Universidade do Minho
Escola de Engenharia

José António Baptista Machado Soares

The role of *Gardnerella vaginalis* biofilms in Bacterial vaginosis

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PhD in Biomedical Engineering

This work was realized under supervision of:
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October 2013

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TITLE OF THE THESIS: The role of *Gardnerella vaginalis* biofilms in Bacterial vaginosis

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PhD in Biomedical Engineering

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"One father is more than a hundred schoolmasters."

George Herbert

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Abstract/Sumário

Abstract

Bacterial vaginosis (BV) is the leading vaginal disorder in women of reproductive age worldwide. BV is characterized by the replacement of beneficial bacteria (lactobacilli) and the augmentation of anaerobic bacteria. *Gardnerella vaginalis* is a predominant bacterial species, however, whether it is a cause or an effect is unclear and the etiology of BV remains unknown. This has consequently led to limitations in the diagnosis and adequate treatment of BV. Aiming to improve BV diagnostic, we designed the first Peptide Nucleic Acid (PNA) Fluorescence *In Situ* Hybridization (FISH) methodology to increase the specificity and sensitivity of the detection of *Lactobacillus* spp. and *G. vaginalis* strains in vaginal samples. We performed a prospective study using a collection of vaginal samples that enabled the validation of the PNA-FISH methodology as a reliable alternative for BV diagnosis, demonstrating a higher specificity and accuracy when compared to classical methods.

We hypothesized that *G. vaginalis* is the initial colonizing species and that its adherence is required before other BV-associated anaerobes are able to interact with the vaginal epithelium. To test this hypothesis, the initial adhesion of *G. vaginalis* and other BV-associated bacteria (*A. vaginae, M. mulieris, P. bivia* and *F. nucleatum*) was analyzed in the presence of two vaginal lactobacilli (*L. crispatus* and *L. iners*) using human epithelial cells as a model. Our results revealed that *G. vaginalis* had the greatest capacity to initially adhere to epithelial cells, in support of the hypothesis, it could be the main candidate for early colonization. Based on the previous results, it was also postulated that *G. vaginalis* could enhance the ability of other bacteria to grow and colonize the vaginal epithelium. Hence, the growth of dual species biofilms, with *G. vaginalis* and other BV-associated anaerobes, was evaluated. Interestingly, the *G. vaginalis* biofilm growth was strongly enhanced by any of the BV-associated anaerobes tested. Furthermore, it also enhanced the growth of certain BV-associated anaerobes (*P. bivia* and *F. nucleatum*). These results suggest *G. vaginalis* as a key role in the early establishment of BV biofilms.

Finally, we performed a study to evaluate the probiotic potential of intra- and extracellular biosurfactants from 86 lactobacilli strains against several clinical *G. vaginalis* strains. We found 6 lactobacilli that were able to inhibit the growth and biofilm formation of

several clinical *G. vaginalis* strains, suggesting their probiotic potential as adjuvants for BV treatment.

Sumário

A vaginose bacteriana (VB) é a principal causa de desordem vaginal em mulheres de idade reprodutiva a nível mundial. A VB é caracterizada pelo decréscimo da flora vaginal saudável (lactobacilos) e pelo aumento de bactérias anaeróbicas, sendo a *Gardnerella vaginalis* a espécie dominante. No entanto, o agente etiológico da VB permanece desconhecido, dificultando o seu diagnóstico e consequentemente o seu tratamento adequado. Com o intuito de melhorar o diagnóstico da VB, desenvolveu-se a primeira metodologia de Hibridação *In Situ* de Fluorescência com sondas de Péptidos de Ácido Nucleico para aumentar a especificidade e a sensibilidade da detecção de *Lactobacillus* spp. e *G. vaginalis* em amostras vaginais. Posteriormente, realizou-se um estudo prospectivo numa coleção de amostras vaginais que permitiu validar a metodologia desenvolvida como um método alternativo e robusto para o diagnóstico correto da VB, demonstrando uma elevada especificidade e precisão quando comparado com os métodos clássicos de diagnóstico.

Adicionalmente, postulamos que a *G. vaginalis* poderá ser o colonizador primário e que a sua adesão inicial é necessária para uma posterior colonização por outros anaeróbicos associados à VB. Por forma a testar esta hipótese, comparou-se a adesão inicial da *G. vaginalis* e de outros anaeróbios associados à VB (*A. vaginae*, *M. mulieris*, *P. bivia* e *F. nucleatum*) contra dois lactobacilos vaginais (*L. crispatus* e *L. iners*) usando células epiteliais humanas como modelo. Conclui-se que *G. vaginalis* teve a maior capacidade de adesão inicial, evidenciando-se como o principal candidato a colonizador primário na VB. Com base nestes resultados, postulou-se que *G. vaginalis* poderá facilitar o crescimento e a colonização secundária de outros anaeróbicos. Deste modo, quantificou-se o crescimento de biofilmes mistos entre *G. vaginalis* e um segundo aneróbio associado à VB. Curiosamente, o biofilme da *G. vaginalis* apresentou um crescimento fortemente incrementado na presença de qualquer um dos outros aneróbios testados. Por sua vez, o biofilme da *G. vaginalis* promoveu também o crescimento de alguns anaeróbios associados à VB (*P. bivia* e *F. nucleatum*). Estes resultados sugerem que a *G. vaginalis* possui de facto um papel preponderante na formação inicial dos biofilmes na VB.

Por último, avaliou-se o potencial probiótico dos biosurfactantes intra- e extracelular de 86 lactobacilos em várias estirpes clínicas de *G. vaginalis*. Este estudo permitiu selecionar 6

espécies de lactobacilos capazes de inibir o crescimento e a formação de biofilmes de *G. vaginalis*, demonstrando assim o potencial destes lactobacilos como probióticos candidatos para o tratamento da VB.

Erratum

Erratum

THESIS TITLE: The role of *Gardnerella vaginalis* biofilms in Bacterial vaginosis

The errors found in this thesis are described in the following tables. The words/symbols/punctuations in bold are the ones that were either corrected, removed or added to the indicated sentence, figure or table.

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xviii	3.2.4 **Fluorescent** *in situ* hybridization and vaginal bacteria quantification	3.2.4 **Fluorescence** *in situ* hybridization and vaginal bacteria quantification

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xxvii	Figure 1.1 Microscopic images of (…) in **Bacteria** vaginosis	Figure 1.1 Microscopic images of (…) in **Bacterial** vaginosis
xxvii	Figure 3.1 Fluorescence microscopy pictures of Lactobacillus ***spp.***,	Figure 3.1 Fluorescence microscopy pictures of Lactobacillus **spp.**,

LIST OF TABLES

PAGE	ORIGINAL TEXT	CORRECTED TEXT
xxx	Table S6.1 Probiotic screening results from intracellular **surfactants**	Table S6.1 Probiotic screening results from intracellular **biosurfactants**
xxx	Table S6.2 Probiotic screening results from extracellular **surfactants**	Table S6.2 Probiotic screening results from extracellular **biosurfactants**

STRUCTURE OF THE THESIS

PAGE	ORIGINAL TEXT	CORRECTED TEXT
xxxix	addresses the **bacteria** vaginosis (BV) relevance	addresses the **bacterial** vaginosis (BV) relevance

CHAPTER 1. GENERAL INTRODUCTION

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
4	FIGURE 1.1	2	"(…) in **Bacteria** vaginosis."	"(…) in **Bacterial** vaginosis."
7	TABLE 1.1.		"*Lactobacillus* spp. **Morphotype**"	"*Lactobacillus* spp. **morphotype**"
12	1	16	"(…)**bacteria** species"	"(…)**bacterial** species"
13	FIGURE 1.5		"(image) **Hibridization**"	"(image) **Hybridization**"
16	1	10	"(…)***L. rhamnosis***"	"(…)***L. rhamnosus***"

CHAPTER 2. DEVELOPMENT OF A PNA-FISH METHODOLOGY FOR THE DETECTION OF *LACTOBACILLUS* AND *GARDNERELLA* SPP.

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
33	1	7	"Nowadays, **Peptid** Nucleic Acid (PNA) probes (…)"	"Nowadays, **Peptide** Nucleic Acid (PNA) probes (…)"
34	2	16	"(…) **Ribosomal Database Project II (**version 10.0; (…)."	"(…) **Ribosomal Database Project II (RDPII,** version 10.0; (…)."
39	TABLE 2.1.		"**No. of *Lactobacillus*** strains detected[a]."	"**No. of target bacteria** strains detected[a]."
39	TABLE 2.1.		"**No. of non-*Lactobacillus*** strains detected[a]."	"**No. of non-target bacteria** strains detected[a]."
44	TABLE 2.2		"***Fusobacteria nucleatum***."	"***Fusobacterium nucleatum***."

CHAPTER 3. APPLICATION OF THE MULTIPLEX PNA-FISH METHODOLOGY FOR DIAGNOSIS OF BACTERIAL VAGINOSIS

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
61	1	8-9	"(…)The results showed a sensitivity of **66.7%** (95% confidence interval (CI), from **49.7 to 80.4%)** and a specificity of **94.2%** (95% CI, from **83.1 to 98.5%**) (…)."	"(…)The results showed a sensitivity of **76.9%** (95% confidence interval (CI), from **60.3 to 88.3%)** and a specificity of **100%** (95% CI, from **91.4 to 100.0%**) (…)."
63	4	28	"(…)our novel PNA **probe** (…)"	"(…)our novel PNA-**FISH**(…)"
64	1	9	"(…) used for ***Gram* stain** (...)"	"(…) used for **Gram staining** (...)"
65		22	**"**3.2.4 **Fluorescent** *in situ* hybridization and vaginal bacteria quantification**"**	**"**3.2.4 **Fluorescence** *in situ* hybridization and vaginal bacteria quantification**"**
70	1	8-14	"Based on the results, an experimental specificity of **94.2%** (95% CI, **83.1 to 98.5%**) and sensitivity of **66.7%** (95% CI, **49.7 to 80.4%**) were obtained for the BV diagnosis by our PNA-FISH method (Table 3.2). As shown in Table 3.2, when compared with the standard Gram staining, PNA-FISH method was able to determine normal flora **in 49 from a total of 52 healthy cases** and capable to categorize **26** from a total of 39 BV cases. This results in an accuracy of BV diagnosis by our novel PNA-FISH method of **82.4%** (95% CI, **72.2 to 88.8%**), evidencing a PLR of **11.56** and a NLR of **0.35**."	"Based on the results, an experimental specificity of **100%** (95% CI, **91.4** to **100.0%**) and sensitivity of **76.9%** (95% CI, **60.3 to 88.3%**) were obtained for the BV diagnosis by our PNA-FISH method (Table 3.2). As shown in Table 3.2, when compared with the standard Gram staining, PNA-FISH method was able to determine normal flora **in all 52 healthy cases** and capable to categorize **30** from a total of 39 BV cases. This results in an accuracy of BV diagnosis by our novel PNA-FISH method of **90.1%** (95% CI, **81.3 to 93.7%**), evidencing a PLR of **infinity value** and a NLR of **0.23**."

70 TABLE 3.2.

PNA-FISH results	Gram results		
	BV+	BV -	Total
BV +	26	3	29
BV -	13	49	62
Total	39	52	91

Statistical analysis of PNA-FISH method

Statistical parameters	Estimated value	Lower limit	Upper limit
Sensitivity	**66.7%**	49.7%	80.4%
Specificity	**94.2%**	83.1%	98.5%
Accuracy	**82.4%**	72.2%	88.8%
Positive likelihood	**11.56**	3.77	35.44
Negative likelihood	**0.35**	0.23	0.55

PNA-FISH results	Gram results		
	BV+	BV -	Total
BV +	30	0	30
BV -	9	52	61
Total	39	52	91

Statistical analysis of PNA-FISH method

Statistical parameters	Estimated value	Lower limit	Upper limit
Sensitivity	**76.9%**	60.3%	88.3%
Specificity	**100%**	91.4%	100.0%
Accuracy	**90.1%**	81.3%	93.7%
Positive likelihood	**Infinity**	-	Infinity
Negative likelihood	**0.23**	0.13	0.41

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
72	2	11	"Moreover, our experimental specificity (**94.2**%) revealed to be (…)."	"Moreover, our experimental specificity (**100%)** revealed to be (…)."
72	2	13	"(…) **94.2%** of those patients previously (…)."	"(…) **100%** of those patients previously (…)."
72	2	15-19	"(…) Despite the experimental sensitivity (**66.7**%) was much lower than the sensitivity of the Gram stain by Nugent score (89%) (25), it was higher than the Amsel criteria sensitivity (60%) determined by Gallo and colleagues (26). Nevertheless, it is important to refer that 5 samples of the **13** false negative results showed a negative result for G. vaginalis in the PCR procedure (see Table 3.1), (…)."	"(…) Despite the experimental sensitivity (**76.9**%) was much lower than the sensitivity of the Gram stain by Nugent score (89%) (25), it was higher than the Amsel criteria sensitivity (60%) determined by Gallo and colleagues (26). Nevertheless, it is important to refer that 5 samples of the **9** false negative results showed a negative result for G. vaginalis in the PCR procedure (see Table 3.1), (…)."
73	1	1-3	"By excluding those 5 samples (more exactly, UM108, UM117, UM120, UM132 and UM255), our PNA-FISH method would show a **76.5%** (95% CI, **58.4 to 88.6%**) and specificity of **94.7%** (95% CI, **84.5 to 98.6%**)."	"By excluding those 5 samples (more exactly, UM108, UM117, UM120, UM132 and UM255), our PNA-FISH method would show a **89.7%** (95% CI, **74.8 to 96.7%**) and specificity of **100%** (95% CI, **91.4 to 100.0%**)."

CHAPTER 4. INITIAL ADHESION OF BACTERIAL VAGINOSIS ANAEROBES IN EPITHELIAL CELLS

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
79	1	5	"Initial adhesion to the **vagina** epithelium (…)."	"Initial adhesion to the **vaginal** epithelium (…)."
79	1	8	"(…) ***Fusobacteria nucleatum*** against *Lactobacillus* ***spp.*** using (…)."	"(…)***Fusobacterium nucleatum*** against *Lactobacillus* **spp.** using (…)."
81	1	7	"(…) and ***Fusobacteria nucleatum*** (7–9)."	"(…) and ***Fusobacterium nucleatum*** (7–9)."
82	1	9	"(…) ***Fusobacteria nucleatum*** 718BVC are (…)."	"(…) ***Fusobacterium nucleatum*** 718BVC are (…)."
83	1	7	"(…) with **300** µL of sterile PBS to (…)."	"(…) with **400** µL of sterile PBS to (…)."
83	2	17	"(…) with **300** µL of sterile PBS and (…)."	"(…) with **400** µL of sterile PBS and (…)."
83	2	19	"(…) with **300** µL of sterile PBS to (…)."	"(…) with **400** µL of sterile PBS to (…)."
85	FIGURE 4.1		"(…) between *L. crispatus* **and anaerobe** by (…)."	"(…) between *L. crispatus* **and a BV anaerobe** by (…)."

CHAPTER 5. INITIAL ATTACHMENT AND BIOFILM FORMATION OF ANAEROBES INVOLVED IN BACTERIAL VAGINOSIS

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
101	1	7	"(…) and ***Fusobacteria nucleatum*** (2–4)."	"(…) and ***Fusobacterium nucleatum*** (2–4)."
101	1	12	"(…) biofilm formation may be more complex and **depend** upon (…)."	"(…) biofilm formation may be more complex and **depends** upon (…)."
102	1	8	"(…) ***Fusobacteria nucleatum*** 718BVC were (…)."	"(…) ***Fusobacterium nucleatum*** 718BVC were (…)."
107	TABLE 5.3		"(…) counting ($7.36 \times 10^7 \pm 9.97 \times 10^4$)."	"(…) counting (7.36×10^7 **per cm^2** $\pm 9.97 \times 10^4$)."
108	1	14	"(…) with *G. vaginalis* **strains**, (…)."	"(…) with *G. vaginalis* **strain**, (…)."
113	REFERENCE 20	26	"(…) between ***gardnerella vaginalis*** and ***prevotella bivia*** (…)."	"(…) between ***Gardnerella vaginalis*** and ***Prevotella bivia*** (…)."

CHAPTER 6. PROBIOTIC ACTIVITY OF LACTOBACILLI BIOSURFACTANTS AGAINST *GARDNERELLA VAGINALIS*

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
119	1	18	"(…) **intra** and extracellular biosurfactants (…)."	"(…) **intra-** and extracellular biosurfactants (…)."
119	1	22	"(…) substances **they secrete** (19–21)."	"(…) substances **usually secreted by them** (19–21)."
125	1	6	"The selected **intra** and (…)."	"The selected **intra-** and (…)."
128	FIGURE 6.2.	PLOT	"Percentage of growth"	"Percentage of growth**, %**"
131	1	24	"(…) formation (***t-student*** statistical (…)."	"(…) formation (***t*-student** statistical (…)."
137	REFERENCE 36	31	"(…)Beuerman D, Poehner **R BA** (…)."	"(…)Beuerman D, Poehner **RBA** (…)."
139-141	Table S6.1.		"Table S6.1 Probiotic screening results from intracellular **surfactants** (…)."	"Table S6.1 Probiotic screening results from intracellular **biosurfactants** (…)."
142-144	Table S6.2.		"Table S6.2 Probiotic screening results from extracellular **surfactants** (…)."	"Table S6.2 Probiotic screening results from extracellular **biosurfactants** (…)."

CHAPTER 7. CONCLUDING REMARKS AND FUTURE WORK

PAGE	PARAGRAPH	LINE	ORIGINAL TEXT	CORRECTED TEXT
147	1	2	"(…) effectiveness of BV **diagnostic** and (…)."	"(…) effectiveness of BV **diagnosis** and (…)."
147	1	7-8	"Aiming to improve BV **diagnostic**, (…)."	"Aiming to improve BV **diagnosis**, (…)."
147	1	17-19	"This methodology showed a sensitivity of **66.7%** and a specificity of **94.2%**, thus demonstrating its (…)"	"This methodology showed a sensitivity of **76.9%** and a specificity of **100%,** thus demonstrating its (…)"
149	2	8	"(…) Gram staining **and Nugent scoring, compared with** our PNA-FISH (…)"	"(…) Gram staining **under Nugent score and** our PNA-FISH (…)"

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Abbreviations

Abbreviations

AEEA - 8-amino-3,6-dioxaoctanoic acid
ATCC - American tissue culture collection
Av - *Atopobium vaginae*
BBA - Brucella blood agar
BHI - Brain heart infusion
BLAST - Basic local alignment search tool
BV - Bacterial vaginosis
CBA - Columbia blood agar
CCUG - Culture collection university of Göteborg
CDM - Chemically defined medium
CECT - Colección española de cultivos tipo
CFU - Colony-forming unit
CI - Confidence interval
CLSM - Confocal laser scanning microscopy
C_t - Threshold cycle number
DAPI - 4',6-diamidino-2-phenylindole
DMEM - Dulbecco's modified Eagle's medium
DNA - Deoxyribonucleic acid
dNTP - Deoxyribonucleotide triphosphate
DSM - Deutsche sammlung von mikroorganismen
FBS - Fetal bovine serum
FDA - Food and drug administration
FISH - Fluorescence *in situ* hybridization
Fu - *Fusobacterium nucleatum*
Gv - *Gardnerella vaginalis*
HIV - Human immunodeficiency virus
HPLC - High-performance liquid chromatography
IRB - Institutional review board
Mm - *Mobiluncus mulieris*
MRS - Man, Rogosa and Sharpe
NCIMB - National collection of industrial, food and marine bacteria
NCTC - National collection of type cultures
NLR - Negative likelihood ratios
NTS - Non-target strains
OD - Optical density
ODbiosurf - Optical density of the biosurfactant

ODcontrol - Optical density of the control
Pb - *Prevotella bivia*
PBS - Phosphate buffer saline
PCR - Polymerase chain reaction
PID - Pelvic inflammatory disease
PLR - Positive likelihood ratios
PNA - Peptide nucleic acid
qPCR - Quantitative Polymerase chain reaction
rDNA - Ribosomal DNA
RDP II - Ribosomal database project II
RNA - Ribonucleic acid
rRNA - Ribosomal RNA
sBHI - supplemented BHI
SD - Standard deviation
STIs - Sexually transmitted infections
TNT - Total of non-target strains
TSA - Trypticase soy agar
Vol - Volume
Wt - Weight

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Publications

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Structure of the thesis

Structure of the thesis

This thesis is organized in seven chapters. The first chapter is a general introduction that addresses the bacteria vaginosis (BV) relevance in women, from Portugal and Worldwide, standard diagnostic methods applied in BV, their advantages and limitations, and the emergence of molecular techniques to increase BV diagnosis accuracy.

Chapters II and III report the development and application of new PNA probes through Fluorescence *In Situ* Hybridization (FISH) methodology for the detection of *Lactobacillus* spp. and *Gardnerella vaginalis* in vaginal swabs. The multiplex PNA-FISH methodology developed constitutes an alternative to the currently classical Nugent score criteria using standard Gram stain used.

Chapter IV and V describe the role of *Gardnerella vaginalis* in the initial adhesion and biofilm formation in BV, respectively. In chapter IV, the initial adhesion of *G. vaginalis* is studied against an epithelial cell line and is further compared with other BV-related anaerobes. Chapter V presents a characterization of *G. vaginalis* dual-species biofilms, showing commensal and synergetic relationships between *G. vaginalis* and other BV-related anaerobes.

The chapter VI demonstrates lactobacilli probiotic activity against *G. vaginalis* growth and biofilm formation. Several lactobacilli biosurfactants from a culture collection and vaginal isolates are evaluated. Finally, chapter VII summarizes the major conclusions of the thesis addressing the role of *G. vaginalis* on the etiology of BV, mixed species biofilms and resistance against probiotic lactobacilli. Furthermore, in this chapter some important issues that should be clarified in future work are discussed.

Chapter I

General Introduction

1.1 Bacterial vaginosis

Bacterial vaginosis (BV) is an imbalance in the vaginal microflora (1). It is the most common vaginal disorder in women of reproductive age and the most common cause of vaginal discharge (1, 2). BV is responsible for more than 60% of vulvovaginal infections and has been linked to serious public health consequences including pelvic inflammatory disease, postoperative infections, acquisition and transmission of the Human immunodeficiency virus (HIV), and preterm birth (1, 3). However, the current knowledge about its etiology remains scarce (4). BV is associated with numerous bacterial species, mainly anaerobes, such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus mulieris*, *Prevotella bivia* and *Fusobacterium nucleatum* (5–7). Current paradigm is that the establishment of a biofilm plays a key role in the pathogenesis of BV (8, 9).

The lack of basic information about BV etiology has led to an ongoing debate between two hypotheses. The first is the polymicrobial hypothesis, which infers that BV is caused by a mixture of pathogenic bacteria, principally anaerobes (10). The second is that a single pathogenic species, in many cases *G. vaginalis*, is the primary pathogenic agent being frequently transmitted via sexual contact (6). In 1955, Gardner and Dukes isolated *G. vaginalis* (originally described as *Haemophilus vaginalis*) from the vaginas of 92% of patients with BV (11, 12). They postulated that *G. vaginalis* was the etiological agent responsible for BV. However, some studies demonstrated that the artificial infection with pure cultures of *G. vaginalis* did not reliably cause BV (13), making the role of *G. vaginalis* in BV establishment less clear. In addition, other bacteria, such as *Atopobium vaginae* and *Mobiluncus mulieris*, were positively associated with BV (2, 8), thus suggesting a polymicrobial role. Nevertheless, the polymicrobial hypothesis does not currently agree with available epidemiological data. Risk factor studies have shown that the BV profile mirrors a sexual transmitted disease (6, 14) or sexually enhanced disease (3). As a sexual transmitted disease, it is highly likely that BV has a single etiological agent, rather than being caused by multiple organisms. However this has not been directly proven. Recently, several studies revealed the virulence potential of *G. vaginalis* and evidenced again this bacterium as main etiological candidate (15–18).

It is generally accepted that the microflora of the healthy adult vagina is dominated by hydrogen peroxide and lactic acid producing lactobacilli, which leads to an acidic pH of the vaginal environment (19). The shift in the composition of vaginal microflora that occurs in BV has been extensively studied (see Figure 1.1) and is characterized by a decrease in these healthy vaginal bacteria and an increase in the numbers of *G. vaginalis*, *A. vaginae*, *M. mulieris* and other anaerobes (2, 8, 20, 21). Nonetheless, it remains unknown if certain anaerobes are capable of acting as primary pathogens in the vaginal microflora (6). Also, increasing number of anaerobes is not specific of BV since it has been also described in other vaginal conditions, such as trichomoniasis (22). Therefore, this anaerobe overgrowth may be a symptom of the infection rather than specifically related to BV etiology (23).

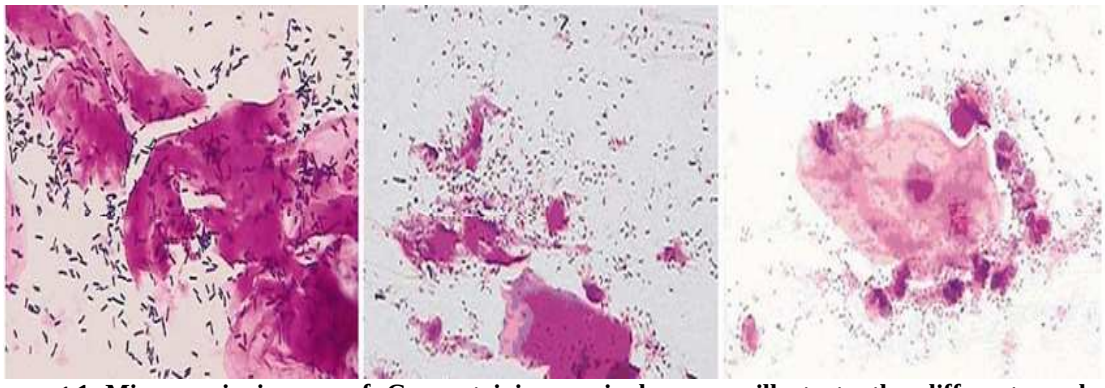

Figure 1.1 Microscopic images of Gram-staining vaginal smears illustrate the different grades of microflora evolution in Bacteria vaginosis (adaptation from 16).

In BV patients, a biofilm can be formed on the vaginal epithelium and *G. vaginalis* is typically the predominant species (8, 24), therefore it was hypothesized that *G. vaginalis*' biofilm induction is needed to induce BV in women. In addition, Patterson *et al*. (25) also demonstrated that *G. vaginalis* biofilms exhibited increased tolerance to hydrogen peroxide and lactic acid when compared to planktonic cells. The distinct gene expression pattern and morphological structure of biofilms increase the bacterial resistance against numerous agents, such as chemical disinfectants, extreme pH values, host immune defenses and antibiotics (26). Also, Patterson *et al*. (17) demonstrated that *G. vaginalis* was the only anaerobe to exhibit three key virulence determinants, including adherence to vaginal epithelial cells, biofilm-producing capacity and cytotoxic activity, when compared with *A. vaginae*, *M. mulieris*, *P. bivia* and *Veillonella* sp. Hence, all these findings suggest that biofilm forming

G. vaginalis plays a key role in BV pathogenesis. It is important to notice that the biofilm phenotype was not previously considered in the studies addressing the single pathogenic species theory, and therefore further studies are required to fully understand BV etiology.

Currently, it is recognized that planktonic cell growth does not accurately reflect bacterial growth in nature or in infectious diseases, where most bacteria grow as biofilms (27). A biofilm is defined as a complex and structured community of bacteria attached to a surface and surrounded by a matrix of extrapolymeric substances, such as proteins, lipids, deoxyribonucleic acids (DNA) and polysaccharides (26). Bacteria may form a biofilm in response to many extrinsic or intrinsic factors, such as cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or even by exposure to sub-inhibitory concentrations of antibiotics (6, 7, 28). However, when bacteria switch to the biofilm mode of growth, it goes through a phenotypic shift in which a large number of genes is differentially regulated (26). The development of biofilm formation is characterized by an initial attachment and maturation stage (see Figure 1.2).

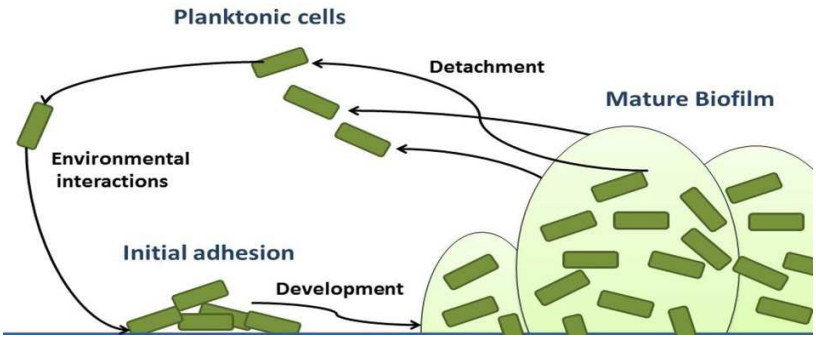

Figure 1.2 Conceptual model about the development of biofilm formation. The biofilm formation is characterized by an initial attachment and a maturation stage (adaptation from 29).

1.2 BV epidemiology and clinic diagnosis

BV is the most cited cause of vaginal symptoms prompting women to seek primary health care (30). However, BV normally appears initially as asymptomatic, developing gradually to the final stage that evidence a characteristic group of symptoms (5). Its prevalence changes with several factors, such as ethnic race, sexual habits and age. Nevertheless, BV prevalence is commonly higher in pregnant and sexually active women from both developed and developing countries (4, 5). In developed countries, the incidence of BV is higher than symptomatic genital candidiasis and trichomoniasis (31), reveling concerns about the cost-effectiveness for BV diagnosis and appropriateness treatment efficiency. Moreover, the epidemiologic studies linked BV with an increased risk for sexually transmitted infections (STIs) acquisition and pelvic inflammatory disease (PID) development (32). In addition, during pregnancy, BV has been related to late fetal loss and premature birth (33, 34). Initial stages of the infection and its absence of clinical symptoms commonly difficult a correct BV diagnosis. Therefore, an improvement of the current standard methods for BV diagnosis is currently needed.

1.2.1 Standard diagnostic methods for BV

The diagnosis of BV is normally based on Amsel clinical criteria or Gram stain under Nugent score system, which are both fairly subjective and thus complicate the research evaluation and clinical practice (30, 32, 35). As previously mentioned, BV is clinically described as a syndrome based on the presence of a collection of clinical symptoms without a defined etiologic agent. In fact, BV diagnosis by Amsel criteria is made through the following criteria: vaginal fluid pH above 4.5; positive “whiff test” (detection of fishy odor upon 10% potassium hydrogen addition); presence of clue cells (vaginal epithelial cells covered by bacteria) on microscopic examination of vaginal fluid; and homogeneous milky vaginal discharge. At least three from four clinical signs must be present to establish a positive BV diagnosis (30). Despite the fact that the Amsel criteria requires the least training and is therefore the most frequently used diagnostic procedure, it is not the most appropriate method to diagnose BV, due to its low specificity (36). Therefore, Nugent and colleagues

attempted to improve the BV diagnosis through Gram stain of vaginal swabs. This technique enabled the observation of the existent vaginal microflora and also the preservation of the clinical sample for further medical evaluation (37). These authors elaborated a Gram stain scoring system (37) based in the evaluation of the following morphotypes: large gram-positive rods (*Lactobacillus* spp. morphotypes); small gram-variable rods (*G. vaginalis* morphotypes); small gram-negative rods (*Bacteroides* spp. morphotypes); and curved gram-variable rods (*Mobiluncus* spp. morphotypes). Each morphotype is quantified from 1 to 4+ with regard to the number of morphotypes observed in the microscopic fields of the Gram-stained vaginal smear (see Table 1.1). The vaginal microflora diagnosis is then based in the sum of each morphotype score, classifying normal microflora (score between 0 – 3), intermediate microflora (score between 4 – 6) and BV (score between 7 – 10; see Table 1.1) (5, 37).

Table 1.1 Scheme for grading Gram-stained vaginal contents by Nugent score system (adaptation from 5).

Nugent's Gram stain scoring system			
Score	***Lactobacillus* spp. Morphotype**	***Gardnerella* and *Bacteroides* spp. morphotypes**	***Mobiluncus* spp. morphotype**
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		**Interpretation**	
0 – 3		Normal vaginal microflora	
4 – 6		Intermediate vaginal microflora	
7 – 10		Bacterial vaginosis in vaginal microflora	

Legend – Morphotypes are scored as the average number see per oil immsersion 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. Total score is the sum of the average classification of *Lactobacillus*, *Gardnerella* and *Bacteroides*, and finally *Mobiluncus* spp.

This Gram stain scoring system has been used for the past 3 decades, allowing also to compare prospective and longitudinal studies in the BV research (14, 38–40). However, Gram-stained vaginal smears require skilled personnel to perform the scoring and are not used as frequently in clinical practice (2, 41). A comparison between Nugent system score and Amsel clinical criteria was performed by Brotman (32), revealing that both methods are effective for the diagnosis of symptomatic BV but neither is capable to diagnose BV in early stages. In addition, the Amsel criteria do not convey information on the composition of vaginal microflora and the Nugent system score provides only morphological information. However, the Nugent score is unable to specifically recognize bacterial species due to an unspecific stain (32).

1.2.2 BV prevalence and diagnosis in Portugal

Numerous epidemiologic studies performed in different countries revealed that BV prevalence varies with geographic location, socioeconomic status, sexual behavior and race (3, 4, 42), as discussed above. In Portugal, studies about BV prevalence are almost non-existent except for two studies elaborated by Guerreiro *et al*. (43) and Henriques *et al*. (44). Guerreiro and colleagues studied the prevalence of several sexual transmitted diseases, including BV, in 840 women from Lisbon region (43). The majority of BV patients were young women at reproductive age (58.7%) with low academic education (59.0%) being middle or working class (85.0%) and having a fixed partner (79.8%) (43). Also, that study correlated BV prevalence to women with high sexual risk behavior, similarly as STIs, being in agreement with several international studies (3, 6, 7, 32, 45, 46). In 2012, Henriques and colleagues conducted a follow up study to assess Portuguese doctors' perception of BV prevalence in the country, as well as of the standard diagnostic methods used, therapies of choice and relapse of BV (44). This study collected the experts perception from 197 gynecologists from continental Portugal (44), and it was found that 42% and 74% of the gynecologists perceived BV as frequent in pregnancy and prevalent in Portuguese women, as illustrated in Figure 1.3A and 1.3B, respectively.

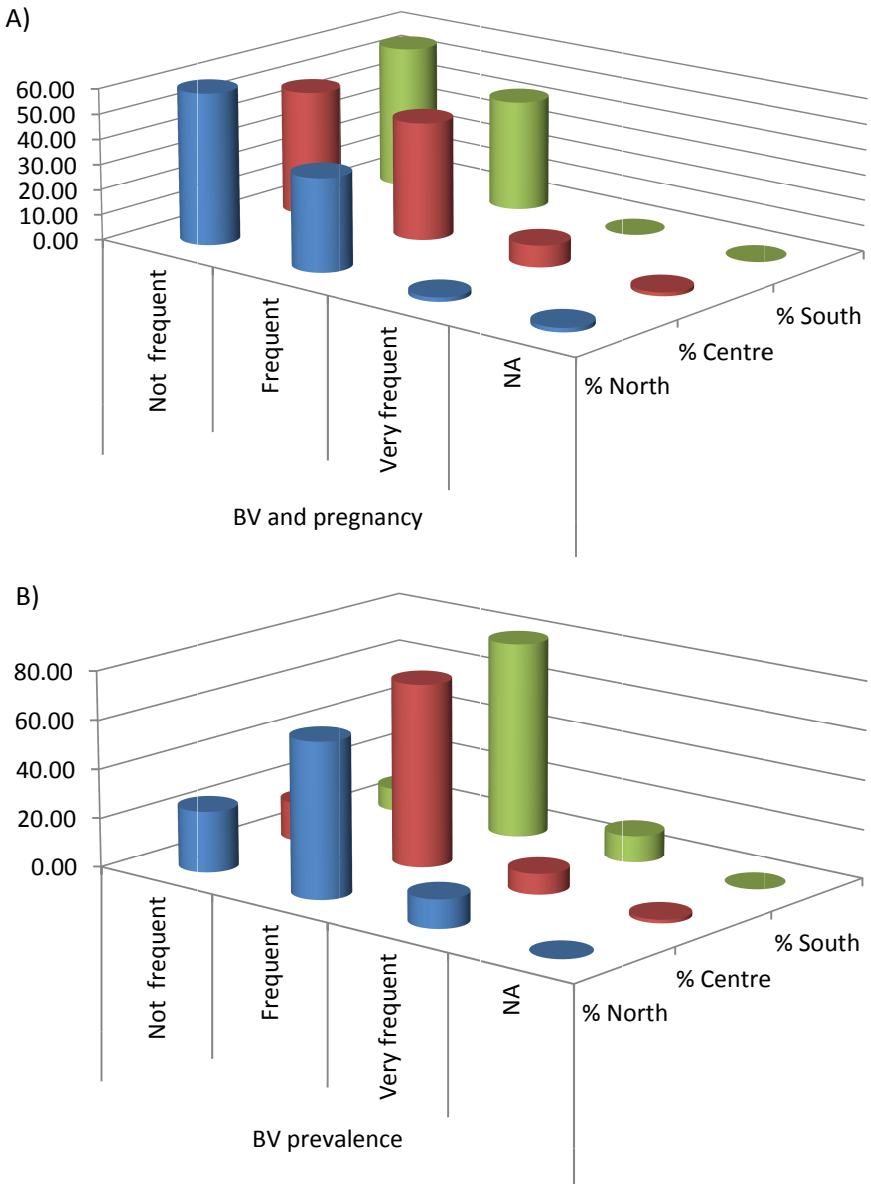

Figure 1.3 Epidemiologic study realized at Portuguese gynaecologists about BV and pregnancy (A) and BV prevalence (B) (adaptation from 44).

In Portugal, most gynecologists use the Amsel clinical criteria to diagnose BV infection (75%; see Figure 1.4A), and consider an augmentation of vaginal exudate (54%) and malodor (43%) as main symptoms for a positive BV diagnosis. Although different BV treatments are applied in each region of Portugal, most of the doctors who participated in the study shared the opinion that BV relapses are not very frequent (62%; see Figure 1.4B) contrasting with epidemiologic studies from other countries (3, 4, 42).

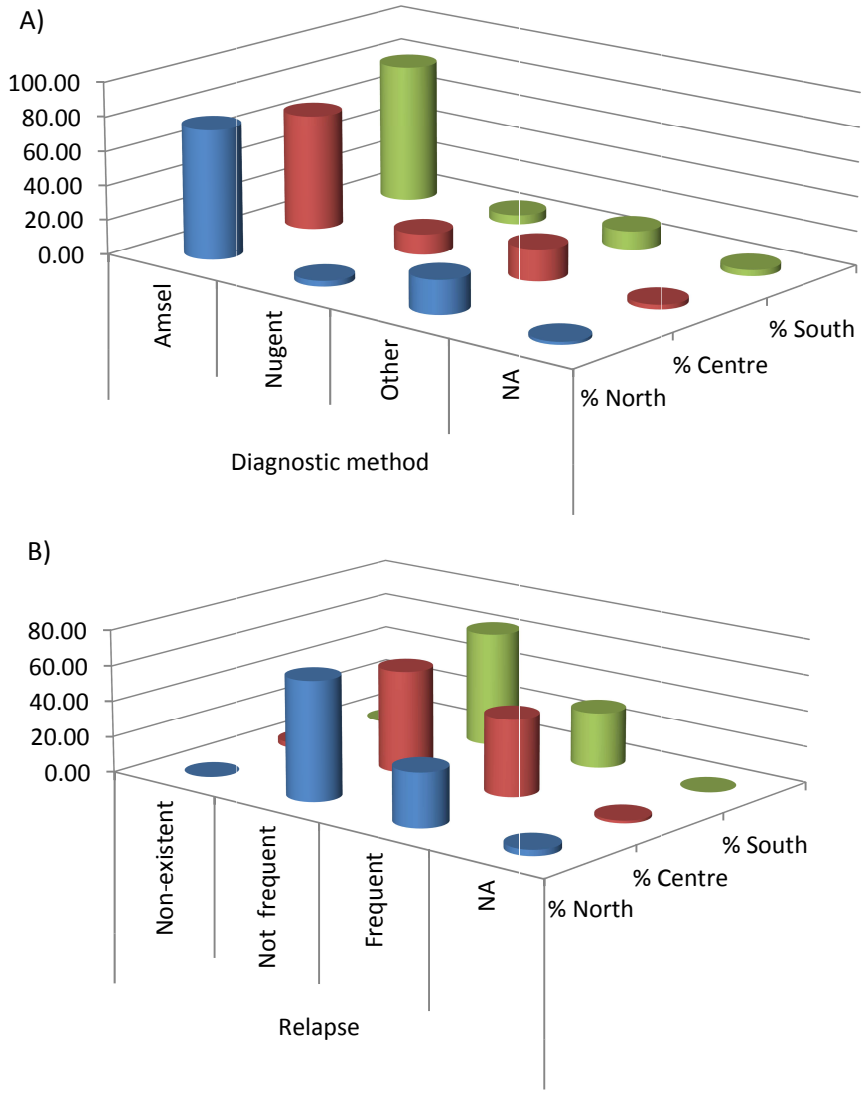

Figure 1.4 Epidemiologic study realized at Portuguese gynaecologists about diagnostic method of choice (A) and BV relapse after treatment (B) (adaptation from 44).

This study demonstrated that BV prevalence remains an important matter in Portuguese women's health and that diagnostic methods currently used are nonspecific and are only capable of diagnosing BV in later staged of infection (43, 44). Therefore, the optimization of BV diagnosis in the early stage would allow an easier treatment avoiding more serious clinical problems in women and higher health costs.

1.2.3 Pitfalls of the standard BV diagnostic methods

Since the first developments of BV research (11), numerous studies were done to attempt a correctly BV diagnosis. In 1983, the first clinical diagnostic criteria worldwide accepted for BV diagnosis was proposed by Amsel and colleagues (30), as previously discussed. However, other score systems continued to be developed towards an increase in accuracy. Spiegel *et al*. (35) latter developed another score system for BV recognition through examination of bacterial morphotypes in Gram-stained smears of vaginal secretion. Further improvement of this score system was obtained by Nugent and colleagues in 1991 (37), gaining also a wide acceptance as BV diagnosis scoring system. Despite the fact that the Amsel criteria had become the for the most frequently used for BV diagnosis, most research studies have been using the Nugent score system since the nineties (47). In addition, Schwebke and colleagues determined the sensitivity (89%) and specificity (83%) of the Nugent's Gram stain scoring system when compared with Amsel criteria, demonstrating that this standard method is more sensitive for BV diagnosis (48). However, the specificity reported in this study still suggests that BV may be underdiagnosed. Others studies also showed some concerns regarding the Nugent score system for BV diagnosis (47, 49, 50) and even with Amsel clinical criteria accuracy (51, 52). For instance, Gallo *et al*. studied the accuracy of clinical BV diagnosis by Amsel criteria, verifying a poor sensitivity (60%) in the conducted study (52). Moreover, this prospective study suggested that the Amsel criteria for routine BV diagnosis is unsuitable for asymptomatic women as previously advised in other studies (53, 54).

Several discrepancies in the Nugent methodology (37) were found by Forsum and colleagues, specifically in the scoring of morphotypes on vaginal smears (47). This international study involved the participation of 13 researchers that scored 238 slides with smears from vaginal fluid, and allowed the recognition of at least three pitfalls in the classification of the morphotype types. First, the fixation method may influence the real number of *Lactobacillus* spp. morphotypes in the slide, leading to a mismatch counting in the microscopy analysis (47). Also, the staining step and selected stain may lead to inaccuracy in distinguishing the *Lactobacillus* morphotypes from the *Gardnerella* and *Bacteroides* spp. morphotypes since old lactobacilli from vaginal microflora tend to lose their Gram-positive appearance (47). Finally, *Gardnerella* and *Bacteroides* spp. may vary in size and form from

round to more elongated, impairing the typical morphotype recognition and consequently the final Nugent score (47). These inaccuracies lead often to mismatches of vaginal smears classification, in particular, intermediate vaginal microflora, conducting to under- or overdiagnosis. It is important to refer that all this discrepancies were already postulated by other studies (49, 55, 56). All the pitfalls from classical standard methods lead to the search of other alternative methods for BV diagnosis. In the last two decades, the development of molecular methodologies allowed to gather new information about normal and BV microflora, highlighting alternative techniques that may replace the classical standard methods for BV diagnosis (40, 57–60).

1.2.4 Novel molecular methodologies in BV diagnosis

BV and normal vaginal microflora are constituted by a multifaceted bacterial consortium and consequently its diagnosis requires a complex analysis (61). Understanding this bacteria consortium in vaginal epithelium appears to be the key for a complete explanation of vaginal health (21, 45). However, the conventional microbiological methods have limited utility in evaluating BV patients (60). The Amsel criteria and Nugent's Gram stain scoring system are unable to identify the bacteria species in the vaginal microflora and their diagnosis often relys, to some degree, on subjective interpretation by observers (62), as discussed above. Alternatively, molecular methodologies have been successfully developed to detect and characterize microbial species, allowing the diagnosis of numerous infectious diseases (63, 64). In fact, the augmentation of the nucleic acid sequence databases allowed the detection of several human pathogens by sequence-based identification (65). The most commonly used molecular methods applied to study BV microflora are based on nucleic acid sequences detection and quantification (60, 61, 63), such as Fluorescence *in situ* hybridization (FISH) (8, 17, 24, 40, 57, 66) and quantitative Polymerase Chain Reaction (qPCR) (60, 67–70). FISH is based in nucleic acid sequences hybridization directly in the bacteria without any kind of extraction procedure (71), enabling the spatial visualization of bacteria consortium from vaginal swabs (8, 24). On the other hand, qPCR is a molecular technique that is also capable of quantifying the phenotypic expression of the bacteria (72), enabling the analysis of the interactions between BV-associated bacteria (69, 73).

These molecular techniques are been used to improve our understanding in host genetic factors, physiological conditions and environmental factors that may influence the vaginal microflora (61). However, molecular methodologies and its implementation in the laboratory for BV diagnosis are time-consuming, costly and also involve rigorous optimization and commercialization of standardized assays (74). Nevertheless, all these requirements from molecular techniques are being overcome and further implementation of these methods will be suitable in the future in all clinical microbiology laboratories (63).

1.2.4.1 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization is a well-established technique that allows whole-bacterial cell detection (75) and, when using a confocal laser scanning microscopy (CLSM), biofilms can also be studied (17). Briefly, FISH is a technique based on the annealing of DNA or ribonucleic acid (RNA) molecules, also known as probes, to a specific target sequence within a cell. To visualize this specific target sequence, the probes are attached to a fluorescent label allowing specific microorganisms identification and visualization of their spatial organization in the studied sample (71, 75, 76). The fluorescence detection is usually performed by fluorescence microscopy or flow cytometry (71, 76, 77). However, the confocal laser scanning microscopy is the best approach for biofilms analysis (77). In spite of other techniques used to study microorganisms, which require cells actively in division, FISH can also be performed on non-dividing cells, making it a highly versatile methodology (71).

The FISH procedure is usually divided in three main steps, more exactly, fixation, hybridization and washing (Figure 1.5).

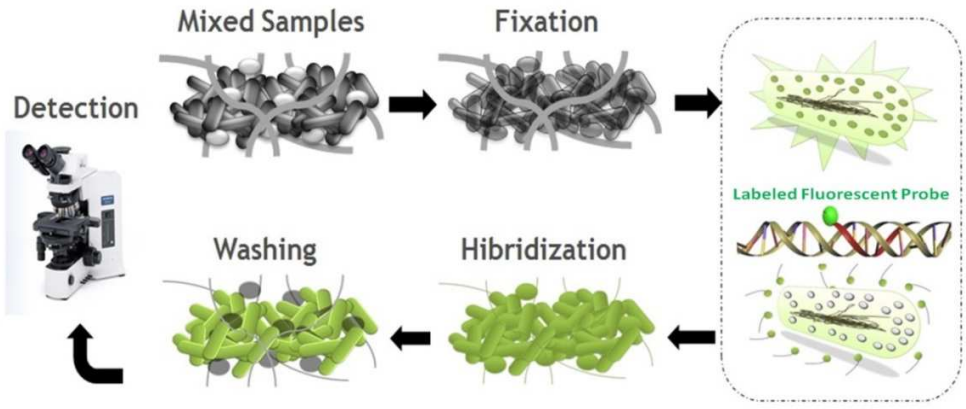

Figure 1.5 Basic steps of fluorescence *in situ* hybridization (adaptation from 78).

As shown in Figure 1.5, the sample is initially fixed to stabilize the cells and permeabilize their membranes. Afterwards, labeled oligonucleotide probes are added to the fixed cells, leading to the hybridization on the desired sequence targets; and then followed by a washing step to remove the unbinding or mismatched probes still present into the cells (79). Finally, fluorescence microscopy, flow cytometry or confocal laser scanning microscopy is used for identification and visualization of bacteria cells target in the sample (80, 81).

For bacterial detection, this methodology commonly used DNA probes to 16S and 23S ribosomal RNA (rRNA) sequences (71). These rRNA target regions are well suited for bacteria identification because all bacteria contain several ribosomes in which target molecules are usually amplified up to 100,000 per cell (71, 76). Thereby, the fluorescence intensity is easily observed due to the result of multiple probe labels and the enormous bacteria ribosomal content. In addition, the fact that the rRNA genes are composed of both highly conserved and highly variable regions, allows identification and classification of large taxonomic entities, such as, phyla, classes, genera or even species (71, 80).

1.3 Standard treatment of BV

BV treatment initially consisted of oral administration of doxycycline or ampicillin simultaneously with application of sulfonamide vaginal cream (82). However, this treatment was acknowledged to be inefficient against anaerobes present in BV infection (83, 84). Later, a therapy with 75% metrodinazole gel once-daily or twice-daily for 5 to 7 days revealed an efficient BV treatment in 99% of clinical cases (85). This treatment could be administrated orally up to 750 mg of metronidazole daily for one month (36). In addition, Austin and colleagues compared the efficacy of BV treatment with metronidazole and clindamycin showing that the single use of metronidazole treatment exhibited a significant decrease in the majority anaerobes of BV (86).

In 1998, Food and Drug Administration (FDA) published a guideline for BV treatment aiming to establish different and efficient therapeutics capable to treat BV (5). The main goal for BV treatment was the BV anaerobes elimination and simultaneously lactobacilli colonization in vaginal epithelium. This colonization has been intended with probiotic lactobacilli products in several studies (1, 5, 20).

In the past decades, as with many other bacterial species, antimicrobial resistance started to appear and novel antibiotics were sought. However, few advances had been achieved in BV treatment using antibiotic or probiotic agents. Tinidazole is the only new antibiotic for BV treatment approved by FDA in the last few years (5, 36). This antibiotic belongs to a second generation being chemically related to metronidazole (first generation nitroimidazole). However, tinidazole has a longer half-life and requires different dosing regimens when compared to metronidazole (36). Due to a longer half-life, tinidazole can be taken with in lower dosages and less frequently per day during the BV treatment. Also, this new antibiotic has been applied in recurrent BV cases in which standard therapy did not show any improvement in patients (36). On the other hand, the use of probiotics is an alternative approach to antibiotics that had been reported in several studies (1, 20, 87, 88). Briefly, this kind of BV treatment use probiotic strains to replace BV anaerobes in the vaginal epithelium through systemic and/or topic applications of certain products (capsules, yogurts, tablets or vaginal suppositories) (20, 89). Although the first choice for BV treatment

is usually the antimicrobial therapy, probiotics did not eradicate the healthy vaginal microflora showing an advantage when compared to conventional antibiotics. Several properties had been studied in probiotic strains for vaginal epithelium, such as, adhesion to human epithelial cells, antimicrobial activity or competition growth towards well-known pathogens, bacteriocins and hydrogen peroxide production (1, 90). *Lactobacillus* spp. is one of the main probiotic candidates for BV treatment (91). This genus is constituted by a heterogeneous microbial group containing more than 100 species and numerous subspecies (92). However, few lactobacilli strains are probiotic candidates for BV treatment (1, 91). Numerous studies in the treatment of BV with several probiotic lactobacilli had been attempted with no significant adverse events, such as *L. fermentum* (RC-14), *L. rhamnosis* GR-1, *L. crispatus*, *L. plantarum*, *L. brevis* CD2, *L. salivarius* FV2 and *L. gasseri* MB335 (90, 93–96). However, probiotic application in BV treatment did not show the same effectiveness as the antimicrobial therapy (1, 20). In 2006, Anukam and colleagues proposed that the combination of metronidazole and probiotic lactobacilli strains could be the most efficient treatment for BV (89).

Moreover, a clinical study in BV women treated with metronidazole administered orally and topic gel application with probiotic lactobacilli products revealed that the combination of the therapies was able to promote lactobacilli colonization in vaginal epithelium, when compared to each therapy individually applied to the BV women (97). It is important to notice that the number of probiotic lactobacilli capable to adhere in the vaginal epithelium and also the amount of antimicrobial substances secreted by them are difficult to control during BV treatment. However, these antimicrobial substances can be concentrated in several probiotic products and then topically applied in appropriate concentrations for BV anaerobes elimination (89, 98). Therefore, lactobacilli products can also be used for alternative treatment of BV (98, 99). All these studies suggested the combination of probiotic lactobacilli and antibiotic therapy as bottom line to achieve an efficient BV treatment and simultaneously a lactobacilli recolonization in the vaginal epithelium.

In Portugal, BV treatment is still performed exclusively by antimicrobial therapy, although doctors from different geographical regions prescribe different antibiotic therapies (see Figure 1.6) (44).

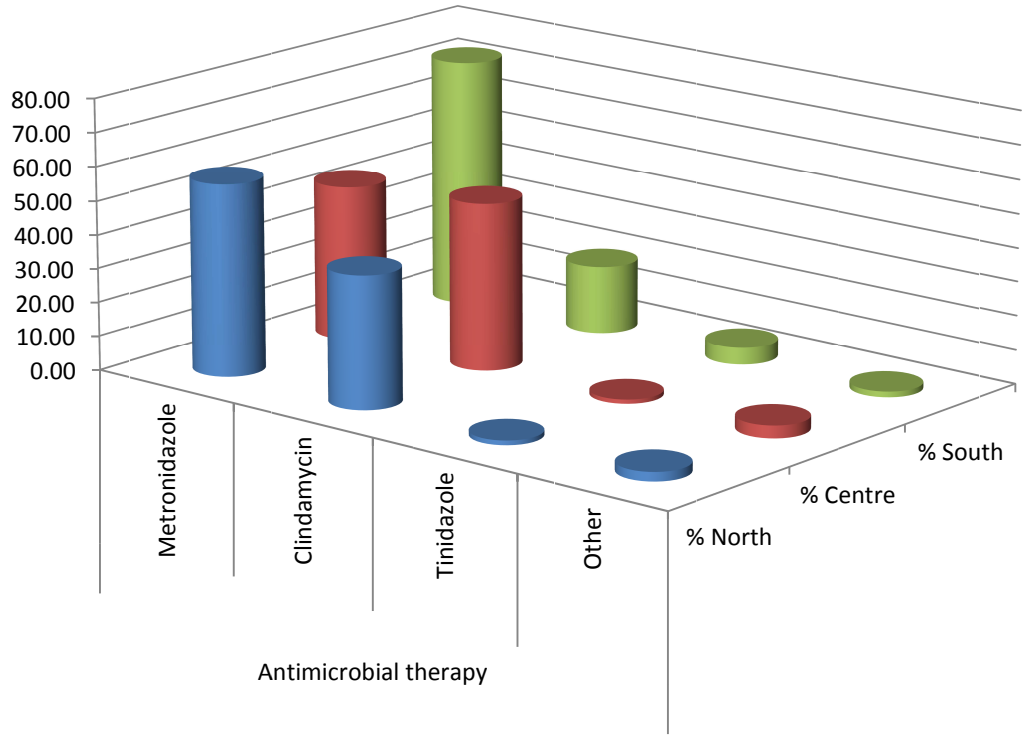

Figure 1.6 Epidemiologic study realized at Portuguese gynaecologists about BV treatment of choice (adaptation from 44).

As shown in Figure 1.6, metronidazole is the first choice of antimicrobial therapy for BV treatment by gynaecologists in Portugal, more specifically at north and south regions of the country (44), as in other countries as well (36). However, gynaecologists of the centre region of Portugal prefer the use of clindamycin (49%) instead of metronidazole (45%) (44).

1.4 Conclusions

In summary, the lack of knowledge of BV etiology led to difficulties in the effectiveness of BV diagnostic and treatment, increasing public health consequences and costs. The current BV diagnostic methodologies are unable to detect the early stages of BV development and so therapy is usually applied in severe clinical stages of the infection, with consequences in the healthy recovery of the patient vaginal microflora. Aiming to improve BV diagnostic, molecular methods (such as FISH and qPCR) are becoming a suitable alternative to the standard methods and allowing also a better characterization of the microbial species in the early stages of BV development.

Finally, current BV treatments are strictly based in antibiotic therapy inducing an antibiotic resistance in BV anaerobes, besides the severe reduction of the healthy lactobacilli strains in vaginal epithelium. A more appropriate treatment is required, aiming to eliminate BV pathogens but simultaneously promoting lactobacilli colonization in BV patients. The combination of antibiotic therapy with enforcement of probiotic lactobacilli products appears to be one of the most viable alternatives to the existing BV treatment.

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Chapter II

Development of a PNA-FISH methodology for the detection of *Lactobacillus* and *Gardnerella* spp.

Abstract

Bacterial vaginosis (BV) is a common vaginal infection occurring in women of reproductive age. It is widely accepted that the microbial switch from normal microflora to BV is characterized by a decrease in vaginal colonization by *Lactobacillus* species together with an increase of *Gardnerella vaginalis* and other anaerobes. Our goal was to develop and optimize a novel Peptide Nucleic Acid (PNA) Fluorescence *in situ* Hybridization assay (PNA-FISH) for the detection of *Lactobacillus* spp. and *G. vaginalis* in mixed samples. Therefore, we evaluated and validated two specific PNA probes by using 36 representative *Lactobacillus* strains, 22 representative *G. vaginalis* strains and 27 other taxonomically related or pathogenic bacterial strains commonly found in vaginal samples. The probes were also tested at different concentrations of *G. vaginalis* and *Lactobacillus* species *in vitro*, in the presence of a HeLa cell line. Specificity and sensitivity of the PNA probes were found to be 98.0% (95% confidence interval (CI), from 87.8 to 99.9%) and 100% (95% CI, from 88.0 to 100.0%), for *Lactobacillus* spp.; and 100% (95% CI, from 92.8 to 100%) and 100% (95% CI, from 81.5 to 100.0%) for *G. vaginalis*. Moreover, the probes were evaluated in mixed samples mimicking women with BV or normal vaginal microflora, demonstrating efficiency and applicability of our PNA-FISH. This quick method accurately detects *Lactobacillus* spp. and *G. vaginalis* species in mixed samples, thus enabling efficient evaluation of the two bacterial groups, most frequently encountered in the vagina.

Key words: Fluorescence *In Situ* Hybridization (FISH); Peptide Nucleic Acid Probe (PNA probe); *Lactobacillus* spp.; *Gardnerella vaginalis*; Bacterial vaginosis.

2.1 Introduction

Fluorescence *in situ* hybridization (FISH) is a molecular method used to identify and quantify microorganisms in a wide range of samples. This technique combines the simplicity of microscopic observation and the specificity of DNA/rRNA hybridization, allowing detection of selected bacterial species and morphologic visualization (1, 2). Nowadays, Peptid Nucleic Acid (PNA) probes are used instead of natural nucleic acids to improve FISH efficiency (3–6), because they enable more rapid and more specific hybridization (6–10). These types of probes are oligomers, in which single bases are linked by a neutral peptide backbone, avoiding repulsion from negative charges or attraction to positive charges (6). In addition, PNA probes can hybridize simultaneously with complementary DNA or RNA sequences and, due to the polyamide backbone, they are also resistant against cytoplasmic enzymes, such as nucleases and proteases (6, 11). Plus, the hybridization step can be performed efficiently under low a salt concentration, which endorses the destabilization of rRNA secondary structures and consequently improves the access to target sequences (6, 12–14). All these advantages became FISH using PNA probes (PNA-FISH) methodology in a new tool for diagnosis and therapy-directing technique (14), providing already a rapid and accurate diagnosis of several microbial infections (14–19).

The main goal of our work was to evaluate the PNA-FISH performance on mixed samples using a multiplex approach to detect *Lactobacillus* spp. and *G. vaginalis*. To validate the PNA probes, we determined both *in silico* and *in vitro* their specificity and sensitivity, using a broad diversity of representative *Lactobacillus* and *Gardnerella* strains, as well as other taxonomically related or pathogenic bacterial strains commonly found in vaginal samples. To confirm the usefulness of our methodology, the efficiency and specificity of the probes was also tested at different concentrations of *Lactobacillus* and *G. vaginalis* strains in the presence of a monolayer of HeLa cells.

2.2 Materials and Methods

2.2.1 Culture of bacterial strains

All strains from *Lactobacillus* spp. were grown in Man, Rogosa and Sharpe agar (MRS; Sigma-Aldrich, Germany), except *Lactobacillus iners* that was grown in Brucella Blood Agar (BBA; Oxoid, United Kingdom), as well as *Atopobium vaginae* and *Gardnerella vaginalis*. The remaining bacterial species were cultured on Brain Heart Infusion agar (BHI; Oxoid, United Kingdom) or Trypticase Soy Agar (TSA; Oxoid, United Kingdom). Each bacterial culture was streaked onto fresh plates every 48-72 h. Plates were incubated at 37 ºC or 30 ºC (in the case of *L. pentosus* strains) under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 24–48 h prior to FISH experiments.

2.2.2 PNA probe design

To identify *Gardnerella* genus potential oligonucleotides-target for the probe design, we used the software Primrose (20), coupled with the 16S rRNA databases from the Ribosomal Database Project II (version 10.0; http://rdp.cme.msu.edu/) (21). Complementarity with a low number of non-target and a high number of target sequences, as well as a higher predicted melting temperature and the absence of self-complementary sequences, were the main criteria for the PNA probe design. The selected sequences were synthesized (Panagene, Daejeon, South Korea) and the oligonucleotides N terminus was attached to an Alexa Fluor 594 molecule via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker (PNA Probe: Gard162, Alexa Fluor 594-OO-CAGCATTACCACCCG; HPLC purified > 90%). The Gard162 probe hybridizes between positions 162 and 176 of the *G. vaginalis* strain 409-05 16S rRNA sequence (RDPII ID: S001872672) and was selected for probe design. For the detection of *Lactobacillus* spp., a sequence between positions 663 and 677 of the *Lactobacillus* sp. strain MDL2 16S rRNA sequence (Genebank ID: HM753265.1) was selected for the PNA probe and consequently it was denominated as Lac663. This probe was attached to an Alexa Fluor 488 molecule, also via an AEEA linker (PNA Probe: Lac663, Alexa Fluor 488-OO-ACATGGAGTTCCACT; HPLC purified > 90%).

2.2.3 *In silico* determination of sensitivity and specificity

The theoretical specificity and sensitivity of both probes were evaluated using updated databases available at the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu/) through the Primrose software, and then were confirmed by a BLAST search at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Theoretical specificity and sensitivity were calculated according to Almeida *et al.* (22). Only target sequences with at least 1200 base pairs and good quality were included. Briefly, theoretical sensitivity was calculated as *ts*/(*Tts*)x100, where *ts* stands for the number of target strains detected by the probe and *Tts* for the total number of target strains present in the RDP II database (http://rdp.cme.msu.edu/probematch/, last accession date, May 2012). Theoretical specificity was calculated as *nts*/(*Tnt*)x100, where *nts* stands for the number of non-target strains that did not react with the probe and *Tnt* for the total of non-target strains examined.

2.2.4 FISH hybridization procedure

Biomass from a single colony of each strain was diluted and homogenised in sterile water, and then 20 µL were spread on epoxy coated microscope glass slides (Thermo Scientific, USA). For mixed samples, 10 µL of the final suspension from each strain suspension (prepared as previously referred) for the selected mixed sample were spread on glass slides. The slides were air-dried prior to fixation. Next, the smears were immersed in 4% (wt/vol) paraformaldehyde (Fisher Scientific, United Kingdom) followed by 50% (vol/vol) ethanol (Fisher Scientific, United Kingdom) for 10 min at room temperature on each solution. After the fixation step, the samples were covered with 20 µL of hybridization solution containing 10% (wt/vol) dextran sulphate (Fisher Scientific, United Kingdom), 10 mM NaCl (Sigma, Germany), 30% (vol/vol) formamide (Fisher Scientific, United Kingdom), 0.1% (wt/vol) sodium pyrophosphate (Fisher Scientific, United Kingdom), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma, Germany), 0.2% (wt/vol) ficoll (Sigma, Germany), 5 mM disodium EDTA (Sigma, Germany), 0.1% (vol/vol) triton X-100 (Sigma, Germany), 50 mM Tris-HCl (at pH 7.5; Sigma, Germany) and 200 nM of the PNA probe. Subsequently, the samples on glass slides were covered with coverslips and incubated in moist chambers at the hybridization temperature under analysis (from 50 ºC to 72 ºC) during a range of hybridization times (from 30 to 180 min). Next, the

coverslips were removed and a washing step was performed by immersing the slides in a pre-warmed washing solution for 30 min at the same temperature of the hybridization step. This solution consisted of 5 mM Tris base (Fisher Scientific, United Kingdom), 15 mM NaCl (Sigma, Germany) and 0.1% (vol/vol) triton X-100 (at pH 10; Sigma, Germany). Finally, the glass slides were allowed to air dry.

A FISH procedure in suspension was developed and optimized according to the previous work of Almeida and colleagues (12, 22) and to the results obtained for the FISH procedure on glass slides described above. Hybridization was perfomed at 60 ºC for 90 min and for washing (60 ºC for 30 min) and a fresh solution was prepared less than 24 h before use. The suspension samples were stored at 4 ºC in the dark for a maximum of 24 h before microscopic observation/visualization. Both hybridization procedures (in glass slides and in suspension) are able to detect lactobacilli and *G. vaginalis* strains. While glass slide hybridization is the more commonly used technique in analytical laboratories (22), hybridization in suspension is frequently used to avoid autofluorescence background in complex matrix samples, besides being the hybridization technique used in flow cytometry (12, 22).

2.2.5 Microscopic visualization

Prior to microscopy, one drop of non-fluorescent immersion oil (Merck, Germany) was added to either slides or filters and covered with coverslips. Microscopic visualization was performed using an Olympus BX51 (Olympus Portugal SA, Portugal) epifluorescence microscope equipped with a CCD camera (DP72; Olympus, Japan) and filters capable of detecting the two PNA probes (BP 470-490, FT500, LP 516 sensitive to the Alexa Fluor 488 molecule attached to the Lac663 probe and BP 530-550, FT 570, LP 591 sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe).

Other filters (such as BP 365-370, FT 400, LP 421) present in the microscope, that are not capable of detecting the probe fluorescent signal were used to confirm the absence of autofluorescence. In each experimental assay, a negative control was performed simultaneously in which all the steps described above were carried out, but where no probe was added in the hybridization step. All images were acquired using Olympus CellB software using a total magnification of ×1000.

2.2.6 Experimental assessment of probe specificity and sensitivity

After the hybridization optimization, the specificity and sensitivity of the PNA Lac663 and Gard162 probes were tested using 36 representative strains from the genus *Lactobacillus*, 22 representative strains from *Gardnerella vaginalis* (the only species of the genus *Gardnerella* (23)) and 27 representative strains from other related genera, of which 16 belonged to the order Lactobacillales and the other are common pathogens usually found in clinical samples, specifically strains from the following genera: *Atopobium*, *Bacillus*, *Lactococcus*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Klebsiella*, *Leuconostoc*, *Listeria*, *Mobiluncus*, *Prevotella*, *Salmonella*, *Shigella*, *Staphylococcus* and *Streptococcus* (24–26). All experiments were performed in triplicate at identical conditions and the experimental specificity and sensitivity were calculated.

2.2.7 Detection of *Lactobacillus* spp. and *G. vaginalis* adhered to HeLa cell line

HeLa epithelial cells (American Tissue Culture Collection, ATCC, USA) were cultured at 37 °C, in 5 % CO_2 (vol/vol), in Dulbecco's modified Eagle's medium (DMEM; Quality Biological, USA) supplemented with 10 % (vol/vol) Fetal bovine serum (FBS; Sigma, USA) and 1 IU penicillin/streptomycin mL^{-1} (MediaTech, Germany). Aliquots of 1mL from HeLa epithelial cells were seeded into 24-well tissue culture plates (Frilabo, Portugal) containing glass slides (12 mm) at a density of 2×10^5cells per well, and incubated at 37 ºC and 5% CO_2 (vol/vol) until the formation of a cell monolayer. The cultures were fed with fresh media every 48 hours. Simultaneously, two *Lactobacillus* (*L. crispatus* and *L. iners*) strains and *G. vaginalis* strain 5-1 were grown in MRS broth and BHI broth as described above. Prior to the adhesion assay, these broth cultures were harvested by centrifugation (4,000 *g*, 12 min, at room temperature) and washed twice with sterile phosphate buffer saline (PBS). Several standard concentrations of the bacteria were prepared in eukaryotic cell media (DMEM) and the optical density at 600 nm was adjusted using a microplate reader (Tecan, Switzerland). When monolayer of HeLa cells was obtained, the cells were washed twice with 500 µL of sterile PBS to remove non-adhered cells and culture media. Next, aliquots of 250 µL of cell culture media with a known concentration of a *Lactobacillus* strain and *G. vaginalis* 5-1 strain (1×10^3 to 1×10^9 CFU/mL) were added to each well of the 24-well tissue culture plate containing the

washed cell monolayer. Then, the plate was incubated for 30 min at 37 °C in anaerobic conditions and 120 rpm. Finally, each well was carefully washed twice with 500 µL of sterile PBS to remove non-adherent bacteria. The glass slides containing the adhered bacteria and eukaryotic cells were fixed and hybridized with both PNA probes and observed in fluorescence microscopy, as referred above. An additional 4',6-diamidino-2-phenylindole (DAPI; Sigma, Germany) staining step was conducted at the end of the hybridization procedure, covering each of the glass slides with 10 µL of DAPI for 5 min at room temperature in the dark, followed by immediate observation in the fluorescence microscope. All these assays were repeated three times on separate days.

2.3 Results

2.3.1 *In silico* analysis of PNA probes

The Lac663 probe showed a theoretical sensitivity and specificity of 91.5% and 99.7% (27), respectively. These results match the best values amongst the existing *Lactobacillus* probes. Gard162 probe presented a theoretical sensitivity of 95.0% and specificity of 100% (28). The theoretical specificity and sensitivity of these two probes and those developed in other studies were calculated as previously described by Almeida *et al.* (22) and are listed in Table 2.1. ProbeMatch tool, from RPDII (http://rdp.cme.msu.edu/probematch/; last accession, May 2012), was used with the following data set options: Strain – Both; Source – Both; Size – > 1200 bp; Quality – Both. For *Lactobacillus* probes, the specificity and sensitivity values previously determined (27), were considered.

Table 2.1 Theoretical specificity and sensitivity of several primers and probes for *Lactobacillus* and *Gardnerella* spp. detection.

Probe	Type	Sequence (5′→3′)	No. of *Lactobacillus* strains detected [a]	No. of non-*Lactobacillus* strains detected [a]	Specificity (%)[a]	Sensibility (%)[a]	Reference or source
Lab158[b]	DNA	GGTATTAGCA(C/T)CTGTTTCCA	11,991	7,165	99.30[g]	92.69[g]	(29)
LGC354A[c]	DNA	TGGAAGATTCCCTACTGC	12,701	12,329	98.79[g]	98.18[g]	(30)
LAB759[e]	DNA	CTACCCATRCTTTCGAGCC	10,371	2,823	99.72[g]	80.17[g]	(31)
Name not available	PNA	CCATTGTGGAAGATTC	12,930	14,880	98.54[g]	99.95[g]	(32)
Lac663	PNA	ACATGGAGTTCCACT	11,837	3,548	99.65[g]	91.50[g]	(27)
GardV	DNA	CCACCGTTACACCGAGAA	20	39	99.99	50.00	(33)
G.vag1008[f]	DNA	CTGCAGAGATGTGGTTTCCYTTCG	39	7	100.00	97.50	(34)
G.vag198	DNA	CCACTAAACACTTTCCCAACAAGA	34	0	100.00	85.00	(35)
GV003	DNA	AGACGGCTCCATCCCAAAAGGGTT	32	0	100.00	80.00	(36)
Gard162	PNA	CAGCATTACCACCCG	38	1	100.00	95.00	(28)

a Calculated through ProbeMatch/, last accession, May 2012) with the following data set options: Strain – Both; Source – Both; Size – > 1200bp; Quality – Both
b DNA probe that also detects members of *Enterococcus*, *Pediococcus*, *Weissella*, *Vagococcus*, *Leuconostoc* and *Oenococcus* spp. used by Lebeer *et al.* (37)
c DNA probe mainly detecting members of Lactobacillales and Bacillales, such as Lactobacillus spp., used in Olsen *et al.* (38)
e DNA probe also detects members of *Ruminococcaceae* sp. and *Pediococcus* sp. used in Quevedo *et al.* (39); The R symbol of the DNA probe sequence may be Adenosine or Guanosine, therefore Quevedo *et al.* (39) used a degenerate base in the sequence of the DNA probe to detect *Lactobacillus* spp.
f The Y symbol of the DNA probe sequence may be Cytidine or Thymidine, therefore Fredricks *et al.* (35) used a degenerate base in the sequence of the DNA probe to detect *G. vaginalis*
g Values determined in Machado *et al.* (27)

2.3.2 FISH Protocol optimization and autofluorescence-related factors

FISH protocols on slides and in suspension were adapted from previous protocols developed by Almeida *et al*. (12), due to the relevance of fixation and hybridization conditions for an efficient multiplex FISH with different probes. From an initial temperature range of 50 to 72 ºC and an incubation time range between 30 and 180 min, the best hybridization conditions were set as a moist chamber temperature of 60 ºC during 90 min of incubation (*data not shown*). Hybridization conditions started to reveal strong signal-to-noise ratio at 59 ºC to 61 ºC from 30 min of incubation up to 120 min, reaching its peak at 60 ºC during 90 min of incubation. Hybridization conditions above 60 ºC and 90 min were also efficient, but the signal-to-noise ratio seemed to decrease beyond the selected values of time and temperature. Both hybridization protocols (on slides and in suspension) revealed the same results and pitfalls, as discussed below (some examples are shown in Figure 2.1).

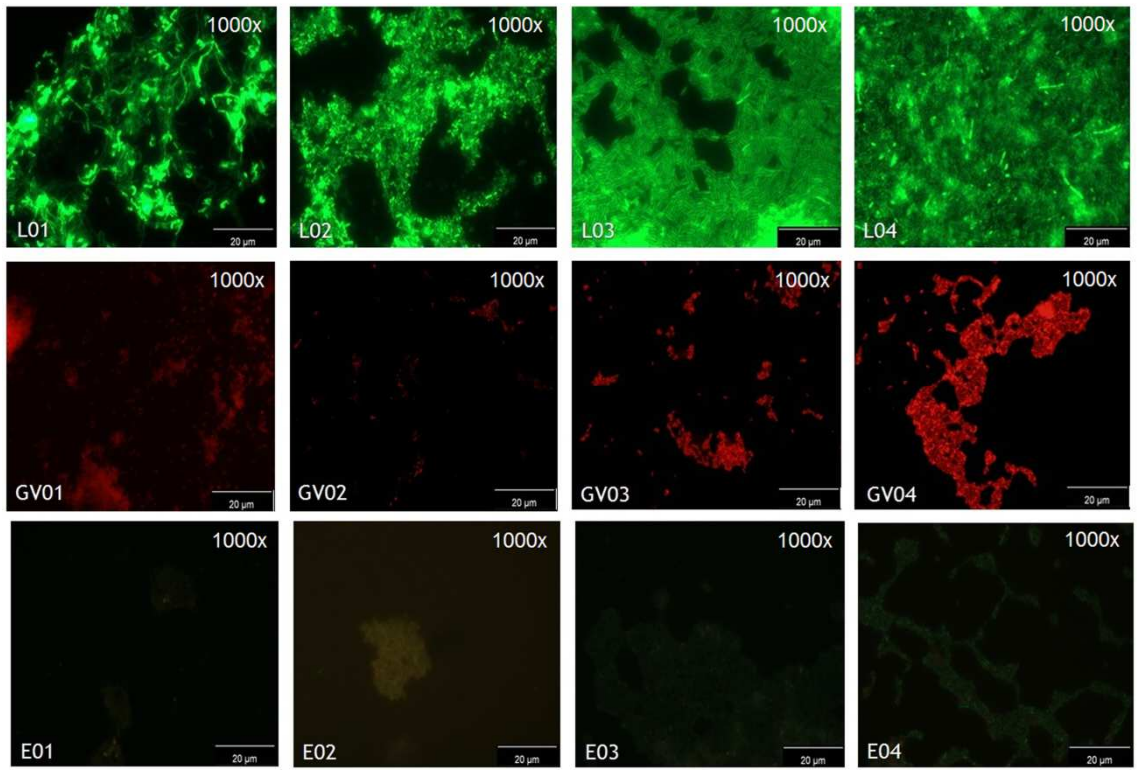

Figure 2.1 Fluorescence microscopy pictures of *Lactobacillus* species, *G. vaginalis* and other related bacteria by PNA probes. L01, *L. paracasei* CECT227; L02, *L. delbrueckii* ATCC9649; L03, *L. murinus* ATCC35020; L04, *L. salivarius* 438; GV01, *G. vaginalis* 5-1; GV02, *G. vaginalis* ATCC; GV03, Belgian *G. vaginalis isolate* 17; GV03, Belgian *G. vaginalis* isolate 18; E01, *Streptococcus thermophilus* A; E02, *Leuconostoc mesenteroides*; E03, *Enterococcus faecium*; E04, *Enterococcus faecalis*. The Lac663 and Gard162 PNA probes were associated with Alexa Fluor 488 and 594 fluorochromes, respectively.

2.3.3 Experimental determination of probe specificity and sensitivity

As shown in Table 2.2, the Lac663 probe was able to detect all *Lactobacillus* strains and cross hybridization was found only for *Streptococcus thermophilus* B. Based on these results, an experimental sensitivity of 100% (95% CI, 88.0 to 100.0%) and specificity of 98.0% (95% CI, 87.8 to 99.9%) were obtained for the Lac663 PNA probe. The Gard162 probe hybridized with all *G. vaginalis* strains, whereas no hybridization was observed for the other species tested. Therefore, this probe revealed a sensitivity of 100% (95% CI, 81.5 to 100.0%) and a specificity of 100% (95% CI, 92.8 to 100%).

Table 2.2 Bacterial strains used in PNA-FISH assays and their specificity with Lac663 and Gard162 probes.

Bacterial species	Collection strain	Lac663 Probe efficiency	Gard162 Probe efficiency
Lactobacillus acidophilus	ATCC 4356^T	++++	-
L. crispatus	ATCC 33820^T	++++	-
L. gasseri	ATCC 9857^T	++++	-
L. reuteri	NCFB 2656^T	+++	-
L. rhamnosus	ATCC 7469^T	++++	-
L. rhamnosus	CECT 288^T	++++	-
L. johnsonii	ATCC 11506^T	++++	-
L. hilgardii	NCFB 962^T	+++	-
L. delbrueckii subsp. *delbrueckii*	ATCC 9649^T	+++	-
L. delbrueckii subsp. *Lactis*	ATCC 12315^T	+++	-
L. pentosus	CECT 4023^T	++++	-
L. casei	CECT 5275^T	++++	-
L. coryniformis subsp. *torquens*	CECT 4129^T	++++	-
L. paracasei	CECT 227^T	++++	-
L. agilis	CCUG 31450^T	++++	-
L. animalis	ATCC 35046^T	+++	-

Table 2.2 Bacterial strains used in PNA-FISH assays and their specificity with Lac663 and Gard162 probes. (**Continuation)**

Bacterial species	Collection strain	Lac663 Probe efficiency	Gard162 Probe efficiency
L. bifermentans	ATCC 35409^T	+++	-
L. brevis	ATCC 14869^T	++++	-
L. buchneri	ATCC 4005^T	+++	-
L. fermentum	ATCC 11739^T	+++	-
L. curvatus subsp. *curvatus*	ATCC 25601^T	++++	-
L. farciminis	DSM 20182T	++++	-
L. fructivorans	ATCC 8288^T	+++	-
L. gallinarum	CCUG 31412^T	++++	-
L. graminis	DSM 20719^T	++	-
L. hamster	ATCC 43851^T	+++	-
L. helveticus	ATCC 15009^T	++++	-
L. intestinalis	ATCC 49335^T	+++	-
L. murinus	ATCC 35020^T	++++	-
L. parabuchneri	ATCC 12936^T	++++	-
L. paracasei subsp. *paracasei*	CCUG 27320^T	+++	-
L. plantarum	NCIMB 8827^T	+++	-
L. ruminis	ATCC 27781^T	++++	-
L. sakei subsp. *carnosus*	CCUG 8045^T	++	-
L. salivarius	DEVRIESE 94/438^T	+++	-
L. plantarum	NCCB 46043^T	+++	-
L. lactis 53	-	-	-
Streptococcus. thermophilus A	-	-	-
S. thermophilus B	-	+++	-
Leuconostoc mesenteroides	-	-	-
Bacillus subtilis	DSM 7-10^T	-	-
Enterococcus faecium	CECT 410^T	-	-
E. faecalis	CECT 184^T	-	-

Table 2.2 Bacterial strains used in PNA-FISH assays and their specificity with Lac663 and Gard162 probes. (**Continuation)**

Bacterial species	Collection strain	Lac663 Probe efficiency	Gard162 Probe efficiency
Gardnerella vaginalis 5-1	-	-	++++
G. vaginalis 101	-	-	++++
G. vaginalis AMD	-	-	++++
G. vaginalis	ATCC	-	++++
G. vaginalis	Belgian isolate 1	-	+++
G. vaginalis	Belgian isolate 2	-	++++
G. vaginalis	Belgian isolate 3	-	++++
G. vaginalis	Belgian isolate 4	-	++++
G. vaginalis	Belgian isolate 5	-	++++
G. vaginalis	Belgian isolate 6	-	++++
G. vaginalis	Belgian isolate 7	-	+++
G. vaginalis	Belgian isolate 8	-	+++
G. vaginalis	Belgian isolate 9	-	++++
G. vaginalis	Belgian isolate 10	-	++
G. vaginalis	Belgian isolate 11	-	++++
G. vaginalis	Belgian isolate 12	-	+++
G. vaginalis	Belgian isolate 13	-	+++
G. vaginalis	Belgian isolate 14	-	++
G. vaginalis	Belgian isolate 15	-	+++
G. vaginalis	Belgian isolate 16	-	+++
G. vaginalis	Belgian isolate 17	-	++++
G. vaginalis	Belgian isolate 18	-	++++
Atopobium vaginae	CCUG 38953^{T}	-	-
A. vaginae	CCUG 42099^{T}	-	-
A. vaginae	CCUG 44116^{T}	-	-
A. vaginae	Clinical isolate	-	-
Bacillus cereus	-	-	-
Enterobacter aerogenes	CECT 684^{T}	-	-
Escherichia coli O157:H7	NCTC 12900^{T}	-	-

Table 2.2 Bacterial strains used in PNA-FISH assays and their specificity with Lac663 and Gard162 probes. (**Continuation)**

Bacterial species	Collection strain	Lac663 Probe efficiency	Gard162 Probe efficiency
Staphylococcus aureus	CECT 976^{T}	-	-
S. aureus	CECT 86^{T}	-	-
Shigella flexneri	ATCC 12022^{T}	-	-
Listeria monocytogenes	-	-	-
L. monocytogenes	CECT 5873^{T}	-	-
L. seeligeri	CECT 917^{T}	-	-
Klebsiella pneumoniae subsp. *Ozaenae*	ATCC 11296^{T}	-	-
Salmonella typhi	-	-	-
S. enterica	-	-	-
Escherichia coli	CECT 434^{T}	-	-
Prevotella bivia	ATCC 29303^{T}	-	-
Mobiluncus mulieris	ATCC 26-9^{T}	-	-
Fusobacteria nucleatum	Clinical isolate	-	-

The PNA Probe (Lac663 and Gard162) efficiencies were tested in triplicate experiments for each strain, with the following hybridization PNA-FISH qualitative evaluation: (-) Absence of hybridization; (+) Poor hybridization; (++) Moderate hybridization; (+++) Good hybridization; (++++) Optimal hybridization. The table shows the median value from the three experiments for each strain.

2.3.4 Detection of *Lactobacillus* spp. and *G. vaginalis* by Multiplex FISH

Once the hybridization procedure was fully optimized, the multiplex methodology was also tested against mixed bacterial cultures (containing *Lactobacillus* or/and *G. vaginalis* cells together with others species, Table 2.3) and infected tissue cell line (Table 2.4). Lac663 and Gard162 probes selectively bound to *Lactobacillus* and *G. vaginalis* strains, respectively.

Table 2.3 Results of the Lac663 and Gard162 probes specificity test in artificial mixed samples.

Species in the artificial mixed samples	Bacteria strain collection codes	Multiplex PNA-FISH assay	
		Lac663 Probe efficiency	Gard162 Probe efficiency
L. pentosus; G. vaginalis 5-1	CECT 4023^T; -	++++	++++
L. casei; G. vaginalis 101	CECT 5275^T; -	++++	++++
L. rhamnosus; G. vaginalis AMD	CECT 288^T; -	++++	++++
L. crispatus; G. vaginalis ATCC	ATCC 33820^T; -	++++	++++
L. delbrueckii; A. vaginae	ATCC 9649^T; CCUG 38953^T	+++	-
L. acidophilus; A. vaginae	ATCC 4356^T; CCUG 42099^T	++++	-
L. gasseri; A. vaginae	ATCC 9857^T; CCUG 44116^T	++++	-
L. paracasei; L. lactis 53	CCUG 27320^T; -	+++	-/+
L. rhamnosus; E. faecium	ATCC 7469^T; CECT 410^T	++++	-
L. reuteri; E. coli O157:H7	NCFB 2656^T; NCTC 12900^T	+++	-
S. aureus; G. vaginalis 5-1	CECT 976^T; -	-	++++
Shigella; G. vaginalis 101	ATCC 12022^T; -	-	++++
L. seeligeri; G. vaginalis AMD	CECT 917^T; -	-	++++
E. aerogenes; G. vaginalis ATCC	CECT 684^T; -	-	++++
L. pentosus; G. vaginalis ATCC*; E. faecalis*	CECT 4023^T; -; CECT 184^T	++++	++++
L. casei; G. vaginalis AMD*; A. vaginae*	CECT 5275^T; -; CCUG 38953^T	++++	++++
L. rhamnosus; G. vaginalis 101*; A. vaginae*	CECT 288^T; -; CCUG 42099^T	++++	++++
L. crispatus; G. vaginalis 5-1*; A. vaginae*	ATCC 33820^T; -; CCUG 44116^T	++++	++++
L. casei; L. mesenteroides; A. vaginae	CECT 5275^T; -; CCUG 38953^T	++++	-

The PNA probe (Lac663 and Gard162) efficiencies were tested in triplicate experiments for each strain, with the following hybridization PNA-FISH qualitative evaluation: (-) Absence of hybridization; (+) Poor hybridization; (++) Moderate hybridization; (+++) Good hybridization; (++++) Optimal hybridization. Median values from the three experiments for each strain are shown in the table.

The fluorescence signal was easily observable (Figure 2.2) and no cross hybridization with other species was detected (Table 2.3). Additionally, the multiplex also performed well in the presence of HeLa cells (Table 2.4) for all the bacterial concentrations evaluated (1×10^3 until 1×10^9 CFU/mL), thus confirming the previous *in silico* analysis of the PNA probes.

Table 2.4 Efficiency of the *Lactobacillus* spp. and *G. vaginalis* detection in adhesion assays with HeLa cell line.

Concentration of cells (CFU/mL)		**Multiplex PNA-FISH assay**	
L. crispatus	***G. vaginalis 5-1***	**Lac663 Probe efficiency**	**Gard162 Probe efficiency**
1×10^9	1×10^9	+++	+++
1×10^5	1×10^5	+++	+++
1×10^3	1×10^3	++++	+++
L. iners	***G. vaginalis 5-1***	**Lac663 Probe efficiency**	**Gard162 Probe efficiency**
1×10^9	1×10^9	+++	+++
1×10^5	1×10^5	+++	+++
1×10^3	1×10^3	++	+++

The PNA probe (Lac663 and Gard162) efficiencies were tested in each sample with the following hybridization PNA-FISH qualitative evaluation: (-) Absence of hybridization; (+) Poor hybridization; (++) Moderate hybridization; (+++) Good hybridization; (++++) Optimal hybridization. The table shows the median value from the three experiments for each sample.

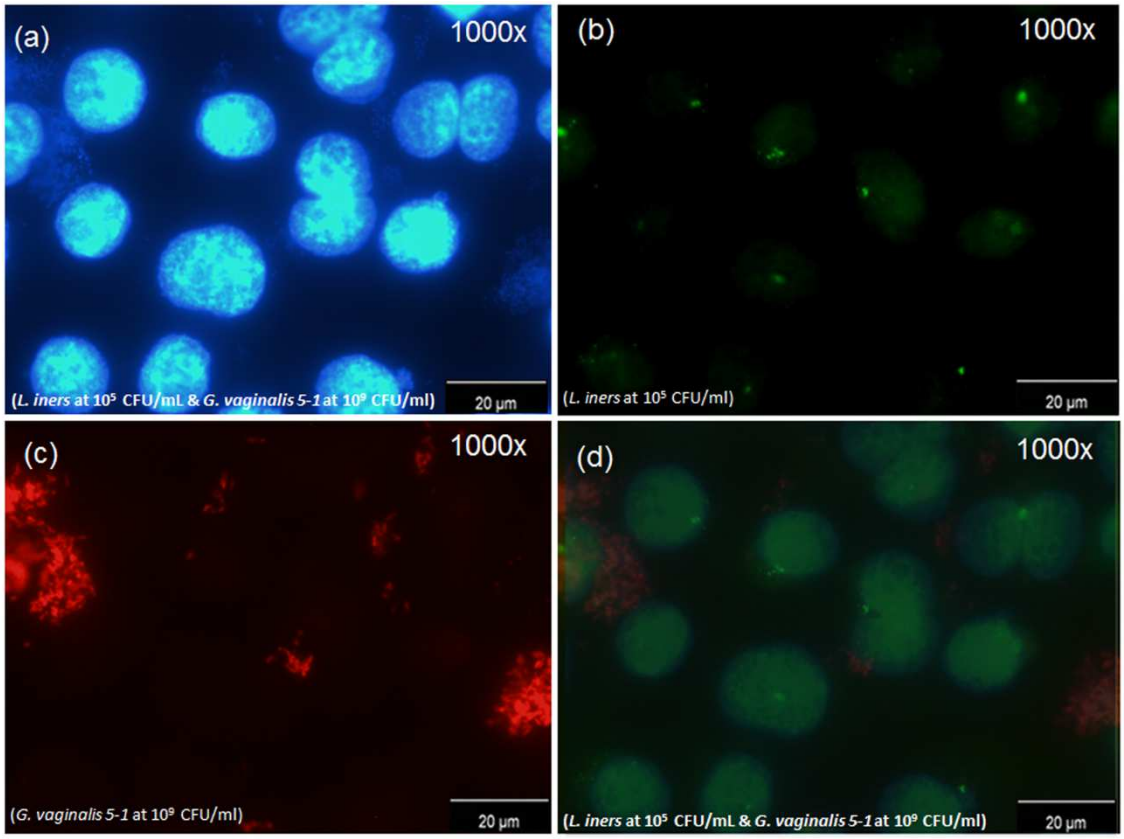

Figure 2.2 Fluorescence microscopy pictures with *Lactobacillus* spp. and *G. vaginalis* at different concentrations against HeLa cell line. (a) blue filter; (b) green filter; (c) red filter; (d) overlay of the three previous filters. These fluorescence microscopy pictures were taken in the same microscopic field with *L. iners* and *G. vaginalis* 5-1 from culture strain collection at different concentrations against HeLa cell line by DAPI staining and specific PNA probes (Lac663 and Gard162), associated with Alexa Fluor 488 and 594 fluorochromes, respectively.

2.4 Discussion

2.4.1 *In silico* and *in vitro* probe specificity and sensibility

Fluorescence microscopy has become a widely used technique for direct detection of bacteria in complex samples. In fact, many authors demonstrated the efficiency of FISH methodology for the analysis of lactobacilli and *G. vaginalis* (33–35, 37, 40–43). However, the herein described multiplex approach may be the simpler to perform and still has high specificity for lactobacilli and *G. vaginalis* detection.

As previously shown in Table 2.2, the Lac663 and Gard162 probes bound with high specificity to each target strain. In fact, Lac663 probe hybridized with all *Lactobacillus* collection strains, whereas no hybridization was observed for the others species used, except for *Lactococcus lactis* 53, *Streptococcus thermophilus* B and *Leuconostoc mesenteroides*, which showed some cross-hybridization with the probe when a washing step of 15 minutes was used. However, extending the washing step to 30 minutes and using fresh washing solution allowed the removal of the Lac663 probe poorly bound from all non-*Lactobacillus* strains, except for *S. thermophilus* B. However, *S. thermophilus* coccus morphology allows a clear differentiation from *Lactobacillus* spp., which has a rod-shaped morphology (with the exception of *L. iners*). Importantly, Lac663 probe showed absence of hybridization with several bacterial species from the Bacilli class, such as *Streptococcus thermophilus* A, *Enterococcus faecium* CECT 410, *Enterococcus faecalis* CECT 184, *Bacillus subtilis* DSM 7-10 and *Bacillus cereus*. Also, Lac663 probe did not hybridize with other common vaginal pathogenic bacteria, providing further evidence of its usefulness for *Lactobacillus* spp. detection in clinical samples.

Furthermore, the Gard162 probe showed hybridization with all *G. vaginalis* strains and no cross-hybridization was observed with other species, including other related pathogenic bacteria which may be present in the vaginal microflora, such as *A. vaginae*, *P. bivia*, *M. mulieris* and *F. nucleatum* (Table 2.2). It is worth to mention that *in silico* analysis of the Gard162 probe only identified one non-target strain as match, more precisely *Bifidobacterium indicum* HM534842 (RDPII ID: S002908348). However, *B. indicum* is not a common bacterium from vaginal microflora, as it is usually present in the

gut (44). Recently, a strong association between the bacterial loads in the vagina and rectum of pregnant women was described (45). Although some gut bacteria such as *Escherichia coli* (44) have been associated with vaginal infections, *B. indicum* has not been described as a pathogenic bacterium (46). The FISH efficiency and hybridization quality for the Gard162 probe, either alone or together with the Lac663 probe, confirmed the applicability of these two probes together in a multiplex PNA-FISH (Figures 2.1 and 2.2).

As shown above in Table 2.1, sensitivity and specificity equations allowed the comparison between our PNA probes and other published ones for *G. vaginalis* detection. For Lac663 probe, theoretical performance was quite similar to what had previously been reported for the other probes mentioned in Table 2.1. Although probes Lab158, LGC354A and the probe described by Burton *et al*. (2003) detected approximately 1 to 8% more *Lactobacillus* strains in comparison with our probe, Lac633 was found to be the probe with the lowest number of false positive hits (Table 2.1). In fact, the Lac663 probe does not cross-react with 3,617, 8,781 and 11,332 non-*Lactobacillus* strains that are detected with the Lab158, LGC354A and Burton *et al*. (2003) probes, respectively. From Table 2.1 it can be concluded that only the LAB759 probe was more specific than the herein developed Lac663 probe. However, the LAB759 probe shows a clearly lower sensitivity percentage (80.17%) compared to our probe (91.50%). It is also important to note that our probe has the shortest oligonucleotide sequence from all the probes for lactobacilli detection listed in Table 2.1, more precisely 1 and 3 nucleotides less than the other PNA probe and the shorter DNA probe (LGC354A), respectively. This implies that the Lac663 probe should penetrate better through the cell wall and that 1 base mismatch can be more easily discriminated (13). Also, Lab158, LGC354 and PNA Burton *et al*. (32) probes were found to cross-hybridize with one strain (RDPII ID: S000536416) from *G. vaginalis*, which might be incompatible with a multiplex approach to be used in vaginal samples. On the other hand, it is possible that this *G. vaginalis* strain was a misidentified *L. iners* strain, because confusion between both species has been reported (47).

Gard162 theoretical performance in specificity (100 %) was found to be similar to other probes for *G. vaginalis* detection that have been previously reported (Table 2.1). G.vag1008 is the only probe with higher sensitivity (97.5%) than our probe, being able to detect an extra *G. vaginalis* strain. This higher sensitivity is due to the presence of a

degenerate oligonucleotide in the sequence of the probe (Table 2.1), allowing G.vag1008 to act as two different sequence probes. However, G.vag1008 has 24 oligonucleotides (i.e. 9 nucleotides more than our probe) and it is a DNA probe, which penetrates the cell wall less efficiently (13) and requires longer hybridization periods.

GardV probe detected species from several bacterial genera present in vaginal samples, such as *Alloscardovia*, *Parascardovia* and *Scardovia* spp. (48). G.vag1008 probe hybridized with *Aeriscardovia* spp. that may also be found in vaginal samples (48) and therefore, this represents an important pitfall for the *G. vaginalis* detection with such probes.

It is important to notice that our Gard162 probe is the first PNA probe specifically designed for *G. vaginalis* detection. Furthermore, other PNA probes for the detection of lactobacilli (32, 42) revealed several disadvantages when compared to the Lac663 probe, as shown before (24).

2.4.2 Multiplex FISH detection

Although numerous authors attempted to correlate differences between healthy and BV vaginal samples (49–52), no consensus was attained, except that biofilm formation of *G. vaginalis* and a decrease in lactobacilli number could be considered as the initial stages in the pathogenesis of BV (33, 53). Swidsinski and colleagues conducted an international follow-up study in which vaginal samples from several BV patients were analyzed by DNA-based FISH and a dense and active bacterial biofilm on vaginal mucosa was detected, primarily consisting of *G. vaginalis* (43). Therefore, multiplex FISH to analyze *G. vaginalis* biofilm establishment and subsequently lactobacilli replacement appeared to be a useful molecular methodology for BV diagnosis in vaginal samples. Although several authors have been developing specific probes for *G. vaginalis* and *Lactobacillus* spp. detection by FISH, our multiplex method presents new improvements on the method (Table 2.1).

To evaluate the efficiency and eventual pitfalls of our multiplex FISH methodology previously to a prospective study using vaginal samples (see chapter III), we devised an *in vitro* experiment mimicking the shift from healthy vaginal flora to BV. HeLa cells were incubated with different concentrations of *G. vaginalis* and *Lactobacillus* strains (*L. crispatus* and *L. iners*), ranging from normal to BV vaginal microflora contents

(1×10^3 to 1×10^9 CFU/mL; Table 2.4). The HeLa cell line is an established tool in experimental research with lactobacilli. It has not only been used to study attachment of several *Lactobacillus* species, but also of other pathogens (54–56). The *Lactobacillus* strains used in this work were selected because high concentrations of *L. crispatus* (in conjugation with low loads or absence of *G. vaginalis*) are usually associated to the normal vaginal microflora, while high concentrations of *L. iners* (in conjugation with high loads of *G. vaginalis*) are commonly associated to the microflora of BV diagnosed women (23, 47, 57). The efficiency of our multiplex PNA-FISH methodology was demonstrated by the ability of the PNA probes to hybridize in a large range of *Lactobacillus* spp. and *G. vaginalis* concentrations, even in the presence of epithelial cells (Table 2.4). As referred above, Swidsinski and colleagues (33, 43) used a multiplex FISH methodology to study BV biolfims and a drawback of their approach is that it requires pre-treatment with lysozyme before fixation and the use of urine or paraffin-embedded samples. These experimental steps increase the analysis time and decrease FISH efficiency for *Lactobacillus* spp. and *G. vaginalis* strains detection, due to the lower number of cells available for hybridization. The advantage of our methodology is that it does not require a pre-treatment for FISH analysis. Another DNA hybridization test for vaginal infection was reported by Witt and colleagues (58). The authors evaluated the Affirm VPIII Kit, which detected *G. vaginalis*, *Candida* spp. and *Trichomonas vaginalis* in clinical samples, using two distinct single-stranded nucleic acid probes for each organism, which makes the analysis more complex and vulnerable to experimental pitfalls. This validated method showed sensitivity and specificity values for *G. vaginalis* of 89.5% and 97.1%, respectively, both lower than our Gard162 experimental values (95.0% and 100%, respectively). Furthermore, Fredricks and colleagues developed a FISH methodology for molecular identification of unknown bacteria associated with BV (35), using DNA probes Eub338-Cy5 and G.vag198-Cy3. However, the Eub338 is an unspecific probe used to detect *Lactobacillus* spp., thus detecting all species of the order Bacillales; and G.vag198 corresponds to a twenty five oligonucleotide probe with high specificity (100%) but with low sensitivity (85.0%) when compared to our probe (Table 2.1). Both these probes worked together at a hybridization temperature of 45 ºC, which may easily lead to the occurrence of false positive results. Moreover, previous studies have shown that probes with Cy fluorochromes present a lower fluorescence signal than those with the corresponding Alexa Fluor (59).

To conclude, our main purpose was achieved by demonstrating the *in vitro* applicability of the PNA multiplex methodology for detection of *Lactobacillus* species and *G. vaginalis* in the presence of HeLa epithelial cells and other taxonomically related or pathogenic bacterial strains commonly found in vaginal samples. These *in vitro* results confirmed the previous *in silico* analysis from Lac663 and Gard162 probes.

2.5 Conclusions

In summary, the use of the PNA multiplex FISH assay herein described significantly increases the specificity and sensitivity of the detection of *Lactobacillus* spp. and *G. vaginalis* strains in mixed samples, and no interference was observed in the presence of human epithelial cells. As previously discussed, there are no consensual agreements regarding BV markers, except for lactobacilli number decrease and initial adherence, and consequent biofilm formation from *G. vaginalis*. Our approach allows a fast identification (approximately 3 hours) of the main bacteria involved in BV establishment. The next steps for the validation of this methodology consist in a prospective study using a collection of vaginal samples isolated by our research group, which will enable the evaluation of PNA-FISH as a BV diagnostic technique, as well as a comparison with the standard BV diagnostic method. Furthermore, our research group will attempt to detect BV biofilm formation in clinical samples and to characterize possible interactions with other unknown bacteria in the biofilm. Finally, the combination of the PNA-FISH methodology with other methodologies, such as confocal laser scanning microscopy (CLSM) and quantitative polymerase chain reaction (qPCR), may help to better understand the BV etiology.

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Chapter III

Application of the Multiplex PNA-FISH methodology for diagnosis of Bacterial vaginosis

Abstract

Bacterial vaginosis (BV) is one of most common vaginal infection and its diagnosis by classical methods reveals low specificity. Our goal was to compare BV diagnosis in vaginal samples with standard methods and our Peptide Nucleic Acid (PNA) probes by Fluorescence *in situ* Hybridization (FISH) methodology. Also, we described the first PNA-FISH methodology for BV diagnosis, providing results in approximately 3 hours. The results showed a sensitivity of 66.7% (95% confidence interval (CI), from 49.7 to 80.4%) and a specificity of 94.2% (95% CI, from 83.1 to 98.5%), demonstrating the higher specificity of the PNA-FISH method and showing false positive results in BV diagnosis commonly obtained by the classical methods. This methodology combines the specificity of PNA probes for *Lactobacillus* species and *G. vaginalis* visualization and the calculation of the microscopic field by Nugent score, allowing a trustful evaluation of the bacteria present in vaginal microflora and avoiding the occurrence of misleading diagnostics. Therefore, the PNA-FISH methodology represents a valuable alternative for BV diagnosis.

Keywords: Fluorescence *In Situ* Hybridization (FISH); Peptide Nucleic Acid Probe (PNA probe); *Lactobacillus* spp.; *Gardnerella vaginalis*; Bacterial vaginosis.

3.1 Introduction

BV often exhibits high prevalence, high relapse rates and associated complications, which makes this infection of paramount global importance (1, 2). As previously referred, the BV etiology remains relatively unknown although it is normally characterized by a decrease in vaginal lactobacilli number and a simultaneous increase of the anaerobes number (2). BV is associated with increased taxonomic richness and diversity (3). Therefore, vaginal bacterial communities differ dramatically between healthy patients and patients with BV, where *G. vaginalis* is present in over 90% of BV cases (4).

The most frequently used method for BV diagnosis is the physician's assessment by Amsel clinical criteria wherein BV diagnosis requires the observation of three of the four clinical criteria already mentioned (see Chapter I), ignoring the vaginal microflora that the patients may exhibit (5). Alternatively, laboratory diagnostic is based on the Nugent score analysis, a microscopic method that quantifies three different bacteria morphotypes present in the smears (see Chapter I) but disregarding the clinical symptoms of the patients (6). Although both methodologies are easy and fast to perform, they do not present a high specificity for BV diagnosis. When combined, these standard tests have a sensitivity and specificity of 81 and 70% (5), respectively. To improve BV diagnosis, several new molecular methodologies have been proposed (7–9).

FISH had been applied in several prospective studies and vaginal microbiome characterization in BV research (10–13). However, these BV studies used FISH with DNA probes, that frequently showed low fluorescent responses due to numerous factors (14), such as difficulties into cell membranes permeability, degradation of the probe by cell enzymes, low affinity to the target sequence (15). As previously described, PNA probes had emerged as more efficient probes, binding to their complementary nucleic acid sequences with higher thermal stability and specificity (13–18).

To determine the feasibility of our novel PNA probe (described in the previous chapter) as a diagnostic method to be used in BV, we selected 91 vaginal samples from Portuguese women and characterized it's microflora using our probe and protocol and compared those results with the laboratory microscopic derived method using the Nugent score. Finally, *G. vaginalis* detection by Polymerase Chain Reaction (PCR) was performed to confirm the presence of this bacterium in the selected vaginal samples.

3.2 Materials and methods

3.2.1 Vaginal sample collection and preparation

A total of 91 samples of vaginal swabs were obtained, after informed consent, as approved by the Institutional Review Board (IRB) of University of Minho. The vaginal swabs were collected for Gram stain, PCR and FISH procedures, using the culture swab transport system (VWR, CE0344, Italy). These swabs were brushed against the lateral vaginal wall to collect the vaginal fluid sample, then placed in the culture swab transport media and immediately conserved at 4 ºC. First, the set of swabs was used for *Gram* stain procedure as described by Nugent and colleagues (6). Next, the collected swabs were immersed in 1 mL of phosphate buffer saline (PBS) and centrifuged at 17,000 *g* during 5 min at room temperature. Afterwards, the pellet was resuspended in 2 mL of saline solution (0.9% NaCl prepared in distilled water) and finally diluted 1:10 in saline solution or PBS to eliminate possible contaminants for PCR and FISH procedures, as previously described (19, 20).

3.2.2 Classification of vaginal swabs under Nugent score

Vaginal swabs evaluation was performed using the Nugent criteria score (6). Briefly, vaginal smear was examined under oil immersion objective (1000x magnification) and through 10-15 microscopic fields. Initially, each smear was graded as per standardized, quantitative, morphological classification developed by Nugent. More specifically, composite score was grouped into three categories, scores 0-3 being normal, 4-6 being intermediate, and 7-10 being definite bacterial vaginosis. Finally, the smears that showed scores between 0-3 and 7-10 were selected for further study, as normal (–) and BV (+) samples, respectively. Meanwhile, the smears with a Nugent score of 4-6 were rejected from our study.

3.2.3 Polymerase chain reaction for identification of bacteria in vaginal samples

A preliminary molecular characterization was performed using PCR detection of *G. vaginalis*. *G. vaginalis* was specifically detected by 16S rDNA amplification PCR using forward primer GV-Ap (5'- TCC TGT CTA CCA AGG CAT CC-3') and reverse primer GV-Sp (5'- CGT GTG ATA ACC GTC AGG TG-3'). This set of primers was previously developed and characterized by our research group (19). The PCR amplification was then performed according to the publication mentioned above. Briefly, all samples were pre-treated during 5 min at 100 ºC and then placed at 4 ºC for 5 min. The conditions for PCR amplification were as follows: 1 µL template; 0.25 µM forward primer; 0.25 µM reverse primer; 0.5 µL deoxyribonudeotide triphosphate (dNTP) mix; 2.5 U Taq DNA polymerase (BioRad, Portugal); 2 µL 10× buffer and 14 µL ultra-pure water for each PCR reaction. PCR parameters in the MJ Mini Personal thermal cycler (BioRad, Portugal) were as follows: denaturation 94 ºC for 30 s; annealing 60 ºC for 30 s; and elongation 72 ºC for 60 s. After 40 cycles the reaction mixture was cooled to 4 ºC.

For each amplification product, a 2 µL sample was analyzed on a 1% (wt/vol) agarose gel electrophoresis followed by ethidium bromide staining. Electrophoreses were carried out on all samples and using an aliquot with no template as negative control, an internal positive PCR control (no *G. vaginalis*) and ladder marker IV (Roche Biochemicals, Germany). Electrophoresis was carried out at 80 mV for 45 min and followed by Gel imaging system (BioRad, Portugal) analysis.

3.2.4 Fluorescent *in situ* hybridization and vaginal bacteria quantification

The glass slides containing vaginal swabs were first fixed and hybridized with Lac663 and Gard162 PNA probes, as described in the previous chapter. Briefly, the glass slides were fixed with 4% paraformaldehyde followed by 50% ethanol. Hybridization was performed at 60 °C for 90 min and then washed with a fresh solution. An additional 4',6-diamidino-2-phenylindole (DAPI; Sigma, Germany) staining step was done at the end of the hybridization procedure. Then microscopic visualization was performed using an Olympus BX51 (Olympus Portugal SA, Portugal) epifluorescence microscope equipped with a CCD camera (DP72; Olympus, Japan). These assays were repeated three times and a negative control was performed simultaneously with each step previous described.

Bacterial cells quantification was conducted through the National Institutes of Health image analysis software ImageJ (version 1.451) (21).

3.2.5 Statistical analysis

The data was analyzed to calculate sensitivity, specificity, accuracy, positive and negative likelihood ratios (PLR and NLR, respectively) and 95% confidence intervals (CI) of the PNA-FISH methodology against the classic Nugent criteria score using a clinical online statistical software (www.vassarstats.net/clin1.html; accessed 2013) (22).

3.3 Results

On this prospective study, we used 91 vaginal swabs that were classified by the classic Nugent criteria score using Gram staining and our PNA-FISH methodology. Also, *G. vaginalis* presence in vaginal samples was confirmed by PCR.

As shown in Table 3.1, 82 vaginal swabs showed the same results in Gram staining or PNA-FISH, being 30 samples positive for BV and 52 samples negative. In addition, *G. vaginalis* was detected by PCR in 22 of these 30 BV positive samples and only 3 of the 52 normal (BV negative) samples. However, some discrepancies were also found between the two methodologies, more exactly in 9 vaginal swabs, which were positive for BV by Gram staining but negative by PNA-FISH evaluation. *G. vaginalis* was detected by PCR in 4 of these 9 vaginal swabs (see Table 3.1).

Table 3.1 Bacterial vaginosis diagnosis by Gram staining and PNA-FISH method using Nugent score criteria. PCR was also performed for *G. vaginalis* detection.

Samples	BV diagnosis by Nugent	BV diagnosis by PNA-FISH method	*G. vaginalis* detection by PCR
UM057	+	+	+
UM059	+	+	+
UM064	+	+	+
UM065	+	+	+
UM066	+	+	+
UM074	+	+	+
UM090	+	+	+
UM104	+	+	+
UM121	+	+	+
UM126	+	+	+
UM127	+	+	+
UM137	+	+	+
UM165	+	+	+
UM209	+	+	+
UM222	+	+	+
UM226	+	+	+
UM230	+	+	+
UM231	+	+	+
UM234	+	+	+
UM235	+	+	+
UM242	+	+	+
UM262	+	+	+
UM056	+	+	-
UM072	+	+	-
UM086	+	+	-
UM163	+	+	-
UM224	+	+	-

Table 3.1 Bacterial vaginosis diagnosis by Gram staining and PNA-FISH method using Nugent score criteria. (Continuation)

Samples	BV diagnosis by Nugent	BV diagnosis by PNA-FISH method	*G. vaginalis* detection by PCR
UM241	+	+	-
UM252	+	+	-
UM278	+	+	-
UM170 *	+	-	+
UM245 *	+	-	+
UM265 *	+	-	+
UM301 *	+	-	+
UM108 *	+	-	-
UM117 *	+	-	-
UM120 *	+	-	-
UM132 *	+	-	-
UM255 *	+	-	-
UM115	-	-	+
UM116	-	-	+
UM118	-	-	+
UM070	-	-	-
UM071	-	-	-
UM075	-	-	-
UM077	-	-	-
UM084	-	-	-
UM093	-	-	-
UM098	-	-	-
UM101	-	-	-
UM105	-	-	-
UM107	-	-	-
UM110	-	-	-
UM112	-	-	-
UM114	-	-	-
UM119	-	-	-
UM122	-	-	-

Table 3.1 Bacterial vaginosis diagnosis by Gram staining and PNA-FISH method using Nugent score criteria. (Continuation)

Samples	BV diagnosis by Nugent	BV diagnosis by PNA-FISH method	*G. vaginalis* detection by PCR
UM125	-	-	-
UM129	-	-	-
UM130	-	-	-
UM133	-	-	-
UM135	-	-	-
UM140	-	-	-
UM143	-	-	-
UM145	-	-	-
UM146	-	-	-
UM148	-	-	-
UM149	-	-	-
UM151	-	-	-
UM152	-	-	-
UM154	-	-	-
UM156	-	-	-
UM183	-	-	-
UM198	-	-	-
UM210	-	-	-
UM211	-	-	-
UM220	-	-	-
UM257	-	-	-
UM266	-	-	-
UM268	-	-	-
UM269	-	-	-
UM271	-	-	-
UM272	-	-	-
UM273	-	-	-
UM274	-	-	-
UM275	-	-	-
UM276	-	-	-

Table 3.1 Bacterial vaginosis diagnosis by Gram staining and PNA-FISH method using Nugent score criteria. (Continuation)

Samples	BV diagnosis by Nugent	BV diagnosis by PNA-FISH method	*G. vaginalis* detection by PCR
UM277	-	-	-
UM300	-	-	-
UM302	-	-	-
UM303	-	-	-

* Discrepancies between Nugent and PNA classification in vaginal sample as Bv+ and Bv–, respectively.

Additionally, PNA-FISH methodology was capable to illustrate clear differences between healthy and BV swabs, showing specific detection of *Lactobacillus* spp. and *G. vaginalis* species directly in clinical samples. In fact, UM300 (healthy) and UM235 (BV) samples exhibited a totally different vaginal microflora (see Figure 3.1), being clue cells and *G. vaginalis* augmentation easily detected in UM235 sample.

Based on the results, an experimental specificity of 94.2% (95% CI, 83.1 to 98.5%) and sensitivity of 66.7% (95% CI, 49.7 to 80.4%) were obtained for the BV diagnosis by our PNA-FISH method (Table 3.2). As shown in Table 3.2, when compared with the standard Gram staining, PNA-FISH method was able to determine normal flora in 49 from a total of 52 healthy cases and capable to categorize 26 from a total of 39 BV cases. This results in an accuracy of BV diagnosis by our novel PNA-FISH method of 82.4% (95% CI, 72.2 to 88.8%), evidencing a PLR of 11.56 and a NLR of 0.35.

Table 3.2 Comparison between PNA-FISH method *versus* Gram staining using Nugent score criteria for BV diagnosis.

PNA-FISH results	Gram results		
	BV+	BV -	Total
BV +	26	3	29
BV -	13	49	62
Total	39	52	91
Statistical analysis of PNA-FISH method			
Statistical parameters	Estimated value	Lower limit	Upper limit
Sensitivity	**66.7%**	49.7%	80.4%
Specificity	**94.2%**	83.1%	98.5%
Accuracy	**82.4%**	72.2%	88.8%
Positive likelihood	**11.56**	3.77	35.44
Negative likelihood	**0.35**	0.23	0.55

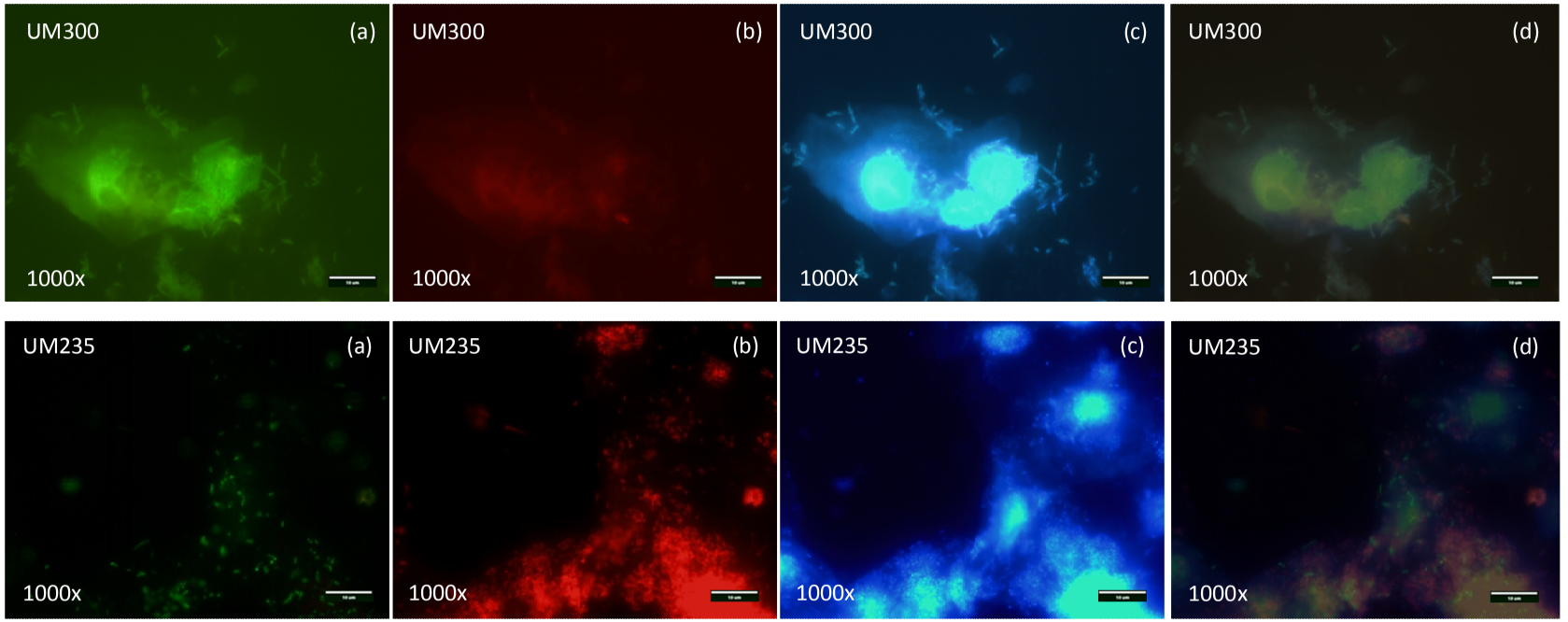

Figure 3.1 Fluorescence microscopy pictures of *Lactobacillus* spp., *Gardnerella vaginalis* and others bacteria species from a healthy (UM300) and BV (UM235) vaginal clinical samples by specific PNA probes (Lac663 and Gard162) associated with Alexa Fluor 488 and 594 fluorochromes and DAPI staining, respectively. (a) green filter; (b) red filter; (c) blue filter; (d) overlay of the three previous filters.

3.4 Discussion

Conventional laboratory BV diagnosis accuracy is highly dependent on the training and experience of the technician due to the unspecific staining of the Gram method (23). As we shown here, FISH methodology arises as an alternative technique for BV diagnosis. Nonetheless, the technique performance depends on several factors (accuracy, specificity, sensitivity, PLR, NLR, among others) and implications of false-negative results on laboratory analysis requires further consideration (24).

The specificity and PLR of PNA-FISH demonstrated a strong association between a positive result for BV diagnostic and the probability of the patient having indeed BV. Moreover, our experimental specificity (94.2%) revealed to be superior than Nugent's Gram stain system specificity (83%) (25). Therefore, our method was able to correctly identify 94.2% of those patients previously classified with normal vaginal flora making PNA-FISH a trustful method to ensure a healthy diagnosis and avoiding false positive results. However, the sensibility and NLR parameters were lower than expected. Despite the experimental sensitivity (66.7%) was much lower than the specificity of the Gram stain by Nugent score (89%) (25), it was higher than the Amsel criteria sensitivity (60%) determined by Gallo and colleagues (26). Nevertheless, it is important to refer that 5 samples of the 13 false negative results showed a negative result for *G. vaginalis* in the PCR procedure (see Table 3.1), thus meaning that other bacterial species with similar Gram stain morphology could be at high number in the samples leading to an incorrect classification of BV according to Nugent criteria. In fact, Verhelst and colleagues presented evidences that infers a lack of accuracy in the interpretation of the results in Gram stain by Nugent score in their clinical results (27). Forsum and colleagues also found discrepancies in scoring bacterial cell types, when pleomorphic lactobacilli and other kinds of bacteria could be regarded as *G. vaginalis* cells, leading to an incorrect BV diagnosis (28, 29). Also, *G. vaginalis* may vary in size and form from round to more elongated where there is no defined border to separate them from the lactobacilli morphotypes (28), thus illustrating again problems in the accuracy of the smears interpretation. These facts suggest that the sensitivity value is likely to be underestimated, since in some false negative results, samples did not seem to contain *G. vaginalis* as could be

seen through PCR analysis. By excluding those 5 samples (more exactly, UM108, UM117, UM120, UM132 and UM255), our PNA-FISH method would show a 76.5% (95% CI, 58.4 to 88.6%) and specificity of 94.7% (95% CI, 84.5 to 98.6%). So, all these results evidenced the need for a molecular methodology capable to recognize specifically the bacteria present in the swab samples.

Overall, despite the cost effective nature of the Nugent score, PNA-FISH appears to be an accurate method for detecting BV from vaginal swabs, maintaining similar complexity as the previous standard method.

3.5 Conclusions

In summary, in this chapter we described the first PNA-FISH methodology applied for BV diagnosis, suggesting a reliable alternative to the Amsel criteria and Gram stain under Nugent score. It is also the only alternative that simultaneously allows the specific ribosome RNA sequence recognition and spatial visualization of the bacterial balance directly in vaginal swabs. This methodology combines the specificity of PNA probes for *Lactobacillus* species and *G. vaginalis* visualization and the calculation of the microscopic field by Nugent score, allowing a trustful evaluation of the bacteria present in vaginal microflora and avoiding false diagnostics.

Our data showed some problems in the accuracy of the smears interpretation and classification by Gram staining under Nugent criteria, thus supporting previous studies (27–29). However, it is important to mention that our evaluation was performed with only 91 vaginal swabs and so further studies including a larger number of samples will be required.

To conclude, our PNA-FISH methodology arises as a trustful alternative for a correct diagnosis of BV.

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Chapter IV

Initial adhesion of Bacterial vaginosis anaerobes in epithelial cells

Abstract

Bacterial vaginosis (BV) is the most prevalent vaginal disorder worldwide, being its etiology still unknown. Multiple microorganisms have been found in BV patients but its virulence potential is not fully understood. Initial adhesion to the vagina epithelium is a crucial step for the development of infection. Our goal was to quantify the initial adhesion of *Gardnerella vaginalis, Atopobium vaginae, Mobiluncus mulieris, Prevotella bivia* and *Fusobacteria nucleatum* against *Lactobacillus spp.* using two *in vitro* competitive and displacement/blockage assays. Our results confirmed previous observations that *G. vaginalis* presented the higher capability of adhesion to human epithelial cells. Furthermore, in competition assays, it was the only species that outcompeted *L. crispatus*. While *A. vaginae* and *M. mulieris* were also able to adhere in high numbers, they were easily outcompeted by *L. crispatus.* The ability of BV-associated pathogens to displace a monolayer of *L. crispatus* and *L. iners* previously adhered to ME-180 cells was also tested. Interestingly, *G. vaginalis* and *P. bivia* showed increased ability to displace *L. crispatus* but no significant displacement was observed in *L. iners*. Finally, *L. iners* was able to enhance *G. vaginalis* 101 adhesion to ME-180 cells.

Keywords: Lactobacilli; *Gardnerella vaginalis*; BV anaerobes; competitive initial adhesion; blockage; displacement; epithelial cells.

4.1 Introduction

Adhesion to host cells or tissues is a necessary early step in the establishment of infection (1–3). As previously referred in Chapter I, BV is characterized by a decrease in beneficial vaginal bacteria (*Lactobacillus* spp.) (4–6) and also by an increase in the number of anaerobic bacteria, such as *Gardnerella vaginalis*, *Mobiluncus mulieris*, *Atopobium vaginae*, *Prevotella bivia* and *Fusobacteria nucleatum* (7–9). In 2005, Swidsinski and colleagues conducted a study in which vaginal epithelial biopsies from healthy subjects and those with BV were analyzed, and found that a multispecies biofilm (see Chapter I), predominated by *G. vaginalis* and *A. vaginae* adhered to the surface of the epithelium in BV (9). They hypothesized that *G. vaginalis* is the initial colonizing species and that its adherence is required before other BV-associated anaerobes are able to colonize the vaginal epithelium. *G. vaginalis* can display resistance to the antimicrobial products produced by *Lactobacillus* spp. including hydrogen peroxide and lactic acid (10, 11). Therefore, it has been proposed that *G. vaginalis* might compete with *Lactobacillus* spp. and enable other anaerobes to incorporate and grow within the biofilm (12). However, convincing evidence that *G. vaginalis* is an initial colonizer requires further study. Evidence indicates that certain *Lactobacillus* species are capable of blocking adhesion of pathogenic bacteria to the vaginal epithelium, and these have been studied for their potential use as probiotics (10, 13–15). The goal of this study was to characterize and quantify the initial adhesion of several of the most common BV-associated anaerobes in the presence of vaginal lactobacilli to ME-180 cervical epithelial cells. Also, we analyzed the ability of these anaerobes to compete for adherence to the cell monolayer when added simultaneously with lactobacilli and when added after the lactobacilli adhesion.

4.2 Materials and Methods

4.2.1 Culture of bacterial strains

L. crispatus EX533959VC06 is a vaginal isolate from a healthy woman (11). It was grown in Man, Rogosa and Sharpe both (MRS; Sigma-Aldrich, Germany) at 37 ºC under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 24–48 h prior to adhesion assays. *L. iners* ATCC 55195, *Atopobium vaginae* FA, *Mobiluncus mulieris* ATCC 26-9, *Prevotella bivia* ATCC 29303 and *Fusobacteria nucleatum* 718BVC are vaginal isolates. *G. vaginalis* 101 is a vaginal isolate from a woman with BV who gave birth preterm (16). All the anaerobes were grown in Brain Heart Infusion (BHI; Oxoid, United Kingdom) supplemented as previously described (16) and incubated at 37 ºC under anaerobic conditions for 24–48 h prior to adhesion assays. Prior to competition or displacement/blockage assays, all strains were harvested by centrifugation (4,000 *g*, 12 min, at room temperature), washed with sterile PBS and then centrifuged again (4,000 *g*, 12 min, at room temperature). The washing step was repeated twice. Then, the bacteria were resuspended in PBS and the concentration was adjusted to 2×10^3 CFU/mL (for competition assays) and 1×10^9 CFU/mL (for displacement/blockage assays) by optical density at 600 nm using a microplate reader (Tecan, Switzerland).

4.2.2 Culture of ME-180 cell line

ME-180 cervical epithelial cells (American Type Culture Collection, ATCC, USA) were cultured in McCoy's 5A medium (Quality Biologic, USA) supplemented with 10 % (vol/vol) Fetal bovine serum (FBS; Sigma, USA) and 1 IU penicillin/streptomycin mL^{-1} (MediaTech, USA), at 37 °C and in 5 % CO_2 (vol/vol). ME-180 cells were seeded into Lab-Tek 8 chamber tissue culture-treated glass slides (0.8 cm^2; Thermo Fisher Scientific, USA). ME-180 cells were incubated at 37 ºC and 5% CO_2 (vol/vol) and were grown until a cell monolayer. Before the interference and blockage adhesion assays, the cells were washed twice with 300 µL of sterile phosphate buffer saline (PBS) to remove non-adhered cells and culture media.

4.2.3 Competition adhesion assays to ME-180 cells

To assess the competition for adhesion, *L. crispatus* and one of the anaerobic species were added at a final cell density of 1×10^3 CFU/mL to the slide chambers containing ME-180 monolayers. Then, the co-cultures were incubated for 30 min at 37 °C in anaerobic conditions and 120 rpm. Finally, each chamber was carefully washed twice with 300 µL of sterile PBS to remove non-adherent bacteria and was allowed to air-dry before FISH hybridization procedure (see section 4.2.5). In each assay, adhesion controls were performed simultaneously in each 8 chamber slide with a monolayer of ME-180 epithelial cells by adding each bacterium individually and maintaining the same experimental conditions.

4.2.4 Displacement and blockage adhesion assays to ME-180 cell lines

For displacement and blockage adhesion assays, aliquots of 400 µL of either *L. crispatus* or *L. iners* were added to the epithelial monolayers in each well of the 8 chamber slides. Afterwards, the chamber slides were incubated for 4 h at 37 °C, in anaerobic conditions and 120 rpm. Non-adherent lactobacilli were removed by washing with 300 µL of sterile PBS and subsequently a second adhesion step was performed, using one BV-associated anaerobe, for 30 min at 37 °C, in anaerobic conditions and 120 rpm. Finally, each chamber was carefully washed twice with 300 µL of sterile PBS to remove non-adherent bacteria and let to air-dry before FISH hybridization procedure (see section 4.2.5). In each assay, adhesion controls were performed simultaneously in each 8 chamber slide by adding each species individually and maintaining the same experimental conditions.

4.2.5 Fluorescence *in situ* hybridization (FISH) procedure

The 8 chamber slides containing epithelial monolayers and adherent bacteria were fixed and hybridized with Lac663 and Gard162 PNA probes, which we optimized in a previous study (17, 18) and then stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, Germany). Briefly, the adhered bacteria slides were fixed with 4% paraformaldehyde followed by 50% ethanol, for 10 min, at room temperature. After the fixation step, the slides were covered with 20 µL of hybridization solution with PNA probe (200 nM). Hybridization was performed at 60 ºC for 90 min and for washing (60 ºC

for 30 min) and a fresh solution was prepared less than 24 h before use. Finally, the slides were allowed to air dry in the dark. An additional DAPI staining step was done at the end of the hybridization procedure, by covering each slide with 20 µL of DAPI (2.5 µg/mL, Sigma, USA) for 5 min at room temperature in the dark, followed by five washing steps with 20 µL of PBS. In each experimental assay, a negative control was performed simultaneously with each step previous described, but no probe or DAPI staining were added in the hybridization step.

4.2.6 Quantification of adhered cells by microscopic visualization

Prior to microscopy, one drop of non-fluorescent immersion oil (Merck, Germany) was added to the slide within each chamber. Microscopic visualization was performed using an EVOS*fl* fluorescence microscope (AMG, USA) equipped with a CCD camera (Sony ICX285AQ color, Japan) and filters capable of detecting the two PNA probes (GFP filter: 470 nm excitation and 525 nm emission, sensitive to the Alexa Fluor 488 molecule attached to the Lac663 probe; and RFP filter: 530 nm excitation and 593 nm emission, sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe). Also, DAPI staining was detected by an appropriate filter (DAPI filter: 360 nm excitation and 447 nm emission) present in the microscope. Finally, 20 images from random regions of each glass slide were taken in each filter previously referred at the same field of view. All images were acquired by AMG EVOS*fl* intrinsic software using a total magnification of ×1000. The lactobacilli and anaerobes adhered cells quantification was evaluated through the National Institutes of Health image analysis software ImageJ (version 1.451, freely available at: http://rsbweb.nih.gov/ij/). All these assays were repeated three times, on separate days, with three fields of view assessed each time.

4.2.7 Statistical analysis

The data was analysed using a two-tailed ANOVA or Student's *t*-test with SPSS statistical software (version 17.0) and expressed as mean ± standard deviation (SD). The *p* values below 0.05 were considered significant.

4.3 Results

4.3.1 Competition between *L. crispatus* and BV anaerobes for adhesion to ME-180 cells

Initially we studied the competition between several BV anaerobes and *L. crispatus*, a species that tends to promote vaginal health and to prevent the growth of other species, to determine its effect on initial adhesion in the ME-180 cell line (some examples are illustrated in Figure 4.1).

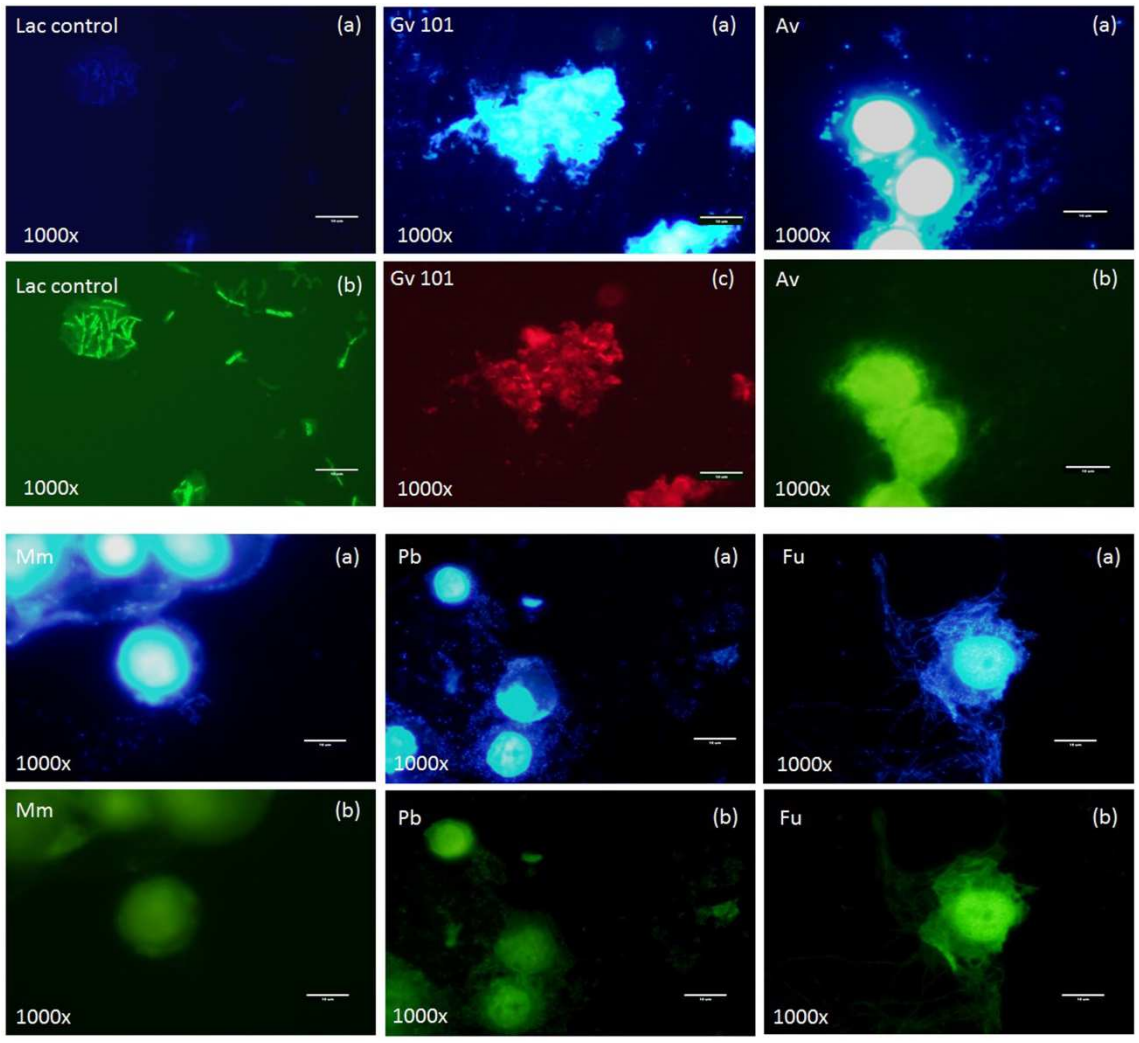

Figure 4.1 Fluorescence microscopy of the initial adhesion competitive assays between *L. crispatus* and anaerobe by 4',6-diamidino-2-phenylindole (DAPI) and specific PNA probes (Lac663 and Gard162) associated with Alexa Fluor 488 and 594 fluorochromes. (a) blue filter; (b) green filter; (c) red filter; Lac control, *L. crispatus*; Gv 101, *G. vaginalis* 101 & *L. crispatus*; Av, *A. vaginae* & *L. crispatus*; Mm, *M. mulieris* & *L. crispatus*; Pb, *P. bivia* & *L. crispatus*; Fu, *F. nucleatum* & *L. crispatus*.

As shown in Figure 4.1, *G. vaginalis* 101 exhibited the greatest capacity for adherence to ME-180 cells, confirming our previous observations (12). Interestingly, the *G. vaginalis* strain also maintained its ability to adhere in the presence of *L. crispatus* which was found to be better than the other species, and there was only a 10% reduction in adherence with respect to the control. This result was statistically different from the ones with other BV anaerobes (*ANOVA Tukey* statistical test values, $p < 0.05$). In the competition assays against *L. crispatus*, *G. vaginalis* adhered approximately 4-fold better than *A. vaginae* or *M. mulieris* and approximately 2-fold better than *P. bivia* (Figure 4.2). Adherence of *L. crispatus* was not significantly inhibited by any of the BV anaerobes tested.

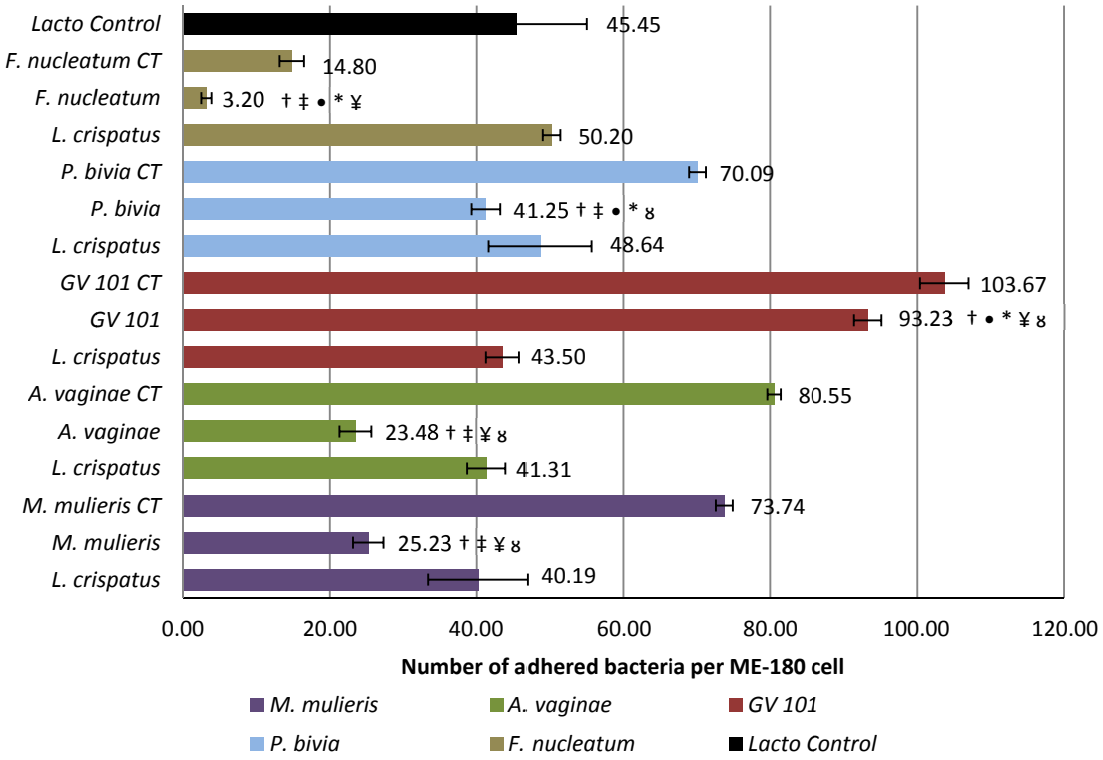

Figure 4.2 Initial adhesion competitive assays realized by equal mixture between *L. crispatus* and a BV anaerobe at low level each one (10^3 CFU/mL) to ME-180 cells during 30min at 100 rpm and anaerobic conditions. † $p<0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay. ‡ $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* 101 tested in the adhesion assay. • $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay. * $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay. ¥ $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay. ȣ $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

4.3.2 Blockage of BV anaerobes adhesion and displacement of lactobacilli in ME-180 cells

In order to simulate the introduction of BV-associated bacteria into a healthy vagina colonized by lactobacilli, we first allowed *L. crispatus* or *L. iners* to adhere to the epithelial monolayers and subsequently we added a BV-associated species to quantify the inhibitory effect of the lactobacilli on secondary colonization. *L. crispatus* inhibited the adherence of *G. vaginalis* 101 by approximately 43% (Table 4.1). Addition of *G. vaginalis* appeared to cause a slight displacement of adherent *L. crispatus*, but this was not found to be statistically significant. *L. crispatus* also reduced the adherence of *A. vaginae* and *M. mulieris* by approximately 50%. *P. bivia* and *F. nucleatum* appeared to be less susceptible to inhibition by *L. crispatus*. Interestingly, *L. iners*, which has been shown in previous studies to be less protective against BV relative to other vaginal lactobacilli (7), had a similar inhibitory effect on the adherence by all the BV-associated species except for *G. vaginalis* (Table 4.2). Indeed the adherence of *G. vaginalis* increased somewhat in the presence of *L. iners*, although this increase was not statistically significant. None of the anaerobes displaced *L. iners* as can be seen in Table 4.2.

Table 4.1 Blockage of adherence of BV-associated anaerobes to ME-180 epithelial cells by adherent *L. crispatus* and its displacement by BV-associated anaerobes. The number of each BV-associated anaerobe, when incubated at high level (1×10^9 CFU/mL), that adhered per ME-180 cell (± standard deviation) is shown on the left and the percentage of bacteria that adhered when the ME-180 monolayer was pre-coated with *L. crispatus* (1×10^9 CFU/mL) relative to the control (± standard deviation) is shown on the middle. Following the addition of a BV-associated anaerobe, the number of remaining *L. crispatus* was counted and compared to the *L. crispatus* control (62.91 per ME-180 cell ±1.96) and the percent (± standard deviation) of *L. crispatus* that remained adherent after addition of each BV anaerobe (1×10^9 CFU/mL) is shown on the right.

	Number of BV anaerobe per ME-180 cell	Percent of BV adherent to *L. crispatus*-coated ME-180 monolayer	Percentage of *L. crispatus* remaining after addition of BV anaerobe
Gardnerella vaginalis 101	232.11 (±6.39)	57.15 % [a,c,d,e,f] (±2.31)	76.37 % [c,d] (±4.93)
Atopobium vaginae FA	16.74 (±1.09)	51.42 % [a,b,f] (±7.28)	95.53 % [b,e,f] (±4.09)
Mobiluncus mulieris ATCC 26-9	16.61 (±1.60)	52.85 % [a,b,f] (±0.46)	95.62 % [b,e,f] (±4.57)
Prevotella bivia ATCC 29303	23.17 (±3.00)	70.11 % [b] (±6.17)	75.41 % [b,c,d] (±12.70)
Fusobacteria nucleatum 718BVC	25.79 (±1.16)	74.34 % [a,b,c,d] (±8.50)	82.94 % [b,c,d] (±12.71)

[a] $p<0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay.
[b] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* 101 tested in the adhesion assay.
[c] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay.
[d] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay.
[e] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay.
[f] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

Table 4.2 Blockage of adherence of BV-associated anaerobes to ME-180 epithelial cells by adherent *L. iners* and its displacement by BV-associated anaerobes. The number of each BV-associated anaerobe, when incubated at high level (1×10^9 CFU/mL), that adhered per ME-180 cell (± standard deviation) is shown on the left and the percentage of bacteria that adhered when the ME-180 monolayer was pre-coated with *L. iners* (1×10^9 CFU/mL) relative to the control (± standard deviation) is shown on the middle. Following the addition of a BV-associated anaerobe, the number of remaining *L. iners* was counted and compared to the *L. iners* control (126.92 per ME-180 cell ±2.25) and the percent (± standard deviation) of *L. iners* that remained adherent after addition of each BV anaerobe (1×10^9 CFU/mL) is shown on the right.

	Number of BV anaerobe per ME-180 cell	Percentage of BV adherent to *L. iners*-coated ME-180 monolayer	Percentage of *L. iners* remaining after addition of BV anaerobe
Gardnerella vaginalis 101	411.91 (±52.90)	116.90 % [c,d,e,f] (±18.34)	96.29 % [c,d] (±4.90)
Atopobium vaginae FA	43.82 (±3.18)	48.62 % [a,b] (±3.86)	87.23 % [b,e,f] (±2.72)
Mobiluncus mulieris ATCC 26-9	47.54 (±3.62)	79.13 % [b] (±8.97)	94.61 % [b,e,f] (±5.83)
Prevotella bivia ATCC 29303	145.34 (±8.38)	79.99 % [b] (±1.24)	93.26 % [b,c,d] (±2.38)
Fusobacteria nucleatum 718BVC	206.32 (±3.44)	48.42 % [a] (±0.15)	94.60 % [b,c,d] (±0.43)

[a] $p<0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay.
[b] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* 101 tested in the adhesion assay.
[c] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay.
[d] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay.
[e] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay.
[f] $p<0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

4.4 Discussion

BV is characterized by a decrease in the number of normal protective lactobacilli and an increase in various anaerobes, but the events leading to this disorder are yet unknown. It is well known that vaginal lactobacilli inhibit the growth of BV anaerobes, largely through the production of lactic acid and hydrogen peroxide (11). However, as far as we know, the effect of lactobacilli on the initial adherence of BV-associated anaerobes, which could be mediated through steric hindrance, competition for receptors, or the secretion of soluble factors, has not been reported. We first tested the initial adherence of common BV-associated microorganisms to a cervical epithelial cell line in the presence of two species of vaginal lactobacilli. Previously, using a semi-quantitative approach, we determined that *G. vaginalis* had a greater capacity for adhesion to ME-180 cells as compared to other known BV-associated bacteria (12). Herein, we confirmed this finding using a quantitative assay to determine adherence of *G. vaginalis*, *A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum* and we determined the effects of *L. crispatus*, which has been shown to be a highly protective vaginal lactobacilli (19), and *L. iners*, which has been associated with risk for BV, on the initial adherence of these anaerobes to epithelial cells (4, 7, 19).

As further evidence of its role in BV, *G. vaginalis* exhibited the greatest capacity for adherence to ME-180 cells, and while adherence was inhibited somewhat by *L. crispatus*, it actually increased slightly in the presence of *L. iners*. The effect of *L. crispatus* on initial adherence to epithelial cells could be related to several factors, such as intra and extracellular probiotic metabolites. Although the majority of lactobacilli are able to produce lactic acid and hydrogen peroxide (11, 20, 21), the time course of the assays used in this study was too short for lactic acid and hydrogen peroxide to build up to bactericidal levels. It is possible that sub-inhibitory concentrations of these compounds, or other compounds secreted by *L. crispatus* inhibit adherence. In sum, inhibition of initial adherence by *L. crispatus* appears to be an additional mechanism by which this vaginal lactobacillus species maintain vaginal health.

Confirming our first experiments, *G. vaginalis* was more able (compared to the other BV-associated species) to adhere to ME-180 cells when *L. crispatus* was first allowed to attach to the cells. In addition, *P. bivia* and *F. nucleatum* were proportionally less affected by *L. crispatus* early colonization (Table 4.1). Interestingly, adherence of *L. iners* to the ME-180 cells did not prevent secondary colonization by *G. vaginalis* (Table 4.2), but it prevented adherence of the other anaerobes as effectively as *L. crispatus.* Evidence suggests that *L. iners* is not very protective against BV, but the reason for this lack of apparent protection role is not clear (19, 22). Our results show that *L. iners* did not have an antagonistic effect on *G. vaginalis*, which may partially explain its failure to prevent BV. Our data also suggest that *L. iners* was not displaced by *G. vaginalis* suggesting that the two species may be tolerant to one another. These results support the idea that *G. vaginalis* is an early colonizer in BV, which can outcompete most bacteria from the vaginal niche, and afterwards allowing other bacteria to co-colonize the human vagina. However, this is a simplified model system and lacks many of the bacteria-specific and host-specific factors that would be present in the vagina.

F. nucleatum adhered poorly in the competitive initial adhesion assays but it was able to adhere more efficiently when it was added after the lactobacilli adhered to the ME-180 cells. This result is in agreement with a study reported by Foster and Konlenbrander (23), demonstrating that *F. nucleatum* is a weak initial adherent bacteria but capable to co-aggregate with other pre-adhered bacteria. Our study is the first to quantify initial adhesion per epithelial cell and clearly demonstrated the greater capacity of *G. vaginalis* for initial adhesion even in the presence of high levels of *L. crispatus* and *L. iners*. Also, it appears that the species of vaginal lactobacilli play an important role not only in preventing the growth of BV-associated anaerobes but also in impairing the adherence of certain species to vaginal epithelial cells.

4.5 Conclusions

In the current work, it was quantitatively proved that *G. vaginalis* has indeed the greatest capacity from all BV-associated anaerobes tested for initial adhesion to epithelial cells. Although *L. crispatus* and *L. iners* have different protective competences in the vaginal epithelium, *G. vaginalis* sustained its high initial adhesion ability against both lactobacilli species at high levels. This study supports the single pathogenic species hypothesis suggesting *G. vaginalis* as a main candidate for early colonizer in BV that could allow other bacteria to grow and colonize vaginal epithelium.

Also, it is important to notice that *A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum* exhibited different initial adhesion competences in the presence of both vaginal lactobacilli species tested, suggesting that certain lactobacilli species are simultaneously capable to avoid initial adhesion and to prevent the growth of BV-associated anaerobes.

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Chapter V

Initial attachment and biofilm formation of anaerobes involved in bacterial vaginosis

Abstract

Certain anaerobic bacterial species are predominant in the vaginal flora during bacterial vaginosis (BV), being *Gardnerella vaginalis* the most commonly found. However, the exact role of *G. vaginalis* in BV has not yet been fully elucidated. The main goal of this study was to test the hypothesis that *G. vaginalis* is an early colonizer, paving the way for intermediate (e.g., *Fusobacterium nucleatum*) and late colonizers (e.g., *Prevotella bivia*). Theoretically, in order to act as an early colonizer, species would need to be able to adhere to the vaginal epithelium, even in the presence of vaginal lactobacilli. Therefore, using our recently developed Peptide Nucleic Acid (PNA) Fluorescence *In Situ* Hybridization (FISH) methodology, we quantified the adherence of *G. vaginalis* and other BV-associated bacteria to an inert surface pre-coated with *Lactobacillus crispatus*. We found that *G. vaginalis* had the greatest capacity to adhere in the presence of *L. crispatus*. Additionally, it is well known that an early colonizer contributes to the adherence and/or growth of additional species, hence using the quantitative Polymerase Chain Reaction (qPCR) technique we next quantified the growth of dual species biofilms with *G. vaginalis* and other BV-associated anaerobes. Interestingly, it was found that, regardless of the species, the *G. vaginalis* growth was promoted by the presence of additional species. Conversely, *G. vaginalis* biofilms enhanced the growth of *P. bivia*, and to a minor extent of *F. nucleatum*. These results contribute to our understanding of BV biofilm formation and the progression of the disorder.

Keywords: *Lactobacillus* spp.; *Gardnerella vaginalis*; BV anaerobes; initial adhesion; epithelial cell line; fluorescence *in situ* hybridization; peptide nucleic acid; quantitative PCR.

5.1 Introduction

Bacterial vaginosis (BV) is the most common vaginal disorder in women of reproductive age but its etiology is still unclear (1). However, BV is characterized by a decrease of the number of beneficial vaginal bacteria, such as *Lactobacillus cripatus*, and by an increase of the number of anaerobic bacteria, such as *Gardnerella vaginalis*, *Mobiluncus mulieris*, *Atopobium vaginae*, *Prevotella bivia* and *Fusobacteria nucleatum* (2–4). BV is typically a polymicrobial condition (5, 6). Recently it has been found that multi-species microbial biofilms are involved in BV (4). However, the process by which this multi-species biofilm is established remains unknown. In general, single-species biofilm formation involves two main independent steps: initial adhesion to the surface and biofilm formation (7). In contrast, multi-species biofilm formation may be more complex and depend upon interactions between the species involved. The most thoroughly studied clinically relevant polymicrobial biofilm is the oral biofilm associated with periodontitis (8). During the development of these biofilms, early colonizers first adhere to the tooth pellicle providing a surface to which intermediate colonizers can adhere, as well as producing better conditions for the growth of successive species (9, 10). This community in turn provides an environment favorable to the adherence and growth of secondary colonizers. Similar to oral biofilms, it has been hypothesized that *G. vaginalis* is the initial colonizing species in BV and that its biofilms are beneficial to the growth, adherence and/or biofilm formation by other BV anaerobes, but this has yet to be demonstrated (4).

The main goal of our work was to assess the potential of bacterial species commonly found in BV as early or late colonizers. We first quantified the initial adhesion of such species to an inert surface pre-coated with *Lactobacillus crispatus* and then compared single-species or dual-species biofilms formation in order to evaluate the potential symbiotic interactions between the BV-associated bacterial species.

5.2 Materials and Methods

5.2.1 Culture of bacterial strains

L. crispatus EX533959VC06 was grown in Man, Rogosa and Sharpe both (MRS; Sigma-Aldrich, Germany) at 37 °C under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 24–48 h prior to adhesion assays. Also, *G. vaginalis* 101, *Atopobium vaginae* FA, *Mobiluncus mulieris* ATCC 26-9, *Prevotella bivia* ATCC 29303 and *Fusobacteria nucleatum* 718BVC were grown in supplemented Brain Heart Infusion (sBHI; Oxoid, United Kingdom) and incubated at 37 °C under anaerobic conditions for 24–48 h prior to adhesion assays. Before the displacement/blockage assays, all strains were harvested by centrifugation (4000*g*, 12 min, at room temperature), washed twice with sterile phosphate buffer saline (PBS). The pellet from each bacteria culture was resuspended in sterile PBS and its concentration was adjusted to 1×10^9 CFU/mL by optical density at 600 nm using a microplate reader (Tecan, Switzerland).

5.2.2 Early adhesion assays

Aliquots of 400 µL of *L. crispatus* culture media with a concentration of 1×10^9 CFU/mL were added to each well of a 8 chamber glass slide developed for the adhesion assays. Then, the chamber glass slides were incubated for 4h at 37 °C, in anaerobic conditions, and 120 rpm. Non-adherent lactobacilli were removed by washing with 400 µL of sterile PBS and subsequently a second adhesion step was performed, using one BV-associated anaerobe (*G. vaginalis*, *A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum*) with two different concentrations (1×10^3 or 1×10^9 CFU/mL), for 30 min at 37 °C, in anaerobic conditions and 120 rpm at the same range of concentrations. Finally, each well of the incubated chamber slides was carefully washed twice with 400 µL of sterile PBS to remove non-adherent bacteria and let to air-dry before conducting the FISH hybridization procedure. Controls were performed simultaneously in each chamber slide by adding each bacterium individually and maintaining the same experimental conditions. All these assays were performed with duplicate samples and each assay was repeated three independent times.

5.2.3 Quantification of the adhered bacteria by Fluorescent *in situ* hybridization

The chamber glass slides containing the adhered bacteria were first fixed and hybridized with the Lac663 and Gard162 PNA probes, that we previously developed and optimized (11). Briefly, the glass slides were fixed with 4% paraformaldehyde followed by 50% ethanol. Hybridization was performed at 60 °C for 90 min and then washed with a fresh solution. An additional 4',6-diamidino-2-phenylindole (DAPI; Sigma, Germany) staining step was done at the end of the hybridization procedure. Then microscopic visualization was performed using an Olympus BX51 (Olympus Portugal SA, Portugal) epifluorescence microscope equipped with a CCD camera (DP72; Olympus, Japan). These assays were repeated three times and a negative control was performed simultaneously with each step previous described. Bacteria cells quantification was performed through the National Institutes of Health image analysis software ImageJ (version 1.451) (12).

5.2.4 Evaluation of the *G. vaginalis* mixed species biofilm by quantitative-PCR

The formation of *G. vaginalis* mixed biofilms were performed in a chemically defined medium (CDM), previously developed by Geshnizgani and Onderdonk (13). An initial 100 µL overnight inoculum of *G. vaginalis* 101 was transferred to 10 mL of fresh CDM. Then, 2 mL of this *G. vaginalis* suspension were transferred to each well of a 6-well plate and incubated for 24 h, at 37 °C, in anaerobic conditions. After 24 h, the media was changed in each well by fresh CDM media and 50 µL of an overnight culture of a different secondary anaerobe was added. Next, the 6-well plates were incubated for another 24 h, at 37 °C, in anaerobic conditions. Finally, CDM media and planktonic cells were removed from all the plates and the DNA was extracted from biofilm samples using a Dneasy blood and tissue kit (Qiagen, The Netherlands) according to the manufacturer instructions. All qPCR assays were performed using a Taq 2× Master Mix (BioLabs, USA) on an iCycler iQ5 real-time detection system (Bio-Rad, USA). Each 25 µL reaction mixture contained 12.5 µL Taq 2× Master Mix, 1.0 µL of 10 µM from forward and reverse primers (Table 5.1), 2 µL template DNA, 8.5 µL of nuclease-free water. Temperature cycling for all assays was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 54 °C for 30 s and 72 °C for 15 s. Negative controls

(no template DNA) were run with every assay to check for contamination. Assay results were expressed as threshold cycle number (C_t) of the 16S rRNA gene copies amplification per template DNA sample. All these assays were performed with duplicate samples and each assay was repeated three independent times.

Table 5.1 Set of primers used in this study according to the Ribosomal Database Project II (RDPII) for quantitative real-time PCR.

Bacteria target	qPCR primers	DNA target	Accession number in RDPII	Localization in RDPII sequence
G. vaginalis	Fw 5'-CACATTGGGACTGAGATACGG-3'	16S rRNA	S002289761	325–345
G. vaginalis	Rv 5'-AGGTACACTCACCCGAAAGC-3'	16S rRNA	S002289761	470–490
M. mulieris	Fw 5'-CGTGCTTAACACATGCAAGTCG-3'	16S rRNA	S000110434	44–65
M. mulieris	Rv 5'-GCTGGCTTTCACGACAGACG-3'	16S rRNA	S000110434	1073–1091
A. vaginae	Fw 5'-TATATCGCATGATGTATATGGG-3'	16S rRNA	S000607439	184–205
A. vaginae	Rv 5'-CATTTCACCGCTACACTTGG-3'	16S rRNA	S000607439	658–677
P. bivia	Fw 5'-CGCACAGTAAACGATGGATG-3'	16S rRNA	S000414458	806–825
P. bivia	Rv 5'-ATGCAGCACCTTCACAGATG-3'	16S rRNA	S000414458	1032–1051
F. nucleatum	Fw 5'-ATTTGTAGGAATGCCGATGG-3'	16S rRNA	S001577261	694–713
F. nucleatum	Rv 5'-TACTTATCGCGTTTGCTTGG-3'	16S rRNA	S001577261	842–861

Searched through RDPII (last accession, December 2012) with the following data set options: Strain—Both; Source—Both; Size—> 1200bp; Quality—Both.

5.2.5 Statistical analysis

All data were analyzed using a two-tailed ANOVA or Student's *t*-test with SPSS statistical software (version 17.0) and expressed as mean ± standard deviation (SD). The *p* values below 0.05 were considered significant.

5.3 Results

5.3.1 Evaluation of the early adhesion potential of BV-associated anaerobes onto a surface pre-coated with *L. crispatus*

The early adhesion assays were performed with known BV-associated anaerobes at different concentrations (1×10^3 and 1×10^9 CFU/mL) onto an inert surface pre-coated with *L. crispatus* (1×10^9 CFU/mL; Figure 5.1). As shown in Table 5.2, for both concentrations, *G. vaginalis* was the most adherent species when compared to the other BV anaerobes (*ANOVA Tukey* statistical test, $p < 0.05$), followed by *F. nucleatum* and *P. bivia*.

Table 5.2 Blockage of adherence of bacterial vaginosis (BV)-associated anaerobes to glass by adherent *L. crispatus*. The number of each BV-associated anaerobes that adhered per cm^2 of glass (± standard deviation) is shown on the left and the percentage of bacteria that adhered when the glass was pre-coated with *L. crispatus* relative to the control (± standard deviation) is shown on the right.

	Number of BV anaerobe per cm^2	**Percentage adherent to *L. crispatus*-coated glass**
High inocula		
G. vaginalis 101	5.71×10^7 ($\pm 2.14 \times 10^4$)	86.86% [c,d,e,f] (±14.14)
A. vaginae FA	6.85×10^6 ($\pm 3.38 \times 10^5$)	48.74% [a,b] (±3.36)
M. mulieris ATCC 26-9	5.76×10^6 ($\pm 1.21 \times 10^5$)	82.22% [a,b] (±0.37)
P. bivia ATCC 29303	1.64×10^7 ($\pm 6.29 \times 10^5$)	101.67% [b] (±28.19)
F. nucleatum 718BVC	2.54×10^7 ($\pm 9.41 \times 10^5$)	68.83% [a,b] (±5.60)
Low inocula		
G. vaginalis 101	6.89×10^6 ($\pm 1.26 \times 10^6$)	72.33% (±4.36)
A. vaginae FA	1.47×10^5 ($\pm 9.65 \times 10^4$)	50.27% [a] (±3.97)
M. mulieris ATCC 26-9	1.33×10^6 ($\pm 5.05 \times 10^4$)	70.15% (±7.80)
P. bivia ATCC 29303	2.99×10^6 ($\pm 1.44 \times 10^5$)	84.17% (±1.57)
F. nucleatum 718BVC	2.68×10^6 ($\pm 5.52 \times 10^4$)	60.15% [a] (±0.28)

High inocula = 1×10^9 CFU/mL, Low inocula = 1×10^3 CFU/mL.

[a] $p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the adhesion assay.

[b] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* strain tested in the adhesion assay.

[c] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay.

[d] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay.

[e] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *P. bivia* strain tested in the adhesion assay.

[f] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *F. nucleatum* strain tested in the adhesion assay.

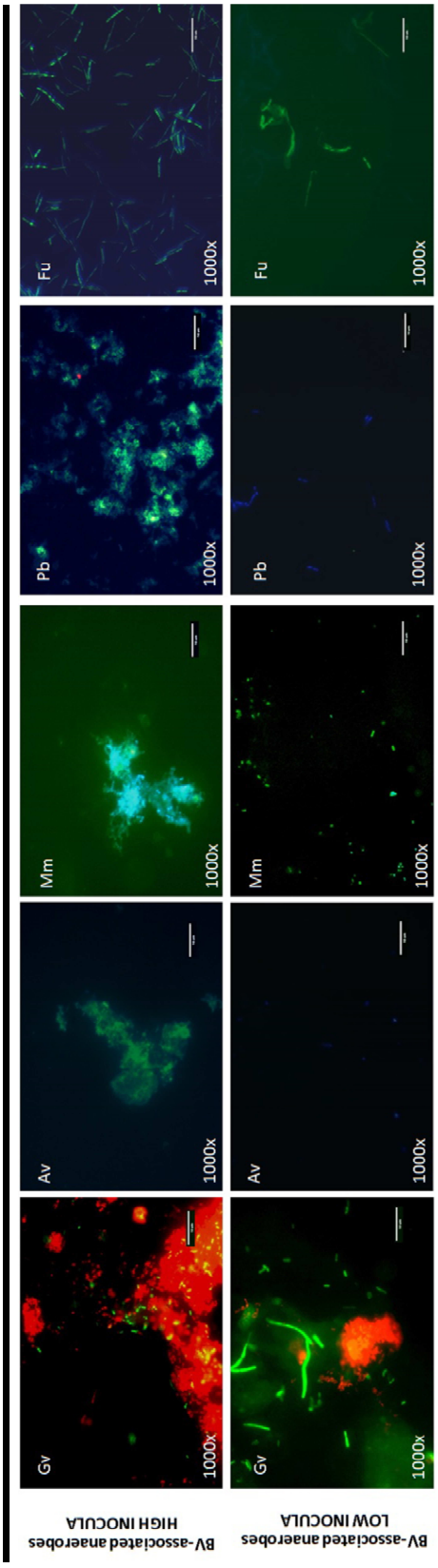

Figure 5.1 Fluorescence microscopy of the early adhesion of several BV-associated anaerobes to surface coated with *L. crispatus* by 4',6-diamidino-2-phenylindole (DAPI) and specific PNA probes (Lac663 and Gard162) associated with Alexa Fluor 488 and 594 fluorochromes. Gv, *G. vaginalis* 101 & *L. crispatus*; Av, *A. vaginae* & *L. crispatus*; Mm, *M. mulieris* & *L. crispatus*; Pb, *P. bivia* & *L. crispatus*; Fu, *F. nucleatum* & *L. crispatus*.

Although *M. mulieris* showed the lowest initial adhesion potential, it was able to displace *L. crispatus* more effectively than any of the other anaerobes tested, including *G. vaginalis* (*ANOVA Tukey* statistical test value, $p < 0.05$; Table 5.3). Nevertheless, it is important to notice that the *L. crispatus* displacement assays conducted with all the BV-associated anaerobes were found to be non-significant as compared to the *L. crispatus* control (see Table 5.3).

Table 5.3 Displacement of adherent *L. crispatus* by BV-associated anaerobes. Following the addition of a BV-associated anaerobe, the number of remaining *L. crispatus* was counted and compared to the *L. crispatus* control counting ($7.36 \times 10^7 \pm 9.97 \times 10^4$). The percentage (± standard deviation) of *L. crispatus* that remained adherent after addition of each BV anaerobe at high or low inocula is shown below.

	Percentage of *L. crispatus* remaining after addition of BV anaerobe
High inocula	
G. vaginalis 101	88.60% [b,c] (±5.14)
A. vaginae FA	99.29% [a] (±7.26)
M. mulieris ATCC 26-9	76.62% [a] (±11.93)
P. bivia ATCC 29303	94.86% (±20.60)
F. nucleatum 718BVC	97.65% (±7.41)
Low inocula	
G. vaginalis 101	101.51% [b,c] (±28.52)
A. vaginae FA	71.18% [a] (±12.54)
M. mulieris ATCC 26-9	68.48% [a] (±12.79)
P. bivia ATCC 29303	97.39% (±2.44)
F. nucleatum 718BVC	98.34% (±9.52)

High inocula = 1×10^9 CFU/mL, Low inocula = 1×10^3 CFU/mL.
[a] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *G. vaginalis* strain tested in the adhesion assay.
[b] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *A. vaginae* strain tested in the adhesion assay.
[c] $p < 0.05$ analysed using ANOVA Tukey statistical test (95% confidence interval) for comparison with *M. mulieris* strain tested in the adhesion assay.

5.3.2 *G. vaginalis* mediated dual species biofilms

In the next experimental step, we analyzed the potential interactions between *G. vaginalis* and other BV anaerobe previously studied in an early-stage *G. vaginalis* biofilm. For that purpose, *G. vaginalis* biofilms were allowed to develop for 24 h, after which a second anaerobe was introduced and co-cultured in the system for an additional 24 h. Then,

qPCR analysis was used to determine the number of *G. vaginalis* and the second species within the biofilm. As shown in Table 5.4, *G. vaginalis* growth was increased by any second anaerobe inoculated after the initial 24 h biofilm formation.

Table 5.4 Results of the qPCR from mixed biofilm formation assays with *Gardnerella vaginalis* 101 and a second BV anaerobe. All experiments were performed in triplicate.

Biofilm	Single specie biofilm		Multi-species biofilm		% GV in mixed biofilm
	GV control CT	2nd anaerobe control CT	GV fold increase	2nd anaerobe fold increase	
G. vaginalis (48 h) & *M. mulieris* (24 h)	14.13 (±0.12)	31.99 (±1.09)	3.78 (±1.10) [a]	0.89 (±0.17)	99.9997
G. vaginalis (48 h) & *A. vaginae* (24 h)	14.13 (±0.12)	26.38 (±0.33)	3.38 (±0.79) [a]	1.37 (±0.17)	99.9844
G. vaginalis (48 h) & *P. bivia* (24 h)	14.13 (±0.12)	24.84 (±0.03)	3.82 (±0.03) [a]	4.20 (±0.92) [a]	99.8960
G. vaginalis (48 h) & *F. nucleatum* (24 h)	14.13 (±0.12)	24.24 (±2.57)	3.39 (±0.28) [a]	1.63 (±0.44)	99.9236

GV, *G. vaginalis* 101; CT, threshold cycle; (± standard deviation), standard deviation from the average values from triplicate assays are in parenthesis after the average value.
[a] $p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of control and bacteria tested in the biofilm assay.

Overall the *G. vaginalis* growth was found to increase around ≈3 fold in the presence of all the secondary anaerobe species studied, although the greatest increase was found in the presence of *P. bivia* (3.83-fold increase) and *M. mulieris* (3.78-fold increase). Interestingly, *F. nucleatum* and *P. bivia* led to higher numbers when co-cultured with *G. vaginalis* strains, showing ≈2 and ≈4 fold increases (Table 5.4), respectively.

5.4 Discussion

In 1983, Spiegel and colleagues postulated that bacterial vaginosis was a polymicrobial infection, in which *G. vaginalis* was the prevalent species (14). However, the etiology of BV remains fairly unknown, and it is still unclear which, if any, of the BV-associated anaerobes are capable of disrupting an established *Lactobacillus* population and initiate colonization on the vaginal epithelium. Several species of lactobacilli may colonize the healthy vagina, however each species differs in its probiotic activity due to differences in their abilities to endure changes in the environmental conditions, that includes pH variations during menstruation or sexual intercourse, as well as due to differences in their abilities to produce antimicrobial compounds such as lactic acid, hydrogen peroxide and bacteriocins (15). *L. crispatus* is able to produce several antimicrobial compounds and it is inversely associated with BV (16). We therefore chose this species as representative lactobacilli for use in our study. Herein, we evaluated the early adhesion of known BV-associated anaerobes at different concentrations to an inert surface pre-coated with *L. crispatus*. As expected, *G. vaginalis* showed greater early adhesion potential than the other BV anaerobes studied. These results are in agreement with several previous studies (17–19) supporting evidence that *G. vaginalis* has a significant initial adhesion potential. These results suggest that *G. vaginalis* could be the early colonizer in the progression of BV. Although *A. vaginae* and *M. mulieris* are often associated with BV (20–22), their capacity to adhere to glass pre-coated with *L. crispatus* was the lowest of all tested anaerobes, thus suggesting that they are not strong candidates as early colonizers in BV. Interestingly, *M. mulieris* displaced *L. crispatus* more effectively than any of the other anaerobes tested. Since this species did not adhere as well as the others, this result suggests that it may secrete some soluble factors that displace the lactobacilli. However, these *in vitro* experiments are limited in that the bacteria were allowed to adhere to glass rather than vaginal epithelium and adherence to vaginal epithelium is likely influenced by a number of host-related and bacteria-specific factors, such as mucus production and the involvement of specific receptors on the epithelial surface (1, 3).

Several studies have shown the prevalence of biofilm formation in BV samples, pointing *G. vaginalis* as a main component of these biofilms, leading to the hypothesis that *G. vaginalis* initiates the biofilm formation allowing successive species to adhere and proliferate (4, 23). However, this has yet to be determined experimentally. We examined whether synergistic or antagonistic interactions would contribute to or prevent growth of BV anaerobes within an early-stage *G. vaginalis* biofilm. Notably, *G. vaginalis* growth was augmented by the incorporation of a second anaerobe after the initial 24 h biofilm formation. In fact, initial *G. vaginalis* biofilm showed a greatest increase with *P. bivia* and *M. mulieris* addition. Also, it is important to notice that *F. nucleatum* and *P. bivia* showed synergistic effects on *G. vaginalis* growth, thus demonstrating the ability of *G. vaginalis* to establish different interactions with others BV-associated anaerobes. This is in agreement with a report from Pybus and Onderdonk that demonstrated the symbiotic relationship between *G. vaginalis* and *P. bivia* (20) and suggesting that symbiotic relationships established between *G. vaginalis* and other anaerobes in BV biofilms could contribute to the progression of BV.

Although *F. nucleatum* has not been extensively studied in BV infection, it plays a key role in the establishment of oral biofilms as a bridging species (24). In fact, Foster and Kolenbrander (24) demonstrated that *F. nucleatum* is capable of co-aggregating with pathogenic bacteria and of becoming a dominant member of the oral multi-species biofilm after several days of incubation although it commonly failed to grow by itself in biofilms. Similarly, our results suggest that *F. nucleatum* is able of joining an initial biofilm and eventually establishes a symbiotic relationship with *G. vaginalis*. Again, our study is limited in its complexity and lacks host-specific factors, but it suggests that certain BV-related species can cooperate and this may provide some insight regarding the ability of these bacterial species to become dominant in an environment normally dominated by lactobacilli.

5.5 Conclusions

The results described in this chapter suggest that *G. vaginalis* may be more suited as an early colonizer relative to the other BV-associated anaerobes studied in the initial adhesion assay and that it may play a key role in the early establishment of BV biofilms.

All anaerobes tested were found to enhance the biofilm formation by *G. vaginalis*. Furthermore, the *G. vaginalis* biofilms were found to enhance the growth of *P. bivia* and to a minor extent of *F. nucleatum.* These observations provide some insights on the ability of each individual BV-associated anaerobe studied to adhere in the presence of a protective layer of lactobacilli, as well as on the ability of *G. vaginalis* biofilms to thrive in presence of other anaerobes.

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Chapter VI

Probiotic activity of lactobacilli biosurfactants against *Gardnerella vaginalis*

Abstract

Current BV treatment is strictly based in antibiotic therapy. However, an increase in antimicrobial resistance has been reported for BV anaerobes, such as *G. vaginalis.* Furthermore, antimicrobial therapy normally reduces the population of the healthy vaginal lactobacilli strains. A more appropriate treatment is being sought, aiming to decrease *G. vaginalis* and also to promote the lactobacilli re-colonization in BV patients. An alternative therapy for BV is the re-colonization of vagina with lactobacilli species. Our goal was to evaluate the probiotic potential of intra- and extracellular biosurfactants from a broad range of lactobacilli strains against several *G. vaginalis* strains. To accomplish our goal, we tested several extracts and supernatants from 86 lactobacilli strains (35 from bacteria collection and 51 isolates from healthy women) through a screening by an agar spot test against 9 *G. vaginalis* strains in order to select the most remarkable probiotic candidates. From the selected candidates, we evaluated their ability to inhibit *G. vaginalis* growth using biosurfactants concentrations ranging 40 to 80% (vol/vol) in the culture medium. Our results showed that the intracellular biosurfactants were unable to reduce *G. vaginalis* proliferation. Nonetheless, the extracellular biosurfactants candidates showed a significant effect on *G. vaginalis* growth and biofilm formation. Overall, from the 86 lactobacilli strains tested, 4 bacteria collection and 6 clinical isolate lactobacilli strains exhibited a broad probiotic activity against all the *G. vaginalis* strains tested. However, only 2 vaginal isolates and 4 lactobacilli collection strains were able to inhibit *G. vaginalis* strains, being their growth of 11% in some cases, when compared to *G. vaginalis* control, thus illustrating an efficient probiotic activity. Interestingly, although none of these lactobacilli collection strains belong to the vaginal microflora, they revealed a much more pronounced activity against *G. vaginalis* as compared to the vaginal isolate lactobacilli tested.

Keywords: *Gardnerella vaginalis*; lactobacilli; probiotic activity; extracellular and intracellular biosurfactants.

6.1 Introduction

Bacterial vaginosis (BV) represents a significant health risk in women in reproductive age because it predisposes women to abnormal pregnancy, pelvic inflammatory disease and an increased risk of sexual transmitted infections (1–3). Despite richness and diversity found in BV anaerobes, *Gardnerella vaginalis* is present in over 90% of the pathologic cases and several studies report its potential as the main etiological candidate (4–7). Although antibiotics constitute the standard BV treatment, their usage had been associated to an increase of BV anaerobes resistance, in particular *G. vaginalis*, and to a decrease in the healthy vaginal microflora, specifically lactobacilli species (8–10). Therefore, other treatments are required to avoid these drawbacks associated with antibiotic therapies. An alternative approach for BV treatment resides in the usage of probiotics strains or their antimicrobial products. Several studies have been conducted in the last decades showing the probiotic potential of lactobacilli in preventing vaginal colonization by pathogens, thus preventing the development of infections (11–14). The *Lactobacillus* genus showed different probiotic mechanisms including auto-aggregation, co-aggregation with pathogenic microorganisms, and adhesion to epithelial cells and/or through some of their metabolites (such as lactic acid, hydrogen peroxide, bacteriocins, intra and extracellular biosurfactants) that may act as growth inhibitors or anti-adhesive agents (15–18). However, for an efficient BV treatment using this approach some requirements have to be met, such as the selection of appropriate lactobacilli strain(s) and the effectiveness of the amounts of antimicrobial substances they secrete (19–21).

Our goal was to select probiotic candidates from a broad range lactobacilli strains and to evaluate their intra- and extracellular biosurfactants, as potential probiotic products, against several *G. vaginalis* strains. Therefore, we evaluated 86 lactobacilli strains through a screening by an agar spot test against 9 *G. vaginalis* strains. The most wide-ranging probiotic lactobacilli strains have been selected. Afterwards, we tested the probiotic activity of the selected lactobacilli biosurfactants against *G. vaginalis* strains, in concentrations ranging 40 to 80% (vol/vol) of the culture medium, in order to determine their probiotic efficiency.

6.2 Materials and Methods

6.2.1 Vaginal sample collection and Gram stain selection

A total of 91 samples of vaginal swabs were obtained, after informed consent, as approved by the Institutional Review Board (IRB) of University of Minho. The vaginal swabs were collected for Gram stain, culture plate's isolation and PCR procedures, using the culture swab transport system (VWR, CE0344, Italy). These swabs were brushed against the lateral vaginal wall to collect the vaginal fluid sample, then placed in the culture swab transport media and immediately conserved at 4 ºC. First, the set of swabs was used for *Gram* stain procedure as described by Nugent and colleagues (22). Next, the collected swabs were immersed in 1 mL of phosphate buffer saline (PBS) and centrifuged at 17,000 *g* during 5 min at room temperature. Afterwards, the pellet was resuspended in 2 mL of saline solution (0.9% NaCl prepared in distilled water) and finally diluted 1:10 in saline solution or PBS to eliminate possible contaminants for lactobacilli isolation and PCR validation, as previously described (23). Vaginal swabs evaluation was performed using the Nugent criteria score (22). Briefly, vaginal smear was examined under oil immersion objective (1000x magnification) and through 10-15 microscopic fields. Initially, each smear was graded as per standardized, quantitative, morphological classification developed by Nugent. More specifically, composite score was grouped into three categories, scores 0-3 being normal, 4-6 being intermediate, and 7-10 being definite BV. Finally, the smears that showed scores between 0-3 were selected for lactobacilli isolation and PCR validation. Meanwhile, the smears with a Nugent score of 4-6 and 7-10 were rejected from our study.

6.2.2 Lactobacilli isolation and its validation by Polymerase chain reaction

All collected samples from vaginal swabs were grown in Columbia Blood Agar (CBA; Sigma-Aldrich, Germany) for 24 h at 37 ^{o}C under anaerobic conditions and were examined for morphological and culture characteristics, following the procedure described by Cappuccino and Sherman (24). The colonies that grew showing frequent rods, pair or chain forming pattern and Gram positive character, were selected for further PCR validation.

Pure colonies of these isolates were finally transferred to de Man, Rogosa and Sharpe agar (MRS agar; Sigma-Aldrich, Germany) and CBA plates and were incubated at the same conditions previously used. Afterwards, a molecular characterization was performed to validate each pure colony selected from culture plates, using PCR detection of *Lactobacillus spp*. The species of this genus were specifically detected by 16S rDNA amplification PCR using the forward primer LactoF (5'-TGG AAA CAG RTG CTA ATA CCG-3') and the reverse primer LactoR (5'-GTC CAT TGT GGA AGA TTC CC-3'). This set of primers and PCR conditions were previously developed and characterized by our research group (23). A total of 51 lactobacilli species were isolated from the collected vaginal swabs for this study.

6.2.3 Culture of bacterial strains

A total of 86 lactobacilli strains were selected in this work (see Table S6.1 and S6.2 in supplementary material section). Lactobacilli were grown in 40 mL of MRS culture broth and supplemented Brain Heart Infusion (sBHI; Oxoid, United Kingdom), respectively. Each bacterial culture was incubated at 37 ºC, except for *L. pentosus* CECT4023, *L. coryniformis* CECT4129, *L. brevis* ATCC14869, *L. curvatus* ATCC25601 and *L. plantarum* NCIMB8827 that were grown at 30 ºC, under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 48–72 h prior to lactobacilli biosurfactants extraction. Anaerobic conditions were used to minimize the formation of hydrogen peroxide and acetic acid as described by Schillinger and Lücke (25). *G. vaginalis* strains (*G. vaginalis* AMD, *G. vaginalis* 5-1, *G. vaginalis* 101 and *G. vaginalis* isolates SH254B, SH222C2, SH92B1, UM23, MM19I and TR1I) were grown in CBA at 37 °C under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 24–48 h prior to probiotic screening and activity assays. *G. vaginalis* isolates were isolated in a previous study (26).

6.2.4 Extraction of intra- and extracellular biosurfactants from lactobacilli strains

The extraction of the intra- and extracellular biosurfactants from all lactobacilli strains was performed as previously reported by Gudiña *et al*. (27) with some modifications. Briefly, a cell-free solution was obtained by centrifuging 40 mL of lactobacilli culture (≈6000 *g*, 10 min, at 4 ºC), followed by filtration of the supernatant through a 0.45 µm-pore-

size cellulose acetate filter (Orange Scientific, Belgium). These extracellular biosurfactants were stored at -80 ºC until their use in the probiotic screening and activity assays. Next, the cells were washed twice in 40 mL of PBS with pH adjusted to 7.0 and harvested again by centrifugation at the same conditions. The pellet was resuspended in 5 mL of PBS (pH 7.0) and left for 2 h at room temperature and 100 rpm for intracellular biosurfactant release. Subsequently, the lactobacilli cells were removed by centrifugation (≈6000 *g*, 10 min, at 4 ºC) and the remaining biosurfactant liquid was filtered through a 0.22 µm-pore-size cellulose acetate filter (Orange Scientific, Belgium). The collected intracellular biosurfactants were stored at -80 ºC until further use for probiotic screening and activity assays.

6.2.5 Probiotic lactobacilli biosurfactants screening by an agar spot test

For screening the probiotic potential of the lactobacilli biosurfactants, an agar spot test was performed (25), with some modifications . More precisely, 250 µL of overnight cultures of each *G. vaginalis* strain were spread onto CBA plates and incubated for 1 h at 37 °C to allow the initial inoculum to dry. These overnight cultures were adjusted to an adequate absorbance range (between 0.100 and 0.200) by measuring the optical density at 620 nm. Then, 50 µL of each intra- and extracellular biosurfactants were spotted into a well previously done in the surface of CBA plates and then the plates were incubated for 48 h at 37 °C under anaerobic conditions. After incubation, the CBA plates were checked for *G. vaginalis* inhibition growth and hemolytic zones around each well. Inhibition was scored positive when a clear or hemolytic zone around the well of the biosurfactant tested was noticeable. All these assays were performed with duplicate samples and each assay was repeated three independent times.

6.2.6 Evaluation of the probiotic activity of lactobacilli biosurfactants against *G. vaginalis*

The evaluation of the probiotic activities against *G. vaginalis* was based on the microdilution method in 96-well culture plates as previously described by Gudiña *et al*. (28) with some modifications. Briefly, 200 µL of sBHI medium with certain percentage of biosurfactant (vol/vol) were dispensed into each row of the 96-well microplate, ranging 40

and 80% of intra- or extracellular biosurfactant. Subsequently, each couple of columns was inoculated with 3 µL of a given overnight *G. vaginalis* culture in sBHI medium, exhibiting an adjusted absorbance at 620 nm between 0.100 and 0.200. Growth control wells did not contain biosurfactant and a negative control with a non-probiotic biosurfactant was simultaneously performed with sBHI medium at all percentage tested against each *G. vaginalis* strain. The 96-well microplates were incubated for 48 h at 37 °C under anaerobic conditions. After 48 h of incubation, the optical density at 620 nm of each well was measured using a microplate reader (Tecan, Switzerland).

The probiotic activities of each biosurfactant tested at different percentages were calculated as *G. vaginalis* growth inhibition compared to their control growth, as followed:

$$\%\, Growth\ inhibition\ = \left(\frac{OD\ \text{biosurf}}{OD\ control}\right) \times 100$$

where ODbiosurf represents the optical density of the well with a given biosurfactant percentage, and ODcontrol is the optical density of the control well (*G. vaginalis* growth without biosurfactant). All these assays were performed with duplicate samples and each assay was repeated two independent times.

6.2.7 *G. vaginalis* biofilm formation evaluation by confocal laser scanning microscopy

In order to assess the changes on *G. vaginalis* biofilm formation as the result of probiotic activity of the selected biosurfactants, we performed confocal laser scanning microscopy (CLSM) analysis of coverslips (Labbox, Spain) with *G. vaginalis* biofilms grown in the presence of extracellular biosurfactants. More exactly, the *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 probiotic activities were tested in a 48 h biofilm of *G. vaginalis* 101 and SH222C2. Briefly, 2 mL of sBHI broth with 80% (vol/vol) of a particular biosurfactant was dispensed into each column of the 6-well microplate, containing a coverslip in each well. A volume of 30 µL of a given overnight *G. vaginalis* culture in sBHI broth was added, exhibiting an adjusted absorbance at 620 nm between 0.100 and 0.200. The 6-well microplates were incubated for 48 h at 37 °C under anaerobic conditions. After 48 h of incubation, the coverslip of each well was washed with PBS and used for biofilm

evaluation by CLSM. A control was performed by allowing *G. vaginalis* biofilm formation in the absence of biosurfactant.

The coverslips containing the *G. vaginalis* biofilms were first fixed and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA), as previously optimized by Almeida *et al*. (29). Briefly, the coverslips were fixed with 100% methanol, then 4% paraformaldehyde and followed by 50% ethanol, for 10 min, at room temperature. A DAPI staining step was done at the end of the fixation procedure and then it was washed three times with a fresh PBS solution. The coverslip images were acquired in an Olympus FluoView FV1000 microscope (Olympus Portugal SA), using a 40× water-immersion objective (40/1.2W). Finally, the maximum height of *G. vaginalis* biofilms was determined by evaluation of the z-stacks grown, analysing 10 different sections of each coverslip through the FluoView application Software package (Olympus, Japan).

6.2.8 Statistical analysis

All data were expressed as mean ± standard deviation (SD) and the maximum height of *G. vaginalis* biofilms data were also analyzed using Student's *t*-test with SPSS statistical software (version 17.0). The *p* values below 0.05 were considered significant.

6.3 Results

6.3.1 Probiotic screening of the lactobacilli biosurfactants against *G. vaginalis*

To select the best lactobacilli candidates for probiotic activity against *G. vaginalis*, we performed an agar spot test to screen the biosurfactants that show a greater number of *G. vaginalis* strains being inhibited and also larger inhibition halos. The selected intra and extracellular biosurfactants for probiotic activity analysis are shown in Table 6.1 and 6.2, respectively.

Table 6.1 Probiotic screening results of the intracellular biosurfactants from lactobacilli against *G. vaginalis* strains. The probiotic screening of the lactobacilli intracellular biosurfactants was tested in duplicate for each *G. vaginalis* strain. The number of *G. vaginalis* inhibited was counted for each probiotic screening assay with the symbol X in the respective column number. The table shows the qualitative results obtained in probiotic screening assays.

Lactobacilli intracellular biosurfactants tested		**Number of *G. vaginalis* strains inhibited**									
Species	**Code**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
L. brevis ATCC 14869	L10		X								
L. buchneri ATCC 4005	L11				X						
L. delbrueckii ATCC 9649	L15				X						
L. parabuchneri ATCC 12936	L28		X								
L. paracasei CCUG 27320	L29		X								
L. rhamnosus ATCC 7469	L32			X							
L. ruminis ATCC 27781	L33				X						
L. salivarius DEVRIESE94/438	L35					X					
Vaginal isolate	SH65D1				X						
Vaginal isolate	SH65K				X						
Vaginal isolate	SH212E		X								
Vaginal isolate	SH212H			X							

Based on the screening results (Table 6.1), 12 intracellular biosurfactants were selected. The maximum number of *G. vaginalis* strains being inhibited was 8 and these biosurfactants were

produced by *L. brevis* ATCC 14869, *L. parabuchneri* ATCC 12936, *L. paracasei* CCUG 27320 and vaginal isolate SH212E.

On the other hand, 10 extracellular biosurfactants were selected, as shown in Table 6.2. Interestingly, the maximum number of *G. vaginalis* strains inhibited was strictly achieved by the selected bacteria collection strains, specifically *L. brevis* ATCC 14869, *L. rhamnosus* ATCC 7469, *L. ruminis* ATCC 27781 and *L. salivarius* DEVRIESE94/438.

Table 6.2 Probiotic screening results of the extracellular biosurfactants from lactobacilli against *G. vaginalis* strains. The probiotic screening of the lactobacilli extracellular biosurfactants was tested in duplicate for each *G. vaginalis* strain. The number of *G. vaginalis* inhibited was counted for each probiotic screening assay with the symbol X in the respective column number. The table shows the qualitative results obtained in probiotic screening assays.

Lactobacilli extracellular biosurfactants tested		**Number of *G. vaginalis* strains inhibited**									
Species	**Code**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
L. brevis ATCC 14869	L10	X									
L. rhamnosus ATCC 7469	L32	X									
L. ruminis ATCC 27781	L33	X									
L. salivarius DEVRIESE94/438	L35	X									
Vaginal isolate	SH40I			X							
Vaginal isolate	SH65G		X								
Vaginal isolate	SH103E				X						
Vaginal isolate	SH130D			X							
Vaginal isolate	SH174A			X							
Vaginal isolate	SH196F				X						

6.3.2 Probiotic activity of the lactobacilli biosurfactants against *G. vaginalis*

Despite of their activity on the agar test, the selected 12 intracellular biosurfactants did not revealed any significant probiotic activity, as determined in the microdilution test assays. However, all the 10 extracellular biosurfactants, with 80% (vol/vol) in the culture medium, were simultaneously capable to inhibit all *G. vaginalis* strains studied (both from bacteria collection and vaginal isolates), as shown in Figures 6.1 and 6.2, respectively.

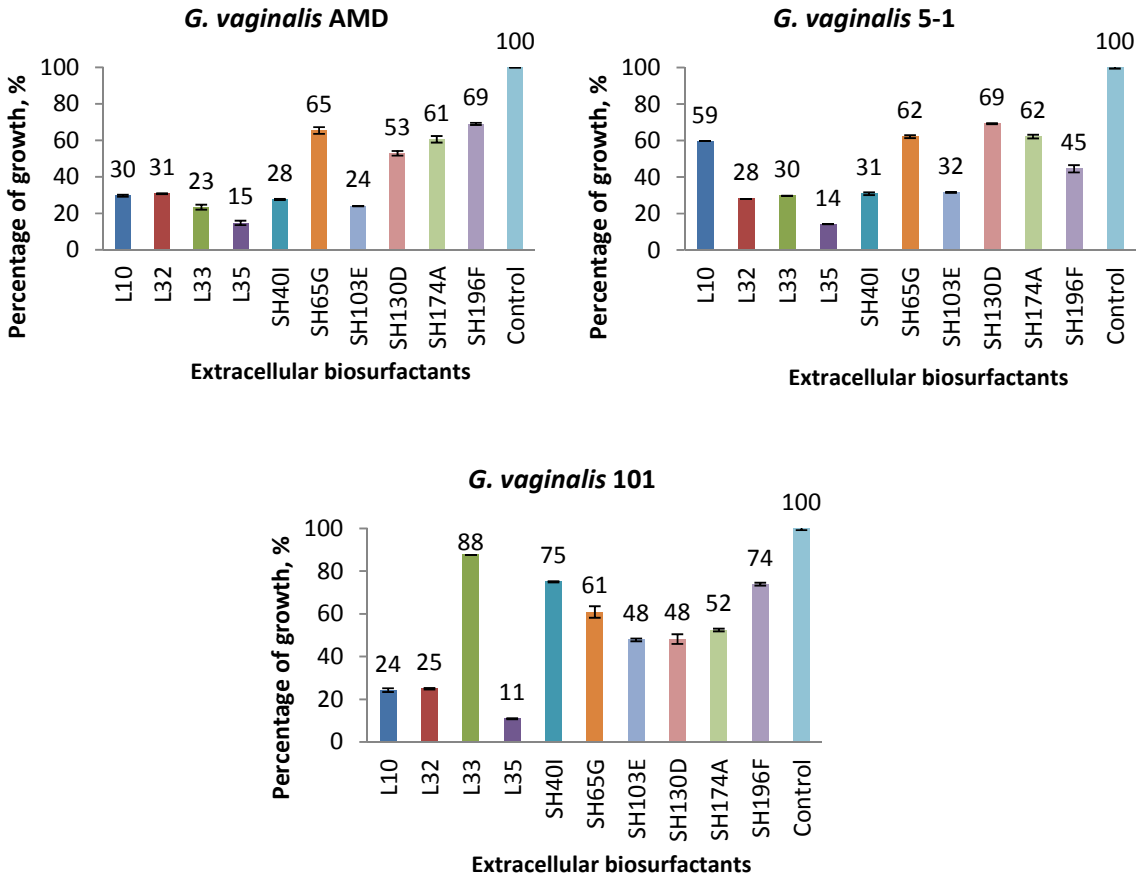

Figure 6.1 Percentage of *G. vaginalis* strains growth inhibition by the extracellular biosurfactants from lactobacilli. The results are the average of duplicate assays for each *G. vaginalis* strain and *error bars* represent the standard deviation. Control corresponds to *G. vaginalis* strain grown with sBHI without adding any extracellular surfactant.

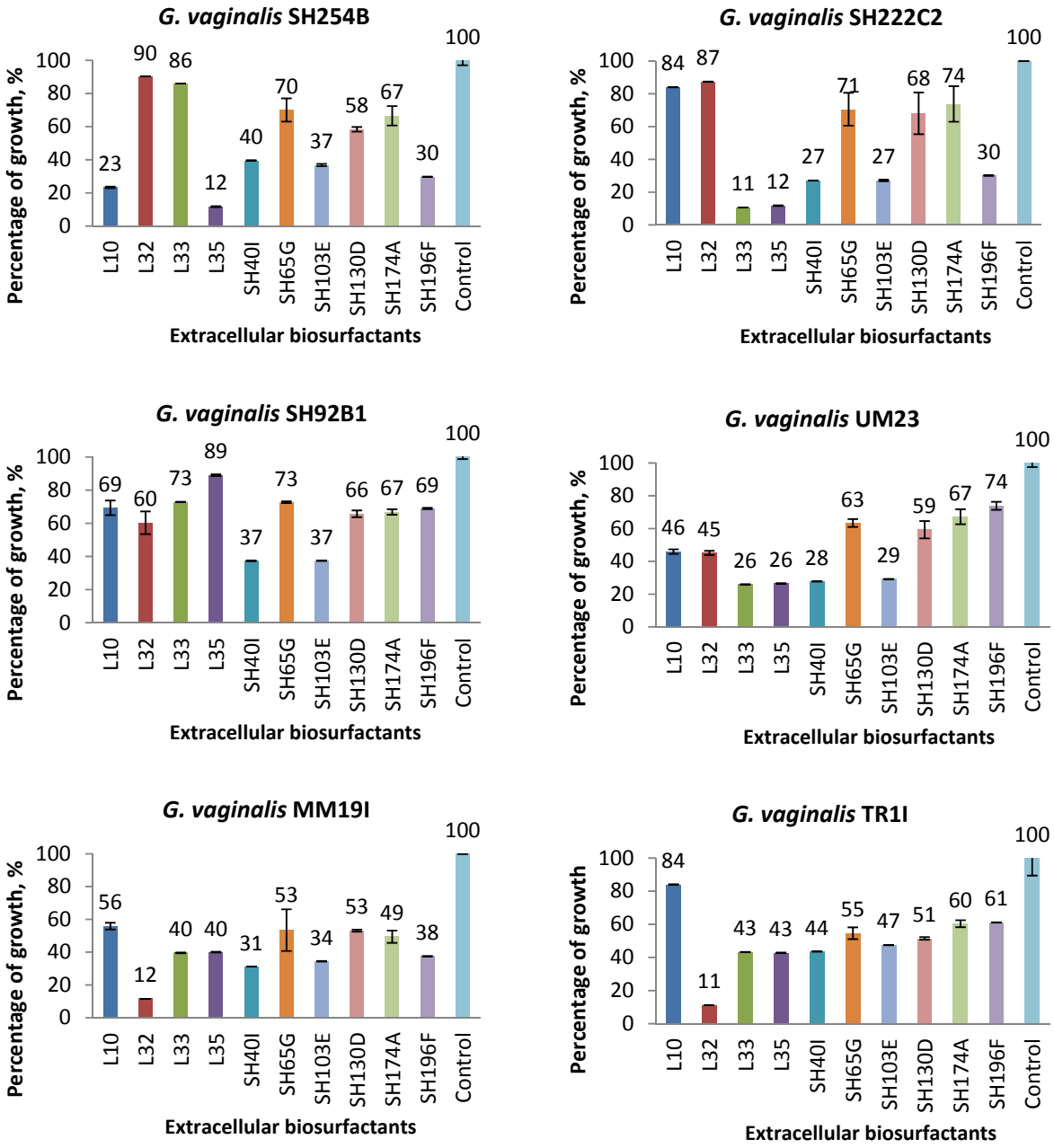

Figure 6.2 Percentage of *G. vaginalis* strains growth inhibition by the extracellular biosurfactants from lactobacilli. The results are the average of duplicate assays for each *G. vaginalis* strain and *error bars* represent the standard deviation. Control corresponds to *G. vaginalis* strain grown with sBHI without adding any extracellular surfactant.

As shown in Figure 6.1, *G. vaginalis* strains from culture collection were more susceptible to extracellular biosurfactants than vaginal isolates, showing a growth range between 15 and 31%. Only *L. brevis* ATCC 14869 (L10) and *L. ruminis* ATCC 27781 (L33) biosurfactants were unable to inhibit *G. vaginalis* 5-1 and 101 strains with the same efficiency. Indeed, these *G. vaginalis* strains were able to growth 59 and 88%, respectively, as compared to the negative control (100%).

Interestingly, the same extracellular biosurfactants from lactobacilli collection exhibited an irregular grade of probiotic activities against *G. vaginalis* isolates (see Figure 6.2), such as *L. rhamnosus* ATCC 7469 (L32) and *L. ruminis* ATCC 27781 biosurfactants against *G. vaginalis* 101 and SH222C2 strains. However, *L. salivarius* DEVRIESE94/438 (L35) were able to inhibit all *G. vaginalis* isolates between 12 to 43%, except for *G. vaginalis* SH92B1 (89%). In addition, the extracellular biosurfactants from lactobacilli isolates revealed lower probiotic activities against *G. vaginalis* isolates (between 30 and 74%; see Figure 6.2), except for SH40I and SH103E isolates. These vaginal isolates revealed probiotic activities comparable to lactobacilli collection strains, ranging between 27 and 47% of growth inhibition (see Figure 6.2).

6.3.3 *G. vaginalis* biofilm formation evaluation by confocal laser scanning microscopy

In order to evaluate the effect of the extracellular biosurfactants on *G. vaginalis* biofilm formation, the *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 probiotic activities were studied on *G. vaginalis* 101 and SH222C2 48 h biofilms through CLSM. More precisely, the variation of the thickness and structure of these *G. vaginalis* biofilms was analyzed in the presence of each biosurfactant (Figure 6.3). CLSM images showed that *G. vaginalis* SH222C2 and 101 strains formed a thick biofilm when grown in the absence of these two extracellular biosurfactants. However, a significant reduction of the biofilm thickness and structure was observed, in particular for *G. vaginalis* 101 and SH222C2 in the presence of the *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 biosurfactants (Figure 6.3 B and F), respectively.

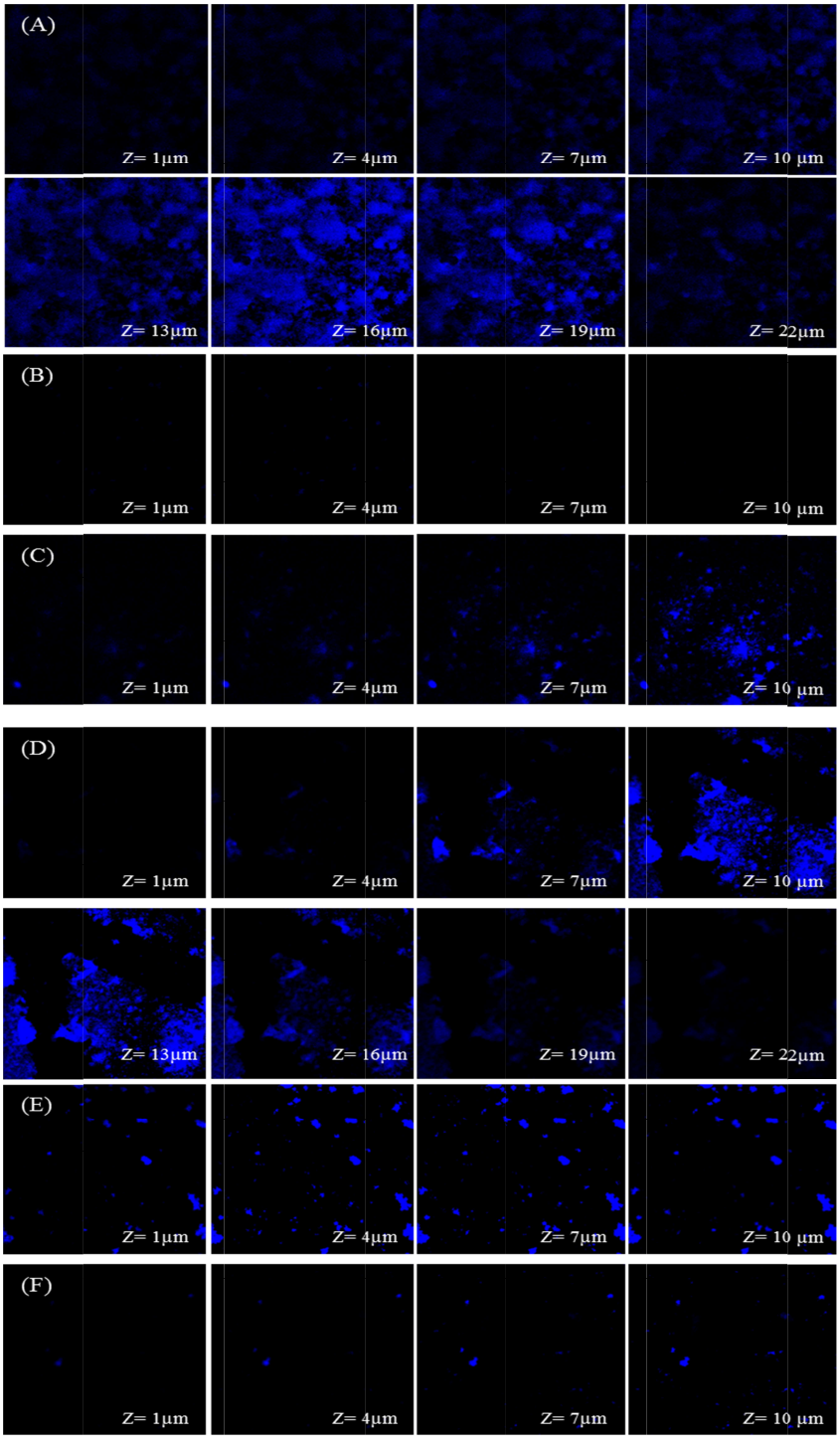

Figure 6.3 Effect of *L. rhamnosus* ATCC 7469 (L32) and *L. ruminis* ATCC 27781 (L33) biosurfactants on a 48h biofilm formed by *G. vaginalis* 101 (GV 101) and SH222C2. Biofilms were stained with 4',6-diamidino-2-phenylindole (DAPI). (A) Biofilm of GV101 without biosurfactant; (B) Biofilm of GV101 with L32 biosurfactant exposure for 48h; (C) Biofilm of GV101 with L33 biosurfactant exposure for 48h; (D) Biofilm of SH222C2 without biosurfactant; (E) Biofilm of SH222C2 with L32 biosurfactant exposure for 48h; (F) Biofilm of SH222C2 with L33 biosurfactant exposure for 48h.

To confirm our initial data from CLSM images, we calculated the maximum biofilm depth average by evaluation of the z-stacks grown between the first and last layers of *G. vaginalis* biofilm through 10 different sections of each coverslip (Figure 6.4).

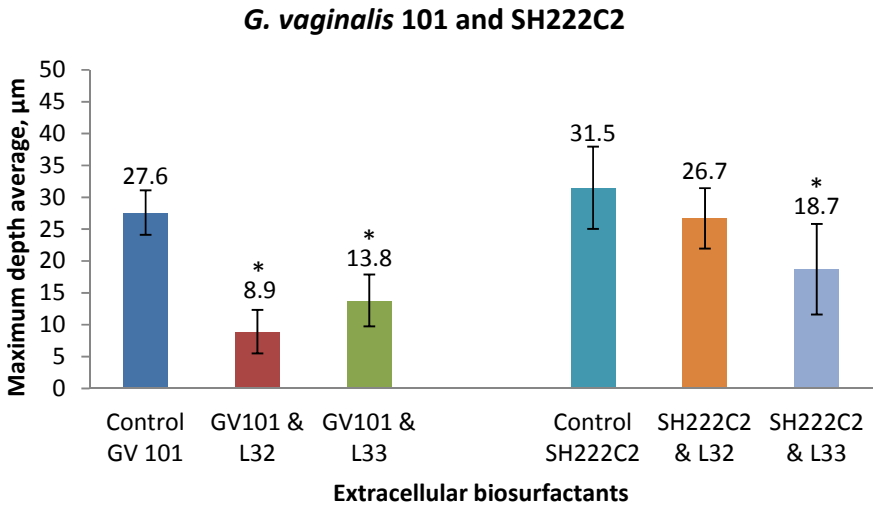

Figure 6.4 Biofilm maximum depth average obtained by CLSM for a 48h biofilm of *G. vaginalis* 101 (GV 101) and SH222C2 growth exposed to 80% of *L. rhamnosus* ATCC 7469 (L32) and *L. ruminis* ATCC 27781 (L33) biosurfactants. Control corresponds to *G. vaginalis* strain growth on sBHI without any extracellular biosurfactant.

* $p < 0.05$ when using *t*-student statistical analysis (95% confidence interval) for comparison of *G. vaginalis* control and *G. vaginalis* with biosurfactant tested in the biofilm assay.

As shown in the figure above, the maximum depth average of the *G. vaginalis* 101 and SH222C2 biofilms are in good agreement with the previous evaluations of *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 probiotic activities by microdilution method in 96-well plates. Although the CLSM analysis evidenced less discrepancy between these extracellular biosurfactants against the *G. vaginalis* strains tested, *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 continued to show a more efficient and statistical reduction of *G. vaginalis* 101 and SH222C2 biofilm formation (*t-student* statistical test value, $p < 0.05$; Figure 6.4), respectively.

6.4 Discussion

Due to the recurrent use of antimicrobial treatments against BV and consequently to the development of highly resistant bacteria, these currently used therapies have become fairly inefficient (17). As a result, an increased interest in the potential use of probiotic lactobacilli as alternatives for BV treatment and prevention has been reported (14, 30–32). Therefore, our goal was to select probiotic lactobacilli strains and to study their biosurfactants activities against a set of clinical *G. vaginalis* strains. For this purpose, we used both vaginal and dairy lactobacilli. Interestingly, the intracellular lactobacilli biosurfactants did not show an efficient inhibition on *G. vaginalis* growth, despite the positive results obtained in the screening by an agar spot test. On the other hand, the extracellular lactobacilli biosurfactants exhibited efficient probiotic activities against a wide-ranging *G. vaginalis* strains. These results are in agreement with a previous study realized by Brzozowski *et al*. (33), in which different activities or properties of the lactobacilli metabolites were found in intra- and extracellular extracts. Nonetheless, we cannot exclude that the absence of activity in the intracellular fraction could be related with the methodology used to recover the intracellular biosurfactants and its eventual low extraction efficiency. In fact, Faijes *et al*. (34) studied five different extraction methodologies to obtain intracellular products from *L. plantarum* species showing that certain intracellular lactobacilli products were less concentrated or even absent due to an inefficient extraction procedure (34).

On the other hand, all extracellular biosurfactants exhibited probiotic activities against the *G. vaginalis* strains tested; although they possessed different growth inhibition efficiencies for each *G. vaginalis* strain (see Figure 6.2 and 6.3). From the pool of lactobacilli studied, we found 6 strains with a higher potential to be used as probiotics, specifically *L. brevis* ATCC 14869, *L. rhamnosus* ATCC 7469, *L. ruminis* ATCC 27781, *L. salivarius* DEVRIESE94/438, SH40I and SH103E isolates. Although the lactobacilli strains from the culture collection were previously reported as probiotic species against several uropathogens (18, 21, 31, 32, 35–37), to the best of our knowledge none of them was specifically tested against a broad range of *G. vaginalis* strains. There is one report, in which *L. brevis* and *L*

salivarius species were used as probiotic tablets against a single *G. vaginalis* strain, being considered good probiotic candidates (38). Since there are numerous *G. vaginalis* strains co-existing in the vaginal epithelium and their pathogenicity is also different (39), an extensive analysis of each lactobacilli probiotic activity against a broad and well-known *G. vaginalis* collection (as in the current study) is useful.

Moreover, SH40I and SH103E vaginal isolates were able to match the probiotic activity shown by the lactobacilli strains from culture collection and therefore they could be also applied for BV prevention or treatment as adjuvants. So, further studies are required to identify these lactobacilli strains. It is also important to note that none of the probiotic lactobacilli strains from the culture collection was isolated from vaginal microflora, suggesting that lactobacilli strains from other sources rather than vaginal epithelium could be better BV probiotic candidates, as advised by Mastromarino *et al*. (38). Finally, CLSM analysis confirmed the probiotic effect of *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 biosurfactants on *G. vaginalis* 101 and SH222C2 biofilms, by diminishing the maximum thickness and structure of the 48 h biofilm formation when compared to the biofilm control. While we only used two strains for the biofilm studies, this suggests that all the probiotic lactobacilli could be good candidates in preventing biofilm formation in BV. An important pitfall of this study lays on the fact that we did not test the ability of the selected probiotics to kill bacteria from a previously formed biofilm. Further studies will be required to test this hypothesis, since a prophylactic approach will be limited to risk pregnancies, as it would not be feasible to administrate the probiotics to the general female population.

6.5 Conclusions

In summary, our study identified 6 lactobacilli strains as good candidates for BV prevention or treatment as adjuvants. We characterized their activities against a large group of *G. vaginalis* strains, inferring a clinical significance.

In addition, we selected 2 vaginal lactobacilli isolates with similar probiotic activities as the ones observed for lactobacilli strains from the culture collection against the tested *G. vaginalis* strains. Finally, CLSM analysis also demonstrated the ability of *L. rhamnosus* ATCC 7469 and *L. ruminis* ATCC 27781 extracellular biosurfactants to inhibit *G. vaginalis* biofilm formation, suggesting their probiotic potential against BV biofilms.

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6.7 Supplementary material

Table S6.1 Probiotic screening results from intracellular surfactants of our lactobacilli strains collection used against *G. vaginalis* strains.

Lactobacilli species	Code	Number of *G. vaginalis* strains inhibited									
		9	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
L. pentosus CECT 4023	L1										XX
L. casei CECT 5275	L2										XX
L. rhamnosus CECT 288	L3										XX
L. coryniformis subsp torquens CECT 4129	L4										XX
L. paracasei CECT 227	L5										XX
L. acidophilus ATCC 4356	L6										XX
L. agilis CCUG 31450	L7				X	X					
L. animalis ATCC 35046	L8										XX
L. bifermentans ATCC 35409	L9								X		X
L. brevis ATCC 14869	L10		XX								
L. buchneri ATCC 4005	L11				XX						
L. cellobiosus/*L. fermentum* ATCC 11739	L12										XX
L. crispatus ATCC 33820	L13										XX
L. curvatus subsp curvatus ATCC 25601	L14						X			X	
L. delbrueckii subsp delbrueckii ATCC 9649	L15				XX						
L. delbrueckii subsp lactis ATCC 12315	L16				X	X					
L. fasciminis DSM 20182	L17							X	X		
L. fructivorans ATCC 8288	L18										XX
L. gallinarum CCUG 31412	L19								X		X
L. gasseri ATCC 9857	L20								X		
L. graminis DSM 20719	L21			X						X	
L. hamsteri ATCC 43851T	L22					X				X	
L. helveticus ATCC 15009	L23								X	X	
L. hilgardii NCFB 962	L24								X	X	
L. instestinalis ATCC 49335	L25								X		X
L. johnsonii ATCC 11506	L26								XX		
L. murinus ATCC 35020	L27						X			X	
L. parabuchneri ATCC 12936	L28		XX								
L. paracasei subsp paracasei CCUG 27320	L29		XX								
L. plantarum NCIMB8827	L30					X					X
L. reuteri NCFB2656	L31					X					X
L. rhamnosus ATCC 7469	L32			XX							

Table S6.1 Probiotic screening results from intracellular surfactants of our lactobacilli strains collection used against *G. vaginalis* strains. (**Continuation)**

Lactobacilli species	Code	Number of *G. vaginalis* strains inhibited									
		9	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
L. ruminis ATCC 27781	L33				XX						
L. sakei subsp carnosus CCUG 8045	L34					X	X				
L. salivarius DEVRIESE94/438	L35					XX					
Vaginal isolate	SH29A										XX
Vaginal isolate	SH29B										XX
Vaginal isolate	SH23J										XX
Vaginal isolate	SH23D										XX
Vaginal isolate	SH40B										XX
Vaginal isolate	SH40I										XX
Vaginal isolate	SH81L										XX
Vaginal isolate	SH81B										XX
Vaginal isolate	SH81E					X					X
Vaginal isolate	SH81H			XX							
Vaginal isolate	SH81M								XX		
Vaginal isolate	SH85A2									X	X
Vaginal isolate	SH85B					XX					
Vaginal isolate	SH85C							XX			
Vaginal isolate	SH103E										XX
Vaginal isolate	SH103G1.1										XX
Vaginal isolate	SH103B			X				X			
Vaginal isolate	SH174A							X		X	
Vaginal isolate	SH174E1							X	X		
Vaginal isolate	SH177E										XX
Vaginal isolate	SH213A2										XX
Vaginal isolate	SH213D										XX
Vaginal isolate	SH212H			XX							
Vaginal isolate	SH212E	XX									
Vaginal isolate	SH65A				X			X			
Vaginal isolate	SH65G						X		X		
Vaginal isolate	SH65K				XX						
Vaginal isolate	SH65D1				XX						
Vaginal isolate	SH65B				X			X			
Vaginal isolate	SH79S			X				X			
Vaginal isolate	SH130A						X	X			
Vaginal isolate	SH130D			X				X			
Vaginal isolate	SH130H						X		X		
Vaginal isolate	SH130I1							X	X		
Vaginal isolate	SH196B				XX						

Table S6.1 Probiotic screening results from intracellular surfactants of our lactobacilli strains collection used against *G. vaginalis* strains. (**Continuation)**

Lactobacilli species	Code	Number of *G. vaginalis* strains inhibited									
		9	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
Vaginal isolate	SH196F					X					X
Vaginal isolate	SH196N			XX							
Vaginal isolate	SH199H				X			X			
Vaginal isolate	SH199A			X			X				
Vaginal isolate	SH199K	X						X			
Vaginal isolate	SH218A		X	X							
Vaginal isolate	SH218B		X		X						
Vaginal isolate	SH218M				X					X	
Vaginal isolate	MM13K3.3	X		X							
Vaginal isolate	MM14L1			X	X						
Vaginal isolate	MM1401.2			X		X					
Vaginal isolate	MM15I1	X	X								
Vaginal isolate	MM15I2		X					X			
Vaginal isolate	MM15Q			X	X						
Vaginal isolate	MM17H			XX							
Vaginal isolate	SH222G2			XX							

The probiotic screening of the lactobacilli intracellular biosurfactants were tested in duplicate for each *G. vaginalis* strain. The number of *G. vaginalis* inhibited was counted for each probiotic screening assay with the symbol X in the respective column number. The table shows the qualitative results obtained in probiotic screening assays.

Table S6.2 Probiotic screening results from extracellular surfactants of our lactobacilli strains collection used against *G. vaginalis* strains.

Lactobacilli species	Code	Number of *G. vaginalis* strains inhibited									
		9	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
L. pentosus CECT 4023	L1							XX			
L. casei CECT 5275	L2						XX				
L. rhamnosus CECT 288	L3				XX						
L. coryniformis subsp torquens CECT 4129	L4					XX					
L. paracasei CECT 227	L5				XX						
L. acidophilus ATCC 4356	L6								X		
L. agilis CCUG 31450	L7			XX							
L. animalis ATCC 35046	L8										XX
L. bifermentans ATCC 35409	L9						XX				
L. brevis ATCC 14869	L10	X	X								
L. buchneri ATCC 4005	L11				XX						
L. cellobiosus/*L. fermentum* ATCC 11739	L12					XX					
L. crispatus ATCC 33820	L13			XX							
L. curvatus subsp curvatus ATCC 25601	L14				XX						
L. delbrueckii subsp delbrueckii ATCC 9649	L15		XX								
L. delbrueckii subsp lactis ATCC 12315	L16					XX					
L. fasciminis DSM 20182	L17					XX					
L. fructivorans ATCC 8288	L18										
L. gallinarum CCUG 31412	L19				XX						
L. gasseri ATCC 9857	L20		XX								
L. graminis DSM 20719	L21				XX						
L. hamsteri ATCC 43851T	L22			XX							
L. helveticus ATCC 15009	L23			XX							
L. hilgardii NCFB 962	L24				XX						
L. instestinalis ATCC 49335	L25				XX						
L. johnsonii ATCC 11506	L26			XX							
L. murinus ATCC 35020	L27					XX					
L. parabuchneri ATCC 12936	L28								XX		
L. paracasei subsp paracasei CCUG 27320	L29	X							X		
L. plantarum NCIMB8827	L30				XX						
L. reuteri NCFB2656	L31					XX					
L. rhamnosus ATCC 7469	L32	XX									
L. ruminis ATCC 27781	L33	XX									

Table S6.2 Probiotic screening results from extracellular surfactants of our lactobacilli strains collection used against *G. vaginalis* strains. (Continuation)

Lactobacilli species	Code	Number of *G. vaginalis* strains inhibited									
		9	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
L. sakei subsp carnosus CCUG 8045	L34		X	X							
L. salivarius DEVRIESE94/438	L35	X	X								
Vaginal isolate	SH29A				X			X			
Vaginal isolate	SH29B							X			
Vaginal isolate	SH23J									X	
Vaginal isolate	SH23D				X						
Vaginal isolate	SH40B					X					
Vaginal isolate	SH40I			X				X			
Vaginal isolate	SH81L						X				
Vaginal isolate	SH81B						X				
Vaginal isolate	SH81E								X		
Vaginal isolate	SH81H								X		
Vaginal isolate	SH81M					X					
Vaginal isolate	SH85A2							X			
Vaginal isolate	SH85B							X			
Vaginal isolate	SH85C						X				
Vaginal isolate	SH103E				X			X			
Vaginal isolate	SH103G1.1							XX			
Vaginal isolate	SH103B							XX			
Vaginal isolate	SH174A			XX							
Vaginal isolate	SH174E1				XX						
Vaginal isolate	SH177E					XX					
Vaginal isolate	SH213A2					XX					
Vaginal isolate	SH213D				XX						
Vaginal isolate	SH212H				X		X				
Vaginal isolate	SH212E				X			X			
Vaginal isolate	SH65A				XX						
Vaginal isolate	SH65G		X	X							
Vaginal isolate	SH65K						XX				
Vaginal isolate	SH65D1			X	X						
Vaginal isolate	SH65B					X				X	
Vaginal isolate	SH79S				XX						
Vaginal isolate	SH130A							X			
Vaginal isolate	SH130D			XX							
Vaginal isolate	SH130H					XX					
Vaginal isolate	SH130I1					XX					
Vaginal isolate	SH196B						XX				
Vaginal isolate	SH196F				X				X		

Table S6.2 Probiotic screening results from extracellular surfactants of our lactobacilli strains collection used against *G. vaginalis* strains. (**Continuation)**

Lactobacilli species	Code	Number of *G. vaginalis* strains inhibited									
		9	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**0**
Vaginal isolate	SH196N							XX			
Vaginal isolate	SH199H					XX					
Vaginal isolate	SH199A					XX					
Vaginal isolate	SH199K					XX					
Vaginal isolate	SH218A					XX					
Vaginal isolate	SH218B				XX						
Vaginal isolate	SH218M						XX				
Vaginal isolate	MM13K3.3							XX			
Vaginal isolate	MM14L1								XX		
Vaginal isolate	MM1401.2						XX				
Vaginal isolate	MM15I1				XX						
Vaginal isolate	MM15I2				XX						
Vaginal isolate	MM15Q						XX				
Vaginal isolate	MM17H				XX						
Vaginal isolate	SH222G2				XX						

The probiotic screening of the lactobacilli extracellular biosurfactants were tested in duplicate for each *G. vaginalis* strain. The number of *G. vaginalis* inhibited was counted for each probiotic screening assay with the symbol X in the respective column number. The table shows the qualitative results obtained in probiotic screening assays.

Chapter VII

Concluding remarks and future work

7.1 Concluding remarks

This thesis intended to answer several key points related to the BV etiology, the effectiveness of BV diagnostic and the most adequate treatment. Nowadays, BV diagnostic methodologies are unable to detect the early stages of BV development and therefore therapy is usually applied in the later clinical stages of the infection. The consequences include a delay in the healthy recovery of the patient vaginal microflora. Aiming to improve BV diagnostic, we developed the first Peptide Nucleic Acid (PNA) Fluorescence *In Situ* Hybridization (FISH) methodology to increase the specificity and sensitivity of the detection of *Lactobacillus* spp. and *G. vaginalis* strains in vaginal samples. We were able to achieve a rapid identification (approximately 3 hours) of these key bacteria involved in BV establishment. In this methodology, the specificity and sensitivity of the designed PNA probes were found to be over 98.0% for *Lactobacillus* spp.; and 100% for *G. vaginalis*. Afterwards, we validated this methodology through a prospective study using a collection of vaginal samples from Portuguese women. This study allowed the validation of the PNA-FISH as a BV diagnostic technique, as well as its comparison with the standard BV diagnostic method. This methodology showed a sensitivity of 66.7% and a specificity of 94.2%, thus demonstrating its higher specificity and showing false positive results in BV diagnosis commonly obtained by the classical methods.

Although *G. vaginalis* has been postulated to be the main early colonizer in BV, studies demonstrating this assumption were scarce. Therefore, using our recently developed PNA-FISH methodology, we quantified the initial adhesion of *G. vaginalis* and other BV-associated bacteria (*A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum*) in the presence of two vaginal lactobacilli (*L. crispatus* and *L. iners*) through competitive and displacement/blockage assays into human epithelial cells. Our study proved that *G. vaginalis* has indeed the greatest capacity from all BV-associated anaerobes tested for initial adhesion to epithelial cells. Although *L. crispatus* and *L. iners* differ greatly in their capacity to protect the health of the vagina and its microbiome, *G. vaginalis* sustained its high initial adhesion ability in the presence of both lactobacilli strains. The results gathered in this study support

the idea that *G. vaginalis* could be an early colonizer in BV, later allowing other bacteria to grow and colonize vaginal epithelium. To test this last hypothesis, we next quantified the growth of dual species biofilms with *G. vaginalis* and other BV-associated anaerobes (*A. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum*) using the quantitative Polymerase Chain Reaction technique. Interestingly, we found that, regardless of the species, *G. vaginalis* biofilm growth was promoted by the presence of additional species (around ≈3 fold increase). On the other hand, *G. vaginalis* biofilms enhanced the growth of *P. bivia* (≈4 fold increase) and to a minor extent of *F. nucleatum* (≈2 fold increase). Thus, this study contributed to our understanding of BV biofilm formation, suggesting *G. vaginalis* as a key role in the early establishment of BV biofilms.

Finally, we performed a study to evaluate the probiotic potential of intra- and extracellular biosurfactants from lactobacilli strains against several *G. vaginalis* strains. Although the intracellular biosurfactants were unable to reduce *G. vaginalis* proliferation, the extracellular biosurfactants candidates showed a significant effect on *G. vaginalis* growth and biofilm formation. To conclude, through Confocal Laser Scanning Microscopy analysis, we confirmed the ability of certain extracellular biosurfactants to reduce *G. vaginalis* biofilm formation, suggesting their probiotic potential against BV biofilms. Accordingly, we were able to select 2 vaginal isolates and 4 lactobacilli strains from a culture collection capable to inhibit a wide range of *G. vaginalis* strains, thus illustrating an efficient probiotic activity.

7.2 Future work

The results gathered in this thesis provided interesting insights on the role of *G. vaginalis* in BV but also raised some questions that should be addressed in future research work.

Initially, when we tested our novel PNA-based methodology for the improved diagnosis of BV, we only included 91 vaginal swabs. Further studies including a larger number of samples should be conducted, in order to better characterize the difference in accuracy between the traditional method of Gram staining and Nugent scoring, compared with our PNA-FISH methodology.

All BV-associated anaerobes tested were found to enhance biofilm formation by *G. vaginalis*, but we also found that *G. vaginalis* biofilms enhanced the growth of *P. bivia* and *F. nucleatum*. However, expression of key genes should be determined to better understand the phenotypic shift from planktonic to biofilm, when grown in mono-culture *versus* multi-species culture containing other BV-associated anaerobes. This study would provide new insights into the ability of each individual BV-associated anaerobe to interact with *G. vaginalis*.

In our last study, the intracellular lactobacilli biosurfactants did not effectively inhibit *G. vaginalis* growth, despite the positive results obtained in the screening by an agar spot test. These negative results could be associated with the methodology used to recover the intracellular biosurfactants and its eventual low extraction efficiency. Further study of the intracellular lactobacilli biosurfactants and testing of other extraction methodologies to improve the efficiency of recovery would be worthwhile and necessary to draw more conclusive remarks about their effects on growth of *G. vaginalis*.

Finally, the probiotic products identification of the selected extracellular biosurfactants and its molecular characterization should be performed to guarantee the lactobacilli candidates' suitability for an alternative BV treatment.