Universidade do Minho
Escola de Engenharia

Aliona Rosca

Multi-species biofilms in bacterial vaginosis: ecological interactions and susceptibility to novel antimicrobial agents

março de 2021

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Biofilmes polimicrobianos na vaginose bacteriana: interações ecológicas e suscetibilidade a novos agentes antimicrobianos

Resumo

A vaginose bacteriana (VB), a causa mais comum de distúrbio vaginal, está associada a complicações ginecológicas e obstétricas graves. Durante a VB, um biofilme polimicrobiano é formado no epitélio vaginal, formado principalmente por espécies de *Gardnerella*, mas contendo também diversos outros microrganismos anaeróbios. No entanto, pouco se sabe sobre o papel destes microrganismos no desenvolvimento de VB, sendo, no entanto, evidente que os biofilmes polimicrobianos de VB apresentam uma maior capacidade de sobreviver ao tratamento clássico por antibióticos. Por essa razão, a busca por novas terapias contra VB tem vindo a aumentar.

Nesta tese, investigou-se as interações entre *Gardnerella vaginalis* e outras espécies associadas à VB, com o objetivo de compreender como estas interações afetam a formação de biofilmes e qual o impacto no tratamento antimicrobiano. Foram usados dois modelos distintos de formação de biofilmes triplos, o que permitiu concluir que, *in vitro*, *G. vaginalis* é a espécie microbiana que constituiu a maior parte da biomassa de todos os consórcios testados, em ambos os modelos. Verificou-se também que, em alguns dos consórcios, a capacidade antimicrobiana quer de antibióticos comuns, quer do óleo essencial (OE) obtido da planta *Thymbra capitata,* sofreram reduções de atividade, o que ajuda a explicar a elevada taxa de recorrência nos casos de VB.

No global deste estudo, destacou-se (i) o papel central da *Gardnerella* na formação de biofilmes polimicrobianos associados à VB, (ii) como as interações entre bactérias em biofilmes polimicrobianos impactam a formação de biofilme e a suscetibilidade antimicrobiana, e (iii) o potencial antimicrobiano do OE de *T. capitata* contra biofilmes polimicrobianos associados à VB. Os resultados obtidos nesta tese podem ajudar a desenvolver novas abordagens terapêuticas com base em OE de *T. capitata*, a fim de prevenir VB e reduzir a sua recorrência.

Palavras-chave: biofilmes polimicrobianos, *Gardnerella*, óleo essencial de *Thymbra capitata*, suscetibilidade antimicrobiana, vaginose bacteriana

Multi-species biofilms in bacterial vaginosis: ecological interactions and susceptibility to novel antimicrobial agents

Abstract

Bacterial vaginosis (BV), the most common cause of vaginal discharge, is associated with serious gynaecologic and obstetric complications. The hallmark of BV is the presence of a polymicrobial biofilm on the vaginal epithelium, presumably initiated and mainly formed by *Gardnerella* species. However, the BV biofilm is also populated by many other anaerobes, but very little is known about their role in BV development. It has been shown that this polymicrobial biofilm may increase the survival of *Gardnerella* and other BV-associated species when exposed to antibiotics, as such causing high recurrence rates of BV. This triggered the interest in exploring agents that have been claimed to disrupt biofilms, such as plant-derived products, namely *Thymbra capitata* essential oil (EO).

In this thesis, we investigated the interactions between *Gardnerella vaginalis* and other BV-associated species in BV biofilms, aiming to understand how they impact BV development and treatment outcome. First, two *in vitro* triple-species biofilm formation models were used, with and without allowing *G. vaginalis* to form an early biofilm before adding the other two species. The data from this study revealed that independent of the model used, all species were able to form triple-species biofilms, in which *G. vaginalis* was always the predominant species. Interestingly, we observed that in some triple-species consortia, synergistic interactions affected the antibiotic treatment outcome, leading to an enhanced tolerance. Second, when applying *T. capitata* EO against multi-species biofilms consisting of six cultivable BV-associated species, we observed that the interactions between the species also influenced, to some extent, the EO antimicrobial activity.

The data collected in this study further highlight (i) the pivotal role of *Gardnerella* in BV polymicrobial biofilms, (ii) how interactions between bacteria in multi-species biofilms impact the biofilm formation and antimicrobial susceptibility, and (iii) the antimicrobial potential of *T. capitata* EO against multi-species BV-associated biofilms. These findings could help to further shape novel treatment approaches based on *T. capitata* EO in order to prevent BV and reduce its recurrence.

Keywords: antimicrobial susceptibility, bacterial vaginosis, *Gardnerella* spp., multi-species biofilms, *Thymbra capitata* essential oil

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List of abbreviations

Aa	L-ascorbic acid
AV	Aerobic vaginitis
BHI	Brain heart infusion broth
BHV	Brucella broth supplemented with Hemin and Vitamin K_1
BHV.Aa	Brucella broth supplemented with Hemin, Vitamin K_1, and L-ascorbic acid
bp	Base pair
BV	Bacterial vaginosis
BVAB	BV associated bacteria
C.Model	Competitive biofilm formation model
CBA	Columbia blood agar
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
CM	Clindamycin
CO_2	Carbon dioxide
CST	Community state type
CV	Crystal violet
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DQC	Dequalinium chloride
EO	Essential oil
FISH	Fluorescence *in situ* hybridization
Fv	*Fannyhessea vaginae*
Fw	Forward
Gv	*Gardnerella vaginalis*
GvPC.Model	*G. vaginalis* pre-conditioned biofilm formation model
HIV	Human immunodeficiency virus
HPV	HPV
HSV	Herpes simplex virus
H_2O_2	Hydrogen peroxide
KOH	Potassium hydroxide
Li	*Lactobacillus iners*
Mc	*Mobiluncus curtisii*
MD	Metronidazole
mGTS	Medium simulating genital tract secretions
MIC	Minimal inhibitory concentration
MLC	Minimal lethal concentration
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information

NYC III	New York City III broth supplemented with 10% (v/v) inactivated horse serum
NYC.Aa	New York City III broth supplemented with 10% (v/v) inactivated horse serum and L-ascorbic acid
Pa	*Peptostreptococcus anaerobius*
Pb	*Prevotella bivia*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNA	Peptide nucleic acid
PSC	Peak serum concentration
qPCR	Quantitative PCR
RNA	Ribonucleic acid
Rv	Reverse
SB	Schaedler broth
SB.Aa	Schaedler broth supplemented with L-ascorbic acid
sBHI	Brain heart infusion broth supplemented with gelatine, starch, and yeast extract
sBHI.Aa	Brain heart infusion broth supplemented with gelatine, starch, yeast extract, and L-ascorbic acid
sld	Sialidase
STI	Sexually transmitted infection
UM	University of Minho
VBNC	Viable but nonculturable bacteria
vly	Vaginolysin
VVC	Vulvovaginal candidiasis

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List of publications

An important part of the work described in this thesis has been published elsewhere.

Articles in peer-reviewed journals:

Castro J, **Rosca AS**, Muzny CA, Cerca N. *Atopobium vaginae* and *Prevotella bivia* are able to incorporate and influence gene expression in a pre-formed *Gardnerella vaginalis* biofilm. Pathogens. 2021;10:247. doi:10.3390/pathogens10020247

Rosca AS, Castro J, Cerca N. Evaluation of different culture media to support *in vitro* growth and biofilm formation of bacterial vaginosis-associated anaerobes. PeerJ. 2020;8:e9917. doi: 10.7717/peerj.9917.

Rosca AS*, Castro J*, Sousa LGV, Cerca N. *Gardnerella* and vaginal health: the truth is out there. FEMS Microbiol Rev. 2020;44(1):73-105. doi: 10.1093/femsre/fuz027. *Both authors contributed equally.

Book chapter

Rosca A, Cerca N. Bacterial Vaginosis. In Diagnostics to Pathogenomics of Sexually Transmitted Infections, ed. Sunit K. Singh PhD. Chapter 13; 2018. p. 257–275. doi: 10.1002/9781119380924.ch13.

Oral presentations

Rosca A*, Vaneechoutte M, Cerca N. Ecological characterization of mixed species biofilms associated with bacterial vaginosis. Third Joint BIOTECnico and AEM PhD programs workshop, Instituto Superior Técnico, Campus Alameda, 11th of June 2018, Lisbon, Portugal (*presenting author).

Poster presentations

Rosca A*, Vaneechoutte M, Cerca N. *Gardnerella* spp. pre-conditioned vs competitive multi-species biofilm growth and the impact on the tridimensional biofilm structure. MICROBIOTEC'19, December 5 – 7, 2019, Coimbra, Portugal (*presenting author).

Rosca A*, Castro J, Cerca N. The effect of culture media on *in vitro* growth and biofilm formation of Bacterial vaginosis (BV)-associated pathogens. MICROBIOTEC'19, December 5 – 7, 2019, Coimbra, Portugal (*presenting author).

Rosca A, Vaneechoutte M, Cerca N*. Probing for bacterial interactions in Bacterial Vaginosis using an *in vitro* tri-species biofilm model. IUSTI 2018 World & European Congress, June 27 – 30, 2018, Dublin, Ireland (*presenting author).

Rosca A*, Cerca N, Vaneechoutte M. Interactions between *Gardnerella vaginalis* and other Bacterial Vaginosis (BV)-related species in an *in vitro* biofilm model. Research Day & Student Research Symposium, Ghent, Belgium, 19th of April 2018 (*presenting author).

Rosca A*, Martins AP, Castro J, Cerca N. Development of an *in vitro* vaginal exudate adhesion model for Bacterial Vaginosis. MICROBIOTEC'17, December 7 – 9, 2017, Porto, Portugal (*presenting author).

To my family.

CHAPTER 1

Introduction

Summary

This chapter provides information on the outline of the thesis. A brief background, research questions, hypothesis, aims, and significance are also presented.

1.1 Background

Bacterial vaginosis (BV) is recognized as one of the most common vaginal infections in women of reproductive age which, if left untreated, may cause serious obstetric and gynecologic complications, including preterm delivery (1,2), spontaneous abortion (3,4), low birth weight (5,6), pelvic inflammatory disease (7,8), infertility (9), and which may also lead to an increased risk of acquisition and transmission of several sexually transmitted infectious agents (10,11). Although the understanding of BV etiology is still limited, it is known that BV is characterized by a shift in the vaginal microbiota from the beneficial lactobacilli to vaginal dysbiosis with a dense biofilm containing a complex mixture of strict and facultative anaerobic bacteria, such as *Gardnerella* spp., *Fannyhessea vaginae* (previously known as *Atopobium vaginae*) (12), *Prevotella bivia*, *Mobiluncus curtisii*, *Peptostreptococcus anaerobius* and/or *Lactobacillus iners* (13–15). It is thought that this biofilm allows BV-associated bacteria to display a high resistance to the protective mechanisms of normal vaginal microbiota (16) as well as an increased tolerance to antibiotics (17), leading, therefore, to treatment failure and high recurrence rates of BV.

Microbiological analysis of BV suggests that the predominant species in this biofilm belong to the genus *Gardnerella*, found in more than 95% of all BV cases (18). It has been shown that *Gardnerella* spp. have a significantly higher virulence potential than many other BV-associated species (19,20) and therefore, it has been suggested that *Gardnerella* plays a pivotal role in BV development (21–24). However, despite this suggestion, there is doubt whether *Gardnerella* spp. alone are capable of causing BV or whether they must interact with other anaerobic species to cause BV. To that matter, some *ex vivo* studies have shown a synergy between *Gardnerella* spp. and *F. vaginae* in BV biofilms (25–27). Recently, it has been also demonstrated that synergistic interactions between *Gardnerella* spp. and other BV-associated species can lead to increased biofilm formation in dual-species biofilms (28–30). These findings paved the way for the further study of bacterial relationships in triple- as well as multi-species *in vitro* BV biofilms.

In this thesis, interactions between *Gardnerella vaginalis* and other BV-associated species in dual-, triple-, and multi-species BV biofilms were investigated. After characterizing the biofilms, we also set out to evaluate the impact of bacterial interactions on the susceptibility of triple-species biofilms to the first-line antibiotics used to treat BV. Furthermore, the effect of *Thymbra capitata* essential oil (EO), a promising new therapeutical agent that could be used to treat BV (31), was also assessed on multi-species BV biofilms. Together, the work performed in this thesis is expected to advance our knowledge on the impact that bacterial cooperation may have on BV etiology and antimicrobial tolerance, since a better

understanding of polymicrobial interactions may be essential for the development of novel treatment approaches to cure BV.

1.2 Research questions

The following questions will be addressed in this thesis:

1. Are non-*Gardnerella* BV-associated species able to form *in vitro* single-species biofilms under the same experimental conditions?

2. Can interactions between *G. vaginalis* and other BV-associated species in triple-species *in vitro* biofilms be key in BV development and antimicrobial susceptibility?

3. Do interactions in multi-species BV-associated biofilms affect *T. capitata* EO treatment outcomes?

Answers to these research questions are expected to provide new insights into importance of microbial interactions in BV and their impact on BV development and antimicrobial therapy.

1.3 Hypothesis and aims

1.3.1 Hypothesis

The importance of microbial interactions within biofilms is established for various polymicrobial biofilm-associated infections (32–35). In this study, considering BV as a polymicrobial infection, it was hypothesized that the interactions established between BV-associated species play a role in BV biofilm development and increase the antimicrobial tolerance.

1.3.2 Aims

The main goal of this study was to better understand the importance of microbial interactions in BV and how they impact BV pathogenesis. To accomplish this, the following specific aims were addressed.

Aim 1: To determine an optimal culture medium for the planktonic and biofilm growth of six cultivable anaerobes frequently associated with BV, namely *F. vaginae*, *Gardnerella* sp., *L. iners*, *M. curtisii*, *P. anaerobius*, and *P. bivia*.

Aim 2: To evaluate the ability of key BV-associated species to incorporate into a pre-formed *G. vaginalis* biofilm in dual- and triple-species consortia.

Aim 3: To assess how *G. vaginalis* pre-formed biofilms influence the ability of known BV-associated species to establish triple-species biofilms.

Aim 4: To determine the impact of triple-species biofilms on antimicrobial tolerance.

Aim 5: To evaluate the effect of *T. capitata* EO on single-species or multi-species biofilms formed by six relevant BV-associated species.

1.4 Significance

It is known that BV is characterized by the presence of a high number of strict and facultative anaerobic bacteria and some of these species have been found to form a polymicrobial biofilm on the vaginal epithelium. Although some *in vivo* and *in vitro* studies have described the occurrence of possible relationships among these bacterial species in BV biofilms, this subject still needs more detailed investigation. The main concern is that the incomplete eradication of this highly structured biofilm by antibiotics or host defences allows BV-associated bacteria to develop resistance to the antibiotic treatment and contribute to recurrent BV. Therefore, it becomes essential to unveil how BV-associated bacteria interact in the vaginal environment and contribute to the formation of the characteristic BV biofilm. This could lead to a better understanding of bacterial interactions during BV as well as shed new light on BV etiology. Furthermore, the results generated by this work could represent a valuable contribution for the development of novel therapeutic strategies to cure BV and consequently to reduce its rates.

1.5 Thesis outline

In this thesis, following this introductory chapter, a literature review (**Chapter 2**) is presented, which summarizes the known information on BV and its association with vaginal biofilms. Special attention is given to polymicrobial interactions present in BV-associated biofilms and their impact on antimicrobial therapy.

Chapters 3 to **6** present all the experimental data obtained after addressing the five aims of this thesis. Each of these chapters can be read independently, providing a summary, brief introduction, materials and methods, results, discussion, and conclusions.

Chapter 3 demonstrates the effect of nine different culture media on the planktonic and biofilm growth of six cultivable BV-associated species considered in this thesis.

Chapter 4 presents the interactions between *G. vaginalis*, *F. vaginae*, and *P. bivia* in *in vitro* dual- and triple-species biofilms. The biomass of these biofilms and their bacterial composition and distribution were first analysed and then the expression of *G. vaginalis* key virulence genes was assessed.

Chapter 5 is focused on evaluating the influence of *G. vaginalis* pre-formed biofilms on the ability of known BV-associated species to establish triple-species biofilms. Two different *in vitro* biofilm formation models where tested, and the differences in bacterial composition and integration was assessed. Special attention is given to the impact of the triple-species biofilms on antimicrobial tolerance.

Chapter 6 addresses the antibacterial activity of *T. capitata* EO against six cultivable BV-associated species grown planktonically and as biofilms. Moreover, the effect of EO was also assessed on a newly described multi-species BV biofilm formed by all six species considered in this chapter.

Finally, this thesis is concluded with **Chapter 7**, which presents a summary of the major findings and their significance as well as limitations and future directions in this field of research.

Figure 1.1 presents the thesis outline with the connection between the different chapters.

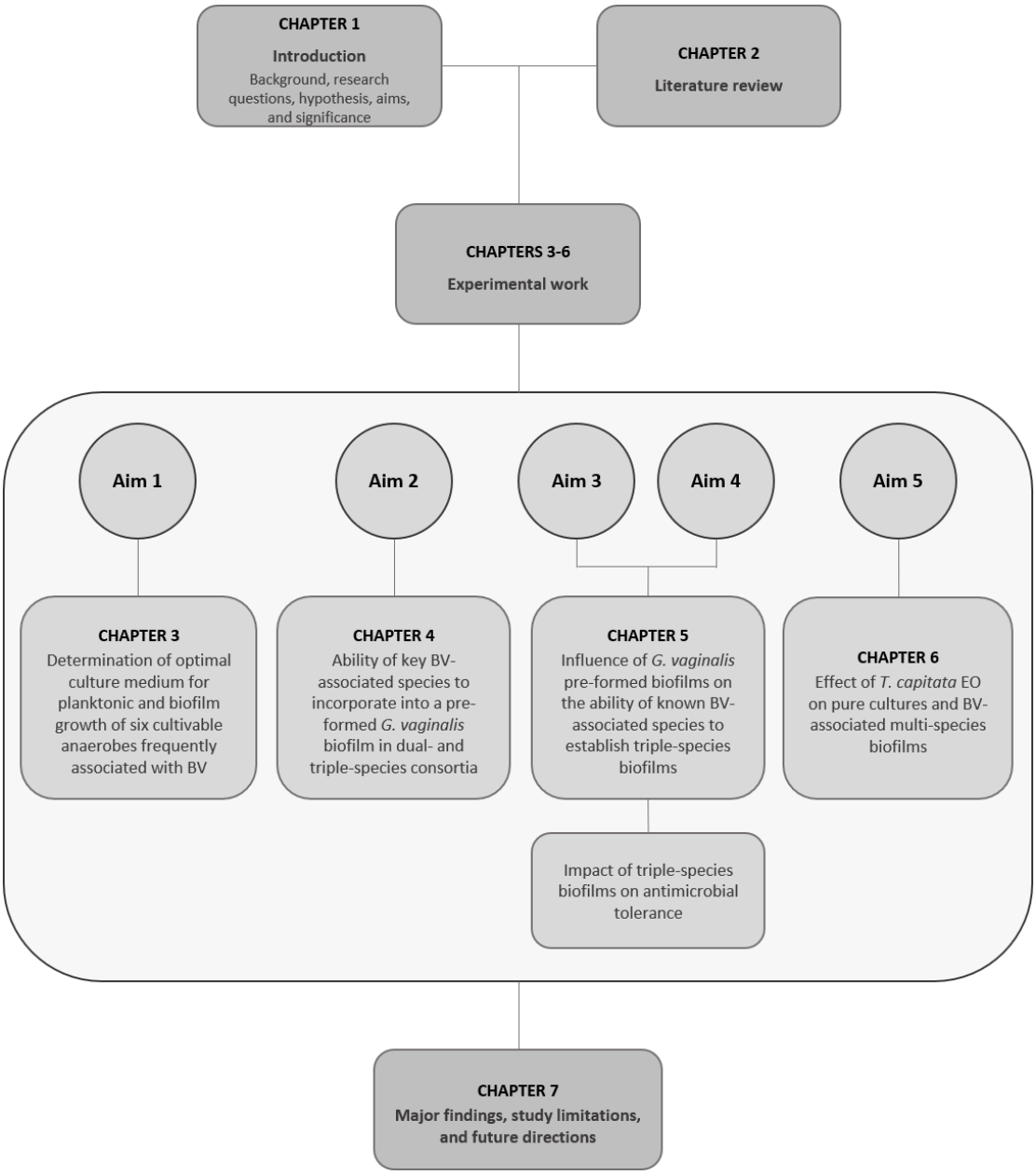

Figure 1.1. Thesis outline.

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CHAPTER 2

Literature review

Summary

This chapter provides important information on BV and its association with biofilm state. Special attention is given to polymicrobial interactions present in BV biofilms and their impact on antimicrobial therapy.

2.1 Introduction

The human vagina is a very adaptable organ of the female reproductive system, colonized by a wide array of bacterial species, that can have a profound effect on the health of women (1), conception (2), pregnancy (3), the mode and timing of delivery (4), and the risk of sexually transmitted infections (STIs) acquisition (5), and as such its function is more important than being a mere passageway for menstrual fluid, sperm, and neonates (6). Structurally, the vagina consists of a stratified squamous non-keratinized epithelium overlying a stratum of smooth muscle and a loose connective tissue stroma (7). The apical layers of the epithelium are comprised of dead cornified cells that, due to their uninfectability, serve as a shield against pathogens (8). However, these protective layers are always challenged and can eventually be disrupted, facilitating the invasion of pathogens (9). Most often, these disruptions are governed by diverse interactions among existing pathogens in the vaginal milieu, as is also the case for BV.

2.2 The healthy vaginal microbiota

The human vagina harbours numerous microorganisms that coexist in a dynamic balance and establish complex connections with each other and with the host (10). In the healthy vaginal ecosystem of most reproductive-age women, Gram-positive bacilli of the genus *Lactobacillus* are the predominant resident bacteria (11). Normally, vaginal colonization with lactobacilli is believed to ensure a protective environment since these bacteria prevent adverse microorganisms from colonizing the vaginal tract, using several mechanisms (12,13). One of the best-studied defence mechanisms is associated with the production of lactic acid by the majority of lactobacilli, which promotes the maintenance of the vaginal pH below 4.5 (12,14–16). This acidic environment renders the vaginal milieu hostile to many other bacteria while favouring the presence of *Lactobacillus* spp. (17,18). Besides lactic acid, lactobacilli also produce broad-spectrum bacteriocins that might play an important role in eliminating non-indigenous bacteria or pathogenic microorganisms through permeabilization of their membrane (19–21). Moreover, lactobacilli are known to produce hydrogen peroxide that could act as a natural microbicide within vaginal environment (22,23). However, its role in vaginal ecosystem is still being debated (12,24) since it has been described that physiological concentrations of this metabolite produced no detectable inactivation of BV-associated bacteria *in vitro*, when these were incubated under optimal, anaerobic growth conditions (25). *Lactobacillus* spp. are also able to interfere with the adhesion of pathogenic bacteria to the vaginal epithelial cells, as has been shown in *in vitro* studies (26,27). Such an ability of lactobacilli plays an essential protective role since the pathogen adhesion and colonization on the host cells often represent the first step of the infection process (28).

In addition to *Lactobacillus* spp., the vaginal microbiota of asymptomatic women of childbearing-age also includes other distinct taxa (29). Based on the differences in the composition and abundance of bacterial species, the vaginal microbiota of reproductive-age women has been devised in five major types, known as community state types (CST). Four of these CST are dominated by *Lactobacillus crispatus* (CST I), *Lactobacillus gasseri* (CST II), *Lactobacillus iners* (CST III), and *Lactobacillus jensenii* (CST V), while the CST IV does not comprise a substantial number of lactobacilli, but contains a wide range of facultative and strict anaerobic bacteria, including *Atopobium, Aerococcus*, *Corynebacterium*, *Eggerthella*, *Finegoldia*, *Gardnerella*, *Megasphaera*, *Mobiluncus*, *Peptoniphilus*, *Prevotella*, and *Sneathia* (11,29). Each CST presents a different bacterial proportion that varies among the four ethnic groups (Asian, white, black, and Hispanic), as illustrated in Figure 2.1. These variations appear to be determined by a combination of genetic, behavioural, cultural, and other undescribed factors (30,31). Nevertheless, all CST are composed by members that have been assigned to genera known to produce lactic acid, including *Lactobacillus*, *Atopobium*, and *Megasphaera*, being suggested that this ability may be conserved among communities (11). Overall, these findings challenged the knowledge that "normal and healthy" is synonymous with the presence of a high number of lactobacilli as almost 30% of healthy women lack appreciable numbers of *Lactobacillus* spp. (11,32,33).

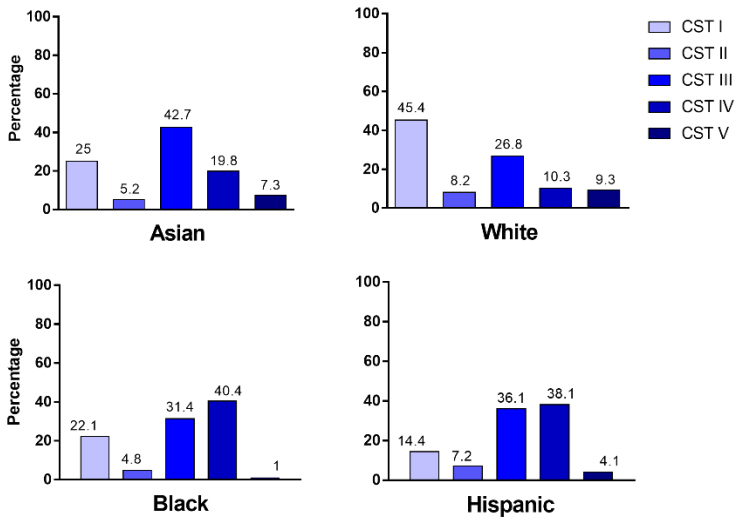

Figure 2.1. Representation of vaginal bacterial community state types (CST) within each ethnic group of women proposed by Ravel and colleagues (11). The study cohort included 96 Asian women, 97 white women, 104 black women, and 97 Hispanic women, showing the relationship between vaginal bacterial community composition and ethnic background.

Besides the protective effect of beneficial vaginal microbiota, the colonization of vaginal environment by pathogenic microbes is also prevented by local components of the immune system (34). In the vagina, the innate immune system, which represents the first line of response to infection (35), consists of several

components that provide specific protective barriers against the invasion of pathogens (36). The mucus lining and epithelial cells act as gatekeepers preventing the entry of pathogens into the vagina and forming a protective physical barrier (37). The mucus layer, besides entrapping the invasive pathogens, it also provides lubrication and serves as a source of nutrition for the vaginal microbiota (36). In addition to the physical barrier, pattern recognition receptors, especially Toll-like receptors (38) and natural antimicrobial peptides (39) form a chemical barrier. Toll-like receptors recognize conserved pathogen-associated molecular patterns synthesized by different microorganisms, and their expression by the vaginal epithelial cells is considered to play an important role in antigen detection and initiation of the immune response (40). As regards the antimicrobial peptides, these present broad-spectrum antibacterial activity as well as additional biological functions including cell proliferation, cytokine induction, chemotaxis, and modulation of innate and adaptive immunity (35). Altogether, the beneficial vaginal microbiota together with the immune system provide protection in the vaginal environment, which has a significant impact on the health of women, their partners, and their newborns (41). Changes in the composition of the vaginal microbiota have been associated to several adverse health outcomes, including BV, as discussed in the next section.

2.3 The unbalanced vaginal microbiota

The dynamic equilibrium of the vaginal microbiota can be altered at any time by environmental factors and external interferences, such as the use of antibiotics (42), vaginal douching (43), sexual intercourse (44) or hormone therapy (45). These changes can lead to periods of increased host susceptibility that negatively impact the ability of the vaginal community to resist pathogen colonization (46). Also, these alterations can determine the occurrence of microbial unbalances or dysbiosis in the urogenital tract, resulting in an infection (47). Vaginal infections are often caused by bacteria (as is the case for BV and for aerobic vaginitis), by fungi (vulvovaginal candidiasis), and by protozoa (trichomoniasis) as listed in Table 2.1. It is also important to note that STIs, including chlamydia, gonorrhoea or viral vaginitis, can also influence the vaginal microbiome (48) and present a certain relationship with BV, which will be further discussed.

Table 2.1. Main characteristics of the normal vaginal microbiota and of the most common vaginal infections.

	Vaginal fluid	Vaginal fluid pH	Clinical inflammation and symptoms	Microscopic features	Sexually transmitted	References
Healthy	White, no or milky odour, variable viscosity along the cycle	3.5 – 4.5	No	Mainly normal intermediate and superficial vaginal cells, numerous lactobacilli, very scarce leukocytes	Not applicable	(49–51)
Bacterial vaginosis	Abundant, greyish white, fishy odour, low viscosity	> 4.5	Odorous discharge (or no symptoms at all), absence of redness; no or slight inflammation	Clue cells, scarce or no lactobacilli, no leukocytes, abundant bacteria	Controversial	(49–51)
Aerobic vaginitis	Abundant watery, yellow, no fishy odour, low viscosity	> 4.5	Erythema	Scarce or no lactobacilli, leukocytes, abundant bacteria	No	(49–52)
Vulvovaginal candidiasis	White, none or ferment odour, “cottage cheese-like”, creamy or floccular, high viscosity	3.5 – 4.5	Diffuse redness, swelling and fissuring to the vulva, burning and pruritus	Presence of vaginal cells from deeper layers, variable number of lactobacilli and leukocytes, blastoconidia and pseudohyphae	No	(49–51,53)
Trichomoniasis	Yellow/ green aqueous discharge, fishy/ putrid odour, low viscosity	> 4.5	Erythema, red plaques, vulvar irritation and pruritus	Protozoa identification, particularly if motile, numerous bacteria and leukocytes, many parabasal cells	Yes	(50,51,54)

2.3.1 Bacterial vaginosis

BV is the most common bacterial vaginal infection among women of childbearing age worldwide, affecting between 23% to 29% of women in the general population (55). Microbiologically, BV is characterized by a change in the vaginal microbiota from the dominant health-associated lactobacilli to a polymicrobial microbiota, including strict and facultative anaerobic pathogens, whereby *Gardnerella vaginalis* plays a key role (11,56–58). It is important to mention that an emended description of *G. vaginalis* was recently proposed with the delineation of 13 genomic species within the genus *Gardnerella* (59). Following this renewed taxonomy of the genus *Gardnerella*, in this thesis, the term *Gardnerella* spp. will be used when discussing previous publications, which use the designation "*G. vaginalis*" to address the 13 different species of the genus *Gardnerella*, as it cannot be excluded the fact that other *Gardnerella* species were involved.

In the last years, BV has emerged as a global issue of concern due to its association with a wide range of adverse outcomes. It has been described that BV notably increases the risk of development of gynaecological postoperative infections (60), pelvic inflammatory disease (61), and infertility (62). Also, BV has been related with adverse pregnancy outcomes such as intra-amniotic infections (63,64), premature rupture of membranes (65), premature labour and delivery (66,67), spontaneous abortion (68), low birth weight (69), and increased neonatal morbidity (70). Moreover, BV enables the transmission of STI agents, including the human immunodeficiency virus (71), human papillomavirus (72), *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* (73).

2.3.1.1 Clinical features and diagnosis of BV

BV is typically characterized by the presence of a profuse, greyish white, thin, and homogenous vaginal discharge with a fishy odour in symptomatic women (49). However, BV has been also reported as being asymptomatic in almost half of the cases (74,75). BV-associated bacteria are responsible for the abnormal vaginal discharge as they produce mucin-degrading enzymes, such as sialidases, that degrade the normal vaginal mucin gel, thus increasing its volume (76). Also, the fishy odour happens because of the presence of BV-associated bacteria that are known to produce volatile polyamines as a result of their metabolism (77). The diagnosis of BV is usually made by using the Amsel criteria, which are assessed based on the presence of at least three out of the following four characteristics: (i) thin, greyish white homogenous vaginal discharge; (ii) vaginal pH exceeding a value of 4.5; (iii) presence of at least 20% of clue cells (vaginal epithelial cells coated with bacteria) on microscopic examination of vaginal fluid; (iv) positive "whiff or sniff test": presence of amine odour that is best induced by mixing vaginal secretion

with a 10% KOH solution on a glass slide (78). However, these characteristics are not always present, making Amsel criteria to some extent subjective (79).

In an effort to improve the accuracy of BV diagnosis, Nugent and colleagues proposed a Gram stain scoring system for examining vaginal smears (80). This method, considered the gold standard for BV diagnosis (79), is based on the Gram staining interpretation of the presence and relative amounts in the vaginal fluid of three bacterial morphotypes: large Gram-positive bacilli (corresponding to lactobacilli), small Gram-negative and Gram-variable rods (assumed to correspond to *Gardnerella* spp. and *Bacteroides* species), and curved Gram-variable rods, before assigned to *Mobiluncus*, but recently suggested to correspond to *Candidatus* Lachnocurva vaginae species, previously known as BV-associated bacteria-1 (BVAB1) (81). Each morphotype is scored in a scale from 0 to 4+, taking into account the number of morphotypes observed per oil immersion field. Therefore, a Nugent score of 0-3 is marked by the presence of a high number of Gram-positive bacilli, or at least no *Gardnerella* spp. and *Bacteroides* spp. or *Candidatus* Lachnocurva vaginae morphotypes and is considered normal (no BV). A Nugent score of 7-10 leads to the diagnosis of BV and is marked by the presence of high concentrations of *Gardnerella* spp. and *Bacteroides* spp. or *Candidatus* Lachnocurva vaginae species and the absence of Gram-positive bacilli. An intermediate microbiota corresponds to a Nugent score of 4-6 and has Gram staining characteristics between these two poles, as summarized in Table 2.2.

Table 2.2. Scoring system for Gram-stained vaginal contents (80).

Score	*Lactobacillus* spp. morphotypes	*Gardnerella* spp. and *Bacteroides* spp. morphotypes	Curved Gram-variable rods
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	-
4	0	4+	-
Vaginal microbiota diagnosis by Nugent score system			
	Total score [a]	**Interpretation**	
	0 – 3	Normal vaginal microbiota	
	4 – 6	Intermediate vaginal microbiota	
	7 – 10	Bacterial vaginosis	

[a] Morphotypes are scored as the average number seen per oil immersion field. Quantification of each individual score: 0 for no morphotype present; 1+ for 1 morphotype present; 2+, 1 to 4 morphotypes present; 3+, 5 to 30 morphotypes present; 4+, 30 or more morphotypes present. The total score = *Lactobacillus* spp. + *Gardnerella* spp. and *Bacteroides* spp. + *Candidatus* Lachnocurva vaginae species.

However, the Nugent score system, as Amsel criteria, has some disadvantages, particularly because it requires a well-trained technician to perform it and is associated with the interobserver variability. Therefore, alternative methods for BV diagnosis have been investigated and used aiming for higher

specificity, sensitivity, and reproducibility. Molecular technologies such as DNA microarray analysis (82,83), polymerase chain reaction (PCR) (56,84), quantitative PCR (qPCR) (85–88) or fluorescence *in situ* hybridization (FISH) (89,90) have permitted the detection or even quantification of the main BV-associated bacteria. However, most of these novel methods are expensive and still require validation (91). Importantly, a recent review about molecular methods for BV diagnosis has concluded that despite the wide variety of diagnostic assays available to diagnose BV, clinicians will need to consider costs, result time, and accuracy in their decision to select a specific assay to test for BV (92).

2.3.1.2 Treatment of BV

The current BV treatment is based on antibiotics and is directed toward relief of symptoms and signs of infection and reduction of the risk of STIs acquisition and BV-associated complications, mainly in pregnancy (93,94). Conventionally, BV is treated with either metronidazole, clindamycin or tinidazole, as described in Table 2.3. Even though certain studies reported successful short-term cure rates of antibiotic therapy (95,96), high levels of recurrence have been noticed within 6-12 months of treatment (97,98). As a result, treatment of recurrent BV can be difficult and may need prolonged courses of antibiotic therapy to obtain a long-lasting cure (99).

Currently, metronidazole, a member of the nitroimidazole drug class, represents the first-line therapy for BV infection, serving as drug of first choice (100). However, various side effects are associated with metronidazole therapy, including nausea, diarrhea, vomiting metallic taste as well as headache and dizziness (100,101). Another antimicrobial agent that can be used to treat BV is clindamycin (94). It was found that topical and oral clindamycin appeared to present a similar effect with that of topical and oral metronidazole (102), with the important advantage of causing a lower rate of adverse side effects (metallic taste in the mouth, nausea, vomiting), when compared to oral metronidazole (95,103). However, due to the oil-based composition of both clindamycin ovules and cream, it has been pointed out that their use might weaken latex condoms and diaphragms for 5 days after use (94). Furthermore, the administration of clindamycin seems to be a risk factor for the development of *Clostridium difficile* infection (104). Finally, tinidazole is currently considered an alternative antimicrobial agent for BV treatment, particularly when metronidazole and clindamycin are not tolerated (94). Tinidazole has a longer half-life than metronidazole and thus, it requires lower dosages and is administered less frequently. Also, its side effects have been reported at half the frequency when compared to metronidazole (105).

Table 2.3. Regimens for BV treatment.

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

[a] The regimens for treatment of BV are according to Workowski and Bolan 2015 (94).

2.3.1.3 Etiology of BV

The exact mechanism of how BV is triggered remains a matter of controversy. The lack of basic information about etiopathogenesis of BV led to the postulation of two main hypotheses. The first is the "primary pathogen" hypothesis, which infers that a single pathogenic species, *Gardnerella* spp., is the etiological agent of BV, usually transmitted by sexual contact (106). In contrast, the second is the "polymicrobial pathogen" hypothesis, which argues that *Gardnerella* spp. act in concert with other bacteria, principally anaerobes, to cause BV (107,108).

Historically, Gardner and Dukes (109) were the first to propose that a "small pleomorphic gram-negative bacillus", which they called *Haemophilus vaginalis* (first classification attributed to *G. vaginalis*), was the etiological agent of BV, as they claimed that *H. vaginalis* fulfilled all the Koch's postulates described in Table 2.4 (110). Nevertheless, a later study carried out by Criswell and colleagues (111) revealed some failures of the previous study, as they demonstrated that the inoculation of the volunteers with a pure culture of *H. vaginalis* did not always cause BV, whereas their inoculation with vaginal discharge infected with *H. vaginalis*, did in most of the cases. Therefore, the assumption was made that *H. vaginalis* was not the specific causative agent of BV, failing one of the Koch's postulates. Subsequently, other anaerobic bacteria were also found during BV episodes (112,113), and this led to the postulation of the "polymicrobial pathogen" hypothesis (114). This hypothesis is supported by the fact that BV is characterized by a high bacterial diversity (115), with other BV-associated species than *Gardnerella* spp. being also able to inhibit the growth of lactobacilli (116). However, as previously revealed in some studies,

many BV anaerobes may not be as virulent as pathogenic strains of *Gardnerella* spp., in terms of cytotoxicity, adherence, and biofilm formation (117–119). It has been also pointed out that current knowledge on BV etiology does not allow to decide whether the presence of multi-species biofilms is the cause or simply a consequence of BV (120). Furthermore, this hypothesis is still conflicting with the epidemiological profile of BV since several studies have been stating that BV reflects the behaviour of a sexually transmitted or sexually enhanced infection (121,122). Therefore, despite all these findings, more evidence is needed to reveal what is, in fact, the primary trigger that initiates BV.

Table 2.4. Koch's postulates.

The etiologic microbe must be present in each case of the disease.
The etiologic microbe can be isolated from a diseased host and grown in pure culture.
The etiologic microbe from the pure culture must cause the disease when inoculated into a healthy, susceptible host.
The etiologic microbe must be reisolated from the experimentally inoculated host and shown to be the same as the originally inoculated pathogen.

2.3.1.4 Epidemiology of BV

As mentioned above, there is evidence that BV might be sexually transmitted (123). Several epidemiologic studies have described many sexual risk factors that may enhance BV acquisition and according to those studies, women are more probable to have BV if they: (i) have a high number of lifetime sexual partners (124,125); (ii) report a new sexual partner (126); (iii) use oral contraception as an alternative to condom (127) or (iv) report a high frequency of intercourse (128). In addition, women who have sex with women (129–132), as well as asymptomatic male carriers (133–135) could be also considered responsible for the sexual transmission of BV (Figure 2.2).

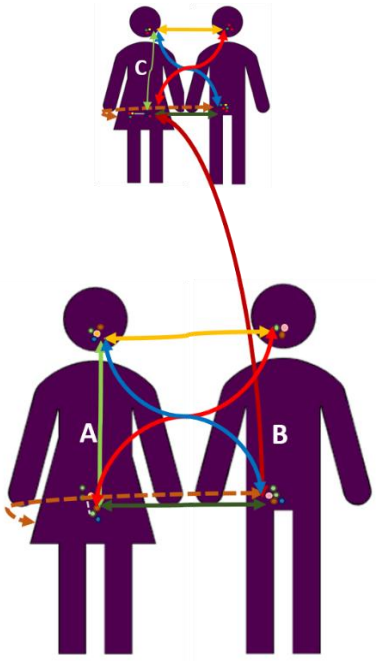

Figure 2.2. A schematic representation for understanding the epidemiology of BV. As proposed by Kenyon and Osbak 2014 (136), the vaginal microbiota of person A is interconnected to her oral and rectal microbiota and can also be connected to the oral and penile microbiota of person B. If person B has a simultaneous relationship with person C, the vaginal microbiota of person A can influence the microbiota of person C. These pathways can be influenced by several factors, including the frequency and type of sex, the use of oral contraception, and circumcision status. Similar connections occur with women who have sex with women.

Although BV might be considered sexually transmitted, there are also some criticisms and controversial studies (124,137). Hence, *Gardnerella* spp. has also been found in adolescent women with no sexual activity (138), and recurrent BV has also been reported in a virgin adolescent woman (139). Further, an alternative model was proposed (121), in which BV was defined as a sexually enhanced rather than a sexually transmitted infection (Figure 2.3). According to this model, it was suggested that the unprotected sexual intercourse is responsible for the alteration of the physico-chemical vaginal environment, thereby also affecting the vaginal microbiota. In particular, as the alkaline ejaculate raises the vaginal pH, it makes the environment less favourable for lactobacilli and more suitable for the growth of BV-associated anaerobes. Condom utilization would protect against BV development by preventing the alkalinisation of the vaginal environment. However, this hypothesis has not been verified yet. Also, it was suggested that both protected and unprotected vaginal sex could, somewhat, promote the transfer of perianal, perineal, and perivulvar bacteria to the vagina, inducing BV (121). In addition, non-coital sexual behaviours, including receptive oral (140) and anal sex (141) and non-penetrative digito-genital contact (142), might also alter the balance of vaginal microbiota through the transfer of BV-associated pathogens from rectal and perineal areas to the vulvar area and vagina, perhaps enhancing BV development.

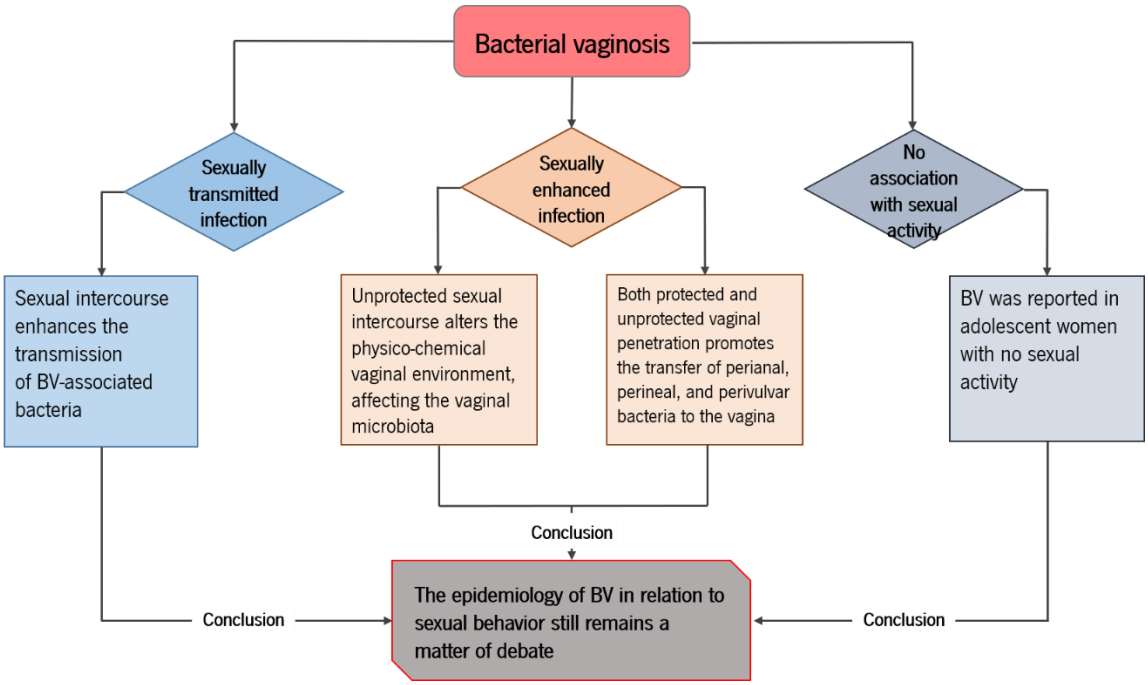

Figure 2.3. Representation of the epidemiological profile of BV in relation to sexual behaviour. This figure was created based on the information presented by Verstraelen and colleagues (121).

Taken together, BV epidemiology in relation to sexual behaviour still remains controversial and it is not surprising that BV has been referred to as "one of the great enigmas in the field of medicine" (143). Even if BV is of high clinical importance, the exact global prevalence is still unknown as it varies according to the characteristics of the studied population (144). As mentioned above, this could be partially explained by the evidence that there exist at least 13 genomic species within the genus *Gardnerella* (59) and that there are differences in virulence potential between virulent and avirulent strains of these species (145), which, therefore, might play distinct roles in vaginal microbiota. Nevertheless, these findings demand further investigation.

2.3.1.5 Bacteria implicated in BV

Although the current understanding of BV etiology is still scarce, the most common agreement is that BV is always associated with the overgrowth of numerous bacterial species, such as *Gardnerella* spp., *F. vaginae*, *P. bivia*, *M. curtisii, Bacteroides fragilis*, *Fusobacterium nucleatum*, *Mobiluncus mulieris*, *Mycoplasma genitalium*, *Mycoplasma hominis* or *Ureaplasma urealyticum* (146). The development of culture-independent profiling methods to detect fastidious or non-cultivable microorganisms has determined the broadening of the spectrum of anaerobes identified in women having BV with the addition of *Bifidobacterium*, *Dialister*, *Eggerthella*, *Leptotrichia*, and *Megasphaera* organisms (147,148), as well

as other bacteria including *Arthrobacter* sp., *Butyrivibrio fibrisolvens*, and *Caulobacter* sp. (149). Furthermore, several bacteria from the order *Clostridiales* have been also detected in BV cases, which were initially designated as BV-associated bacteria (BVAB): BVAB1, BVAB2, and BVAB3 (46,150). Up to now, only BVAB1 and BVAB3 have been phylogenetically characterized, being given the species name as *Candidatus* Lachnocurva vaginae (81) and *Mageeibacillus indolicus* (151), respectively. Significant differences were found when comparing the BV microbiome profiles of African American women and women of European ancestry, with African American women being more likely colonized by BVAB1 and BVAB3, *Bulleidia*, *Dialister*, *F. vaginae, Gemella*, *Megasphaera, Parvimonas, Prevotella,* and *Sneathia*, whereas women of European ancestry were more likely colonized by *Dialister micraerophilus, M. hominis*, and an undefined *Gemella* species (152).

A particular species that is often found in large numbers in BV is *L. iners* (153,154), being thus evident that not all vaginal *Lactobacillus* spp. are necessarily beneficial and protective. Indeed, *L. iners* is different from other lactobacilli, not growing on Man, Rogosa and Sharpe agar (selective for lactobacilli), staining Gram-variable, with a very small genome and cell size, not producing D-lactic acid (155,156), and carrying some pathogenicity factors, such as inerolysin (157), a pore-forming cytolytic toxin, related to vaginolysin of *Gardnerella* spp., which was found to be up-regulated at least six-fold in women presenting BV (158,159). Moreover, *L. iners* has been often identified in the intermediate vaginal microbiota (Table 2.2) (160,161) and also dominates the microbiota after treatment of BV (162). Still, as mentioned previously, *L. iners* has also been detected in the vaginal microbiota of healthy women (11,163). Therefore, to date, the role that this species plays in the vaginal econiche remains controversial and further investigations are needed to clarify this matter.

Although the development of the culture-independent approaches has facilitated comprehensive analyses of the composition of vaginal microbial communities, the importance of these findings remains uncertain, as it is not known whether these bacteria are pathogens that cause BV or if they are just opportunistic microorganisms that take advantage of the temporarily elevated pH and therefore increase numerically (30).

2.3.2 Association of BV with vaginal infections

Vaginal dysbiosis has also been associated with the co-infection of BV-associated bacteria and other microbes responsible for other vaginal infections (164). Moreover, vaginal dysbiosis is also associated with an increased risk of acquisition of STIs (165). Unfortunately, the simultaneous occurrence of different

vaginal infections not only confuses the diagnostic approach but also increases the risk of not treating them optimally. Consequently, the persistence of symptoms and signs has often been observed after the recommended treatment regimen of the supposed identified clinical entity (50,164).

2.3.2.1 BV and aerobic vaginitis

Aerobic vaginitis (AV) was the name given in 2002 to a vaginal condition that was until then misdiagnosed as BV, which contributed, to some extent, to the treatment failures in some women (52). AV is characterized by dysbiotic vaginal microbiota containing aerobic enteric commensals or pathogens, including Group B *Streptococcus* (*S. agalactiae*), *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus aureus*, and by deficient epithelial maturation (166–168). Although AV and BV share some characteristics, such as a reduced number or absence of lactobacilli, increased discharge (fishy smelling in BV, while a foul, rather rotten smell in severe forms of AV), and increased pH (often more pronounced in AV), there are also significant differences between the two. There is no or low-level of inflammation in women with BV, whereas the vagina of women with AV often appears red and edematous and may even display small erosions or ulcerations (52). The colour of the discharge in BV is usually whitish or grey and of a watery consistency, while in AV it is yellow to green and rather thick and mucoid (169). Although still largely undiagnosed, many researchers and clinicians are increasingly considering AV as a cause of symptomatic vaginitis. In some cases, mixed situations (AV and BV) can be found, representing either a transitory form or prolonged co-infection (170,171). Indeed, the vaginal milieu cannot be seen as a static system, but, rather, as a complex dynamic system (12).

2.3.2.2 BV and vulvovaginal candidiasis

Vulvovaginal candidiasis (VVC) is usually caused by an overgrowth of *Candida* spp., particularly *C. albicans*, associated with symptoms of inflammation (53,172). VVC is one of the most common types of infectious vaginitis, being diagnosed in up to 40% of women with vaginal complaints in the primary care settings (173). Approximately 75% of women experience at least one episode of VVC during their lives, most commonly between the age of 20 and 40 (53). However, an estimated 5% of women with VVC experience recurrent VVC, which is defined as four or more distinct episodes in a single year (172,174). Although there have been many studies regarding the host immunity and pathogenesis of *Candida* spp., little attention has been given to the vaginal microbiota, one of the most important parts of the vaginal environment (175). There is some evidence that VVC is a common side effect of BV treatment with

antibiotics: the antibiotics disturb the vaginal microbiota and decrease the protection against *Candida* spp. which, as a consequence, grow excessively, leading to VVC (176,177).

2.3.2.3 BV and trichomoniasis

Trichomoniasis has also been strongly associated with BV (178). In the 2001–2004 National Health and Examination Survey, the co-occurrence of these two infections was found in approximately half of the women infected with *Trichomonas vaginalis* (179). *T. vaginalis* is an anaerobic flagellated parasitic protozoan that adheres to epithelial cells of the urogenital tract (180) and is able to alter vaginal pH (181). Moreover, it has been associated with lower levels of vaginal lactobacilli and an increase of Nugent score (181). Furthermore, *in vitro* evidence indicates that *T. vaginalis* presence reduces epithelial-associated lactobacilli but not BV-associated species (182).

Recent longitudinal analyses have demonstrated that a Nugent score higher than 3 was associated with a significantly increased risk of acquiring *T. vaginalis* (183). Studies addressing *T. vaginalis* infection in vaginal microbiome using high throughput sequencing techniques are limited (184,185). However, one study found that vaginal dysbiosis (dubbed CST-IV) was significantly associated with *T. vaginalis* detection (184).

2.3.2.4 BV and chlamydia/ gonorrhea

Genital *Chlamydia trachomatis* is a common bacterial STI that is spread through oral, anal or vaginal sex in both women and men, although it often goes undiagnosed (186). If left untreated, chlamydia infection leads to pelvic inflammatory disease, which increases a woman's risk of infertility, pelvic adhesions, chronic pelvic pain, and ectopic pregnancy (187). This form of vaginitis is most commonly diagnosed in young women between the ages of 18 and 35 who have multiple sexual partners (188). Vaginitis can also be caused by the bacterium *Neisseria gonorrhoeae*, which causes gonorrhea, a curable but very contagious infection transmitted through genital and anal sex and less frequently through oral sex (189).

There are several studies reporting that BV is a strong predictor of chlamydial infection and gonorrhea (73,190–193). According to a data analysis of 535 women at high-risk for STIs, it was observed that BV severity, as defined by a high Nugent score (8–10), was associated with incident STIs (chlamydia/ gonorrhea), experiencing a 2-fold increased risk for STIs compared to women with normal vaginal microbiota (192). Also, Wiesenfeld and colleagues (73) reported a strong relationship between BV and chlamydial and gonococcal infections in women who reported recent sexual contact with a male partner

in whom either gonococcal or chlamydial urethritis or nongonococcal urethritis was diagnosed. In that study, women with BV were 3.4 times more likely to test positive for *C. trachomatis* and 4.1 times more likely to test positive for *N. gonorrhoeae* compared to women without BV. More recently, a study carried out among U.S. Army women showed that antecedent episodes of BV were associated with an increased risk of subsequent chlamydial and gonococcal infections (193).

2.3.2.5 BV and viral vaginitis

Herpes simplex virus (HSV) and BV have been epidemiologically linked in multiple cross-sectional and prospective studies (194,195). On a population level, Nugent scores of 4 or higher were significantly associated with an 8% increase in HSV type 1 (HSV-1) and a 32% increase in concurrent HSV type 2 (HSV-2) (194). In a meta-analysis of 16 cross-sectional studies, the authors found that the pooled odds of prevalent BV were 60% greater among HSV-2-positive women when compared with HSV-2-negative women, implying HSV-2 infection is an important BV risk factor. Based on these studies, it was hypothesized that pharmacologic HSV-2 suppression may reduce BV incidence and BV-associated adverse events (195).

Another source of viral vaginitis is the human papillomavirus (HPV), a virus that is also transmitted through sexual contact (196). Longitudinal studies have shown an increased association of prevalent and incident HPV in women with both intermediate and BV microbiota (197,198). Interestingly, two follow up molecular analyses described that women who were HPV-positive had a lower proportion of protective vaginal *Lactobacillus* spp. when compared with HPV-negative women (199,200).

Furthermore, there is also considerable evidence associating vaginal dysbiosis with increased risk of acquisition and transmission of human immunodeficiency virus type 1 (HIV-1) (201–203). A meta-analysis of 23 studies showed that BV was associated with a 60% increase in the risk of acquiring HIV-1 (204).

2.4 Vaginal biofilms in BV

Multi-species biofilms are considered the most common state of growth in nature and are characteristic for many microbial species (205). These polymicrobial communities have been found in aquatic environments (206), on artificial industrial structures (207), on implanted medical devices (208), and also on plant and mammalian tissues (209,210), causing serious problems. The microorganisms

embedded in multi-species biofilms often obtain numerous advantages, thereby creating more competitive and beneficial living conditions (211–214).

Not surprisingly, BV was also found to be associated with the presence of a polymicrobial biofilm when back in 2005, Swidsinski and colleagues (215) by using FISH technique observed high concentrations of diverse bacterial groups adhered to the vaginal epithelial cells, with *Gardnerella* spp. as the most prevalent member. These findings were further substantiated by other several studies (216–218) and it is now accepted that BV-biofilms are strongly related with the presence of *Gardnerella* spp.

Up to now, the exact process of BV biofilm development is still unclear (218,219). Nevertheless, there is evidence supporting that the first step of biofilm formation, corresponding to microbial adhesion to the vaginal epithelium, is an important factor in BV development (215). This process reduces the contact of microbes with potentially damaging antibodies and extracellular enzymes as well as decreases their chances of being flushed away in vaginal fluid or urine (220,221). It is noteworthy that the ability of *Gardnerella* spp. to colonize vaginal cells was already acknowledged in the eighties (222,223). Actually, vaginal epithelial cells covered with bacteria, which represent the so-called clue-cells mentioned as one of the Amsel criteria, are exactly what is expected to be noticed in case of biofilm formation. Moreover, these clue-cells have been observed for decades (78,224,225), but only recently they were described as being related to the biofilm formation process (215). Additionally, it has been shown that *Gardnerella* spp. are capable to attach to epithelial cells and displace the pre-adhered *L. crispatus* bacteria, while the other BV anaerobes, such as *F. vaginae*, *F. nucleatum*, *M. mulieris*, and *P. bivia* were outcompeted by the protecting lactobacilli (118). Subsequently, another study confirmed that *Gardnerella* spp. present a higher virulence potential and ability to attach to epithelial cells than 29 other BV-associated species (119). Recently, Muzny and colleagues (107,108) proposed that *Gardnerella* spp. initiates the BV biofilm formation, being followed by *P. bivia* in these lower layers of the biofilm. The synergy between *Gardnerella* spp. and *P. bivia* occurs in the vaginal environment with the production of metabolites favouring their growth, and sialidase and other enzymes that promote the breakdown of the mucous layer of the vaginal epithelium. Loss of the protective mucous layer leads to increased adherence of other BV-associated colonizers, including *F. vaginae* and *Sneathia* spp., to the developing, polymicrobial BV biofilm (Figure 2.4). Still, even though this proposed hypothesis sheds light on the BV development process, further investigations are needed for better understanding of its clinical importance.

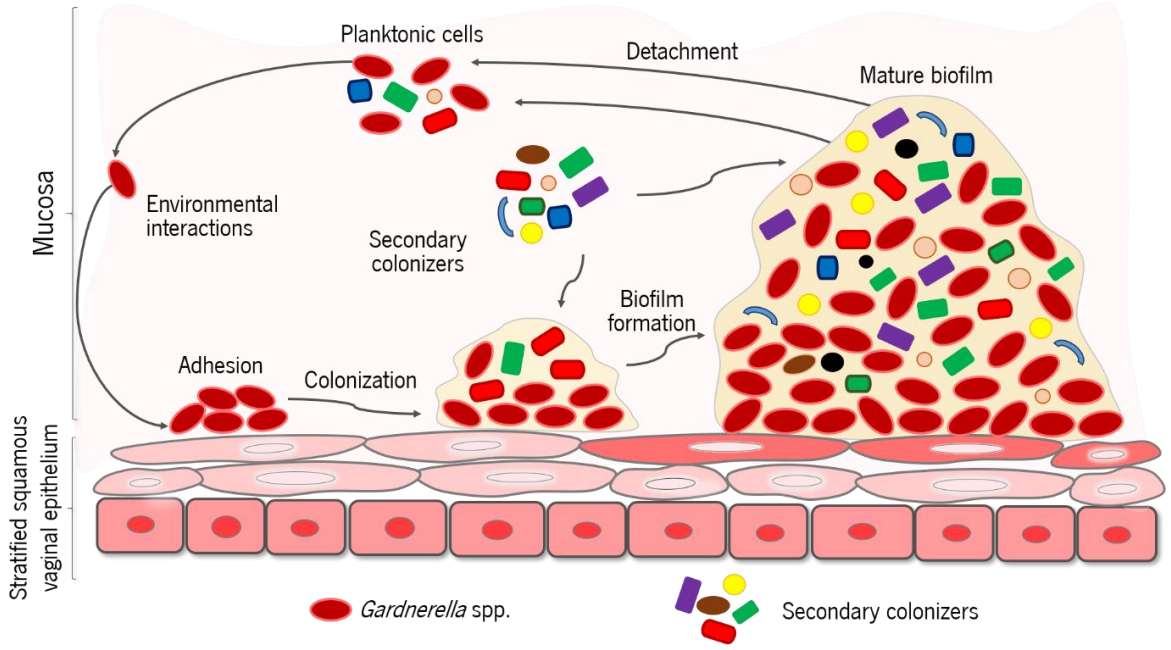

Figure 2.4. The multi-species model proposed for BV-associated biofilm development. In multi-species BV-associated biofilms, secondary pathogens are able to incorporate the initially formed biofilm by *Gardnerella* spp. which is already adhered to the vaginal epithelial cells. Subsequently, a synergistic relationship can be formed, allowing the biofilm to prosper.

2.4.1 Microbial interactions in the vaginal environment

Interspecies interactions within biofilm communities have been described for bacteria present in the oral cavity (226), gastrointestinal tract (227), lung environment (228), as well as in the vaginal ecosystem (218). These interactions can be either synergistic, exerting their effect by changing the environment so it becomes appropriate for proximate species or by producing specific metabolites that stimulate the growth of other microorganisms (229), or antagonistic (230). The antagonistic interactions can result in competition over nutrients and growth inhibition. Considering microbial interactions in the vaginal ecosystem, our understanding is still in its early stages (218). Nevertheless, this type of interactions might have a major impact on vaginal environment, influencing the success of the antimicrobial therapy.

2.4.1.1 Interactions between Gardnerella *spp., BV-associated anaerobes, and commensal bacteria*

Recognizing BV as a polymicrobial infection, multiple studies have suggested that interactions among BV-associated bacteria may influence its progression and pathogenesis, as described in Table 2.5. Correspondingly, bacterial interactions within dual-species biofilms have been started to be studied using an *in vitro* model that allows *Gardnerella* spp. to initiate the biofilm, as an early colonizer during BV, after which a second species is introduced. Interestingly, it was found that some of the tested BV-associated species had the ability to establish synergistic interactions and increase the pre-formed biofilm by

Gardnerella spp., while others presented antagonistic activity (231). Subsequently, by performing confocal laser scanning microscopy, it was possible to observe among bacterial consortia differentiated biofilm structures in at least three unique dual-species biofilm morphologies (232). Also, the impact of the second BV-associated species on *Gardnerella* spp. virulence was assessed by quantifying the key genes, such as genes encoding for vaginolysin and sialidase, and significant variations were found, suggesting that some, but not all species, can modulate the virulence features of *Gardnerella* spp. as well as contribute to enhanced symptoms associated with BV (232).

Several studies addressing synergistic interactions in BV have described specific nutritional pathways involving BV-associated bacteria. Back in 1979, an *in vitro* study identified nutritional pathways to maintain the synergistic relationship observed between *Gardnerella* spp. and *P. bivia*. Growth of *P. bivia* in a medium supplemented with amino acids or peptone resulted in ammonia production while the growth of *Gardnerella* spp. under the same conditions was accompanied by ammonia utilization (233). As a result, ammonia flow from *P. bivia* to *Gardnerella* spp. was suggested as a mechanism to support this interaction (234). Moreover, such commensal interaction was supported by another *in vitro* study in which it was demonstrated that *Gardnerella* spp. growth increased in the presence of *P. bivia*, and *P. bivia* reached higher numbers when incubated together with *Gardnerella* spp. (235). More recently, using a mice model, it has been shown that the presence of *Gardnerella* spp. enhanced the invasive potential of *P. bivia*, supporting its ascension into the uterus (236).

Also, growth of *Peptostreptococcus anaerobius* has been shown to be enhanced in the presence of *P. bivia*, but not in pure culture (237). After analysing *P. bivia* culture supernatant, an elevated concentration of amino acids was observed compared to controls, followed by the growth of *P. anaerobius* and amino acids consumption. Moreover, the addition of amino acids to the growth medium, in concentrations similar to those produced after prior growth with *P. bivia*, had a stimulatory effect on the growth of *P. anaerobius*. Consequently, it has been proposed that the increased availability of amino acids supports the commensal synergism between *P. bivia* and *P. anaerobius*.

In addition to these *in vitro* observations, there are studies conducted *in vivo* also demonstrating the existence of potential synergies among vaginal microorganisms present in BV. By investigating the composition and spatial distribution of bacteria in biopsy specimens from patients with BV, Swidsinski and colleagues (215) found that *F. vaginae* was homogeneously intermixed with *Gardnerella* spp. in an adherent specific biofilm. Later, Hardy and colleagues (238) confirmed the synergy between *Gardnerella* spp. and *F. vaginae* in samples containing biofilms from women with BV. Additionally, synergistic

interactions between *Gardnerella* spp. and *M. hominis* (239) or *F. vaginae* and *Prevotella* spp. (240) have been also identified in clinical samples.

Contrary to synergistic interactions that are beneficial for the microorganisms, antagonistic interactions result in a negative effect for at least one species (230). The occurrence of antagonistic interactions among microorganisms within a community is almost unavoidable due to competition for nutrients, with effects on the viability and growth of competitors, or preference for colonization of new surfaces (241). In the vaginal environment, this type of interrelationships has been also identified, whereby the production of lactic acid by lactobacilli had a negative effect on many BV-associated pathogens (6). Although this effect has been only addressed in a few *in vivo* studies, there are many *in vitro* experiments that have demonstrated the antagonistic effect between lactobacilli and BV anaerobes, as presented in Table 2.5.

2.5 Polymicrobial interactions: impact on antimicrobial therapy

Considering BV as a polymicrobial biofilm-associated infection, there is an emergent need to start focusing on exploring the effect of antibiotics on *in vivo* and *in vitro* BV biofilms in order to improve treatment options. Unfortunately, existing studies addressing this matter are still limited, and up to now, to our knowledge, no investigations have been reported about how polymicrobial interactions can increase the antimicrobial resistance of BV-associated bacteria (218,242). Nevertheless, important information can be deduced from studies that have explored antimicrobial activity in other polymicrobial infections, such as otitis media or cystic fibrosis.

Studying otitis media, Perez and colleagues (243) showed that *Moraxella catarrhalis* and *Streptococcus pneumoniae* presented a higher resistance to the applied antibiotics when grown in polymicrobial biofilms, as compared to single-species biofilms. They demonstrated that a β-lactamase produced by *M. catarrhalis* provided passive protection to *S. pneumoniae* from β-lactam antibiotic killing, while *S. pneumoniae* protected *M. catarrhalis* from azithromycin killing. Similarly, as demonstrated by Lopes and colleagues (244), *Dolosigranulum pigrum* and *Inquilinus limosus*, two species isolated from the airways of patients with cystic fibrosis, became significantly more resistant to several antibiotics upon culture in dual-species biofilms with *Pseudomonas aeruginosa*. Also, multi-species biofilms composed of *P. aeruginosa*, *Pseudomonas protegens*, and *Klebsiella pneumoniae* were more resistant to tobramycin and sodium dodecyl sulfate compared to single-species biofilms, suggesting that enhanced resistance derives from a cross-protection that is beneficial to the entire community (245).

Table 2.5. Interactions between bacteria in BV and their predictive ecological effects.

Microorganisms	Interaction	Mechanism	Effect in host	References
SYNERGISTIC INTERACTIONS BETWEEN MICROORGANISMS FROM VAGINAL ECOSYSTEM				
Gardnerella spp. and *Prevotella bivia*	*P. bivia* produced ammonia which was utilized by *Gardnerella* spp. which produced amino acids that were utilized by *P. bivia*	Ammonia and amino acids cycle	Presence of high vaginal pH	(234)
Gardnerella spp. and *P. bivia*	*Gardnerella* spp. facilitated uterine infection by *P. bivia*	The presence of *Gardnerella* spp. enhanced the invasive potential of *P. bivia*, facilitating its ascension into the uterus	BV bacteria may actively inhibit inflammatory responses	(236)
Gardnerella spp. and *Fannyhessea vaginae*	*F. vaginae* was homogeneously intermixed with *Gardnerella* spp. in specific BV-associated biofilms	Unknown	Presence of clue cells	(215)
Gardnerella spp. and *F. vaginae*	*Gardnerella* spp. and *F. vaginae* are important constituents of the vaginal biofilm	Unknown	Presence of clue cells	(238)
Gardnerella spp. and *Peptostreptococcus anaerobius*	*Gardnerella* spp. strains were able to enhance the growth of *P. anaerobius*	Production of synergistic compounds by *Gardnerella* spp.	Bacterial interactions have an important role in the ecology of vaginal microbiota	(246)
Gardnerella spp. and *Eggerthella, Dialister* sp. type 2*, F. vaginae*, and *Aerococcus christensenii*	Metabolic co-dependencies between these bacteria	Unknown	Possible contribution to enhance the incidence of BV	(115)
F. vaginae and *Prevotella* spp.	Both bacterial species might have metabolic co-dependencies	Unknown	The combination of *Prevotella* spp. and/ or *F. vaginae* seems to help diagnose BV with high accuracy	(240)

Table 2.5. Continued

Microorganisms	Interaction	Mechanism	Effect in host	References
SYNERGISTIC INTERACTIONS BETWEEN MICROORGANISMS FROM VAGINAL ECOSYSTEM				
Gardnerella spp. and *Fusobacterium nucleatum, Mobiluncus mulieris, F. vaginae* or *P. bivia*	*Gardnerella* spp. biofilms derived a growth benefit from the addition of a second species, regardless of the species, in *in vitro* dual-species biofilms. Presence of *Gardnerella* spp. in the biofilms enhanced the growth of *P. bivia* and to a minor extent of *F. nucleatum*	*F. nucleatum* was shown to be able to join an initial *Gardnerella* spp. biofilm (intermediate colonizer)	The symbiotic relationships established between *Gardnerella* spp. and other anaerobes in BV biofilms could contribute to the progression of BV	(235)
Gardnerella spp. and *Actinomyces neuii, Brevibacterium ravenspurgense, Corynebacterium amycolatum, Corynebacterium tuscaniense, Enterococcus faecalis, Escherichia coli, Nosocomiicoccus ampullae, Propionibacterium acnes, Staphylococcus saprophyticus, Staphylococcus simulans, Staphylococcus warnerii* and *Streptococcus anginosus*	These bacterial species were able to cause an enhancement of the biomass of a pre-formed *Gardnerella* spp. biofilm	Unknown	Could be associated with a high number of clue cells	(231)
Gardnerella spp. and *E. coli* or *E. faecalis*	*E. coli* and *E. faecalis* were able to incorporate and enhance a pre-formed *Gardnerella* spp. biofilm	In dual-species biofilms, each of these bacterial species seems to be able to co-aggregate with *Gardnerella* spp.	Uropathogens can associate in BV biofilm	(247)
Gardnerella spp. and *Mycoplasma hominis*	Strong association between *Gardnerella* spp. and *M. hominis* were found in women with BV	A potential quorum sensing-like interaction or co-response to an environmental stimulus	The transmission of one of these bacteria could trigger the outgrowth of the other and start a process leading to BV	(239)

Table 2.5. Continued

Microorganisms	Interaction	Mechanism	Effect in host	References
SYNERGISTIC INTERACTIONS BETWEEN MICROORGANISMS FROM VAGINAL ECOSYSTEM				
Gardnerella spp. and *F. vaginae, A. neuii, C. tuscaniense, M. mulieris, S. anginosus, P. bivia, C. amycolatum, N. ampullae, P. acnes, B. ravenspurgense, E. faecalis, S. saprophyticus, S. simulans, S. hominis, S. warnerii*	Despite all BV-associated species were able to increase the cell number of a pre-formed *Gardnerella* spp. biofilm, not all bacterial species enhanced *Gardnerella* spp. virulence, according to transcriptomic findings	Increased expression of genes associated with cytotoxicity, biofilm formation, antimicrobial resistance, and evasion of immune response by *Gardnerella* spp. in the presence of specific BV-associated bacteria in dual-species biofilms	Bacterial interactions between co-infecting bacteria can profoundly affect the progress of BV and its clinical outcome	(232)
P. anaerobius and *P. bivia*	Amino acids accumulation in *P. bivia* culture supernatants and subsequent growth of *P. anaerobius* in the conditioned supernatants	*P. anaerobius* enhanced its growth in the presence of *P. bivia*, but not in pure culture. Amino acids served as a growth source for *P. anaerobius*	Increased risk for female pelvic infections and adverse pregnancy outcome	(237)
ANTAGONISTIC INTERACTIONS BETWEEN MICROORGANISMS FROM VAGINAL ECOSYSTEM				
Lactobacillus spp. and *Gardnerella* spp., *Mobiluncus* spp., *Bacteroides*, and anaerobic cocci	*Lactobacillus* inhibited the growth of bacteria isolated from women with BV	The capacity of *Lactobacillus* to acidify the medium with a consequent decrease of pH and inhibition of pathogens growth	Lactobacilli prevent the growth of bacteria associated with BV	(248)
Lactobacillus spp. and *Gardnerella* spp., *Mobiluncus* spp., *Peptostreptococcus* spp., *Bacteroides* spp.	*Lactobacillus* inhibited the growth of *Peptostreptococcus*, *M. curtisii*, *Gardnerella* spp., and other anaerobes	The inhibition by *Lactobacillus* was influenced by the pH of the growth medium	The interactions between *Lactobacillus* and other bacteria may regulate the microbiological ecosystem of the vagina	(116)
Lactobacillus spp. and *Gardnerella* spp., *Bacteroides* spp., *P. bivia*	*Lactobacillus* inhibited the growth of BV-associated bacteria	Production of acids and hydrogen peroxide (H_2O_2) by lactobacilli	Lactobacilli would prevent vaginal colonization by other bacteria associated with BV	(249)

Table 2.5. Continued

Microorganisms	Interaction	Mechanism	Effect in host	References
ANTAGONISTIC INTERACTIONS BETWEEN MICROORGANISMS FROM VAGINAL ECOSYSTEM				
Lactobacillus acidophilus and *Gardnerella* spp.	*L. acidophilus* produced a bacteriocin that inhibited the growth of *Gardnerella* spp. isolates	Production of a bacteriocin by *L. acidophilus*	Lactobacilli, by the production of bacteriocins, have the capacity to prevent the growth of pathogenic bacteria	(250)
Lactobacillus helveticus and *Gardnerella* spp. and *P. bivia*	*L. helveticus* inhibited the growth and viability of *Gardnerella* spp. and *P. bivia* and also decreased the capacity of adhesion of *Gardnerella* spp. to HeLa cells	The antagonistic activity is due to the compounds produced by *L. helveticus*	*L. helveticus* is a potential probiotic	(251)
Lactobacillus spp. and *Gardnerella* spp. and *P. bivia*	*Lactobacillus* strains isolated from vaginal cavity of healthy women showed antagonistic activity against *Gardnerella* spp. and *P. bivia* in co-culture and also inhibited viability and adhesion of bacteria to HeLa cells	Production of H_2O_2 and proteolytic enzyme-resistant compounds by *Lactobacillus* spp.	*Lactobacillus* can control the vaginal microbiota and compete with other organisms for the adherence to epithelial cells	(252)
Lactobacillus spp. and *Gardnerella* spp.	*Lactobacillus* has the capacity to displace and kill *Gardnerella* spp. growing as biofilm	The production of H_2O_2 by some *Lactobacillus* strains seems to be the primary effect, however for some non-producer strains, the production of biosurfactants, bacteriocins and signalling molecules may have effect on the displacement and viability of *Gardnerella* spp.	*Lactobacillus* strains have the ability to disrupt biofilms that occur during BV and potentially reduce the need to antibiotics. Indigenous lactobacilli may have a restorative function to maintain a healthy vaginal microbiota	(253)

Table 2.5. Continued

Microorganisms	Interaction	Mechanism	Effect in host	References
ANTAGONISTIC INTERACTIONS BETWEEN MICROORGANISMS FROM VAGINAL ECOSYSTEM				
Lactobacillus rhamnosus and *Gardnerella* spp. and *P. bivia*	*Lactobacillus* showed bactericidal activity against *Gardnerella* spp. and *P. bivia*	It probably includes the production of H_2O_2, lactic acid, and antibacterial compounds by *Lactobacillus*	*L. rhamnosus* is considered a probiotic strain - a promising candidate for use in BV therapy	(254)
Lactobacillus spp. and *Gardnerella* spp., *P. bivia*, *Mobiluncus* spp., and *Bacteroides fragilis*	*Lactobacillus* species inhibited the growth of *Gardnerella* spp., *P. bivia*, and *Mobiluncus* spp., but did not show effect against *B. fragilis*	Production by *Lactobacillus* spp. of lactic acid, H_2O_2, and bacteriocins	Potential role of lactobacilli against BV pathogens	(255)
Lactobacillus johnsonii, *Lactobacillus gasseri* and *Gardnerella* spp.	Lactobacilli inhibited the growth of *Gardnerella* spp.	Production by lactobacilli of lactic acid, H_2O_2, and heat-stable molecules	The main metabolites of *Lactobacillus* spp. act cooperatively to kill BV-associated bacteria	(22)
Lactobacillus spp. and *Gardnerella* spp.	*Lactobacillus* showed antagonistic activity against *Gardnerella* spp.	Unknown	Success in the BV development depends on the presence of *Lactobacillus* species	(256)
Lactobacillus crispatus and *Gardnerella* spp.	*L. crispatus* produced lactic acid and inhibited the growth of *Gardnerella* spp. in an *ex vivo* porcine vaginal mucosal model	Production by *L. crispatus* of antimicrobial compounds	A stable *L. crispatus* colonization of live vaginal mucosa is able to prevent colonization by *Gardnerella* spp. in a pH-dependent manner	(257)
L. acidophilus, *L. rhamnosus*, and *Gardnerella* spp. and *F. vaginae*	*Lactobacillus* was able to inhibit the growth of both *Gardnerella* spp. and *F. vaginae*	The effect could be due to the production of lactic acid, H_2O_2, and bacteriocins	*L. acidophilus* alone or combined with *L. rhamnosus* can be used in probiotic products to prevent bacterial infections	(258)

In addition to bacterial-bacterial interactions, studies on bacterial-fungal interactions, also showed increased resistance to antibiotics. In this regard, by using an *in vitro* dual-species biofilm model of *P. aeruginosa* and *Aspergillus fumigatus*, both microorganisms highly prevalent in the airways of cystic fibrosis patients, Manavathu and colleagues (259) showed that *P. aeruginosa* cells associated with dual-species biofilms were less susceptible to cefepime compared to those in the single-species biofilm, whereas *A. fumigatus* presented similar antifungal drug susceptibility in single- and dual-species biofilms. Increased antimicrobial resistance was also observed in the studies with *E. coli* and *C. albicans* (260) and *S. aureus* and *C. albicans* (261). Accordingly, *E. coli* and *S. aureus* cells embedded within *C. albicans* biofilm were found to have increased resistance to ofloxacin and vancomycin, respectively, when compared to their single-species biofilms.

Taking into account the previously mentioned studies, we hypothesize that in BV biofilms similar interactions could also occur. Furthermore, several *in vivo* studies support this possibility. Bradshaw and colleagues (97) followed up 139 women with BV that were treated with oral metronidazole and found that recurrence rates of BV were significantly higher in women colonized with both *Gardnerella* spp. and *F. vaginae*, when compared to women infected with *Gardnerella* spp. only, suggesting that the association between these two bacterial taxa increased the tolerance to metronidazole, with direct impact on treatment failure. In another *in vivo* study, in which 18 patients diagnosed with BV were treated with metronidazole for 1 week, it was observed that the vaginal polymicrobial *Gardnerella* spp. biofilm was provisionally inhibited, but it quickly recovered its activity following treatment interruption (262). Remarkably, the authors found that high numbers of *Gardnerella* spp. and *F. vaginae* were present on the vaginal epithelium during follow-up analysis, further suggesting a potential synergism between these two bacterial taxa.

Regarding the effect of clinically approved antibiotics, only a few studies on BV-associated *in vitro* biofilms have been reported so far. The first study to determine the effect of clindamycin on *Gardnerella* spp. biofilms showed that the concentration of 1600 $\mu g \cdot mL^{-1}$ was able to reduce up to 2-log of the viable cell count in the pre-established biofilms (263). Higher concentrations of both clindamycin (20000 $\mu g \cdot mL^{-1}$) and metronidazole (2000 $\mu g \cdot mL^{-1}$) were able to kill biofilm-associated *Gardnerella* spp. cells after 8 h of incubation (264). Subsequently, another study demonstrated that concentrations of 100 $\mu g \cdot mL^{-1}$ and 600 $\mu g \cdot mL^{-1}$ of clindamycin and metronidazole respectively, administered on 72 h *Gardnerella* spp. biofilms were sufficient to achieve 100% mortality (265). Although these *in vitro* experiments showed promising results, the used concentrations were significantly higher than the peak serum concentrations (266,267)

and thus, cannot be taken into consideration as treatment options. Indeed, when Gottschick and colleagues (268) used clinically achievable concentrations, they found that metronidazole (0.001 μg·mL^{-1}) was able to prevent the formation of *Gardnerella* spp. biofilms, when used preventively, but could not disrupt the existing biofilms and did not affect the viability of the cells.

Interestingly, the information obtained from the *in vitro* biofilm studies was supported by a recent investigation, which found that the expression of genes involved in antimicrobial resistance was upregulated in *Gardnerella* spp. biofilm cells (214). In addition, it was observed that this up-regulation of genes was also enhanced in dual-species biofilms (232), as such providing some mechanistic evidence which clarifies to a certain degree why some polymicrobial communities might have enhanced antimicrobial resistance, and consequently, lead to BV recurrence. Taken together, understanding the molecular basis and biological influence of these bacterial interrelationships may offer new information essential for defining novel therapeutics for BV control.

2.5.1 Novel therapeutic strategies in the treatment of BV

As it is acknowledged, increased antimicrobial resistance is responsible for high rates of BV recurrence (269). This is of particular concern as we are already heading toward a post-antibiotic era in which many bacterial infections will be untreatable (270). In relation to this issue, there are several attempts to use diverse compounds as alternative therapeutic strategies in order to treat and prevent BV.

One of the most suggested non-antibiotic therapies for BV are oral or vaginal probiotics which have the aim to restore and maintain the normal vaginal microbiota (41). In the vaginal environment, some lactobacilli species can behave as probiotics, inhibiting the growth of pathogenic microorganisms by the production of antimicrobial compounds (12), but also by a competition for adherence, combined with a general stimulation of the immune system (271). Based on this, various pharmaceutical preparations (e.g. vaginal probiotic capsules) containing lactobacilli are generally well-tolerated and used to control BV symptoms and restore the physiological vaginal pH (272). Probiotics have been also used in an attempt to specifically deal with BV biofilms, and it was shown that lactobacilli were able to infiltrate BV biofilms and cause bacterial cells death (273). In addition, probiotics have been also recommended as a complementary approach to antibiotic therapy. Some studies evaluated the use of vaginal probiotics after metronidazole and/ or clindamycin treatment in order to manage and prevent BV recurrence, with promising results (274,275).

Other studies have also described the use of antiseptics as an alternative therapy for BV. These compounds present an antibacterial activity against a large number of bacteria, causing disruption of their cell membrane, followed by cell death (276). A wide range of antiseptics including octenidine hydrochloride/ phenoxyethanol (277), nifuratel (278) or benzydamine hydrochloride (279), have been used in the last few years to treat BV, and most interest has gone to dequalinium chloride (DQC) which was recently listed in an international guideline as an alternative treatment for BV (51). DQC demonstrated an *in vitro* antimicrobial activity against different pathogens that are relevant for vaginal infections, such as anaerobic and aerobic bacteria, as well as *Candida* species (280). In addition, Lopes dos Santos Santiago and colleagues (281) found in their study that DQC not only inhibited the growth of *F. vaginae*, but also killed the bacterial cells at concentrations similar to those of clindamycin and lower than those of metronidazole. More recently, another study also showed that DQC presented an antibacterial effect on BV comparable to that of clindamycin therapy, with no systemic effects on the patient (282).

Another alternative therapeutic approach used in the treatment of vaginal infections is represented by plant-derived compounds. One of the primary findings regarding this subject dates back three decades ago when Blackwell (283) reported the first therapeutic success of using plant extracts to cure BV. Other studies have been performed in this regard, including the one of Braga and colleagues (284), which showed that thymol, one of the major components of thyme oil and a widely known antimicrobial and antifungal agent, had an *in vitro* inhibitory effect on both newly formed and mature *Gardnerella* spp. biofilms. In an *in vivo* study, thymol was combined with eugenol and the efficacy of the mixture was compared with that of metronidazole. After 7 days of application, a similar reduction in symptoms with the mixture of thymol and eugenol was observed as that obtained with metronidazole (285). In addition, more recently, the antibacterial activity of *Thymbra capitata* essential oil was evaluated against *Gardnerella* spp. grown planktonically and as biofilms. The obtained results showed that *T. capitata* essential oil exhibited strong activity against *Gardnerella* spp. planktonic cells and had an evident inhibitory effect against *Gardnerella* spp. biofilms with reduced action on lactobacilli (286). Taken together, these studies support the importance of exploring essential oils and their main constituents as a therapeutic alternative to treat BV.

Taking into account the above-mentioned potential therapies against BV as well as others listed in Table 2.6, it becomes clear that still many of them only address a reduction of the symptoms, but do not target directly the causative agents, with little attention being paid to the microbial interactions. As described before, the vaginal milieu throughout infection is a complex niche being governed by still poorly

understood interactions among present microorganisms. Consequently, it is of the highest importance to focus attention on how microbial interrelationships in BV affect antimicrobial therapies, in order to accelerate the process of finding novel treatment strategies effective against recurrent BV. This issue will be addressed in **Chapters 5** and **6** of this thesis.

Table 2.6. Potential treatment strategies used against BV.

Product	Main results	Reference
Benzoyl peroxide	The highest tested concentrations of 250 and 125 μg/mL were not sufficient to inhibit completely the growth of *Gardnerella* spp., but they prevented biofilm formation by inhibiting the bacterial quorum-sensing system in the pathogen	(287)
Benzoyl peroxide formulated polycarbophil/ carbopol 934P hydrogel	*Gardnerella* spp. showed 6-log reduction; three of the tested *Lactobacillus* spp. were not inhibited while *L. acidophilus* growth was slightly delayed	(288)
Cationic amphiphiles	Effective against the vaginal pathogen *Gardnerella* spp. while preserving the commensal microbiota	(289)
Hydrogen peroxide	Elimination of the main symptoms of bacterial vaginosis in 89% of cases at 3 months after the end of treatment, a result that is comparable to that obtained using metronidazole or clindamycin as a vaginal cream; facilitation of the normal vaginal bacterial microbiota restoration in 100% of cases and normal acid pH (pH < 4.5) in 98% of cases.	(290)
Lactocin 160	Inhibition of the BV-associated vaginal pathogens such as *Gardnerella* spp. and *Prevotella bivia* without affecting the healthy microbiota	(291)
Lauramide arginine ethyl ester	Strong antimicrobial effect on established biofilms of *Gardnerella* spp.	(263)
Octenidine hydrochloride/ phenoxyethanol	Octenidine hydrochloride/ phenoxyethanol spray was as effective as the standard therapy with metronidazole, with 63.2% of the women being without indications of BV after therapy	(292)
Povidone iodine	Improvement of clinical parameters, condition of secretions and subjective state of health	(293)
Silicon-coated tablets containing 250 mg vitamin C	Vaginal application of vitamin C has an effective and long-lasting vaginal pH-lowering effect	(294,295)
Subtilosin and glycerol monolaurate, lauric arginate, and ε-poly-L-Lysine	Subtilosin synergized with all three of the tested natural antimicrobials against BV-associated pathogens but not vaginal lactobacilli	(296)
TOL-463 (boric acid-based vaginal anti-infective with enhanced antibiofilm activity)	The clinical cure rate of BV was 59% for TOL-463 insert and 50% for TOL-463 gel; TOL-463, especially in vaginal insert form, was effective and safe in treating BV	(297)

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CHAPTER 3

Influence of culture media on *in vitro* growth and biofilm formation of bacterial vaginosis-associated species

Summary

The polymicrobial biofilm present on vaginal epithelium during BV plays an important role in the progress and recurrence of this infection. Several studies have demonstrated that the BV biofilm contains mainly *Gardnerella* spp. and in a minor part *F. vaginae*, being suggested that the *in vivo* detection of both bacteria is a strong indicator of BV development. However, this biofilm is often populated by many other facultative or strict anaerobes, but very little is known about their role in BV evolution. Thus, more studies are needed to address the interactions between these species. One issue facing researchers that work with BV-associated species is that most species are fastidious or even uncultivable. Furthermore, *in vitro* biofilm formation requirements are often different from planktonic growth. Therefore, considering these important matters, the current study was undertaken aiming to evaluate the effect of nine different culture media on planktonic growth and biofilm formation of six cultivable anaerobes frequently found in BV, using an *in vitro* model. Our data revealed that BV-associated species had variable ability to grow planktonically or as biofilm in the different tested culture media. Interestingly, New York City III broth was able to significantly support planktonic growth and biofilm formation of most tested species, showing to be a good candidate for future studies addressing multi-species biofilm formation.

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3.1 Brief introduction

The importance of *Gardnerella* spp. in the development of BV as well as its significant ability to form biofilms, have been discussed in detail in **Chapter 2**. If successful initiating the BV biofilm, *Gardnerella* spp. is further followed by other anaerobes, including *F. vaginae*, *P. bivia*, *M. curtisii*, *P. anaerobius* or *L. iners*, with which perhaps establish several interactions, favouring, therefore, the BV progress (1). It has been previously demonstrated that certain BV-associated species are able to modulate the virulence potential of *Gardnerella* spp. in an *in vitro* biofilm model (2), however, their complete role in BV process has been never determined. Moreover, very little information exists regarding their ability to form *in vitro* biofilms, an issue that may delay the understanding of BV etiology. Therefore, in this chapter, it was aimed to define the optimal growth conditions for future co-culture studies of BV-associated species, intending to explore the interactions that might exist between these species and their impact on BV development.

3.2 Material and methods

3.2.1 Bacterial species and growth conditions

Six cultivable bacterial species associated with BV were used in the current study, namely *F. vaginae*, *Gardnerella* sp., *L. iners*, *M. curtisii*, *P. anaerobius*, and *P. bivia* (Table 3.1). These species were preserved frozen in Brain Heart Infusion broth (BHI) (Liofilchem, Italy) with 23% (v/v) glycerol (Panreac, Spain) at -80 °C. Each species was inoculated from the -80 °C bacterial stock on plates containing Columbia Blood Agar (CBA) (Oxoid, UK) supplemented with 5% (v/v) defibrinated horse blood (Oxoid, UK) and incubated at 37 °C under anaerobic conditions [controlled atmosphere composed of 10% carbon dioxide (CO_2), 10% helium and 80% nitrogen generated by a cylinder (Air Liquid, Algés, Portugal) coupled to an anaerobic incubator (Plas-Labs, Lansing, MI, USA)] for 2-4 days. For planktonic and biofilm assays, Brain heart infusion broth supplemented with yeast extract, starch, and gelatine (sBHI), Brucella broth supplemented with hemin and vitamin K_1 solutions (BHV), New York City III broth supplemented with 10% inactivated horse serum (NYC III), Schaedler broth (SB), and a medium simulating genital tract secretions (mGTS) were used as culture media with the mentioned composition, but also supplemented with 0.1% (w/v) L-ascorbic acid (Sigma-Aldrich, UK), excepting mGTS which already contains L-ascorbic acid. The addition of L-ascorbic acid to the culture media was designated with the abbreviation "Aa", added at the end of each medium's name mentioned above (e.g. sBHI supplemented with L-ascorbic acid became sBHI.Aa). The detailed information about each tested medium is presented in Table 3.2. In order to prepare hemin solution, 0.1 g of hemin was dissolved in 2 mL of 1 N NaOH and afterwards distilled water was added to reach the final volume of 10 mL. The hemin solution was autoclaved for 15 min at 121 °C. Vitamin K_1

solution was prepared by adding 0.05 mL of vitamin K_1 stock solution to 4.95 mL of absolute ethanol. The prepared solutions of hemin and vitamin K_1 were used with a concentration of 0.0005% (w/v) and 0.0001% (w/v), respectively. In mGTS, Part III consisted of a vitamin mixture, Sigma K3129, from Sigma-Aldrich (UK) with the stock solution of 100X that was used at a concentration of 0.5% (v/v).

Table 3.1. BV-associated species used for planktonic and biofilm growth assays.

Species	Strain	Origin	Association with BV[1]
Fannyhessea vaginae	ATCC BAA-55^T	Isolated from vaginal microbiota of a healthy woman (3)	Often described
Gardnerella sp.	UM241[2]	Isolated from women diagnosed with BV	Often described
Lactobacillus iners	CCUG 28746^T	Isolated from human urine (4)	Commonly described
Mobiluncus curtisii	ATCC 35241^T	Isolated from women with BV (5)	Commonly described
Peptostreptococcus anaerobius	ATCC 27337^T	Isolated from female genital tract (6)	Commonly described
Prevotella bivia	ATCC 29303^T	Isolated from endometrium (7)	Commonly described

[1] To determine how often the selected cultivable species have been reported in BV, a query in PubMed was performed by using a specific combination of keywords as "bacterial vaginosis" and "name of each species" (e.g. "*Gardnerella*" and "bacterial vaginosis"). We designated as "often described" those species referred in more than 50 articles in the last 25 years, while "commonly described" had at least 10 articles in the same period. Of note, the selected bacterial species used herein have been pointed out in several studies (8–10) as potential microbial pathogens since they are reported as being implicated in BV development.

[2] The partial 16S ribosomal RNA gene sequences of *Gardnerella* sp. is downloadable from NCBI. UM: University of Minho, Portugal. In addition, the strain was phenotypically and genotypically characterized by Castro and colleagues (11–13). Of note that *Gardnerella* sp. UM241 did not match any of the *Gardnerella* species described by Vaneechoutte and colleagues (14) (i.e. *G. vaginalis*, *G. piotii*, *G. leopoldii* and *G. swidsinskii*), belonging to a yet unidentified *Gardnerella* species (13).

3.2.2 Planktonic growth assessment

For the evaluation of planktonic growth, the inoculums were prepared by transferring fresh bacterial colonies from CBA plates to 8 mL of each culture medium described above. The obtained bacterial suspensions were adjusted by optical density (OD) at 620 nm to 0.10 ± 0.05 (Biochrom EZ Read 800 Plus, UK) and equally distributed in two sterile 15 mL falcon tubes (Orange Scientific, Belgium). Afterward, the inoculums were incubated at 37 °C under anaerobic conditions for 48 h, as above-mentioned. Following, planktonic growth was assessed by OD_{620nm}, being normalized as a fold change difference between the final OD (at time 48 h) and the starting OD (at time 0 h). The assays were repeated at least three times on separate days, with four technical replicates considered each time.

3.2.3 Biofilm formation and biomass quantification

Single-species biofilms of each tested species were initiated by inoculating bacterial suspensions of 48 h cultures adjusted to an OD_{620nm} of 0.10 ± 0.05 in sterile 96-well tissue culture plates (Orange Scientific)

and incubated for 72 h, at 37 °C under anaerobic conditions. To quantify the biofilm biomass, we used the crystal violet (CV) method, which is the most frequently employed approach for this purpose (15,16). In brief, following 72 h of incubation, the biofilms were washed once with 200 µL of 1X phosphate-buffered saline (PBS) and allowed to air dry. After, the biofilms were fixed with 100 µL of 100% (v/v) methanol (Thermo Fisher Scientific, UK) for 20 min, and then stained with 100 µL of 1% (v/v) CV solution (Merck, Darmstadt, Germany) for 20 min. Subsequently, each well was washed twice with 200 µL of 1X PBS, and the bound CV was released with 150 µL of 33% (v/v) acetic acid (Thermo Fisher Scientific). To assess the biomass, the OD of the resulting solution was measured at 595 nm. Biofilm experiments were repeated at least three times with eight technical replicates.

3.2.4 Statistical analysis

The data were analysed using the statistical package GraphPad Prism version 7 (La Jolla, CA, USA) by one-way ANOVA (Dunnett's multiple comparison test) and two-way ANOVA (Sidak's multiple comparisons test). Values with a $p < 0.05$ were considered statistically significant.

3.3 Results

3.3.1 Planktonic growth assays

As shown in Figure 3.1, BV-associated anaerobes had variable ability to grow planktonically in the tested culture media. Accordingly, *P. anaerobius* and *P. bivia* had higher metabolic flexibility and were able to grow in most of the tested media, while *M. curtisii* had more restrictive growth requirements and presented low levels of growth in all of them. Interestingly, NYC III broth showed high levels of planktonic growth for the tested species, being overpassed only by NYC.Aa for *L. iners*, SB and SB.Aa for *P. anaerobius*, and by sBHI.Aa for *P. bivia*. The mGTS supported very low levels of bacterial growth, with only *G. vaginalis*, *P. anaerobius*, and *P. bivia* showing moderate levels of growth.

Since it was previously shown that L-ascorbic acid could enhance the growth of several anaerobic bacteria, including *F. vaginae*, *Finegoldia magna*, *Fusobacterium necrophorum*, *Prevotella nigrescens*, *Ruminococcus gnavus*, and *Solobacterium moorei* (17), we repeated the experiments with the culture media supplemented with 0.1% (w/v) L-ascorbic acid. However, contrary to what was described before, the addition of 0.1% (w/v) L-ascorbic acid had a very variable effect on bacterial growth, with only 33.3% (n=8; cut-off ≥ 1.25 – fold change) of the total combinations tested yielding a significant increase in bacterial growth, while in 4.17% (n=1; cut-off < 0.75-fold change) an inhibition of the growth was observed.

In most of the tested combinations (n=15; 0.75 ≤ fold change < 1.25) no effect was observed (Figure 3.1). The most notable case was observed for *P. bivia* growth in SB, in which L-ascorbic acid increased almost seven-fold the growth rate.

3.3.2 Biofilm assays

We observed that similar to planktonic growth, biofilm formation was also strongly affected by the culture media composition, as depicted in Figure 3.2. Importantly, there was not a direct relationship between higher planktonic growth and higher biofilm formation, which further confirms that the requirements for biofilm formation are distinct than the requirements for planktonic growth, as showed before for many other bacterial species (18–21). Further differences between biofilm formation and planktonic growth were observed when adding L-ascorbic acid to the growth media, with 20.8% (n=5) of the tested combinations species/ growth medium resulting in a statistically significant decrease in the biofilm-forming capacity ($p < 0.05$) and 37.5% (n=9) of the situations also presenting a visible biofilm reduction, however not statistically significant. Moreover, in none of the combinations, a significant increase in biofilm biomass was noted upon addition of L-ascorbic acid to the culture media (Figure 3.2).

Table 3.2. Culture media used for the growth of BV-associated bacteria.

Culture medium	Composition	Supplementation	Abbreviation
Brain heart infusion broth (Liofilchem, Italy)	As described by the manufacturer	2% (w/w) Gelatine (Liofilchem, Italy) 0.1% (w/w) Starch (Panreac, Spain) 0.5% (w/w) Yeast extract (Liofilchem, Italy)	sBHI/ sBHI.Aa
Brucella broth (Liofilchem, Italy)	As described by the manufacturer	0.0005% (w/v) Hemin (Sigma, China) 0.0001% (w/v) Vitamin K_1 (Sigma, China)	BHV/ BHV.Aa
New York City III broth	1.5% (w/v) Bacto proteose peptone no. 3 (BD, France) 0.5% (w/v) Glucose (Fisher Scientific, UK) 0.24% (w/v) HEPES (VWR, USA) 0.5% (w/v) NaCl (VWR, USA) 0.38% (w/v) Yeast extract (Liofilchem, Italy)	10% (v/v) Inactivated horse serum (Biowest, France)	NYC III/ NYC.Aa
Schaedler broth (Liofilchem, Italy)	As described by the manufacturer	-	SB/ SB.Aa
Chemically defined medium simulating genital tract secretions (22)	**Part I:** 0.35% NaCl; 0.15% KCl; 0.174% K_2HPO_4; 0.136% KH_2PO_4; 1.08% glucose; 0.05% cysteine HCl **Part II:** 0.1% glycogen; 0.03% mucin; 0.02% tween 20; 0.05% urea; 0.0005% hemin; 0.0001% vitamin K_1; 0.2% bovine serum albumin; 0.03% $MgSO_4$; 0.004% $NaHCO_3$; 0.1% sodium acetate; 0.005% $MnCl_2$ **Part III:** 0.0005% biotin; 5.0% *myo*-inositol; 0.05% niacinamide; 0.05% pyridoxine HCl; 0.05% thiamine HCl; 0.05% D-calcium pantothenate; 0.05% folic acid; 0.001% *p*-aminobenzoic acid; 0.05% choline chloride; 0.01% riboflavin; 0.1% L-ascorbic acid; 0.0005% vitamin A (retinol); 0.0005% vitamin D (cholecalciferol); 0.001% vitamin B_{12} **Part IV** (amino acids): 0.032% alanine; 0.008% arginine; 0.076% aspartic acid; 0.036% glutamic acid; 0.04% glutamine; 0.02% glycine; 0.016% histidine; 0.012% isoleucine; 0.02% leucine; 0.02% lysine; 0.004% methionine; 0.004% phenylalanine; 0.028% proline; 0.012% serine; 0.012% threonine; 0.004% tryptophan; 0.02% tyrosine; 0.068% valine **Part V** (UPI): 0.05% uracil; 0.01% sodium pyruvate; 0.02% inosine	-	mGTS

3.4 Discussion

Despite the fact that BV is an increasingly important health problem, there is a lack of studies addressing multi-species interactions that might occur during BV and their role in its development. Most attempts to understand the microbiology behind BV have been focused mainly on *Gardnerella* spp., perhaps because this species has long been associated with BV development (23,24) and it has been now hypothesized that this microorganism is the initial colonizer of the vaginal epithelium, being able to establish an early biofilm structure to which other BV-associated species can attach (1). However, the role of these species in the development and progress of BV is still poorly understood and therefore, more studies are needed to unravel this matter.

It has been previously shown that BV-associated species have different abilities to grow as biofilms, and this was strongly dependent on the growth media (18). As such, the first step in facilitating BV multi-species biofilm studies is to determine optimal culture medium conditions suitable for multiple BV-associates species, considering to further investigate the interactions that might exist between them in BV multi-species biofilms and their implications in BV process.

Although sBHI has been widely used as a medium that supports *Gardnerella* spp. growth (25–31), it did not facilitate the planktonic growth or biofilm formation for some of the tested species, including *F. vaginae*, *L. iners*, and *M. curtisii*. The same was observed for these three species in SB medium, even though the manufacturer describes it as a medium suitable for the cultivation of anaerobic microorganisms, providing them an important amount of amino acids, nitrogen, vitamins as well as the energy necessary for growth. In an early study, after evaluating nine broth media in varied CO_2 atmospheres for their ability to support growth of anaerobic bacteria including *Bacteroides fragilis* subspecies *fragilis*, *Clostridium perfringens*, *Eubacterium alactolyticum*, and *Peptostreptococcus* CDC group 2, Stalons and colleagues (32) found that SB in an atmosphere of 5% CO_2, 10% hydrogen, and 85% nitrogen exhibited the fastest and highest growth response. However, in our *in vitro* conditions, we obtained high levels of planktonic growth only for *P. anaerobius*, probably because this medium is not appropriate for the growth of all species of anaerobic microorganisms. Still, SB was a good medium to support *in vitro* biofilm formation with high levels of the biomass for *Gardnerella* sp., *P. anaerobius*, and *P. bivia*.

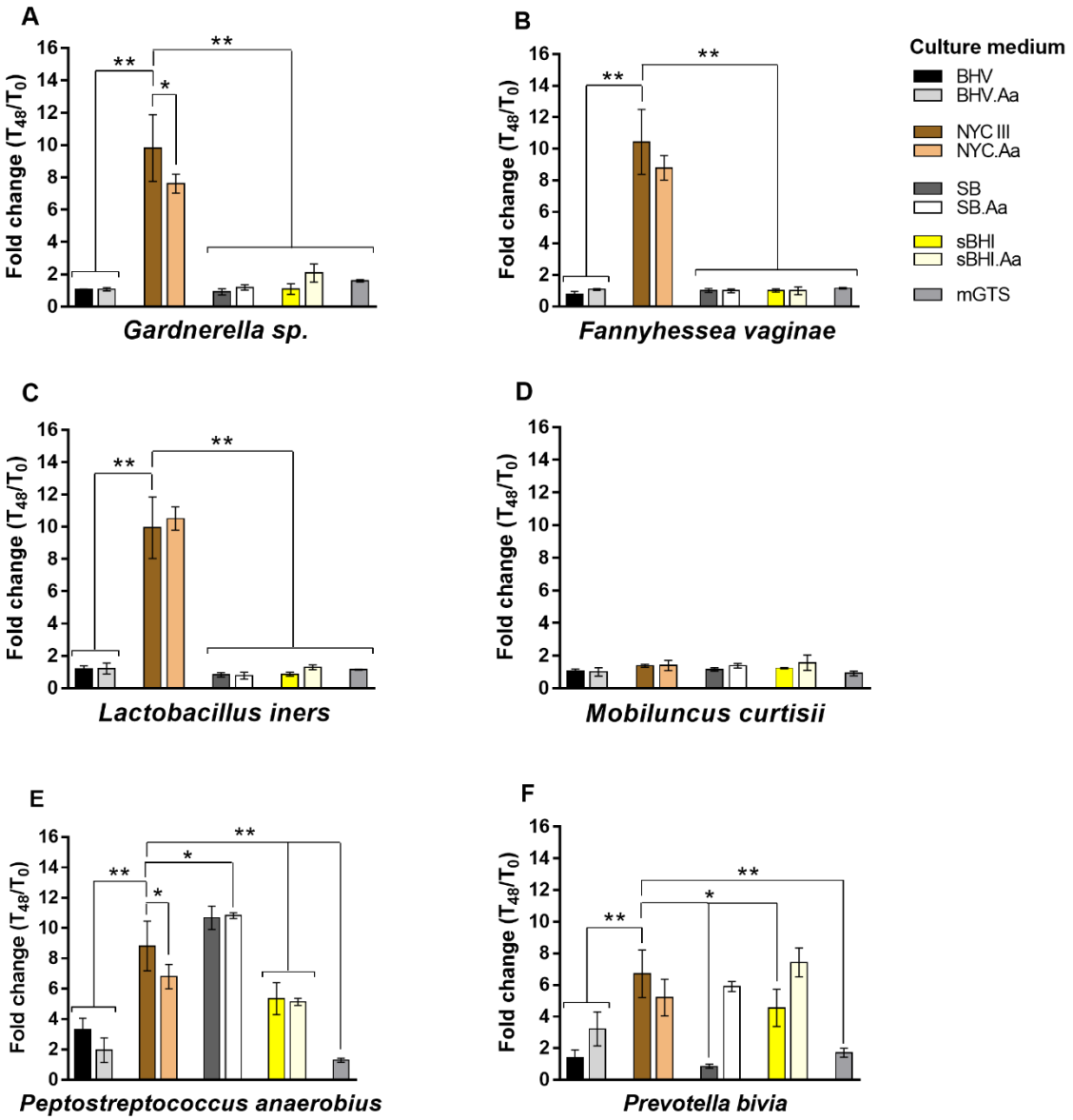

Figure 3.1. Fold change in planktonic growth of BV-associated bacteria in the nine different culture media relative to OD_{620nm} values measured at T 0h. (A) Experiments performed with *Gardnerella* sp. **(B)** Experiments performed with *F. vaginae*. **(C)** Experiments performed with *L. iners*. **(D)** Experiments performed with *M. curtisii*. **(E)** Experiments performed with *P. anaerobius*. **(F)** Experiments performed with *P. bivia*. Results represent the average ± the standard deviation of at least three independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test. Significant differences between NYC III medium (our culture medium of choice) and other culture media are depicted with $*p < 0.05$ and $**p < 0.01$. The effect of L-ascorbic acid on bacterial growth is presented as fold change relative to the growth in the medium without L-ascorbic acid (fold change = 1, control). This effect was classified as inhibitory (cut-off < 0.75 – fold change), neutral (0.75 ≤ fold change < 1.25), and stimulatory (cut-off ≥ 1.25 – fold change).

Curiously, *Gardnerella* sp. and *P. bivia* showed in SB the lowest levels of planktonic growth, but the highest biofilm formation ability. As mentioned, SB is a complex medium and perhaps the presence of certain growth factors determined these two species to turn on the expression of biofilm-related genes. Another of the tested media, BHV, also described by the manufacturer as suitable for the cultivation of anaerobes,

was not appropriate for the growth of the tested species, with the exception of planktonic growth by *P. anaerobius* and biofilm formation by *P. bivia*.

Interestingly, NYC III facilitated the planktonic growth of all tested species, despite *M. curtisii* presented a very slow growth rate. Nevertheless, even *M. curtisii* was able to form high biofilm biomass in this medium. In fact, together with *F. vaginae* and *L. iners,* significant biofilm formation was only detected in NYC III (Figure 3.2). A particularity of NYC III medium, compared to the other tested media, is the presence of proteose peptone no. 3, which has been described by the manufacturer as offering high nutritional benefits to fastidious anaerobic species by providing the necessary amount of nitrogen, carbon, amino acids, and essential growth factors. To assess if, in fact, the enhancement of biofilm formation in NYC III was mainly due to the presence of proteose peptone no. 3, we carried out an experiment by evaluating the biofilm-formation ability of the six tested bacterial species in the original recipe of NYC III versus an altered version of NYC III [with regular peptone from meat (Acros Organics, UK) replacing the proteose peptone no. 3]. Interestingly, while we did find that proteose peptone no. 3 was essential to the biofilm formation by *M. curtisii*, no significant differences were found for the other species (Figure 3.3), which suggests that the ability of NYC III to enhance biofilm formation is not only related to the presence of proteose peptone no. 3.

Besides the commercially available media, we also tested a chemically defined medium that simulates the genital tract fluid, mGTS (22). Since mGTS is a minimal medium without rich nutrient sources, it was not surprising that the growth of the tested BV-associated species was negligent or very slow in this medium. Nevertheless, biofilm formation by *Gardnerella* sp. and *P. anaerobius* was significant under mGTS, further being confirmed that biofilm formation requires specific conditions, different from planktonic growth.

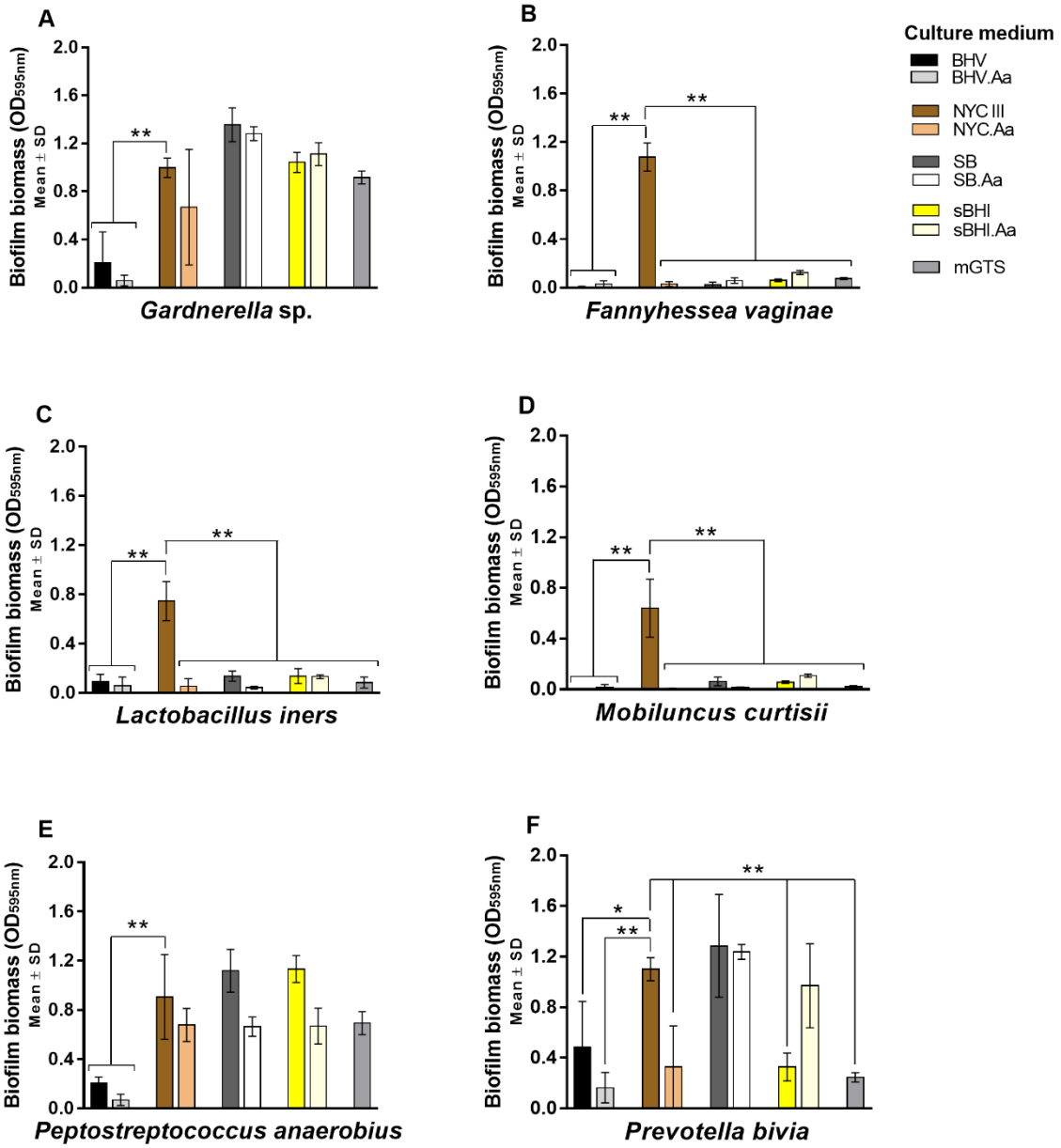

Figure 3.2. Biofilm formation of BV-associated bacteria in the nine different culture media over a 72 h period. Biofilm biomass was quantified using the CV staining assay. **(A)** represents the total biofilm biomass formed by *Gardnerella* sp. in the tested growth media. **(B)** represents the total biofilm biomass formed by *F. vaginae*. **(C)** represents the total biofilm biomass formed by *L. iners*. **(D)** represents the total biofilm biomass formed by *M. curtisii*. **(E)** represents the total biofilm biomass formed by *P. anaerobius*. **(F)** represents the total biofilm biomass formed by *P. bivia*. Results are expressed as average ± standard deviation of at least three independent experiments performed with eight technical replicates. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test. Significant differences between biofilm biomass formed in NYC III medium (our culture medium of choice) and other culture media are represented with $*p < 0.05$ and $**p < 0.01$.

We also tested another variable in our growth conditions optimization. The addition of L-ascorbic acid had the advantage of reducing the oxidation potential of the growth media by removing the oxygen (17). However, the effect of adding L-ascorbic acid was very variable, depending not only on the bacterial

species but also on the respective growth media. Nevertheless, there was a tendency to slightly or highly suppress biofilm formation. Interestingly, the inhibition of biofilm formation by ascorbic acid has been described before in biofilms of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* (33) as well as of methicillin-resistant *Staphylococcus aureus* (34). It should be noted that at higher concentrations, L-ascorbic acid has been reported as a possible adjuvant for antibiotic treatment of BV, playing a role in maintaining a low vaginal pH, which favours the recolonization of the vaginal environment with lactic acid-producing bacteria, decreasing, thereby, the risk of BV recurrence (35,36). Our data further expand these previous findings by demonstrating that, while sometimes favouring planktonic growth, L-ascorbic acid often impairs biofilm formation.

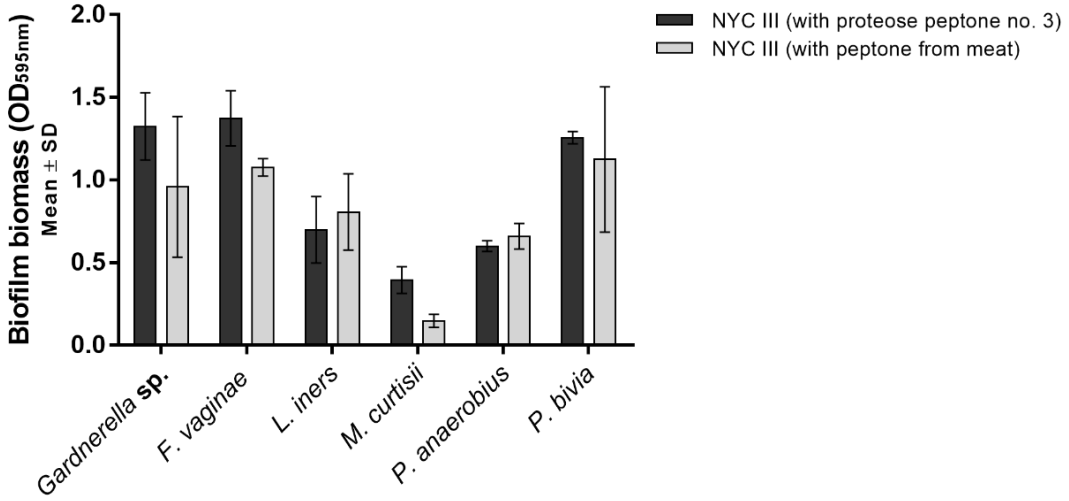

Figure 3.3. Biofilm formation of BV-associated bacteria in NYC III (with proteose peptone no. 3) and altered version of NYC III (with peptone from meat) over a 72 h period. Biofilm biomass was quantified using the CV staining assay. Results represent the average ± the standard deviation of three independent experiments performed with eight technical replicates. Statistical analysis was performed using two-way ANOVA and Sidak's multiple comparisons test. No significant difference was found between biofilm-formation ability of BV-associated bacteria in the two tested culture media.

A limitation of this study was the fact that we only tested a yet unidentified *Gardnerella* sp. isolate, but at least three new species have been recently reported. Previously, it was assessed biofilm formation by seven clinical isolates from BV-women and seven from healthy microbiota and found no significant differences between the ability to form biofilms by the 2 groups, using different growth media (11). We now know that from those 14 isolates, some belong to *G. vaginalis*, *G. leopoldii*, *G. piotii*, and *G. swidsinskii* (13). As such, we hypothesized that the four *Gardnerella* species would have similar biofilm formation abilities in our growth medium of choice: NYC III. To test this hypothesis, we selected one isolate of each species, previously found to form similar biofilms in sBHI (13,14) and compared its biofilms with NYC III medium. As shown in Figure 3.4, all the tested species had a similar biofilm-formation ability as compared

to *Gardnerella* sp. UM241, with *G. leopoldii* showing a slight decrease in biomass, but within the expected variation found in different *Gardnerella* strains (11).

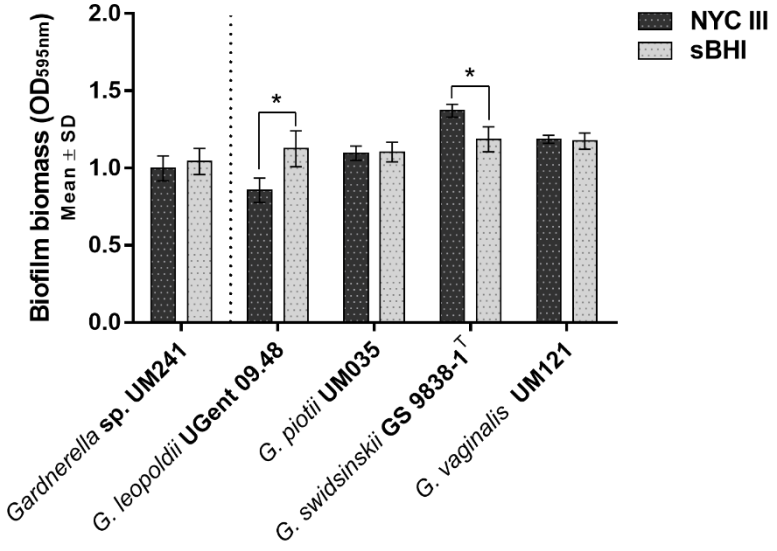

Figure 3.4. Biofilm formation of *Gardnerella* sp., *G. leopoldii*, *G. piotii*, *G. swidsinskii*, and *G. vaginalis* in NYC III and sBHI over a 72 h period. Biofilm biomass was quantified using the CV staining assay. Results represent the average ± the standard deviation of three independent experiments performed with eight technical replicates. Statistical analysis was performed using two-way ANOVA and Sidak's multiple comparisons test. Significant differences between biofilm biomass formed in NYC III and sBHI are represented with $*p < 0.05$.

Overall, our work has shed new light on the optimal conditions required for *in vitro* growth and biofilm formation of bacteria associated with BV. Although we tested nine different growth conditions, including a medium simulating genital tract secretions (mGTS), none of them is able to account for all growth factors present in the vaginal environment, including components of the host immune system, that are known to interfere in bacterial growth (12). Nevertheless, this work highlighted that under the appropriate *in vitro* conditions, some of the most common species found in BV can form single-species biofilms, contrary to what was shown before (13,26). NYC III medium revealed to be an ideal candidate for future studies addressing multi-species biofilm formation since this growth medium allowed significant levels of single-species biofilm formation. Understanding microbial interactions that occur during BV development is crucial for the development of novel antimicrobial strategies, and future work will help to clarify some of these crucial interactions in multi-species biofilms.

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CHAPTER 4

Deciphering bacterial interactions among *Gardnerella vaginalis*, *Fannyhessea vaginae*, and *Prevotella bivia* in BV-associated multi-species biofilms

Summary

The impact of *F. vaginae*, *P. bivia* or both species on a pre-established *G. vaginalis* biofilm was evaluated by first determining the total biofilm biomass using the CV method. Further, bacterial populations were also quantified using a validated Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA FISH) approach. In addition, biofilm structure by confocal laser scanning microscopy was analysed as well as the expression of key *G. vaginalis* virulence genes. Under the experimental conditions tested herein, our results revealed that although *F. vaginae*, *P. bivia* or both species together did not significantly enhance the dual- or the triple-species biomass when compared to 48 h *G. vaginalis* single-species biofilms, these species were able to incorporate the pre-formed *G. vaginalis* biofilms. Furthermore, our gene expression studies seem to indicate that significant changes were only observed for the triple-species consortium. This study highlights the importance of microbial interactions between BV-associated bacteria, contributing to a better understanding of the BV-associated biofilms, and demands more studies addressing the polymicrobial bacterial communities found in BV and their role in BV development.

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4.1 Brief introduction

Gardnerella spp., found in more than 95% of all BV cases (1), were originally thought to be the sole etiologic agent of this infection (2). Compared to other BV-associated species, *Gardnerella* spp. have a strong ability to adhere to vaginal cells (3) and a great tendency to form a biofilm (4), features that could allow them to function as an early colonizer on the vaginal epithelium. Still, vaginal *Gardnerella* spp. colonization does not always induce BV (5), being indicated that *Gardnerella* spp. alone may be essential, but not enough, for BV development (6). Consequently, other BV-associated species could also play an important role in BV establishment, but this topic is not well documented, and thus, more detailed studies are needed.

An interesting example is the case of *F. vaginae* and *P. bivia*. Evidence of a possible dependent relationship between *Gardnerella* spp. and *F. vaginae* has been demonstrated on BV-associated *in vivo* biofilms (7–9). As regards *P. bivia*, *in vitro* studies have suggested a potential growth synergy between *Gardnerella* spp. and *P. bivia* (10). Additionally, a positive association between *Gardnerella* spp. and several *Prevotella* species, including *P. bivia*, has been noted in women with BV (11). Also, recent data suggested the existence of a potentially important synergistic relationship between *Gardnerella* spp., *F. vaginae*, and *P. bivia* in BV pathogenesis, in particular in cases of incident BV, which is defined as the first occurrence of a Nugent score of 7-10 in a woman with a previous Nugent score of less than 7 (6). Nevertheless, studies on microbial interactions in BV-associated biofilms are still scarce, and therefore, in the experiments described in this chapter, it was intended to analyse the interactions between *G. vaginalis*, *F. vaginae*, and *P. bivia*, allowing first *G. vaginalis* to form a single-species biofilm and then adding the other species following a previously described *in vitro* dual-species biofilm formation model (12,13) as well as a newly tested triple-species biofilm formation model.

4.2 Material and methods

4.2.1 Bacterial species and culture conditions

G. vaginalis ATCC 14018^T (13), *F. vaginae* ATCC BAA-55^T (**Chapter 3**), and *P. bivia* ATCC 29303^T (**Chapter 3**) were used in this study. The inoculum of each species was grown in NYC III supplemented with 10% (v/v) inactivated horse serum (as described in **Chapter 3**) and incubated anaerobically at 37 °C using AnaeroGen sachets (Thermo Fisher Scientific) in sealed jars (Oxoid) for 24 h.

4.2.2 Biofilm formation and biomass quantification

Dual- and triple-species biofilms were initiated by inoculating *G. vaginalis* culture adjusted to a concentration of approx. 1.0 x 10^7 colony-forming unit (CFU).mL^{-1} into 24-well tissue culture plates (Orange Scientific, Belgium) and incubated for 24 h at 37 °C under anaerobic conditions using AnaeroGen sachets with plastic pouches (Thermo Fisher Scientific). After 24 h, the spent medium was removed, and suspensions of approx. 1.0 x 10^7 CFU.mL^{-1} of *F. vaginae* or *P. bivia*, respectively (for dual-species biofilms), or both (for triple-species biofilms), in fresh medium were inoculated on the pre-formed *G. vaginalis* biofilms and incubated anaerobically for an additional 24 h. A control was included by allowing *G. vaginalis* to grow for another 24 h. Other controls consisted of single-species biofilms grown for 24 h, as described above. To quantify the biomass of single-, dual-, and triple-species biofilms, we used the CV method. Biofilms were fixed with 1 mL of 100% (v/v) methanol (Thermo Fisher Scientific) for 20 min and then stained with 1 mL of CV solution 1% (v/v) (Merck) for 20 min. Following, each well was washed twice with 1X PBS and bound CV was released with 1 mL of 33% (v/v) acetic acid (Thermo Fisher Scientific). To estimate the biofilm biomass, OD of the resulting solution was measured at 595 nm. The biofilm biomass quantification assays were repeated at least three times with two technical replicates.

4.2.3 Testing specificity and efficiency of the PNA probes Gard162 and AtoITM1 using PNA FISH

Although the specificity of the peptide nucleic acid (PNA) probes Gard162 (14) and AtoITM1 (8) has been previously determined, the analysis performed for some of the selected species was only done *in silico*. Therefore, to confirm the *in silico* analysis, we performed an experiment to observe if any of the two probes would cross-hybridize with any of the tested species considered herein. The evaluation of PNA FISH hybridization was based on a qualitative score, as previously described (14): (–) absence of hybridization, (++) moderate hybridization, (+++) good hybridization, and (++++) optimal hybridization. Next, we analysed the efficiency of both PNA probes by performing several dilutions for pure bacterial suspensions obtained from *G. vaginalis* and *F. vaginae* single-species biofilms. To determine the efficiency of each probe, the same sample was hybridized with the species-specific probe and then stained with 4′,6-diamidino-2-phenylindole (DAPI, 2.5 μg.mL^{-1}) to account for nonhybridized bacteria; defined as double staining. After the double staining, the bacteria were enumerated at two different wavelengths at the same position within the sample. Based on both data, we performed a correlation between the FISH counts and the DAPI counts that allowed us to obtain the equations shown in Figure 4.2. These experiments were carried out in triplicate.

4.2.4 Quantification of bacterial populations in dual- and triple-species biofilms by PNA FISH

The bacterial populations within the biofilms were also discriminated by FISH method using the PNA probes specific for *G. vaginalis* (Gard162) (14) and for *F. vaginae* (AtoITM1) (8), as well as DAPI. Therefore, before counting the cells detected by PNA FISH, any non-adherent cells were removed by a gentle wash with 1X PBS and, thereafter, biofilms were scraped vigorously from the wells. For dual- and triple-cultures, 30 μL of each bacterial suspension was spread on epoxy-coated microscope glass slides (Thermo Fisher Scientific) and the slides were air dried. Further, cells were fixed with 45 μL of 100% (v/v) methanol (Thermo Fisher Scientific) for 20 min, followed by 45 μL of 4% (w/v) paraformaldehyde (Thermo Fisher Scientific) for 10 min, and then by 45 μL of 50% (v/v) ethanol (Thermo Fisher Scientific) for 10 min. After, the wells of the slides were covered with 10 μL of each PNA probe and incubated in a hybridization oven (Nahita, drying oven, model 631/2) in humid conditions at 60 °C during 90 min. Next, the slides were immersed in a washing solution containing 5 mM Tris base (Thermo Fisher Scientific), 15 mM NaCl (VWR), and 0.1% (v/v) Triton X-100 (Fisher Bioreagents, Pittsburgh, PA) for 30 min at 60 °C. After this washing step, the slides were air dried in the dark, at room temperature. Finally, each well was covered with 10 μL of DAPI (2.5 $\mu g.mL^{-1}$) and the slides were let in the dark for 5 min. Microscopic visualization was effectuated using an Olympus BX51 (Olympus Portugal SA, Porto, Portugal) epifluorescence microscope equipped with a CCD camera (DP72; Olympus) and filters capable of detecting the PNA probe Gard162 (BP 530-550, FT570, LP 591 sensitive to Alexa Fluor 594 molecule attached to the Gard162 probe), AtoITM1 probe (BP 470-490, FT500, LP 516 sensitive to Alexa Fluor 488 molecule attached to the AtoITM1 probe), and DAPI (BP 365–370, FT 400, LP 421). Twenty fields were randomly acquired in each sample. The number of bacteria was counted using *ImageJ Software* (15). To reduce any possible overestimation of DAPI counts as the probe efficiency was not 100%, we then applied the equations from Table 4.3 to obtain more accurate relative values. All assays were repeated three times on separate days.

4.2.5 Confocal laser scanning microscopy analysis of biofilm bacterial distribution

To analyse bacterial distribution in dual- and triple-species biofilms, the intact biofilm structure was analysed by confocal laser scanning microscopy (CLSM) using the PNA Gard162 and PNA AtoITM1 coupled to DAPI staining, as described above. For this experiment, the biofilms were formed in 8-well chamber slides (Thermo Scientific™ Nunc™ Lab-Tek™, Rochester, UK) in anaerobic conditions, as mentioned above, at 37 °C for 48 h with replacement of NYC III medium at 24 h of growth and the addition of fresh medium together with *F. vaginae* or *P. bivia* (for the dual-species biofilms), or both (for

the triple-species biofilms). The CLSM images were acquired with an Olympus™ FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) using a 40× objective. Microscopic visualization was performed using lasers capable of detecting the PNA Gard162 probe (Laser 559, excitation wavelength 559 nm, emission wavelength 618 nm, BA575-675, sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe), the PNA AtoITM1 probe (Laser 488, excitation wavelength 488 nm, emission wavelength 520 nm, BA505-540, sensitive to the Alexa Fluor 488 molecule attached to the AtoITM1 probe), and DAPI (Laser 405, excitation wavelength 405 nm, emission wavelength 461 nm, BA430-470). Images were acquired with 640 × 640 resolution of each surface analysed. The CLSM images were analysed using the *FV10-ASW 4.0 Viewer Software* (Olympus). The assays were repeated three times with two technical replicates.

4.2.6 Gene expression quantification

Gene expression of three potential *G. vaginalis* virulence genes, namely vaginolysin (*vly*), sialidase (*sld*), and *HMPREF0424_0821*, was determined in 48 h single-, dual-, and triple-species biofilms. For each tested condition, total RNA was extracted using an E.Z.N.A.® Bacterial RNA Kit (Omega Bio-tek, GA, USA) with minor changes, as optimized before (16). Following, genomic DNA was degraded with one step of DNase treatment (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. RNA concentration, purity, and integrity were determined as previously described (17). The same amount of total RNA (300 ng.μL^{-1}) was reverse transcribed using the RevertAid™ First Strand cDNA synthesis kit (Fermentas), as previously optimized, and gene-specific reverse transcription primers as a priming strategy. Quantitative PCR (qPCR) was prepared by mixing together 5 μL of iQ SYBR green supermix (Bio-Rad, CA, USA), 2 μL of 1:100 diluted cDNA, 0.5 μL of 5 μM Forward and Reverse primers (Table 4.1), and water up to 10 μL. The run was performed in a CFX96TM thermal cycler (Bio-Rad) with the following cycling parameters: 3 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C, and 15 s at 72 °C. Reaction efficiency was determined by the dilution method (18). At 60 °C, all set of primers used (Table 4.1) had similar efficiencies. Furthermore, the analysis of the obtained melting curves confirmed the presence of a single peak, demonstrating the specificity of the tested primers. Normalized gene expression was determined by using the delta C_t method ($E^{\Delta C_t}$), a variation of the Livak method, where $\Delta C_t = C_t$ (reference gene) − C_t (target gene) and E stands for the reaction efficiency experimentally determined. A non-reverse transcriptase control was included in each reaction. All assays were repeated at least three independent times with three technical replicates.

Table 4.1. Primers used in qPCR experiments.

Target gene	Gene description	Primer sequence (5' to 3')	$T_{melting}$ (°C)	Efficiency[a] (%)	Amplicon size (bp)
16S RNA	16S ribosomal RNA of *G. vaginalis*	Fw TGAGTAATGCGTGACCAACC	55.2	100	167
		Rv AGCCTAGGTGGGCCATTACC	59.3		
vly	Thiol-activated cytolysin vaginolysin	Fw GAACAGCTGGGCTAGAGGTG	60.01	100	153
		Rv AATTCCATCGCATTCTCCAG	60.04		
sld	Sialidase	Fw CCGAATTTGCGATTTCTTCT	54.00	86	189
		Rv CGTACGGAAGTTTTGGAAGC	58.00		
HMPREF0424_0821	Glycosyltransferase, group 2 family protein	Fw CAACGAAGGCATAGGTTTCC	59.57	100	156
		Rv GCGCTTGGAACTGCTTTAAC	60.02		

[a] PCR amplification efficiency (E) for each gene was determined from the slope of a standard curve (E=10 [-1/slope]), generated with a 10-fold dilution series of cDNA.

4.2.7 Statistical analysis

The data were analysed using the statistical package GraphPad Prism version 7 (La Jolla) by Paired t test, Mann-Whitney U test, or one-way ANOVA with Dunnett's multiple comparison test. Values with a $p < 0.05$ were considered statistically significant.

4.3 Results

4.3.1 Biofilm biomass quantification

We observed that in our *in vitro* conditions, *G. vaginalis* was able to form a biofilm with significantly higher biomass than *F. vaginae* or *P. bivia* after 24 h of incubation (Figure 4.1A). Furthermore, dual-species biofilms total biomass was not significantly augmented after adding *F. vaginae* or *P. bivia* to the pre-formed *G. vaginalis* biofilms (when compared to 48 h single-species biofilms of *G. vaginalis*) (Figure 4.1B). Interestingly, similar results were obtained for the triple-species biofilms after *F. vaginae* and *P. bivia* were simultaneously added to the pre-established *G. vaginalis* biofilms.

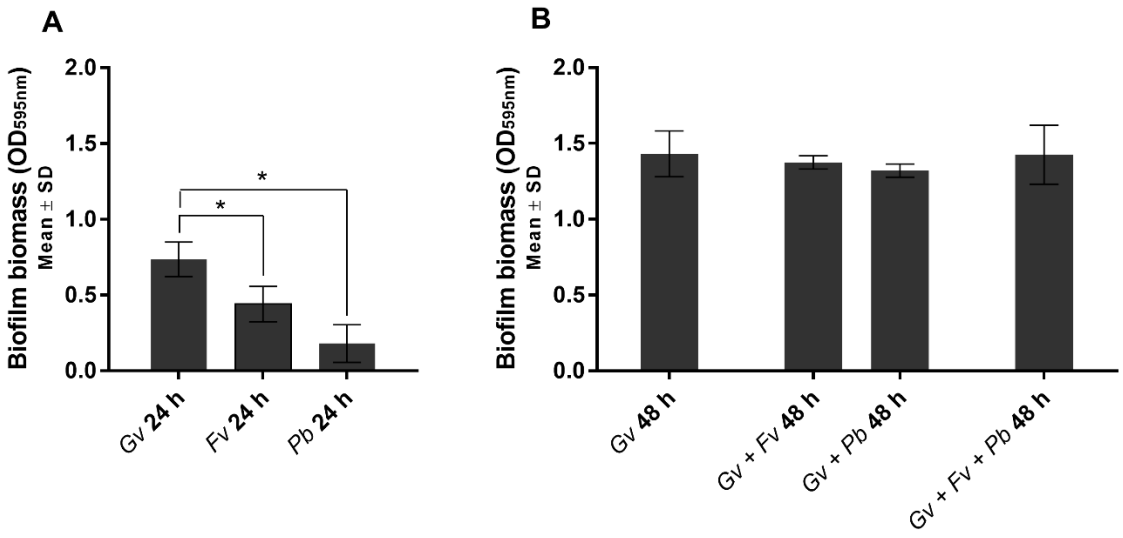

Figure 4.1. Single-, dual-, and triple-species biofilms biomass quantification by CV staining. (**A**) represents the 24 h single-species biofilms controls. (**B**) represents the 48 h *G. vaginalis* single-species biofilms control as well as the 48 h dual- and triple-species biofilms. Results are expressed as average ± standard deviation of three independent assays, with two technical replicates assessed each time. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test. * Values are significantly different between 24 h *G. vaginalis* single-species biofilms and 24 h *F. vaginae* or *P. bivia* single-species biofilms ($p < 0.05$). *Fv*. *Fannyhessea vaginae*; *Gv*. *Gardnerella vaginalis*; *Pb*. *Prevotella bivia*.

4.3.2 Testing specificity and efficiency of the PNA probes Gard162 and AtoITM1

To confirm that the PNA probes Gard162 and AtoITM1 could differentially detect the *G. vaginalis* and *F. vaginae* strains used in this study, we analysed if any of these two probes would cross-hybridize with any of the tested strains. According to the FISH results (Table 4.2), Gard162 probe hybridized with *G. vaginalis* while AtoITM1 probe hybridized with *F. vaginae*, and no cross-hybridization was observed with other species, confirming the high specificity as before reported (8,14).

Table 4.2. Bacterial species used in PNA FISH assays and their specificity with PNA probes Gard162 and AtoITM1.

Species	Gard162 probe specificity[a]	AtoITM1 probe specificity [a]
G. vaginalis ATCC 14018[T]	++++	-
F. vaginae ATCC BAA-55[T]	-	+++
P. bivia ATCC 29303[T]	-	-

[a] The specificity of PNA probes Gard162 and AtoITM1 was tested for each species with the following hybridization PNA FISH qualitative evaluation: (–) absence of hybridization; (++) moderate hybridization; (+++) good hybridization; (++++) optimal hybridization.

Since no *P. bivia* PNA probe currently exists, the estimation of *P. bivia* counts could only be evaluated indirectly by DAPI counterstaining, assuming that all cells with unlabelled PNA probes were *P. bivia*; however, this needs to be experimentally determined (19). Therefore, we compared the data obtained for FISH and DAPI counts for both *G. vaginalis* and *F. vaginae* single-species biofilms. Not surprisingly, each

probe failed to detect 100% of the respective total cells. By performing serial dilutions of each sample, calibration curves were obtained for *G. vaginalis* (Figure 4.2A) and for *F. vaginae* (Figure 4.2B).

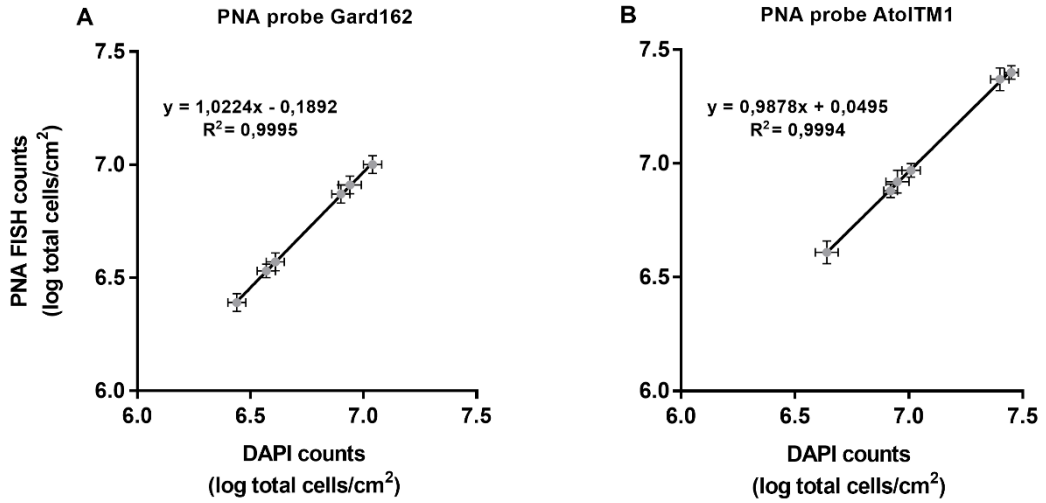

Figure 4.2. Correlation between FISH and DAPI counts for single-species biofilms of *G. vaginalis* and *F. vaginae* at different bacterial concentrations. (A) *G. vaginalis* biofilm cells that were identified indirectly by DAPI coincided with the populations quantified by PNA FISH using the PNA probe Gard162. **(B)** *F. vaginae* biofilm cells that were identified indirectly by DAPI coincided with the populations quantified by PNA FISH using the PNA probe AtoITM1. Each data point represents the average ± standard deviation of three independent assays. For each assay, twenty fields were randomly acquired in each sample and the number of bacteria per image was counted using *ImageJ Software*.

Taking into consideration these results, it was possible to calculate the efficiency of each probe and obtain an equation that would correct FISH counts, to prevent the overestimation of DAPI counts and consider them as *P. bivia* counts (Table 4.3).

Table 4.3. Equations used to quantify bacterial populations in biofilms.

Bacteria	Equation	Efficiency of PNA probe (%)
G. vaginalis	*G. vaginalis* counts = [log (FISH counts/area) + 0.1892]/ 1.0224	92.08
F. vaginae	*F. vaginae* counts = [log (FISH counts/area) – 0.0495]/ 0.9878	91.59

4.3.3 Discriminating bacterial populations in dual- and triple-species biofilms by PNA FISH

Our results showed that although *F. vaginae* and *P. bivia* did not significantly enhance the dual-species biofilms biomass, as aforesaid, these species were able to incorporate *G. vaginalis* pre-formed biofilm, comprising for up to respectively 25% and 40% of the total number of cells in the dual-species biofilms (Figure 4.3). Interestingly, these percentages changed in the triple-species biofilms with *F. vaginae*

showing only about 8% biofilm cells while *P. bivia* about 22%, and *G. vaginalis* maintaining almost the same number of cells as in the dual-species biofilms.

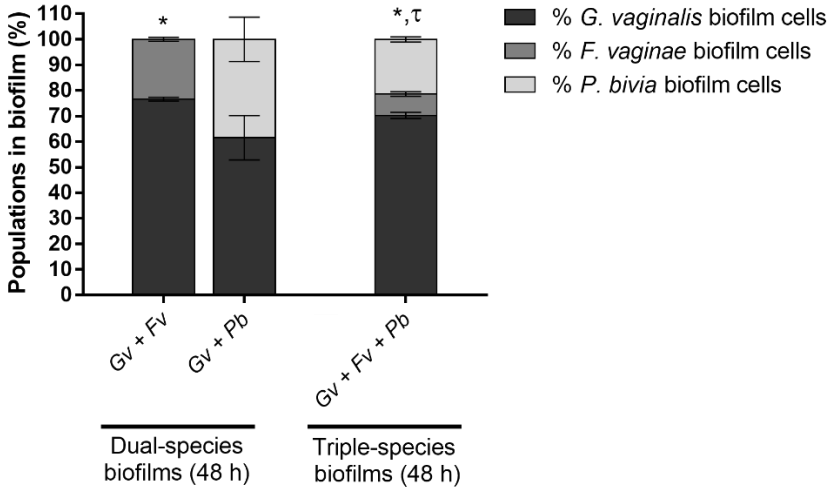

Figure 4.3. Total percentage of cells detected by PNA FISH for 48 h dual- and triple-species biofilms. Results are expressed as average ± standard deviation. For each assay, twenty fields were randomly acquired in each sample and the number of bacteria per image was counted using *ImageJ Software*. * Values are significantly different between bacterial populations of *G. vaginalis* and *F. vaginae* in dual-species (Paired t test, $p < 0.05$) and triple-species (one-way ANOVA and Dunnett's multiple comparisons test, $p < 0.05$) biofilms. $^{\tau}$ Values are significantly different between bacterial populations of *F. vaginae* and *P. bivia* in triple-species biofilms (one-way ANOVA and Dunnett's multiple comparisons test, $p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Pb*: *Prevotella bivia*.

4.4.4 Analysis of biofilm populations' distribution by CLSM

After determining the percentage of each species in the dual- and triple-species biofilms, we sought to analyse their spatial distribution in these biofilms taking advantage of the robustness of PNA FISH/ DAPI method combined with CLSM. As shown in Figure 4.4, on average, in both dual- and triple-species biofilms, *F. vaginae* and *P. bivia* were found well distributed across *G. vaginalis* biofilm, in small clusters of cells.

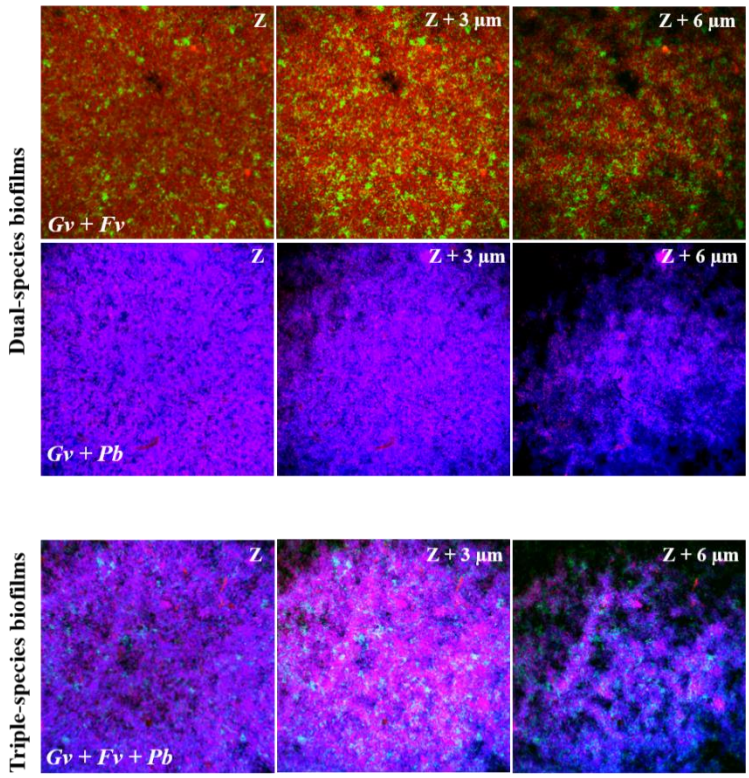

Figure 4.4. An example of data set on the organization of the 48 h dual- and triple-species biofilms by CLSM. *G. vaginalis* is labelled with PNA probe Gard162 (red/ purple colour when coupled with DAPI), *F. vaginae* is labelled with PNA probe AtoITM1 (green/ green-blue colour when coupled with DAPI), and *P. bivia* is stained with DAPI (blue colour). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Pb*: *Prevotella bivia*.

4.4.5 Quantification of the expression of virulence genes in G. vaginalis

In order to elucidate the influence of *F. vaginae*, *P. bivia* or both species on *G. vaginalis* virulence, we evaluated the expression of *G. vaginalis* genes associated to cytotoxicity, vaginal epithelial exfoliation, and biofilm formation in cells from single-, dual-, and triple-species biofilms. It is known that *G. vaginalis* produces vaginolysin, a pore-forming toxin that might induce vaginal cells lysis (20,21). Under our tested conditions, the expression levels of the gene for vaginolysin, *vly*, was slightly reduced only in the triple-species biofilms consortium (Figure 4.5A). Regarding sialidase, *sld*, which is known to facilitate the destruction of the protective mucus layer on the vaginal epithelium (22), similar results were obtained whenever *F. vaginae* was included in the dual- or triple-species consortia (Figure 4.5B). Conversely, the expression of *HMPREF0424_0821* transcript, which codes type II glycosyltransferase, likely to be important for the biosynthesis of exopolysaccharide which in turn might be important for biofilm formation (23), was upregulated in all consortia, however statistically significant only for the triple-species biofilms (Figure 4.5C).

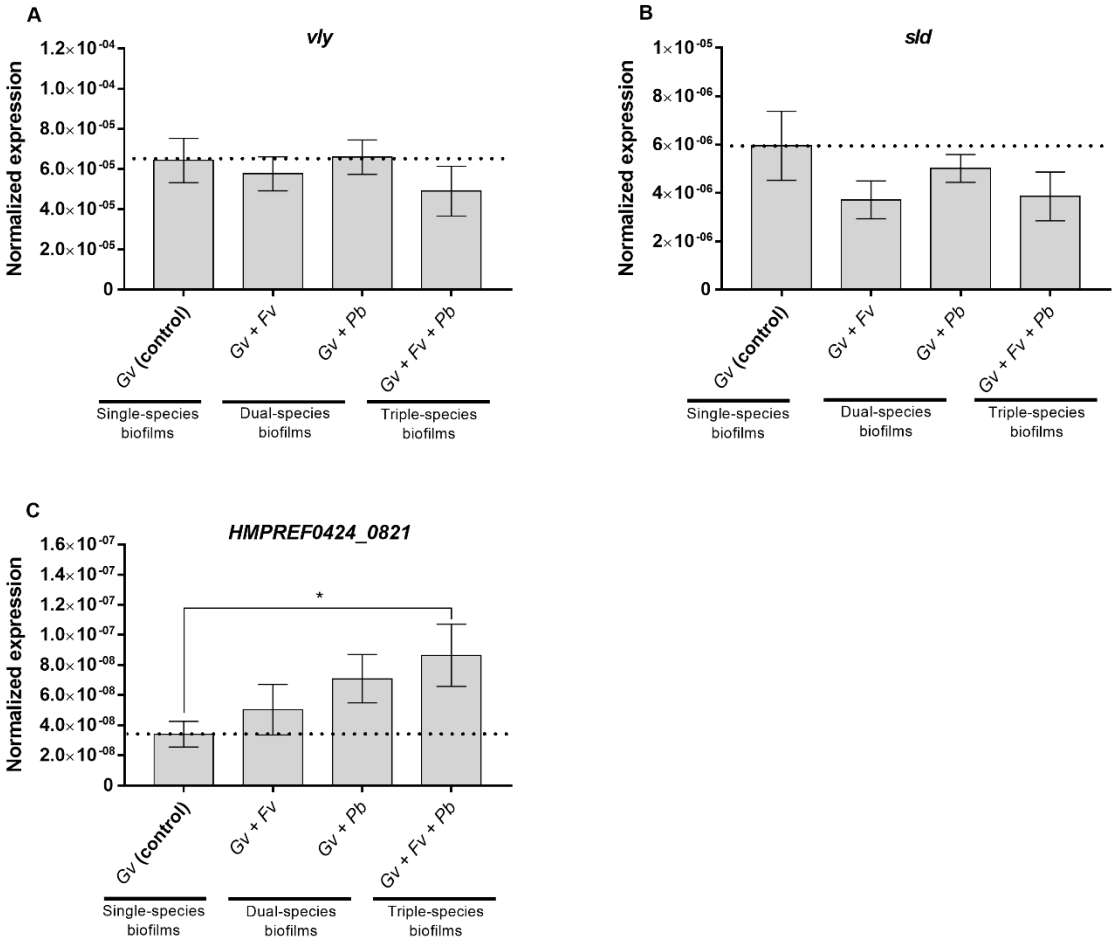

Figure 4.5. Quantification of the expression of virulence genes related to cytotoxicity, vaginal epithelial exfoliation, and biofilm formation, by *G. vaginalis* cultured in 48 h single-, dual-, and triple-species biofilms. (A) Quantification of vaginolysin (*vly*) transcript. **(B)** Quantification of sialidase (*sld*) transcript. **(C)** Quantification of *HMPREF0424_0821* transcript, which codes type II glycosyltransferase. The data show the fold-change expression of genes in *G. vaginalis* single- compared to dual- and triple-species biofilm cells. For qPCR experiments, the bars represent the mean, and the error bars the standard error of the mean (mean ± SEM). * Values are significantly different between the single- and the triple-species *G. vaginalis* biofilm under the same conditions (Mann-Whitney U test, $p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Pb*: *Prevotella bivia*.

4.4 Discussion

While BV is often associated with multiple bacterial species, most *in vitro* biofilms are focused on *Gardnerella* spp. single-species studies (24). A dual-species biofilm model has been described before, where *Gardnerella* spp. is first allowed to form a biofilm and then other species are incorporated in this early stage biofilm (12,13,25,26). However, *in vivo* BV biofilms are composed of more than two species (7,8,27), and as such, it is important to develop more complex biofilm models. Here, we selected three prominent bacterial species associated with BV (28) to develop the first *in vitro* triple-species biofilms.

Our results indicate that even *F. vaginae* and *P. bivia* were able to incorporate the pre-established *in vitro* *G. vaginalis* biofilms in both dual- and triple-species consortia, *G. vaginalis* was the predominant species

in all consortia tested, similar to what occurs *in vivo* (7,29). In addition, the relative composition of *F. vaginae* and *P. bivia* decreased in the triple-species biofilms comparing to the dual-species biofilms, suggesting that different relationships are established in these distinct consortia. Furthermore, if comparing the ability of *F. vaginae* and *P. bivia* to incorporate the pre-formed *G. vaginalis* biofilm in dual-species biofilms, in our tested conditions, *P. bivia* was better fit to grow in the biofilm than *F. vaginae*. This suggests that *G. vaginalis* modifies the local environment, making it more favourable for the growth of *P. bivia.* This might not be surprising, since an early *in vitro* study reported nutritional pathways that maintain a synergistic relationship observed between *Gardnerella* spp. and *P. bivia*. Growth of *P. bivia* in a vaginal defined medium supplemented with amino acids or peptone resulted in ammonia production while the growth of *Gardnerella* spp. under the same conditions was accompanied by ammonia utilization (30). Also, more evidence of such bacterial cooperation was supported by a more recent study, where it was demonstrated that *Gardnerella* spp. growth increased in the presence of *P. bivia*, and *P. bivia* reached higher numbers when co-cultured with *Gardnerella* spp. (25). In our study, *F. vaginae* and *P. bivia* were simultaneously added to the pre-formed *G. vaginalis* biofilm, and perhaps this led to a competition between these two species over the metabolites produced by *G. vaginalis*, being therefore somewhat explained why *F. vaginae* was present in such low concentration in the triple-species biofilms. However, these results are to some extent contradictory to what has been described *in vivo* BV biofilms. A study conducted by Swidsinski and colleagues (7) on vaginal biopsies specimens using a broad range of FISH probes demonstrated that the adherent BV-biofilms were mainly composed by *Gardnerella* spp. (~60%) and *F. vaginae* (~40%). Afterwards, Hardy and colleagues (8) carried out a study on vaginal samples in which they observed that when *F. vaginae* was part of the BV biofilm, compared to a biofilm of only *Gardnerella* spp., both species were present in higher concentrations. In a following study, also carried on vaginal samples, Hardy and colleagues (9) showed that *F. vaginae* is almost always accompanied by *Gardnerella* spp. in BV, but that *Gardnerella* spp. can be found without *F. vaginae* in the BV vaginal milieu. Therefore, all these findings support that *Gardnerella* spp. and *F. vaginae* could indeed establish a relationship in BV-associated biofilms (9), but to better understand the obtained results and also bacterial interactions in BV biofilms, further detailed studies are needed.

In this study, we selected a very rich nutrient culture medium (NYC III), that we recently showed to be an ideal candidate to form single-species *in vitro* biofilms of fastidious bacteria (31). This selection was based on the purpose to better compare dual- and triple-species biofilms with single-species biofilms. However, being such a rich medium, NYC III might be masking possible synergistic growth effects that have been reported in other *in vitro* conditions (13). Still, despite no growth synergistic effects were found under

these conditions, relevant microbial interactions were in fact observed: our PNA FISH demonstrated that in the triple-species consortium, *F. vaginae* decreased the relative concentration of *P. bivia*, as compared with the other consortium. These data support the idea that the development of a BV-associated biofilm reflects the interactions established by the different species over time. Further interactions were also detected when analysing key *G. vaginalis* gene expression. As observed by our qPCR experiments, the expression of *HMPREF0424_0821*, a gene coding for a type II glycosyltransferase thought to be involved in biofilm formation, was increased in *G. vaginalis* in the presence of the other two species in both dual- and triple-species consortia. Interestingly, the higher expression levels were found when in the presence of both *F. vaginae* and *P. bivia.*

Taken together, this study highlights the idea that the interactions between BV-associated bacteria can impact the biofilm structure, which will likely influence BV progress as well as the clinical outcome. Since BV etiology is poorly understood and there is still a lack of studies addressing the polymicrobial bacterial communities found in BV, further studies are needed to investigate the complex interplay between BV-associated species.

4.5 References

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CHAPTER 5

How *in vitro* models influence the composition of triple-species biofilms and impact biofilm tolerance to antibiotics?

Summary

Following the findings described in **Chapter 4**, we established and characterized several triple-species consortia in this chapter. To culture these consortia, two different experimental designs were used, namely the *G. vaginalis* pre-conditioned biofilm formation model described in **Chapter 4** versus a new biofilm formation model, whereby the three species were co-incubated simultaneously (the competitive biofilm formation model). By using these two *in vitro* models, it was possible to understand if the presence of a *G. vaginalis* pre-established biofilm would influence the ability of known BV-associated species to establish triple-species biofilms as well as if it would impact antimicrobial tolerance. Our data revealed that total biofilm biomass synergistic effects were only observed when using the competitive biofilm-forming model. Differences in bacterial compositions were found between the two models as well as in bacterial organization in the biofilm structure. Moreover, antimicrobial tolerance was slightly different between the two models, however only for one antibiotic tested, namely metronidazole. These results provide important evidence on bacterial interactions in BV and their impact on biofilm formation and response to the antibiotic treatment. Better understanding of these microbial interactions during BV may enable to devise more effective treatment options.

5.1 Brief introduction

BV represents an important health problem and yet its etiology is still a matter of controversy (1). In this regard, over the years, two main hypotheses have been proposed for BV development (as discussed in **Chapter 2**): the single agent hypothesis (2), which postulates that BV is caused by one specific agent, previously described as *G. vaginalis* (3). Alternatively, the polymicrobial hypothesis that assumes that BV is caused by a mixture of anaerobic pathogenic bacteria (4). However, in both hypotheses, *Gardnerella* supposedly plays a pivotal role in BV development (1,5–7). Indeed, *Gardnerella* has been shown to have a significantly higher virulence potential than many other BV-related species (8,9). Accordingly, it has been hypothesized that *Gardnerella* acts as the initial colonizer on the vaginal epithelium and then enables other BV-associated bacteria to subsequently adhere and incorporate the early started biofilm (10), with *F. vaginae* identified as a key species associated with *Gardnerella* biofilms during BV (11–13). Based on this hypothesis, our previous dual- and triple-species *in vitro* models followed an experimental design whereby *Gardnerella* was first allowed to establish a single-species biofilm before the other BV-associated species were added to the culture (14–19). However, recent data suggest that at least in some cases, *P. bivia* is detected in incident BV cases before or at the same time as *Gardnerella* (20).

The purpose of this study was, therefore, to investigate triple-species biofilms formation, using two different experimental designs. On the one hand, we allowed a pre-formed *G. vaginalis* biofilm to be established before inoculating the other species; we refer to this approach as the *G. vaginalis* pre-conditioned model. On the other hand, we allowed the different species of each consortium to be incubated simultaneously, whereby all three species compete for nutrients and contact surface during all stages of biofilm formation. We refer to this approach as the competitive model. Triple-species biofilm biomass and their bacterial composition and distribution were first analysed and then their susceptibility to the first-line antibiotics used to treat BV were assessed.

5.2 Material and methods

5.2.1 Bacterial species and growth conditions

G. vaginalis (**Chapter 4**) together with *F. vaginae*, *L. iners*, *M. curtisii*, *P. anaerobius*, and *P. bivia* (**Chapter 3**) were used in this study. The inoculum of each species was grown in NYC III supplemented with 10% (v/v) inactivated horse serum (as described in **Chapter 3**) and incubated in anaerobic conditions generated by AnaeroGen sachets (Thermo Fisher Scientific) in sealed jars (Oxoid) at 37 °C for 24 h.

5.2.2 Normalization of bacterial concentration

For all biofilm formation assays, initial bacterial concentrations were adjusted by OD and confirmed by Neubauer chamber counting and flow cytometry (Table 5.1). Briefly, the OD at 620 nm of the 24 h inoculum was adjusted in fresh NYC III to a value of ~ 0.1 (Biochrom EZ Read 800 Plus) and from this suspension, 30 µL were stained with 10 µL of DAPI (2.5 µg.mL^{-1}) and let at room temperature for 5 min, in the dark. Subsequently, 10 µL of the obtained bacterial suspensions were used for the Neubauer chamber coupled with an Olympus BX51 fluorescence microscope equipped with a CCD colour camera DP71 (Olympus) and the cells were detected and counted using a DAPI filter (BP 365–370, FT 400, LP 421) present in the microscope. In parallel, 20 µL of these suspensions were mixed with 180 µL of 1X PBS and transferred into the flow cytometer tubes, and the cells were quantified on the flow cytometer (EC 800 BR, Sony Biotechnology). A relation between OD and the bacterial concentration was established on the basis of the data from the two approaches. Both measurements were repeated at least three independent times with technical replicates.

Table 5.1. Correlation between OD_{620nm} and both flow cytometry and Neubauer counting for bacterial suspensions of the strains used in this chapter.

Species	OD at 620nm~0.1 correlated with total cells.mL^{-1} [a]
F. vaginae ATCC BAA-55^T	8.55 x 10^7 ± 4.51 x 10^7
G. vaginalis ATCC 14018^T	6.07 x 10^7 ± 2.36 x 10^7
L. iners CCUG 28746^T	5.27 x 10^7 ± 4.70 x 10^6
M. curtisii ATCC 35241^T	3.36 x 10^7 ± 3.38 x 10^7
P. anaerobius ATCC 27337^T	3.91 x 10^7 ± 1.67 x 10^7
P. bivia ATCC 29303^T	4.59 x 10^7 ± 4.08 x 10^6

[a] The results are expressed as the average of three measurements obtained by the flow cytometry and Neubauer chamber counting ± standard deviation.

5.2.3 Biofilm formation

5.2.3.1 G. vaginalis *pre-conditioned biofilm formation model*

G. vaginalis culture was adjusted to a concentration of ~1.0 × 10^7 cells.mL^{-1} in NYC III, as explained above, homogenized by gentle vortexing and distributed in 24-well tissue culture plates (Orange Scientific) or 8-well chamber slides (Thermo Fisher Scientific™ Nunc™ Lab-Tek™) and further incubated for 24 h at 37 °C under anaerobic conditions as described above. After 24 h, the culture medium together with planktonic cells covering the biofilms were carefully removed. Then, bacterial suspensions of ~1.0 × 10^7 cells.mL^{-1} of *F. vaginae* and ~1.0 × 10^7 cells.mL^{-1} of a third BV-associated species, prepared in fresh

medium, were inoculated onto the pre-formed *G. vaginalis* biofilms and incubated anaerobically for an additional 24 h (Figure 5.1A). Subsequently, the triple-species biofilms were carefully washed once with sterile 1X PBS. *G. vaginalis* single-species biofilms were grown as controls for 24 h and for 48 h in which fresh medium was added to the respective wells after the first 24 h of biofilm formation. Additionally, dual-species biofilms of *G. vaginalis* pre-grown for 24 h and then adding *F. vaginae* were also included as controls.

5.2.3.2 Competitive biofilm formation model

Cultures of *G. vaginalis*, *F. vaginae*, and each of the third BV-associated species were adjusted to a concentration of ~ 1.0×10^7 cells.mL^{-1} in NYC III broth, as explained above, homogenized by gentle vortexing and co-incubated simultaneously in 24-well tissue culture plates (Orange Scientific) or 8-well chamber slides (Thermo Fisher Scientific™ Nunc™ Lab-Tek™) for 24 h at 37 °C under anaerobic conditions, as previously mentioned (Figure 5.1B). Afterwards, the medium covering the biofilms was carefully removed and the biofilms were washed once with sterile 1X PBS. Single- as well as dual-species biofilms of *G. vaginalis* and *F. vaginae* were used as controls.

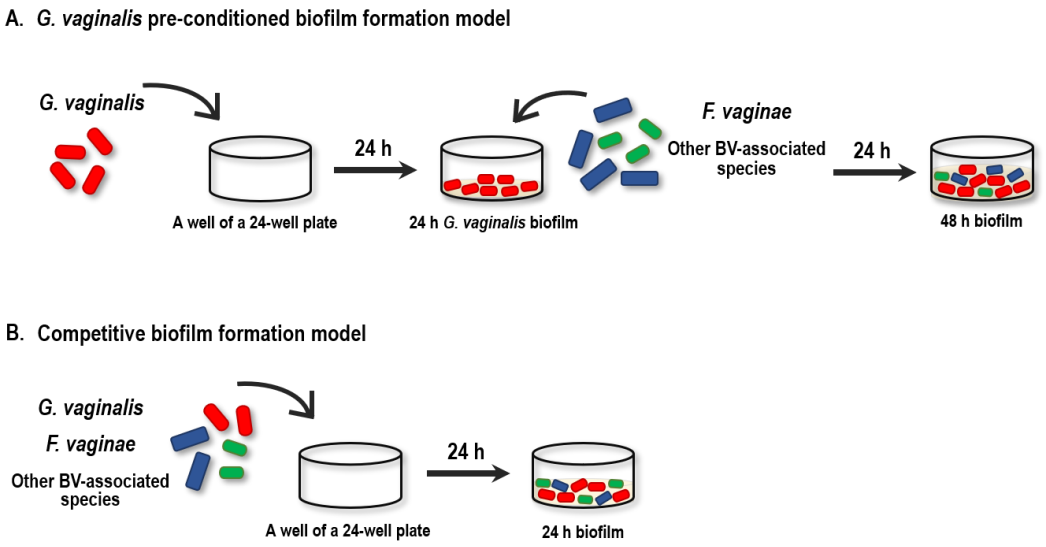

Figure 5.1. Schematic representation of the experimental models used for the biofilm formation assays in this chapter. (A) presents the steps performed for *G. vaginalis* pre-conditioned biofilm formation model. **(B)** presents the steps performed for the competitive biofilm formation model.

5.2.4 Biofilm biomass quantification

The biomass of single-, dual-, and triple-species biofilms formed in 24-well tissue culture plates was quantified by the CV method. Biofilms were fixed with 1 mL of 100% (v/v) methanol (Thermo Fisher

Scientific) for 20 min and then stained with 1 mL of CV solution 1% (v/v) (Merck) for 20 min. Following, each well was washed twice with 1X PBS after which bound CV was released with 1 mL of 33% (v/v) acetic acid (Thermo Fisher Scientific). To quantify the biofilm biomass, OD of the resulting solution was measured at 595 nm. The biofilm biomass quantification assays were repeated at least three times with two technical replicates.

5.2.5 Testing specificity and efficiency of the PNA probes Gard162 and AtoITM1

The specificity of PNA probes Gard162 (21) and AtoITM1 (13) was determined for the species considered in this chapter, following the procedure described in **Chapter 4**. The efficiency of the PNA probes Gard162 and AtoITM1 were also determined in **Chapter 4**, and the obtained equations from the calibration curves used previously were also applied in the current chapter, aiming to correct the FISH counts and prevent the overestimation of the DAPI counts.

5.2.6 Discriminating bacterial populations in triple-species biofilms by PNA FISH

To discriminate bacterial populations within triple-species biofilms from both *in vitro* models, we used the FISH method that was previously described in **Chapter 4**. In brief, after fixing the biofilm suspensions by 100% (v/v) methanol, 4% (w/v) paraformaldehyde, and 50% (v/v) ethanol, PNA probes Gard162 (21) and AtoITM1 (13) specific for *G. vaginalis* and *F. vaginae*, respectively, were added to each well of epoxy coated microscope glass slides (Thermo Fisher Scientific). An additional staining step was done at the end of the hybridization procedure, covering each well of the glass slides with DAPI. Microscopic visualization was achieved using filters capable of detecting the PNA probe Gard162, AtoITM1 probe, and DAPI. All assays were repeated three times on separate days.

5.2.7 CLSM analysis of the in vitro *developed biofilms*

To analyse the integration of bacterial species in the triple-species biofilms, we used CLSM as described in **Chapter 4**. Briefly, after staining the fixed intact biofilms with PNA Gard162, AtoITM1, and DAPI, images were then acquired with an Olympus™ FluoView FV1000 (Olympus) confocal laser scanning microscope using a 10× objective and with a resolution of 800 × 800 pixels. The CLSM images were analysed using the *FV10-ASW 4.0 Viewer Software* (Olympus). All assays were repeated three times with two technical replicates.

5.2.8 Antibiotics

Clindamycin and metronidazole, two antibiotics recommended for the treatment of BV (22), were used in this study. Antibiotic solutions were prepared on the day of use according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (23). The mechanisms of action and the peak serum concentration (PSC) value of each antibiotic used herein are shown in Table 5.2.

Table 5.2. Mechanisms of action and peak serum concentration (PSC) of clindamycin and metronidazole.

Antibiotics	Mechanism of action	PSC ($\mu g.mL^{-1}$) [a]
Clindamycin	Inhibition of protein synthesis (24)	4.8 (25)
Metronidazole	Inhibition of DNA replication (26)	11.5 (27)

[a] In this study, for biofilm assays, each antibiotic was used in a concentration of 4 × PSC.

5.2.9 Determination of the minimal inhibitory concentration (MIC) and minimal lethal concentration (MIC) of antibiotics

MIC and MLC values of clindamycin and metronidazole were determined by the broth microdilution method in 96-well tissue culture plates (28), with some minor modifications. Both antibiotics were initially diluted in NYC III broth. Then, serial dilutions of clindamycin and metronidazole, ranging between 0.008 $\mu g.mL^{-1}$ and 16 $\mu g.mL^{-1}$ and respectively between 0.125 $\mu g.mL^{-1}$ and 256 $\mu g.mL^{-1}$, were also prepared in NYC III broth. Bacterial suspensions of 24 h cultures corresponding to each tested species were adjusted to an OD of 0.10 ± 0.05 at 620 nm. Subsequently, the adjusted bacterial suspensions were added to 96-well tissue culture plates containing the prepared antibiotics dilutions. Also, MIC determination assays included a negative (only NYC III) and a positive (bacterial suspensions without antibiotics) control. The 96-well tissue culture plates were incubated for 48 h at 37 °C in anaerobic conditions created by the anaerobic gas generating sachets, as described above. After incubation, the MICs were determined by reading the OD at 620 nm of bacterial suspensions from the 96-well plates. MIC value was defined as the lowest concentration of the antibiotic that inhibited the growth of treated bacteria (23). All assays were repeated at least three times on separate days with technical replicates.

5.2.10 Determination of the antibiotics effect on triple-species biofilms biomass

Biofilms were formed in 24-well tissue culture plates as mentioned above and afterward challenged with clindamycin and metronidazole. Clindamycin was adjusted to a final concentration of 19.2 $\mu g.mL^{-1}$ (4 × PSC), while metronidazole to a final concentration of 46 $\mu g.mL^{-1}$ (4 × PSC) (Table 5.2), in NYC III broth, being, therefore added to the biofilms, and incubated for 24 h at 37 °C under anaerobic conditions as

previously mentioned. Next, the medium with planktonic cells was removed and biofilms biomass were quantified by the CV method. Negative and positive controls, represented by NYC III and triple-species biofilms without exposition to the antibiotics, respectively, were included in each experimental assay. All assays were repeated at least three times with technical replicates.

5.2.11 Statistical analysis

All numerical data were analysed using statistical package GraphPad Prism version 7 (La Jolla) by one-way and two-way ANOVA with multiple comparisons tests. Values with a $p < 0.05$ were considered statistically significant.

5.3 Results

5.3.1 Biofilm biomass quantification

In order to better understand the role of *G. vaginalis* in multi-species biofilms formation, we first started to quantify total biomass formed in triple-species biofilms using two distinct models, whereby the main difference was allowing (or not) the establishment of a *G. vaginalis* biofilm before introducing the other species. Triple-species biofilms were compared to both dual-species biofilms of *G. vaginalis* and *F. vaginae* and single-species biofilms of *G. vaginalis*, incubated for the same period. It should be noted that a direct comparison between the two experimental models is not possible due to the first 24 h of *G. vaginalis* incubation in the pre-conditioned model, that could not be compensated in the competitive model.

Interestingly, in the pre-conditioned model, no differences were observed between the total biomass accumulated in the triple- or dual-species consortia as compared with 48 h *G. vaginalis* single-species biofilms, suggesting that the other two species did not enhance the ability of *G. vaginalis* biofilm formation, under these experimental conditions (Figure 5.2A). Conversely, in the competitive model, a significant increase of the total biomass was observed for all four triple-species consortia when comparing to the 24 h *G. vaginalis* single-species biofilms but only in the triple-species consortium with *P. anaerobius* this increase was significant when compared to the *G. vaginalis* and *F. vaginae* dual-species biofilms (Figure 5.2B).

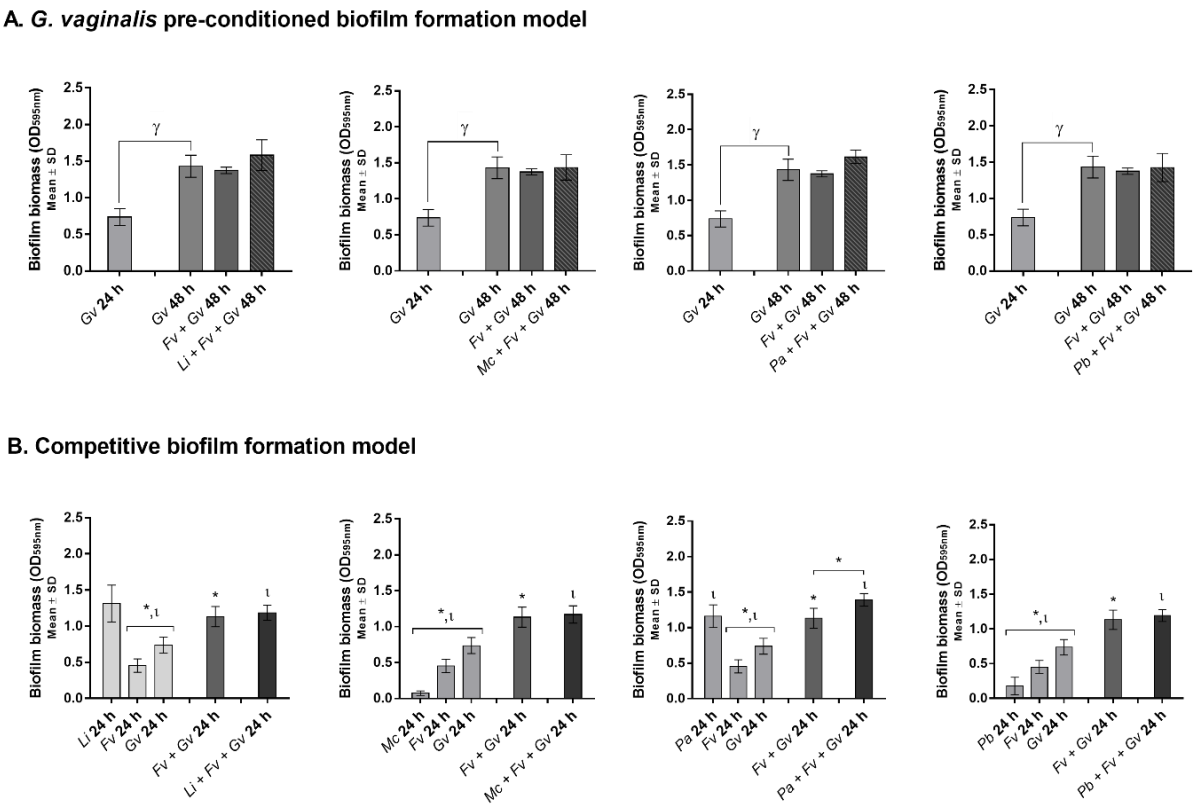

Figure 5.2. Biomass quantification of the single-, dual-, and triple-species biofilms using CV method. (A) represents the total biomass of 24 h and 48 h *G. vaginalis* single-species biofilms as well as the 48 h dual- and triple-species biofilms formed using the *G. vaginalis* pre-conditioned model. **(B)** represents the total biomass of 24 h single-species biofilms as well as the 24 h dual- and triple-species biofilms formed using the competitive model. Results are expressed as average OD_{595nm} ± standard deviation of at least three independent experiments performed with two technical replicates. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test. γ Values are significantly different between 24 h and 48 h *G. vaginalis* single-species biofilms for the pre-conditioned model ($p < 0.05$). * Values are significantly different between dual-species biofilms of *Fv* + *Gv* and single- or triple-species biofilms for the competitive model ($p < 0.05$). τ Values are significantly different between triple-species biofilms and single-species biofilms for the competitive model ($p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Li*: *Lactobacillus iners*; *Mc*: *Mobiluncus curtisii*; *Pa*: *Peptostreptococcus anaerobius*; *Pb*: *Prevotella bivia*.

While the pre-conditioned model yielded biofilms with higher biomass, it should be taken into consideration that those biofilms benefit from the added biomass of the 24 h initial *G. vaginalis* biofilms. We hypothesized that this increased yield was not a result of a specific contribution by *G. vaginalis*, but a direct consequence of enhanced incubation time, as show before (29). To test this hypothesis, we performed another experiment where we allowed the triple-species biofilms formed in the competitive model to be incubated for another 24 h (total of 48 h), which led to an enhanced biofilm formation in all consortia tested, superior to the total biomass obtained in the pre-conditioned model (Figure 5.3). Again, attention should be given when directly comparing both models, since in the 48 h pre-conditioned biofilms, non-*G. vaginalis* isolates were only incubated for 24 h.

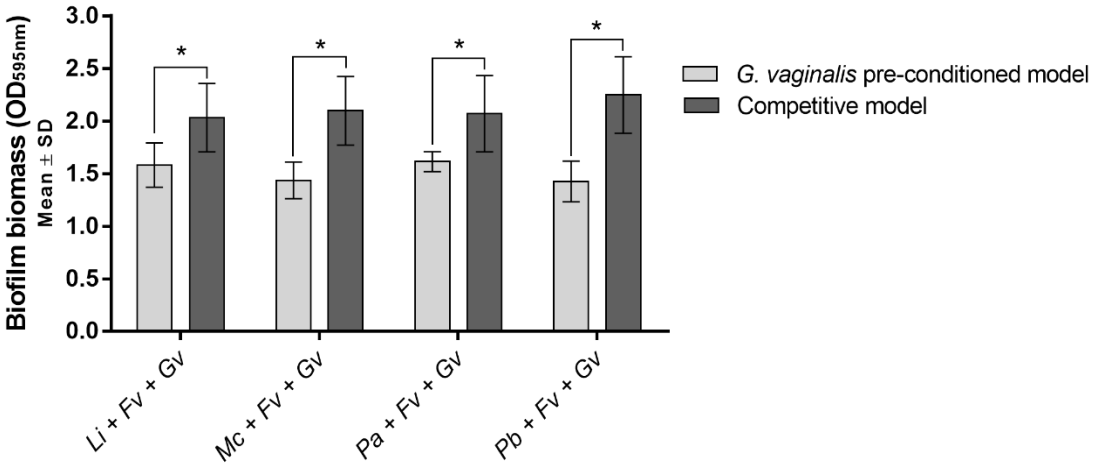

Figure 5.3. Comparison of total biofilm biomass after 48 h of incubation in the two different experimental models. The biofilm biomass was quantified by the CV method. Results are expressed as average of the OD at 595 nm ± standard deviation of at least three independent experiments performed with two technical replicates. * Represents a statistically significant difference (two-way ANOVA and Sidak's multiple comparisons test, $p < 0.05$).

5.3.2 Testing specificity and efficiency of the PNA probes Gard162 and AtoITM1

As determined in **Chapter 4**, Gard162 and AtoITM1 probes hybridized with *G. vaginalis* and *F. vaginae*, respectively, and no cross-hybridization was observed with *P. bivia*. Thus, it was also important to verify if the two PNA probes would cross-hybridize with any of the other species used in this chapter. Based on FISH results, no hybridization of the two probes was observed for *L. iners*, *M. curtisii* or *P. anaerobius* strains used herein, showing a specificity of 100% as previously reported (13,21).

5.3.3 Discriminating bacterial populations in triple-species biofilms by PNA FISH

Taking into consideration each PNA probe efficiency (as described in **Chapter 4**), we quantified *G. vaginalis*, *F. vaginae*, and the third BV-associated species in each consortium from both experimental models. Interestingly, as shown in Figure 5.4, for all consortia and for both models tested, *G. vaginalis* was the dominant species. Furthermore, different bacterial compositions were observed within the same consortium when grown in each of the two distinct *in vitro* models. Also, *G. vaginalis* presented a significant higher number of cells in all consortia of the pre-conditioned model, while *F. vaginae*, conversely, showed a significant higher number of cells in all consortia of the competitive model. Curiously, the other tested species were able to better integrate the triple-species biofilms formed under the pre-conditioned model, suggesting that perhaps in the competitive model, these species might be outcompeted by *F. vaginae*, which prospers better in this model.

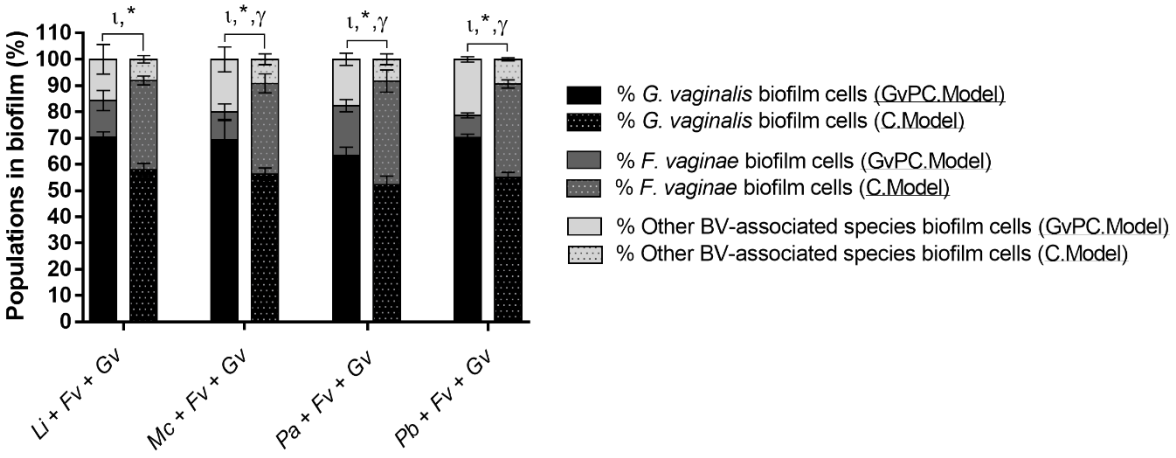

Figure 5.4. Relative composition of each triple-species biofilm consortium as quantified by PNA FISH. Results are expressed as average of cells ± standard deviation. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparisons test. Statistically significant differences between both models are represented with τ for *G. vaginalis*, * for *F. vaginae*, and with γ for the other BV-associated species in each consortium ($p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Li*: *Lactobacillus iners*; *Mc*: *Mobiluncus curtisii*; *Pa*: *Peptostreptococcus anaerobius*; *Pb*: *Prevotella bivia*. GvPC.Model: *G. vaginalis* pre-conditioned biofilm formation model; C.Model: competitive biofilm formation model.

5.3.4 CLSM in situ *observation of the bacterial species integration in the triple-species biofilms*

By using PNA FISH/ DAPI method combined with CLSM, we sought to analyse the integration of the tested bacterial species in the triple-species biofilms. We noted that, on average, bacterial species appeared more equally distributed in the pre-conditioned model, while in the competitive model, they had more the tendency to aggregate in clusters (Figure 5.5A). The three microscopic fields whose superposition resulted in the triple-species biofilm organization for each consortium are shown in Figure 5.5B.

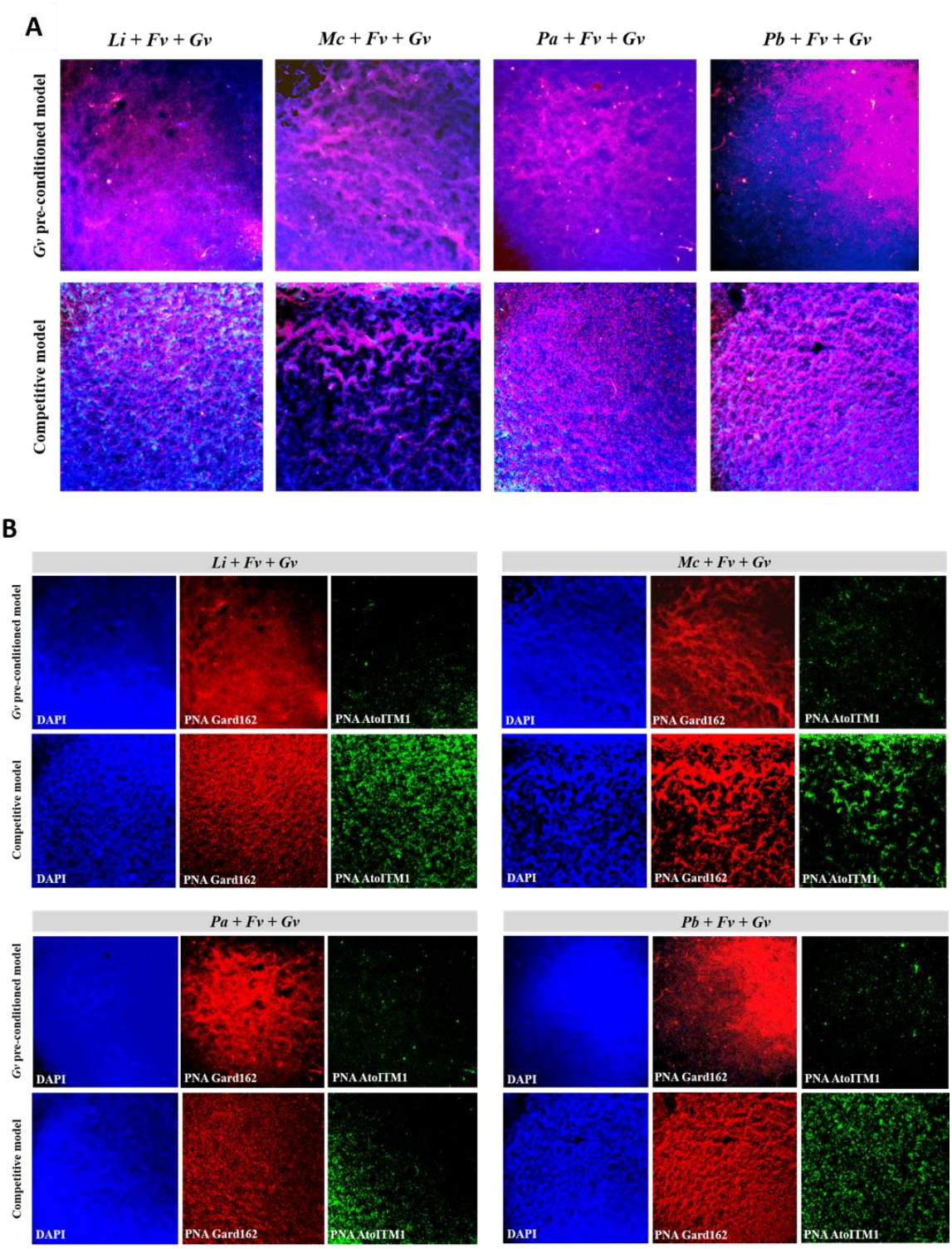

Figure 5.5. CLSM analysis of bacterial distribution in the intact structure of the triple-species BV-associated biofilms (A). Separate microscopic fields that form the structure of the triple-species biofilms **(B).** *G. vaginalis* is labelled with PNA probe Gard162 (red/ purple colour when coupled with DAPI), *F. vaginae* is labelled with PNA probe AtoITM1 (green/ green-blue colour when coupled with DAPI), and DAPI (blue colour) is used as a counterstain. The images were acquired with a 10× objective and with a resolution of 800 × 800 pixels. *Fv*. *Fannyhessea vaginae*; *Gv*. *Gardnerella vaginalis*; *Li*. *Lactobacillus iners*; *Mc*. *Mobiluncus curtisii*; *Pa*. *Peptostreptococcus anaerobius*; *Pb*. *Prevotella bivia*.

5.3.5 Impact of clindamycin and metronidazole on the different biofilm consortia biomass

Taking into consideration the differences observed between the two biofilm-forming models, we further wanted to determine whether those differences could influence the outcome of bacterial susceptibility to antibiotics. First, we determined the MIC and MLC of the planktonic cells of the six different strains used herein for clindamycin and metronidazole (Table 5.3), and then assessed their effect against all consortia characterized above.

Table 5.3. Susceptibility to clindamycin and metronidazole of the species used in this chapter.

Species	[a] Clindamycin, μg.mL^{-1}		[a] Metronidazole, μg.mL^{-1}	
	MIC [b]	MLC [c]	MIC	MLC
F. vaginae ATCC BAA-55^{T}	< 0.0625	0.0625	≥ 128	> 128
G. vaginalis ATCC 14018^{T}	< 0.0625	0.125	> 128	> 128
L. iners CCUG 28746^{T}	1	1	> 128	> 128
M. curtisii ATCC 35241^{T}	> 128	> 128	> 128	≥ 128
P. anaerobius ATCC 27337^{T}	> 128	> 128	4	4
P. bivia ATCC 29303^{T}	< 0.0625	< 0.0625	[4-8]	8

[a] To interpret the MIC results, the microbiological susceptibility and resistance breakpoints for clindamycin (≤ 2 μg.mL^{-1} and ≥ 8 μg.mL^{-1}) and metronidazole (≤ 8 μg.mL^{-1} and ≥ 32 μg.mL^{-1}) were used as defined by CLSI (23).

[b] MIC, minimal inhibitory concentration

[c] MLC, minimal lethal concentration.

Hence, we determined if the addition of each antibiotic would have an influence on the reduction of the total biomass of the mature biofilms (Figure 5.6). Interestingly, despite the differences in total biomass and relative bacterial composition in both models, no significant differences were observed for the antibiotics used between the two models, in most tested conditions, with two exceptions. The most striking difference occurred in the *P. anaerobius* consortium treated with metronidazole: while in the competitive model metronidazole only slightly reduced the growth rate of the biofilm, in the pre-conditioned model it was able to significantly reduce the total biomass of the consortium. The other exception occurred for the *P. bivia* consortium, where metronidazole slightly reduced the growth rate of the biofilm in the competitive model while in the pre-conditioned model, it only prevented the increase of total biomass growth. Not surprisingly, none of the antibiotics was able to completely eradicate the biofilms at the tested concentrations.

We also compared the impact of these two antibiotics on each consortium to the impact on *G. vaginalis* 24 h or 48 h single-species biofilm controls (Figure 5.6). Interestingly, when comparing to *G. vaginalis* single-species biofilms, having more species in the biofilm resulted in a lower metronidazole efficiency.

This was observed in all consortia formed in the competitive model and in two out of four consortia formed in the pre-conditioned model. Conversely, the same effect was not observed when using clindamycin.

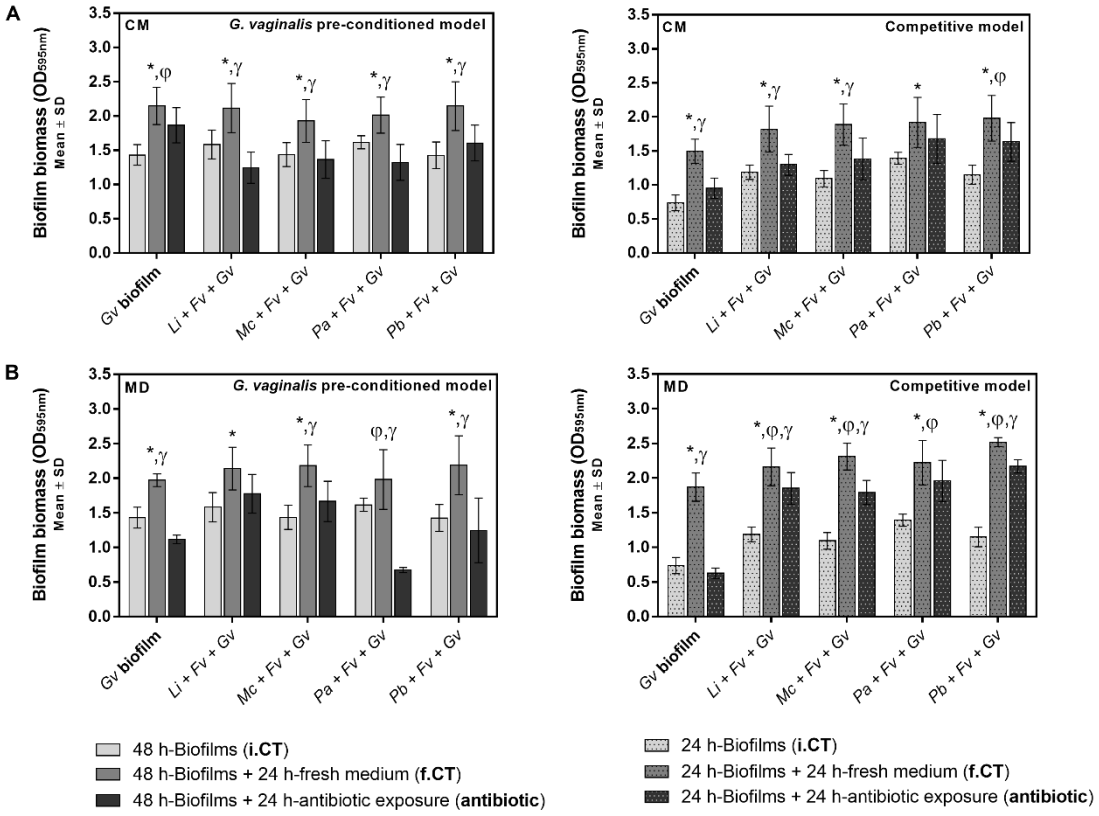

Figure 5.6. Effect of clindamycin (A) or metronidazole (B) on total biomass of *G. vaginalis* single-species biofilms as well as triple-species biofilms from both *in vitro* models. Biofilm biomass was quantified by the CV method and results are expressed as average OD at 595 nm ± standard deviation. The i.CT stands for initial control, before the medium replacement while the f.CT stands for final control, after incubation with fresh medium. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparisons test. * Values are significantly different between i.CT and f.CT ($p < 0.05$). φ Values are significantly different between i.CT and antibiotic ($p < 0.05$). γ Values are significantly different between f.CT and antibiotic ($p < 0.05$). CM: clindamycin; MD: metronidazole. *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Li*: *Lactobacillus iners*; *Mc*: *Mobiluncus curtisii*; *Pa*: *Peptostreptococcus anaerobius*; *Pb*: *Prevotella bivia*.

5.4 Discussion

The vaginal environment hosts a multitude of microbial species in variable quantities and relative proportions, which form a dynamic ecosystem and provide defence against infections (30). In BV, the components of this ecosystem undergo changes, usually being a lactobacilli-dominated microbiota replaced by a polymicrobial microbiota, consisting of strict and facultative anaerobic bacteria. It has been suggested that *Gardnerella* spp. play a pivotal role, initiating BV biofilm on the vaginal epithelium (1,11,13). It has also been described that *F. vaginae* is often associated with *Gardnerella* spp. biofilms during BV and is rarely detected without *Gardnerella* (11–13). Moreover, recent data indicate that at least in some cases, *P. bivia* is also detected in women with BV before or at the same time as *Gardnerella* (20).

However, not much is known how, in fact, bacterial species interact and contribute to the formation of the multi-species BV biofilm. An important limitation preventing such relevant studies is the fact that many BV-associated species are currently uncultivable (31–33).

It has been previously revealed that bacterial interactions within dual-species BV-biofilms are very specific, with some species enhancing biofilm growth, some reducing it, and others showing no interactions (15,18). These interactions also occur at the molecular level, as observed by up-regulation of key virulence gene expression in *Gardnerella* spp. (16). Furthermore, in **Chapter 4** we found that the triple-species biofilms of *G. vaginalis*, *F. vaginae*, and *P. bivia* resulted in a unique consortium that promoted a relevant shift in the overall bacterial biofilm composition, as compared to dual-species biofilms. As BV is a polymicrobial condition, more complex biofilms need to be characterized, since it is expected that with bigger consortia, interactions might become more complex, with less predictable outcomes.

Accordingly, in the present study, we selected *G. vaginalis*, *F. vaginae*, and one representative out of four of a third prominent species in BV (32,34) and aimed to understand how the interactions that might occur between these species would impact biofilm formation and antibiotic susceptibility. Furthermore, to better understand the role of *G. vaginalis* in the process of multi-species biofilm formation during BV, we introduced a new biofilm formation model that does not allow preceding biofilm formation by *G. vaginalis*. Interestingly, we showed that independently of the model used, (i) all three species in each consortium were able to form triple-species biofilms and (ii) *G. vaginalis* formed between 50 - 70% of the total number of cells in any of the biofilm conditions tested. These results suggest that, at least *in vitro*, the role of *G. vaginalis* in BV multi-species biofilm formation might be more relevant than just as the initial colonizer, as previously proposed (10). It should be taken into consideration that biofilm formation can generally be divided in 3 main stages: initial adhesion, biofilm maturation, and detachment (35). It was previously shown that while many BV-associated species can form a mature biofilm *in vitro*, they lack the ability to strongly adhere to a HeLa cell line (9), especially if HeLa cells were previously coated with *L. crispatus* (36,37).

Due to technical limitations, namely the effect of bacterial cytotoxicity in cell cultures (36), it is not feasible to incubate 24 h biofilms on human vaginal cell lines. As such, current biofilm formation models fail to address, simultaneously, the ability to displace the resident lactobacilli from the vaginal epithelium (initial adhesion stage) and the ability to grow in clusters of cells (biofilm maturation stage). Despite these limitations, our current work provided further evidence of the pivotal role of *G. vaginalis* in BV

development, not only due to its ability to dominate all tested consortia, but also taken into consideration the antimicrobial susceptibility experiments performed, as discussed next.

It has been suggested that interactions between species in biofilms can influence bacterial survival within the biofilm when it is exposed to antimicrobial compounds (38). In an *in vitro* dual-species biofilm model containing *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two major species associated with cystic fibrosis biofilms, it was observed that antimicrobial tolerance in the dual-species biofilms could not be predicted from the single-species susceptibility testing (39). In the current study, the MIC and MLC determinations failed to predict the outcome of the antimicrobial activity on biofilms. For instance, despite most isolates being sensitive, clindamycin was only able to prevent an increase in total biofilm biomass growth. Furthermore, when comparing the triple-species biofilms to *G. vaginalis* single-species biofilm, we observed evidence of synergistic interactions between the species, which promoted an increased tolerance to antibiotics: while metronidazole significantly reduced the total biomass of *G. vaginalis* biofilms, it was only able to slightly reduce the total biomass of the triple-species biofilms. *In vivo*, evidence of bacterial synergism towards antimicrobial increased tolerance has been previously pointed out. Bradshaw and colleagues (40) followed up 139 women with BV that were treated with oral metronidazole and examined at 1, 3, 6, and 12 months, and they concluded that recurrence rates of BV were very high (83%) in women colonized with both *Gardnerella* spp. and *F. vaginae*, suggesting that the association between these two bacteria enhanced the tolerance to metronidazole, with direct impact on treatment failure. Swidsinski and colleagues (41) also found high numbers of *Gardnerella* spp. and *F. vaginae* present on the vaginal epithelial cells after completion of metronidazole treatment, which led to BV recurrence.

Collectively, the evidence from this study points towards the idea that the way bacteria interact in BV together with the way the multi-species BV biofilms are formed can profoundly affect the treatment outcome. Therefore, interventions that could modify or block the synergistic relationships between co-infecting bacteria should be the target of future research.

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CHAPTER 6

Evaluation of *Thymbra capitata* essential oil antimicrobial activity in pure cultures and BV-associated multi-species biofilms

Summary

From the biofilm formation models characterized in **Chapter 5**, we selected the competitive biofilm formation model and used it herein to establish a multi-species biofilm consisting of six BV-associated species, which have not been previously described. We first aimed to characterize this biofilm consortia and then assess the effect of *Thymbra capitata* essential oil (EO) on its biomass and biofilm cells. The effect of EO was also evaluated against individual bacterial species grown planktonically and as biofilms. Under the experimental conditions considered in this chapter, our results revealed that *G. vaginalis* was the most prominent species in the multi-species biofilms, accounting for up to 65% of the total number of biofilm cells. Although *T. capitata* EO exhibited a high antibacterial effect against single-species biofilms biomass, it only presented a moderate effect on the biomass of the multi-species biofilms. Additionally, under the tested conditions, EO also manifested a moderate effect on the viability of cells from multi-species biofilms, mainly in the lower layers of their structure. This study demonstrates that bacterial consortia can act synergistically against antimicrobial stresses, which might help to explain the high recurrence rates associated with BV.

6.1 Brief introduction

A major problem for BV is the presence of a polymicrobial biofilm on the vaginal epithelium, which usually protects BV-associated species when exposed to antibiotics, often making these antibiotics ineffective against BV, resulting thus, in recurrent episodes (1). In addition, the frequent ineffectiveness of standard antibiotics against BV has been also related with high rates of bacterial resistance (2). Consequently, in an attempt to overcome BV treatment failure, natural compounds have been proposed as a promising and effective approach to treat this infection (3,4). Plant-derived products, such as essential oils (EOs), represent a therapy on the rise (5). EOs are very complex natural mixtures of volatile compounds produced by aromatic plants, which have been used since ancient times due to their medicinal properties (6). Furthermore, the use of EOs in case of human infections has been related to a low risk of development of antimicrobial resistance (7), which represents a great advantage compared to antibiotics.

Thymbra capitata (L.) Cav. [*Coridothymus capitatus* (L.) Rchb. F.] is a circum-Mediterranean plant belonging to Lamiaceae family, widespread in southern Portugal (Algarve). This plant is traditionally considered to show powerful antiseptic properties, being used for the treatment of cutaneous infections, such as acne (8), and in mouthwashes against gum infections (9). Several *in vitro* studies have found that *T. capitata* EO exhibited high antimicrobial activity against *Candida* spp. (10), *Listeria monocytogenes* (11), and *Aspergillus* species (12). More important, this EO also showed potent antibacterial activity against *Gardnerella* spp. planktonic cultures and biofilms (13). Nevertheless, besides *Gardnerella* spp., BV is also associated with a wide panoply of other anaerobic bacteria and thus, it is of utmost importance to also evaluate their susceptibility to *T. capitata* EO. Therefore, in the experiments presented in this chapter, it was intended to evaluate the antibacterial activity of *T. capitata* EO against six cultivable BV-associated species grown planktonically and as biofilm. Moreover, the effect of the EO was also tested against a multi-species BV biofilm in order to assess if biological interactions that might establish between bacterial species in these biofilms would affect the EO treatment outcomes.

6.2 Material and methods

6.2.1 Bacterial species and growth conditions

G. vaginalis (**Chapter 4**) and other five cultivable bacterial species used for the studies performed in **Chapter 3**, were also used in this chapter, namely *F. vaginae*, *L. iners*, *M. curtisii*, *P. anaerobius*, and *P. bivia*. The inoculum of each species was grown in NYC III broth supplemented with 10% (v/v) inactivated

horse serum (as described in **Chapter 3**) and incubated anaerobically using anaerobic gas generating sachets (Thermo Fisher Scientific) in sealed jars (Oxoid) at 37 °C for 24 h.

6.2.2 Single- and multi-species biofilm formation

Cultures of all six species considered in the present study were adjusted to a concentration of ~1.0×10^7 cells.mL^{-1} in NYC III broth (as mentioned in **Chapter 5**) and used for the single- and multi-species biofilm formation in 24-well tissue culture plates (Orange Scientific) or 8-well chamber slides (Thermo Fisher Scientific™ Nunc™ Lab-Tek™) for 24 h or 48 h at 37 °C under anaerobic conditions, as above mentioned. The multi-species biofilms consisted of all six species co-incubated simultaneously, being thus, formed by following the competitive biofilm formation model described in **Chapter 5**. The 48 h biofilms represented the biofilms to which fresh medium was added after the first 24 h of biofilm formation. After incubation, the medium covering the biofilms was carefully removed and the biofilms were washed once with sterile 1X PBS, being prepared for the further experiments.

6.2.3 Biofilm biomass quantification

The biomass of single- and multi-species biofilms formed in 24-well tissue culture plates was quantified by the CV method, as already described. Briefly, biofilms were fixed with 1 mL of 100% (v/v) methanol (Thermo Fisher Scientific) for 20 min and then stained with 1 mL of CV solution 1% (v/v) (Merck) for 20 min. Subsequently, the bound CV was released with 1 mL of 33% (v/v) acetic acid (Thermo Fisher Scientific) and the OD of the resulting solution was measured at 595 nm. All assays were repeated at least three times with two technical replicates.

6.2.4 Discrimination of G. vaginalis *population in multi-species biofilms by PNA FISH*

G. vaginalis population within the multi-species biofilms was discriminated by FISH method, which was previously described in **Chapter 4**. In brief, after fixing the biofilm suspension, PNA probe Gard162 was added on each well of epoxy coated microscope glass slides (Thermo Fisher Scientific). Before the microscopic visualization, each well of the glass slides was also covered with DAPI. Filters capable of detecting the PNA probe Gard162 and DAPI were used to randomly acquire twenty images for each sample. The number of bacteria was counted using *ImageJ Software* (14). All assays were repeated three independent times with technical duplicates.

6.2.5 Analysis of G. vaginalis *distribution in multi-species biofilms by CLSM*

To analyse the distribution of *G. vaginalis* in the intact structure of the multi-species biofilms, we used CLSM as described in **Chapter 4**. Briefly, after fixing the intact biofilms, these were first stained with PNA probe Gard162 and DAPI and then analysed by an Olympus™ FluoView FV1000 (Olympus) confocal laser scanning microscope, using a 40× objective and with a resolution of 640 × 640 pixels. The CLSM images were analysed using the *FV10-ASW 4.0 Viewer Software* (Olympus). All assays were repeated three times with two technical replicates.

6.2.6 EO extracted from T. capitata

T. capitata EO was used in this study in order to evaluate its antimicrobial activity against BV-associated species tested herein grown planktonically and as biofilms. The EO was provided by the Chemical Process Engineering and Forest Products Research Centre (CIEPQPF), Faculty of Pharmacy, University of Coimbra. The composition of the tested EO is described in Table 6.1. The EO was stored in glass vials at 4 °C, protected from light.

Table 6.1. Composition of the EO extracted from *T. capitata*.

Thymbra capitata EO	Composition of the EO (%)				
	Carvacrol	α-Terpinene	γ-Terpinene	Linalool	ρ-Cymene
A [a]	75	1.5	5.1	2.0	5.0

[a] Sample **A** of the EO extracted from *T. capitata*.

6.2.7 Determination of the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of T. capitata *EO*

MIC and MLC values of *T. capitata* EO were determined by broth macrodilution method in glass flasks (McCartney type), as previously performed (15), with some minor modifications. *T. capitata* EO in a concentration of 5 μL.mL^{-1} was diluted together with 5 μL.mL^{-1} of dimethyl-sulfoxide (DMSO, Scharlau, Spain) in 990 μL of NYC III broth. DMSO had the role to improve the solubility of EO in the culture medium. Then, serial dilutions of EO ranging between 2.5 μL.ml^{-1} and 0.08 μL.ml^{-1} were prepared in 500 μL of NYC III medium. Bacterial suspensions of 24 h cultures corresponding to each species considered in this study were adjusted to an OD of 0.10 ± 0.05 (Biochrom EZ Read 800 Plus) at 620 nm. Afterwards, 500 μL of the adjusted bacterial suspensions were added into the glass flasks with the prepared EO dilutions resulting a total volume of 1 mL. All glass flasks were incubated for 48 h at 37 °C in anaerobic conditions created by the anaerobic gas generating sachets (Thermo Fisher Scientific), as described

above. Negative and positive controls were also included being represented by, respectively, NYC III medium and bacterial suspensions. After incubation, the MICs were evaluated by observing the turbidity, macroscopically, compared to the negative and positive controls. MIC value was defined as the lowest concentration of EO that inhibited visible bacterial growth (absence of turbidity). Moreover, MIC results were confirmed by reading the OD at 620 nm of the tested dilutions. Subsequently, 10 µL from each dilution were inoculated on CBA plates in order to determine the MLC. The CBA plates were further incubated for up to 72 h at 37 °C in anaerobic condition, as aforementioned. MLC value was defined as the lowest concentration of EO that prevented the growth of treated bacteria on agar plates. The MIC and MLC assays were repeated at least three times on separate days.

6.2.8 Effect of T. capitata *EO on single- and multi-species biofilm biomass*

Biofilms were formed in 24-well tissue culture plates as above described and challenged with *T. capitata* EO in a concentration of 0.63 µL.mL^{-1} (Table 6.2) for 24 h at 37 °C under anaerobic conditions, as previously mentioned. Next, the spent medium was carefully removed, and the biofilms biomass were quantified by the CV method. Initial and final controls represented by single- and multi-species biofilms were included in each experimental assay. All assays were repeated at least three independent times with technical duplicates.

6.2.9 Effect of T. capitata *EO on cell viability from multi-species biofilms assessed by Live/ Dead staining combined with CLSM*

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Thermo Fisher Scientific), consisting of SYTO 9 and propidium iodide, was used to determine the viability of cells from multi-species biofilms exposed to EO. The multi-species biofilms were formed in 24-well culture plates for a period of 24 h, as previously described, and then, challenged with EO (Table 6.2) for 24 h at 37 °C in anaerobic conditions. At the bottom of each well of the 24-well culture plate, a sterile plastic coverslip with a diameter of 13 mm (Thermo Fisher Scientific™ Nunc™ Thermanox™) was placed. After incubation, the biofilms coating the coverslips were gently washed with 1X PBS and then, the coverslips were removed from the wells and placed on microscope glass slides (VWR). Two types of controls represented by live and dead biofilm cells not treated with EO were considered for this experiment. The dead control was obtained by covering the coverslips with the biofilms with 200 µL of 100% (v/v) methanol (Thermo Fisher Scientific) for 30 min. Then, all the coverslips with the live, dead, and EO treated biofilms were covered with 100 µL of the Live/ Dead staining mix, with SYTO 9 and propidium iodide used each in a concentration of 3 µL.mL^{-1}.

Subsequently, the coverslips were incubated for 15 min. in the dark at room temperature. Biofilm image stacks were acquired with an Olympus™ FluoView FV1000 (Olympus) confocal laser scanning microscope, using a 40× objective and a resolution of 640 × 640 pixels. Microscopic visualization was performed using lasers capable of detecting SYTO 9 (Laser 488, excitation wavelength 488 nm, emission wavelength 520 nm, BA505-540) and propidium iodide (Laser 559, excitation wavelength 559 nm, emission wavelength 618 nm, BA575-675). The CLSM images were analysed using the *FV10-ASW 4.0 Viewer Software* (Olympus). The experiment was performed in duplicate and repeated three times.

6.2.10 Effect of T. capitata *EO on cell culturability from single- and multi-species biofilms assessed by CFU counting*

To determine the culturability of cells from biofilms exposed to EO, we used CFU counting method. The single- and multi-species biofilms were formed in 24-well culture plates as mentioned above and exposed to EO (Table 6.2) for 24 h at 37 °C in anaerobic conditions. After 24 h, the biofilms were gently washed with 1X PBS, disrupted, and resuspended in NYC III. From each obtained biofilm suspension, 100 µL were transferred to a 1.5 mL tube with 900 µL of 0.9% (v/v) NaCl (VWR) and then, 10-fold serial dilutions were performed and plated onto CBA plates to allow CFU counting. The CBA plates were further incubated for up to 72 h at 37 °C in anaerobic condition, as aforementioned. Controls were represented by biofilms not exposed to the EO. At least three independent assays, with technical duplicates, were performed.

6.2.11 Statistical analysis

Statistical analysis was performed with GraphPad Prism version 7 (La Jolla). For comparisons among different groups, one-way and two-way ANOVA with multiple comparisons tests were used. A $p < 0.05$ was considered statistically significant.

6.3 Results

6.3.1. Multi-species biofilms characterization

As the multi-species biofilm consortia used in this chapter were not previously described, we first performed a preliminary characterization of the multi-species biofilms and then assess their response to the *T. capitata* EO. We started by quantifying the biomass of the multi-species biofilms in comparison to the single-species biofilms. We observed that in our *in vitro* conditions, independent of the incubation time used, the multi-species biofilms presented a significantly higher total biomass than the single-species biofilms, with exception of *L. iners* grown for 24 h (Figure 6.1A). Interestingly, *G. vaginalis* was the

dominant species in the multi-species biofilms, accounting for up to ~65% of the total number of cells, as determined by PNA FISH (Figure 6.1B), similar to what we found in triple-species biofilms (**Chapter 5**). CLSM analysis further confirmed the dominance of *G. vaginalis* in the biofilm structure and revealed that this species was well distributed across the biofilm, in small clusters of cells (Figure 6.1C).

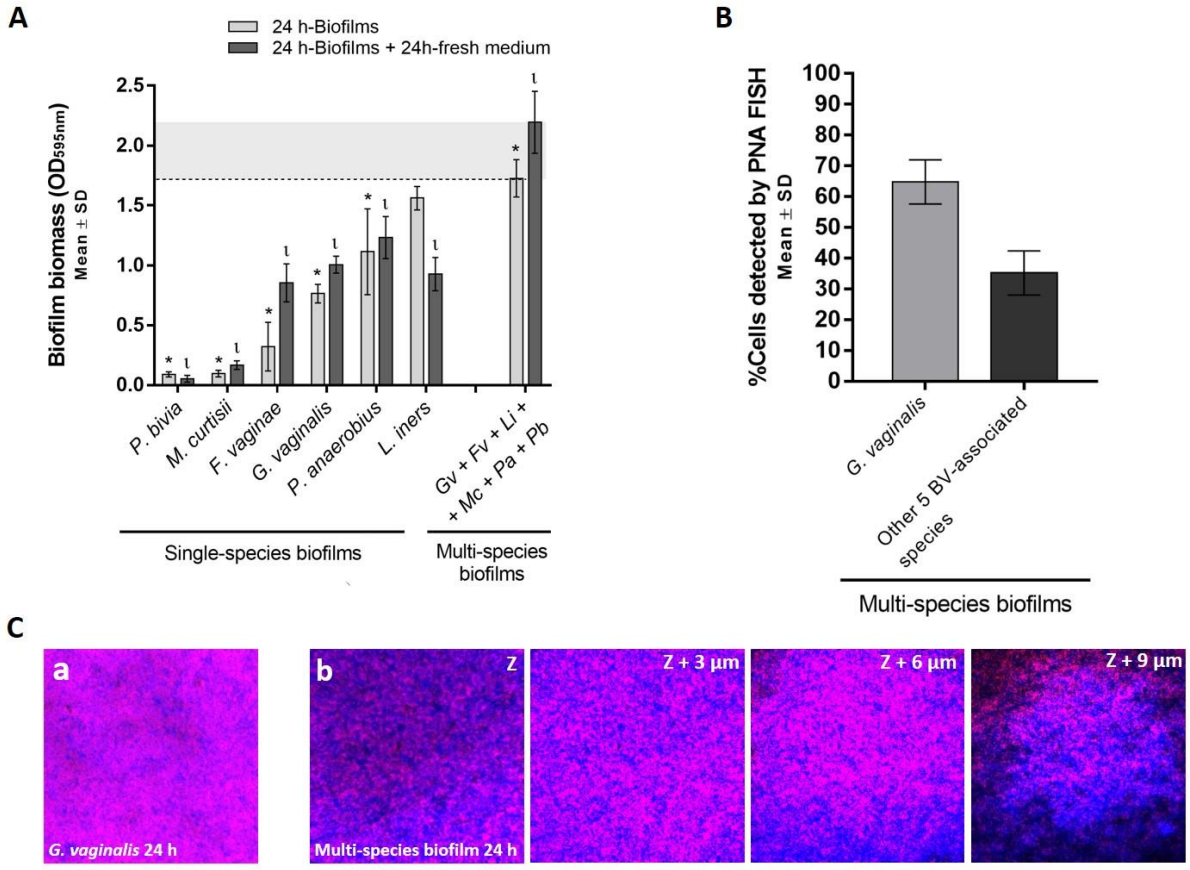

Figure 6.1. Characterization of single- and BV multi-species biofilms grown under *in vitro* conditions. (A) Total biomass of single- and multi-species BV-associated biofilms was determined by staining with CV. Results represent the mean ± standard deviation of at least three independent assays, with two technical replicates assessed each time. **(B)** Percentage of *G. vaginalis* cells detected by PNA FISH from 48 h multi-species biofilm. Results are expressed as mean ± standard deviation of three independent assays. **(C)** Example of data set on the organization of the multi-species BV-associated biofilms by CLSM. **a** *G. vaginalis* single-species biofilm labelled with PNA-probe Gard162 (purple colour when combined with DAPI) and DAPI (blue). **b** CLSM images of z-stacks acquired from multi-species biofilms stained with the probe Gard162 for *G. vaginalis* (purple colour when combined with DAPI) and DAPI (blue) for other BV-associated species. Statistically significant differences between multi-species and single-species biofilms are represented with * for 24 h incubation time and with τ for 48 h incubation time (one-way ANOVA and Dunnett's multiple comparisons test, $p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Li*: *Lactobacillus iners*; *Mc*: *Mobiluncus curtisii*; *Pa*: *Peptostreptococcus anaerobius*; *Pb*: *Prevotella bivia*.

6.3.2 Susceptibility of BV-associated species planktonic cells to T. capitata *EO*

In vitro antibacterial activity of the EO against each species tested herein was evaluated by determining both MIC and MLC values. As can be seen in Table 6.2, the EO showed a moderate antimicrobial effect against BV-associated bacteria, with slight variations in some cases. Taking into account that for all six

species tested, the EO highest MLC value was 0.63 µL.mL^{-1}, this was the concentration further considered for the work performed in this chapter.

Table 6.2. Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of *T. capitata* EO for planktonic cells of BV-associated bacteria.

Bacteria	MIC (µL.mL^{-1})	MLC (µL.mL^{-1}) [a]
F. vaginae ATCC BAA-55^{T}	[0.16 - 0.31]	[0.31 - 0.63]
G. vaginalis ATCC 14018^{T}	0.31	0.63
L. iners CCUG 28746^{T}	0.31	[0.31 - 0.63]
M. curtisii ATCC 35241^{T}	[0.16 - 0.31]	[0.31 - 0.63]
P. anaerobius ATCC 27337^{T}	0.63	0.63
P. bivia ATCC 29303^{T}	0.31	[0.31 - 0.63]

[a] The MLC concentration of 0.63 µL.mL^{-1} was considered for the work performed in this chapter.

6.3.3 Impact of T. capitata *EO on biomass of single- and multi-species biofilms*

As BV is a biofilm-associated infection, we further sought to determine whether EO at MLC concentration (0.63 µL.mL^{-1}) could have an impact on the total biomass of *in vitro* BV-associated biofilms, assessing first the effect of EO on biomass of single-species biofilms. We observed that for all tested species, the reduction of the total biomass of the single-species biofilms was significantly elevated, with exception of *P. bivia* and *M. curtisii*, for which it was not possible to determine the EO effect due to a very low amount of biofilm biomass formed under these conditions (Figure 6.2). Additionally, our results revealed that the EO at MLC concentration had a significant reduction effect on the total biomass of the multi-species biofilms, however only when compared to the corresponding multi-species biofilm controls.

6.3.4 T. capitata *EO effect on cell viability from multi-species biofilms assessed by Live/ Dead staining*

Despite *T. capitata* EO strong ability to reduce most single-species biofilms biomass, a significant amount of biomass remained in the multi-species biofilms. To better address this phenomena, multi-species biofilms were observed with CLSM using Live/ Dead staining method. As can be observed in Figure 6.3, while the most of the remaining cells (after EO challenge) within the multi-species biofilms showed some level of cell wall damage (as noted by the yellow or orange colour), there were still some totally viable cells (as noted by the green colour), especially in the lower layers of the biofilm.

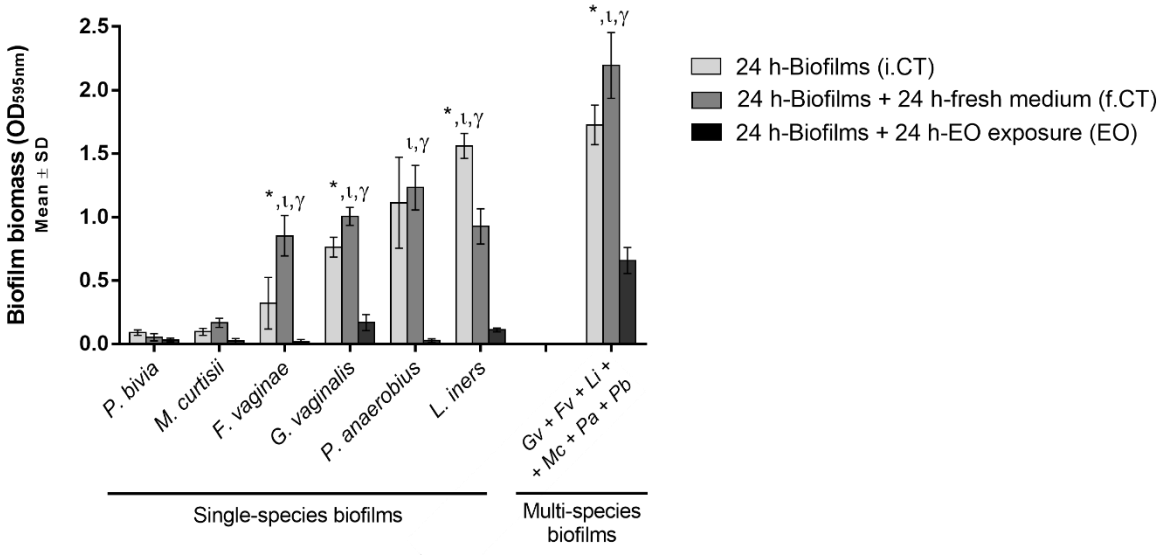

Figure 6.2. Effect of *T. capitata* EO on biomass of single- and multi-species biofilms of BV-associated bacteria. Biofilm biomass was quantified using the CV staining assay. i.CT stands for initial control, before the medium replacement; f.CT stands for final control, after incubation with fresh medium. Results represent the mean ± standard deviation of at least three independent assays. Values are significantly different for * i.CT versus f.CT, $^{\tau}$i.CT versus EO, and $^{\gamma}$f.CT versus EO (two-way ANOVA and Tukey's multiple comparisons test, $p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Li*: *Lactobacillus iners*; *Mc*: *Mobiluncus curtisii*; *Pa*: *Peptostreptococcus anaerobius*; *Pb*: *Prevotella bivia*.

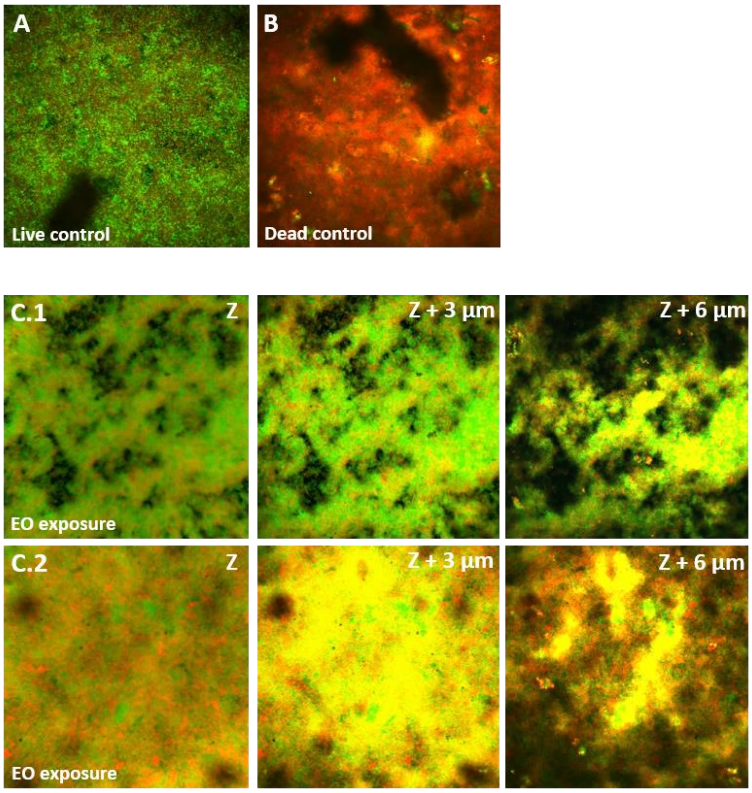

Figure 6.3. Effect of *T. capitata* EO on multi-species biofilms assessed by Live/ Dead staining. (A) 48 h multi-species biofilm without EO treatment with viable cells visualized in fluorescent green. **(B)** 48 h multi-species biofilm without EO treatment with dead cells appearing in red/ orange. **(C)** Multi-species biofilm exposed to EO at 0.63 µL.mL^{-1} for 24 h. Example of two sets (C.1 and C.2) of z-stack CLSM images acquired with a 40× objective in different biofilm regions.

6.3.5 T. capitata *EO influence on cell culturability from single- and multi-species biofilms*

As we showed that multi-species biofilms exposed to the *T. capitata* EO still contained viable cells, we decided to also quantify bacterial culturability after EO challenge. Interestingly, both on single- and multi-species biofilms, the EO had the ability to reduce in 100% cell culturability (Figure 6.4).

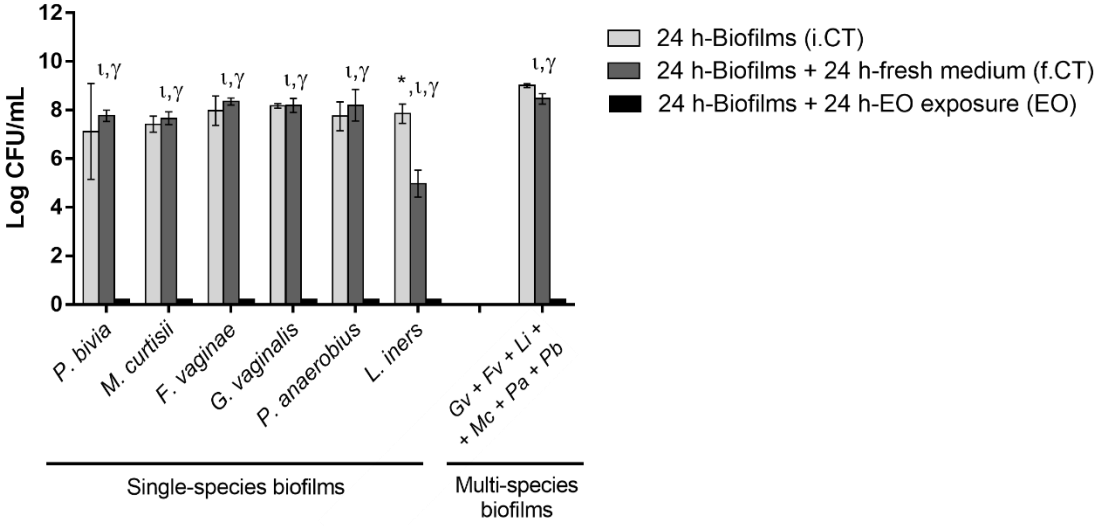

Figure 6.4. Effect of *T. capitata* EO on cell culturability from single- and multi-species biofilms of BV-associated bacteria. The effect of EO on cell culturability was determined by performing CFU. i.CT stands for initial control, before the medium replacement; f.CT stands for final control, after incubation with fresh medium. Results represent the mean ± standard deviation of at least three independent assays. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparisons test. Values are significantly different for * i.CT versus f.CT, $^{\tau}$i.CT versus EO, and $^{\gamma}$f.CT versus EO ($p < 0.05$). *Fv*: *Fannyhessea vaginae*; *Gv*: *Gardnerella vaginalis*; *Li*: *Lactobacillus iners*; *Mc*: *Mobiluncus curtisii*; *Pa*: *Peptostreptococcus anaerobius*; *Pb*: *Prevotella bivia*.

6.4 Discussion

In spite of the fact that existing antibiotics against BV are somewhat effective, management of this infection continue to be challenging (16,17). The polymicrobial BV biofilm is considered one of the major factors responsible for the treatment failure since it becomes metabolically inactive upon treatment, leading to decreased susceptibility to antibiotics, and this may be further contributing to high BV recurrence rates (18).

As described in **Chapter 2**, some alternative approaches to existing antibiotics are being studied against BV, showing promising results (19–21). One of these therapies emerges from plant-derived compounds, namely *T. capitata* EO. As this EO previously showed a potent antibacterial activity against *Gardnerella* spp. growing in planktonic cultures and in biofilms (13), in this chapter, we aimed to assess its effect on other BV-associated species also grown planktonically and as biofilms. Furthermore, in order to

understand if bacterial interactions in BV polymicrobial biofilms would influence the EO effect, we used herein a multi-species biofilm consisting of six BV-associated species and tested the EO against it.

As such, we first performed a preliminary characterization of the multi-species biofilms since these were not previously described. Interestingly, we found that independent of the incubation time (i) the multi-species biofilms showed a considerably higher biofilm biomass than the single-species biofilms, with only one exception, and (ii) *G. vaginalis* was the predominant species in the multi-species biofilms, similar to what has been described for dual- and triple-species biofilms (**Chapter 4** and **Chapter 5)**. Unfortunately, since no PNA FISH probes exist for most of the other species, we could not differentiate the relative composition of all species using this experimental technique. While there are other alternative techniques for bacterial discrimination (22–24), due to time limitations, those experiments could not be performed. Thus, while all six species were inoculated in the biofilm, we cannot claim that after the 24 h and 48 h incubation period, all six species were present. Indeed, cumulating evidence from experimental and metabolic model-based studies demonstrated that often microorganisms compete for limited resources, such as space and nutrients (25–27). However, cooperation between certain species in the community are still present, leading to enhanced biomass production (28,29), access to complex nutrient sources (30) or stress resistance (31,32). Therefore, in the future, we need to experimentally determine if all six species were present at the end of the incubation period.

As shown in **Chapter 5**, interactions between the species in some triple-species biofilm consortia promoted an increased tolerance to antibiotics, and, as such, we hypothesized that a similar effect could happen in the present study. Indeed, *T. capitata* EO at 0.63 $\mu L.mL^{-1}$ was effective in reducing most biomass of single-species biofilms, but lost some efficiency when applied in multi-species biofilms, which further demonstrates synergisms between BV-associated species that enhance tolerance to antimicrobial agents. A similar effect has been demonstrated for other multi-species bacterial biofilms after exposure to various antibacterial agents (29,32).

It is acknowledged that CV staining method used in this study for biofilm quantification is an easy and fast-performing procedure to analyse bacterial biofilm formation ability (33). However, as it stains the extracellular matrix and all cells, including the dead ones, CV staining does not give a measure of biofilm cells viability and therefore, other methods must be used to evaluate the EO antimicrobial effect on multi-species BV biofilms (34). Consequently, we further aimed to reveal if there are still viable cells, using the Live/ Dead staining method combined with CLSM, in particular in the multi-species biofilms, which had a higher amount of total biomass after EO challenge. The Live/ Dead kit consists of a mixture of two

stains, SYTO 9 and propidium iodide, which differ both in their spectral characteristics and in their ability to penetrate bacterial cells. While, when both dyes are used, SYTO 9 labels bacteria with intact cell membranes which then become fluorescent green, propidium iodide penetrates only bacteria with damaged membranes, staining them in fluorescent red/ yellow-orange (35). According to this method, we noticed that in our tested conditions, the EO affected the multi-species biofilms in a relative different way: while upper biofilm layers had regions with a high number of damaged cells, the bottom layers still presented some regions with viable cells. These results might explain the high recurrence rates of BV after the antimicrobial treatment. It is acknowledged that the biofilm can serve as a protective barrier, and its thickness and chemical composition can limit the perfusion and/ or activity of antimicrobial compounds (36). This specific feature may lead to the protection of a minor fraction of cells which, in most cases, will be further able to reinitiate the biofilm formation, and as such, contribute to recurrent infections.

The presence of viable cells in the biofilms challenged with EO led us to perform the CFU counting method in order to assess bacterial culturability. Curiously, the EO inhibited in 100% the culturability of biofilm cells, being thought that perhaps the viable cells noticed with the CLSM were cells in a viable but non-culturable (VBNC) state, as has been demonstrated for other bacterial biofilms after antibiotic exposure (37). It is believed that VBNC cells are cells in a stage preceding cell death or adaptation to stress (38), but can eventually recover and initiate cell division (38).

Taken together, our data suggest that the relationships among co-infecting bacteria present in the vaginal environment during BV can impact the treatment outcome. Our results also show that *T. capitata* EO may represent a potent agent against BV biofilm. However, further investigations are required to determine if the EO would maintain the antimicrobial activity *in vivo*. In this sense, an *ex vivo* vaginal mucosa model would be an adequate approach to assess that.

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CHAPTER 7

Conclusions and future perspectives

Summary

This chapter presents the main findings and limitations of the studies conducted in this thesis as well as addresses future perspectives.

7.1 Conclusions

Despite advances in understanding BV, there is still a gap in knowledge regarding the relationships that bacteria might establish in the BV polymicrobial biofilm and their impact on BV pathogenesis and treatment outcome. The presence of this biofilm makes BV difficult to treat, as it favours the development of antibiotic resistance, leading to serious concerns for women due to the recurrent episodes they usually present after therapy. Uncovering how BV-associated bacteria interact in the vaginal environment and contribute to the BV biofilm formation could facilitate the development of new therapeutic strategies to cure BV.

As such, the main goal of this study was to better understand the importance of microbial interactions in BV and how they impact BV pathogenesis and the treatment result. The evidence from this work suggests that microbial interactions in BV multi-species biofilms impact the biofilm structure and composition and influence the effect of antimicrobial challenge, which will likely influence BV outcome.

The findings described in this thesis provided answers to the main three research questions formulated in **Chapter 1**.

1. **Are non-*Gardnerella* BV-associated species able to form *in vitro* single-species biofilms under the same experimental conditions?**

While most previously *in vitro* studies focused on *Gardnerella*-mediated biofilm formation, herein it was demonstrated that under appropriate conditions, other cultivable BV-associated species, namely *F. vaginae*, *L. iners*, *M. curtisii*, *P. anaerobius*, and *P. bivia*, could also form single-species biofilms. This was an optimization step needed to better compare the integration of each species in the biofilm consortia formed and characterized throughout this thesis.

2. **Can interactions between *G. vaginalis* and other BV-associated species in triple-species *in vitro* biofilms be key in BV development and antimicrobial susceptibility?**

Since it has been suggested that *Gardnerella* spp. play a pivotal role, initiating BV biofilm on the vaginal epithelium (1–3), we aimed to understand if the presence of a *G. vaginalis* pre-established biofilm would influence the ability of known BV-associated species to form triple-species biofilms. To assess that, two *in vitro* biofilm formation models were used, with and without allowing *G. vaginalis* to form an early biofilm before adding the other two species. We found that independent of the model used, all species were able to form triple-species biofilms. At the same time, *G. vaginalis* maintained predominance in all consortia

from both tested models, suggesting that, at least *in vitro*, the role of *G. vaginalis* in BV multi-species biofilm formation might be more significant than just as the initial colonizer.

Interestingly, when comparing *G. vaginalis* single-species biofilm to the triple-species biofilms, we noticed evidence of synergistic interactions between the species in some consortia, which promoted an increased tolerance to antibiotics, mainly metronidazole. Therefore, while metronidazole was able to significantly reduce the total biomass of *G. vaginalis* biofilm, it only slightly reduced the total biomass of the triple-species biofilms. Collectively, this suggests that, indeed, interactions between *G. vaginalis* and other species in BV biofilm can impact BV development and antimicrobial susceptibility.

3. Do interactions in multi-species BV-associated biofilms affect *T. capitata* EO treatment outcomes?

T. capitata EO was previously shown as a potent anti-biofilm agent against *Gardnerella spp*. (4). As interactions in some of the tested triple-species biofilms contributed to an enhanced tolerance to antibiotics, we hypothesized that a similar effect could occur in the case of the multi-species biofilms exposed to EO. Not surprisingly, we noticed that *T. capitata* EO was effective in reducing almost totally the biomass of single-species biofilms but lost some efficiency against the multi-species biofilms biomass, suggesting that the cooperation between BV-associated bacteria may increase the tolerance to antimicrobial agents. Importantly, we also found that the multi-species biofilms, in some regions, still presented viable cells in the lower layers after EO exposure, and this might explain the high recurrence rates of BV.

7.2 Study limitations

The results from our study should be interpreted considering some limitations. The first is that our work on triple-species biofilm formation models should have included more triple-species consortia taking into account other bacterial species associated with BV as the third species (5–7), however, due to time restrictions, we had to focus on some of the most common BV species. Furthermore, many other BV-associated species are unculturable, or difficult to isolate and work with *in vitro* conditions. Second, even if our culture medium of choice, NYC III, is a nutritionally rich growth medium (as described in **Chapter 3**), it did not contain all the factors found *in vivo*, and some *in vivo* key components may influence microbial interactions and BV biofilm formation. Third, as also mentioned in **Chapter 5**, because of the bacterial cytotoxicity in cell cultures (8), the biofilms were formed in polystyrene 24-well tissue culture plates rather than on cells from a human vaginal cell line previously coated with *L. crispatus*. In addition,

the formed *in vitro* biofilms were not subjected to host defence mechanisms. Collectively, these limitations point towards the idea that a more complex biofilm model should be used for further research to reflect better the *in vivo* conditions.

7.3 Future perspectives

Although this work provided answers to different aspects about the importance of microbial interactions in BV and how they affect BV development and treatment outcome, many other questions remain open to be addressed in the near future.

First, as evaluated by CV staining method, an increase in the total biofilm biomass was observed only for the triple-species biofilms for the competitive biofilm-forming model. As previously mentioned, CV staining is an easy and fast procedure to quantify total bacterial biofilm mass (9). However, a limitation of this method is that total biomass direct comparison between species is not feasible, since different species produce distinct biofilm matrices and have different cell sizes (10,11). Also, within multiple-species biofilms, this method does not enable to quantify the contribution of each species. Therefore, for a better assessment of how multi-species biofilms are established, total cells count approach (12) should be used in future works.

Second, while bacterial discrimination in the triple-species biofilms was performed for comparison between pre-conditioned and competitive biofilm forming models, there was not enough time to perform a similar analysis on the biofilms after exposure to antibiotics. This experiment would likely reveal why only some of the triple-species biofilms showed an increased tolerance to antibiotics, highlighting the individual roles of each species on the antimicrobial susceptibility profile.

A similar approach should be performed on the multi-species biofilms after *T. capitata* EO exposure. However, since we cannot use PNA FISH to perform this work due to the unavailability of PNA probes for most of the species, genomic DNA extraction and quantification by quantitative real time PCR (qRT-PCR) could be used (13–15) in order to determine the percentage distribution of each species in these biofilms prior and after EO treatment.

Third, *G. vaginalis* was used for most of our studies in this thesis. However, considering the recent description of three new species in the genus *Gardnerella*, mainly *G. leopoldii*, *G. piotii*, and *G. swidsinskii* (16), future work needs to be done including isolates of these species (and combinations of these species)

in order to assess the possible differences between them in terms of bacterial interactions with the other BV-associated species and role in multi-species biofilm formation, compared to *G. vaginalis*. Lastly, to confirm the findings from this study as well as to perform future work, an *ex vivo* vaginal model should be used, such as porcine vaginal mucosa (17), which could allow a closer imitation of *in vivo* conditions.

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