault, L., Le Loir, Y., Even, S. and Nader-Macias, M.E.F. **(2017)**. *In vitro* effect of vaginal lactobacilli on the growth and adhesion abilities of uropathogenic *Escherichia coli*. *Arch Microbiol* 199, 767-774.

[25] Ma, B., Forney, L.J. and Ravel, J. **(2012)**. Vaginal microbiome: rethinking health and disease. *Annu Rev Microbiol* 66, 371-89.

[26] Borgdorff, H. et al. **(2017)**. The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. *PLoS One* 12, e0181135.

[27] Amjadi, F., Salehi, E., Mehdizadeh, M. and Aflatoonian, R. **(2014)**. Role of the innate immunity in female reproductive tract. *Adv Biomed Res* 3, 1.

[28] Yarbrough, V.L., Winkle, S. and Herbst-Kralovetz, M.M. **(2015)**. Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. *Hum Reprod Update* 21, 353-77.

[29] Nasu, K. and Narahara, H. **(2010)**. Pattern recognition via the toll-like receptor system in the human female genital tract. *Mediators Inflamm* 2010, 976024.

[30] Wiesner, J. and Vilcinskas, A. **(2010)**. Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* 1, 440-64.

[31] Wira, C.R., Patel, M.V., Ghosh, M., Mukura, L. and Fahey, J.V. **(2011)**. Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am J Reprod Immunol* 65, 196-211.

[32] Doss, M., White, M.R., Tecle, T. and Hartshorn, K.L. **(2010)**. Human defensins and LL-37 in mucosal immunity. *J Leukoc Biol* 87, 79-92.

[33] Ganz, T. **(2003)**. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 3, 710-20.

[34] Baranger, K., Zani, M.L., Chandenier, J., Dallet-Choisy, S. and Moreau, T. **(2008)**. The antibacterial and antifungal properties of trappin-2 (pre-elafin) do not depend on its protease inhibitory function. *FEBS J* 275, 2008-20.

[35] Sallenave, J.-M. **(2000)**. The role of secretory leukocyte proteinase inhibitor and elafin (elastase-specific inhibitor/skin-derived antileukoprotease) as alarm antiproteinases in inflammatory lung disease. *Respir Res* 1, 87-92.

[36] Sallenave, J.M. **(2010)**. Secretory leukocyte protease inhibitor and elafin/trappin-2: versatile mucosal antimicrobials and regulators of immunity. *Am J Respir Cell Mol Biol* 42, 635-43.

[37] Doumas, S., Kolokotronis, A. and Stefanopoulos, P. **(2005)**. Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. *Infect Immun* 73, 1271-4.

[38] Chen, B., Wen, Y. and Polan, M.L. **(2004)**. Elastolytic activity in women with stress urinary incontinence and pelvic organ prolapse. *Neurourol Urodyn* 23, 119-26.

[39] Scott, A., Weldon, S. and Taggart, C.C. **(2011)**. SLPI and elafin: multifunctional antiproteases of the WFDC family. *Biochem Soc Trans* 39, 1437-40.

[40] Simpson, A.J., Wallace, W.A., Marsden, M.E., Govan, J.R., Porteous, D.J., Haslett, C. and Sallenave, J.M. **(2001)**. Adenoviral augmentation of elafin protects the lung against acute injury mediated by activated neutrophils and bacterial infection. *J Immunol* 167, 1778-86.

[41] Fahey, J.V. and Wira, C.R. **(2002)**. Effect of menstrual status on antibacterial activity and secretory leukocyte protease inhibitor production by human uterine epithelial cells in culture. *J Infect Dis* 185, 1606-13.

[42] Williams, S.E., Brown, T.I., Roghanian, A. and Sallenave, J.M. **(2006)**. SLPI and elafin: one glove, many fingers. *Clin Sci (Lond)* 110, 21-35.

[43] Abbott, D.S., Chin-Smith, E.C., Seed, P.T., Chandiramani, M., Shennan, A.H. and Tribe, R.M. **(2014)**. Raised trappin2/elafin protein in cervico-vaginal fluid is a potential predictor of cervical shortening and spontaneous preterm birth. *PLoS One* 9, e100771.

[44] Pillay, K., Coutsoudis, A., Agadzi-Naqvi, A.K., Kuhn, L., Coovadia, H.M. and Janoff, E.N. **(2001)**. Secretory leukocyte protease inhibitor in vaginal fluids and perinatal human immunodeficiency virus type 1 transmission. *J Infect Dis* 183, 653-6.

[45] Andrault, P.M. et al. **(2015)**. Antimicrobial Peptide LL-37 is both a substrate of cathepsins S and K and a selective inhibitor of cathepsin L. *Biochemistry* 54, 2785-98.

[46] Bandurska, K., Berdowska, A., Barczynska-Felusiak, R. and Krupa, P. **(2015)**. Unique features of human cathelicidin LL-37. *Biofactors* 41, 289-300.

[47] Valenti, P., Rosa, L., Capobianco, D., Lepanto, M.S., Schiavi, E., Cutone, A., Paesano, R. and Mastromarino, P. **(2018)**. Role of lactobacilli and lactoferrin in the mucosal cervicovaginal defense. *Front Immunol* 9, 376.

[48] Sinha, M., Kaushik, S., Kaur, P., Sharma, S. and Singh, T.P. **(2013)**. Antimicrobial lactoferrin peptides: the hidden players in the protective function of a multifunctional protein. *Int J Pept* 2013, 390230.

[49] Dial, E.J. and Lichtenberger, L.M. **(2002)**. Effect of lactoferrin on *Helicobacter felis* induced gastritis. *Biochem Cell Biol* 80, 113-7.

[50] Wakabayashi, H., Oda, H., Yamauchi, K. and Abe, F. **(2014)**. Lactoferrin for prevention of common viral infections. *J Infect Chemother* 20, 666-71.

[51] Niyonsaba, F. and Ogawa, H. **(2005)**. Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *J Dermatol Sci* 40, 157-68.

[52] Davis, K.M. and Weiser, J.N. **(2011)**. Modifications to the peptidoglycan backbone help bacteria to establish infection. *Infect Immun* 79, 562-70.

[53] Nash, J.A., Ballard, T.N., Weaver, T.E. and Akinbi, H.T. **(2006)**. The peptidoglycan-degrading property of lysozyme is not required for bactericidal activity *in vivo*. *J Immunol* 177, 519-26.

[54] During, K., Porsch, P., Mahn, A., Brinkmann, O. and Gieffers, W. **(1999)**. The non-enzymatic microbicidal activity of lysozymes. *FEBS Lett* 449, 93-100.

[55] Ragland, S.A. and Criss, A.K. **(2017)**. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathog* 13, e1006512.

[56] Lee-Huang, S. et al. **(2005)**. Structural and functional modeling of human lysozyme reveals a unique nonapeptide, HL9, with anti-HIV activity. *Biochemistry* 44, 4648-55.

[57] Ellison, R.T. and Giehl, T.J. **(1991)**. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest* 88, 1080-91.

[58] Agerberth, B. and Gudmundsson, G.H. **(2006)**. Host antimicrobial defence peptides in human disease. *Curr Top Microbiol Immunol* 306, 67-90.

[59] Kenyon, C., Colebunders, R. and Crucitti, T. **(2013)**. The global epidemiology of bacterial vaginosis: a systematic review. *Am J Obstet Gynecol* 209, 505-23.

[60] Onderdonk, A.B., Delaney, M.L. and Fichorova, R.N. **(2016)**. The human microbiome during bacterial vaginosis. *Clin Microbiol Rev* 29, 223-238.

[61] Martin, D.H. **(2012)**. The microbiota of the vagina and its influence on women's health and disease. *Am J Med Sci* 343, 2-9.

[62] Jung, H.S., Ehlers, M.M., Lombaard, H., Redelinghuys, M.J. and Kock, M.M. **(2017)**. Etiology of bacterial vaginosis and polymicrobial biofilm formation. *Crit Rev Microbiol*, 1-17.

[63] Zhou, X., Bent, S.J., Schneider, M.G., Davis, C.C., Islam, M.R. and Forney, L.J. **(2004)**. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiology* 150, 2565-73.

[64] Lin, L. et al. **(1999)**. The role of bacterial vaginosis in infection after major gynecologic surgery. *Infect Dis Obstet Gynecol* 7, 169-74.

[65] Ness, R.B., Kip, K.E., Hillier, S.L., Soper, D.E., Stamm, C.A., Sweet, R.L., Rice, P. and Richter, H.E. **(2005)**. A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *Am J Epidemiol* 162, 585-90.

[66] Harmanli, O.H., Cheng, G.Y., Nyirjesy, P., Chatwani, A. and Gaughan, J.P. **(2000)**. Urinary tract infections in women with bacterial vaginosis. *Obstet Gynecol* 95, 710-2.

[67] Salah, R.M., Allam, A.M., Magdy, A.M. and Mohamed, A. **(2013)**. Bacterial vaginosis and infertility: cause or association? *Eur J Obstet Gynecol Reprod Biol* 167, 59-63.

[68] Isik, G., Demirezen, S., Donmez, H.G. and Beksac, M.S. **(2016)**. Bacterial vaginosis in association with spontaneous abortion and recurrent pregnancy losses. *J Cytol* 33, 135-40.

[69] Svare, J.A., Schmidt, H., Hansen, B.B. and Lose, G. **(2006)**. Bacterial vaginosis in a cohort of Danish pregnant women: prevalence and relationship with preterm delivery, low birthweight and perinatal infections. *BJOG* 113, 1419-25.

[70] Dingens, A.S., Fairfortune, T.S., Reed, S. and Mitchell, C. **(2016)**. Bacterial vaginosis and adverse outcomes among full-term infants: a cohort study. *BMC Pregnancy Childbirth* 16, 278.

[71] Haddad, L. et al. **(2018)**. Bacterial vaginosis modifies the association between hormonal contraception and HIV acquisition. *Aids* 32, 595-604.

[72] Gillet, E., Meys, J.F.A., Verstraelen, H., Bosire, C., De Sutter, P., Temmerman, M. and Broeck, D.V. **(2011)**. Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC Infect Dis* 11, 10.

[73] Wiesenfeld, H.C., Hillier, S.L., Krohn, M.A., Landers, D.V. and Sweet, R.L. **(2003)**. Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin Infect Dis* 36, 663-8.

[74] Eschenbach, D.A., Hillier, S., Critchlow, C., Stevens, C., DeRouen, T. and Holmes, K.K. **(1988)**. Diagnosis and clinical manifestations of bacterial vaginosis. *Am J Obstet Gynecol* 158, 819-28.

[75] Gibbs, R.S. **(2007)**. Asymptomatic bacterial vaginosis: is it time to treat? A*m J Obstet Gynecol* 196, 495-6.

[76] Turovskiy, Y., Sutyak Noll, K. and Chikindas, M.L. **(2011)**. The aetiology of bacterial vaginosis. *J Appl Microbiol* 110, 1105-28.

[77] Olmsted, S.S., Meyn, L.A., Rohan, L.C. and Hillier, S.L. **(2003)**. Glycosidase and proteinase activity of anaerobic Gram-negative bacteria isolated from women with bacterial vaginosis. *Sex Transm Dis* 30, 257-61.

[78] Wolrath, H., Forsum, U., Larsson, P.G. and Borén, H. **(2001)**. Analysis of bacterial vaginosis-related amines in vaginal fluid by gas chromatography and mass spectrometry. *J Clin Microbiol* 39, 4026-4031.

[79] Amsel, R., Totten, P.A., Spiegel, C.A., Chen, K.C., Eschenbach, D. and Holmes, K.K. **(1983)**. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. *Am J Med* 74, 14-22.

[80] Sha, B.E., Chen, H.Y., Wang, Q.J., Zariffard, M.R., Cohen, M.H. and Spear, G.T. **(2005)**. Utility of Amsel criteria, Nugent score, and quantitative PCR for *Gardnerella vaginalis, Mycoplasma hominis*, and *Lactobacillus* spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *J Clin Microbiol* 43, 4607-12.

[81] Nugent, R.P., Krohn, M.A. and Hillier, S. **(1991)**. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 29, 297-301.

[82] Spiegel, C.A., Amsel, R. and Holmes, K.K. **(1983)**. Diagnosis of bacterial vaginosis by direct Gram stain of vaginal fluid. *J Clin Microbiol* 18,170-7.

[83] Livengood, C.H. **(2009)**. Bacterial vaginosis: an overview for 2009. *Rev Obstet Gynecol* 2, 28-37.

[84] Fredricks, D.N., Fiedler, T.L., Thomas, K.K., Oakley, B.B. and Marrazzo, J.M. **(2007)**. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol* 45, 3270-6.

[85] Hilbert, D.W. et al. **(2016)**. Development and validation of a highly accurate quantitative real-time PCR assay for diagnosis of bacterial vaginosis. *J Clin Microbiol* 54, 1017-24.

[86] Machado, A., Castro, J., Cereija, T., Almeida, C. and Cerca, N. **(2015)**. Diagnosis of bacterial vaginosis by a new multiplex peptide nucleic acid fluorescence *in situ* hybridization method. *PeerJ* 3, e780.

[87] Africa, C.W. **(2013)**. Efficacy of methods used for the diagnosis of bacterial vaginosis. *Expert Opin Med Diagn* 7, 189-200.

[88] Schwebke, J.R. **(1997)**. Bacterial vaginosis-more questions than answers. *Genitourin Med* 73, 333-4.

[89] Allsworth, J.E. and Peipert, J.F. **(2007)**. Prevalence of bacterial vaginosis: 2001-2004 national health and nutrition examination survey data. *Obstet Gynecol* 109, 114-20.

[90] Paul, K., Boutain, D., Manhart, L. and Hitti, J. **(2008)**. Racial disparity in bacterial vaginosis: the role of socioeconomic status, psychosocial stress, and neighborhood characteristics, and possible implications for preterm birth. *Soc Sci Med* 67, 824-33.

[91] Hoffmann, J.N., You, H.M., Hedberg, E.C., Jordan, J.A. and McClintock, M.K. **(2014)**. Prevalence of bacterial vaginosis and *Candida* among postmenopausal women in the United States. *J Gerontol B Psychol Sci Soc Sci* 69 Suppl 2, S205-14.

[92] Hellberg, D., Nilsson, S. and Mardh, P.A. **(2000)**. Bacterial vaginosis and smoking. *Int J STD AIDS* 11, 603-6.

[93] Verstraelen, H., Verhelst, R., Vaneechoutte, M. and Temmerman, M. **(2010)**. The epidemiology of bacterial vaginosis in relation to sexual behaviour. *BMC Infect Dis* 10, 81.

[94] Schwebke, J.R., Desmond, R.A. and Oh, M.K. **(2004)**. Predictors of bacterial vaginosis in adolescent women who douche. *Sex Transm Dis* 31, 433-6.

[95] Ranjit, E., Raghubanshi, B.R., Maskey, S. and Parajuli, P. **(2018)**. Prevalence of bacterial vaginosis and its association with risk factors among nonpregnant women: a hospital based study. *Int J Microbiol* 2018, 8349601.

[96] Chiaffarino, F., Parazzini, F., De Besi, P. and Lavezzari, M. **(2004)**. Risk factors for bacterial vaginosis. *Eur J Obstet Gynecol Reprod Biol* 117, 222-6.

[97] Wang, L.Y. et al. **(2016)**. The effect of contraceptive methods on reproductive tract infections risk: a cross-sectional study having a sample of 52,481 women. *Arch Gynecol Obstet* 294, 1249-56.

[98] Fethers, K.A., Fairley, C.K., Hocking, J.S., Gurrin, L.C. and Bradshaw, C.S. **(2008)**. Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clin Infect Dis* 47, 1426-35.

[99] Fethers, K.A., Fairley, C.K., Morton, A., Hocking, J.S., Hopkins, C., Kennedy, L.J., Fehler, G. and Bradshaw, C.S. **(2009)**. Early sexual experiences and risk factors for bacterial vaginosis. *J Infect Dis* 200, 1662-70.

[100] Marrazzo, J.M. **(2011)**. Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused? *Anaerobe* 17, 186-90.

[101] Guerreiro, D., Gigante, M.A. and Teles, L.C. **(1998)**. Sexually transmitted diseases and reproductive tract infections among contraceptive users. *Int J Gynaecol Obstet* 63 Suppl 1, S167-73.

[102] Henriques, A.F., Martinez-de-Oliveira, J. and Cerca, N. **(2012).** Doctor's perception on bacterial vaginosis in Portugal: prevalence, diagnostic methods and choice of treatment. *Sex Transm Infect* 88, 421-421.

[103] Bradshaw, C.S. et al. **(2006)**. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* 193, 1478-86.

[104] Silva, D., Henriques, A., Cereija, T., Martinez-de-Oliveira, J., Miranda, M. and Cerca, N. **(2014)**. Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in Portuguese women and association with risk factors for bacterial vaginosis. *Int J Gynaecol Obstet* 124, 178-9.

[105] Cristiano, L., Rampello, S., Noris, C. and Valota, V. **(1996)**. Bacterial vaginosis: prevalence in an Italian population of asymptomatic pregnant women and diagnostic aspects. *Eur J Epidemiol* 12, 383-90.

[106] Gratacos, E., Figueras, F., Barranco, M., Ros, R., Andreu, A., Alonso, P.L. and Cararach, V. **(1999)**. Prevalence of bacterial vaginosis and correlation of clinical to Gram stain diagnostic criteria in low risk pregnant women. *Eur J Epidemiol* 15, 913-6.

[107] Machado, D., Castro, J., Martinez-de-Oliveira, J., Nogueira-Silva, C. and Cerca, N. **(2017)**. Prevalence of bacterial vaginosis in Portuguese pregnant women and vaginal colonization by *Gardnerella vaginalis*. *PeerJ* 5, e3750.

[108] Muzny, C. and Schwebke, J. **(2013)**. *Gardnerella vaginalis*: still a prime suspect in the pathogenesis of bacterial vaginosis. *Curr Infect Dis Rep* 15:130-5.

[109] Josey, W.E. and Schwebke, J.R. **(2008)**. The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. *Int J STD AIDS* 19, 152-4.

[110] Gardner, H.L. and Dukes, C.D. **(1955)**. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *Am J Obstet Gynecol* 69, 962-76.

[111] Criswell, B.S., Ladwig, C.L., Gardner, H.L. and Dukes, C.D. **(1969)**. *Haemophilus vaginalis*: vaginitis by inoculation from culture. *Obstet Gynecol* 33, 195-9.

[112] Evans, A.S. **(1993)**. Limitations of the Henle - Koch Postulates. *Causation and Disease*, pp. 123-46.

[113] Spiegel, C.A., Davick, P., Totten, P.A., Chen, K.C., Eschenbach, D.A., Amsel, R. and Holmes, K.K. **(1983)**. *Gardnerella vaginalis* and anaerobic bacteria in the etiology of bacterial (nonspecific) vaginosis. *Scand J Infect Dis Suppl* 40, 41-6.

[114] Holst, E., Svensson, L., Skarin, A., Westrom, L. and Mardh, P.A. **(1984)**. Vaginal colonization with *Gardnerella vaginalis* and anaerobic curved rods. *Scand J Urol Nephrol Suppl* 86, 147-52.

[115] Chen, K.C., Forsyth, P.S., Buchanan, T.M. and Holmes, K.K. **(1979)**. Amine content of vaginal fluid from untreated and treated patients with nonspecific vaginitis. *J Clin Invest* 63, 828-35.

[116] Machado, A. and Cerca, N. **(2015)**. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *J Infect Dis* 212, 1856-61.

[117] Leppaluoto, P.A. **(2011)**. Bacterial vaginosis: what is physiological in vaginal bacteriology? An update and opinion. *Acta Obstet Gynecol Scand* 90, 1302-6.

[118] Romero, R. et al. **(2014)**. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2, 4.

[119] Muzny, C.A. et al. **(2018)**. Identification of key bacteria involved in the induction of incident bacterial vaginosis: a prospective study. *J Infect Dis* jiy243.

[120] Huang, B., Fettweis, J.M., Brooks, J.P., Jefferson, K.K. and Buck, G.A. **(2014)**. The changing landscape of the vaginal microbiome. *Clin Lab Med* 34, 747-61.

[121] Fredricks, D., Fiedler, T. and Marrazzo, J. **(2005)**. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med* 353, 1899-911.

[122] Austin, M.N., Rabe, L.K., Srinivasan, S., Fredricks, D.N., Wiesenfeld, H.C. and Hillier, S.L. **(2015)**. *Mageeibacillus indolicus* gen. nov., sp. nov.: a novel bacterium isolated from the female genital tract. *Anaerobe* 32, 37-42.

[123] Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. **(2004)**. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2, 95-108.

[124] Bryers, J.D. **(2008)**. Medical Biofilms. *Biotechnol Bioeng* 100, 1-18.

[125] Verstraelen, H. and Swidsinski, A. **(2013)**. The biofilm in bacterial vaginosis: implications for epidemiology, diagnosis and treatment. *Curr Opin Infect Dis* 26, 86-9.

[126] Johnson, A. and Boustouller, Y. **(1987)**. Extra-vaginal infection caused by G*ardnerella vaginalis*. *Epidemiol Infect* 98, 131-37.

[127] Scott, T.G., Curran, B. and Smyth, C.J. **(1989)**. Electron microscopy of adhesive interactions between *Gardnerella vaginalis* and vaginal epithelial cells, McCoy cells and human red blood cells. *J Gen Microbiol* 135, 475-80.

[128] Mardh, P.A., Holst, E. and Moller, B.R. **(1984)**. The grivet monkey as a model for study of vaginitis. Challenge with anaerobic curved rods and *Gardnerella vaginalis*. *Scand J Urol Nephrol Suppl* 86, 201-5.

[129] Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L.P. and Lochs, H. **(2005)**. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 106, 1013-23.

[130] Swidsinski, A. et al. **(2013)**. Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). *Histol Histopathol* 29, 567-87.

[131] Swidsinski, A., Loening-Baucke, V., Swidsinski, S. and Verstraelen, H. **(2015).** Polymicrobial *Gardnerella* biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. *Arch Gynecol Obstet* 291, 605-9.

[132] Swidsinski, A., Verstraelen, H., Loening-Baucke, V., Swidsinski, S., Mendling, W. and Halwani, Z. **(2013)**. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS One* 8, e53997.

[133] Schilling, J., Loening-Baucke, V. and Dorffel, Y. **(2014)**. Increased *Gardnerella vaginalis* urogenital biofilm in inflammatory bowel disease. *J Crohns Colitis* 8, 543-9.

[134] Swidsinski, A., Dorffel, Y., Loening-Baucke, V., Schilling, J. and Mendling, W. **(2011)**. Response of *Gardnerella vaginalis* biofilm to 5 days of moxifloxacin treatment. *FEMS Immunol Med Microbiol* 61, 41-6.

[135] Machado, A., Salgueiro, D., Harwich, M., Jefferson, K.K. and Cerca, N. **(2013)**. Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. *Anaerobe* 23,1-4.

[136] Alves, P., Castro, J., Sousa, C., Cereija, T.B. and Cerca, N. **(2014)**. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *J Infect Dis* 210, 593-6.

[137] Patterson, J.L., Stull-Lane, A., Girerd, P.H. and Jefferson, K.K. **(2010)**. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 156, 392-99.

[138] Workowski, K.A. and Bolan, G.A. **(2015)**. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm Rep* 64, 1-137.

[139] Sherrard, J., Donders, G., White, D. and Jensen, J.S. **(2011)**. European (IUSTI/WHO) guideline on the management of vaginal discharge, 2011. *Int J STD AIDS* 22, 421-9.

[140] Nygren, P., Fu, R., Freeman, M., Bougatsos, C., Klebanoff, M. and Guise, J.M. **(2008)**. Evidence on the benefits and harms of screening and treating pregnant women who are asymptomatic for bacterial vaginosis: an update review for the U.S. Preventive Services Task Force. *Ann Intern Med* 148, 220-33.

[141] Schwebke, J.R. **(2000)**. Asymptomatic bacterial vaginosis: response to therapy. *Am J Obstet Gynecol* 183, 1434-9.

[142] Sociedade Portuguesa de Ginecologia. **(2012)** Revisão dos Consensos em Infeções Vulvovaginais.

[143] Sobel, R. and Sobel, J.D. **(2015)**. Metronidazole for the treatment of vaginal infections. *Expert Opin Pharmacother* 16, 1109-15.

[144] Miljkovic, V., Arsic, B., Bojanic, Z., Nikolic, G., Nikolic, L., Kalicanin, B. and Savic, V. **(2014)**. Interactions of metronidazole with other medicines: a brief review. *Pharmazie* 69, 571-7.

[145] Paavonen, J., Mangioni, C., Martin, M.A. and Wajszczuk, C.P. **(2000)**. Vaginal clindamycin and oral metronidazole for bacterial vaginosis: a randomized trial. *Obstet Gynecol* 96, 256-60.

[146] Menard, J.-P. **(2011)**. Antibacterial treatment of bacterial vaginosis: current and emerging therapies. *Int J Womens Health* 3, 295-305.

[147] Smieja, M. **(1998)**. Current indications for the use of clindamycin: a critical review. The *Can J Infect Dis* 9, 22-28.

[148] Wood, B.A. and Monro, A.M. **(1975)**. Pharmacokinetics of tinidazole and metronidazole in women after single large oral doses. *Br J Vener Dis* 51, 51-3.

[149] Nikolov, A., Shopova, E., Nashar, S. and Dimitrov, A. **(2008)**. Application of sumamed in treatment of bacterial vaginal infections during pregnancy. *Akush Ginekol (Sofiia)* 47, 7-10.

[150] Thulkar, J., Kriplani, A. and Agarwal, N. **(2012)**. A comparative study of oral single dose of metronidazole, tinidazole, secnidazole and ornidazole in bacterial vaginosis. *Indian J Pharmacol* 44, 243-5.

[151] Nyirjesy, P. and Schwebke, J.R. **(2018)**. Secnidazole: next-generation antimicrobial agent for bacterial vaginosis treatment. *Future Microbiol* 13,507-24.

[152] Bohbot, J.M., Vicaut, E., Fagnen, D. and Brauman, M. **(2010)**. Treatment of bacterial vaginosis: a multicenter, double-blind, double-dummy, randomised phase III study comparing secnidazole and metronidazole. *Infect Dis Obstet Gynecol* 2010.

[153] Bilardi, J. et al. **(2016)**. Women's management of recurrent bacterial vaginosis and experiences of clinical care: a qualitative study. *PLoS One* 11, e0151794.

[154] Gunn, J.S., Bakaletz, L.O. and Wozniak, D.J. **(2016)**. What's on the outside matters: the role of the extracellular polymeric substance of Gram-negative biofilms in evading host immunity and as a target for therapeutic intervention. *J Biol Chem* 291, 12538-46.

[155] Cerca, N., Jefferson, K.K., Oliveira, R., Pier, G.B. and Azeredo, J. **(2006)**. Comparative antibody-mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the planktonic state. *Infect Immun* 74, 4849-55.

[156] Jefferson, K.K. **(2004)**. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett* 236, 163-73.

[157] Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S. and Ciofu, O. **(2010)**. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35, 322-32.

[158] Machado, D., Castro, J., Palmeira-de-Oliveira, A., Martinez-de-Oliveira, J. and Cerca, N. **(2015)**. Bacterial vaginosis biofilms: challenges to current therapies and emerging solutions. *Front Microbiol* 6, 1528.

[159] Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., Lochs, H. and Verstraelen, H. **(2008)**. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 198, 97. e1-97. e6.

[160] Stewart, P.S. and William Costerton, J. **(2001)**. Antibiotic resistance of bacteria in biofilms. *The Lancet* 358, 135-38.

[161] Hymes, S.R., Randis, T.M., Sun, T.Y. and Ratner, A.J. **(2013)**. DNase Inhibits *Gardnerella vaginalis* biofilms *in vitro* and *in vivo*. *J Infect Dis* 207, 1491-97.

[162] Hooven, T.A., Randis, T.M., Hymes, S.R., Rampersaud, R. and Ratner, A.J. **(2012)**. Retrocyclin inhibits *Gardnerella vaginalis* biofilm formation and toxin activity. *J Antimicrob Chemother* 67, 2870-2.

[163] Mur Perez, A.M., Mateo Segura, Z., Ramirez Dominguez, N. and Vela Condon, P. **(2017)**. Use of probiotics in bacterial vaginosis. *Semergen* 43, 394-98.

[164] Mendling, W., Weissenbacher, E.R., Gerber, S., Prasauskas, V. and Grob, P. **(2016)**. Use of locally delivered dequalinium chloride in the treatment of vaginal infections: a review. *Arch Gynecol Obstet* 293, 469-84.

[165] Machado, D., Gaspar, C., Palmeira-de-Oliveira, A., Cavaleiro, C., Salgueiro, L., Martinez-de-Oliveira, J. and Cerca, N. **(2017)**. Thymbra capitata essential oil as potential therapeutic agent against *Gardnerella vaginalis* biofilm-related infections. *Future Microbiol* 12, 407-16.

[166] Turovskiy, Y., Ludescher, R.D., Aroutcheva, A.A., Faro, S. and Chikindas, M.L. **(2009)**. Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis. Probiotics Antimicrob Proteins* 1, 67-74.

[167] Bradshaw, C.S. and Brotman, R.M. **(2015)**. Making inroads into improving treatment of bacterial vaginosis - striving for long-term cure. *BMC Infect Dis* 15, 292.

[168] Koumans, E.H., Sternberg, M., Bruce, C., McQuillan, G., Kendrick, J., Sutton, M. and Markowitz, L.E. **(2007)**. The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sex Transm Dis* 34, 864-9.

[169] Catlin, B.W. **(1992)**. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clin Microbiol Rev* 5, 213-37.

[170] Zozaya-Hinchliffe, M., Lillis, R., Martin, D.H. and Ferris, M.J. **(2010)**. Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. *J Clin Microbiol* 48, 1812-9.

[171] Leopold, S. **(1953)**. Heretofore undescribed organism isolated from the genitourinary system. *U S Armed Forces Med J* 4, 263.

[172] Zinnemann, K. and Turner, G. **(1963)**. The taxonomic position of "*Haemophilus vaginalis*" [Corynebacterium vaginale]. *The Journal of Pathology* 85, 213-19.

[173] Greenwood, J.R. and Pickett, M.J. **(1980)**. Transfer of *Haemophilus vaginalis* Gardner and Dukes to a New Genus, *Gardnerella*: *G. vaginalis* (Gardner and Dukes) comb. nov. *Int J Syst Evol Microbiol* 30, 170-78.

[174] Piot, P., van Dyck, E., Goodfellow, M. and Falkow, S. **(1980)**. A taxonomic study of *Gardnerella vaginalis* (*Haemophilus vaginalis*) Gardner and Dukes 1955. *J Gen Microbiol* 119, 373-96.

[175] Sadhu, K., Domingue, P.A., Chow, A.W., Nelligan, J., Cheng, N. and Costerton, J.W. **(1989**). *Gardnerella vaginalis* has a Gram-positive cell-wall ultrastructure and lacks classical cell-wall lipopolysaccharide. *J Med Microbiol* 29, 229-35.

[176] Cereija, T.B., Castro, J., Alves, P. and Cerca, N. **(2013)**. Influence of anaerobic conditions on vaginal microbiota recovery from bacterial vaginosis patients. *Sex Transm Infect* 89, 307-307.

[177] Schellenberg, J.J., Patterson, M.H. and Hill, J.E. **(2017)**. *Gardnerella vaginalis* diversity and ecology in relation to vaginal symptoms. *Res Microbiol* 68, 837-44.

[178] Malone, B.H., Schreiber, M., Schneider, N.J. and Holdeman, L.V. **(1975)**. Obligately anaerobic strains of *Corynebacterium vaginale* (*Haemophilus vaginalis*). *J Clin Microbiol* 2, 272-5.

[179] Schwebke, J.R., Muzny, C.A. and Josey, W.E. **(2014)**. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis* 210, 338-43.

[180] Hickey, R.J. and Forney, L.J. **(2014)**. *Gardnerella vaginalis* does not always cause Bacterial Vaginosis. *J Infect Dis* 210, 1682-3.

[181] Schwebke, J.R., Flynn, M.S. and Rivers, C.A. **(2014)**. Prevalence of *Gardnerella vaginalis* among women with *Lactobacillus*-predominant vaginal flora. *Sex Transm Infect* 90, 61-3.

[182] Hickey, R.J. et al. **(2015)**. Vaginal microbiota of adolescent girls prior to the onset of menarche resemble those of reproductive-age women. *MBio* 6, pii: e00097-15.

[183] Shen, J., Song, N., Williams, C.J., Brown, C.J., Yan, Z., Xu, C. and Forney, L.J. **(2016)**. Effects of low dose estrogen therapy on the vaginal microbiomes of women with atrophic vaginitis. *Sci Rep* 6, 24380.

[184] Piot, P., Van Dyck, E., Peeters, M., Hale, J., Totten, P.A. and Holmes, K.K. **(1984)**. Biotypes of *Gardnerella vaginalis*. *J Clin Microbiol* 20, 677-9.

[185] Briselden, A.M. and Hillier, S.L. **(1990)**. Longitudinal study of the biotypes of G*ardnerella vaginalis*. *J Clin Microbiol* 28, 2761-4.

[186] Aroutcheva, A., Simoes, J., Behbakht, K. and Faro, S. **(2001)**. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin Infect Dis* 33, 1022-27.

[187] Pedraza-Aviles, A.G., Inzunza-Montiel, A.E., Ortiz-Zaragoza, M.C., Morales-Espinosa, M.R. and Ponce-Rosas, E.R. **(1995)**. *Gardnerella vaginalis* biotypes: modification of a proposed system. *Rev Latinoam Microbiol* 37, 19-26.

[188] Gonzalez-Pedraza Aviles, A., Ortiz-Zaragoza, M.C., Inzunza-Montiel, A.E. and Ponce-Rosas, E.R. **(1996)**. Biotypes of *Gardnerella vaginalis* isolated from urinary tract. *Rev Latinoam Microbiol* 38, 75-80.

[189] Tosun, I., Alpay, K.S., Ciftçi, H., Buruk, C.K., Aydin, F., Kilic, A.O. and Ertürk, M. **(2007)**. Biotypes and antibiotic resistance patterns of *Gardnerella vaginalis* strains isolated from healthy women and women with bacterial vaginosis. *Mikrobiyoloji bülteni* 41, 21.

[190] Jayaprakash, T.P., Schellenberg, J.J. and Hill, J.E. **(2012)**. Resolution and characterization of distinct *cpn60*-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PloS One* 7, e43009.

[191] Schellenberg, J.J., Paramel Jayaprakash, T., Withana Gamage, N., Patterson, M.H., Vaneechoutte, M. and Hill, J.E. **(2016)**. *Gardnerella vaginalis* subgroups defined by *cpn60* sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PLoS One* 11, e0146510.

[192] Pleckaityte, M., Janulaitiene, M., Lasickiene, R. and Zvirbliene, A. **(2012)**. Genetic and biochemical diversity of *Gardnerella vaginalis* strains isolated from women with bacterial vaginosis. *FEMS Immunol Med Microbiol* 65, 69-77.

[193] Benito, R., Vazquez, J., Berron, S., Fenoll, A. and Saez-Nieto, J. **(1986)**. A modified scheme for biotyping *Gardnerella vaginalis*. *J Med Microbiol* 21, 357-59.

[194] Yeoman, C.J. et al. **(2010)**. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS One* 5, e12411.

[195] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

[196] Ahmed, A. et al. **(2012)**. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol* 194, 3922-37.

[197] Lopes dos Santos Santiago, G., Deschaght, P., El Aila, N., Kiama, T.N., Verstraelen, H., Jefferson, K.K., Temmerman, M. and Vaneechoutte, M. **(2011)**. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. *Am J Obstet Gynecol* 204, 450. e1-450. e7.

[198] Balashov, S.V., Mordechai, E., Adelson, M.E. and Gygax, S.E. **(2014)**. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* 63, 162-75.

[199] Hilbert, D.W., Schuyler, J.A., Adelson, M.E., Mordechai, E., Sobel, J.D. and Gygax, S.E. **(2017)**. *Gardnerella vaginalis* population dynamics in bacterial vaginosis. *Eur J Clin Microbiol Infect Dis* 36, 1269-78.

[200] Vodstrcil, L.A. et al. **(2017)**. The influence of sexual activity on the vaginal microbiota and *Gardnerella vaginalis* clade diversity in young women. *PLoS One* 12, e0171856.

[201] Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A. and Pleckaityte, M. **(2017)**. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infect Dis* 17, 394.

[202] Knupp de Souza, D.M. et al. **(2016)**. Antimicrobial susceptibility and vaginolysin in *Gardnerella vaginalis* from healthy and bacterial vaginosis diagnosed women. *J Infect Dev Ctries* 10, 913-19.

[203] Hardy, L., Jespers, V., Van den Bulck, M., Buyze, J., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M. and Crucitti, T. **(2017)**. The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PLoS One* 12, e0172522.

[204] Cornejo, O.E., Hickey, R.J., Suzuki, H. and Forney, L.J. **(2018)**. Focusing the diversity of *Gardnerella vaginalis* through the lens of ecotypes. *Evol Appl* 11, 312-24.

[205] Fettweis, J.M., Serrano, M.G., Girerd, P.H., Jefferson, K.K. and Buck, G.A. **(2012)**. A new era of the vaginal microbiome: advances using next-generation sequencing. *Chem Biodivers* 9, 965-76.

[206] Huang, B., Fettweis, J.M., Brooks, J.P., Jefferson, K.K. and Buck, G.A. **(2014)**. The changing landscape of the vaginal microbiome. *Clin Lab Med* 34, 747-61.

[207] Lewis, W.G., Robinson, L.S., Gilbert, N.M., Perry, J.C. and Lewis, A.L. **(2013)**. Degradation, foraging, and depletion of mucus sialoglycans by the vagina-adapted *Actinobacterium Gardnerella vaginalis*. *J Biol Chem* 288, 12067-79.

[208] Cohan, F.M. **(2006)**. Towards a conceptual and operational union of bacterial systematics, ecology, and evolution. *Philos Trans R Soc Lond B Biol Sci* 361, 1985-996.

[209] Swidsinski, A. et al. **(2010)**. *Gardnerella* biofilm involves females and males and is transmitted sexually. *Gynecol Obstet Invest* 70, 256-63.

[210] Lianou, A. and Koutsoumanis, K.P. **(2013)**. Strain variability of the behavior of foodborne bacterial pathogens: a review. *Int J Food Microbiol* 167, 310-21.

[211] Castro, J., Martins, A.P., Rodrigues, M.E. and Cerca, N. **(2018)**. *Lactobacillus crispatus* represses vaginolysin expression by BV associated *Gardnerella vaginalis* and reduces cell cytotoxicity. *Anaerobe* 50, 60-3.

CHAPTER 3

Using an in-vitro biofilm model to assess the virulence potential of Bacterial Vaginosis or non-Bacterial vaginosis Gardnerella vaginalis isolates

Summary

In an effort to better understand the differences between *Gardnerella vaginalis* isolated from women with bacterial vaginosis (BV) *versus* normal healthy flora (non-BV), we compared the virulence potential of 7 BV and 7 non-BV *G. vaginalis* isolates by assessing the initial adhesion capacity and cytotoxic effect, biofilm accumulation, susceptibility to antibiotics and transcript levels of known virulence genes (vaginolysin and sialidase). Furthermore, we also determined the ability of *G. vaginalis* to displace lactobacilli previously adhered to HeLa cells. Our results showed that non-BV strains were less virulent than BV strains, as suggested by the lower cytotoxicity and initial adhesion to Hela cells. Significant differences in expression of known virulence genes were also detected, further suggesting a higher virulence potential of the BV associated *G. vaginalis*. Importantly, we demonstrated that BV associated *G. vaginalis* were able to displace pre-coated vaginal protective lactobacilli and we hypothesize this to be a trigger for BV development.

The work presented in this chapter was published in *Scientific Reports (2015) 5: 11640.*

3.1 Brief introduction

Despite being the most prevalent and virulent species found in BV, *G. vaginalis* can also be a part of the vaginal microbiota in healthy women [1,2]. Consequently, there has been much debate in the literature concerning the contribution of *G. vaginalis* to the etiology of BV [3,4]. Phenotypic diversity within *G. vaginalis* has been described in terms of virulence factors, particularly production of sialidase [5], cytotoxicity [3] and ability to adhere and establish a biofilm on the vaginal epithelium [3,6]. Furthermore, full genome sequencing of different *G. vaginalis* strains revealed significant differences between BV and non-BV isolates [3]. This raised the question of whether there are distinct pathogenic and commensal lineages within this species. Thus, the present study aimed to isolate BV and non-BV associated *G. vaginalis* strains and to evaluate their virulence potential, using an *in vitro* biofilm model, by determining their ability to adhere to epithelial cells, to interfere with the displacement of healthy lactobacilli on epithelial cells, to grown as biofilm, to induce cytotoxic changes on epithelial cells, to express known virulence genes, and finally by determining their susceptibility to the antibiotics commonly used in BV treatment.

3.2 Material and methods

3.2.1 Subject selection and sample collection

Vaginal samples were obtained from volunteers during private gynecology consult. All sampling was conducted in accordance with relevant guidelines and regulations and research approved by the University of Minho Institutional Review Board (approval number: SESVC 003-2013) in accordance with the Declaration of Helsinki and the guidelines of Good Clinical Practice. Written informed consent was obtained from all study participants prior to enrolment. Women were excluded from the study if they had any chronical disease. Classification of samples was done as before [7]. Briefly, BV diagnosis was first performed by the clinician, using the Amsel criteria [8]. Then based on the criteria for BV assessment developed by Nugent and colleagues [9], participants with the Gram stain score of ≥7 were finally confirmed as BV (Supplementary Table S3.1). We also probed the samples with a novel PNA-FISH probe against *G. vaginalis* [10].

3.2.2 Bacterial isolation and identification

The presence of *G. vaginalis* in vaginal samples was further confirmed by PCR using an optimized protocol, as we previously described [11]. Samples positive for *G. vaginalis* were plated in columbia blood agar medium (Liofilchem, Roseto degli Abruzzi, Italy) with 5% (v/v) defibrinated horse blood (Oxoid Ltd., Basingstoke, Hants, United Kingdom) and incubated

under anaerobic conditions, as described before [4,12]. Isolated bacteria were analyzed by Gram stain and subsequently identified by partial sequencing of 16S rRNA coding gene as described before [13] (Eurofins, Germany). Nucleotide sequences obtained were compared to known sequences through BLAST software (NCBI, Bethesda, MD, USA). The primers used are listed in Table 3.1. The accession number for these 14 strains are listed in Supplementary Table S3.2.

Table 3.1 Primer sequences used for by partial sequencing of 16S rRNA

Target	Primers sequence (5'to 3')	$T_{melting}$ (ºC)	Amplicon size (bp)	Reference
16s RNA (Bacteria)	Fw AGA GTT TGA TCC TGG CTC AG	55	789	[13]
16s RNA (Bacteria)	Rv GGA CTA CCA GGG TAT CTA AT	55	789	[13]

3.2.3 Initial adhesion to epithelial cells and cytotoxicity assays

Initial adhesion to human cervical HeLa cells (ATCC CCL-2) and cytotoxicity assays were performed as described previously [4]. Briefly, for the adhesion assays, blind bacterial suspensions with a concentration of 1×10^8 colony-forming units (CFU)/mL were added to a monolayer of HeLa cells for 30 minutes at 37ºC under anaerobic conditions. After washing the non-adherent bacteria, cells were fixed with methanol and adhesion was microscopically quantified as we previously described [4]. For the cytotoxicity assays, blind bacterial suspensions adjusted to 2.9×10^7 CFU/mL were added to a monolayer of HeLa cells for 3 hours. Cytotoxicity was scored on a 0 to 5 scale [14]. Numeric scores were assigned as follows: 0, no difference between the test and the control; 1, 25% of the cells were rounded; 2, 25 – 50% of the cells were rounded; 3, 50% of the cells were rounded; 4, 50% cells were rounded, with partial disruption of the monolayer; and 5, complete disruption or absence of the monolayer. All experiments were performed in triplicate with technical replicates.

3.2.4 Quantification of biofilm formation

Bacteria were grown in 9 different commercially available culture media, commonly used for biofilm growth: LB [composed by 10 g/L Tryptone (Liofilchem), 5 g/L yeast extract (Liofilchem) and 10 g/L of NaCl (Liofilchem)], MRS (Liofilchem), TSB (Liofilchem), sBHI [BHI (Liofilchem) supplemented with 2% (w/w) gelatin (Oxoid), 0.5% (w/w) yeast extract, 0.1% (w/w) starch (Thermo Fisher Scientific, Lenexa, KS, USA)], sBHIF [sBHI with 10% (v/v) FBS], and finally LBG, MRSG, TSBG and sBHIG supplemented with 0.25% (w/v) of glucose (Liofilchem) [4]. Biofilm formation assays were performed as described previously [3,14]. In brief, 200 µL of

each bacterial suspension adjusted to 1×10^6 CFU/mL was incubated in 96-well flat-bottom tissue culture plates (Orange Scientific, Braine L'Alleud, Belgium) at 37ºC for 48 hours under anaerobic conditions. Biofilms were first qualitatively evaluated with safranin staining [14]. Subsequently, the intrinsic ability of *G. vaginalis* strains to grow as biofilms was quantified, using the equation optical density $(OD)_{600nm}$ biofilm / (OD_{600nm} biofilm + OD_{600nm} planktonic) as described by Harwich *et al.* [3], for the 3 media that promoted the greatest biofilm growth. The biofilm formation index (BFI) was defined as the average biofilm quantity in the 3 selected growth media [4]. All assays were repeated 3 times with technical replicates.

3.2.5 Antibiotic susceptibility

The susceptibility of *G. vaginalis* to antibiotics was evaluated by determining the minimal inhibitory concentration (MIC) of metronidazole, tinidazole and clindamycin. A pre-culture was first prepared for each isolate in sBHI by incubating at 37ºC under anaerobic conditions. After 24 hours, growth was confirmed by measuring the OD at 600 nm. MIC was determined by microdilution method in 96-well tissue culture plates [15]. All assays were repeated 3 times with technical replicates.

3.2.6 *G. vaginalis* ability to induce displacement of lactobacilli pre-adhered to epithelial cells

The ability of *G. vaginalis* to displace *Lactobacillus crispatus* pre-adhered to epithelial cells was assessed using a protocol that we previously optimized [16] with minor changes. Briefly, a suspension of 1.0×10^9 CFU/mL of *L. crispatus* EX533959VC06 was added to each well of the 24-well plate containing the monolayer of HeLa cells. The plates were incubated for 4 hours at 37°C in anaerobic conditions, at 0.081 *g (*PSU-10i, Biosan, Latvia). Subsequently, *G. vaginalis* strains (1.0×10^8 CFU/mL) were added for 30 minutes under the same conditions as described above. Bacterial quantification was done as previously described [17].

3.2.7 PCR detection of virulence genes

Oligonucleotide primers for the detection of *vly* and *sld* genes were designed using the Primer3 software [18] using the complete genome of *G. vaginalis* strain ATCC 14019 as a template. The 16S rRNA was used as internal control. Negative PCR results were confirmed using a second pair of independent primers. All primers used are listed in Table 3.2. Genomic DNA was extracted as described before [11] and the thermocycling program (Mini-MJ, Bio-Rad, Hercules, CA, USA) was performed using the DreamTaq PCR Master Mix 2x (Finnzymes, Espoo, Finland) and consisted on the following steps: 94°C for 2 minutes followed by 40 cycles

of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 60 seconds and finally 72°C for 5 minutes. The PCR product was then kept hold at 4°C. PCR products were analyzed by gel electrophoresis with 1.5% agarose (Bioron, Ludwigshafen, Germany) and Orange G DNA loading dye (Thermo Fisher Scientific). All assays were repeated 3 times.

Table 3.2 Primer sequences used for PCR and qPCR assays

Target	Primers sequence (5'to 3')	$T_{melting}$ (ºC)	Amplicon size (bp)	Reference
16s RNA (G. vaginalis)	Fw CTC TTG GAA ACG GGT GGT AA	62	300	[11]
16s RNA (G. vaginalis)	Rv TG CTC CCA ATC AAA AGC GGT	62	300	[11]
Vaginolysin	Fw 1 CTCGCATGCAGTACGATTCT	58	187	This study
Vaginolysin	Rv 1 TCTGGTGCATCAACGCTTAC	58	187	This study
Vaginolysin	Fw 2 GCCAGACAGCTTGAAGAACC	60	116	This study
Vaginolysin	Rv 2 CAGTGCTCTTGCTGGTGGTA	60	116	This study
Sialidase	Fw 1 CCGAATTTGCGATTTCTTCT	54	189	This study
Sialidase	Rv 1 CGTACGGAAGTTTTGGAAGC	58	189	This study
Sialidase	Fw 2 GGGTTTATGCACACGCTTTT	56	131	This study
Sialidase	Rv 2 GAAAATGCAGACAACGCAGA	58	131	This study

3.2.8 Gene expression quantification

G. vaginalis strains were grown as described for the adhesion assays. Total RNA was extracted as previous described [19]. Briefly, genomic DNA was degraded with one step of DNase treatment (Fermentas, Lithuania) following manufacturer's instructions. RNA concentration, purity and integrity was determined as described before [20]. Quantitative PCR (qPCR) was performed as previously described [19] with some modifications. Briefly, qPCR was done using a CFX96^{TM} thermal cycler (Bio-Rad) with the following cycling parameters: 3 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C, 10 seconds at 58°C and 15 seconds at 72°C. The primer efficiency and the normalized gene expression was determined by using the delta Ct method ($2^{\Delta Ct}$), a variation of the Livak method, where $\Delta C_t = C_t$ (reference gene) - C_t (target gene). All primer pairs had similar efficiencies. A control lacking the reverse transcriptase enzyme was included in each reaction. Gene expression assays were performed 3 independent times and in each time we had 3 qPCR wells per gene.

3.2.9 Statistical analysis

The data were analyzed using the independent samples *t*-test, one-way analysis of variance (ANOVA), or non-parametric Wilcoxon matched-pairs rank test for the data that did not follow a normal distribution according Kolmogorov-Smirvon's test, with the statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL). The data were represented as mean ± standard deviation (SD) or as mean ± standard error of mean (SEM) at least 3 independent experiments. *P*-values of less than 0.05 were considered significant.

3.3 Results

3.3.1 Initial adhesion to human cervical HeLa cells and cytotoxic effect

After isolating 7 BV and 7 non-BV associated strains of *G. vaginalis* (Supplementary Table S3.2) we first determined the ability of all strains to adhere to a monolayer of HeLa epithelial cells. As can be seen in Figure 3.1, variations in adhesion were observed among the 14 isolates, with statistical differences between the 2 groups ($p < 0.05$). Importantly, BV isolates showed a greater ability to adhere to epithelial cells than non-BV isolates, with an average of 14.83 and 2.89 bacteria per HeLa cell, respectively.

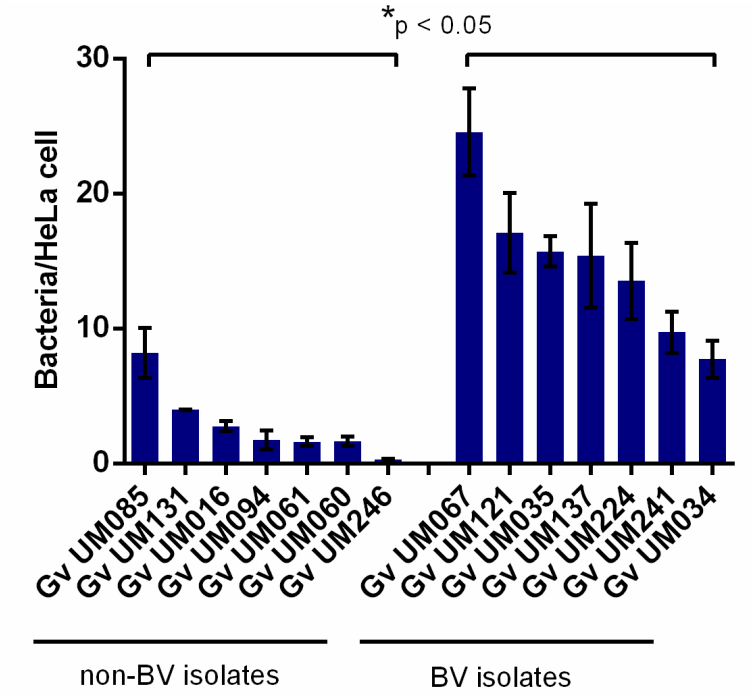


Figure 3.1 Initial adhesion of non-BV and BV *G. vaginalis* isolates to HeLa cells. Adhesion was microscopically quantified and expressed as the average ± SD number of bacteria per epithelial cell. *Denotes significance differences between the 2 groups of *G. vaginalis* strains at same conditions (one-way ANOVA, $p < 0.05$).

Cytotoxicity was also quantified in order to determine the capacity of the 2 groups of bacteria to induce cytotoxic changes in cell morphology on HeLa cells. Similar to the initial adhesion assays, BV isolates had a higher cytotoxicity score than non-BV isolates ($p < 0.05$; Figure 3.2), which were only capable of causing slight morphological changes in HeLa monolayer.

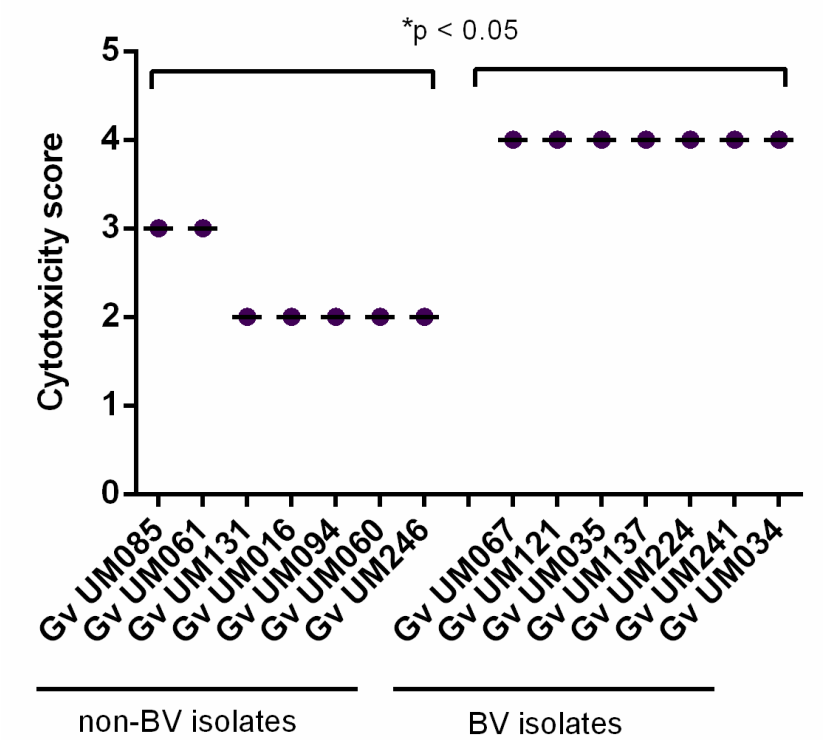


Figure 3.2 Cytotoxicity score of non-BV and BV *G. vaginalis* isolates. Cytotoxicity was scored as follows: 0, no difference between the experimental well and the control; 1, <25 % cells were rounded; 2, 25-50 % cells were rounded; 3, >50 % cells were rounded; 4, > 50% were rounded, with partial disruption of the monolayer; 5, complete disruption/absence of the monolayer. *Values are significantly different between the 2 groups of *G. vaginalis* strains under the same conditions (one-way ANOVA, $p < 0.05$).

3.3.2 Biofilm formation

In order to determine the optimal medium for *in vitro* biofilm formation, all isolates were initially cultured anaerobically in 9 different media (Table 3.3). As expected, *G. vaginalis* isolates formed different amounts of biofilm, depending on the growth media.

Table 3.3 Qualitative analysis[a] of biofilm formed by *G. vaginalis* strains in 9 different media

	Media[b]								
Strain	**LB**	**LBG**	**MRS**	**MRSG**	**TSB**	**TSBG**	**sBHI**	**sBHIG**	**sBHIF**
non-BV associated									
G. vaginalis UM085	+-	+-	+-	+-	+-	+-	+-	+-	+-
G. vaginalis UM061	-	+-	-	-	+-	+-	++	+++	+-
G. vaginalis UM131	+-	+-	+-	+-	+-	+-	+-	+-	+-
G. vaginalis UM016	+-	+-	-	-	+-	+-	++	++	++
G. vaginalis UM094	+-	+-	+-	+-	+-	+-	++	++	+-
G. vaginalis UM060	-	+-	-	-	-	-	+-	+-	+-
G. vaginalis UM246	+-	+-	+-	+-	+-	+-	+-	+-	+-
BV associated									
G. vaginalis UM067	-	-	-	-	-	-	+-	+++	+-
G. vaginalis UM121	+-	+-	+-	+-	+-	+-	+-	++	+-
G. vaginalis UM035	-	-	-	-	+-	+-	++	++	++
G. vaginalis UM137	-	+-	-	-	+-	+-	++	+-	+-
G. vaginalis UM224	-	-	+-	+-	+-	+-	++	+++	++
G. vaginalis UM241	+-	+-	+-	+-	+-	+-	++	++	++
G. vaginalis UM034	-	-	-	-	+-	+-	+-	+-	+-

[a] Biofilm formation was classified using the following scale: (-) no biofilm formed, (+-) formed medium biofilm, (++) good biofilm formation, (+++) strong biofilm formation in all tests. [b] LB: luria broth, LBG: LB supplemented with 0.25% (w/v) glucose, MRS: de man-rogosa and sharpe agar, MRSG: MRS supplemented with 0.25% (w/v) glucose, TSB: tryptic soy broth, TSBG: TSB supplemented with 0.25% (w/v) glucose, sBHI: BHI supplemented brain heart infusion broth supplemented with 2% (w/w) gelatin, 0.5% (w/w) yeast extract, and 0.1% (w/w) starch, sBHIG: sBHI supplemented with 0.25% (w/v) glucose, sBHIF: sBHI supplemented with 10% (v/v) fetal bovine serum.

To minimize the bias introduced by the growth media, we defined the biofilm formation index (BFI) as the average of growth in the 3 best growth media. Interestingly, our results showed that there were no significant differences in BFI between the 2 groups ($p > 0.05$), although there was a trend of higher BFI levels associated with the BV isolates (Figure 3.3).

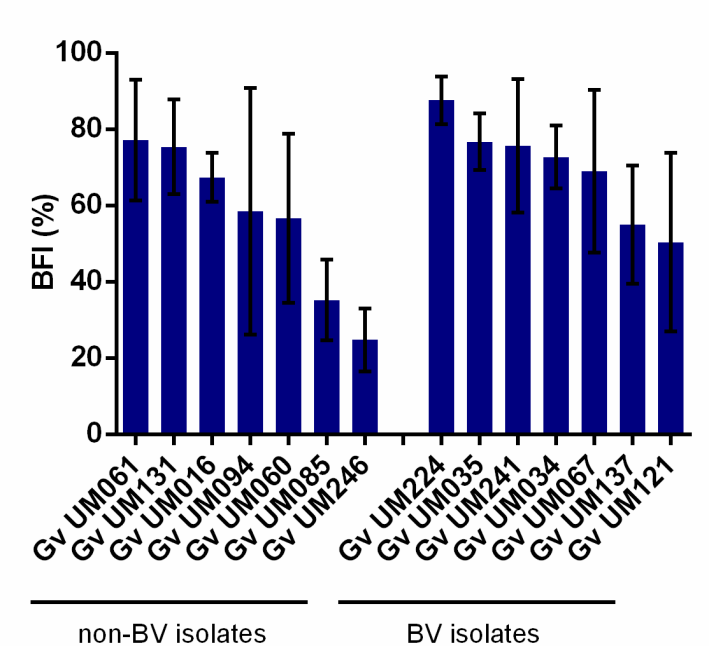


Figure 3.3 Intrinsic ability of non-BV and BV *G. vaginalis* isolates to form biofilms. The biofilm formation index (BFI) was defined as the average percentage of bacteria grown as biofilms, in the 3 media with higher biofilm growth for each *G. vaginalis* strains. The growth percentage as a biofilm for the 3 media was calculated using the equation OD_{600nm} biofilm/ (OD_{600nm} biofilm + OD_{600nm} planktonic) and represented as mean ± SD. No significant differences between non-BV and BV isolates were found to BFI (non-parametric Wilcoxon matched-pairs rank test, $p > 0.05$).

3.3.3 Antimicrobial susceptibility

In vitro antimicrobial susceptibility of *G. vaginalis* was evaluated by determining the MIC of metronidazole, tinidazole and clindamycin. Similar to the BFI determinations, no significant differences were found in antimicrobial susceptibility profiles between non-BV and BV isolates ($p > 0.05$; Table 3.4). Interestingly, all *G. vaginalis* strains tested exhibited intermediate resistance or resistance to metronidazole. Similarly, the strains exhibited intermediate resistance or resistance to tinidazole (86% of strains) while only 36% of strains were resistant to clindamycin.

Table 3.4 Minimum inhibitory concentration (MIC) of metronidazole, tinidazole and clindamycin for planktonic cells of *G. vaginalis* isolates. Statistical analysis: no significant differences were found in antimicrobial susceptibility profiles between non-BV and BV associated isolates by the non-parametric Wilcoxon matched-pairs rank test ($p > 0.05$)

Strain	MIC range		
	Metronidazole	**Tinidazole**	**Clindamycin**
non-BV associated			
G. vaginalis UM085	>[128]	[16]	<[0-01]
G. vaginalis UM061	[16]-[32]	[8]-[16]	<[0.01]
G. vaginalis UM131	>[128]	>[128]	>[128]
G. vaginalis UM016	[32]	[8]-[16]	<[0.01]
G. vaginalis UM094	>[128]	[32]	[0.5]
G. vaginalis UM060	>[128]	>[128]	<[0.01]
G. vaginalis UM246	[16]-[32]	[16]-[32]	>[128]
BV associated			
G. vaginalis UM067	[16]-[32]	[8]-[16]	<[0.01]
G. vaginalis UM121	[32]-[64]	[16]	>[128]
G. vaginalis UM035	[64]-[128]	[4]-[8]	<[0.01]
G. vaginalis UM137	[32]-[64]	[16]-[32]	>[128]
G. vaginalis UM224	[32]	[16]-[32]	>[128]
G. vaginalis UM241	[32]	[2]-[4]	<[0.01]
G. vaginalis UM034	>[128]	[32]-[64]	<[0.01]

3.3.4 *G. vaginalis* ability to induce displacement of lactobacilli pre-adhered to epithelial cells

We recently reported on the capacity of *G. vaginalis* to displace adherent vaginal lactobacilli from epithelial cells [16]. We sought to determine whether the non-BV and BV strains of *G. vaginalis* differed in their abilities to displace adherent lactobacilli populations. We found that, on average, BV isolates had a stronger ability to cause displacement of *L. crispatus* (63.78%) than non-BV isolates (19.05%, $p < 0.05$), as shown in Figure 3.4. Also, similar to our previous

observations [16], *L. crispatus* inhibited the adherence of BV *G. vaginalis* isolates to the epithelial cells but failed to antagonize the adherence of non-BV isolates.

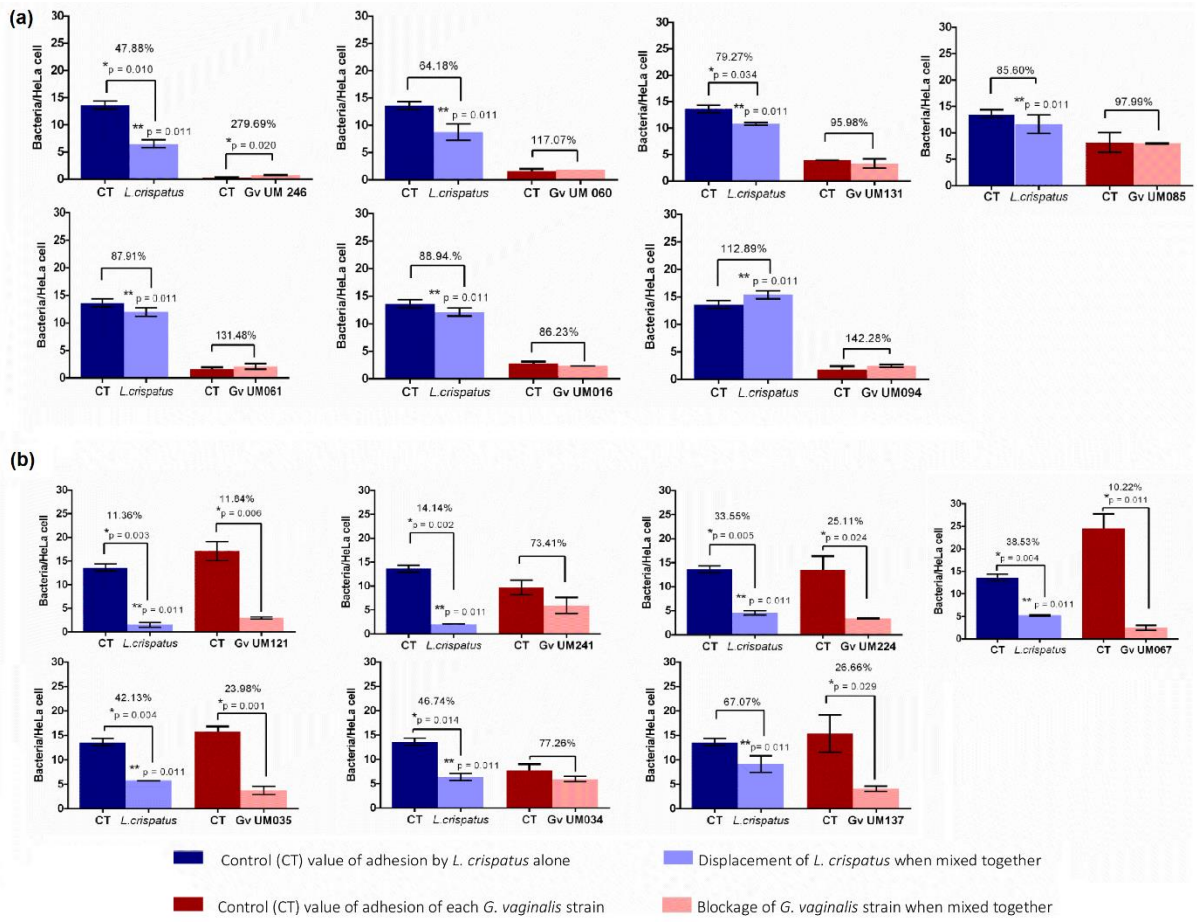


Figure 3.4 Influence of *L. crispatus* on *G. vaginalis* initial adhesion to HeLa cells. *L. crispatus* was pre-adhered to the epithelial cells. Subsequently, each *G. vaginalis* strain was added. **(a)** Represents the non-BV isolates. **(b)** Represents the BV isolates. Results are expressed as mean ± SD of bacteria/HeLa cell. The percentage indicated is the result of the variation in the final adhesion of *L. crispatus* and *G. vaginalis*, after *G. vaginalis* challenge to the pre-coated *L. crispatus*, as compared to the adhesion levels of each strain independently. *Values are significantly different from the respective control (independent samples t-test, $p < 0.05$). **Significant differences in the displacement of *L. crispatus* by two groups of *G. vaginalis* strains were found (one-way ANOVA, $p < 0.05$). No significant differences in the adherence of *G. vaginalis* were found between non-BV and BV isolates when mixed with *L. crispatus* (one-way ANOVA, $p > 0.05$).

3.3.5 Presence and expression of virulence genes

To understand the role of virulence genes in non-BV and BV isolates of *G. vaginalis*, we initially determined whether the vaginolysin (*vly*) and sialidase (*sld)* genes were present in all 14

strains. As shown in Table 3.5, no differences were found between the groups. Surprisingly, we verified that *vly* was absent in strains UM035 and UM224, as determined by PCR amplification with 2 independent pairs of primers, contrary to what was been described before [3,21].

Table 3.5 Detection by PCR of the *vly* and *sld* genes in *G. vaginalis* isolates

Strain	Presence of virulence genes	
	vly	***sld***
non-BV associated		
G. vaginalis UM085	+	+
G. vaginalis UM061	+	+
G. vaginalis UM131	+	+
G. vaginalis UM016	+	+
G. vaginalis UM094	+	–
G. vaginalis UM060	+	+
G. vaginalis UM246	+	+
BV associated		
G. vaginalis UM067	+	+
G. vaginalis UM121	+	+
G. vaginalis UM035	–	+
G. vaginalis UM137	+	+
G. vaginalis UM224	–	–
G. vaginalis UM241	+	+
G. vaginalis UM034	+	–

Furthermore, this data was confirmed by amplifying (Supplementary Table S3.3) and sequencing the flanking regions of *vly* (Supplementary Figure S3.1). Since we did not find differences in the presence of these virulence genes between the 2 groups, we then analyzed the expression of those genes using a selection of 6 *G. vaginalis* strains (3 of each group) in which all strains carried the 3 genes of interest. Our data revealed differences in the expression of the tested genes (Figure 3.5). Interestingly, the biggest difference found between the 2 groups was related to *vly* expression, in which BV isolates of *G. vaginalis* showed, on average, an expression 2-fold higher than non-BV isolates ($p < 0.05$). Nevertheless, no significant differences in expression of *sld* ($p > 0.05$) were detected between the 2 groups.

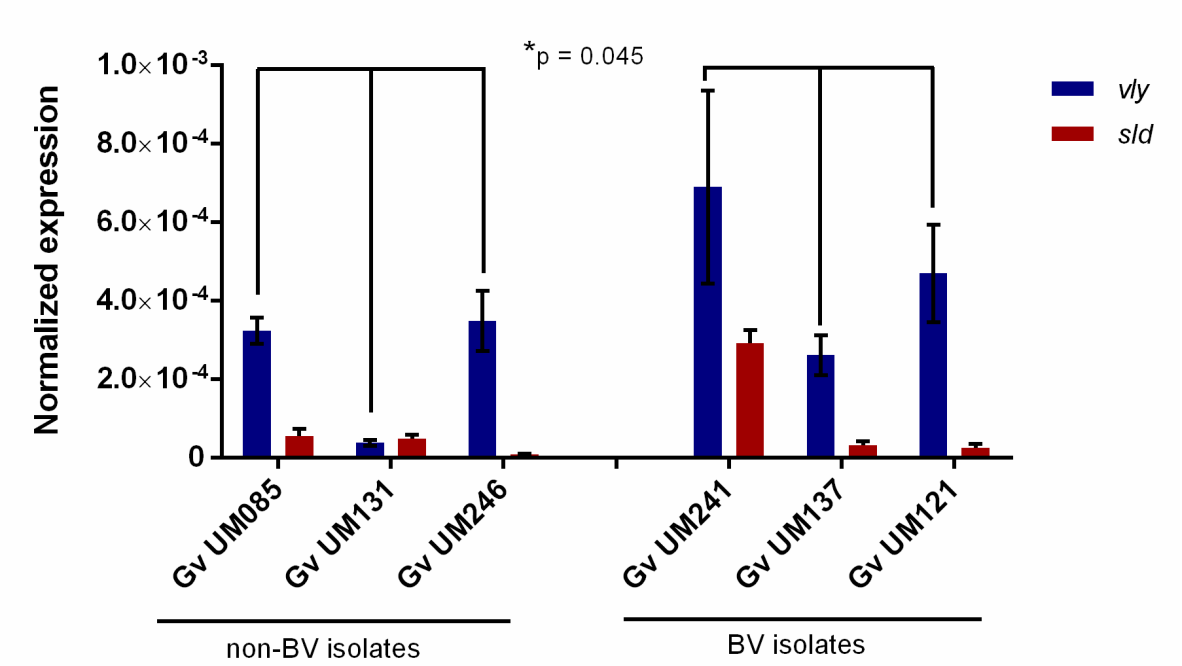


Figure 3.5 Expression of vaginolysin (*vly*) and sialidase (*sld)* by *G. vaginalis* isolates. Transcript levels within planktonic culture of the *G. vaginalis* strains were quantified. Results are expressed as normalized expression in relation to 16S rRNA and represented as mean ± SEM. *Values are significantly different between non-BV and BV *G. vaginalis* strains to *vly* gene expression (one-way ANOVA, $p < 0.05$). No significant differences between two groups were found to *sld* gene expression (one-way ANOVA, $p > 0.05$).

3.4 Discussion

This study provides a more comprehensive understanding of the different *G. vaginalis* strains that can be found in the vaginal bacterial ecosystem, in health or disease. Clearly, all 7 strains isolated from women with BV were more virulent than the 7 non-BV strains. However, contrary to what was previously hypothesized, this increased virulence was not directly related to biofilm accumulation [3], since all of our strains had similar biofilm formation, assessed in distinct growth media. On the other hand, the higher initial adhesion and cytotoxicity, as well as the ability to displace pre-adherent healthy vaginal lactobacilli, were important features of BV associated *G. vaginalis,* suggesting that the trigger for BV development could occur during the early stages of biofilm formation.

G. vaginalis is the most thoroughly studied BV associated microorganism but the fact that it is frequently present in healthy women casts doubt on its role in the etiology of BV [22,23]. Interestingly, it has been reported that certain biotypes of *G. vaginalis* are more frequently associated with BV [24]. However, functional microbiological studies addressing virulence properties of BV or non-BV strains are still scarce and often do not account for strain to strain

variability [3,16]. We designed a series of *in vitro* experiments to compare the relative virulence capacities of BV and non-BV isolates of *G. vaginalis*. We used 7 different strains per group, to increase the confidence of the results.

We started by quantifying *G. vaginalis* initial adhesion to HeLa cells, since initial adhesion to the vaginal epithelium is a crucial step in BV development [25] and the first step of biofilm formation [26]. Importantly, our data clearly showed that BV isolates adhered more avidly to the epithelial cells. Because the vagina is commonly colonized by *Lactobacillus* species [22,27-29], we also explored the interaction between different *G. vaginalis* isolates and protective lactobacilli. The pathogenesis of BV is poorly understood and two different chains of events leading to BV have been proposed. One suggests that the population of lactobacilli is drastically reduced, by yet unknown factors, thus allowing the colonization by the multiple bacterial species associated with BV, while the other proposes that a single bacterial agent competes with lactobacilli, resulting in its overgrowth, later allowing other species to colonize the vaginal epithelium [27]. Recently, we showed that while one BV associated *G. vaginalis* strain was able to displace a protective layer of vaginal lactobacilli and colonize HeLa epithelium cells, this did not occur with a non-BV strain [16]. To confirm those findings, we analysed the ability of the *G. vaginalis* panel used in this study to displace *L. crispatus* previously adhered to the HeLa cells. Strengthening our previous observations, only BV associated strains of *G. vaginalis* were able to displace around 80% of the pre-coated lactobacilli (5 out of 7 strains). On the other hand, *L. crispatus* had a more pronounced effect in impeding the colonization by BV associated *G. vaginalis*. This data suggests that BV associated variants of *G. vaginalis* could be the primary pathogens in BV development, since this subset of strains have the ability to significantly displace vaginal lactobacilli, supporting one of the BV development models proposed [27].

We also analyzed the ability of *G. vaginalis* to cause cytopathogenic changes in HeLa epithelial cells. We found that the BV isolates were significantly more cytotoxic, inducing rounding and lysis of HeLa epithelial cells, while non-BV *G. vaginalis* were unable to cause such cytopathogenic changes. The cytotoxicity activity of BV isolates could be due to a pore-forming toxin produced by *G. vaginalis*, vaginolysin, which is able to induce cell death and is thus a virulence factor [21]. Interestingly our data revealed that on average, BV isolates expressed 2-fold more *vly* than non-BV strains. However, strain to strain variability suggests that *vly* expression is not exclusive of BV associated *G. vaginalis*. Furthermore, sialidase could increase the cytotoxic activity of *G. vaginalis* and contribute to exfoliation and detachment of vaginal epithelial cells, by degrading mucins, which normally protect the epithelium [5]. Our studies did not reveal a direct relationship between sialidase expression and cytotoxicity,

however, the epithelial monolayers used in our model do not produce mucins. Therefore, a different model system would be required to test this hypothesis.

It has also been described that as BV progresses, a highly structured polymicrobial biofilm develops on the vaginal epithelium and a major component of the biofilm is *G. vaginalis* [6,14,25,30,31]. Taking into consideration the differences in adhesion to epithelial cells, and the fact that initial adhesion does not always correlate to biofilm accumulation [32], we characterized the intrinsic ability of *G. vaginalis* strains to grow as biofilms. Curiously, in our *in vitro* assay, BV isolates generally presented a higher BFI, however, differences in biofilm formation between the 2 groups did not reach statistical significance. Nevertheless, only 5 out of 7 non-BV isolates were able to grow preferentially as a biofilm (BFI>50%) while all 7 BV isolates analyzed showed a BFI>50%. Biofilm formation is an important virulence factor because it confers increased tolerance to antibiotics [33] and antimicrobial byproducts produced by lactobacilli normally associated with the healthy vagina [14]. Importantly, we detected high levels of antimicrobial tolerance in all isolates analyzed, confirming our previous reports [4]. Surprisingly, similar to the biofilm assay, no differences were detected between the two groups. Overall, *G. vaginalis* strains were more susceptible to clindamycin than to metronidazole or tinidazole, which was unexpected based on previous reports [34,35].

This work clearly demonstrates strain differences between *G. vaginalis* isolates that could impact the ability of this organism to cause disease. However, the *in vitro* model of adherence used in this study is limited by the fact that cell monolayers of HeLa cells are not polarized, as are vaginal epithelial cells *in vivo*. The assay for biofilm formation was limited by the fact that the growth medium did not contain all of the factors found *in vivo*, and some *in vivo* cues may turn on expression of biofilm-related genes. Nevertheless, these limitations aside, *in vitro* models can be very informative, and are key to furthering our understanding of virulence potential of *G. vaginalis.*

Taking in consideration our novel findings and our previous observations [4,16,36,37] we hypothesize that colonization by a subset of *G. vaginalis* is the trigger for BV development. By displacing lactobacilli, adhered *G. vaginalis* will then start to form a biofilm that will subsequently promote the incorporation of secondary colonizers and this mixed biofilm will ultimately become recalcitrant to antimicrobial therapy, similar to what has been described for oral biofilms [38]. Future genomic characterization of the non-BV and BV isolates of *G. vaginalis* will unveil the molecular mechanisms involved in these reported virulence differences. We envision that this will later impact novel diagnostic procedures and therapeutic options to treat BV.

3.5 References

[1] Aroutcheva, A.A., Simoes, J.A., Behbakht, K. and Faro, S. **(2001)**. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin Infect Dis* 33, 1022-27.

[2] Hickey, R.J. and Forney, L.J. **(2014)**. *Gardnerella vaginalis* does not always cause Bacterial Vaginosis. *J Infect Dis* 210, 1682-3.

[3] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

[4] Alves, P., Castro, J., Sousa, C., Cereija, T.B. and Cerca, N. **(2014)**. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with Bacterial Vaginosis, using in an *in vitro* biofilm formation model. *J Infect Dis* 210, 593-6.

[5] Cauci, S., Thorsen, P., Schendel, D.E., Bremmelgaard, A., Quadrifoglio, F. and Guaschino, S. **(2003)**. Determination of immunoglobulin A against *Gardnerella vaginalis* hemolysin, sialidase, and prolidase activities in vaginal fluid: implications for adverse pregnancy outcomes. *J Clin Microbiol* 41, 435-38.

[6] Swidsinski, A. et al. **(2013)**. Infection through Structured Polymicrobial *Gardnerella* biofilms (StPM-GB). *Histol Histopathol* 29, 567-87.

[7] Ling, Z. et al. **(2010)**. Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. *BMC Genomics* 11, 488.

[8] Amsel, R., Totten, P.A., Spiegel, C.A., Chen, K., Eschenbach, D. and Holmes, K.K. **(1983)**. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. *Am J Med* 74, 14-22.

[9] Nugent, R.P., Krohn, M.A. and Hillier, S. **(1991)**. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 29, 297-301.

[10] Machado, A., Castro, J., Cereija, T., Almeida, C. and Cerca, N. **(2015)**. Diagnosis of bacterial vaginosis by a new multiplex peptide nucleic acid fluorescence *in situ* hybridization method. *PeerJ* 3, e780.

[11] Henriques, A., Cereija, T., Machado, A. and Cerca, N. **(2012)**. *In silico* vs *in vitro* analysis of primer specificity for the detection of *Gardnerella vaginalis*, *Atopobium vaginae* and *Lactobacillus* spp. *BMC Res Notes* 5, 637.

[12] Cereija, T.B., Castro, J., Alves, P. and Cerca, N. **(2013)**. Influence of anaerobic conditions on vaginal microbiota recovery from bacterial vaginosis patients. *Sex Transm Infect* 89, 307-07.

[13] Wilson, K.H., Blitchington, R.B. and Greene, R.C. **(1990)**. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J Clin Microbiol* 28, 1942-6.

[14] Patterson, J.L., Stull-Lane, A., Girerd, P.H. and Jefferson, K.K. **(2010)**. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 156, 392-99.

[15] Reller, L.B., Weinstein, M., Jorgensen, J.H. and Ferraro, M.J. **(2009)**. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin infect Dis* 49, 1749-55.

[16] Castro, J., Henriques, A., Machado, A., Henriques, M., Jefferson, K.K. and Cerca, N. **(2013)**. Reciprocal Interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on Initial Adherence to Epithelial Cells. *Int J Medical Sci* 10, 1193.

[17] Machado, A. et al. **(2013)**. Fluorescence *in situ* Hybridization method using Peptide Nucleic Acid probes for rapid detection of *Lactobacillus* and *Gardnerella* spp. *BMC Microbiol* 13, 82.

[18] Rozen, S. and Skaletsky, H. **(2000)**. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132, 365.

[19] França, A., Freitas, A.I., Henriques, A.F. and Cerca, N. **(2012)**. Optimizing a qPCR Gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol. *PloS One* 7, e37480.

[20] Carvalhais, V., Delgado-Rastrollo, M., Melo, L.D. and Cerca, N. **(2013)**. Controlled RNA contamination and degradation and its impact on qPCR gene expression in *S. epidermidis* biofilms. *J Microbiol Methods* 95, 195-200.

[21] Gelber, S.E., Aguilar, J.L., Lewis, K.L. and Ratner, A.J. **(2008)**. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis. J Bacteriol* 190, 3896-903.

[22] Schwebke, J.R., Muzny, C.A. and Josey, W.E. **(2014)**. Role of *Gardnerella vaginalis* in the pathogenesis of Bacterial Vaginosis: a conceptual model. *J Infect Dis* 210, 338-43.

[23] Silva D, e.a. **(2013)**. Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in northern Portuguese women and its association with contraception choices. *Int J Gynecol Obstet* 124, 178-9.

[24] Piot, P., Van Dyck, E., Peeters, M., Hale, J., Totten, P. and Holmes, K. **(1984)**. Biotypes of *Gardnerella vaginalis*. *J Clin Microbiol* 20, 677-79.

[25] Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L.P. and Lochs, H. **(2005)**. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 106, 1013-23.

[26] Joo, H.-S. and Otto, M. **(2012)**. Molecular basis of *in vivo* biofilm formation by bacterial pathogens. *Chem Biol* 19, 1503-13.

[27] Srinivasan, S. and Fredricks, D.N. **(2009)**. The human vaginal bacterial biota and bacterial vaginosis. *Interdiscip Perspect Infect Dis* 2008.

[28] Petricevic, L., Kaufmann, U., Domig, K.J., Kraler, M., Marschalek, J., Kneifel, W. and Kiss, H. **(2014)**. Molecular detection of *Lactobacillus* species in the neovagina of male-to-female transsexual women. *Sci Rep* 4, 3746.

[29] Petricevic, L. et al. **(2014)**. Characterisation of the vaginal *Lactobacillus* microbiota associated with preterm delivery. *Sci Rep* 4, 5136.

[30] Swidsinski, A. et al. **(2010)**. *Gardnerella* biofilm involves females and males and is transmitted sexually. *Gynecol Obstet Invest* 70, 256-63.

[31] Swidsinski, A., Verstraelen, H., Loening-Baucke, V., Swidsinski, S., Mendling, W. and Halwani, Z. **(2013)**. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PloS One* 8, e53997.

[32] Cerca, N., Pier, G.B., Vilanova, M., Oliveira, R. and Azeredo, J. **(2005)**. Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. *Res Microbiol* 156, 506-14.

[33] Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., Lochs, H. and Verstraelen, H. **(2008)**. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 198, 97. e1-97. e6.

[34] Austin, M., Beigi, R., Meyn, L. and Hillier, S. **(2005)**. Microbiologic response to treatment of bacterial vaginosis with topical clindamycin or metronidazole. *J Clin Microbiol* 43, 4492-97.

[35] Eschenbach, D.A. **(2007)**. Bacterial vaginosis: resistance, recurrence, and/or reinfection? *Clin Infect Dis* 44, 220-21.

[36] Machado, A., Salgueiro, D., Harwich, M., Jefferson, K.K. and Cerca, N. **(2013)**. Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. *Anaerobe* 23, 1-4.

[37] Machado, A., Jefferson, K.K. and Cerca, N. **(2013)**. Interactions between *Lactobacillus crispatus* and Bacterial Vaginosis (BV)-Associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 14, 12004-12.

[38] Jakubovics, N.S., Yassin, S.A. and Rickard, A.H. **(2014)**. Community interactions of oral streptococci. *Adv Appl Microbiol* 87, 43-110.

3.6 Supplementary data

Supplementary Table S3.1 Characterization of vaginal samples

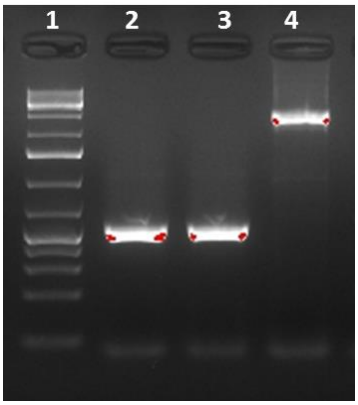
BV positive samples			BV negative samples		
Strain	**Nugent score**	**Women age**	**Strain**	**Nugent score**	**Women age**
UM034	7	66	UM016	0	45
UM035	7	43	UM060	1	24
UM067	9	54	UM061	3	38
UM121	7	23	UM085	1	24
UM137	9	22	UM094	2	30
UM224	9	33	UM131	1	28
UM241	8	20	UM246	4	19

Supplementary Table S3.2 Accession code of *G. vaginalis* strains

Strain	Accession number
non-BV associated	
G. vaginalis UM016	KP996686.1
G. vaginalis UM060	KP996673.1
G. vaginalis UM061	KP996674.1
G. vaginalis UM085	KP996679.1
G. vaginalis UM094	KP996680.1
G. vaginalis UM131	KP996676.1
G. vaginalis UM246	KP996677.1
BV associated	
G. vaginalis UM034	KP996684.1
G. vaginalis UM035	KP996685.1
G. vaginalis UM067	KP996675.1
G. vaginalis UM121	KP996681.1
G. vaginalis UM137	KP996682.1
G. vaginalis UM224	KP996678.1
G. vaginalis UM241	KP996683.1

Supplementary Table S3.3 Primer sequences used for amplifying the flanking regions of *vly*

Target	Primers sequence (5'to 3')	$T_{melting}$ (ºC)	Amplicon size (bp)	Reference
Reverse flanking region of *vly*	GGCGGAATTATGTGCGTTATTGG	55	3334	This study
Forward flanking region of *vly*	CATCTTCGCCAGCAACTTCC	55	3334	This study



Supplementary Figure S3.1 The absence of *vly* gene in *G. vaginalis* UM035 (2) and UM224 (3) strains was confirmed by PCR its flanking regions. *G. vaginalis* UM034 (4) was used as a positive control. 1Kb+ ladder was included (1). Subsequently the upper flanking regions were sequenced to confirm the identity of the genes. The sequencing results of the upper region are the following:

UM224:
TAATTCGCCTTGCAAGCGACTGATTTCTTCGTCGCTAAGCGCAATCTGGGATAGAACGCCCAGAT
GCTCAATTTCTTCGCGTGTGAATGTTGGCATAACCTCAACTATATGTGTGATGCGTGACCTTTGCT
ATAGCAAAAATTATGCAAAAAGGGCTTTATCTTGTAGTCAAATCTTTTGAATCTACAGAGTAAAGCC
CTTATTGTTTTTGCAAAATATTATTTGCAGAATATTTAAATATTTTAGATATCGCGATGCTTTTCAAC
AACGTGGCCAATTGCATACATGACTACGCCCCAAGCGAGAACCACCAAGCCTGATTGCCACCAT
GTGAAAATATATGCGTTTGGTGGAAGCTGTGCACCCGCATTAGGGGAGCCGCCCAAGAATTTCC
CCACCGCTGTGGCTGGCAAAAGCTGTATAAGTATTGAATTCCACTTCGCGAAATTGCTTGCAAAC
ATAATAATGCTAAGAACACTAGGCAAAATCACCACGGCTCCAATAACGCACATAAATTCCCGCCAA
AAAA

UM035:
TGACGTTTATTCGCCTTGCAAGCGACTGATTTCTTCGTCGCTAAGCGCAATCTGGGATAGAACGC
CCAGATGCTCAATTTCTTCGCGTGTGAATGTTGGCATAACCTCAACTATATGTGTGATGCGTGACC
TTTGCTATAGCAAAAATTATGCAAAAAGGGCTTTATCTTGTAGTCAAATCTTTTGAATCTACAGAGT
AAAGCCCTTATTGTTTTTGCAAAATATTATTTGCAGAATATTTAAATATTTTAGATATCGCGATGCTT
TTCAACAACGTGGCCAATTGCATACATGACTACGCCCCAAGCGAGAACCACCAAGCCTGATTGCC
ACCATGTGAAAATATATGCGTTTGGTGGAAGCTGTGCACCCGCATTAGGGGAGCCGCCCAAGAA
TTTCCCCACCGCTGTGGCTGGCAAAAGCTGTATAAGTATTGAATTCCACTTCGCGAAATTGCTTG
CAAACATAATAATGCTAAGAACACTAGGCAAAATCACCACGGCTCCAATAACGCACATAATTCCGC
CAAA

CHAPTER 4

Comparative analysis of the influence of host innate immune components in bacterial vaginosis and non-bacterial vaginosis Gardnerella vaginalis isolates

Summary

Mucosal surfaces of the female reproductive tract contain a variety of antimicrobials that provide the first line of defense against bacteria involved in the development of BV. Microbiological analysis of BV has shown *Gardnerella vaginalis* to be a keystone species in BV development. However, *G. vaginalis* colonization does not always lead to BV. Over the last decade, phenotypic and genotypic studies have demonstrated the existence of strain variants. Therefore, this study aimed to investigate the effect of major components of the vaginal immune response, specifically lysozyme, lactoferrin, and human β-defensin 2, on *G. vaginalis* strains isolated from healthy women and women with BV to highlight virulence differences. In order to do, 7 non-BV and 7 BV associated *G. vaginalis* strains were first genotypically and then phenotypically characterized. Subsequently, we determined the minimal inhibitory concentration of the innate immune components. Using a growth medium simulating genital tract secretions supplemented with the tested molecules at physiological concentrations, we then examined the bacterial fitness profile, its ability to adhere to HeLa cells and the biofilm-forming capacity. Our results revealed that the tested isolates could not be differentiated using the clade-genotyping approach despite key differences in initial adhesion were found in both groups. Importantly, we found that growth, initial adhesion and biofilm formation were strongly affected by lysozyme, but at similar levels in both groups. The response to the other components was also similar between both groups, suggesting that a better adaptation to the host immune components is not a key factor differentiating between isolates from women with BV and from healthy women.

4.1 Brief introduction

The mucosal lining of the female reproductive tract (FRT), which contains a variety of antimicrobials, acts as the primary barrier against bacteria involved in the development of BV [1]. As referred in chapter 3, the phenotypic and genetic content differences among non-BV and BV associated *G. vaginalis* strains underlie the capacity for this bacterial species to survive in the face of host defenses and that these differences determine the diverse pathological features, outcomes, and sequelae that are associated with this species [2-6].

Understanding the interactions between antimicrobial peptides (AMPs), residing at the forefront of host barrier defense, and *G. vaginalis* is of extreme importance to help unravel the pathogenesis and progression of BV. Importantly, lysozyme (LYS), lactoferrin (LF) and human β-defensin 2 (HBD2), which are categorized as AMPs, have been identified in the FRT [7, 8]. Since AMPs act as "natural antimicrobial agents", some efforts have been conducting in evaluating their efficacy in preventing or resolving BV [9]. Importantly, *in vitro* studies have shown that LYS, a glycoside hydrolase, was able to degrade biofilms produced by *G. vaginalis* [10, 11]. Similarly, a recent study led by Pino and colleagues showed that vaginal LF administration (100 mg and 200 mg vaginal pessaries) modified the vaginal microbiota composition in patients with BV, significantly decreasing the occurrence of BV associated bacteria, and increasing the occurrence of *Lactobacillus* species [9]. To date, a single study was carried out analysing a specific type of defensin, the retrocyclins (Θ-defensin), against *G. vaginalis*, showing that this class of defensins markedly decreases biofilm formation and the cytolytic activity of vaginolsyin, a toxin produced by *G. vaginalis* [12]. It is noteworthy that most of these studies attempt to analyse the antimicrobial capacity of innate immune molecules to be effective against BV associated bacteria, while, in the present study, our aim was to analyse the physiological adaptation of *G. vaginalis* to the vaginal niche, which contains such soluble molecules.

Remarkably, according to our *in vitro* model described in chapter 3, we observed that there are crucial differences in the virulence potential between non-BV and BV associated *G. vaginalis* strains. As the vaginal immune response is likely to influence the physiological adaptation of bacteria, herein, we hypothesized that those differences could be further highlighted in the presence of innate immune components, which could explain why only some strains are able to induce the development of BV. Thus, to better understand the virulence traits of *G. vaginalis*, we first genotyped 7 non-BV and 7 BV isolates and we then performed a phenotypic analysis to compare the effect of LYS, LF and HBD2 at physiological vaginal concentrations found in healthy subjects, on the two groups.

4.2 Materials and methods

4.2.1 Bacterial strains and culture conditions

G. vaginalis strains originally obtained from women diagnosed with BV (*n* = 7) or without BV (*n* = 7) were used herein. The strains were preserved and cultured as described in chapter 3. In brief, planktonic cells were grown in supplemented brain heart infusion (sBHI) for 24 or 48 hours at 37ºC with 10% CO_2 (Shel Lab, Cornelius, Oregon, USA).

4.2.2 *G. vaginalis* clade-specific PCR assays

Four *G. vaginalis* clades were detected by amplification of the genes, whose primer sequences are described in Table 4.1 [5].

Table 4.1 Primer sequences used for the detection of the clade-specific group of *G. vaginalis* strains

	Primer	Protein	Primers sequence (5'to 3')	Amplicon size (bp)
Clade 1	Gv1_fuc1_Fw	Putative a-L-fucosidase	CCAGTCATAAGTTTGCGTTTTACC	139
	Gv1_fuc1_Rv		TGGCACTGGCAAAGTTTACAAC	
	Gv1_galK_Fw	Galactokinase	TTCTAGATTATTCGCCGCCAAATC	108
	Gv1_galK_Rv		TTGCGATGTGTTGAAGGTAATGC	
Clade 2	Gv2_hyp_Fw	Hypotetical protein	GCAAAGCAGACTGAGCGTATTAG	124
	Gv2_hyp_Rv		GTAATAATCAGGCTCCTCATCGC	
	Gv2_cel_Fw	Cellulosome anchoring protein	GCTTGGGGTTCATATGGTGATGG	137
	Gv2_cel_Rv		TCTTTATCAGACACGCCCTTAGC	
Clade 3	Gv3_thi_Fw	Thioredoxin	TTCTGCTTCTTCTGCTATTTGCTG	142
	Gv3_thi_Rv		TTCGTTGACTTTTGGGCAACATG	
	Gv3_a-b_Fw	α/β Hydrolase fold protein	TGATTACGCTCACGCTCTCG	149
	Gv3_ a-b_Rv		CGGCAACAGCTTTAGGAAGAAG	
Clade 4	Gv4_cic_Fw	Chloride transporter	CCTACGCAAGCTCCAGACGAC	74
	Gv4_ cic_Rv		ACAAGTTGCACTCTTCGAGCTGG	
	Gv4_all_Fw	Allantoate amidohydrolase	CACGCTGGCACAACAATGATG	139
	Gv4_all_Rv		TTGGAACTACGCTGATTCTACCG	

Identification of clades by conventional PCR was performed using genomic DNA from both characterized non-BV and BV associated *G. vaginalis* isolates as described before [13]. In

brief, genomic DNA was extracted and the thermocycling program (Mini-MJ, Bio-Rad, Hercules, CA, USA) was performed using the DreamTaq PCR Master Mix 2x (Finnzymes, Espoo, Finland). The reaction mixture was subjected to an initial denaturation of 95 °C for 2 minutes, followed by 38 cycles of denaturation at 95ºC for 30 seconds, primer annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds [13]. The last cycle included a 7 minutes extension step. The PCR product was then kept hold at 4°C. PCR products were separated on 1.7% agarose gels (Bioron, Ludwigshafen, Germany) stained with RedSafe loading dye (Thermo Fisher Scientific, Lenexa, KS, USA).

4.2.3 Minimal inhibitory concentration

The susceptibility of *G. vaginalis* to innate immune molecules was evaluated by determining the minimal inhibitory concentration (MIC) of LYS (Sigma-Aldrich, St. Louis, MO, USA), LF (Sigma-Aldrich) and HBD2 (Innovagen, Lund, Sweden) by microdilution method in 96-well plates (Orange Scientific, Braine L'Alleud, Belgium) [14]. Briefly, innate immune components were initially prepared according to manufacturer's instructions. Then, serial dilutions of immune components were prepared using a medium simulating genital tract secretions (mGTS) [15]. Bacterial suspensions were prepared by dilution of a 24 hours-old culture, to a final concentration of 5.0×10^5 colony-forming units (CFU)/mL, estimated using optical density (OD) at 620 nm (Bio-Tek Synergy HT, VT, USA). Afterward, 100 µL of *G. vaginalis* suspensions were dispensed into each well of 96 well-plates containing 100 µL of each dilution of innate immune molecules. Plates were then incubated for 48 hours at 37°C in a 10% CO_2 atmosphere. After incubation, the MICs were evaluated by reading the $OD_{620\ nm}$. All assays were repeated 3 times with technical replicates.

4.2.4 Adhesion assays

Initial adhesion assays to human cervical HeLa cells (ATCC CCL-2) were firstly optimized after 48 hours of planktonic growth of *G. vaginalis,* using two different models: *(i)* bacterial adhesion without any pre-conditioning of bacteria to innate molecules; and *(ii)* bacterial adhesion after pre-conditioning of bacteria to mGTS containing 166.7 µg/mL of LYS [16], 72.7 µg/mL of LF [16], or 10 ng/mL of HBD2 [17] corresponding to the physiological concentrations found in vaginal secretions of healthy subjects, for 3 hours at 37 °C, 10% CO_2. Briefly, for both adhesion models, blind bacterial suspensions, with a final concentration of 1×10^8 CFU/mL, diluted in mGTS supplemented with the innate molecules, were added to a monolayer of HeLa cells for 30 minutes at 37°C at 5% CO_2. Importantly, for each *G. vaginalis* strain, a bacterial suspension without any compound was used as a control. After washing the non-adherent bacteria, cells

were fixed with methanol and then stained with 4'-6-Diamidino-2-phenylindole (DAPI, 2.5 µg/mL) [18]. Microscopic visualization was performed using an Olympus BX51 epifluorescence microscope equipped with a CCD camera (DP72; Olympus, Lisboa, Portugal) and a filter capable of detecting the DAPI staining (BP 365-370, FT 400, LP 421). Twenty fields were randomly counted in each sample. Thereafter, it was counted the number of bacteria adhered to epithelial cells and also eukaryotic cells per image, using the *ImageJ Software*. Results were expressed as bacteria per HeLa cells. Adherence assays were repeated three times on separate days, with two technical replicates assessed each time.

4.2.5 Biofilm formation and quantification

For biofilm formation quantification, the cell concentration of 24 hours-old cultures was adjusted by OD_{620nm} to a final concentration of approximately 10^6 CFU/mL, in mGTS supplemented with LYS, LF, and HBD2 at the concentrations described above. A control was included using just mGTS. After homogenization, 200 µL of *G. vaginalis* suspensions, were dispensed into each well of 96-well flat-bottom tissue culture plates. The tissue culture plates were then incubated at 37 °C in 10% CO_2 for 24 hours. Twenty-four hours biofilms were then washed once with 1 × phosphate buffered saline (PBS). Finally, biofilm biomass was quantified according to the crystal violet (CV) staining method described by Peeters *et al*. [19]. In brief, after fixation step, biofilms were stained with 0.5% (wt/v) CV (Acros Organics, NJ, USA). Next, biofilms were washed twice with PBS and bound CV was released with 33% (v/v) acetic acid (Thermo Fisher Scientific). Finally, OD_{590nm} was measured using the 96-well microplate reader. All assays were repeated at least three-times with eight technical replicates.

4.2.6 Study of growth kinetics

Planktonic cells were grown as mentioned in section 4.2.1. Afterward, bacteria were diluted to an $OD_{620nm} \sim 0.1$, which corresponds approximately 5.5×10^5 CFU/mL, in mGTS supplemented with the innate immune molecules. A control was included using just mGTS. Thereafter each suspension was added to 96-well plates and incubated at 37 °C, 10% CO_2. OD_{620nm} was measured after 2, 4, 6, 8, 10, 24, 28, 32, 48, 52, 55 and 72 hours of incubation. Three independent experiments were performed for each tested condition.

4.2.7 Statistical analysis

The data were analysed using the Manny Whitney test, Kruskal-Wallis H test, or non-parametric Wilcoxon matched-pairs rank test since the data that did not follow a normal

distribution according to Kolmogorov-Smirvon's test, with the statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL). The data were represented as the mean ± standard deviation (SD) or as the mean ± standard error of mean (SEM) at least 3 independent experiments. P-values of less than 0.05 were considered significant.

4.3 Results

4.3.1 Subtyping of *G. vaginalis*

The clade-specific genes that have previously proposed by Balashov and colleagues [5] were used to subtype *G. vaginalis* strains isolated from women with or without BV. Based on this genotyping system, we observed that we cannot associate a specific clade to a BV status, as presented in Table 4.2, since both groups were similarly distributed between clades 1, 2 and 4. Furthermore, we confirmed that clade 4 strains lack the sialidase coding gene (*sld*), similarly to what has been described in other studies [20-22].

Table 4.2 *G. vaginalis* genotyping based on clade classification system

Bacteria	**Clade classification**	**Presence of virulence genes [a]** ***sld***
non-BV associated		
G. vaginalis UM085	1	+
G. vaginalis UM061	1	+
G. vaginalis UM131	2	+
G. vaginalis UM016	1	+
G. vaginalis UM094	4	-
G. vaginalis UM060	1	+
G. vaginalis UM246	1	+
BV associated		
G. vaginalis UM067	2	+
G. vaginalis UM121	1	+
G. vaginalis UM035	2	+
G. vaginalis UM137	1	+
G. vaginalis UM224	4	-
G. vaginalis UM241	1	+
G. vaginalis UM034	4	-

[a] The results regarding the presence or absence of sialidase (*sld*) gene on the tested *G. vaginalis* strains were presented in chapter 3.

4.3.2 Susceptibility to antimicrobial peptides

In vitro antimicrobial susceptibility of *G. vaginalis* to LYS, LF, and HBD2 was evaluated by determining the MIC. Similar to what was described in chapter 3, regarding the response to standard antibiotics used to treat BV, herein, significant differences were also not found in antimicrobial peptides susceptibility profiles between non-BV and BV isolates ($p > 0.05$; Table 4.3). Interestingly, all *G. vaginalis* strains tested showed a high tolerance to LYS, LF and HBD2, at normal physiological concentrations.

Table 4.3 Minimum inhibitory concentration (MIC) of lysozyme (LYS), lactoferrin (LF), and human β-defensin 2 (HBD2) for planktonic cells of *G. vaginalis* isolates. Statistical analysis: no significant differences were found in antimicrobial peptides susceptibility profiles between non-BV and BV associated isolates by the non-parametric Wilcoxon matched-pairs rank test

	MIC range		
Bacteria	**LYS (µg/mL)**	**LF (µg/mL)**	**HBD2 (ng/mL)**
non-BV associated			
G. vaginalis UM085	>1280	> 256	> 128
G. vaginalis UM061	>1280	>256	> 128
G. vaginalis UM131	>1280	> 256	> 128
G. vaginalis UM016	1280	>256	> 128
G. vaginalis UM094	>1280	>256	> 128
G. vaginalis UM060	>1280	>256	> 128
G. vaginalis UM246	[640-1280]	>256	> 128
BV associated			
G. vaginalis UM067	[640-1280]	>256	> 128
G. vaginalis UM121	1280	>256	> 128
G. vaginalis UM035	>1280	>256	> 128
G. vaginalis UM137	1280	>256	> 128
G. vaginalis UM224	>1280	>256	> 128
G. vaginalis UM241	>1280	> 256	> 128
G. vaginalis UM034	[640-1280]	>256	> 128

4.3.3 Initial adhesion to human cervical HeLa cells

Some factors have been shown to interfere with the capacity of *G. vaginalis* to adhere to vaginal cells [23]. However, to date, no information exists regarding the effect of LYS, LF, and HBD2 on initial adhesion of *G. vaginalis* to epithelial cells. Two different *in vitro* models were tested: in half of the experiments, *G. vaginalis* was pre-conditioned with each of the tested components, before the adhesion assays, and in the other half, the influence of the tested components occurred only during adhesion. As can be seen in Figure 4.1, no overall significant differences ($p > 0.05$) were found between the bacterial adhesion with or without a pre-conditioning to innate molecule. As such, in the subsequent experiments, the bacterial adhesion was performed without the pre-conditioning of the bacteria with innate molecules.

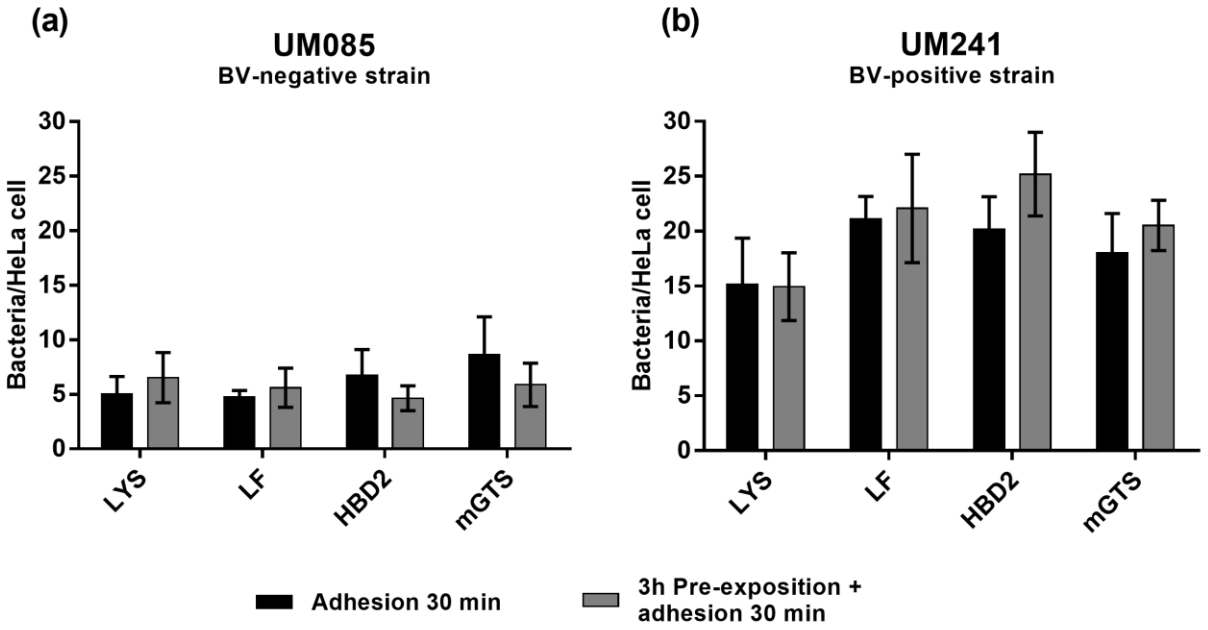


Figure 4.1 Comparison of the bacterial adhesion ability to HeLa cells in presence of LYS, LF or HBD2 using 2 different models. Bacteria per HeLa cell were quantified after 30 minutes of adhesion. In the first model, bacterial suspensions were directly exposure to HeLa cells, while in the second model, bacterial suspensions were firstly subject to a pre-conditioning to the innate molecules for 3 hours. For both models, each bacterial strain diluted in mGTS without any innate immune molecule was used as a control. **(a)** *G. vaginalis* UM085 was isolated from a healthy woman. **(b)** *G. vaginalis* UM241 was isolated from a woman with BV. No significant differences in the adherence of *G. vaginalis* were found in presence of each innate compound using the two tested adhesion models (Manny Whitney test, $p > 0.05$).

Afterward, we selected 6 representative strains (3 of each group) from our collection based on the results of chapter 3. Interestingly, we verified that LYS was able to slightly decrease the

capacity of the majority of strains to adhere to HeLa cells (Figure 4.2). Similarly, LF also led to a slight decrease in the bacterial adhesion capacity of 4 strains. However, these differences did not achieve statistical significance. On the contrary, HBD2 did not demonstrate any effect on bacterial adhesion. It is noteworthy that independent of the presence or absence of innate immune compounds, BV associated strains showed a greater ability to adhere to epithelial cells than non-BV strains, corroborating our previous results (chapter 3).

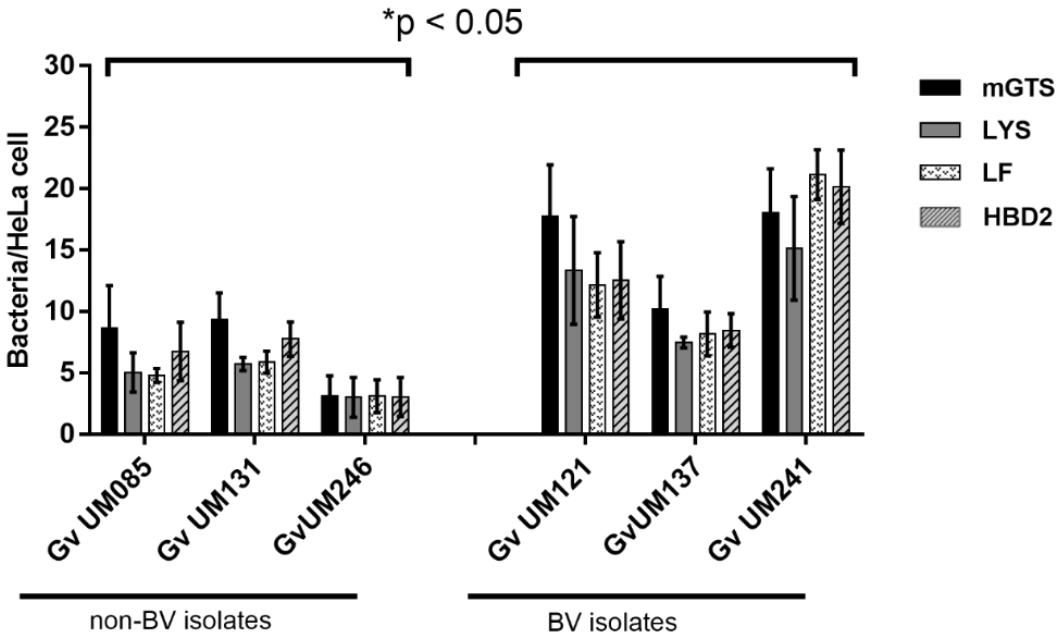


Figure 4.2 Initial adhesion of non-BV and BV isolates to HeLa cells in presence of physiological vaginal concentrations of LYS, LF or HBD2. Adhesion was microscopically quantified and expressed as the average ± SD number of bacteria per epithelial cell. No significant differences in the adherence of *G. vaginalis* were found in the presence of each innate compound when compared with the bacterial adhesion only in mGTS (Manny Whitney test, $p > 0.05$). *Denotes significance differences between the 2 groups of *G. vaginalis* strains at same conditions (Kruskal-Wallis H test, $p < 0.05$).

4.3.4 Influence of innate molecules on biofilm formation

Following initial adherence to vaginal cells, the invading *G. vaginalis* multiply and may produce a biofilm community as a means of future survival [3]. Thus, in this study, we analysed the influence of LYS, LF and HBD2 on biofilm formation. Similar to what was shown in chapter 3, our results presented that there were no significant differences in biofilm biomass between the 2 *G. vaginalis* groups ($p > 0.5$) as presented in Figure 4.3. Furthermore, despite significant differences between LYS and mGTS have been only found with *G. vaginalis* UM085, we also noted that LYS was able to decrease the biofilm-forming capacity of all strains.

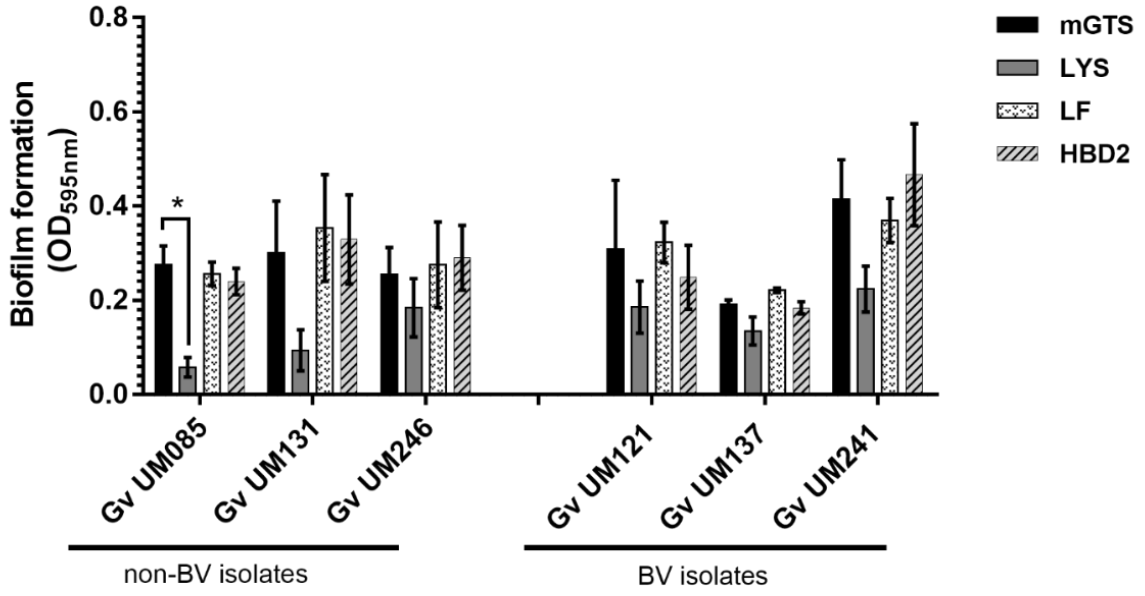


Figure 4.3 Biofilm formation of non-BV and BV isolates in presence of physiological vaginal concentrations of LYS, LF or HBD2. Biofilm biomass was quantified by CV method and expressed as the average ± SEM. No significant differences between non-BV and BV isolates were found to biofilm biomass (Kruskal-Wallis H test, $p > 0.05$). *Denotes significance differences between the biofilm formation in the presence of LYS and mGTS, which corresponds to a control (Manny Whitney test, $p < 0.05$).

4.3.5 Effects of LYS, LF and HBD2 on planktonic growth of *G. vaginalis*

Given the pivotal role of innate immune molecules in host defense [7, 24], it was hypothesized that effects observed on biofilm formation particularly in presence of LYS, could be attributable to an impaired cell growth. To assess this, planktonic cells were grown under the exact same conditions (Figure 4.4). We observed that HBD2 was not detrimental to planktonic growth at physiological vaginal concentrations. Conversely, LF was able to somewhat interfered with bacterial growth for some strains, but it was not significantly different from to what observed just with mGTS (control). Furthermore, LYS effect is in line with the previous results, since it was able to attenuate the bacterial growth of both groups of *G. vaginalis.*

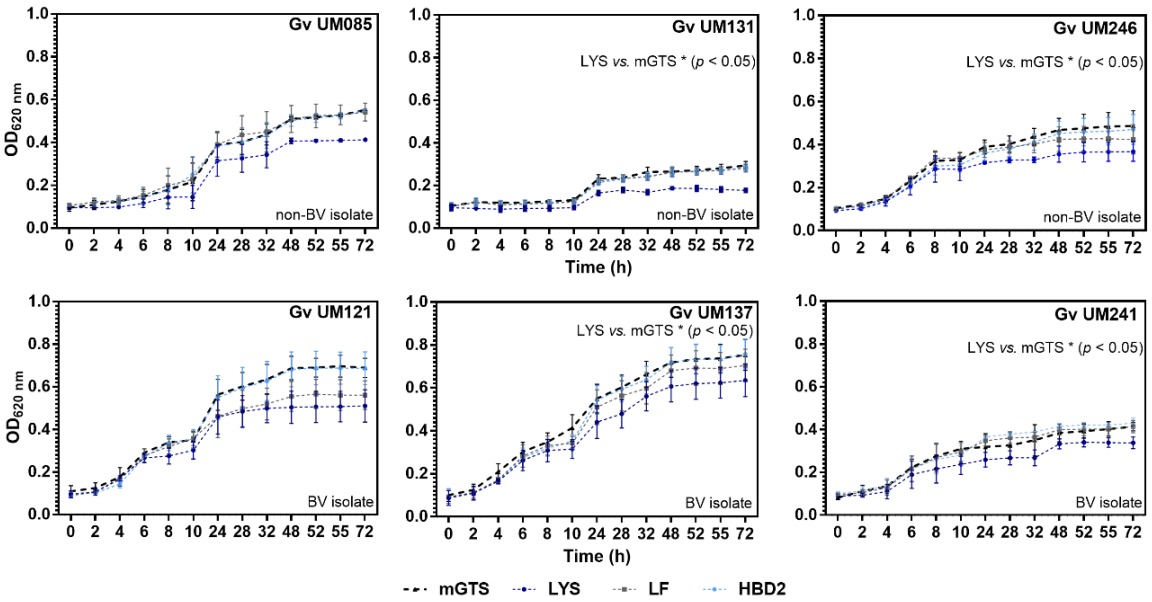


Figure 4.4 Bacterial fitness profile of non-BV and BV isolates in the presence of physiological vaginal concentrations of LYS, LF or HBD2. *Denotes significance differences between the bacterial growth in presence of LYS and mGTS, which corresponds to a control (Manny Whitney test, $p < 0.05$).

4.4 Discussion

The interplay between non-BV or BV *G. vaginalis* strains and the mucosal immune soluble factors is a challenging research topic and can lead to new insights into bacterial virulence [25]. The innate components of the immune system provide immediate protection against pathogens by recognizing the presence of microorganisms, thus preventing from tissue invasion and/or eliciting a host response to limit microbial proliferation [8, 26-28]. In the FRT, most published work focuses on the genital mucosal immune response to BV and how that response alters the risk of reproductive health complications such as human immunodeficiency virus acquisition [29] or preterm birth [30]. However, there is less knowledge about the influence of the host innate immune molecules on *G. vaginalis* strains and whether non-BV and BV isolates could differ in the manner in which they respond to these molecules. We hypothesized that such differences could play a key role in BV development.

To shed further light on this question, in this study we characterized the virulence properties of non-BV and BV associated *G. vaginalis,* using a functional *in vitro* model, which represents a significant improvement over our previous study (see chapter 3), since we used a chemically-defined medium simulating genital tract secretions supplemented with LYS, LF, or HBD2 at physiological concentrations. Given the fact that *in vitro* conditions can strongly influence bacterial phenotypes [31], and that salt concentrations and other parameters influence AMP function [32], we hypothesized that under these conditions, the previous virulence differences

found between both *G. vaginalis* groups might be enhanced, and as such, this would provide evidence that a better adaptation to host immune components could explain why only some variants of *G. vaginalis* might contribute for BV development. However, this was not the case and the effect of the innate immune molecules was similar between non-BV and BV associated *G. vaginalis* isolates. Subsequently, we performed a more in-depth genotypic analysis when compared with chapter 3, but again no differences between the two groups were found.

Interestingly, our data clearly showed that physiological concentrations of LYS were sufficient to attenuate the planktonic growth of *G. vaginalis,* its adhesion to HeLa cells and the biofilm formation capacity. An earlier study carried out by Thellin and colleagues showed that LYS added to 24 hours pre-formed biofilms significantly reduced the biomass of 8 out the 9 biofilms produced by different strains of *G. vaginalis* [11]. Recent research supports these findings, showing that LYS (0.5 mg/mL) when added at the beginning of the biofilm culture, was able to prevent biofilm formation and also inhibited biofilm viability [10]. This antimicrobial activity was linked to the cleavage of the peptidoglycan layer of the bacterial cell wall, causing bacterial lysis, since bacteria cannot resist the osmotic pressure in the medium [33, 34].

Interestingly, Ellison and colleagues showed that LYS displays synergism with LF, an iron-binding protein, which promotes innate immune protection in the FRT*,* by binding and regulating the iron needed for bacterial proliferation [35]. Curiously, LF is elevated in the vaginal discharge of women with BV [36]. Iron is an essential micronutrient for *G. vaginalis*, since its proliferation is dependent on exogenous iron [2, 37-39]. Based on our results, we concluded that the normal physiological vaginal concentration of LF found in healthy subjects seems to cause a slightly inhibitory effect on bacterial initial adhesion and planktonic growth, whereas no such effect was observed in biofilm formation. It is well known that *G. vaginalis* has developed several high-affinity mechanisms to overcome this iron-withholding capacity of the host and obtain this essential nutrient, as the production of siderophores [39] and the expression of high-affinity iron transporters [2].

Similarly, an upregulation of HBD2 was observed after infection of a three-dimensional vaginal epithelial cell culture with anaerobic bacteria commonly found in BV [40]. HBD2 is produced by epithelial cells and has been reported to be predominantly effective against Gram-negative bacteria such as *Pseudomonas aeruginosa* [41, 42] and *Escherichia coli* [43], but antimicrobial activity against Gram-positive bacteria found in the oral cavity has been also reported [44]. Our data pointed out that the antimicrobial effect of HBD2 was ineffective for both groups of *G. vaginalis* strains at physiological concentrations found in the vaginal environment.

This work has led us to conclude that the effect of the LYS, LF and HBD2 was similar between non-BV and BV associated *G. vaginalis* strains, suggesting that a better adaptation to the host

innate immune molecules is not a key factor differentiating between strains isolated from women with BV and from healthy women. Therefore, there remains a great deal of work to be done in clarifying the possible differences in the virulence factors of non-BV and BV associated *G. vaginalis* and its interplay with the host.

Overall, the findings of the present study support the data described in chapter 3, in which the most relevant phenotypic difference between the 2 groups of isolates was the bacterial adherence capability. Furthermore, given that the genotypic findings from chapter 3 were only based on analysis of presence/absence of 2 virulence genes of *G. vaginalis*, here a more detailed genotypic characterization of non-BV and BV associated strains was carried out. Genotyping was performed according to a recent clade system based on the presence of specific genes [5]. We found that clade 1 was the most frequently detected (57.1%), followed by clade 2 (21.4%) and 4 (21.4%). Clade 3 was not found in our collection of isolates (0%). Similar to what was described before [22], herein, the gene for sialidase, a virulence factor of *G. vaginalis*, was also detected in all isolates of clade 1 and clade 2, but not in clade 4 isolates. This enzyme is known to facilitate the destruction of the protective mucus layer on the vaginal epithelium [45]. However, it is not clear whether clade 4 strains produce other mucinases whose activity may affect clinical status [22]. As referred in chapter 1, a tricky issue of clade-system classification is the huge discrepancy between the association of a specific clade to healthy, intermediate or BV associated microflora [5, 22, 46]. In fact, these disagreements might have been due to the small number of clinical samples analysed in these studies, or, to the fact that ethnicity and geographical location of subjects in the different studies [22]. Further work on the analysis of the genotypic differences between the 2 groups of strains might be elucidated with a combination of omics and deep sequencing methods that could examine *G. vaginalis* in the context of the entire microbiome.

4.5 References

[1] Valenti, P., Rosa, L., Capobianco, D., Lepanto, M.S., Schiavi, E., Cutone, A., Paesano, R. and Mastromarino, P. **(2018)**. Role of lactobacilli and lactoferrin in the mucosal cervicovaginal Defense. *Front Immunol* 9, 376.

[2] Yeoman, C.J. et al. **(2010)**. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS One* 5, e12411.

[3] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

[4] Ahmed, A. et al. **(2012)**. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol* 194, 3922-37.

[5] Balashov, S.V., Mordechai, E., Adelson, M.E. and Gygax, S.E. **(2014)**. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* 63, 162-75.

[6] Castro, J., Martins, A.P., Rodrigues, M.E. and Cerca, N. **(2018)**. *Lactobacillus crispatus* represses vaginolysin expression by BV associated *Gardnerella vaginalis* and reduces cell cytotoxicity. *Anaerobe* 50, 60-3.

[7] Yarbrough, V.L., Winkle, S. and Herbst-Kralovetz, M.M. **(2015)**. Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. *Hum Reprod Update* 21, 353-77.

[8] Wira, C.R. and Fahey, J.V. **(2004)**. The innate immune system: gatekeeper to the female reproductive tract. *Immunology* 111, 13-5.

[9] Pino, A., Giunta, G., Randazzo, C.L. and Caruso, S. **(2017)**. Bacterial biota of women with bacterial vaginosis treated with lactoferrin: an open prospective randomized trial. *Microb Ecol Health Dis* 28, 1357417.

[10] Gottschick C, S.S., Kunze B, Sztajer H, Masur C, Abels C, Wagner-Döbler I. **(2016)**. Screening of compounds against *Gardnerella vaginalis* biofilms. *Plos One* 11, e0154086.

[11] Thellin, O., Zorzi, W., Zorzi, D., Delvenne, P., Heinen, E., ElMoualij, B. and Quatresooz, P. **(2016)**. Lysozyme as a cotreatment during antibiotics use against vaginal infections: an *in vitro* study on *Gardnerella vaginalis* biofilm models. *Int Microbiol* 19, 101-07.

[12] Hooven, T.A., Randis, T.M., Hymes, S.R., Rampersaud, R. and Ratner, A.J. **(2012)**. Retrocyclin inhibits *Gardnerella vaginalis* biofilm formation and toxin activity. *J Antimicrob Chemother* 67, 2870-2.

[13] Henriques, A., Cereija, T., Machado, A. and Cerca, N. **(2012)**. *In silico* vs *in vitro* analysis of primer specificity for the detection of *Gardnerella vaginalis*, *Atopobium vaginae* and *Lactobacillus* spp. *BMC Res Notes* 5, 637.

[14] Reller, L.B., Weinstein, M., Jorgensen, J.H. and Ferraro, M.J. **(2009)**. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis* 49, 1749-55.

[15] Stingley, R.L., Liu, H., Mullis, L.B., Elkins, C.A. and Hart, M.E. **(2014)**. *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) production and *Lactobacillus* species growth in a defined medium simulating vaginal secretions. *J Microbiol Methods* 106, 57-66.

[16] Bard, E., Laibe, S., Bettinger, D., Riethmuller, D., Biichle, S., Seilles, E. and Meillet, D. **(2003)**. New sensitive method for the measurement of lysozyme and lactoferrin for the assessment of innate mucosal immunity. part I: time-resolved immunofluorometric assay in serum and mucosal secretions. *Clin Chem Lab Med* 41, 127-33.

[17] Fan, S.R., Liu, X.P. and Liao, Q.P. **(2008)**. Human defensins and cytokines in vaginal lavage fluid of women with bacterial vaginosis. *Int J Gynaecol Obstet* 103, 50-4.

[18] Castro, J., Henriques, A., Machado, A., Henriques, M., Jefferson, K.K. and Cerca, N. **(2013)**. Reciprocal Interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on initial adherence to epithelial cells. *Int J Med Sci* 10, 1193.

[19] Peeters, E., Nelis, H.J. and Coenye, T. **(2008)**. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Methods* 72, 157-65.

[20] Schellenberg, J.J., Paramel Jayaprakash, T., Withana Gamage, N., Patterson, M.H., Vaneechoutte, M. and Hill, J.E. **(2016)**. *Gardnerella vaginalis* subgroups defined by *cpn60*

sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PLoS One* 11, e0146510.

[21] Jayaprakash, T.P., Schellenberg, J.J. and Hill, J.E. **(2012)**. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PloS One* 7, e43009.

[22] Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A. and Pleckaityte, M. **(2017)**. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infect Dis* 17, 394.

[23] Peeters, M. and Piot, P. **(1985)**. Adhesion of *Gardnerella vaginalis* to vaginal epithelial cells: variables affecting adhesion and inhibition by metronidazole. *Genitourin Med* 61, 391-5.

[24] Smith, S.B. and Ravel, J. **(2017)**. The vaginal microbiota, host defence and reproductive physiology. *J Physiol* 595, 451-63.

[25] Murphy, K. and Mitchell, C.M. **(2016)**. The Interplay of host immunity, environment and the risk of bacterial vaginosis and associated reproductive health outcomes. *J Infect Dis* 214, S29-S35.

[26] Amjadi, F., Salehi, E., Mehdizadeh, M. and Aflatoonian, R. **(2014)**. Role of the innate immunity in female reproductive tract. *Adv Biomed Res* 3, 1.

[27] Quayle, A.J. **(2002)**. The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. *J Reprod Immunol* 57, 61-79.

[28] Wira, C.R., Patel, M.V., Ghosh, M., Mukura, L. and Fahey, J.V. **(2011)**. Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am J Reprod Immunol* 65, 196-11.

[29] Pellett Madan, R. et al. **(2015)**. Innate Antibacterial Activity in Female Genital Tract Secretions Is Associated with Increased Risk of HIV Acquisition. *AIDS Res Hum Retroviruses* 31, 1153-9.

[30] Trend, S. et al. **(2016)**. Levels of innate immune factors in preterm and term mothers' breast milk during the 1st month postpartum. *Br J Nutr* 115, 1178-93.

[31] Tan, C., Smith, R.P., Tsai, M.C., Schwartz, R. and You, L. **(2014)**. Phenotypic signatures arising from unbalanced bacterial growth. *PLoS Comput Biol* 10, e1003751.

[32] Donnarumma, G., Paoletti, I., Fusco, A., Perfetto, B., Buommino, E., de Gregorio, V. and Baroni, A. **(2016).** beta-Defensins: Work in Progress. *Adv Exp Med Biol* 901, 59-76.

[33] Davis, K.M. and Weiser, J.N. **(2011)**. Modifications to the peptidoglycan backbone help bacteria to establish infection. *Infect Immun* 79, 562-70.

[34] Nash, J.A., Ballard, T.N., Weaver, T.E. and Akinbi, H.T. **(2006)**. The peptidoglycan-degrading property of lysozyme is not required for bactericidal activity *in vivo*. *J Immunol* 177, 519-26.

[35] Ellison, R.T. and Giehl, T.J. **(1991)**. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest* 88, 1080-91.

[36] Rein, M.F., Shih, L.M., Miller, J.R. and Guerrant, R.L. **(1996)**. Use of a lactoferrin assay in the differential diagnosis of female genital tract infections and implications for the pathophysiology of bacterial vaginosis. *Sex Transm Dis* 23, 517-21.

[37] Jarosik, G.P. and Land, C.B. **(2000)**. Identification of a human lactoferrin-binding protein in *Gardnerella vaginalis. Infect Immun* 68, 3443-47.

[38] Jarosik, G.P. and Land, C.B. **(2001)**. Binding of heme by *Gardnerella vaginalis. J Basic Microbiol* 41, 37-43.

[39] Jarosik, G.P., Land, C.B., Duhon, P., Chandler, R., Jr. and Mercer, T. **(1998)**. Acquisition of iron by *Gardnerella vaginalis*. *Infect Immun* 66, 5041-7.

[40] Doerflinger, S.Y., Throop, A.L. and Herbst-Kralovetz, M.M. **(2014)**. Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. *J Infect Dis* 209, 1989-99.

[41] Wu, M., McClellan, S.A., Barrett, R.P. and Hazlett, L.D. **(2009)**. Beta-defensin-2 promotes resistance against infection with *P. aeruginosa. J Immunol* 182, 1609-16.

[42] Routsias, J.G., Karagounis, P., Parvulesku, G., Legakis, N.J. and Tsakris, A. **(2010)**. *In vitro* bactericidal activity of human beta-defensin 2 against nosocomial strains. *Peptides* 31, 1654-60.

[43] Baricelli, J., Rocafull, M.A., Vazquez, D., Bastidas, B., Baez-Ramirez, E. and Thomas, L.E. **(2015)**. Beta-defensin-2 in breast milk displays a broad antimicrobial activity against pathogenic bacteria. *J Pediatr* (Rio J) 91, 36-43.

[44] Joly, S., Maze, C., McCray, P.B. and Guthmiller, J.M. **(2004)**. Human β-Defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. *J Clin Microbiol* 42, 1024-29.

[45] Hardy, L., Jespers, V., Van den Bulck, M., Buyze, J., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M. and Crucitti, T. **(2017)**. The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PLoS One* 12, e0172522.

[46] Vodstrcil, L.A. et al. **(2017)**. The influence of sexual activity on the vaginal microbiota and *Gardnerella vaginalis* clade diversity in young women. *PLoS One* 12, e0171856.

CHAPTER 5

Comparative transcriptomic analysis of Gardnerella vaginalis biofilms versus planktonic cultures using RNA-seq

Summary

Recent data indicates that *Gardnerella vaginalis* biofilms are more tolerant to antibiotics and are able to incorporate other BV associated species, yielding a multi-species biofilm. However, despite its apparent role in BV, little is known regarding the molecular determinants involved in biofilm formation by *G. vaginalis*. To gain insight into the role of *G. vaginalis* biofilms in the pathogenesis of BV, we carried out comparative transcriptomic analysis between planktonic and biofilm phenotypes, using RNA-sequencing. Significant differences were found in the expression levels of 815 genes. A detailed analysis of the results obtained was performed based on direct and functional gene interactions. Similar to other bacterial species, expression of genes involved in antimicrobial resistance were elevated in biofilm cells. In addition, our data indicate that *G. vaginalis* biofilms assume a characteristic response to stress and starvation conditions. The abundance of transcripts encoding proteins involved in glucose and carbon metabolism was reduced in biofilms. Surprisingly, transcript levels of vaginolysin were reduced in biofilms relative to planktonic cultures. Overall, our data revealed that gene-regulated processes in *G. vaginalis* biofilms resulted in a protected form of bacterial growth, characterized by low metabolic activity. This phenotype may contribute towards the chronic and recurrent nature of BV. This suggests that *G. vaginalis* is capable of drastically adjusting its phenotype through an extensive change of gene expression.

The work presented in this chapter was published in *NPJ Biofilms and Microbiomes (2017)* 3:3.

5.1 Brief introduction

Biofilm formation represents a protected mode of growth that allows cells to survive in the acidic vaginal environment [1]. *G. vaginalis* can also adopt a planktonic phenotype that differs greatly from biofilm lifestyle [2]. It is postulated that a biofilm provides an ecological advantage over planktonic bacteria [3]. Importantly, biofilm infections are particularly problematic because sessile bacteria are generally much more tolerant to antibiotics than planktonic cells [4]. Evidence suggests that biofilm formation contributes significantly to BV treatment failure and high recurrence rates [4,5]. Targeting virulence factors represents a new paradigm in the development of new and effective treatments to prevent and treat biofilm-associated infections [6]. Therefore, a better understanding of BV associated *G. vaginalis* biofilm physiology and virulence is needed to understand the high persistence and resistance of biofilm cells.

The purpose of this study was, therefore, to identify the major transcriptomic features of BV associated *G. vaginalis* biofilms, as compared to their planktonic counterparts, using high-throughput RNA-sequencing (RNA-seq). Transcriptomic comparisons between biofilm and planktonic cultures that have been carried out for *Staphylococcus aureus* [7], *Staphylococcus epidermidis* [8], *Streptococcus mutans* [9] and *Streptococcus pneumoniae* [10], indicate that gene-regulated processes in the biofilm led to a protective mode of growth by developing an effective cellular response to stress and decreasing metabolic activity.

Herein, we sequenced the transcriptome of BV associated *G. vaginalis* biofilms and planktonic cultures and used a data analysis approach based on direct and functional gene interactions, namely gene set enrichment and cluster analysis.

5.2 Materials and methods

5.2.1 Bacterial strains

G. vaginalis strain AMD, isolated from a woman diagnosed with BV based on Amsel criteria at VCU Women's Health Clinic [11], was used for RNA-seq analysis. *G. vaginalis* strains UM121, UM137 and UM241, also isolated from women with BV based on Amsel and Nugent criteria (see chapter 3), were used for subsequent analysis.

5.2.2 Planktonic growth

Planktonic cells were grown in supplemented brain heart infusion (sBHI) for 24 hours at 37ºC with 10% CO_2 (Shel Lab, Cornelius, Oregon, USA), as previously described (see chapter 3). At this time, planktonic cells were still in the exponential growth phase. Thereafter, 18 mL of

planktonic cells were harvested by centrifugation (20 minutes, 7197 *g*) and suspended in 1 mL of RNA protect [diluted 2:1 in phosphate buffered saline (PBS); QIAGEN, Germany].

5.2.3 Biofilm formation

For biofilm formation, the cell concentration of 24 hours old cultures was assessed by optical density (OD) at 600 nm (Model Sunrise, Tecan, Switzerland) and was further diluted in order to obtain a final concentration of approx. 10^6 colony-forming units (CFU)/mL. After homogenization, 200 µL of *G. vaginalis* suspensions were dispensed into each well of three 96-well flat-bottom tissue culture plates (Orange Scientific, Braine L'Alleud, Belgium). The tissue culture plates were then incubated at 37ºC in 10% CO_2. After 24 hours, the culture medium covering the biofilms was removed, replaced by fresh sBHI and allowed to grow, under the same conditions, for an additional 24 hours. This time was required for this strain to develop a notable biofilm. Forty-eight hours biofilms were then washed once with 1× PBS, scraped from the bottom of 96-well plates in sBHI and pooled together. Finally, biofilm cells were harvested by centrifugation (20 minutes, 7197 *g*) and suspended in 1 mL of RNA protect (as described above).

5.2.4 RNA extraction

Total RNA was extracted using a combination of mechanical lysis (3.0 mm zirconium beads, Sigma-Aldrich Inc., St. Louis, MO, USA) and the columns of the RNeasy Mini kit (QIAGEN), as optimized before [12]. To remove genomic DNA, TURBO DNA-free™ kit (Ambion, Austin, TX, USA) was used as indicated by the manufacturer followed by acid-phenol:chloroform:isoamyl alcohol (125:24:1) treatment. RNA integrity was determined using an Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA, USA), and samples with RNA Quality Indicator (RQI) above eight were selected for complementary DNA (cDNA) library preparation.

5.2.5 cDNA library preparation and sequencing

cDNA libraries were constructed using the kit ScriptSeq™ Complete Kit - low input (Illumina, San Diego, WI, USA), which already includes the kit for ribosomal RNA (rRNA) depletion: Ribo-Zero™ Kit (Bacteria) – Low Input (Illumina, Madison, WI, USA). The construction of the libraries was rigorously validated by quantitative PCR and Hi-Sensitivity D1K TapeStation (Agilent 2200 TapeStation). Libraries were then multiplexed and sequencing data generated from paired-end reads (2×150 bp) using a MiSeq® system (Illumina).

5.2.6 RNA-sequencing data analysis

After sequencing, adapters were trimmed by MiSeq® internal software during the base calling. Quality, ambiguity and length trimming, as well as mapping to the reference genome, and normalization of gene expression were performed using CLC Genomics Workbench version 8 (MA, USA). Quality, ambiguity and length trimming were performed using the CLC genomics workbench default settings. RNA-seq reads were aligned to the reference genome of *G. vaginalis* strain 409-05 (GenBank accession number NC_013721). Gene expression was normalized using reads per kilobase per million (RPKM), that account for both library size and gene length, as described by Mortazavi and colleagues [13]. To detect significant gene expression alterations, Kal's test [14] with false discovery rate (FDR) [15] correction was applied. A p-value ≤ 0.05 was considered statistically significant. Transcripts uniquely expressed in each condition were identified using BioinfoGP [16]. Data were deposited at Gene Expression Omnibus database (accession number GSE8012, available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80127).

5.2.7 Biological interactions

In order to determine the function of differentially expressed genes, gene ontology (GO) [17] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [18] assignations and enrichment analysis were performed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (version 10) [19]. In addition, UniProt repository [20] was used to determine the function of proteins that were not identified by STRING. Classes with p-value ≤ 0.05, FDR-adjusted, were considered statistically significant for enrichment. Further analysis was carried out using Cytoscape (version 3.2.1) [21], in which a gene interaction network including all differentially expressed genes and neighbors created by STRING [19] was imported. Gene clusters (regions of high connectivity) were obtained in Cytoscape with the MCODE plugin [22]. Default parameters (score value above two and at least four nodes) were used as the cut-off criteria for network module screening. Thereafter an enrichment analysis of clusters was performed using STRING with a threshold of $p < 0.05$, FDR-adjusted [19].

5.2.8 Quantitative PCR

In order to validate RNA-seq data, quantitative PCR (qPCR) was performed to quantify the transcription of 8 randomly selected genes, by using the same total RNA utilized for libraries construction (technical validation) and new total RNA obtained from independent experiments performed under the same biological conditions (biological validation). Furthermore, the gene

expression profile of known virulence genes was also addressed. Oligonucleotide primers were designed using Primer3 [23] having *G. vaginalis* 409-05 genome as template (Table 5.1).

qPCR was performed as described in chapter 3 with minor modifications. Briefly, qPCR was done using a CFX96^{TM} thermal cycler (Bio-Rad) with the following cycling parameters: 3 minutes at 95ºC, followed by 45 cycles of 10 seconds at 95ºC, 10 seconds at 60ºC and 15 seconds at 72ºC. Reaction efficiency was determined by the dilution method [24]. At 60ºC all set of primers used had the highest and more similar efficiencies. Normalized gene expression was determined by using the delta C_t method ($E^{\Delta Ct}$), a variation of the Livak method, where $\Delta C_t = C_t$ (reference gene) - C_t (target gene) and E stands for the reaction efficiency experimentally determined. A non-reverse transcriptase control was included in each reaction. Three biologic replicates of each condition were analysed.

Table 5.1 Primers used in qPCR experiments

Target gene	Gene description	Primer sequence (5' to 3')	$T_{melting}$ (ºC)	Amplicon size (bp)
16S RNA	16S ribosomal RNA of *G. vaginalis*	Fw TGAGTAATGCGTGACCAACC	55.20	167
		Rv AGCCTAGGTGGGCCATTACC	59.30	
HMPREF0424_0103 (*vly*)	Thiol-activated cytolysin vaginolysin	Fw GAACAGCTGGGCTAGAGGTG	60.01	153
		Rv AATTCCATCGCATTCTCCAG	60.04	
HMPREF0424_0471 (gap)	Glyceraldehyde 3-phosphate dehydrogenase domain-containing protein	Fw AAGAACCAGCGGAAACAATG	60.11	192
		Rv ATGGCGTTGAATTCGTTCTC	60.08	
HMPREF0424_0343 (pgi)	Gucose-6-phosphate isomerase	Fw ATCGCGTGGATAAGTTGAGC	60.24	184
		Rv TGCAAAACTGCACGATCTTC	60.00	
HMPREF0424_1220	Aspartate transaminase	Fw TCGTCAAGCAACATTTCAGC	60.00	174
		Rv TAGACGCAAAGCAATTGTGG	59.87	
HMPREF0424_0125	TadE-like protein	Fw GGTTCTGGCACTATGCTTGG	58.90	171
		Rv ACACGCATTATCCTCCATCC	57.45	
HMPREF0424_0821	Glycosyltransferase, group 2 family protein	Fw CAACGAAGGCATAGGTTTCC	59.57	156
		Rv GCGCTTGGAACTGCTTTAAC	60.02	

Table 5.1 Continued

Target gene	Gene description	Primer sequence (5' to 3')	$T_{melting}$ (ºC)	Amplicon size (bp)
HMPREF0424_1336	Periplasmic binding protein and sugar binding domain of the LacI family protein	Fw ATGGCACCTAATGCCATCTC	59.92	173
		Rv GGCAAAGGATTCAAAGATCG	59.65	
HMPREF0424_1286 (thiO)	Glycine oxidase	Fw AATGCCGTGACGGAAGTAAC	60.00	200
		Rv ATGACCGCGATATTCCAAAG	59.92	
HMPREF0424_1122	Multidrug resistance ABC transporter	Fw CAGCACCTGTAGCTCCAACA	60.05	195
		Rv TGGCTCAAGAGATTGTGTGC	59.99	
HMPREF0424_0156	Bacitracin transport ATP-binding protein BcrA	Fw CCGACCGCATACCTATTTTG	60.34	178
		Rv GCAAGACGGTCTCCAAACTC	59.85	
HMPREF0424_0354	Drug resistance MFS transporter	Fw AACCAAGCAATTCCACAAGC	60.12	199
		Rv CCGTCGTTTTGGCAGTATTT	60.00	
HMPREF0424_1196	LPXTG-motif cell wall anchor domain-containing protein	Fw TGCAAAGACAGGCGATAGTG	60.00	173
		Rv TAATCGTTGCGGTTGTTTCA	60.11	

5.3 Results

5.3.1 Transcriptome analysis

A total of 561 302 (planktonic phenotype) and 311 643 (biofilm phenotype) sequencing reads were obtained for the cDNA libraries. Before trimming the raw data, we identified the genes, with the RPKM above 1.00, expressed in each condition. We only detected 3 genes uniquely expressed in biofilm cells, whereas 11 genes were found uniquely in planktonic cells. However, the majority of gene transcripts that were only detected in planktonic or biofilm cells, encoded uncharacterized proteins or tRNA, as shown in Supplementary Table S5.1.

Our data indicated that within the 1045 genes that were transcribed in both conditions, 815 (78%) were differentially expressed between planktonic and biofilm cells. For downstream analysis, only genes with fold-changes above two were considered. Transcript levels of 309 (30%) genes were elevated, whereas 36 (3%) were reduced in biofilms. Among the transcripts

that were more abundant in biofilms, 78 encoded hypothetical proteins. In an effort to find homology with known proteins, we performed a BLAST analysis, a search in the Pfam database (version 29.0) for Pfam domains [25] and used the PSORTb program (v.3.0) [26] to predict their subcellular localization. The results are shown in Supplementary Table S5.2. Interestingly, 53% of these proteins might have cytoplasmic membrane localization, suggesting that part of these proteins could have a transporter function.

In order to confirm the results obtained by RNA-seq, transcripts detected in greater or lesser abundance in biofilms were randomly selected and their relative levels quantified by qPCR. Both RNA used for cDNA libraries construction (technical validation) and RNA obtained by performing new experiments (biological validation) were used for validation. As can be seen in Figure 5.1, the same trend was observed in all measurements (qPCR and RNA-seq).

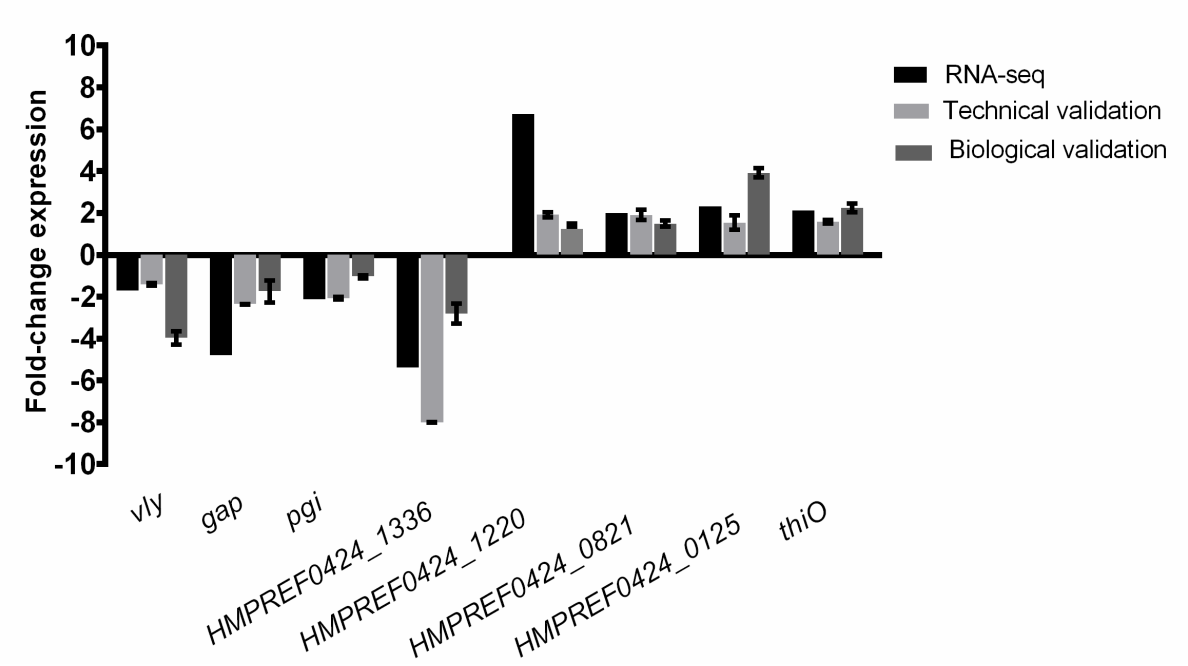


Figure 5.1 qPCR validation of the transcription of differentially expressed genes randomly selected. Technical validation means that we used the same total RNA utilized for libraries construction. Biological validation means that we used new total RNA obtained from independent experiments performed under same biological conditions. The data indicate the fold-change expression of genes in *G. vaginalis* biofilms cells compared to planktonic cells. For qPCR experiments, the bars represent the mean and the error bars the standard error of the mean (Mean ± SEM).

5.3.2 Enrichment analysis of genes with increased and decreased transcription

GO annotation, placement of genes on KEGG pathways, and enrichment analysis of the genes with down and upregulated transcription was accomplished using STRING [19]. Significant enrichment was only found ($p < 0.05$, FDR-corrected) in KEGG pathways (Figure 5.2). As could

be expected, classes associated with metabolism were found significantly enriched among the genes with decreased transcription, suggesting that biofilm cells were less metabolically active than planktonic cells. Conversely, protein export was found to be enriched among transcripts that were elevated in biofilm cells.

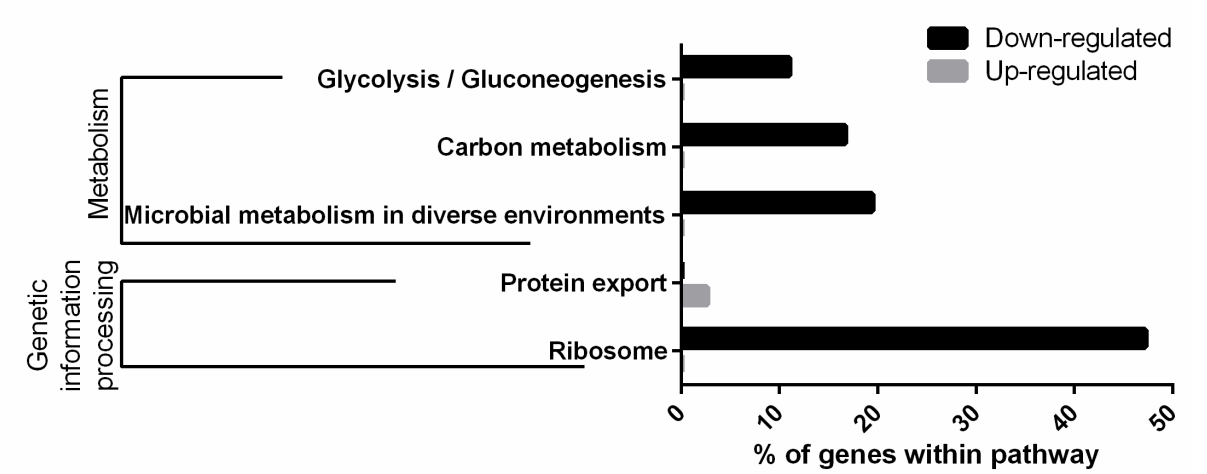


Figure 5.2 KEGG pathways found significantly enriched ($p < 0.05$) within the genes with increased and decreased transcription in biofilm cells.

5.3.3 Cluster analysis

Gene clustering analysis was based on direct and functional gene interactions using Cytoscape [27]. Cytoscape was used to create a gene interaction network including all differently expressed genes and neighbors, yielding a total of 764 nodes and 7685 edges (complete gene network of differently expressed genes is shown in Figure 5.3).

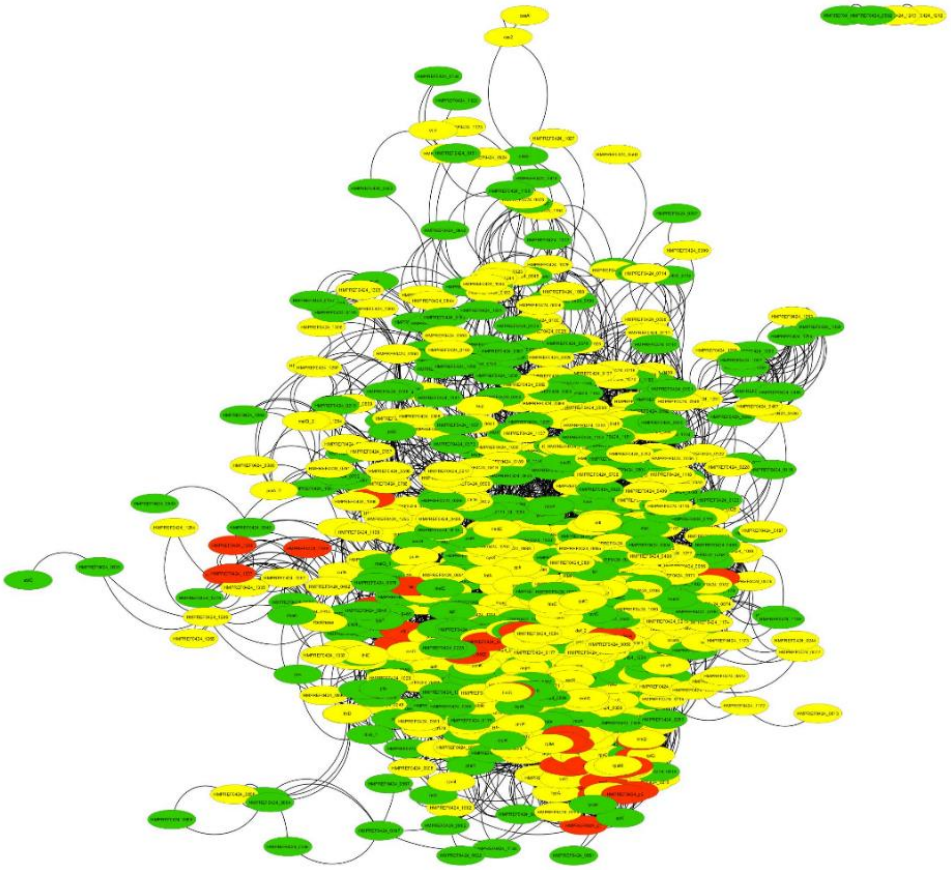


Figure 5.3 Gene interaction network generated using Cytoscape, showing downregulated transcripts (fold-change ≤ -2) in red and upregulated transcripts (fold-change ≥ 2) in green. Yellow circles correspond to transcripts differentially expressed with a fold-change between -2 and 2.

Among the differently expressed genes, we found 22 clusters. Significant enrichment was found ($p < 0.05$, FDR-corrected) in biological processes or KEGG pathways associated with translation and metabolic process (Figure 5.4 a), cell-wall biogenesis and mismatch repair (Figure 5.4 b), and antimicrobial resistance (Figure 5.4 c).

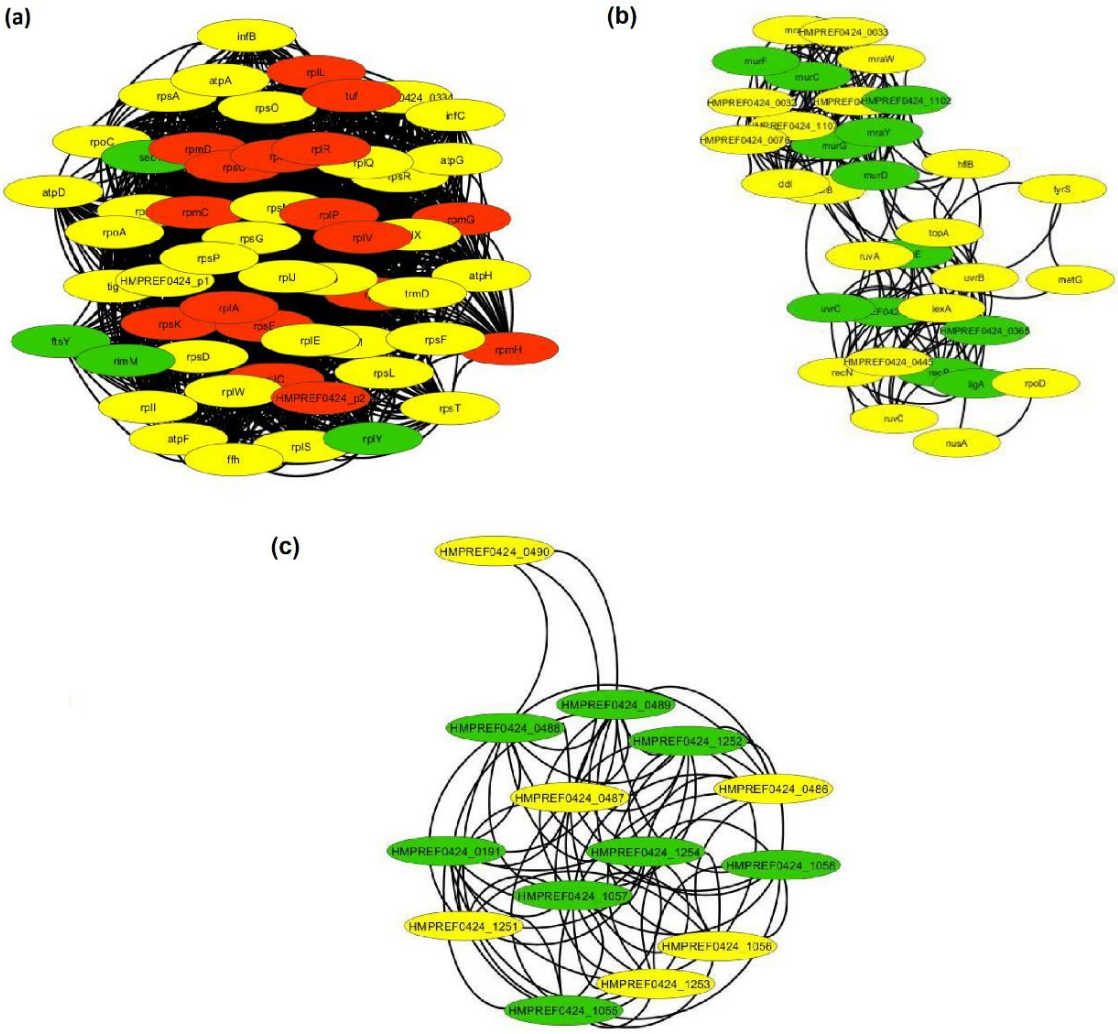


Figure 5.4 Clusters generated by the MCODE plugin in Cytoscape. Red, green, and yellow circles represent fold change values under −2, above 2, and between −2 and 2, respectively. Biological process indicates enrichment ($p < 0.05$) in translation and metabolic process **(a)**; cell-wall biosynthesis biogenesis and mismatch repair **(b)**; and KEGG indicates β-Lactamase resistance **(c).**

5.3.4 The top 10 most significantly down or upregulated genes in biofilms

Table 5.2 lists the 10 transcripts with the greatest increase and the 10 with the greatest decrease in biofilm cells.

Among the transcript decreased, we found ribosomal proteins suggesting that biofilms had decreased level of translation. Furthermore, BV associated *G. vaginalis* biofilm cells showed decreased transcript levels of genes encoding several factors involved in energy production, such as *HMPREF0424_1336,* a gene encoding primary receptors for chemotaxis and transport of many sugar based solutes.

Table 5.2 List of the 10 genes with lowest and highest fold-change values among the differentially expressed genes in *G. vaginalis* cultured under biofilm *versus* planktonic conditions

	Gene	Definition	Fold-change (Biof *vs* Plank cells)
Rank	**Downregulated**		
1	*HMPREF0424_0046*	50S ribosomal protein L34	-21.93
2	*HMPREF0424_0269*	50S ribosomal protein L30	-8.99
3	*HMPREF0424_0429 (xseA)*	Exodeoxyribonuclease VII large subunit	-6.73
4	*HMPREF0424_0260*	30S ribosomal protein S3	-5.43
5	*HMPREF0424_1336*	Periplasmic binding protein and sugar binding domain of the LacI family protein	-5.39
6	*HMPREF0424_0259*	50S ribosomal protein L22	-5.36
7	*HMPREF0424_0258*	50S ribosomal protein L2	-4.93
8	*HMPREF0424_0471 (gap)*	Glyceraldehyde 3-phosphate dehydrogenase domain-containing protein	-4.81
9	*HMPREF0424_0276*	30S ribosomal protein S11	-3.86
10	*HMPREF0424_0394 (gpmA)*	Phosphoglycerate mutase	-3.62
Rank	**Upregulated**		
1	*HMPREF0424_0510*	Uncharacterized protein	14.41
2	*HMPREF0424_0563*	Pyroglutamyl-peptidase I	9.57
3	*HMPREF0424_1220*	Aminotransferase, class I/II	6.74
4	*HMPREF0424_0420*	LPXTG-motif cell wall anchor domain-containing protein	6.30
5	*HMPREF0424_0397*	Uncharacterized protein	5.76
6	*HMPREF0424_0573*	LysM domain-containing protein	5.51
7	*HMPREF0424_0943*	ComEA protein	5.15
8	*HMPREF0424_0166*	Uncharacterized protein	4.28
9	*HMPREF0424_0888*	NLPA lipoprotein	4.24
10	*HMPREF0424_0797*	Uncharacterized protein	4.12

In addition, the expression levels of genes associated with glucose metabolic pathways were also lower in biofilms cells, namely *gap*, that also has a role in oxidoreductase activity, and *gpmA,* that displays an important role in a subpathway of the glycolysis pathway (glycolysis/gluconeogenesis pathway of *G. vaginalis* 409-05 is shown in Figure 5.5), which itself is part of carbohydrate degradation. Taken together, these results imply that *G. vaginalis*

biofilm cells are characterized by the reduction of basic cell processes (translation) and metabolism (glycolysis and carbon metabolism).

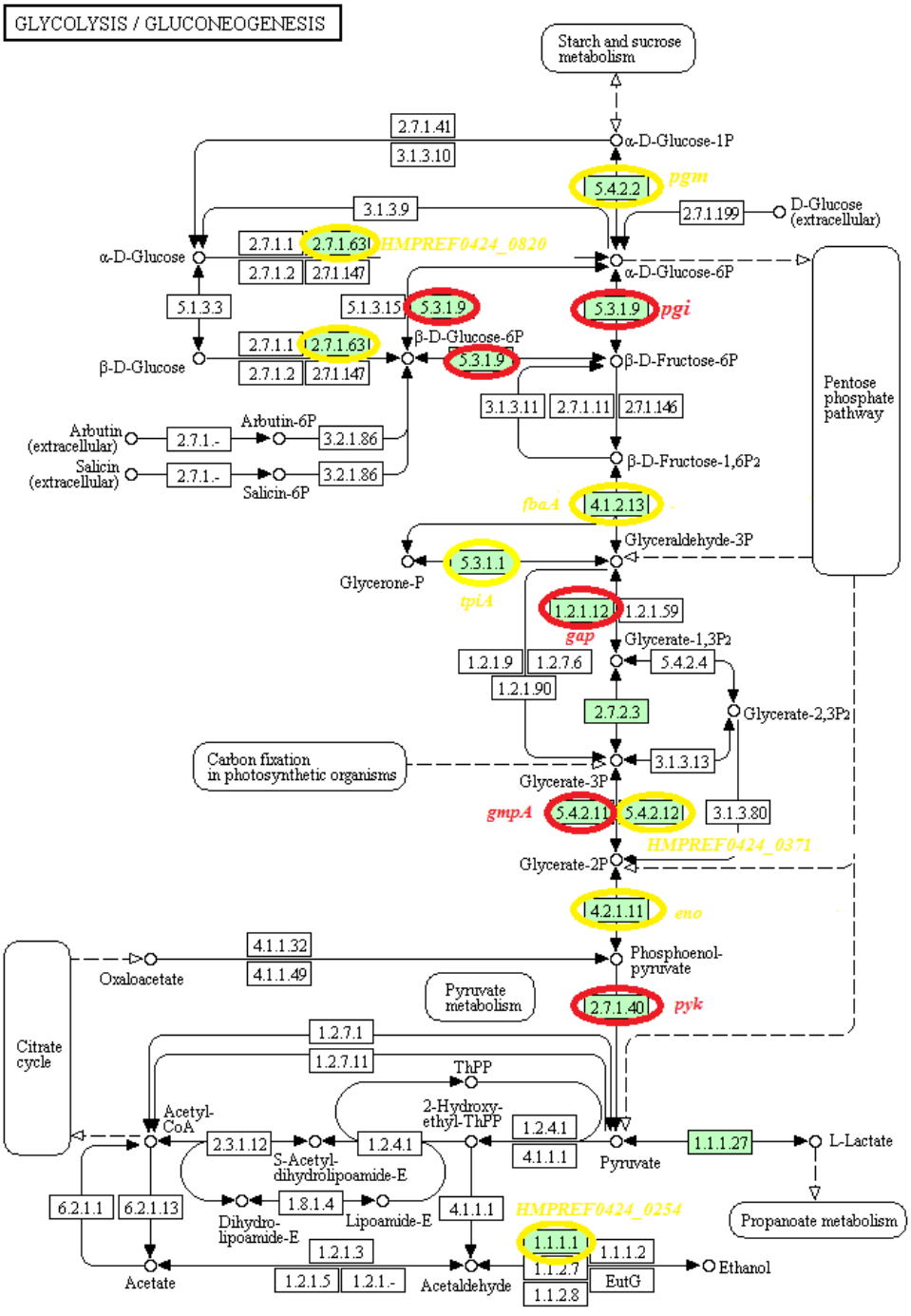


Figure 5.5 Glycolysis/gluconeogenesis pathway of *G. vaginalis* 409-05 by KEGG Pathway Maps. Available at: http://www.genome.jp/kegg-bin/show_pathway?map00010. Green boxes correspond the known pathways in *G. vaginalis* 409-05. Downregulated transcripts (fold-change ≤ -2) are represented in red. Yellow circles correspond to transcripts differentially expressed with a fold-change between -2 and 2. The genes responsible in the pathways 2.7.23 (*pgk*) and 1.1.1.27 (*HMPREF0424_0663*) are annotated in *G. vaginalis* 409-05, but they were not transcribed in our experiments.

Among the transcripts elevated in biofilm cells, we found *HMPREF0424_0563,* a gene with a molecular function related to hydrolase activity. Furthermore, in biofilm cells we found an overexpression of the *HMPREF0424_1220* gene encoding an aminotransferase involved in amino acid biosynthesis. A similar trend was reported for *Neisseria meningitidis* [28]. Interestingly, we found *HMPREF0424_0420*, a gene that encodes the LPXTG-motif cell anchor domain-containing protein, which can be involved in biofilm formation, as described in Gram-positive bacteria [29]. Moreover, in *G. vaginalis* biofilm cells, transcript levels of the gene *HMPREF0424_0573*, which encodes a LysM domain-containing protein possibly associated with autoaggregation of *G. vaginalis,* were also increased, similar to what was observed for *Lactobacillus reuteri* biofilms [30]. Transcripts encoding the ComEA protein (*HMPREF0424_0943),* which is involved in DNA repair and NLPA lipoprotein (*HMPREF0424_0888* gene), which is involved in ABC transporters were also found in greater abundance in biofilms cells.

5.3.5 Upregulation of the transcription of potential virulence genes in *G. vaginalis* biofilms

Biofilm formation by pathogenic bacteria is often associated with altered virulence. Bacterial biofilms may suppress certain virulence factors while others are activated in order to evade immune defenses, and survive challenging conditions [31]. It was, therefore, of interest to determine the expression levels of previously annotated potential virulence genes [32]. As can be seen in Figure 5.6, we found a slight increase in the *HMPREF0424_0125* transcript, which encodes *TadE*-like protein. This might play an important role in adhesion to vaginal epithelial cells, similarly to what has been described in *Actinobacillus actinomycetemcomitans* [33]. Furthermore, our data supported the previous hypothesis that *G. vaginalis* biofilm development is likely associated with type II glycosyltransferase [32]. Of note, glycosyltransferases are likely to be important for the biosynthesis of exopolysaccharide which in turn is important for biofilm formation. The ability to grow as a biofilm would likely confer an increase in antibiotic tolerance and resistance to mucosal immune defenses [34]. Herein, levels of transcripts encoding antimicrobial-specific resistance proteins belonging to efflux pump families were increased. In addition, the *HMPREF0424_1196* transcript, which encodes a Rib-protein, was elevated in biofilm cells. Rib proteins belong to the α-like protein (Alp)-family of highly repetitive surface antigens and are commonly found in Gram-positive pathogens [35]. These proteins elicit protective immunity through their inter-strain size variability [32].

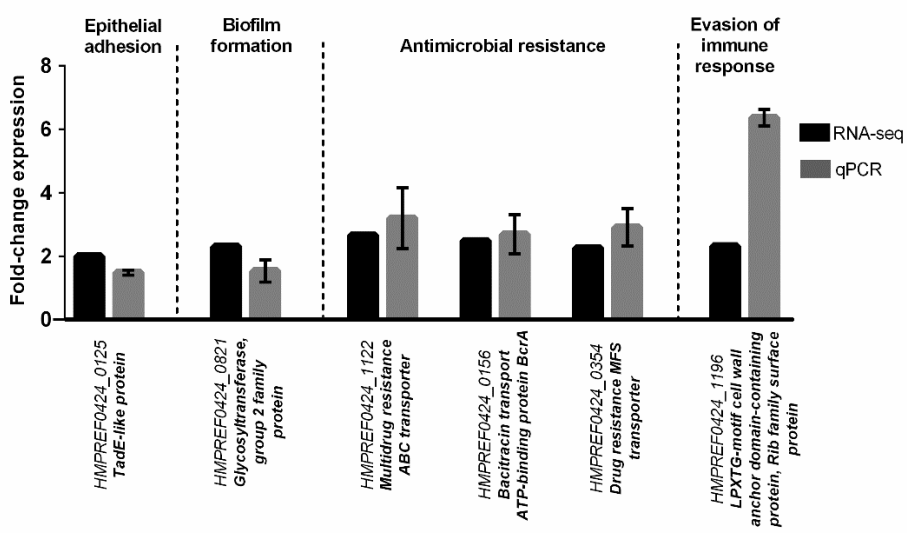


Figure 5.6 Quantification of the transcription of known virulence genes in *G. vaginalis* cultured under biofilm and planktonic conditions. Bars represent the mean and the error bars the standard error of the mean (Mean ± SEM).

5.3.6 Differential expression of vaginolysin in BV associated *G. vaginalis* biofilms

G. vaginalis produces a thiol-activated cholesterol-dependent cytolysin, vaginolysin (*vly*), which might induce vaginal cells lysis. Strikingly, in our experiments, the expression levels of *vly* (*HMPREF0424_0103)* were significantly lower in biofilm cells (Figure 5.1). In order to determine whether this was a strain-specific variation, we evaluated *vly* gene expression, by qPCR, in three other biofilm forming isolates, which were previously characterized (see chapter 3). Interestingly, as shown in Figure 5.7, the downregulation of the transcription of *vly* was observed in all different isolates.

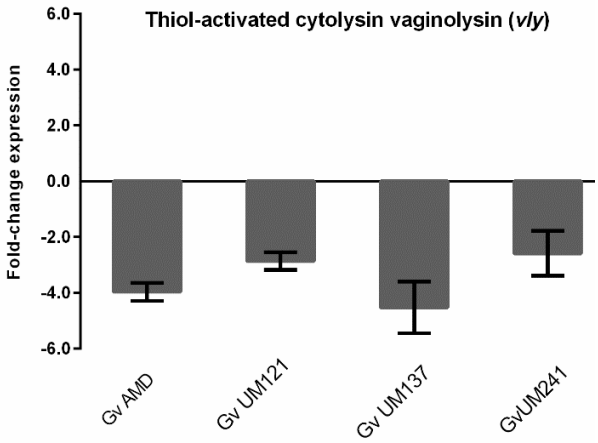


Figure 5.7 Quantification of thiol-activated cytolysin vaginolysin (*vly*) transcription in *G. vaginalis* strains cultured under biofilm or planktonic conditions. Bars represent the mean and the error bars the standard error of the mean (Mean ± SEM).

5.4 Discussion

As noted elsewhere, gene expression profiles can reveal essential information about the adaptation of a bacterial species to a particular environmental niche. Therefore, adaptation to a given host environment is an extremely important factor and underlies the capacity of a colonizing species or a pathogen to persist in a host [3]. In the present study, we analysed the transcriptome of a BV associated *G. vaginalis* cultivated under biofilm and planktonic conditions. Our results demonstrated that more transcripts were increased in biofilm relative to planktonic cells. Importantly, our findings provide key insights into the development of biofilms and the pathogenicity of *G. vaginalis*, the predominant bacterial species isolated in women with BV [36,37].

Here, we showed that BV associated *G. vaginalis* biofilm cells alter their gene expression profile, namely transcript levels of genes involved in metabolism (with downregulation of genes associated with glycolysis and carbon metabolism) and translation (with downregulation of genes encoding ribosomal proteins), as also reported for other microorganisms such as *S. epidermidis* [8], *S. aureus* [7] and *S. mutans* [9]. In *G. vaginalis* biofilms, cell density is substantially higher than in planktonic culture [1]. As a consequence, most biofilm cells are likely to encounter restricted availability of nutrients [38]. Similar to what was found for *S. mutans* [39] and *S. pneumoniae* [10], we also observed that the transcripts encoding ABC transporter proteins were elevated in biofilm cells. In addition, our study revealed that transcripts of genes involved in the synthesis of peptidoglycan and cell wall were also greater in biofilms. This has also been shown for *P. aeruginosa* [40] and *S. aureus* [7]. It has been hypothesized that the cell envelope is a highly dynamic and active component of biofilm cells, contributing to its persistence [7,10]. However, the reasons for the overexpression of genes involved in cell wall biogenesis require further investigation.

Notably, transcripts of other potential virulence genes, previously annotated by Yeoman and colleagues [32], were also more abundant in biofilm cells, with the exception of *vly*. Several studies have highlighted the role of *vly* gene in *G. vaginalis* virulence [41,42]. The *vly* gene belongs to the cholesterol-dependent cytolysins, a family of pore-forming toxins, which cause cytotoxicity on vaginal epithelium [42]. Our previous findings showed that planktonic cultures of BV isolates of *G. vaginalis* expressed 2-fold more *vly* than planktonic cultures of non-BV isolates (see chapter 3). Herein, we found that *vly* transcript levels were higher in planktonic than in biofilms cells. The low levels of expression of *vly* in biofilms might reflect the more chronic nature of vaginal colonization by BV associated *G. vaginalis* and serve as a means towards preventing a host immune response. Similarly, Resch and colleagues showed that the production of various *S. aureus* toxins were significantly upregulated in planktonic rather than

in biofilm cells [7] suggesting that toxins may not be conducive to biofilm persistence in the host.

Similar to what was observed in other microorganisms, BV associated *G. vaginalis* biofilm phenotype might induce a quiescent mode of growth that is less sensitive to antibiotics, as the efficacy of many antibiotics relies on active cell metabolism and the cell-wall construction process [8]. Here, we observed that efflux pumps and ABC transporters, reported as mechanisms responsible for antimicrobial resistance [43], were upregulated in biofilms cells. Comparable evidence for the role of efflux pumps in biofilm resistance has been found in several microorganisms such as *Pseudomonas aeruginosa* [44], *Escherichia coli* [45] and *Candida albicans* [46].

Taken together, these data indicated that BV associated *G. vaginalis* changes its transcriptomic profile when growing as a biofilm. These changes are likely important for biofilm persistence and, consequently, for the virulence of this bacterium. Furthermore, the fact that *vly* is downregulated in biofilms represents an important finding, that might contribute towards a better understanding of the pathogenesis of BV. However, this study is limited by the fact that the growth medium did not contain all the factors found *in vivo*, and some *in vivo* cues may turn on the expression of biofilm-related genes. Nevertheless, as animal models for BV are lacking, *in vitro* models can be very informative, and are key to furthering our understanding of virulence potential of *G. vaginalis*. In conclusion, our findings showed that the gene expression profile of BV associated *G. vaginalis* biofilms characterizes a distinct physiologic status that may promote the chronic and recurrent nature of BV.

5.5 References

[1] Patterson, J.L., Girerd, P.H., Karjane, N.W. and Jefferson, K.K. **(2007)**. Effect of biofilm phenotype on resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. *Am J Obstet Gynecol* 197, 170.e1-7.

[2] Verstraelen, H. and Swidsinski, A. **(2013)**. The biofilm in bacterial vaginosis: implications for epidemiology, diagnosis and treatment. *Curr Opin Infect Dis* 26, 86-9.

[3] Jefferson, K.K. **(2004)**. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett* 236, 163-73.

[4] Swidsinski, A., Loening-Baucke, V., Swidsinski, S. and Verstraelen, H. **(2015)**. Polymicrobial *Gardnerella* biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. *Arch Gynecol Obstet* 291, 605-9.

[5] Muzny, C.A. and Schwebke, J.R. **(2015)**. Biofilms: an underappreciated mechanism of treatment failure and recurrence in vaginal infections. *Clin Infect Dis* 61, 601-6.

[6] Cegelski, L., Marshall, G.R., Eldridge, G.R. and Hultgren, S.J. **(2008)**. The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol* 6, 17-27.

[7] Resch, A., Rosenstein, R., Nerz, C. and Gotz, F. **(2005)**. Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Appl Environ Microbiol* 71, 2663-76.

[8] Yao, Y., Sturdevant, D.E. and Otto, M. **(2005)**. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *J Infect Dis* 191, 289-98.

[9] Shemesh, M., Tam, A. and Steinberg, D. **(2007)**. Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. *Microbiology* 153, 1307-17.

[10] Yadav, M.K., Kwon, S.K., Cho, C.G., Park, S.W., Chae, S.W. and Song, J.J. **(2012)**. Gene expression profile of early in vitro biofilms of *Streptococcus pneumoniae*. *Microbiol Immunol* 56, 621-9.

[11] Patterson, J.L., Stull-Lane, A., Girerd, P.H. and Jefferson, K.K. **(2010)**. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 156, 392-99.

[12] França, A., Freitas, A.I., Henriques, A.F. and Cerca, N. **(2012)**. Optimizing a qPCR gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol. *PloS One* 7, e37480.

[13] Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B. **(2008)**. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 5, 621-8.

[14] Kal, A.J. et al. **(1999)**. Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. *Mol Biol Cell* 10, 1859-72.

[15] Pawitan, Y., Michiels, S., Koscielny, S., Gusnanto, A. and Ploner, A. **(2005)**. False discovery rate, sensitivity and sample size for microarray studies. *Bioinformatics* 21, 3017-24.

[16] Oliveros, J.C. **(2007)**. VENNY. An interactive tool for comparing lists with Venn Diagrams, available at http://bioinfogp.cnb.csic.es/tools/venny/index.html.

[17] Ashburner, M. et al. **(2000)**. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 25, 25-9.

[18] Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y. and Hattori, M. **(2004)**. The KEGG resource for deciphering the genome. *Nucleic Acids Res* 32, D277-80.

[19] Franceschini, A. et al. **(2013)**. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res* 41, D808-15.

[20] Pundir, S., Martin, M.J. and O'Donovan, C. **(2016)**. UniProt Tools. *Curr Protoc Bioinformatics* 53, 1.29.1-1.29.15.

[21] Shannon, P. et al. **(2003)**. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13, 2498-504.

[22] Bader, G.D. and Hogue, C.W. **(2003)**. An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics* 4, 2.

[23] Rozen, S. and Skaletsky, H. **(2000)**. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132, 365.

[24] Pfaffl, M.W. **(2004)**. AZ of quantitative PCR (ed Bustin S. A., Series 5), Ch.3 (International University Line).

[25] Punta, M. et al. **(2012)**. The Pfam protein families database. *Nucleic Acids Res* 40, D290-D301.

[26] Nancy, Y.Y. et al. **(2010)**. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26, 1608-15.

[27] Cline, M.S. et al. **(2007)**. Integration of biological networks and gene expression data using Cytoscape. *Nat Protoc* 2, 2366-82.

[28] Schoen, C., Kischkies, L., Elias, J. and Ampattu, B.J. **(2014)**. Metabolism and virulence in *Neisseria meningitidis*. *Front Cell Infect Microbiol* 4, 114.

[29] Lévesque, C.M., Voronejskaia, E., Huang, Y.-C.C., Mair, R.W., Ellen, R.P. and Cvitkovitch, D.G. **(2005)**. Involvement of sortase anchoring of cell wall proteins in biofilm formation by *Streptococcus mutans*. *Infect Immun* 73, 3773-77.

[30] Frese, S.A. et al. **(2013)**. Molecular characterization of host-specific biofilm formation in a vertebrate gut symbiont. *PLoS Genet* 9, e1004057.

[31] Kostakioti, M., Hadjifrangiskou, M. and Hultgren, S.J. **(2013)**. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med* 3, a010306.

[32] Yeoman, C.J. et al. **(2010)**. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS one* 5, e12411.

[33] Tomich, M., Fine, D.H. and Figurski, D.H. **(2006)**. The TadV protein of *Actinobacillus actinomycetemcomitans* is a novel aspartic acid prepilin peptidase required for maturation of the Flp1 pilin and TadE and TadF pseudopilins. *J Bacteriol* 188, 6899-914.

[34] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

[35] Lindahl, G., Stalhammar-Carlemalm, M. and Areschoug, T. **(2005)**. Surface proteins of *Streptococcus agalactiae* and related proteins in other bacterial pathogens. *Clin Microbiol Rev* 18, 102-27.

[36] Gardner, H.L. and Dukes, C.D. **(1955)**. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *Am J Obstet Gynecol* 69, 962-76.

[37] Kusters, J.G., Reuland, E.A., Bouter, S., Koenig, P. and Dorigo-Zetsma, J.W. **(2015)**. A multiplex real-time PCR assay for routine diagnosis of bacterial vaginosis. *Eur J Clin Microbiol Infectious Dis* 34, 1779-85.

[38] Mikkelsen, H., Duck, Z., Lilley, K.S. and Welch, M. **(2007)**. Interrelationships between colonies, biofilms, and planktonic cells of *Pseudomonas aeruginosa. J Bacteriol* 189, 2411-6.

[39] Nagayama, K., Fujita, K., Takashima, Y., Ardin, A.C., Ooshima, T. and Matsumoto-Nakano, M. **(2014)**. Role of ABC transporter proteins in stress responses of *Streptococcus mutans*. *Oral Health Dent Manag* 13, 359-65.

[40] El Zoeiby, A., Sanschagrin, F., Havugimana, P.C., Garnier, A. and Levesque, R.C. **(2001)**. *In vitro* reconstruction of the biosynthetic pathway of peptidoglycan cytoplasmic precursor in Pseudomonas aeruginosa. *FEMS Microbiol Lett* 201, 229-35.

[41] Cauci, S., Monte, R., Ropele, M., Missero, C., Not, T., Quadrifoglio, F. and Menestrina, G. **(1993)**. Pore-forming and haemolytic properties of the *Gardnerella vaginalis* cytolysin. *Mol Microbiol* 9, 1143-55.

[42] Gelber, S.E., Aguilar, J.L., Lewis, K.L. and Ratner, A.J. **(2008)**. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *J Bacteriol* 1190, 3896-903.

[43] Soto, S.M. **(2013)**. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence* 4, 223-9.

[44] Hancock, R.E. **(1998)**. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clin Infect Dis* 27 Suppl 1, S93-9.

[45] Lynch, S.V. et al. **(2007)**. Role of the rapA gene in controlling antibiotic resistance of *Escherichia coli* biofilms. *Antimicrob Agents Chemother* 51, 3650-8.

[46] Ramage, G., Bachmann, S., Patterson, T.F., Wickes, B.L. and Lopez-Ribot, J.L. **(2002)**. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother* 49, 973-80.

5.6 Supplementary data

Supplementary Table 5.1 List of genes uniquely expressed in *G. vaginalis* cultured under planktonic or biofilm conditions and their known functions

Gene	Description
Planktonic unique genes	
HMPREF0424_RS02025	tRNA-Arg
HMPREF0424_RS05465	tRNA-Asp
HMPREF0424_RS04945	tRNA-Gln
HMPREF0424_RS00345	tRNA-Lys
HMPREF0424_RS05080	tRNA-Arg
HMPREF0424_RS00030	tRNA-Gly
HMPREF0424_RS05015	tRNA-His
HMPREF0424_RS01165	tRNA-Tyr
SrpB	Signal recognition particle RNA
HMPREF0424_RS06130	Uncharacterized protein
HMPREF0424_RS02080	Exodeoxyribonuclease 7 small subunit. Bidirectionally degrades single-stranded DNA into large acid-insoluble oligonucleotides, which are then degraded further into small acid-soluble oligonucleotides
Biofilm unique genes	
HMPREF0424_RS03150	Uncharacterized protein
HMPREF0424_RS05930	tRNA-Trp
HMPREF0424_RS02715	Pyroglutamyl-peptidase I. Removes 5-oxoproline from various penultimate amino acid residues except L-prolin

Supplementary Table 5.2 Differentially expressed genes encoding hypothetical proteins with significant pfam domain, including predicted localization by PSORTb and pBLAST results

Gene [a]	Predicted localization	Protein family (Pfam) domain match	Blastp [b]
HMPREF0424_0510	Cytoplasmatic	Domain of unknown function	Hypothetical protein
HMPREF0424_0397	Cytoplasmatic membrane	Protein of unknown function	Hypothetical protein
HMPREF0424_0166	Unknown	Uncharacterized protein family	FMN-binding protein
HMPREF0424_0797	Unknown	MerR HTH family regulatory protein	MerR family transcriptional regulator
HMPREF0424_0712	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_1216	Cell Wall	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0922	Cytoplasmatic membrane	Inner membrane component domain	Membrane protein
HMPREF0424_0502	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_1106	Extracellular	Uncharacterized protein family	UDP-N-acetylmuramyl peptide synthase
HMPREF0424_0796	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0123	Unknown	Domain of unknown function: conserved EYA sequence motif	Hypothetical protein
HMPREF0424_0135	Cytoplasmatic	Protein of unknown function	Endonuclease
HMPREF0424_0150	Unknown	Uncharacterized protein family	Hypothetical protein
HMPREF0424_1257	Unknown	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0868	Cytoplasmatic	Possible lysine decarboxylase	Rossman fold protein, TIGR00730 family
HMPREF0424_0377	Unknown	YceG-like family	Hypothetical protein
HMPREF0424_0567	Unknown	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0460	Cytoplasmatic membrane	ABC-type cobalt transport system, permease component	Hypothetical protein
HMPREF0424_1007	Cytoplasmatic membrane	EamA-like transporter family	Transporter
HMPREF0424_0833	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0912	Cytoplasmatic	Uncharacterized protein family	Primosome assembly protein PriA

Supplementary Table S5.2 Continued

Gene [a]	Predicted localization	Protein family (Pfam) domain match	Blastp [b]
HMPREF0424_0219	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0230	Unknown	Uncharacterized protein family	Hypothetical protein
HMPREF0424_1136	Cytoplasmatic membrane	Uncharacterized protein family	Membrane protein
HMPREF0424_0378	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein/peptidase A24
HMPREF0424_0851	Cytoplasmatic membrane	Uncharacterized protein family	Membrane protein
HMPREF0424_0301	Cytoplasmatic membrane	Uncharacterized conserved protein	Hypothetical protein
HMPREF0424_0119	Unknown	Uncharacterized protein family	ATPase
HMPREF0424_0186	Cytoplasmatic membrane	Acyltransferase family	Acyltransferase
HMPREF0424_1130	Cytoplasmatic membrane	Domain of unknown function	Hypothetical protein
HMPREF0424_0162	Cytoplasmatic membrane	Domain of unknown function, predicted membrane protein	Membrane protein
HMPREF0424_0557	Unknown	Nucleotidyl transferase AbiEii toxin, Type IV TA system	Hypothetical protein
HMPREF0424_1246	Cytoplasmatic	HD domain, conserved protein domain	Phosphohydrolase
HMPREF0424_1202	Cytoplasmatic membrane	Uncharacterized protein family	Beta-carotene 15,15'-monooxygenase
HMPREF0424_0231	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0713	Cytoplasmatic	Protein of unknown function	Hypothetical protein
HMPREF0424_1139	Cytoplasmatic membrane	Domain of unknown function	AI-2E family transporter
HMPREF0424_0016	Cytoplasmatic membrane	Predicted permease	Permease
HMPREF0424_0939	Cytoplasmatic	Glycoprotease family	tRNA threonylcarbamoyladenosine biosynthesis
HMPREF0424_0015	Cytoplasmatic membrane	Uncharacterized protein family	Membrane protein
HMPREF0424_0792	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0579	Unknown	Uncharacterized protein family	Hypothetical protein

Supplementary Table S5.2 Continued

Gene [a]	Predicted localization	Protein family (Pfam) domain match	Blastp [b]
HMPREF0424_0352	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein / ABC transporter
HMPREF0424_1154	Cytoplasmatic membrane	UvrD-like helicase C-terminal domain	Helicase
HMPREF0424_0170	Cytoplasmatic membrane	Uncharacterized protein family	Histidine kinase
HMPREF0424_0823	Cytoplasmatic membrane	Protein of unknown function	Membrane protein
HMPREF0424_0719	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0158	Cytoplasmic membrane	ABC-2 family transporter protein	hypothetical protein
HMPREF0424_1108	Unknown	Uncharacterized protein family	hypothetical protein
HMPREF0424_0799	Cytoplasmatic membrane	Bacterial protein of unknown function	Membrane protein
HMPREF0424_0157	Cytoplasmatic membrane	ABC-2 family transporter protein	Lantibiotic ABC transporter permease
HMPREF0424_0229	Cytoplasmatic	Uncharacterized protein family	Helicase
HMPREF0424_1301	Cytoplasmatic membrane	Protein of unknown function, DUF624	Beta-carotene 15,15'-monooxygenase
HMPREF0424_0249	Cytoplasmatic	Uncharacterized protein family	IMPACT family protein
HMPREF0424_0418	Unknown	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0592	Cytoplasmatic	Zinicin-like metallopeptidase	Peptidase
HMPREF0424_0909	Extracellular	WhiA N-terminal LAGLIDADG-like domain	DNA-binding protein WhiA
HMPREF0424_0200	Cytoplasmatic	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0801	Unknown	Bacterial protein of unknown function	Hypothetical protein
HMPREF0424_0208	Unknown	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0283	Cytoplasmic	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0727	Extracellular	Uncharacterized protein family	DNA methyltransferase
HMPREF0424_0505	Cytoplasmatic membrane	Uncharacterized protein family	Exodeoxyribonuclease V
HMPREF0424_0857	Cytoplasmatic membrane	UPF0126 domain	Membrane protein
HMPREF0424_1293	Cytoplasmatic membrane	TraX protein	Endonuclease VII

Supplementary Table S5.2 Continued

Gene [a]	Predicted localization	Protein family (Pfam) domain match	Blastp [b]
HMPREF0424_0595	Cytoplasmatic membrane	Uncharacterized protein family	Hypothetical protein
HMPREF0424_0389	Cytoplasmatic	Protein of unknown function	Hypothetical protein
HMPREF0424_0591	Unknown	Protein of unknown function	Hypothetical protein
HMPREF0424_0325	Cytoplasmatic	KH domain	RNA-binding protein
HMPREF0424_0159	Cytoplasmatic	Type I restriction and modification enzyme - subunit R C terminal	Hypothetical protein
HMPREF0424_1160	Cytoplasmatic membrane	Iron permease FTR1 family	Iron permease
HMPREF0424_1037	Cytoplasmatic membrane	Uncharacterized conserved protein	Hypothetical protein
HMPREF0424_0818	Cytoplasmatic membrane	Uncharacterized conserved protein	Beta-carotene 15,15'-monooxygenase
HMPREF0424_0250	Unknown	Uncharacterized conserved protein	AbrB family transcriptional regulator
HMPREF0424_1150	Cytoplasmatic	Beta-lactamase superfamily domain	RNase J family beta-CASP ribonuclease
HMPREF0424_0373	Cytoplasmatic membrane	Uncharacterized conserved protein	Hypothetical protein
HMPREF0424_1176	Cytoplasmatic membrane	Uncharacterizsed conserved protein	Hypothetical protein
HMPREF0424_0492	Cytoplasmatic membrane	Protein of unknown function	Zinc ABC transporter permease

[a] All uncharacterized genes encoding hypothetical proteins were upregulated in biofilms cells

[b] Protein-protein BLAST (Blastp) results indicated 100% identity with *G. vaginalis*

CHAPTER 6

BV and non-BV associated Gardnerella vaginalis establish similar synergistic interactions with other BV associated microorganisms in dual-species biofilms

Summary

BV is presumably triggered by *Gardnerella vaginalis* ability to adhere to the vaginal epithelium and then becoming the scaffolding to which other vaginal colonizers incorporate a multi-species biofilm. Nevertheless, *G. vaginalis* colonization does not always lead to BV. Thus, this study aimed to determine if BV associated *G. vaginalis* presented any advantage over non-BV isolates in biofilm enhancement by other BV associated microorganisms, using an *in vitro* dual-species biofilm formation model. Our findings failed to demonstrate significant differences in biofilm enhancement between the two *G. vaginalis* groups, with the exception of dual-species biofilms formed with *Mobiluncus mulieris*, suggesting that the key difference in virulence potential between non-BV and BV associated *G. vaginalis* strains seems not to be related to biofilm maturation.

The work presented in this chapter was published in *Anaerobe (2015)* 36*, 56-59.*

6.1 Brief introduction

During BV, there is a complex interplay between pathogenic species, endogenous vaginal microbiota and the vaginal epithelium [1-3]. These interactions become more complex when microbes are adhered to the epithelium, forming biofilms, and communicate via "quorum-sensing", a cell-density dependent bacterial intercellular signalling mechanism [4]. However, *G. vaginalis* can also be a part of the vaginal microbiota in healthy women [5]. This raised the question of whether there are pathogenic and commensal lineages within this species. Jayaprakash and colleagues provided genomic evidence that all *G. vaginalis* strains had the potential to form biofilm but not all strains had the potential to cause BV symptoms, namely due to the absence of sialidase gene [6]. We recently also provided *in vitro* evidence that supports Jayaprakash hypothesis [7]. However, only the BV isolates demonstrated higher cytotoxicity and were able to adhere in high density clusters to a HeLa cell line (see chapter 3), a condition necessary to foster *in vivo* biofilm development [4]. Another important insight providing evidence that not all *G. vaginalis* have the same virulence potential was derived from recent *in vivo* observations by Swidsinski and colleagues. They demonstrated the presence of adherent bacterial biofilms in 90% of biopsies from women with BV, while only 10% of healthy women exhibited similar biofilms [8]. Subsequently, they proposed that the mere presence of loosely adherent *G. vaginalis* on the vaginal epithelium was of lesser clinical significance than the presence of high density clusters of *G. vaginalis* [9].

In effort to better understand the differences between virulent and non-virulent *G. vaginalis* strains, the aim of the present study was to analyse the interactions between non-BV (*n* = 3) or BV associated (*n* = 3) *G. vaginalis* isolates with other BV associated species (*n* = 24) using a dual-species biofilm assembly, consisting in the combination of *G. vaginalis* and secondary BV associated species.

6.2 Material and methods

6.2.1 Bacterial strains and culture conditions

G. vaginalis strains UM121, UM137 and UM241 originally obtained from women with BV and strains UM085, UM131 and UM246 from women without BV were used herein. These 6 representative strains were selected from our culture collection based on the results of chapter 3. In addition, 24 other BV associated species previously characterized [7,10] were also included in our study. More details on the species used here are found in Table 6.1. Bacterial species were grown in supplemented brain heart infusion (sBHI) and incubated at 37ºC in 10% CO_2 (Shel Lab, Cornelius, Oregon, USA) for 24 hours, as described by Alves *et al.* [8]. The

exceptions were *M. mulieris* and *P. bivia* that were grown in sBHI and incubated at 37ºC, under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 48 hours [11].

Table 6.1 GenBank accession numbers of strains used in this study

Bacteria[a]	Genes	Accession numbers[b]
Actinomyces neuii UM067An	*16S rRNA*	KT805271.1
Actinomyces turicensis UM066At	*16S rRNA*	KT805270.1
Aerococcus christensenii UM137Ac	*16S rRNA*	KT805273.1
Bacillus firmus UM034Bf	*16S rRNA*	KT805263.1
Brevibacterium ravenspurgense UM066Br	*16S rRNA*	KT805269.1
Corynebacterium amycolatum UM065Ca	*16S rRNA / rpoB*	KT805275.1 / KT923481.1
Corynebacterium tuberculostearicum UM137Ct2	*16S rRNA / rpoB*	KT805279.1 / KT923486.1
Corynebacterium tuscaniense UM137Ct	*16S rRNA / rpoB*	KT805278.1 / KT923485.1
Enterococcus faecalis UM035	*16S rRNA*	KT614045.1
Escherichia coli UM056	*16S rRNA*	KT614048.1
Gardnerella vaginalis UM085	*16S rRNA*	KP996679.1
Gardnerella vaginalis UM121	*16S rRNA*	KP996681.1
Gardnerella vaginalis UM131	*16S rRNA*	KP996676.1
Gardnerella vaginalis UM137	*16S rRNA*	KP996682.1
Gardnerella vaginalis UM241	*16S rRNA*	KP996683.1
Gardnerella vaginalis UM246	*16S rRNA*	KP996677.1
Gemella haemolysans UM034Gh	*16S rRNA*	KT805264.1
Lactobacillus vaginalis UM062Lv	*16S rRNA*	KT805268.1
Mobiluncus mulieris ATCC 35239	whole genome	NZ_GL405260.1
Nosocomiicoccus ampullae UM121Na	*16S rRNA*	KT805272.1
Prevotella bivia ATCC 29303	*16S rRNA*	L16475.1
Propionibacterium acnes UM034Pa	*16S rRNA*	KT805265.1
Streptococcus agalactiae UM035Sa	*16S rRNA*	KT805266.1
Staphylococcus epidermidis UM066Se	*16S rRNA / rpoB*	KT805277.1 / KT923483.1
Staphylococcus haemolyticus UM066Sh	*16S rRNA / rpoB*	KT805276.1 / KT923482.1
Staphylococcus hominis UM224Sh	*rpoB*	KT923487.1
Staphylococcus saprophyticus UM121Ss	*rpoB*	KT923484.1
Staphylococcus simulans UM059Ss	*16S rRNA*	KT805267.1
Staphylococcus warnerii UM224Sw	*rpoB*	KT923488.1
Streptococcus anginosus UM241b	*16S rRNA*	KT805274.1

[a] Due to NCBI sequence deposition regulations, the designation of the strains previously used in Alves *et al.* [8], were updated (highlighted in blue). [b]The accession numbers of partial *16S ribosomal RNA* or *rpoB* gene sequence of vaginal isolates are downloadable from NCBI.

6.2.2 Dual-species biofilm formation and quantification

The dual-species biofilm formation model used was the same as described by Machado and colleagues [12], with some minor modifications. Briefly, *G. vaginalis* cultures were adjusted to 1×10^7 colony-forming units (CFU)/mL by optical density (OD) at 600 nm (Model Sunrise, Tecan). After homogenization, 100 µL of each bacterial suspension of *G. vaginalis* isolates was dispensed into each well of 96-well flat-bottom tissue culture plate (Orange Scientific, Braine L'Alleud, Belgium). The tissue culture plates were then placed in an incubator at 37ºC in 10% CO_2. Following 24 hours, the culture medium covering the biofilm was carefully removed and replaced by fresh medium. A second inoculation with 1×10^7 CFU/mL of each BV associated strain was performed and biofilms were allowed to grow for another 24 hours. Quantification of biofilm was performed by the crystal violet staining, as previously described [13]. All assays were repeated at least 3 times with 8 technical replicates.

6.2.3 Statistical analysis

The data were analysed using the non-parametric Kruskal-Wallis test, since the data did not follow a normal distribution according Kolmogorov-Smirvon's test, with the statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL). *P*-values of less than 0.05 were considered significant.

6.3 Results

As shown in Figure 6.1, our results revealed that 54% (n = 13) of the BV associated species tested had a synergistic effect in most of *G. vaginalis* strains. However, only 6 species caused an increase in biofilm formation in all tested conditions: *Actinomyces neuii, Brevibacterium ravenspurgense, Corynebacterium amycolatum, Corynebacterium tuscaniense, Staphylococcus hominis* and *Staphylococcus saprohyticus*. Conversely, we observed that 42% of the tested species showed variable interactions dependent on the specific *G. vaginalis* strain used. However, no link ($p > 0.05$; Kruskal-Wallis) was found between non-BV and BV associated *G. vaginalis* strains, with the exception of *M. mulieris,* which showed an antagonistic effect when added to the biofilm formed by BV strains, whereas a synergistic interaction was verified in presence biofilms formed by non-BV *G. vaginalis* isolates. Our data also revealed an antagonistic interaction between all *G. vaginalis* strains tested and *Lactobacillus vaginalis*.

	BV-negative *G. vaginalis* strains			BV-positive *G. vaginalis* strains		
Strains (*n* = 24)	UM131	UM085	UM246	UM241	UM121	UM137
Actinomyces neuii	3.51	3.30	9.87	1.61	2.49	3.46
Brevibacterium ravenspurgense	3.19	4.26	3.73	1.36	2.33	2.02
Corynebacterium amycolatum	1.91	2.59	7.34	1.82	1.66	1.49
Corynebacterium tuscaniense	2.41	3.04	1.67	3.36	1.41	1.59
Staphylococcus hominis	2.93	2.61	7.95	2.10	1.51	3.31
Staphylococcus saprohyticus	4.42	4.33	12.52	2.17	2.56	4.99
Enterococcus faecalis	2.54	1.24	1.44	1.47	1.49	1.37
Nosocomiicoccus ampullae	1.81	0.79	5.06	1.36	2.53	1.72
Staphylococcus simulans	1.61	1.25	1.30	5.91	0.78	2.08
Staphylococcus warnerii	2.02	1.26	1.27	4.87	0.81	1.55
Streptococcus anginosus	1.20	1.72	2.62	1.69	1.89	1.21
Propiobacterium acnes	2.66	1.06	2.56	1.57	1.85	0.80
Escherichia coli	1.04	1.07	7.44	0.77	1.16	1.31
Bacillus firmus	1.32	1.65	1.58	0.70	1.31	1.55
Prevotella bivia	0.50	3.03	3.97	1.66	1.53	2.17
Staphylococcus haemolyticus	1.57	1.28	1.10	1.10	1.35	0.64
Streptococcus agalactiae	1.24	1.29	1.00	1.37	1.66	0.66
Actinomyces turicensis	1.10	1.19	1.89	0.70	1.03	0.86
Gemella haemolysans	0.53	1.02	0.99	0.87	1.05	0.88
Aerococcus christensenii	0.78	0.46	1.79	0.66	1.68	0.78
Corynebacterium tuberculosteraricum	1.02	0.37	1.26	0.73	1.08	0.82
Staphylococcus epidermidis	0.84	1.13	0.59	0.62	1.00	0.68
Mobiluncus mulieris	1.26	1.96	1.84	0.06	0.67	0.07
Lactobacillus vaginalis	0.30	0.21	0.02	0.68	0.23	0.42

Synergistic interaction Neutral interaction Antagonistic interaction

Figure 6.1 Synergistic, antagonistic or neutral interactions detected in dual-species biofilms in relation to single biofilms of non-BV or BV *G. vaginalis* isolates. The data are presented as fold change relative to the single *G. vaginalis* biofilm (fold change = 1, control). Interactions were classified as antagonistic (cut-off < 0.75-fold changes), neutral (0.75 ≤ fold changes < 1.25) and synergistic (cut-off ≥ 1.25 - fold changes). Results represent at least 3 independent experiments performed with 8 technical replicates. No significant differences between non-BV and BV *G. vaginalis* strains were found in a dual-species biofilm formation ($p > 0.05$; Kruskal-Wallis), with exception to *M. mulieris* ($p < 0.05$; Kruskal-Wallis).

6.4 Discussion

The most recent model for the pathogenesis of BV suggests that *G. vaginalis* adhered to vaginal epithelium might be acting as a scaffold for the attachment of a subsequent species [1,14]. However, the role of BV associated bacteria in multi-species biofilms is still poorly understood. An early study by Machado and colleagues demonstrated that a few secondary

BV associated anaerobes, such as *P. bivia*, were able to increment the concentration of cells within the biofilm when added to a pre-formed *G. vaginalis* biofilm [12].

Herein, we were interested to determine if similar synergistic interactions occurred when using BV or non-BV *G. vaginalis* isolates. Surprisingly, with the exception of one species (*M. mulieris*), no differences were found between BV and non-BV associated *G. vaginalis* mediated dual-species biofilm augmentation. These results suggest that the key difference in BV or non-BV *G. vaginalis* virulence potential seems not to be related to biofilm maturation, at least in a dual-species model. We propose that once specific strains of *G. vaginalis* are able to outcompete the resident *Lactobacillus* species and start to grow in clusters, secondary anaerobes will easily incorporate the biofilm. This might be the key difference in virulence potential of *G. vaginalis*.

A particular example of synergistic interaction in dual-species biofilms is the case of *G. vaginalis* and *P. bivia*. It has been previously shown that *G. vaginalis* produces amino acids through its metabolism and *P. bivia*, a strict anaerobe, uses amino acids as its fuel source and as a result produces ammonia, which in turn is used by *G. vaginalis* [15]. Nevertheless, our data also showed that *L. vaginalis* had an antagonistic effect in the presence of all tested *G. vaginalis* biofilms. Boskey and colleagues have shown that the growth limiting factor for *L. vaginalis* was a depletion of a metabolite or the buildup of an unspecified toxic waste product [16], that might also be toxic to *G. vaginalis* causing a disruption of the biofilm. Curiously, our findings revealed that *M. mulieris* was the only bacterial species with opposing interactions in the presence of either non-BV or BV pre-formed *G. vaginalis* biofilms. Nevertheless, further work is required to explore the bacterial interactions between these bacterial species.

The results from our study should be interpreted considering several limitations. First, initial adhesion by *G. vaginalis* was performed in polystyrene microtiter plate wells rather than vaginal epithelium, where the presence of host-derived factors (e.g. mucus production, specific receptors on the epithelial surface) can influence bacterial adherence and biofilm formation. This technical limitation is not easy to overcome since, as we shown before, *G. vaginalis* quickly induces cytotoxic changes and detachment of pre-adhered epithelial cultures (see chapter 3). Furthermore, the growth medium did not contain all the factors found *in vivo*, and some *in vivo* cues may turn on expression of biofilm-related genes. However, these limitations aside, *in vitro* models can be very informative and are key to furthering our understanding of multi-species biofilms and the development of BV.

In conclusion, this study provides direct evidence that confirms synergistic roles of many secondary or late colonizers in BV multi-species biofilm development, but reveals that those interactions are not specific for more virulent BV associated *G. vaginalis*.

6.5 References

[1] Schwebke, J.R., Muzny, C.A. and Josey, W.E. **(2014)**. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis* 210, 338-43.

[2] Marrazzo, J.M. **(2011)**. Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused? *Anaerobe* 17, 186-90.

[3] Redelinghuys, M.J., Ehlers, M.M., Dreyer, A.W. and Kock, M.M. **(2015)**. Normal flora and bacterial vaginosis in pregnancy: an overview. *Crit Rev Microbiol*, 1-12.

[4] Peters, B.M., Jabra-Rizk, M.A., O'May, G.A., Costerton, J.W. and Shirtliff, M.E. **(2012)**. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev* 25, 193-213.

[5] Marrazzo, J.M., Koutsky, L.A., Eschenbach, D.A., Agnew, K., Stine, K. and Hillier, S.L. **(2002)**. Characterization of vaginal flora and bacterial vaginosis in women who have sex with women. *J Infect Dis* 185, 1307-13.

[6] Jayaprakash, T.P., Schellenberg, J.J. and Hill, J.E. **(2012)**. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PloS One* 7, e43009.

[7] Alves, P., Castro, J., Sousa, C., Cereija, T.B. and Cerca, N. **(2014)**. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *J Infect Dis* 210, 593-6.

[8] Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L.P. and Lochs, H. **(2005)**. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 106, 1013-23.

[9] Swidsinski, A. et al. **(2013)**. Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). *Histol Histopathol* 29, 567-87.

[10] Patterson, J.L., Stull-Lane, A., Girerd, P.H. and Jefferson, K.K. **(2010)**. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 156, 392-99.

[11] Cereija, T.B., Castro, J., Alves, P. and Cerca, N. **(2013)**. Influence of anaerobic conditions on vaginal microbiota recovery from bacterial vaginosis patients. *Sex Transm Infec*t 89, 307.

[12] Machado, A., Jefferson, K.K. and Cerca, N. **(2013)**. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 14, 12004-12.

[13] Machado, D., Palmeira-de-Oliveira, A. and Cerca, N. **(2015)**. Optimization of culture conditions for *Gardnerella vaginalis* biofilms formation. *J Microbiol Methods*, 118:143-6.

[14] Machado, A. and Cerca, N. **(2015)**. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *J Infect Di*s 212, 1856-61.

[15] Pybus, V. and Onderdonk, A.B. **(1997)**. Evidence for a commensal, symbiotic relationship between *Gardnerella vaginalis* and *Prevotella bivia* Involving ammonia: potential significance for bacterial vaginosis. *J Infect Di*s 175, 406-13.

[16] Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R. and Cone, R.A. **(1999)**. Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. *Infect Immun* 67, 5170-5.

CHAPTER 7

Unveiling Gardnerella vaginalis role in Bacterial vaginosis (BV) polymicrobial biofilms: the impact of other vaginal pathogens living as neighbors

Summary

BV is characterized by a highly structured polymicrobial biofilm, strongly adhered to the vaginal epithelium, primarily consisting of *Gardnerella vaginalis.* However, despite the presence of other BV associated bacteria, little is known regarding the impact of other species in BV development. To gain insight about BV progress, we analysed the ecological interactions between *G. vaginalis* and 15 BV associated microorganisms using a dual-species biofilm model. Bacterial populations were quantified using a validated peptide nucleic acid fluorescence *in situ* hybridization approach. Furthermore, biofilm structure was analysed by confocal laser scanning microscopy. In addition, the bacterial coaggregation ability was determined as well as the expression of key virulence genes. Remarkably, our results revealed distinct biofilm structures between each bacterial consortium, leading to at least 3 unique dual-species biofilm morphotypes. Furthermore, our transcriptomic findings seem to indicate that an important role can be attributed to *Enterococcus faecalis* and *Actinomyces neuii* in the enhancement of *G. vaginalis* virulence, while the other tested species had a lower or no impact in the expression of virulence genes by *G. vaginalis*. This study cast a new light on how BV associated species can modulate the virulence aspects of *G. vaginalis,* contributing to better understanding the development of BV associated biofilms.

Part of the work presented in this chapter was published in *Pathogens and Disease (2016) 74, ftw007*

7.1 Brief introduction

The specific roles of the multiple microorganisms found in BV are largely unknown [1,2] being *Gardnerella vaginalis* currently the best studied species [3-6]. *G. vaginalis* role in BV is not without controversy. First proposed as the sole etiological agent by Gardner and Dukes [7], its presence in healthy women casted doubt on its virulence potential [8]. Nevertheless, in the past decade, it has been demonstrated that *G. vaginalis* had significant higher virulence potential than many other BV associated species [9-11].

Due to its strong adherence to vaginal cells and biofilm-forming capacities, it has been suggested that BV associated *G. vaginalis* initiates the colonization of the vaginal epithelium and serves a scaffolding to which other species subsequently can attach [2,12-14]. As result, during BV, there is a complex interplay between pathogenic species, endogenous vaginal microbiota and the vaginal epithelium [5,15]. Due to the presumably central role of *G. vaginalis* in BV development, it is crucial to assess how secondary BV associated species interact with BV associated *G. vaginalis*. The study of these microbial interactions is extremely important for obtaining knowledge of the pathogenicity of microbes in the host and for the development of effective treatments without relapses, a common problem in BV [16,17]. Some studies have already evaluated the interplay between *G. vaginalis* and other BV associated species in biofilms [18,19]. However, all these studies were carried out by observing a few phenotypic aspects of the interactions between *G. vaginalis* and BV associated species, and, as such, more detail is needed. We recently showed that *G. vaginalis* exhibits a specific gene expression behaviour according to its phenotype form, probably to overcome the host defenses and allow the colonization of mucosal tissue (see chapter 5). However, hardly any information exists on how BV associated *G. vaginalis* gene expression is influenced by the presence of other BV associated bacteria. Thus, in an effort to better understand the virulence of BV associated *G. vaginalis* in polymicrobial communities, the aim of the present study was to analyse the interactions between 15 BV associated species and BV associated *G. vaginalis* using a dual-species biofilm assembly.

7.2 Material and methods

7.2.1 Bacterial strains and culture conditions

G. vaginalis strain UM241 was isolated from a woman diagnosed with BV (see chapter 3). Fifteen BV associated species previously phenotypically characterized [9,10], namely: *Actinomyces neuii, Atopobium vaginae, Brevibacterium ravenspurgense, Corynebacterium amycolatum, Corynebacterium tuscaniense, Enterococcus faecalis, Mobiluncus mulieris, Nosocomicoccus ampullae, Prevotella bivia*, *Propionibacterium acnes, Staphylococcus*

hominis, Staphylococcus saprohyticus, Staphylococcus simulans, Staphylococcus warnerii and *Streptococcus anginosus,* were included in this study. Each inoculum was grown in supplemented brain heart infusion (sBHI) and incubated at 37ºC in 10% CO_2 (Shel Lab, Cornelius, Oregon, USA) for 24 hours, as described by Alves *et al.* [9]. The exceptions were *A. vaginae*, *M. mulieris* and *P. bivia* that were grown in sBHI and incubated at 37ºC, under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 48 hours.

7.2.2 Coaggregation assays

To determine the extent of the coaggregation between *G. vaginalis* and BV associated bacteria we used an experimental model developed by Reid and colleagues [20]. In brief, 500 µL of *G. vaginalis* (10^7 CFU/mL) was combined with 500 µL of each BV associated specie (10^7 CFU/mL) in 24-well plates (Thermo Fisher Scientific, Lenexa, KS, USA). Then, bacteria were incubated for 4 hours, at 37ºC, in 10% CO_2. The aggregates were visualized using an inverted light microscope Leica DMI 3000B (Leica Microsystems GmbH, Wetzlar, Germany) and the score was evaluated as following: 0, no aggregation; 1, small aggregates comprising small visible clusters of bacteria; 2, aggregates comprising larger numbers of bacteria, settling to the center of the well; 3, macroscopically visible clumps comprising larger groups of bacteria which settle to the center of the well; 4, maximum score allocated to describe a large, macroscopically visible clump in the center of the well. Auto-aggregation was assessed for each bacterial strain. All assays were performed in duplicated and repeated in three different days.

7.2.3 Biofilm formation

Dual-species biofilms were performed as described previously [11]. Briefly, the cell concentration of *G. vaginalis* was assessed by optical density (OD) and this initial culture was further diluted in order to obtain a final concentration of approx. 10^7 CFU/mL. After homogenization, 500 µL of *G. vaginalis* suspensions were dispensed into each well of 24-well flat-bottom tissue culture plate (Orange Scientific, Braine L'Alleud, Belgium). The tissue cultured plates were then placed in an incubator at 37ºC in 10% CO_2. Following 24 hours of biofilm formation, the planktonic cells were removed carefully and 500 µL of fresh medium was added to each well. At the same time, the suspension of second BV-isolate was added (in a concentration approx. 10^7 or 10^5 CFU/mL) to each well and the plates were further incubated for 24 hours. Then, dual-species biofilms were washed once with phosphate buffer saline (PBS) solution. The 24 or 48 hours mono-specie biofilm of *G. vaginalis* was used as a control.

7.2.4 PNA FISH hybridization and DAPI staining

To quantify the total cells of mono- and dual-species biofilms, we used the method suggested by Freitas and colleagues [21]. In brief, biofilms were scraped and resuspended in PBS. The total cells of the mono- or dual-species biofilms were quantified using a Neubauer chamber coupled with Olympus BX51 epifluorescence microscope equipped with a CCD camera (DP72; Olympus, Lisboa, Portugal). Cell suspensions were stained with 4'-6-Diamidino-2-phenylindole (DAPI, 2.5 µg/mL). DAPI staining was detected in a specific filter, BP 365-370, FT 400, LP 421 present in the microscope. Next, we discriminated the bacterial population of biofilm by using the Peptide Nucleic Acid Fluorescence *in situ* hybridization (PNA FISH) method as previously described [22]. Briefly, after fixing the biofilm suspension, a PNA probe specific for *G. vaginalis* (Gard 162) was added to each well of epoxy coated microscope glass slides (Thermo Fisher Scientific). An additional staining step was done at the end of the hybridization procedure, covering each glass slide with DAPI. Microscopic visualization was performed using filters capable of detecting the PNA probe (BP 530-550, FT 570, LP 591 sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe) and DAPI (as described above). An external control was performed to determine the sensibility of the PNA probe for several dilutions of 48 hours *G. vaginalis* mono-species biofilm cells, correlating the DAPI with PNA FISH counts.

7.2.5 Confocal laser scanning microscopy analysis of biofilm bacterial distribution

To analyse the bacterial distribution of dual-species biofilms, the biofilm structure was evaluated by confocal laser scanning microscopy (CLSM) using the PNA Gard162 probe coupled to DAPI staining as we described above. For this experiment, biofilms were formed on 8-well chamber slide (Thermo Fisher Scientific™ Nunc™ Lab-Tek™, Rochester, NY, USA) at 37°C in 10% CO_2 for 48 hours with replacement of sBHI medium at 24 hours of growth and the addition of the respective second BV associated bacteria. The CLSM images were acquired in an Olympus™ FluoView FV1000 (Olympus) confocal scanning laser microscope, using a 40 × objective. Images were acquired with 512 × 512 resolutions at four different regions of each surface analysed.

7.2.6 Gene expression quantification

Dual-species biofilms were grown as described above. Gene expression of six potential virulence genes was determined according to our previous study (see chapter 5). All primers used here are listed in Table 7.1. Total RNA was extracted using an ExtractME RNA Bacteria & Yeast kit (Blirt S.A., Poland) with minor changes, as optimized before [23]. Next, genomic DNA was degraded with one step of DNase treatment (Fermentas, Lithuania) following

manufacturer's instructions. RNA concentration, purity and integrity was determined as described before [24]. qPCR was performed as described in chapter 5. Normalized gene expression was determined by using the delta C_t method ($E^{\Delta Ct}$), a variation of the Livak method, where $\Delta C_t = C_t$ (reference gene) - C_t (target gene) and E stands for the reaction efficiency experimentally determined. A non-reverse transcriptase control was included in each reaction. At least three biologic replicates of each condition were performed.

Table 7.1 Primers used in qPCR experiments

Target gene	Gene description	Primer sequence (5' to 3')	T melting (ºC)	Amplicon size (bp)
16S RNA	16S ribosomal RNA of *G. vaginalis*	Fw TGAGTAATGCGTGACCAACC	55.2	167
		Rv AGCCTAGGTGGGCCATTACC	59.3	
HMPREF0424_0103 (*vly*)	Thiol-activated cytolysin vaginolysin	Fw GAACAGCTGGGCTAGAGGTG	60.01	153
		Rv AATTCCATCGCATTCTCCAG	60.04	
sld	Sialidase	Fw CCGAATTTGCGATTTCTTCT	54.00	189
		Rv CGTACGGAAGTTTTGGAAGC	58.00	
HMPREF0424_0821	Glycosyltransferase, group 2 family protein	Fw CAACGAAGGCATAGGTTTCC	59.57	156
		Rv GCGCTTGGAACTGCTTTAAC	60.02	
HMPREF0424_1122	Multidrug resistance ABC transporter	Fw CAGCACCTGTAGCTCCAACA	60.05	195
		Rv TGGCTCAAGAGATTGTGTGC	59.99	
HMPREF0424_0156	Bacitracin transport ATP-binding protein BcrA	Fw CCGACCGCATACCTATTTTG	60.34	178
		Rv GCAAGACGGTCTCCAAACTC	59.85	
HMPREF0424_1196	LPXTG-motif cell wall anchor domain-containing protein	Fw TGCAAAGACAGGCGATAGTG	60.00	173
		Rv TAATCGTTGCGGTTGTTTCA	60.11	

7.2.7 Statistical analysis

The data were analysed using the independent samples t-test, paired sample t-test, or non-parametric Mann-Whitney U test for the data that did not follow a normal distribution according Kolmogorov-Smirvon's test, with the statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL). The data were represented as mean ± standard deviation (SD) or as mean ± standard error of mean (SEM) at least 3 independent experiments. P-values of less than 0.05 were considered significant.

7.3 Results

7.3.1 Co-aggregation between *G. vaginalis* & other BV associated isolates

It has been described that coaggregation is highly specific and considered a virulence factor since microbial aggregates are a common mechanism of the survival of bacteria in nature [25,26]. Thus, our first aim was to analyse whether BV associated *G. vaginalis* and other BV associated bacteria could co-aggregate. As shown in Figure 7.1, our data demonstrated that the distinct BV associated species co-aggregate with BV associated *G. vaginalis* in different degrees, having *A. vaginae*, *C. tuscaniense*, *M. mulieres* and *S. anginosus* the most pronounced effect in increasing microbial aggregates in dual-species cultures.

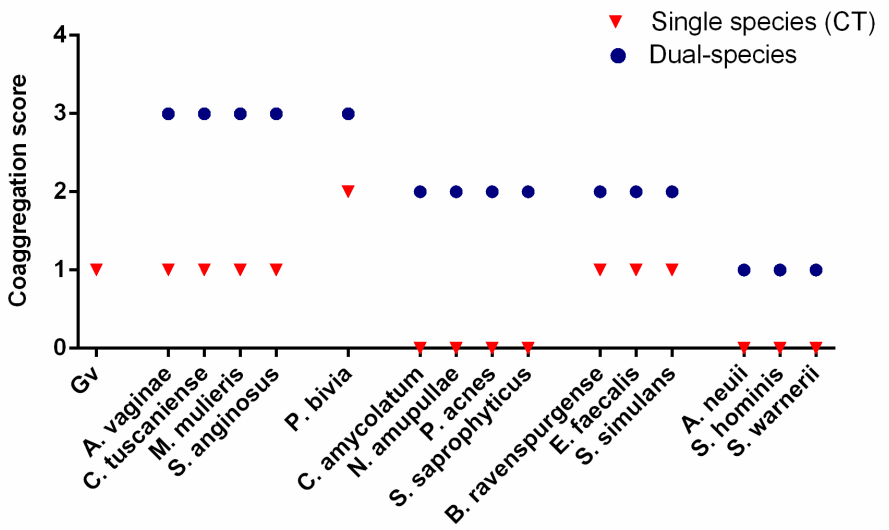


Figure 7.1 Coaggregation score of mono- or dual- bacterial species. Coaggregation score was evaluated as following: 0, no aggregation; 1, small aggregates comprising small visible clusters of bacteria; 2, aggregates comprising larger numbers of bacteria, settling to the center of the well; 3, macroscopically visible clumps comprising larger groups of bacteria which settle to the center of the well; 4, maximum score allocated to describe a large, macroscopically visible clump in the center of the well. Each data point represents the mode.

7.3.2 In *vitro* PNA Gard162 probe specificity

Despite the PNA Gard162 specificity has been previously tested for 22 representative *G. vaginalis* strains and 27 other taxonomically related or pathogenic bacterial species commonly found in vaginal samples [22], it was necessary to analyse the probe specificity for the bacterial species used here, that were not tested before. Thus, we carried out an experiment in order to detect any possible cross-hybridization with any of the BV associated species used herein (Table 7.2). Based on these results, Gard162 probe hybridized with a *G. vaginalis* strain, whereas no hybridization was observed for the other species tested, showing a specificity of 100% as previously reported [22].

Table 7.2 Bacterial species used in PNA-FISH assays and their specificity with PNA Gard162 probe

Bacteria (*n* = 16)	Accession numbers [a]	Gard162 Probe efficiency [b]	Reference
Actinomyces neuii UM067An	KT805271.1	-	This study
Atopobium vaginae FA	absence [c]	-	[22]
Brevibacterium ravenspurgense UM066Br	KT805269.1	-	This study
Corynebacterium amycolatum UM065Ca	KT805275.1	-	This study
Corynebacterium tuscaniense UM137Ct	KT805278.1	-	This study
Enterococcus faecalis UM035	KT614045.1	-	This study
Gardnerella vaginalis UM241	KP996683.1	++++	This study
*Mobiluncus mulieris*ATCC 35239	NZ_GL405260.1	-	[22]
Nosocomiicoccus ampullae UM121Na	KT805272.1	-	This study
Prevotella bivia ATCC 29303	L16475.1	-	[22]
Propionibacterium acnes UM034Pa	KT805265.1	-	This study
Staphylococcus hominis UM224Sh	KT923487.1	-	This study
*Staphylococcus saprohyticus*UM121Ss	KT923484.1	-	This study
Staphylococcus simulans UM059Ss	KT805267.1	-	This study
Staphylococcus warnerii UM224Sw	KT923488.1	-	This study
Streptococcus anginosus UM241b	KT805274.1	-	This study

[a] The accession numbers of partial *16S ribosomal RNA* or *rpoB* gene sequence of vaginal isolates are downloadable from NCBI. [b] The PNA Probe (Gar162) efficiency was tested for each strain, with the following hybridization PNA FISH qualitative evaluation: (−) Absence of hybridization; (++) Moderate hybridization; (+++) Good hybridization; (++++) Optimal hybridization. The table shows the median value from the ten fields of view of each strain. [c] Strain isolated from a woman with BV based on Amsel criteria at Virginia Commonwealth University (VCU) Women's Health Clinic and it was kindly offered by Dr. Kimberly Jefferson.

7.3.3 Quantification of bacterial populations in dual-species biofilms by PNA FISH

Taking advantage of the robustness of the PNA FISH/DAPI method for the differentiation between *G. vaginalis* and other BV associated species (Figure 7.2), we discriminated the bacterial populations in dual-species BV associated biofilms.

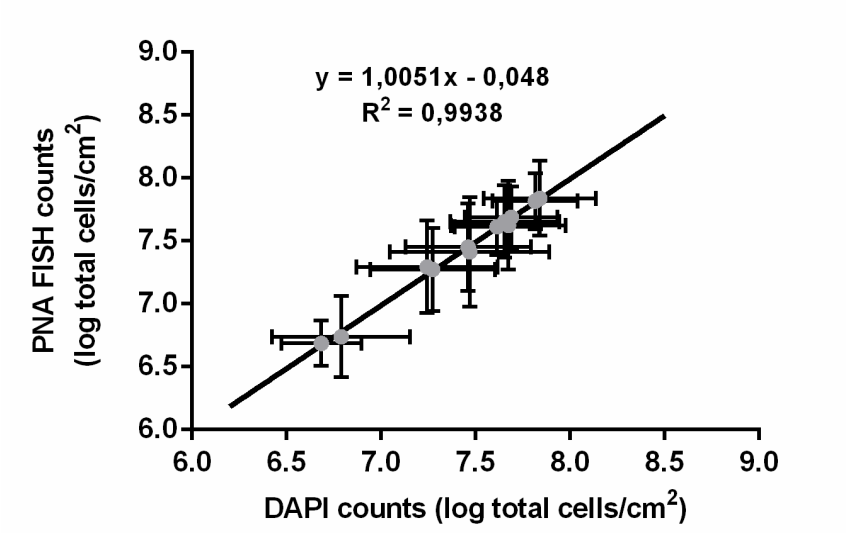


Figure 7.2 Correlation between the PNA FISH counts and the DAPI counts for *G. vaginalis* at different bacterial concentrations. *G. vaginalis* biofilm cells that were identified indirectly by DAPI coincided with the populations quantified by PNA FISH. Each data point represents the mean ± SD.

Initially, we assessed the total cells number in each consortium by DAPI staining. Our results showed that all tested dual-species biofilms had a considerable enhancement on the total number of cells, as compared with mono-species *G. vaginalis* biofilm (Figure 7.3 a). However, under our *in vitro* conditions, we showed that most of the dual-species biofilms were composed majority by the second BV associated species (Figure 7.3 b), and not BV associated *G. vaginalis*, as showed *in vivo* [12,27].

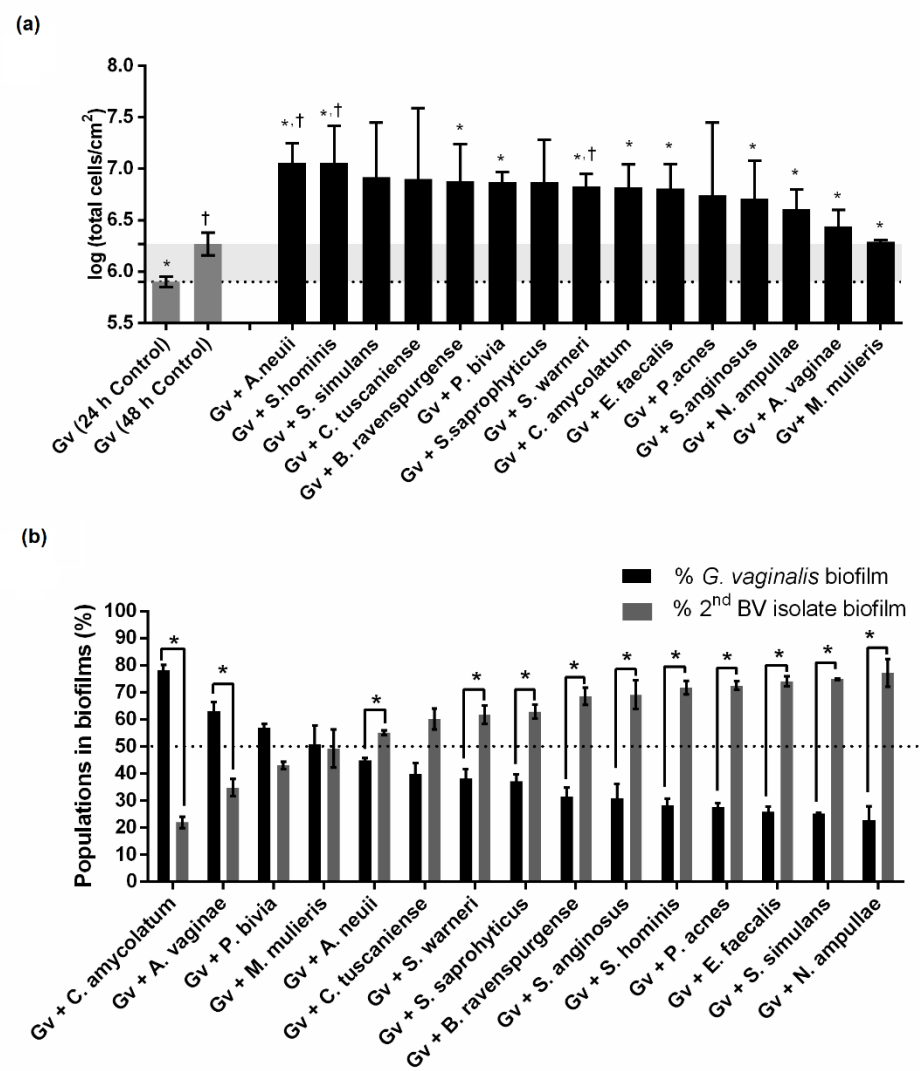


Figure 7.3 Biofilm formation profiles for each BV associated species consortium (10^7 CFU/mL of BV associated *G. vaginalis* & 10^7 CFU/mL of other BV associated bacteria) on dual-species biofilms. (a) Total cells counts by DAPI for mono- (*G. vaginalis* controls) and dual-species biofilms. **(b)** Total percentage of cells detected by PNA FISH for 48 hours biofilms. Each data point represents the mean ± SD. *, †Values are significantly different between the dual-species consortium and the mono- *G. vaginalis* biofilm for 24 hours and 48 hours, respectively (independent samples t-test, $p < 0.05$ for Figure 7.3 a). *Values are significantly different between the bacterial populations of *G. vaginalis* and second BV associated in dual-species biofilms (paired samples t-test for Figure 7.3 b, $p < 0.05$).

Nonetheless, the different optimal conditions of bacterial growth can lead to discrepant bacterial growth-rates [28], and consequently directly impact the composition of *in vitro* BV biofilms. To minimize this, we repeated the co-incubation experiments, using a lower bacterial concentration in order to mimic the vaginal microflora [29], but the overall results did not change significantly (Figure 7.4).

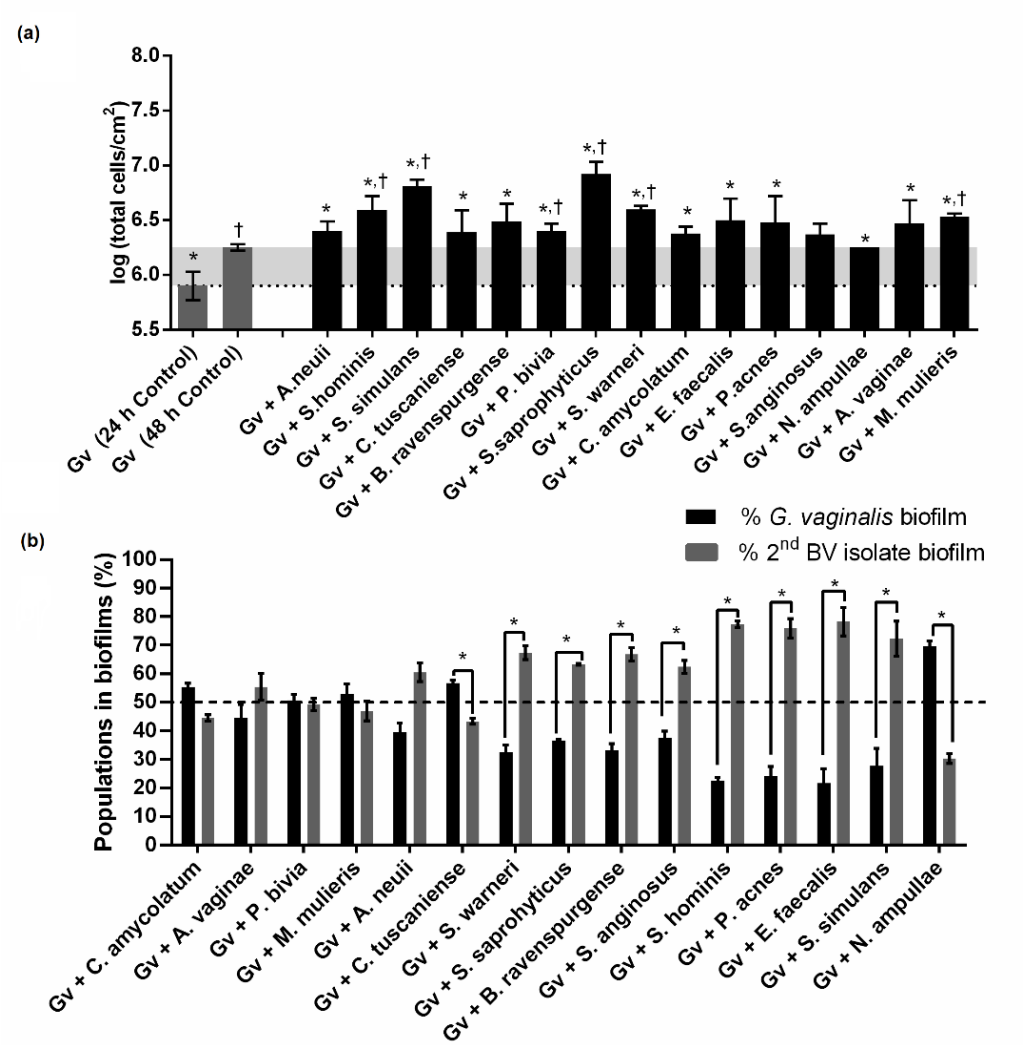


Figure 7.4 Biofilm formation profiles for each BV associated species consortium (10^7 CFU/mL of BV associated *G. vaginalis* & 10^5 CFU/mL of other BV associated bacteria) on dual-species biofilms. (a) Total cells counts by DAPI for mono- (*G. vaginalis* controls) and dual-species biofilms. **(b)** Total percentage of cells detected by PNA FISH for 48 hours biofilms. Each data point represents the mean ± SD. *, † Values are significantly different between the dual-species consortium and the single-*G. vaginalis* biofilm for 24 hours and 48 hours, respectively (independent samples t-test, $p < 0.05$ for Figure 7.4 a). * Values are significantly different between the bacterial populations of *G. vaginalis* and second BV associated in dual-species biofilms (paired samples t-test for Figure 7.4 b, $p < 0.05$).

7.3.4 Analysis of dual-species biofilms by scanning and confocal microscopy

The combined use of FISH with CLSM has been a useful tool to provide a better understanding of the distribution of bacterial population within the multi-species biofilms [30,31]. Thus, in order to visualize the spatial distribution and different architectures of the tested dual-species biofilms, we analysed different z-stacks among the 15 bacterial consortia by FISH/CLSM.

As shown in Figure 7.5, we were able to conclude that a second-BV species could differentially associate with a pre-established *G. vaginalis* biofilm.

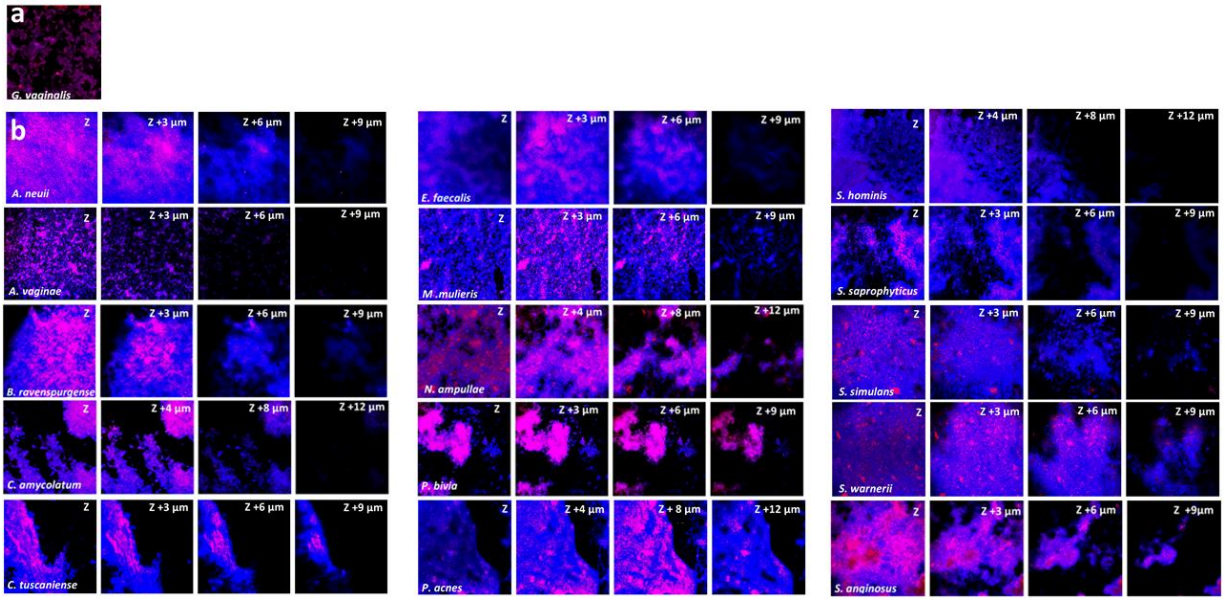


Figure 7.5 An example of data set on the organization of the dual-species BV associated biofilm for 48 hours by confocal laser scanning microscopy (CLSM). (a) *G. vaginalis* mono-species biofilm labeled with PNA-probe Gard162 and DAPI staining corresponding to an experimental control. **(b)** CLSM images of dual-species biofilms for all 15 bacterial consortia. Images were acquired with 512 × 512 resolutions at four different regions of each surface analysed.

We grouped the bacterial consortia with an apparent similar spatial arrangement in the dual-species biofilm, using 3 criteria for bacterial distribution: presence in the top (*T*)*;* and bottom (*B*)*;* layers of the biofilm, as well as the relative distribution and aggregation within the biofilm (*D*). For each criterion, we found two main phenotypes, as represented in a schematic Figure 7.6.

Taken together, these observations indicate that only in 27% of the tested bacterial consortia, the biofilm bottom was predominantly composed by BV associated *G. vaginalis,* with rare spots of second BV associated bacteria (see *B1* in Figure 7.6). Otherwise, we noted that in the majority of the consortia, the secondary BV associated species were able to incorporate the lower layers of this *in vitro* dual-species pre-formed *G. vaginalis* biofilm (see *B2* in Figure 7.6). Conversely, in 33% of the consortia, *G. vaginalis* was absent in the top layers of the biofilm (see *T2* in Figure 7.6), while in the remaining cases it was observed a reduced concentration from the bottom to the top of the biofilm (see *T1* in Figure 7.6). Interestingly, from the bottom to the top layer of the biofilm, we observed that the majority of bacterial consortia (80%) were not well distributed in a typical co-aggregation structure [32,33] (see *D2* in Figure 7.6), but were

rather characterized by separate spatial clusters of *G. vaginalis* (see *D1* in Figure 7.6), leading to the incorporation of BV associated bacteria in low numbers.

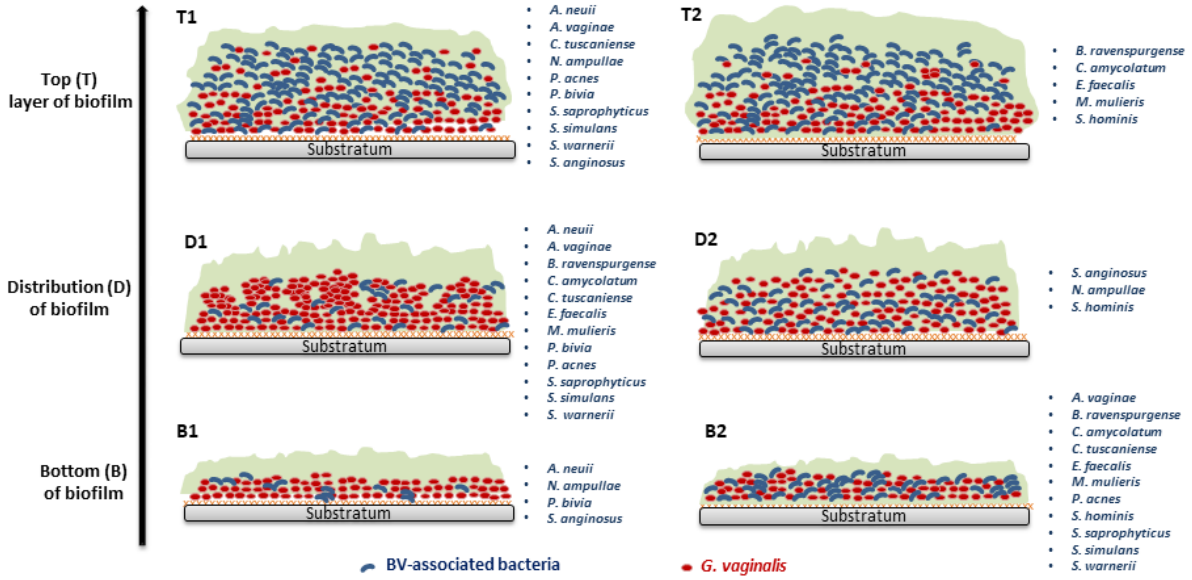


Figure 7.6 Schematic representation of the distribution of dual-species BV associated biofilm structure from the bottom to the biofilm top. (Bottom 1 – B1) Predominantly *G. vaginalis* with rare spots of second BV-isolate in the bottom; (Bottom 2 – B2) Both species in the bottom; (Distribution 1 – D1) *G. vaginalis* exists on clusters in the biofilm; (Distribution 2 – D2) *G. vaginalis* is well distributed in the biofilm; (Top 1 – T1) *G. vaginalis* is reduced from the bottom to the top; (Top 2 – T2) *G. vaginalis* is absence on the top layer of biofilm.

7.3.5 Expression of critical genes related with *G. vaginalis* virulence can be altered in dual-species biofilms

Changes in *G. vaginalis* transcriptome during the establishment of polymicrobial BV biofilm could be a key for unravelling whether the interplay between inter-species enhance *G. vaginalis* virulence. Thus, to decipher the impact of the second-BV species on *G. vaginalis* pathogenicity, we analysed the expression of genes related to cytotoxicity, biofilm formation, antimicrobial resistance and evasion of the immune system (see chapter 5), in cells from mono- and dual-species biofilms.

G. vaginalis produces vaginolysin (*vly*), which might induce vaginal cells lysis [34]. Notably, our results indicated that in dual-species biofilms, the expression levels of *vly* were greatly upregulated when *G. vaginalis* was associated with *A. neuii* or *E. faecalis* ($p < 0.05$; Figure 7.7 a). Furthermore, most of the other tested species also induced a slight increase in *vly* expression, being *B. ravenspurgense* the only species that repressed *G. vaginalis vly*

expression. Regarding sialidase (*sld*), which facilitate the destruction of the protective mucus layer on the vaginal epithelium [35], *E. faecalis*, *B. ravenspurgense* or *A. neuii* considerably upregulated its expression on BV associated *G. vaginalis*. Conversely, *S. anginosus* caused a statistically significant ($p < 0.05$) reduction of *sld* expression (Figure 7.7 b).

It has been proposed that glycosyltransferases are likely to be important for the biosynthesis of exopolysaccharide which in turn is important for biofilm formation required for full virulence of *G. vaginalis* [36]. Not surprisingly, the expression of *HMPREF0424_0821* transcript, which encodes glycosyltransferases type II, was upregulated in all consortia, with statistical significance in 73% of the tested dual-species biofilms (Figure 7.7 c).

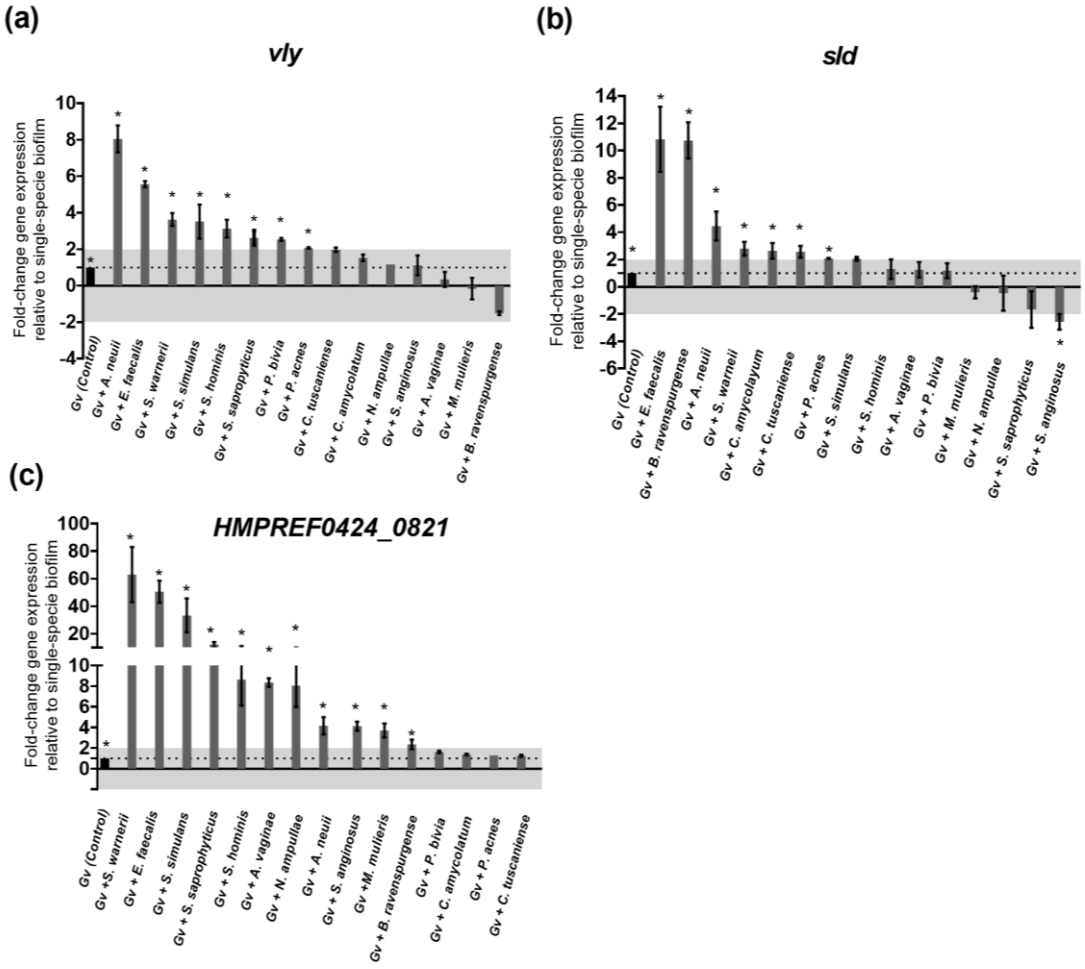


Figure 7.7 Quantification of the transcription of virulence genes, related to cytotoxicity, exfoliation of vaginal epithelium or biofilm formation, in *G. vaginalis* cultured under dual and mono-species biofilms. **(a)** Quantification of vaginolysin (*vly*) transcription. **(b)** Quantification of sialidase (*sld*) transcription. **(c)** Quantification of *HMPREF0424_0821* transcript, which encodes type II glycosyl-transferase. The data indicate the fold-change expression of genes in *G. vaginalis* dual- compared to mono-species *G. vaginalis* biofilm cells. For qPCR experiments, the bars represent the mean and the error bars the standard error of the mean (mean ± SEM). *Values are significantly different between the dual-species consortium and the mono- *G. vaginalis* biofilm under the same conditions (non-parametric Mann-Whitney U, $p < 0.05$).

We also tested the expression of transcripts encoding antimicrobial-specific resistance proteins belonging to efflux pump families (*HMPREF0424_1122* and *HMPREF0424_0156*), since it has been proposed that dual-species biofilm would likely confer an increase in antibiotic tolerance and resistance to mucosal immune defences [37]. Herein, the biggest difference found on *G. vaginalis* transcriptomic profile was caused by *E. faecalis*, in which we observed, on average, an expression of approx. 12-fold higher in the dual-species biofilms than in the mono-species biofilm ($p < 0.05$; Figure 7.8 a, and Figure 7.8 b). Contrariwise, only *S. anginosus* promoted a significantly ($p < 0.05$) reduction of the of transcription levels of *HMPREF0424_0156* gene (Figure 7.8 b).

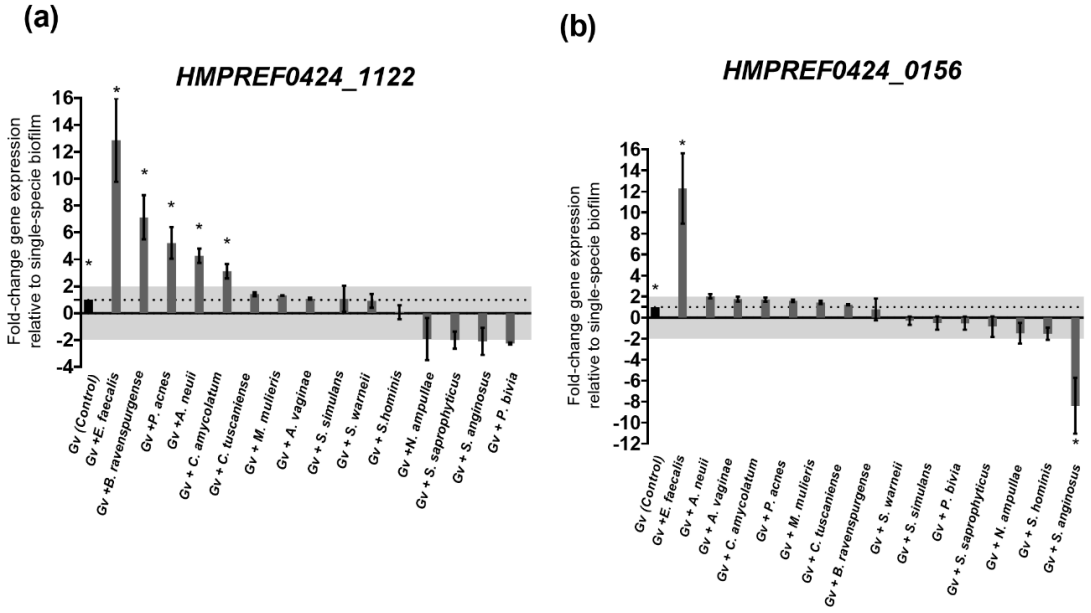


Figure 7.8 Quantification of the transcription of virulence genes, related to antimicrobial resistance, in *G. vaginalis* cultured under dual and mono-species biofilms. **(a)** Quantification of *HMPREF0424_1122* transcript, which encodes a multidrug ABC transporter. **(b)** Quantification of *HMPREF0424_0156* transcript, which encodes Bacitracin transport, ATP-binding protein BcrA. The data indicate the fold-change expression of genes in *G. vaginalis* dual- compared to mono-specie *G. vaginalis* biofilm cells. For qPCR experiments, the bars represent the mean and the error bars the standard error of the mean (mean ± SEM). *Values are significantly different between the dual-species consortium and the mono- *G. vaginalis* biofilm under the same conditions (non-parametric Mann-Whitney U, *p < 0.05).*

Finally, we analysed the expression of *HMPREF0424_1196* transcript, which encodes a Rib-protein that belongs to the α-like protein (Alp)-family of highly repetitive surface antigens [38]. These proteins elicit protective immunity through their inter-strain size variability [36]. Importantly, it was found that *HMPREF0424_1196* transcript levels were greatly elevated ($p < 0.05$) when *E. faecalis* or *S. warneii* was co-cultured with the BV associated *G. vaginalis* pre-established biofilm (Figure 7.9). It is also worthwhile noting that the remaining BV associated

bacteria incited a more slight alteration in transcription levels of *HMPREF0424_1196* gene by BV associated *G. vaginalis* biofilm cells.

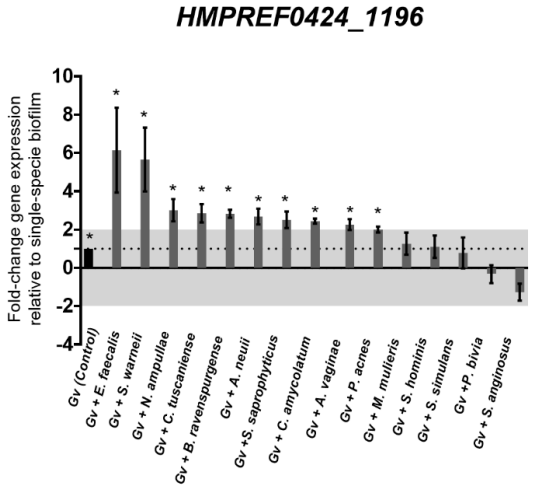


Figure 7.9 Quantification of the transcription of virulence genes, related to evasion of immune response, in *G. vaginalis* cultured under dual and mono-species biofilms. Quantification of *HMPREF0424_1196* transcript, which encodes a Rib-protein. The data indicate the fold-change expression of genes in *G. vaginalis* dual- compared to mono-species *G. vaginalis* biofilm cells. For qPCR experiments, the bars represent the mean and the error bars the standard error of the mean (mean ± SEM). *Values are significantly different between the dual-species consortium and the mono- *G. vaginalis* biofilm under the same conditions (non-parametric Mann-Whitney U, $p < 0.05$).

7.4 Discussion

Microbial cell-cell interactions in the vaginal flora are believed to play an integral role in the development of biofilms and ultimately, they can also generate an array of serious gynaecological and obstetric complications [39-41]. The description of a polymicrobial biofilm on the epithelial surface from BV vaginal biopsy specimens puts *G. vaginalis*, the major component of these multi-species communities, at the centre of BV pathogenesis [12,13,27,35,42]. However, the effect of other species found in BV associated microflora on biofilm formation and its impact in *G. vaginalis* pathogenicity, the presumably primary etiologic agent of BV, are still poorly known [11,13,18]. Importantly, we have previously shown that, by themselves, some BV associated species lack key virulent traits [9]. Therefore, herein, we hypothesized that some, but not all BV associated species could enhance BV associated *G. vaginalis* biofilms mediated virulence. We selected 15 BV associated species previously characterized [9] and assessed their interactions with a BV associated *G. vaginalis* isolate using a dual-species biofilm model.

As we have demonstrated in chapter 6, most of the tested BV-secondary species were able to enhance the total biomass of pre-established *G. vaginalis* biofilms. Curiously, contrary to what has been described *in vivo* [12,27,43], most of our dual-species biofilms were composed by less than 50% of *G. vaginalis.* Discrepancies from *in vitro* and *in vivo* biofilms have been previously reported in other infections [44] and can be attributed to several factors. First, biofilm formation by *G. vaginalis* was pre-formed in tissue-culture plates rather than vaginal epithelium, where the presence of host-derived factors (e.g. mucus production, specific receptors on the epithelial surface) can influence the biofilm development. Unfortunately, this technical limitation is not easy to overcome since, as shown in chapter 3, *G. vaginalis* quickly induces cytotoxic changes and detachment of pre-adhered epithelial cultures.

In an effort to better understand the ecological interactions between BV associated *G. vaginalis* and other BV associated species, we also analysed the architecture and bacterial spatial organization of *in vitro* BV biofilms, since this remains unclear. It has been shown before that microorganisms are not randomly organized within a multi-species biofilm, but follow a pattern that contributes to the fitness of the whole community [45,46] e.g., bacteria are organized in layers, clusters, or are well-mixed [47]. This spatial organization partially determines bacterial survival when the biofilm is exposed to toxic compounds [48]. This depends to a great extent on interactions between the species and their local micro-environments in the matrix with respect to nutrient, oxygen, and metabolite gradients [49]. To date, some studies have been shedding new light on the arrangement and spatial distribution of BV associated biofilms through the analysis of vaginal specimens by FISH [12,13,17,18,27,43]. These studies have mainly focused on *G. vaginalis* and *A. vaginae*. It has been proposed that the vaginal biofilm creates a favourable environment for anaerobic bacteria, due to the presence of an oxygen gradient within the biofilm. By embedding itself within the biofilm, *A. vaginae* can take advantage of the anaerobicity, proliferates and exists in a mutualistic relationship with *G. vaginalis*. Remarkably, our present study provides new insights into the spatial distribution of multiple dual-species biofilms, since we found striking differences in the different consortia, suggesting that the type of bacterial interaction is species-specific in the presence of a polymicrobial community. Interestingly, the most predominant dual-species biofilm phenotype was characterized by the presence of both species on the biofilm bottom, with *G. vaginalis* present in clusters in the intermediate layers, with higher concentration in the lower biofilm layers. This *G. vaginalis* spatial distribution in mixed biofilms could reflect a protective mode for *G. vaginalis* maintenance in adverse conditions, such as in presence of antimicrobial compounds [50].

Noteworthy, bacterial biofilms may also suppress certain virulence factors while others are activated in order to evade immune defenses and survive challenging conditions. Therefore,

we also analysed how the different consortia could influence *G. vaginalis* key virulence genes [35,36,51]. Several studies have highlighted the role of *vly* gene in *G. vaginalis* virulence [3,34,52-54]. The *vly* gene belongs to the cholesterol-dependent cytolysins (CDCs), a family of pore-forming toxins, which cause cytotoxicity on vaginal epithelium [34]. Interestingly, we recently showed that *vly* expression can vary according to *G. vaginalis* phenotype, in which it was found higher *vly* transcript levels in a planktonic than in mono-BV associated *G. vaginalis* biofilm cells (see chapter 5). The lower levels of expression of *vly* transcript in single biofilms might reflect the more chronic nature of vaginal colonization by BV associated *G. vaginalis* and serve as a means towards preventing a host immune response. Importantly, based on our present study, we also propose that under specific ecological conditions, some BV associated bacteria, in particular *A. neuii* or *E. faecalis*, can trigger an overexpression of *vly* transcript by *G. vaginalis* cells. Consequently, the complex interplay between BV associated *G. vaginalis* and specific BV associated species can enhance vaginal desquamation and eventual formation of clue cells (Figure 7.10).

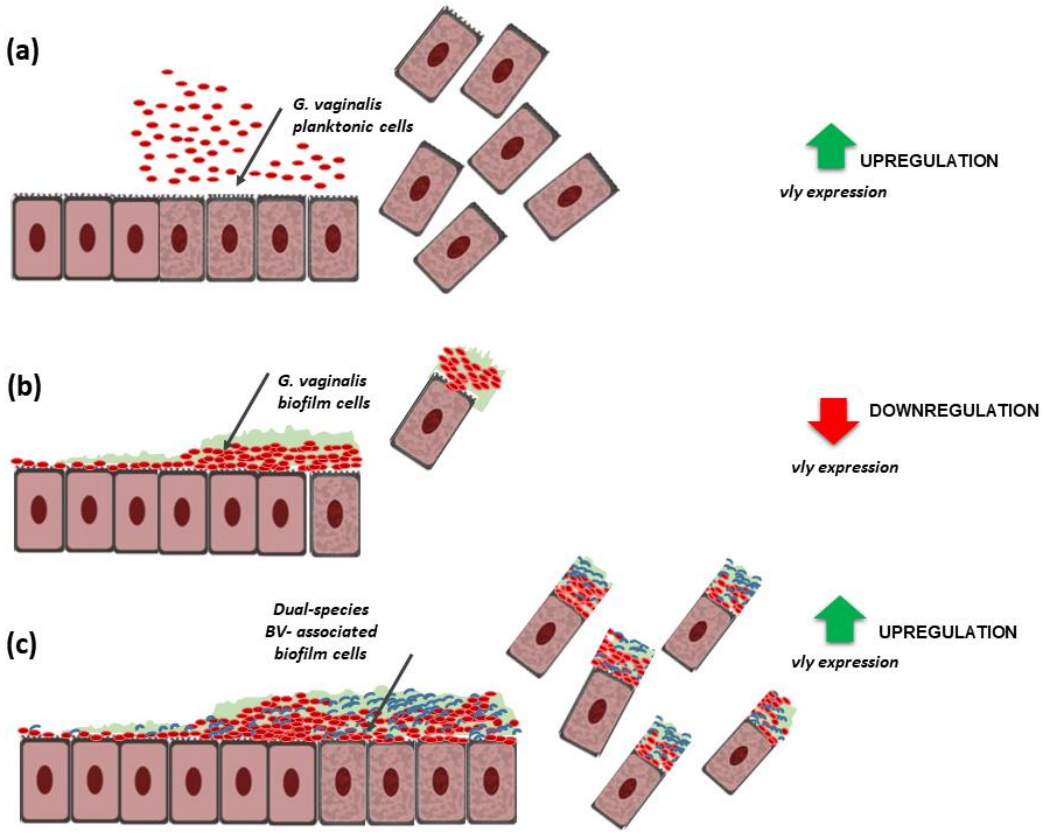


Figure 7.10 Hypothetical model of *G. vaginalis* vaginolysin (*vly*)-mediated cytotoxicity in different bacterial phenotypes. (a) Planktonic cells. **(b)** *G. vaginalis* mono-species biofilm. **(c)** Dual-species biofilms, corresponding to a pre-formed BV associated *G. vaginalis* biofilm in association with second-BV associated bacteria.

The same trend was observed with *sld*, which is known to facilitate the destruction of the protective mucus layer on the vaginal epithelium by hydrolysis of sialic acid on the glycans of mucous membranes. This process possibly facilitates adhesion of bacteria on the vaginal epithelium since it has been linked with the development of biofilm [35]. Moreover, the biofilm formation was also likely affected by the glycosyltransferases type II [36]. Furthermore, similar to what was described in other mixed biofilm studies [37,55], in the present study we also observed that some second BV associated species might likely confer an increase in antibiotic tolerance and resistance to mucosal immune defenses, contributing to its persistence, and BV recurrence.

Taken together, this study reveals that molecular interactions were very specific to each consortium, confirming our original hypothesis that not all BV-secondary bacteria contribute to the enhancement of BV pathogenesis by influencing *G. vaginalis* virulence. Importantly, our results reline the importance of *E. faecalis* and *A. neuii,* since these BV associated bacteria were able to significantly induce the expression of all tested virulence genes in dual-species biofilms (Figure 7.11).

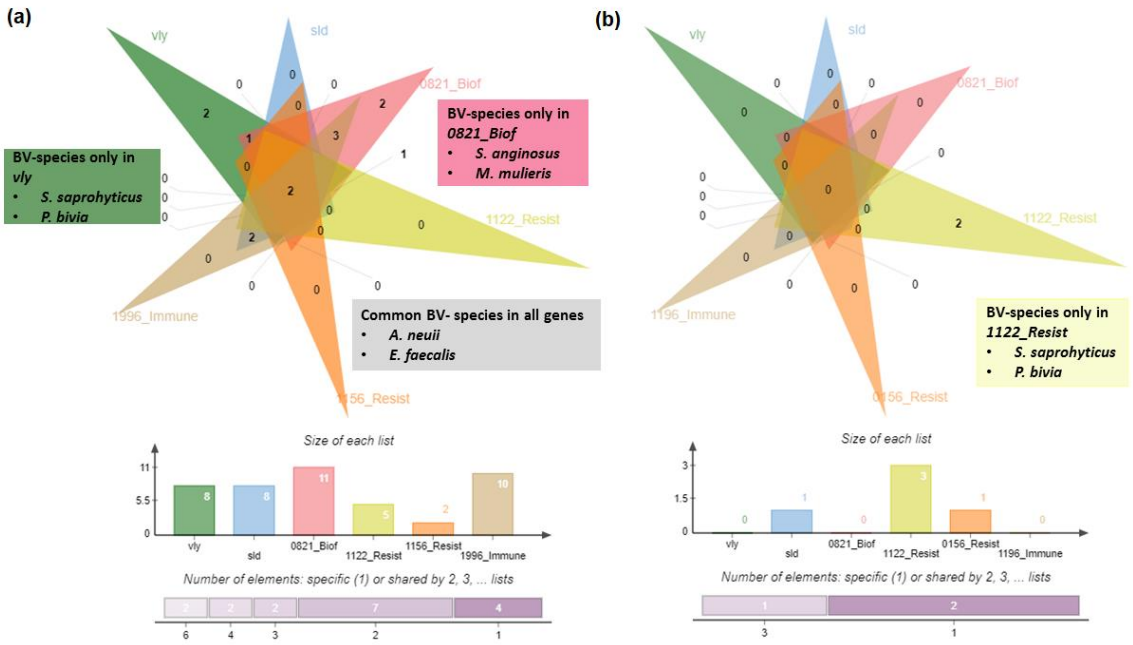


Figure 7.11 Venn diagram summarize the transcript levels of all six potential virulence genes of *G. vaginalis* among 15 bacterial consortia (a) *G. vaginalis* upregulation in dual-species biofilms (fold-change difference ≥ 2), in which *A. neuii* and *E. faecalis* caused an overexpression of all 6 transcripts in *G. vaginalis*. Specific molecular interactions of some consortia were found, leading to a unique change in *G. vaginalis* transcriptome for vly (Gv/*P. bivia* or Gv/*S. saprophyticus* dual-species biofilms) and *HMPREF0424_0821* transcripts (Gv/*M. mulieris* or Gv/ *S. anginosus* dual-species biofilms). **(b)** Downregulation in dual-species biofilms (fold-change ≤ -2). A specific downregulation of *HMPREF0424_1122* transcript was found to Gv/*S. saprophyticus* o Gv/*P. bivia* dual-species biofilms.

Of note that *E. faecalis* have also been isolated from patients with urinary tract infections [56] and vaginitis [57-59], whereas *A. neuii* can be isolated from a variety of infections [60], including genitourinary infections [61,62]. Both bacterial species have different factors implicated in the pathogenesis [59,63], which may contribute to aggravate the outcomes, sequelae and recurrence of BV. In any case, more basic research in need to fully understand the pathway and functions of these potential virulence genes [64].

Overall, the evidence from this study points towards the idea that social networking between BV associated bacteria can profoundly affect the BV progress and clinical outcome. However, more research is needed to provide a better mechanistic insight into the complex interplay between *G. vaginalis*, other BV associated species, and their eukaryotic hosts. Understanding the molecular basis and biological effect of these inter-bacterial processes may provide novel information necessary to define new targets and strategies for BV control.

7.5 References

[1] Hardy, L., Cerca, N., Jespers, V., Vaneechoutte, M. and Crucitti, T. **(2017)**. Bacterial biofilms in the vagina. *Res Microbiol* 168, 865-74.

[2] Jung, H.S., Ehlers, M.M., Lombaard, H., Redelinghuys, M.J. and Kock, M.M. **(2017)**. Etiology of bacterial vaginosis and polymicrobial biofilm formation. *Crit Rev Microbiol* 43, 651-67.

[3] Abdelmaksoud, A.A. et al. **(2017)**. Association between statin use, the vaginal microbiome, and *Gardnerella vaginalis* vaginolysin-mediated cytotoxicity. *PLoS One*12, e0183765.

[4] Hilbert, D.W., Schuyler, J.A., Adelson, M.E., Mordechai, E., Sobel, J.D. and Gygax, S.E. **(2017)**. *Gardnerella vaginalis* population dynamics in bacterial vaginosis. *Eur J Clin Microbiol Infect Dis* 36, 1269-78.

[5] Schwebke, J.R., Muzny, C.A. and Josey, W.E. **(2014)**. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis* 210,338-43.

[6] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

[7] Gardner, H.L. and Dukes, C.D. **(1955)**. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *Am J Obstet Gynecol* 69, 962-76.

[8] Hickey, R.J. and Forney, L.J. **(2014)**. *Gardnerella vaginalis* does not always cause bacterial vaginosis. *J Infect Dis* 210,1682-3.

[9] Alves, P., Castro, J., Sousa, C., Cereija, T.B. and Cerca, N. **(2014)**. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *J Infect Dis* 210, 593-6.

[10] Patterson, J.L., Stull-Lane, A., Girerd, P.H. and Jefferson, K.K. **(2010)**. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 156, 392-99.

[11] Machado, A., Jefferson, K.K. and Cerca, N. **(2013)**. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 14, 12004-12.

[12] Swidsinski, A. et al. **(2013)**. Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). *Histol Histopathol* 29, 567-87.

[13] Hardy, L., Jespers, V., Dahchour, N., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M. and Crucitti, T. **(2015)**. Unravelling the bacterial vaginosis-associated biofilm: a multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence *in situ* hybridization assay using peptide nucleic acid probes. *PLoS One* 10, e0136658.

[14] Machado, A. and Cerca, N. **(2015)**. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *J Infect Dis* 212,1856-61.

[15] Marrazzo, J.M. **(2011)**. Interpreting the epidemiology and natural history of bacterial vaginosis: are we still confused? *Anaerobe* 17, 186-90.

[16] Bradshaw, C.S. et al. **(2006)**. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* 193, 1478-86.

[17] Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., Lochs, H. and Verstraelen, H. **(2008)**. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 198, 97. e1-97. e6.

[18] Hardy, L., Jespers, V., Abdellati, S. and De Baetselier, I. **(2016)**. A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm. *Sex Transm Infect* pii: sextrans-2015-052475.

[19] Cox, C., Watt, A.P., McKenna, J.P. and Coyle, P.V. **(2016)**. *Mycoplasma hominis* and *Gardnerella vaginalis* display a significant synergistic relationship in bacterial vaginosis. *Eur J Clin Microbiol Infect Dis* 35, 481-7.

[20] Reid, G., McGroarty, J.A., Domingue, P.G., Chow, A.W., Bruce, A.W., Eisen, A. and Costerton, J.W. **(1990)**. Coaggregation of urogenital bacteria *in vitro* and *in vivo*. *Current Microbiology* 20, 47-52.

[21] Freitas, A.I., Vasconcelos, C., Vilanova, M. and Cerca, N. **(2014)**. Optimization of an automatic counting system for the quantification of *Staphylococcus epidermidis* cells in biofilms. *J Basic Microbiol* 54, 750-7.

[22] Machado, A. et al. **(2013)**. Fluorescence *in situ* Hybridization method using Peptide Nucleic Acid probes for rapid detection of *Lactobacillus* and *Gardnerella* spp. *BMC Microbiology* 13, 82.

[23] França, A., Freitas, A.I., Henriques, A.F. and Cerca, N. **(2012)**. Optimizing a qPCR gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol. *PloS One* 7, e37480.

[24] Carvalhais, V., Delgado-Rastrollo, M., Melo, L.D. and Cerca, N. **(2013)**. Controlled RNA contamination and degradation and its impact on qPCR gene expression in *S. epidermidis* biofilms. *J Microbiol Methods* 95, 195-200.

[25] Peters, B.M., Jabra-Rizk, M.A., O'May, G.A., Costerton, J.W. and Shirtliff, M.E. **(2012)**. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev* 25, 193-213.

[26] Reardon-Robinson, M.E., Wu, C., Mishra, A., Chang, C., Bier, N., Das, A. and Ton-That, H. **(2014)**. Pilus hijacking by a bacterial coaggregation factor critical for oral biofilm development. *Proc Natl Acad Sci U S A* 111, 3835-40.

[27] Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L.P. and Lochs, H. **(2005)**. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 106, 1013-23.

[28] Aldea, M., Jenkins, K. and Csikász-Nagy, A. **(2017)**. Growth rate as a direct regulator of the start network to set cell size. *Front Cell Dev Biol* 5, 57.

[29] Xiao, B., Niu, X., Han, N., Wang, B., Du, P., Na, R., Chen, C. and Liao, Q. **(2016)**. Predictive value of the composition of the vaginal microbiota in bacterial vaginosis, a dynamic study to identify recurrence-related flora. *Sci Rep* 6, 26674.

[30] Palmer, R.J., Jr. and Sternberg, C. **(1999)**. Modern microscopy in biofilm research: confocal microscopy and other approaches. *Curr Opin Biotechno*l 10, 263-8.

[31] Karygianni, L., Follo, M., Hellwig, E., Burghardt, D., Wolkewitz, M., Anderson, A. and Al-Ahmad, A. **(2012)**. Microscope-based imaging platform for large-scale analysis of oral biofilms. *Appl Environ Microbiol* 78, 8703-11.

[32] Almstrand, R., Daims, H., Persson, F., Sörensson, F. and Hermansson, M. **(2013)**. New methods for analysis of spatial distribution and coaggregation of microbial populations in complex biofilms. *Appl Environ Microbiol* 79, 5978-87.

[33] Katharios-Lanwermeyer, S., Xi, C., Jakubovics, N.S. and Rickard, A.H. **(2014)**. Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development. *Biofouling* 30, 1235-51.

[34] Gelber, S.E., Aguilar, J.L., Lewis, K.L. and Ratner, A.J. **(2008)**. Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *J Bacteriol* 190, 3896-903.

[35] Hardy, L., Jespers, V., Van den Bulck, M., Buyze, J., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M. and Crucitti, T. **(2017)**. The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PLoS One* 12, e0172522.

[36] Yeoman, C.J. et al. **(2010)**. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS One* 5, e12411.

[37] Nair, N., Biswas, R., Götz, F. and Biswas, L. **(2014)**. Impact of *Staphylococcus aureus* on pathogenesis in polymicrobial infections. *Infection and Immun* 82, 2162-9.

[38] Lindahl, G., Stalhammar-Carlemalm, M. and Areschoug, T. **(2005)**. Surface proteins of *Streptococcus agalactiae* and related proteins in other bacterial pathogens. *Clin Microbiol Rev* 18, 102-27.

[39] Menard, J.P., Mazouni, C., Salem-Cherif, I., Fenollar, F., Raoult, D., Boubli, L., Gamerre, M. and Bretelle, F. **(2010)**. High vaginal concentrations of *Atopobium vaginae* and *Gardnerella vaginalis* in women undergoing preterm labor. *Obstet Gynecol* 115, 134-40.

[40] Leitich, H., Bodner-Adler, B., Brunbauer, M., Kaider, A., Egarter, C. and Husslein, P. **(2003)**. Bacterial vaginosis as a risk factor for preterm delivery: A meta-analysis. *Am J Obstet Gynecol* 189, 139-47.

[41] Mania-Pramanik, J., Kerkar, S.C. and Salvi, V.S. **(2009)**. Bacterial vaginosis: a cause of infertility? *Int J STD AIDS* 20, 778-81.

[42] Verstraelen, H. and Swidsinski, A. **(2013)**. The biofilm in bacterial vaginosis: implications for epidemiology, diagnosis and treatment. *Curr Opin Infect Dis* 26, 86-9.

[43] Swidsinski, A. et al. **(2010)**. *Gardnerella* biofilm involves females and males and is transmitted sexually. *Gynecol Obstet Invest* 70, 256-63.

[44] Lebeaux, D., Chauhan, A., Rendueles, O. and Beloin, C. **(2013)**. From *in vitro* to *in vivo* models of bacterial biofilm-related infections. *Pathogens* 2, 288-356.

[45] Marsh, P. and Bradshaw, D. **(1995)**. Dental plaque as a biofilm. *J Ind Microbiol* 15, 169-75.

[46] Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E. and Handley, P.S. **(2003)**. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 11, 94-100.

[47] Elias, S. and Banin, E. **(2012)**. Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol Rev* 36, 990-1004.

[48] Simoes, M., Simões, L.C. and Vieira, M.J. **(2009)**. Species association increases biofilm resistance to chemical and mechanical treatments. *Water Res* 43, 229-37.

[49] Stewart, P.S. and Franklin, M.J. **(2008)**. Physiological heterogeneity in biofilms. *Nat Rev Microbiol* 6, 199-210.

[50] Jefferson, K.K. **(2004)**. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett* 236, 163-73.

[51] Ahmed, A. et al. **(2012)**. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol* 194, 3922-37.

[52] Cauci, S., Monte, R., Ropele, M., Missero, C., Not, T., Quadrifoglio, F. and Menestrina, G. **(1993)**. Pore-forming and haemolytic properties of the *Gardnerella vaginalis* cytolysin. *Mol Microbiol* 9, 1143-55.

[53] Randis, T.M., Kulkarni, R., Aguilar, J.L. and Ratner, A.J. **(2009)**. Antibody-based detection and inhibition of vaginolysin, the *Gardnerella vaginalis* cytolysin. *PLoS One* 4, e5207.

[54] Castro, J., Martins, A.P., Rodrigues, M.E. and Cerca, N. **(2018)**. *Lactobacillus crispatus* represses vaginolysin expression by BV associated *Gardnerella vaginalis* and reduces cell cytotoxicity. *Anaerobe* 50, 60-63.

[55] Burmølle, M., Webb, J.S., Rao, D., Hansen, L.H., Sørensen, S.J. and Kjelleberg, S. **(2006)**. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol* 72, 3916-23.

[56] Kline, K.A. and Lewis, A.L. **(2016)**. Gram-positive uropathogens, polymicrobial urinary tract infection, and the emerging microbiota of the urinary tract. *Microbiol Spectr* 4, 2.

[57] Fan, A., Yue, Y., Geng, N., Zhang, H., Wang, Y. and Xue, F. **(2013)**. Aerobic vaginitis and mixed infections: comparison of clinical and laboratory findings. *Arch Gynecol Obste*t 287, 329-35.

[58] Tansarli, G.S., Skalidis, T., Legakis, N.J. and Falagas, M.E. **(2017)**. Abnormal vaginal flora in symptomatic non-pregnant and pregnant women in a Greek hospital: a prospective study. *Eur J Clin Microbiol Infect Dis* 36, 227-232.

[59] Wong, V.K., Turmezei, T.D. and Weston, V.C. **(2011)**. Actinomycosis. *BMJ* 343, d6099.

[60] Zelyas, N., Gee, S., Nilsson, B., Bennett, T. and Rennie, R. **(2016)**. Infections caused by *Actinomyces neuii*: a case series and review of an unusual bacterium. *Can J Infect Dis Med Microbiol* 2016, 6017605.

[61] Hall, V., Talbot, P.R., Stubbs, S.L. and Duerden, B.I. **(2001)**. Identification of clinical isolates of actinomyces species by amplified 16S ribosomal DNA restriction analysis. *J Clin Microbiol* 39, 3555-62.

[62] Mann, C., Dertinger, S., Hartmann, G., Schurz, R. and Simma, B. **(2002)**. *Actinomyces neuii* and neonatal sepsis. *Infection* 30, 178-80.

[63] Saffari, F., Dalfardi, M.S., Mansouri, S. and Ahmadrajabi, R. **(2017)**. Survey for correlation between biofilm formation and virulence determinants in a collection of pathogenic and fecal *Enterococcus faecalis* isolates. *Infect Chemother* 49, 176-83.

[64] Pundir, S., Martin, M.J. and O'Donovan, C. **(2016)**. UniProt Tools. *Curr Protoc Bioinformatics* 53, 1.29.1-1.29.15.

CHAPTER 8

Concluding remarks and future work

Summary

This chapter presents a summary of the thesis findings, major outcomes, limitations and suggestions for future directions.

8.1 Concluding remarks

This aim of this work was to provide novel insight into the pathogenic potential of *Gardnerella vaginalis* strains isolated from women with BV relative to those isolated from healthy women. The role of *G. vaginalis* in BV is not without controversy, since *G. vaginalis* colonization does not always lead to BV development. Understanding *G. vaginalis* physiology and its role on the etiology and pathogenesis of BV could enable the development of strategies to improve the clinical success of treatment.

It is likely that differences between strains of *G. vaginalis* that determine whether a commensal *versus* a pathologic relationship with the host will ensue, include multiple genes or pathways. Importantly, recent genomic evidence reveals that *G. vaginalis* is so genetically heterogeneous, that the taxon might, in fact, harbour different sub-species or even distinct species, with different virulence potential [1-5]. Furthermore, the association of *G. vaginalis* with different clinical phenotypes could be explained by different cytotoxicity and biofilm-forming capacities of the strains [6]. Thus, we undertook an *in vitro* characterization of the phenotypic and genotypic features of *G. vaginalis* strains isolated from women with BV and from healthy women. Additionally, a transcriptomic analysis was also conducted comparing the gene expression profile of planktonic and biofilm cells of a BV associated *G. vaginalis* strain. Moreover, the impact of other BV associated species on pre-established *G. vaginalis* biofilms was also addressed.

This thesis intended to answer several key points related to virulence aspects of *G. vaginalis*, as proposed in chapter 1.

I. Can BV associated *G. vaginalis* isolates exhibit more virulence factors than non-BV isolates?

Our findings clearly demonstrate phenotypic differences between non-BV and BV associated *G. vaginalis* isolates that could impact the ability of this organism to cause disease. We hypothesize that colonization by a subset of *G. vaginalis* is the trigger for BV development (Figure 8.1). By displacing lactobacilli, adhered *G. vaginalis* will then start to form a biofilm that will subsequently promote the incorporation of secondary colonizers and this mixed biofilm will ultimately become recalcitrant to antimicrobial therapy. It is noteworthy that the BV and non-BV associated *G. vaginalis* strains that were tested could not be differentiated in relation to its presence in the healthy vaginal microflora (avirulent state) or in the BV associated vaginal microflora (virulent state) using the clade-genotyping approach, as previously suggested [2].

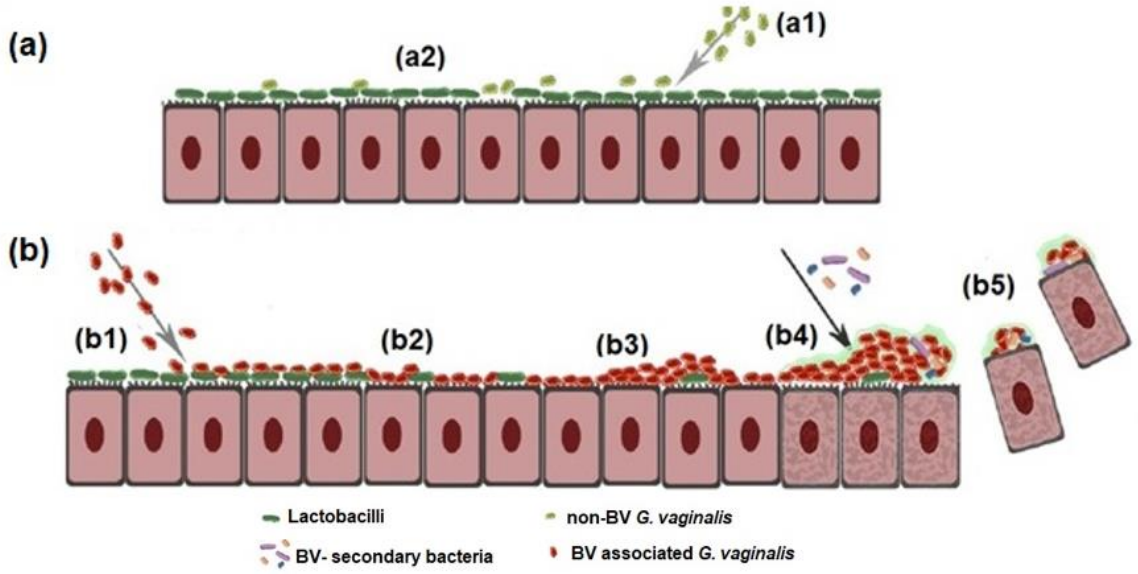


Figure 8.1 Representative model for *G. vaginalis* vaginal colonization. (a) Non-virulent *G. vaginalis* can adhere at small numbers to lactobacilli dominated vaginal epithelium (a1), and can successfully colonize the human vagina (a2) at low numbers. **(b)** Virulent *G. vaginalis* can adhere at high numbers to lactobacilli dominated vaginal epithelial (b1) and can successfully displace lactobacilli from the epithelium (b2). Without the competition of lactobacilli, *G. vaginalis* starts to multiply (b3) and eventually develops a biofilm (b4). At this stage, other microorganisms will incorporate the biofilm and the activity of specific enzymes will result in damaging of the epithelium, resulting on the release of the characteristic "clue cells" (b5). Adapted from Cerca [7].

II. Can the differential response to innate immune components by non-BV and BV associated *G. vaginalis* isolates be key in BV development?

Because *in vitro* conditions can strongly influence bacterial phenotypes, we also characterized virulence properties of non-BV and BV associated isolates in the presence of innate immune molecules (LYS, LF and HBD2). We hypothesized that under these conditions, the previous virulence differences found between both *G. vaginalis* groups could be enhanced. However, this was not the case and the effect of the 3 components was similar between non-BV and BV associated *G. vaginalis* isolates, suggesting that a better adaptation to the host immune components is not a key factor differentiating between isolates from women with BV and from healthy women. Strikingly, we found that growth, initial adhesion and biofilm formation were strongly affected by LYS, but at similar levels in both *G. vaginalis* groups.

III. What happens to *G. vaginalis* virulence profile when growing as a biofilm?

Our data indicated that BV associated *G. vaginalis* changes its transcriptomic profile when growing as a biofilm, resulted in a distinct physiologic status that may promote the chronic and recurrent nature of BV. These changes are likely important for biofilm persistence and, consequently, for the virulence of this bacterium, suggesting that biofilms indeed play a key role in BV development.

IV. Are other BV associated species cooperating with *G. vaginalis* and enhancing its virulence?

The evidence from this work points towards the idea that social networking between BV associated bacteria can profoundly affect the BV progress and clinical outcome. Importantly, our transcriptomic findings seem to indicate that a significant role can be attributed to *Enterococcus faecalis* and *Actinomyces neuii* in the enhancement of *G. vaginalis* virulence, while the other tested species had a lower or no impact in the expression of virulence genes by *G. vaginalis*. Finally, this *in vitro* dual-species biofilm study cast a new light on how BV associated species can modulate the virulence aspects of *G. vaginalis,* contributing to better understanding the development of BV associated biofilms.

8.2 Study limitations

This study has two main limitations. The first is that our dual-species biofilm study did not include all of the bacterial species that have been found to be associated with BV [8-10], because many bacteria are unculturable, or difficult to isolate and maintain under *in vitro* conditions. Furthermore, it is now well known that women with BV can be colonized with multiple strains of *G. vaginalis* [3]. However, we only isolated one single *G. vaginalis* strain from each vaginal sample. The second is the fact that the growth medium did not contain all of the factors found *in vivo*, and some *in vivo* cues may influence *G. vaginalis* phenotype. In addition, in our *in vitro* model, *G. vaginalis* did not deal with immune cells. Together, these limitations highlight the challenges in assessing *G. vaginalis* role in BV development using *in vitro* models.

8.3 Future perspectives

The described work highlighted several aspects regarding the differences of the pathogenic potential of commensal *versus* clinical *G. vaginalis* isolates. However, several questions remain open and may be taken into consideration in the near future.

Firstly, we would like to confirm our phenotypic observations of both *G. vaginalis* groups using an *ex vivo* vaginal model, such as porcine vaginal tissue, that would allow mimicking more closely the *in vivo* conditions.

Secondly, the dynamic equilibrium of the vaginal microbiome can be altered by environmental factors and external interferences (*e.g.,* antibiotics, vaginal hygiene, sexual intercourse, hormone therapy). These alterations can result in microbial imbalances or dysbiosis in the female reproductive tract (FRT). The normally commensal bacterial communities present in the FRT can, under certain circumstances, become pathogenic if a shift in the equilibrium favours their competitiveness [11]. Thus, future work needs to be done so as to clarify the impact of the environmental factors and external interferences in the vaginal microbiome. Furthermore, the individual or collective contribution of bacteria to the development of BV should be elucidated with a combination of omics and deep sequencing methods that examine *G. vaginalis* in the context of the entire microbiome. These findings might elucidate whether only certain lineages of *G. vaginalis* are pathogenic and others are natural commensals; or whether *G. vaginalis* is an opportunistic pathogen present in vaginal microflora, that under specific conditions might turn into in a more virulent state [12].

Thirdly, more research is needed to understand the molecular basis and biological effect of the complex interplay between *G. vaginalis*, other BV associated species, and their eukaryotic hosts. In this thesis a limited transcriptome analysis was performed but ideally, a RNA-sequencing analysis of BV associated dual-species biofilms, consisting of *G. vaginalis* & *Enterococcus faecalis* and *G. vaginalis* & *Actinomyces neuii,* formed in vaginal cells pre-coated with endogenous bacteria might shed a new light on virulence-related genes and provide novel information necessary to define new targets and strategies for BV control.

Lastly, despite tremendous research efforts, our current understanding of the physiology and complexity of BV associated biofilms is still inadequate, especially as it is based mostly on studies of mono- or dual-species biofilms. Therefore, there is a pressing need for more research directed at delineating ecological interactions within multi-species BV associated biofilms and the effects of such interactions on the development, nature and survival of the biofilm community.

This is just a small fraction of all the work that can be done…

8.4 References

[1] Ahmed, A. et al. **(2012)**. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol* 194, 3922-37.

[2] Balashov, S.V., Mordechai, E., Adelson, M.E. and Gygax, S.E. **(2014)**. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* 63, 162-75.

[3] Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A. and Pleckaityte, M. **(2017)**. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infect Dis* 17, 394.

[4] Schellenberg, J.J., Paramel Jayaprakash, T., Withana Gamage, N., Patterson, M.H., Vaneechoutte, M. and Hill, J.E. **(2016)**. *Gardnerella vaginalis* subgroups defined by *cpn60* sequencing and sialidase activity in Isolates from Canada, Belgium and Kenya. *PLoS One* 11, e0146510.

[5] Yeoman, C.J. et al. **(2010)**. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS One* 5, e12411.

[6] Harwich, M., Alves, J., Buck, G., Strauss, J., Patterson, J., Oki, A., Girerd, P. and Jefferson, K. **(2010)**. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 11, 375.

[7] Cerca, N. **(2015)**. A vaginose bacteriana e o seu impacto nas mulheres portuguesas. *Magazine da SPM* 4, 1-4.

[8] Oakley, B.B., Fiedler, T.L., Marrazzo, J.M. and Fredricks, D.N. **(2008)**. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Appl Environmental Microbiol* 74, 4898-909.

[9] Srinivasan, S. and Fredricks, D.N. **(2009)**. The human vaginal bacterial biota and bacterial vaginosis. *Interdiscip Perspect Infect Dis* 2008, 2008.

[10] Srinivasan, S., Liu, C., Mitchell, C.M., Fiedler, T.L., Thomas, K.K., Agnew, K.J., Marrazzo, J.M. and Fredricks, D.N. **(2010)**. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One* 5, e10197.

[11] Huang, B., Fettweis, J.M., Brooks, J.P., Jefferson, K.K. and Buck, G.A. **(2014)**. The changing landscape of the vaginal microbiome. *Clin Lab Med* 34, 747-61.

[12] Schellenberg, J.J., Patterson, M.H. and Hill, J.E. **(2017)**. *Gardnerella vaginalis* diversity and ecology in relation to vaginal symptoms. *Res Microbiol* 168, 837-44.