Universidade do Minho
Escola de Engenharia

Ângela Martins Lima

Assessing triggers of bacterial vaginosis development by assessing microbial interactions during initial adhesion

julho de 2026

Ângela Martins Lima **Assessing triggers of bacterial vaginosis development by assessing microbial interactions during initial adhesion**

Uminho | 2026

Universidade do Minho
Escola de Engenharia

Ângela Martins Lima

Assessing triggers of bacterial vaginosis development by assessing microbial interactions during initial adhesion

Tese de Doutoramento
em Engenharia Biomédica

Trabalho efetuado sobre a orientação do
Doutor Nuno Cerca
da
Doutora Christina A. Muzny
E da
Doutora Joana Castro

julho de 2026

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.
Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.
Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.



Atribuição-NãoComercial-SemDerivações
CC BY-NC-ND

https://creativecommons.org/licenses/by-nc-nd/4.0/

ACKNOWLEDGEMENTS

Ao longo desta jornada foram várias as pessoas que contribuíram para a realização do meu doutoramento, as quais eu quero muito agradecer. Começo por agradecer ao meu orientador, **Doutor Nuno Cerca**, por me ter dado a oportunidade de realizar o meu doutoramento com ele e a sua equipa. Agradeço pelo acompanhamento regular do meu trabalho, pela partilha de conhecimento e sugestões dadas com o propósito de tornar o meu trabalho melhor. To **Doctor Christina Muzny**, I want to thank for the good suggestions made to my work and the motivational messages that are so important during such challenging times. Sou também grata à **Doutora Joana Castro**, por me ter recebido tão bem no laboratório do grupo NC no início do meu doutoramento, pela partilha de conhecimento laboratorial, e por ao longo destes anos ter estado sempre disponível para me ajudar. Quero também agradecer às minhas colegas do **grupo NC**, atuais e antigas, pelo bom ambiente de trabalho sempre presente no laboratório e pelo espírito de equipa. Agradeço à **Lúcia**, **Ângela França**, **Inês Lameira**, **Ana Sofia** e **Filipa Macedo**, pela contribuição no trabalho aqui apresentado. Sou grata à Lúcia também por muitas vezes me ter ajudado a esclarecer dúvidas relacionadas com o meu trabalho. Agradeço também pela amizade e bons momentos vividos durante o meu doutoramento às minhas amigas do laboratório: **Nathalie**, **Lúcia**, **Inês Santos**, **Sofia**, **Vânia Pinheiro**, **Susana**, **Paula**, **Tânia**, **Taynara**, **Filipa Castro** e **Ana Paula**. Sem vocês, esta jornada teria sido bem mais difícil! Um agradecimento especial à **Doutora Nicole** e ao **Doutor Luís Melo**, pelo auxílio no uso do citómetro, por todas as horas que passaram comigo a explicar-me o seu funcionamento. Por fim, quero agradecer à minha **mãe** por me ter sempre motivado para concluir este trabalho e especialmente ao meu **irmão Vasco**, por ser o meu pilar e o meu melhor amigo! Aproveito para agradecer aos meus amigos pela força que me deram.

This study was supported by the Portuguese Foundation for Science and Technology (FCT) through an individual PhD scholarship (2022.13112.BD). This work was also funded by FCT under the strategic funding of the unit UID/BIO/04469/2020. This study was also supported through funds of the National Institute Allergy and Infectious Diseases, the funded project PR01AI146065-01.













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.

Avaliação dos fatores desencadeantes do desenvolvimento da vaginose bacteriana através da análise das interações microbianas durante a adesão inicial

RESUMO

Apesar dos esforços significativos desenvolvidos para esclarecer a etiologia da vaginose bacteriana (VB), a sequência de eventos subjacente à transição de uma microbiota vaginal dominada por espécies de *Lactobacillus* para uma microbiota dominada por anaeróbios permanece por elucidar. Atendendo à associação da VB a complicações obstétricas, ao aumento da suscetibilidade a infeções sexualmente transmissíveis e a impactos negativos na saúde mental, o desenvolvimento de estratégias terapêuticas eficazes constitui uma prioridade dependente de uma compreensão mais aprofundada da sua etiologia. Estudos recentes demonstram que múltiplos subgrupos de *Gardnerella* coexistem frequentemente na microbiota vaginal de mulheres com VB, tendo sido proposto que espécies de *Gardnerella* atuem como colonizadores iniciais do epitélio vaginal, contudo, a evidência experimental permanece limitada. Com o objetivo de aprofundar o conhecimento sobre a colonização inicial por espécies de *Gardnerella* e as interações estabelecidas, avaliou-se a adesão de combinações pareadas de quatro espécies de *Gardnerella* num modelo *in vitro* de células HeLa, na presença e ausência de *L. crispatus* previamente aderido, bem como o seu potencial efeito protetor durante a adesão inicial de duas espécies de *Gardnerella*. Para tal, foi desenvolvido um método de quantificação de Lactobacilos baseado em microscopia de fluorescência e aperfeiçoada uma abordagem molecular por qPCR, permitindo a quantificação precisa de lactobacilos e de espécies de *Gardnerella* utilizadas nos ensaios de adesão; a microscopia de fluorescência indireta possibilitou a quantificação rigorosa de bactérias fastidiosas e o método molecular foi otimizado para melhorar o desempenho. Nos ensaios de adesão sem *L. crispatus* previamente aderido, observou-se efeito sinergístico entre a maioria das combinações das espécies de *Gardnerella*. Todas as combinações, com exceção das que incluíam *G. leopoldii*, evidenciaram capacidade sinérgica para destacar *L. crispatus* das células HeLa. Adicionalmente, tanto culturas simples como culturas de duas espécies de *Gardnerella* apresentaram níveis de adesão inferiores quando *L. crispatus* se encontrava previamente aderido, sugerindo um possível efeito protetor face às espécies de *Gardnerella*. Embora o presente estudo in vitro não demonstre de forma conclusiva que a VB seja desencadeada pela disrupção da microbiota vaginal normal por espécies de *Gardnerella*, fornece evidência substancial que sustenta esta hipótese.

Palavras-chave: Adesão, *Gardnerella*, *Lactobacillus*, qPCR, vaginose bacteriana.

Assessing triggers of bacterial vaginosis development by assessing microbial interactions during initial adhesion

SUMMARY

Despite extensive efforts to uncover the etiology of bacterial vaginosis (BV), the sequence of events leading from a Lactobacillus-dominated to an anaerobe-dominated vaginal microbiota remains unclear. As BV is associated with pregnancy complications, increased susceptibility to sexually transmitted infections, and adverse mental health effects, developing effective therapies is crucial and depends on a clearer understanding of its etiology. Recent studies show that multiple *Gardnerella* subgroups often coexist in the vaginal microbiota of women with BV. A conceptual framework proposes that *Gardnerella* species act as early colonizers of the vaginal epithelium, however, experimental evidence remains limited. To better understand the initial colonization of *Gardnerella* spp. and their interactions, we examined the adhesion of pairwise combinations of four *Gardnerella* spp. using an in vitro HeLa cell model, with and without pre-adhered *L. crispatus*. We also assessed the potential protective effect of *L. crispatus* during the initial adhesion of dual *Gardnerella* species. To support these experiments, we developed a fluorescence microscopy-based method for *Lactobacilli* quantification and further refined a qPCR molecular approach to accurately quantify *Lactobacillus* and *Gardnerella* species used in adhesion assays. The indirect fluorescence microscopy technique enabled accurate quantification of fastidious bacteria, and the molecular method was optimized for improved performance. In adhesion assays without pre-adhered *L. crispatus*, synergistic effect was observed among most combinations of *Gardnerella* spp. All pairwise combinations, except those including *G. leopoldii*, showed synergistic ability to displace *L. crispatus* from HeLa cells. Additionally, both single- and dual-species *Gardnerella* cultures adhered at lower levels when *L. crispatus* was pre-adhered. These findings suggest a protective effect of *L. crispatus* against *Gardnerella* spp. Although this in vitro study does not definitively demonstrate that BV is triggered by disruption of the normal vaginal microbiota by *Gardnerella* spp., it provides substantial evidence supporting this hypothesis.

Keywords: Adhesion, bacterial vaginosis, *Gardnerella*, *Lactobacillus*, qPCR.

INDEX

ACKNOWLEDGEMENTS ..... **III**

RESUMO ..... **V**

SUMMARY ..... **VI**

LIST OF FIGURES ..... **XI**

LIST OF TABLES ..... **XV**

LIST OF ABBREVIATIONS ..... **XVI**

LIST OF PUBLICATIONS ..... **XVII**

PAPERS IN PEER-REVIEWED JOURNALS ..... **XVII**

POSTERS COMMUNICATIONS IN SCIENTIFIC CONFERENCES ..... **XVIII**

ORAL COMMUNICATION IN SCIENTIFIC CONFERENCE ..... **XVIII**

CHAPTER 1 Introduction **1**

1.1. Background 2

1.2. Main research question 2

1.3. Hypothesis and aims 3

1.3.1. Hypothesis 3

1.4. Significance 4

1.5. Thesis outline 4

1.6. References 7

CHAPTER 2 Literature Review **9**

2.1. The vaginal microbiome 10

2.2. Bacterial vaginosis (BV) 11

2.2.1. Clinical features and diagnosis of BV 12

2.2.2. Epidemiology 13

2.2.2.1. Epidemiology of BV in Portugal 15

2.2.3. Etiology 16

2.2.4. Bacterial species in BV 17

2.2.4.1. Gardnerella spp. 18

2.2.4.1. Presence of Gardnerella spp. in the vaginal microbiota of healthy women 19

2.2.4.1.2. Genotyping, biotyping, ecotyping of *Gardnerella* spp. 20
2.2.4.1.3. Cooccurrence of Gardnerella subgroups/clades in vaginal microbiota 23
2.2.4.1.4. Functional analyses of virulence factors of *Gardnerella* spp. 24
2.2.4.2. Prevotella spp. 26
2.2.4.2.1. *Prevotella* spp. in the vaginal microbiota 27
2.2.4.2.2. Functional analysis of virulence factors of vaginal *Prevotella* spp. 27
2.2.4.3. Fannyhessea vaginae 28
2.2.4.3.1. *Fannyhessea vaginae* in the vaginal microbiota 28
2.2.4.3.2. Functional analysis of virulence factors of *Fannyhessea vaginae* 29
2.2.5. The role of biofilm in BV 29
2.2.5.1. Interactions between BV-associated bacteria within the polymicrobial biofilm 32
2.3. Treatment of BV 35
2.4. The nature of vaginal bacteria and the methods used to quantify them 38
2.5. References 39

CHAPTER 3 Development of an indirect fluorescence microscopy method to quantify vaginal lactobacilli 63

3.1. Contextualization of the experimental chapter 64
3.2. Materials and Methods 65
3.2.1. Culture conditions and strains 65
3.2.2. Colony-forming units counting method 65
3.2.3. Flow cytometry (FC) quantification 65
3.2.4. Visualization of bacteria and yeast in the Neubauer chamber 66
3.2.5. Fluorescence microscopy (FM) quantification of total counts 66
3.2.6. Statistical analysis 66
3.3. Results 67
3.3.1. Comparison of CFU and FC in the quantification of vaginal lactobacilli 67
3.3.2. Neubauer chamber to quantify bacteria vs yeast 67
3.3.3. Correlation of surface area bacterial measurement with initial bacterial density 68
3.3.4. Quantification of Lactobacillus spp. concentration by an indirect microscopy method 68
3.4. Discussion 70
3.5. References 73

CHAPTER 4 Optimizing qPCR method to accurately quantify bacteria in mixed cultures77
4.1. Contextualization of the experimental chapter 78
4.2. Methods 78
4.2.1. Culture conditions and strains 78
4.2.2. Assessment of the accuracy of different calibration strategies 79
4.2.2.1. Preparation of the bacterial cultures standards 79
4.2.2.2. Preparation of Mock Cultures 79
4.2.2.3. DNA extraction 79
4.2.2.4. qPCR 79
4.2.3. Assessment of the efficiency of different exogenous controls as calibrators for qPCR quantification 80
4.2.3.1. Preparation of the bacterial cultures standards 80
4.2.3.2. DNA extraction 80
4.2.3.3. qPCR 81
4.2.4. Assessment of the efficiency of adding an exogenous culture as calibrator for qPCR quantification 81
4.2.4.1. Preparation of the bacterial cultures standards 81
4.2.4.2. Bacterial loss during centrifugation 81
4.2.4.3. Preparation of Mock cultures 81
4.2.4.4. Biofilm formation 82
4.2.4.5. DNA extraction 82
4.2.4.6. qPCR 82
4.2.5. Statistical analysis 83
4.3. Results 84
4.3.1. Assessment of the accuracy of different calibration strategies 84
4.3.2. Assessment of the efficency of different exogenous controls 86
4.3.3 Assessment of the efficiency of adding an exogenous culture 87
4.4. Discussion 91
4.5. References 94

CHAPTER 5 Evaluation of the interactions between pairwise combinations of *Gardnerella* spp. on the adhesion to HeLa cells with and without *Lactobacillus crispatus*..... 97

5.1. Contextualization of the experimental chapter..... 98

5.2. Methods..... 98

5.2.1. Growth conditions and strains..... 98

5.2.2. Adhesion assays..... 99

5.2.3. Construction of calibration curves for quantitative Polymerase Chain Reaction (qPCR).... 99

5.2.4. Genomic DNA (gDNA) extraction and Bacterial quantification by qPCR..... 100

5.2.5. Statistical analysis..... 100

5.3.1. Calibration curves..... 101

5.3.2. Adhesion of pairwise combinations of *Gardnerella* spp. on HeLa..... 101

5.3.3. Displacement of *L. crispatus* by pairwise combinations of *Gardnerella* spp..... 102

5.3.4. Adhesion of pairwise combinations of *Gardnerella* spp. on HeLa covered with *L. crispatus* 104

5.5. Discussion..... 105

5.6 References..... 107

CHAPTER 6 Conclusion and Future work perspectives 109

6.1. Concluding remarks..... 110

6.2. Study limitations..... 111

6.3. Future work..... 111

6.4. References..... 112

LIST OF FIGURES

Figure 1.1. Thesis outline. 6

Figure 2.1. Representation of BV prevalence of the different ethnic groups in the United States, according to a survey conducted from 2001 and 2004 [66]. 15

Figure 2.2. Schematic representation of the work developed by Criswell et al., in which it was verified that the inoculation of a pure culture of Gardnerella spp. isolated from infected women did not cause the development of BV in all the women included in the study did not cause the development of BV in all the women included in the study [94]. 17

Figure 2.3. The association proposed by Schellenberg et al. [145] between *cpn 60* subgroups and clades and the respective *Gardnerella* spp. [51], adapted from Castro et al. [128]. 22

Figure 2.4. Schematic representation of the steps that lead to monospecies (A) and polymicrobial (B) biofilms formation. From Machado et al. [243] 32

Figure 2.5. Conceptual model for the development of polymicrobial biofilm of BV. From Muzny et [251]. 33

Figure 3.1. *7Quantification of bacterial concentrations of L. crispatus, L. jensenii, L. gasseri, and L. iners* suspensions with an OD of 0.5, by culture dependent (CFU) and independent (FC) techniques. The bars represent the mean and the standard deviation of 3 independent experiments. *Represents statistical significance (T- test paired samples, parametric, p≤0.05). 67

Figure 3.2. 8Comparison of the use of Neubauer chamber for (a) *L. crispatus* and (b) *C. albicans* using three z-stacks. The observation of these microorganisms was performed using fluorescence microscopy. For *L. crispatus* (a), the variation of focal points demonstrates different numbers of counts, while for *C. albicans* (b) the number of cells is the same for the three z-stacks. The red circles surround the bacteria that are not visible in the first focal plan. 69

Figure 3.3. The relationship between E. coli CFUs and total cell counts using FM for the same OD. Three bacterial suspensions initially adjusted to different OD_{620nm}, namely, 0.5, 0.25 and 0.125, were quantified by both FM and by plate counting. The dots correspond to three independent assays for each OD. 70

Figure 3.4. 10Lactobacillus spp. concentrations obtained through total counts. Four bacterial suspensions adjusted to OD_{620}nm= 0.5 were quantified by FC and the results obtained were converted into CFUs, as mentioned above. The dots correspond to three independent assays for each *Lactobacillus* spp. 70

Figure 3.5. Quantification of bacterial concentrations of L*. crispatus, L. jensenii, L. gasseri, and L. iners* suspensions with an OD of 0.5, using FM and FC techniques. The bars represent the mean and the standard deviation of 3 independent experiments. *Represents statistical significance (T-test paired samples, parametric, p≤0.05). 72

Figure 4.1. Schematic representation of the three performed experiments. 84

Figure 4.2. Comparison of different calibration strategies to evaluate the relationship between cycle threshold (Ct) values and bacterial load. (i) Calibration based on serial dilution of gDNA from a known bacterial concentration (orange line); (ii) calibration using gDNA extracted from bacterial suspensions of varying known concentrations (blue line); (iii) calibration using gDNA extracted from bacterial suspensions of varying concentrations with Ct values adjusted for exogenous control (luciferase) loss (green line). Each point represents an independent biological extraction, with qPCR performed in triplicate. 85

Figure 4.3. Variability of gDNA extraction efficiency of *G. vaginalis* cultures with different bacterial concentrations for each exogenous control and for the two different extraction methods. 87

Figure 4.4. Quantitative differences between DNA extraction methods and the recovery rate of the exogenous controls. The bars represent the mean and the standard deviation of 3 independent assays. * represents a statistical significance (Two-way ANOVA, Turkey's multiple comparisons test, $p < 0.05$). 88

Figure 4.5. Bacterial losses from pure cultures of *G. vaginalis*, *P. bivia*, and *F. vaginae* after 10, 20, and 30 min of centrifugation. The concentrations of bacteria remaining in the supernatant after centrifugation of 1×10^8 CFU/mL suspensions for 10, 20, and 30 min are shown for *G. vaginalis* (A), *P. bivia* (B), and *F. vaginae* (C). Boxes indicate the mean ± standard deviation of at least three independent experimental replicates. Statistical comparisons were performed using a t-test. 89

Figure 4.6. Calibration curves illustrating the relationship between cycle threshold values (ΔCt) and bacterial load quantification. Curves were generated for *G. vaginalis* (A), *P. bivia* (B), and *F. vaginae* (C) by adding *E. coli* at 1×10^8 CFU/mL as an exogenous control. 89

Figure 4.7. Accuracy of bacterial quantification in mock cultures of *G. vaginalis, P. bivia, and F. vaginae.* Quantification of each species was performed using the three calibration curves described above. Mock culture C contained 1×10^8 CFU/mL of each species, mock culture D contained 1×10^3 CFU/mL of each species, and mock culture E contained 1×10^5 CFU/mL of *G. vaginalis* and *F. vaginae* together with 1×10^3 CFU/mL of *P. bivia.* In all cases, 1×10^8 CFU/mL of *E. coli* was added as an exogenous control prior to gDNA extraction. Dots represent the mean ± standard deviation of at least three independent replicates. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test; no significant differences were observed. Abbreviations: Gv, *G. vaginalis;* Pb*, P. bivia*; Fv, *F. vaginae.*..... 90

Figure 4.8. Triple-species quantification of 24 h, 48 h, and 72 h biofilms of *G. vaginalis, P. bivia,* and *F. vaginae.* Bacterial concentrations within the biofilms were determined by qPCR, using the calibration curves described above. Statistical analysis was performed using a t-test, *p <0.05. Abbreviations: Gv – *G. vaginalis;* Pb – *P. bivia;* Fv – *F. vaginae.*..... 91

Figure 5.1. Calibration curves to relate cycle threshold detection (ΔCt) and bacterial load quantification. Each dot represents the average of 3 independent assays and the standard deviation.. 102

Figure 5.2. Adhesion of dual combinations of Gardnerella spp. to HeLa cells. The bars represent the *Gardnerella* spp. concentration alone or in dual combinations that adhered to HeLa cells. The bars also represent the average and the standard deviation of 3 independent experiments. * Represents statistical significance between *Gardnerella* spp. alone; δ represents statistical significance between *G. vaginalis* (Gv) alone and the mix; λ represents statistical significance between *G. piotii* (Gp) alone and the mix; ε represents statistical significance between *G. leopoldii* (Gl) alone and the mix; α represents statistical significance between *G. swidsinskii (Gs)* alone and the mix; Φ represents statistical significance between *Gv* alone and *Gv* in the mix and β represents statistical significance between Gs alone and Gs in the mix (t-test, paired samples, $p \leq 0.05$). Gv= *G. vaginalis;* Gp= *G. piotii,* Gl= *G. leopoldii* and Gs= *G. swidsinskii.* 103

Figure 5.3. Displacement of *L. crispatus* by dual combinations of *Gardnerella spp.* The bars represent *L. crispatus* concentrations before (Control) and after being challenged with *Gardnerella* spp. The bars also represent the mean and the standard deviation of 3 independent assays. * Represents statistical significance (multiple t-test, $p \leq 0.05$). Gv= *G. vaginalis;* Gp= *G. piotii,* Gl= *G. leopoldii and Gs= G. swidsinskii.* 104

Figure 5.4. *Gardnerella* spp. dual combination adhesion to HeLa cells with pre-adhered *L. crispatus.* The bars represent the *Gardnerella spp.* concentration alone or in dual combinations that adhered to HeLa cells. The bars also represent the mean and the standard deviation of 3 independent experiments. * Represents statistical significance between *Gardnerella spp.* alone; δ represents statistical significance between Gv alone and the mix;; θ represents statistical significance between Gp alone and Gp in the mix; ε represents statistical significance between Gl alone and Gl in the mix; α represents statistical significance between Gs alone and Gs in the mix and β represents statistical significance between Gs alone and Gs in the mix (t-test, paired samples, $p \leq 0.05$). Gv= G. vaginalis; Gp= G. piotii, Gl= G. leopoldii and Gs= G. swidsinskii. 106

LIST OF TABLES

Table 2.1. Scheme for grading Gram-stained vaginal content according to Nugent..... 14

Table 2.2. Koch´s postulates. Adapted from Evans [92]..... 16

Table 2.3. Main characteristics of the four known species of the genus Gardnerella [51,123]..... 20

Table 2.4. Studies of assessing sialidase and vaginolysin gene presence or activity in the four Gardnerella clades/subgroups 25

Table 2.5. Studies on the interactions between Gardnerella subgroups/clades 26

Table 2.6. Studies of Gardnerella spp. and P. bivia interactions..... 33

Table 2.7. International guidelines for BV treatment. 37

Table 4.1. Primer sequences..... 83

Table 4.2. Accuracy of bacterial quantification in mock mixed-species cultures containing approximately 1 × 108 CFU/mL (A) or 3 × 106 CFU/mL (B) of each tested species, evaluated using three different calibration strategies: (i) based on reaction efficiency; (ii) b 85

Table 5.1. Primers sequence..... 101

LIST OF ABBREVIATIONS

ARDRA – amplified ribosomal DNA restriction analysis

bp – base pair

BV – Bacterial vaginosis

BVAB – BV associated bacteria

CBA – Colombia Blood agar

CFU – colony-forming units

DNA – deoxyribonucleic acid

FC – Flow cytometry

FISH – fluorescence *in situ* hybridization

FM – Fluorescence microscopy

Gl – *Gardnerella leopoldii*

Gp – *Gardnerella piotii*

Gs – *Gardnerella swidsinskii*

Gv – *Gardnerella vaginalis*

Lc – *Lactobacillus crispatus*

Lg – *Lactobacillus gasseri*

Lj – *Lactobacillus jensenii*

Li – *Lactobacillus iners*

OD – optical density

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PNA – peptide nucleic acid

qPCR – quantitative PCR

rRNA – ribossomal ribonucleic acid

TSA – Tryptic Soy agar

LIST OF PUBLICATIONS

The work presented in this thesis has been previously published.

PAPERS IN PEER-REVIEWED JOURNALS

Lima Â., Sousa L. G. V., Macedo F., Muzny C. A., Cerca N. Assessing recovery rates of distinct exogenous controls for gDNA extraction efficiency using phenol-chloroform or silica-column based extractions. J Microbiol Methods **203**, 2022.

Lima Â., Muzny C. A., Cerca N. An indirect fluorescence microscopy method to assess vaginal *Lactobacillus* concentrations. Microorganisms **12**, 2024.

Lima Â., Castro J., Muzny C. A., Cerca N. *Gardnerella* species exhibit synergy in their ability to displace *Lactobacillus crispatus* adhered to HeLa cells. PeerJ **13**:e20076, 2025.

Cerca, N., **Lima, Â.**, França, A. Accurate qPCR quantification in polymicrobial communities requires assessment of gDNA extraction efficiency. Journal of Microbiological Methods **194**:106421, 2022.

Lameira, I., Pinto, A. S., **Lima, Â.**, Muzny, C. A., Cerca, N. "Optimized bacterial absolute quantification method by qPCR using an exogenous bacterial culture as a normalization strategy in triple-species BV-like biofilms". Journal of Microbiological Methods **219**, 2024.

POSTERS COMMUNICATIONS IN SCIENTIFIC CONFERENCES

Lima Â., Castro, J., Muzny C. A., Cerca N. (2023) Quantification of vaginal lactobacilli using an optimized fluorescence microscopy method. Microbiotec 23 – Congress of Microbiology and Biotechnology 2023, December 11-13, Covilhã, Portugal.

Lima Â., Muzny C. A., Cerca N. (2025) Selective displacement of *Lactobacillus crispatus* by *Prevotella* spp. supports a role in early bacterial vaginosis development. Microbiotec 25 – Congress of Microbiology and Biotechnology 2025, December 4-6, Azores, Portugal.

ORAL COMMUNICATION IN SCIENTIFIC CONFERENCE

Lima Â., Muzny C. A., Cerca N. (2025) Selective displacement of *Lactobacillus crispatus* by *Prevotella* spp. supports a role in early bacterial vaginosis development. ISIDOG Congress 2025 – 6th International Society for infectious diseases in obstetrics and gynecology, October 23-26, Belgrade, Serbia.

CHAPTER 1

Introduction

Summary

This chapter presents the research questions and hypotheses that guided the development of this thesis. It also provides a concise background, highlighting the study's significance and offering an overview of the thesis structure.

1.1. Background

Bacterial vaginosis (BV) is a vaginal infection associated with serious women reproductive health complications, with negative impact in quality of life [1]. Its prevalence is around 23-29 % in women of reproductive age [2], while more than 50% of the women experience BV recurrence between 3 to 6 months after finishing the recommended antibiotic treatment [3,4]. This justifies the importance of prevention and the search for effective treatments. However, the fact that BV etiology remains uncertain hinders the development of effective treatments [5].

This infection results in a dysbiotic state in the vaginal microbiota, marked by a reduction in *Lactobacillus* spp. and an overgrowth of anaerobic bacteria, including *Gardnerella* spp [6]. BV is also characterized by a polymicrobial biofilm [7], and deciphering the sequence of events that drives the displacement of lactobacilli and the subsequent development of this biofilm remains a critical area of investigation. The research group of Dr. Nuno Cerca had previously proposed that the onset of BV is triggered by virulent *Gardnerella* spp., which outcompete and displace protective *Lactobacillus* species from the vaginal epithelium [8–10]. Moreover, in 2019, the taxonomy of the *Gardnerella* genus was revised, leading to the proposal of three additional species (*Gardnerella leopoldii*, *Gardnerella piotii*, and *Gardnerella swidsinskii*) isolated from the vaginal microbiota, alongside the previously recognized *Gardnerella vaginalis* [11]. Recently, 2 more bacteria were renamed as *Gardnerella greenwoodii* and *Gardnerella pickettii* [12]. Some studies also showed that multiple *Gardnerella* subgroups frequently coexist in the vaginal microbiota of women with BV [13–15].

1.2. Main research question

The central question of this dissertation focuses on the potential interactions between *Gardnerella* species during the early stages of colonization of the vaginal epithelium:

Is there synergism between *Gardnerella* spp. in the displacement of vaginal *Lactobacillus* species from the epithelial surface?

This question lies at the core of understanding the mechanisms that may facilitate the transition from a healthy vaginal microbiota, dominated by protective *Lactobacillus* species, to a dysbiotic state characterized by *Gardnerella* overgrowth. Exploring the cooperative behavior among *Gardnerella* strains during the displacement process could help explain how these bacteria gain a competitive advantage and initiate biofilm formation, a hallmark of BV.

1.3. Hypothesis and aims

1.3.1. Hypothesis

Studies indicate that several subgroups of *Gardnerella* spp. often coexist within the vaginal microbiota of women affected by BV. Based on this observation, our hypothesis is that different combinations of known *Gardnerella* spp., isolated from BV cases, display an increased capacity to outcompete *L. crispatus* pre- adhered to HeLa cells, thus promoting the initial steps of BV pathogenesis.

1.3.2. Aims

The study of the vaginal microbiota involves both facultative and strictly anaerobic microorganisms, many of which display fastidious growth requirements. These characteristics often limit the reliability of culture-dependent quantification methods [16], creating the need for accurate, reproducible, and standardized molecular and microscopy-based approaches. Given that BV has a major impact on women's health and quality of life, the development, comparison, and optimization of such methodologies are essential to ensure the robustness of experimental data and to advance our understanding of microbial interactions in the vaginal ecosystem.

Accordingly, this work was structured into three main aims:

Aim 1: To accurately quantify the vaginal lactobacilli that will be used in this work.

- To compare vaginal lactobacilli quantification by colony-forming unit (CFU), fluorescence microscopy and flow cytometry.
- To investigate the relation between surface area colonization, in fluorescence microscopy, and bacteria concentration in suspension.

Aims 2: To improve quantitative PCR (qPCR) method in order to accurately quantify bacteria in mixed cultures from adhesion assays.

- To compare a traditional DNA extraction method with silica-column based kits, generally used in research.
- To analyze exogenous controls for DNA extraction with different sizes.
- To create a qPCR method that normalize results, considering the bacteria loss during centrifugation step, before DNA extraction.

Aim 3: To determine the interactions (neutral, synergism or antagonism) in dual combinations of *Gardnerella* spp. in the initial adhesion to HeLa cells with and without *L. crispatus* adhered, by quantifying these bacteria after adhesion assays using qPCR.

- To assemble pairwise combinations between *G. vaginalis*, *G. piotii*, *G. leopoldii* and *G. swidsinskii*, and investigate their when adhering to HeLa cells.
- To study the interactions between the pairwise combinations between *G. vaginalis*, *G. piotii*, *G. leopoldii* and *G. swidsinskii*, on the displacement of *L. crispatus* adhered to HeLa cells.

1.4. Significance

This study aims to advance our understanding on the initial adhesion of *Gardnerella* spp. to the vaginal epithelium, which is a crucial step in the development of BV biofilm. For this reason, it is important to investigate the interactions between *Gardnerella* spp., in order to know more about BV etiology. Without the knowledge of how BV starts, the development of effective therapies is much tougher.

In addition, this work also intends to study adequate methods to quantify vaginal lactobacilli. Since these bacteria present a fastidious growth and they are facultative anaerobe, which makes culture dependent methods inappropriate to quantify them. Furthermore, DNA extraction and qPCR will be optimized to quantify the bacteria used in adhesion assays. Thus, with these improvements, our study will contribute to the molecular research community.

1.5. Thesis outline

Chapter 2 presents a literature review that provides information on important features of BV. Prominence will be given to the composition of vaginal microbiota, specifically to *Gardnerella* spp., biofilm role in BV and the techniques used to quantify vaginal bacteria.

Chapters from 3 to 5 present all the experimental data obtained in this work. Each experimental chapter is structured as an independent unit, containing a summary, a concise contextualization of the experimental chapter, a description of materials and methods, the results obtained and a discussion.

Chapter 3 describes the development of an indirect fluorescence microscopy method to quantify vaginal lactobacilli. These lactobacilli will be used in Chapter 5, thus, we needed to quantify them to prepare our inoculum. Through the use of an easily cultivable and well-known bacterium, *Escherichia coli*, a relation between CFU/mL and total counts/cm^2 was created and vaginal lactobacilli quantified.

In Chapter 4, an analysis was made to the correct use of qPCR to quantify bacteria. Starting by comparing three types of possible qPCR calibration curves, then comparing a traditional DNA extraction method with a commercial kit, besides testing three DNA molecules with different sizes as exogenous control. In the end of the Chapter, we demonstrate the loss of bacteria during the centrifugation step that precedes DNA extraction, which leads to the optimization of qPCR method by using an exogenous control that accounts for that loss.

Chapter 5 constitutes the core of the thesis, where the optimized methodologies developed in the previous chapters are applied to study the interactions between *Gardnerella* species and their interplay with *Lactobacillus crispatus,* during the initial adhesion to HeLa cells. This chapter directly addresses the main research question, evaluating whether *Gardnerella* species exhibit synergistic or antagonistic relationships, and whether *L. crispatus* exerts a protective effect against their adhesion or displacement. The findings presented here provide new insights into the early mechanisms underlying bacterial vaginosis pathogenesis.

This dissertation is concluded in Chapter 6, where the major findings and limitations are described, and future work is proposed.

The thesis outline is depicted in Figure 1.1.

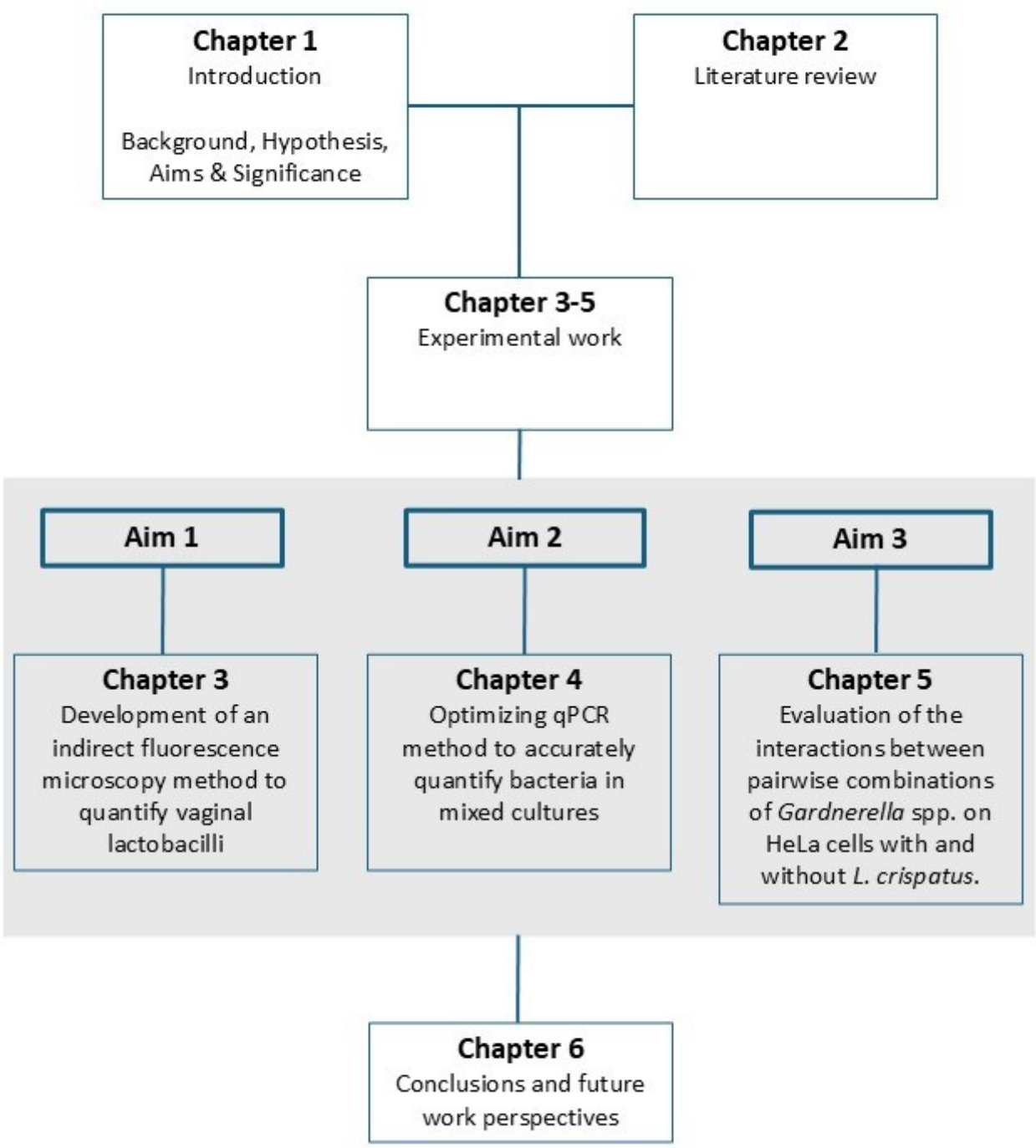


Figure 1.1.

Thesis outline.

1.6. References

1. Bradshaw, C. S., Plummer, E., Muzny, C. A., Mitchell, C., Fredricks, D., Herbst-Kralovetz, M., Vodstrcil, L. Bacterial vaginosis. *Nat Rev Dis Primers* 11, 1-23 (2025).

2. Peebles, K., Velloza, J., Balkus, J., McClelland, R. & Barnabas, R. *et al.* High Global Burden and Costs of Bacterial Vaginosis: A Systematic Review and Meta-Analysis. *Sex Transm Dis* 46, 304–311 (2019).

3. Bradshaw, C., Morton, A., Hocking, J. & Garland, S., Morris, M., Moss, L., Horvath, L., Kuzevska, I. & Fairley, C. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *Journal of Infectious Diseases* 193, 1478–1486 (2006).

4. Clindamycin, T., Sobel, J. D., Schmitt, C. & Meriwether, C. Long-Term Follow-Up of Patients with Bacterial Vaginosis Treated with Oral Metronidazole. *The Journal of Infectious Diseases* vol. 167 (1993).

5. Khedkar, R. & Pajai, S. Bacterial Vaginosis: A Comprehensive Narrative on the Etiology, Clinical Features, and Management Approach. *Cureus* (2022) doi:10.7759/cureus.31314.

6. Abbe, C. & Mitchell, C. M. Bacterial vaginosis: a review of approaches to treatment and prevention. *Frontiers in Reproductive Health* 5, (2023).

7. Sousa, L. G. V., Pereira, S. A. & Cerca, N. Fighting polymicrobial biofilms in bacterial vaginosis. *Microb Biotechnol* 16, 1423–1437 (2023).

8. Castro, J., Henriques, A., Henriques, M. & Jefferson, K. K. Reciprocal Interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on Initial Adherence to Epithelial Cells. *International Journal of Medical Microbiology* 10, 1193–1198 (2013).

9. Alves, P., Castro, J., Sousa, C., Cereija, T. B. & Cerca, N. *Gardnerella vaginalis* Outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an in vitro biofilm formation model. *Journal of Infectious Diseases* 210, 593–596 (2014).

10. Castro, J. *et al.* Using an in-vitro biofilm model to assess the virulence potential of Bacterial Vaginosis or non-Bacterial Vaginosis *Gardnerella vaginalis* isolates. *Sci Rep* 5, 1–10 (2015).

11. Vaneechoutte, M. *et al.* Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. Nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella*. *Int J Syst Evol Microbiol* 69, 679–687 (2019).

12. Sousa, M. *et al. Gardnerella pickettii* sp. nov.(formerly *Gardnerella* genomic species 3) and *Gardnerella greenwoodii* sp. nov. (formerly *Gardnerella* genomic species 8) isolated from female urinary microbiome. *Int J Syst Evol Microbiol* 73, (2023).

13. Lynch, T. *et al.* Molecular diagnosis of vaginitis: Comparing quantitative PCR and microbiome profiling approaches to current microscopy scoring. *J Clin Microbiol* 57, 1–13 (2019).

CHAPTER 2

Literature Review

Summary

This chapter offers an overview of key aspects of bacterial vaginosis (BV), with particular focus on the initial adhesion of BV-associated bacteria (BVAB) to the vaginal epithelium during the early stages of BV development. Moreover, this chapter examines the methodological challenges involved in the microbiological study of BV.

2.1. The vaginal microbiome

The microbial composition of the human vagina has a dynamic nature [1]. The compositional changes occur over time and can derive from several host factors, such as hormonal changes [2], personal hygiene [3], pregnancy [4], sexual intercourse [5], the presence of disease [6] and the use of antibiotics [7]. These microbial fluctuations occur within a unique anatomical and biochemical environment, which also plays a crucial role in shaping microbial communities. The vaginal tract is composed of a stratified squamous epithelium that is lined with a mucus layer [8]. Importantly, the mucus layer protects the epithelial cells from direct contact with exogenous microbes, being formed by mucins, a family of glycosylated proteins with the ability to entrap pathogens facilitating their binding to antibodies [9,10]. Generally, bacterial communities coexist with the human host in mutualistic relationship, maintaining vaginal health and defending against potential pathogens, while the host provides nutrients that support bacterial growth [11]. Since the first microbiological study of the human vagina, published by Döderlein in 1892 [12], the vaginal microbiota of healthy premenopausal women [13] has been described as constituted predominantly by *Lactobacillus* spp. [14]. These bacteria are proposed to provide protection against infections, producing lactic acid through glycogen fermentation [9], which leads to acidification of the environment (pH≈3.9) [15] and consequently limits the colonization of pathogenic bacteria [16]. Another antimicrobial substance produced by *Lactobacillus* spp. is hydrogen peroxide, however, under hypoxia conditions present in the vagina, the amount of hydrogen peroxide produced by lactobacilli is very low, unlikely able to exert a significant antimicrobial effect on pathogenic bacteria [17]. On the other hand, hydrogen peroxide has been suggested to enhance the antimicrobial activity of vaginal epithelial cells [18]. Bacteriocins, which are also produced by *Lactobacillus* spp., are peptide toxins that may play a significant role in fending off non-indigenous bacteria or pathogenic microorganisms, thereby contributing to the vaginal microbiome homeostasis [19]. In addition, according to He and coworkers, lactobacilli have the ability to adhere to vaginal epithelial cells preventing the pathogens colonization [20].

The introduction of culture-independent methods, such as 16S ribosomal RNA (rRNA) sequencing, has allowed researchers to learn the vaginal microbial composition in depth. The microorganisms that inhabit the vaginal epithelium are grouped in community state types (CSTs), according to their composition. As shown by Ravel et al. [21] through a study that analyzed the microbial composition of the vaginal microbiome of 396 asymptomatic women, bacteria can be grouped into five Community State Types (CSTs). The CSTs were established based on different species composition and their relative abundance, being CST I communities dominated by *L. crispatus*, CST II characterized by *L. gasseri*, CST III characterized by *L. iners* and CST V presented predominance of *L. jensenii*. At last, CST

IV is composed of polymicrobial communities, including strictly and anaerobic bacteria, such as *Fannyhessea* (previously known as *Atopobium* [22]), *Gardnerella*, *Prevotella*, *Megasphaera*, *Sneathia* and *Mobiluncus*, and it does not contain a significant number of lactobacilli [21]. Later, in another study [23] whereby the bacterial composition of vaginal samples from 32 women (collected over 16 weeks) was evaluated, the researchers redefine the classification of CST IV into IV-A and IV-B. Usually, CST IV-A is composed by *L. crispatus*, *L. iners* and other *Lactobacillus* spp., with low proportions of strictly anaerobic bacteria, including, for example, *Streptococcus* and *Corynebacterium*. While CST IV-B presented higher proportions of anaerobic bacteria such as *Fannyhessea*, *Prevotella*, *Gardnerella*, *Sneathia* and *Parvimonas*, and low proportions of lactobacilli [23]. It is also important to highlight that according to the authors these findings do not agree with the assumption that only *Lactobacillus*-dominated vaginal bacterial communities are normal and healthy, since some women with a low number of *Lactobacillus* spp. are healthy [21,23]. Furthermore, a more recent study suggests the addition of one more CST at the previous classification established by Ravel and colleagues [21], namely, a CST with high proportions of *Bifidobacterium* [24]. The bacteria from this genus are lactic acid producers and were found in the vaginal microbiome of healthy-aged women [25]. As mentioned above, the fluctuations in the vaginal microbial communities are influenced by several factors. Some of these fluctuations may cause increased host susceptibility, leading to microbial unbalances in the vagina that contribute to infections [26]. The microbial dysbiosis in the vagina is frequently associated with several gynecological infections, such as bacterial vaginosis (BV) [27], among others [28–30].

2.2. Bacterial vaginosis (BV)

Worldwide, BV is the most common vaginal infection in women of reproductive age [31], with annual global economic costs estimated at 4.8 billion dollars [32]. BV is clinically relevant since it affects millions of women annually and is associated with multiple complications [33,34]. Some of them related to adverse pregnancy outcomes, namely, preterm delivery [35], miscarriage [36], and post-partum endometritis [37]. In addition, BV is linked to infertility [38], pelvic inflammatory disease [39], and to the increased risk to acquire sexually transmitted infections (STIs) such as HIV [40], trichomoniasis [41], gonorrhea, and chlamydia [42]. BV also affects women psychologically, being reported that many BV-positive women lack self-esteem and feel frustrated [43]. Consequently, this vaginal infection has a negative impact on the sexual and social life of women [44].

Regarding BV symptoms, women with BV commonly experience increased thin white vaginal discharge and vaginal malodor [45]. However, it is important to highlight that BV remains asymptomatic in

approximately 50% of BV cases [46]. Microbiologically, BV is generally defined by the disruption of the optimal vaginal microbiota, characterized by lactobacilli predominance, with the overgrowth of strictly and facultative anaerobic bacteria [47], where *Gardnerella* spp. play a pivotal role [48]. The dramatic depletion of *Lactobacillus* spp. results in a decrease of lactic acid content, which leads to an increase in the pH of the vaginal milieu [49]. Curiously, some strains of *Fannyhessea* spp., *Leptotrichia* spp. and *Megasphaera* spp. are reportedly capable of producing lactic acid [26]. Therefore, the lack of beneficial lactobacilli and the presence of non-lactobacilli vaginal microbiota may not necessarily be sufficient to cause BV [50]. In spite of over six decades of medical research, the BV etiology still remains controversial. Thus, it is very important to conduct research into this field trying to unravel the progress of this infection.

In 2019, Vaneechoutte and colleagues proposed an emended description of *G. vaginalis*, with the definition of 13 species within the genus *Gardnerella* [51]. In this sense, throughout this chapter, *G. vaginalis* will be referred as "*Gardnerella* spp." whenever previous articles are mentioned.

2.2.1. Clinical features and diagnosis of BV

Generally, the most adopted methods to diagnose BV are the clinical Amsel criteria [52] and the laboratorial Nugent score [53]. According to the Amsel criteria, developed in 1983, a woman is diagnosed with BV when three out of four signs or symptoms are confirmed, namely, (i) vaginal pH superior to 4.5, (ii) the presence of clue cells (vaginal epithelial cells coated with bacteria), (iii) thin, homogenous, grey/white vaginal discharge and (iv) positive whiff-amine test result, derived from the fishy smell of vaginal secretions after a solution of 10% potassium hydroxide is added [52]. Since BV symptoms are not always detectable, the Amsel criteria can often fail BV diagnosis [54]. Nugent score was created in 1991 aiming to improve the accuracy in BV diagnosis. This method assesses the bacterial morphotypes present in the vaginal samples through the Gram staining technique [55]. It is, thus, a standard scoring system that evaluates the number of morphotypes present in vaginal smears, specifically, Gram-positive rods (*Lactobacillus* morphotypes), small Gram-variable and Gram-negative rods (*Gardnerella* spp./bacteroides spp. morphotypes) and curved Gram-variable rods (*Mobiluncus* morphotypes). The vaginal smears are observed microscopically and the morphotypes of each oil immersion field are counted, the sum of the three scores indicates the state of the vaginal microbiota, as described in Table 2.1[55].

Comparing the two traditional BV diagnostic methods, the Nugent score system is more sensitive, while Amsel criteria are more specific [53]. Furthermore, molecular techniques have shown that the level of optimal vaginal microbiota deviation to a vaginal microbiota characteristic of BV, correlates well with the Nugent score and vaginal pH, but not with the Amsel criteria [56]. However, Nugent score is time-consuming

and subjective since the presence of Gram-variable morphotypes can make the microscopic examination difficult [57]. In order to improve BV diagnosis, several alternatives have been proposed. In the United States, there are commercially available diagnostic tests for BV, namely, DNA direct probes and nucleic acid amplification assays (NAATS) [58]. Regarding DNA direct probes, a DNA probe is introduced in a vaginal fluid sample, binding to a specific sequence of certain bacterium, namely BD *AffirmTM VPIII Microbial Identification Test,* being able to detect BV [59]. In the case of nucleic acid amplification assays, the polymicrobial nature of BV has driven the development of quantitative multiplex PCR tests. Currently, four tests of this type are available: *NuSwab®, SureSwab® BV DNA quantitative real-time PCR, BD MaxTM vaginal panel* and *BV panel TM* [60].

Other developing molecular techniques included microarray analysis, [61,62] and next-generation sequencing [63] since these techniques have allowed the detection of known and unknown microorganisms linked to BV. Another technique is Peptide Nucleic Acid (PNA) probes by Fluorescence *in situ* Hybridization (FISH), whose accuracy was compared to Nugent score system to detect BV in vaginal samples [64]. PNA-FISH method proved to be a good alternative to commonly used methods, with a sensitivity of 84.6% and a specificity of 97.6%.

2.2.2. Epidemiology

In 2019, a systematic review and meta-analysis of global BV prevalence among reproductive-aged women in the general population was conducted. As result, it was concluded that BV prevalence is high globally [32]. In addition, it was found that the prevalence of BV varies with ethnicity [65]. For instance, a survey was conducted from 2001 to 2004 in the United States, in order to determine the prevalence of BV in the population. The outcomes revealed that among women aged 14-49 years, the BV prevalence rate in this country was approximately 29%, varying with ethnicity as shown in Figure 2.1 [66]. In line with previous evidence, Borgdoff et al. used 16S rRNA gene sequencing in a study conducted in Amsterdam and observed higher BV prevalence in women of sub-Saharan African descent compared with ethnically Dutch women [67]. Moreover, in a cross-sectional study performed in sub-Saharan Africa it was determined a BV prevalence of 38%, using the Nugent score system [68]. Another example is the prevalence of BV among women from South and East Africa that presented higher rates of BV, than women from West Africa, 68 % in Mozambique [69] and 6% in Burkina Faso [70], respectively. It is also noteworthy that BV prevalence has been shown to vary depending on the diagnostic method applied [71–73].

Table 2.1.

Scheme for grading Gram-stained vaginal content according to Nugent

Organism morphotype	Number/oil immersion field	Score
Gardnerella morphotype	>30	4
	5-30	3
	1-4	2
	<1	1
	0	0
Curved Gram-Variable rods	>5	2
	<1-4	1
	0	0
Lactobacillus morphotype	>30	0
	5-30	1
	1-4	2
	<1	3
	0	4

The total score is the sum of the average classification of *Lactobacillus*, *Gardnerella* and *Bacteroid*es, and finally *Mobiluncus* spp. Total score: 0-3 is negative for BV; 4-6 is intermediate and ≥7 is BV positive. Adapted from Nugent and colleagues [55].

Several risk factors associated with BV have been identified, including, ethnicity [74], smoking [75], socioeconomic status [76], young age at first intercourse and a new partner [77] a high number of lifetime sexual partners [78], and regular vaginal douching [79]. Regarding contraceptive methods, women using cooper intra-uterine device have a higher risk to develop BV [80]. The use of oral contraceptives containing estrogen has been demonstrated to have a protective effect against BV [81]. This can be explained by the fact that estrogen increases the production of glycogen by the vaginal epithelium which leads to an increase in lactobacilli growth [81]. The same protective effect was observed for the use of condom [82]. A systematic review from 2008 on the sexual risk factors involved with BV showed that this infection is significantly affected by sexual contact with new and multiple partners and additionally it was noted that BV has an epidemiological profile similar to chlamydia and gonorrhea, two STIs [83].

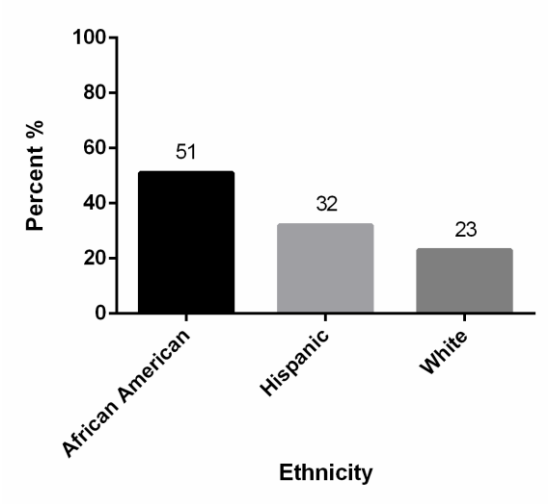


Figure 2.1.

Representation of BV prevalence of the different ethnic groups in the United States, according to a survey conducted from 2001 and 2004 [66].

2.2.2.1 Epidemiology of BV in Portugal

Few studies have been conducted on the epidemiology of BV among Portuguese women. An earlier study, conducted in 2000, compared the Amsel criteria with the Nugent score using 74 Gram-stained vaginal smears, finding a BV prevalence of 28% and 31%, respectively [84]. After a questionnaire carried out in 2012 to 197 Portuguese gynecologists from different regions, it was found that the Amsel criteria is the method most used to detect BV, and most of the doctors consider that this infection is frequent in Portugal (74%) with lower prevalence in pregnancy [85]. Later, Silva et al. [86] performed a study to determine the prevalence of BV, *Gardnerella* spp. and *Fannyhessea vaginae* [22] in 260 women. The results showed that 20% of the women were diagnosed with BV, with 36.9% of the vaginal samples containing *Gardnerella* spp. and 11.9% *F. vaginae* [86]. In another research, Machado and his co-workers [64] determined a BV prevalence of about 17% in 150 vaginal samples, when comparing the accuracy of their PNA probes by FISH to Nugent score system to detect BV. A cross-sectional study was conducted by Vieira-Baptista et al. from 2014 to 2016, where 959 women were evaluated, and a BV prevalence of 15.5% was determined [87]. Moreover, a report was designed to obtain information about the epidemiology of BV in pregnant women. The prevalence of BV, in this case, was low, 3.88%, in contrast to the presence of *Gardnerella* spp., which was detected in 67.48% of samples [88].

2.2.3 Etiology

After more than 60 years of intense research, the etiology of BV is still unknown [89]. *Gardnerella* spp. are present in more than 90 % of BV cases [90] and were firstly considered by Gardner and Dukes as the major causative agent of this pathology [91]. These investigators stated that *Gardnerella* spp. met all Koch's postulates, presented in Table 2.2 [92].

Table 2.2. Koch´s postulates. Adapted from Evans [92]

i)	The etiological pathogen must be found in abundance in every case of disease;
ii)	The etiological pathogen should not be found in organisms that do not suffer from the disease;
iii)	The cultured etiological pathogen must be capable of causing disease when inoculated in a susceptible host;
iv)	The etiological pathogen should be re-isolated from the inoculated experimental host.

This finding is on the basis of the single pathogen hypothesis, which considers *Gardnerella* spp. as the sole pathogen to induce BV, being transmitted by sexual contact [93]. Nevertheless, the discovery of Gardner and Dukes was later rejected, since a follow up study demonstrated that it failed one of Koch's postulates, namely, the inoculation of a pure culture of the etiological microbe into a host agent did not always induce BV, as shown in Figure 2.2 [94]. Later, other bacterial species were found in women with BV, leading to a new hypothesis suggesting that BV is caused by a consortium of anaerobic bacteria, with *Gardnerella* spp. having an essential role [95]. This postulate is generally known as the polymicrobial hypothesis, being supported by the evidence that multiple anaerobic species are present in the vaginal microbiota of women suffering from BV [96].

Due to the doubts regarding the nature of BV, it is important to debate whether BV is an STI or a sexually enhanced infection in order to better understand its etiology [97]. The possible confirmation of BV as an STI could indicate that associated anaerobic bacteria are only opportunistic bacteria that take advantage of the environmental conditions created by the etiological agent [95]. Moreover, knowing the etiology of BV is important since it will lead to improvements in the diagnosis and treatment of this infection.

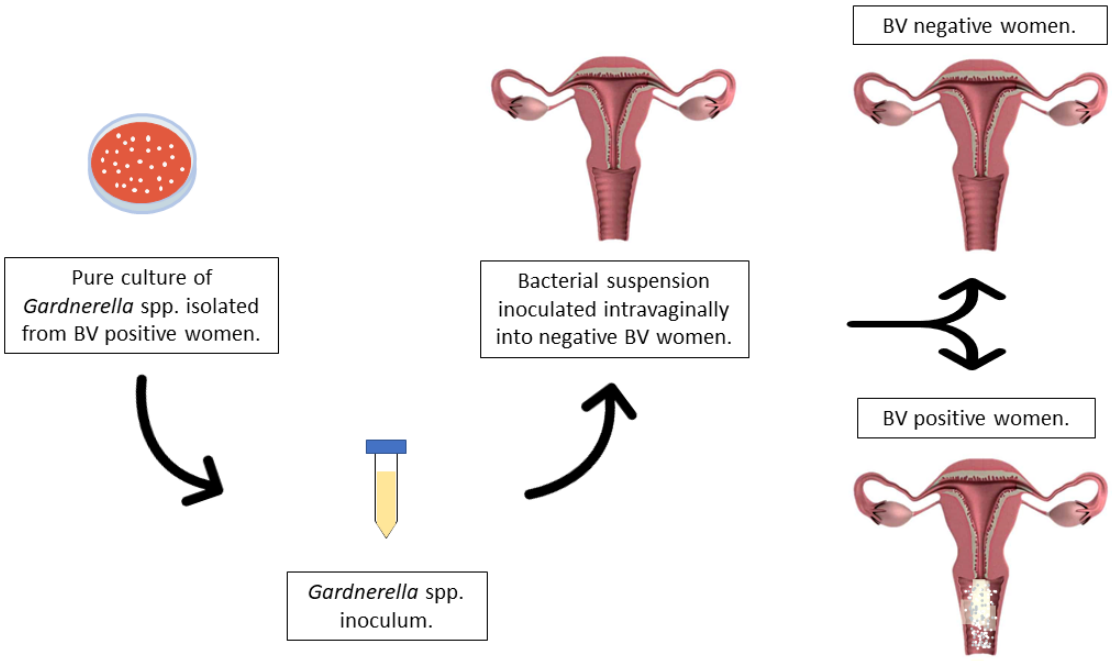


Figure 2.2.

Schematic representation of the work developed by Criswell et al., in which it was verified that the inoculation of a pure culture of Gardnerella spp. isolated from infected women did not cause the development of BV in all the women included in the study did not cause the development of BV in all the women included in the study [94].

2.2.4 Bacterial species in BV

The polymicrobial nature of the vaginal microbiota associated with BV is well known, being characterized by diverse, heterogenous communities, with a wide variety of anaerobic species [98,99]. A relevant bacterial species consistently associated with BV is *F. vaginae*, first linked to this pathology in 2004 [100]. Interestingly, in some studies *F. vaginae* has been detected in more than 90% of women with BV [101,102]. *F. vaginae* have been associated with three out of four Amsel criteria, not being associated with amine smell [103]. *Prevotella bivia* is also common in the vaginal microbiota of BV-positive women, and it was reported that its concentration increased significantly as the Nugent score increased [104]. Curiously, *L. iners* is commonly present in the vaginal microbiota of healthy women and also in women with BV [105]. This microorganism may present clonal variants, some of which appear to support a healthy vaginal environment, whereas others are associated with dysbiosis [106]. Regarding *Mobiluncus* genus, *M. curtisii* is associated with BV while *M. mulieris* is more prevalent in healthy women [107]. Moreover, it was shown that the prevalence of *Sneathia* spp. enhanced with the increased sexual activity [108]. Furthermore,

Mycoplasma hominis, *Ureaplasma urealitycum* [109], and the genus *Parvimonas*, *Anaerococcus*, *Finegoldia*, *Gallicola*, *Peptoniphilus* and *Megasphaera* types 1 and 2 were also detected in women with BV [110,111]. In 2005, researchers discovered three new bacteria present in the vaginal fluid of women with BV using PCR and FISH. These three groups of bacteria are designated as "Bacterial vaginosis associated bacteria 1"(BVAB1), BVAB2 and BVAB3, and all belong to *Clostridium* phylum [102]. It was reported that BVAB3 was isolated by culture and biochemically characterized, being *Mageeilbacillus indolicus* the name proposed for this species [112]. Through sequence homology and phylogenetic analyses, BVAB1 and BVAB2 were recently classified as *Clostridiales* genomosp. BVAB-1 and *Oscillospiraceae* bacterium strain CHIC02, respectively [113].

The advances in molecular-based techniques allowed us to decipher a wide range of species present in the vaginal microbiome during BV. However, BV etiology remains unclear probably due to the complexity of the vaginal microbiota, host immunity, and the variability of the individual response to inflammatory mediators produced by several microorganisms [89].

2.2.4.1 *Gardnerella* spp.

Gardnerella spp. was firstly identified as "*Haemophilus*-like" species by Leopold in 1953, being isolated from cases of cervicitis and prostatitis [114]. In 1955, Gardner and Dukes proposed the name *Haemophilus vaginalis* after isolation and microbiologic study of this bacterium [91]. Later, based on microscopic morphology and metabolic requirements, this bacterium was removed from the genus *Haemophilus* and included in the genus *Corynebacterium* [115]. However, Greenwood and Pickett verified through DNA-DNA hybridization that there were no genetic relationships between "*Haemophilus vaginalis*" and members of *Haemophilus* and other genera. Therefore, it was proposed a new genus, *Gardnerella*, and the name *Gardnerella vaginalis* for the bacterium [116]. *G. vaginalis* was characterized as pleomorphic rods, nonencapsulated, non-motile, non-spore-forming and small with average dimensions of 0.4 by 1 to 1.5 μm [117]. Regarding Gram staining, *G. vaginalis* is Gram-variable, since in vaginal smears its Gram reaction may vary from positive to negative [118]. Furthermore, these bacteria are fastidious, requiring complex media and 5% to 10% of carbon dioxide (CO_2) to grow, at 37 ℃ [117]. *Gardnerella* is a genus generally composed of facultative anaerobic bacteria, but strict anaerobic strains have been detected [119]. The surface of these cells is covered with fimbriae, which are thought to facilitate the attachment of *Gardnerella* to the vaginal epithelium [120]. Biochemically, *G. vaginalis* is catalase, oxidase, and β-glucosidase-negative [117].

Importantly, Vaneechoutte et al. updated the description of *G. vaginalis*, after determining the existence of 13 distinct species within the genus *Gardnerella*, describing 3 new species, namely, *G. leopoldii*, *G. swidsinskii* and *G. piotii*. This discovery was accomplished by the comparison of whole-genome sequences, biochemical properties and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis [51]. Moreover, the authors described the characteristics of the four known species, being listed in Table 2.3 [51]. This study even demonstrated that *Gardnerella* species cannot be distinguished using 16S rRNA sequencing, since none of 81 genomes analyzed showed less than 98.5% similarity, and 97% is the threshold for bacterial speciation [51,121]. A more recent study performed by Hill and colleagues showed that the amplification and sequencing of the 'universal target' region of the gene encoding the 60 kDa chaperonin (*60cpn*) can differentiate the new *Gardnerella* species [122]. In 2023, Sousa et al. also named *Gardnerella* genomic species 3 and 6 as *Gardnerella pickettii* and *Gardnerella greenwoodii*, respectively [123].

2.2.4.1 Presence of Gardnerella spp. in the vaginal microbiota of healthy women

Although some *Gardnerella* spp. are undoubtedly associated with BV, they are also detected in the vaginal microbiota of healthy women [124], however, normally at lower concentrations [56]. Therefore, the presence of *Gardnerella* spp. does not always induce BV [125]. For this reason, the role of *Gardnerella* spp. in BV etiology remains controversial, since it is not clear how an opportunistic pathogen can be a constituent of the vaginal microbiota of healthy women. One of the hypotheses to answer this question is the possibility that only some of the lineages or species are pathogenic [126]. Another hypothesis is that all *Gardnerella* spp. encode virulence factors, but these are conditionally expressed depending on the conditions present in the vagina [127]. In this sense, several studies have been performed to determine the characteristics involved with *Gardnerella* spp. virulence, analyzing the diversity of this genus through phenotypic properties, genotyping differences, ecotypes, and *in vitro* functional properties [128].

Table 2.3.

Main characteristics of the four known species of the genus Gardnerella [51,123].

Species	Colonies	Sialidase activity	β-galactosidase activity	DNA G+C content
G. vaginalis	Pinpoint white to greyish with a smooth surface	+/-		41.0 mol% to 42.8 mol%
G. leopoldii	Pinpoint white to greyish with a smooth surface	-	-	41.9 mol% to 43.2 mol%
G. piotii	Pinpoint white to greyish with a smooth surface	+	-	41.1 mol% to 42.3 mol%
G. swidsinskii	Pinpoint white to greyish with a smooth surface	-	-	41.4 mol% to 42.3 mol%
G. pickettii	Pinpoint white to greyish with a smooth surface	+	*	42.3 mol% to 42.6 mol%
G. greenwoodii	Pinpoint white to greyish with a smooth surface	-	*	43.2 mol% to 43.4 mol%

*Not known.

2.2.4.1.2. Genotyping, biotyping, ecotyping of *Gardnerella* spp.

Over the past decades, several investigations have focused on the assessment of the diversity within *Gardnerella* spp., trying to delineate subgroups based on biochemical tests and molecular methods, and subsequently, correlate them with BV cases [129–131]. A biotyping scheme was created by Piot et al., based on phenotypic properties, namely, hydrolysis hippurate, lipase and β-galactosidase activities of *Gardnerella* spp. strains, which resulted in the identification of 8 distinct biotypes [129]. Later, another study used a modified scheme including the fermentation of galactose, xylose and arabinose, in addition to the tested reactions of Piot and colleagues. The authors observed 33 biotypes, with

significantly different distributions, verifying the predominance of certain biotypes in women with BV [132]. In addition to significant differences in the abundance of biotypes in patients with and without BV, a positive association was also observed between the presence of lipase-positive biotypes and BV cases [133,134]. However, other investigations did not find any correlation between a single biotype and BV [129,135,136]. Moreover, it was verified the absence of an association between *Gardnerella* spp. biotype and virulence factors, such as adherence to the vaginal epithelium, biofilm production, phospholipase C activity, vaginolysin and sialidase production [137,138]. Furthermore, the possible correspondence between genotypes and specific biotypes was evaluated, showing inconsistent results [138,139]. Three *Gardnerella* spp. genotypes were firstly established by Ingianni et al. using amplified ribosomal DNA restriction analysis (ARDRA) [130]. These genotypes were then confirmed based on ARDRA and rapid amplified polymorphic DNA analyses (RAPD) and a link between sialidase production and two of the genotypes was also verified [140]. However, again no correlation was observed between any genotype and BV [130,140]. In 2005, four clusters of *Gardnerella* spp. sequences were observed in vaginal microbiome profiles based on sequencing of amplified *cpn60* barcode [141]. Later, Jayaprakash and colleagues identified the same four *Gardnerella* spp. subgroups (A, B, C and D) through amplification and sequence of *cpn60* gene, analyzing the vaginal microbiome profiles of Kenyan women, and between subgroups, whole-genome average nucleotide identity values were less than 90% [142]. The authors also showed that *Gardnerella* spp. subgroups were consistent with ADRA genotypes and sialidase presence [143]. Confirmation of the results obtained using *cpn60* gene was performed by Ahmed and colleagues, who recognized the presence of four subgroups/clades based on whole-genome sequencing of 17 *Gardnerella* spp. isolates [144]. This genomic analysis revealed that *Gardnerella* is highly heterogeneous and taxonomically diverse, with only 52% of the genome of each isolate consisting of conserved genes [144]. In 2016, Schellenberg and colleagues proposed the reconciliation of *cpn60* subgroups [143] with the whole-genome sequence clades [144], as shown in Figure 2.3, whereby *cpn60* subgroups A, B, C and D correspond to clades 4, 2, 1 and 3, respectively [145]. Interestingly, in the 2019 study carried out by Vaneechoutte et al. [51], 9 of the 13 delineated *Gardnerella* spp. were grouped into the clades defined by Ahmed et al. as described in Figure 2.3 [144]. *G. vaginalis* and an undefined species were recognized by clade 1, *G. piotii* and an undefined species by clade 2, three undefined species belong to clade 3, and clade 4 contained *G. leopoldi* and *G. swidsinskii* [51]. More recently, this distribution of species in clades [51] was confirmed by Hill and colleagues through amplification and sequence of *cpn60* gene, with the exception of clade 1 that was observed to only contain *G. vaginalis* and clade 2 that corresponded only to *G. piotii* [122].

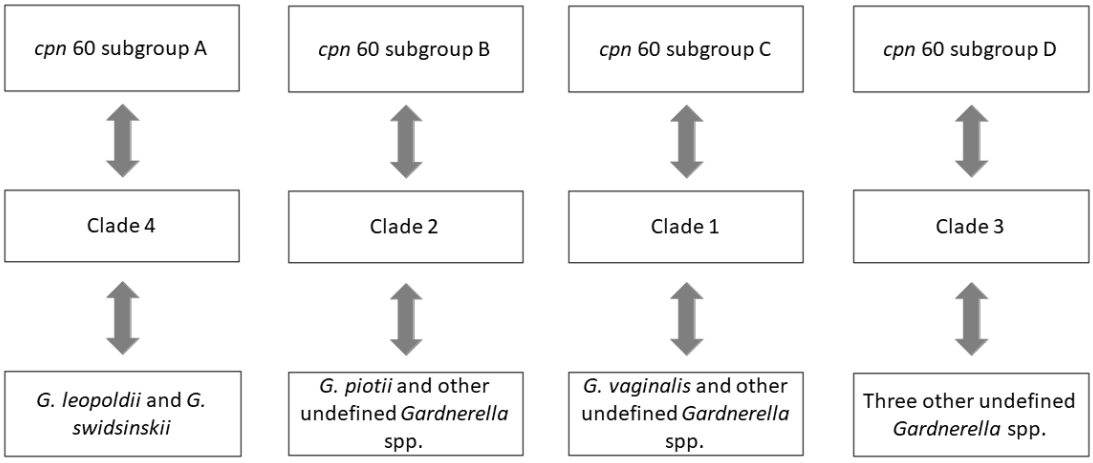


Figure 2.3.

The association proposed by Schellenberg et al. [145] between cpn 60 subgroups and clades and the respective Gardnerella spp. [51], adapted from Castro et al. [128].

The identification of a potential link between genotypes or clades with the expression of virulence factors has been explored, due to its possible role in BV pathogenesis [131,138,146]. According to Janulaitiene et al., *Gardnerella* spp. clades could be best described by two phenotypic features, namely, sialidase activity and vaginolysin production [146], as described in Table 2.4. Both factors may enable *Gardnerella* spp. adherence and colonization of vaginal epithelium [147]. The presence of high loads of sialidase gene expression was strongly associated with BV [148]. It was found that *Gardnerella* spp. can possess three sialidase genes, namely, sialidase A gene (NanH1), NanH2 and NanH3, the latter two being the primary sources of sialidase activity in *Gardnerella* spp. [149]. Regarding vaginolysin production, Pleckaityte et al. demonstrated that the level of this toxin production was not correlated with any *Gardnerella* spp. genotype [138]. Recent findings showed that there are 5 types of vaginolysin, classified according to species-specific variations in their amino acid sequences.[150]. According to this study, vaginolysin Type 1 was the most abundant among the vaginal microbiome profiles, being associated with BV symptoms, while the subjects dominated by vaginolysin Type 2 presented a high abundance of *Gardnerella* spp. [150].

In addition to the phenotypic and genotypic characterization of *Gardnerella* spp., the diversity of this genus was also ecologically evaluated, by using the concept of ecotype [151]. An ecotype can be defined as a group of strains that are genetically similar to one another but ecologically distinct from others [152]. A study carried out by Cornejo et al. demonstrated the existence of three distinct *Gardnerella* spp. ecotypes through phylogenetic and functional analyses. The authors verified that ecotype 1 only encodes glycosidases, and it has augmented galactose and pentose sugar metabolism pathways. The genomes of

ecotype 2 possess at least two distinct genes encoding sialidase (a type of glycosidase) and the majority of genomes in ecotype 3 are characterized by the absence of genes for any of these enzymes [151].

Despite attempts to define the groups of isolates that are more pathogenic, this issue remains to be fully understood. The study of phenotypic and genotypic characterization of *Gardnerella* spp. should be deepened to gain a clear insight into the species belonging to *Gardnerella* genus, as well as their potential role in the development of BV.

2.2.4.1.3 Cooccurrence of *Gardnerella* subgroups/clades in vaginal microbiota

Several studies have shown that the vagina is generally colonized by multiple *Gardnerella* clades/subgroups [153–155]. A study developed by Balashov et al. verified that multiple clades were detected in 70% of the vaginal samples and it was strongly associated with BV [156]. It was also observed that clades 1 and 3 were positively associated with BV, clade 2 was shown to be associated with intermediate vaginal microbiota and clade 4 had no association with the infection [157]. Nevertheless, more recent studies failed to validate that finding [223–225]. Clades distribution was also studied in a cohort of Australian women who have sex with women, where it was verified that clade 3 was only detected in multiple clades communities [158]. In addition, it was reported that most vaginal samples had multiple clades and only 5 of 181 vaginal specimens, positive for a *Gardnerella* spp. clade, contained the four clades [158]. Furthermore, another study [122] demonstrated that multiple clades were detected in most vaginal microbiome profiles as shown before by Balashov et al. [157] It was also observed the significant co-occurrence of *G. vaginalis* and *G. swidsinskii* and its link to BV symptoms, namely, vaginal discharge and abnormal odor [122]. In a recent study, Tortelli and colleagues evaluated the strain level variation among vaginal microbiomes, and they also found that most samples with *Gardnerella* spp. harbored multiple strains from different groups/clades. Interestingly, they suggested that the co-occurrence of different groups can be explained based on the ecotype concept, whereby different strains of the same species may occupy the same niche if they function as different ecological species [160].

Few studies have investigated the interactions between *Gardnerella* spp. clades/subgroups. The aim of these studies was to understand how such interactions could influence the population dynamics in vaginal microbiota as shown in Table 2.5 [161–163]. The three studies presented were performed by the same authors, and in their last research, they suggested that the rarity of subgroup D, along with its generalist nutritional strategy, may enable it to compete for and occupy the vaginal epithelium [163].

2.2.4.1.4 Functional analyses of virulence factors of *Gardnerella* spp.

In addition to the comparative genomic methods, several culture-based studies have also investigated the diversity of *Gardnerella* spp., highlighting the wide variety of virulence factors observed for their isolates [126,138,164,165]. As previously mentioned, *Gardnerella* spp. are also isolated from the vaginal microbiota of women in whom BV has been clinically excluded [166]. In this sense, Harwich and colleagues evaluated the virulence potential of two *Gardnerella* spp. isolates, one from a BV-positive woman and another from a healthy woman using *in vitro* assays [126]. According to this study, the BV isolate exhibited greater adherence and cytotoxicity than the non-BV isolate. The BV isolate biofilm growth was also significantly higher, and it was verified that a biofilm-associated protein (BAP) family gene was harbored by both isolates, presenting substantial differences in the protein sequence [126]. Similarly, an *in vitro* study performed by Nisha et al. verified that *Gardnerella* spp. isolates from the genital tract of women with BV possess a significantly higher ability to form biofilm as well as to adhere to vaginal epithelial cells than isolates from women without BV. Furthermore, the authors observed that BV isolates exhibited more virulence factors than non-BV isolates, specifically, phospholipase C production, protease production and agglutination of human red blood cells [165]. Conversely, Castro et al. demonstrated that the differences found in biofilm formation of *Gardnerella* spp. isolated from women with and without BV were not statistically different. However, BV isolates were capable of displacing lactobacilli preadhered to epithelial cells and exhibited higher initial adhesion and cytotoxicity to Hela cells [167].

Table 2.4.

Studies of assessing sialidase and vaginolysin gene presence or activity in the four Gardnerella clades/subgroups.

Type of assay	Number (N) of strains or vaginal samples	Clade(s)/ subgroup(s)	Association with BV	Reference
Sialidase gene/activity				
Targeting clades specific genes and detecting the presence of sialidase gene	N=109 vaginal samples	- Sialidase gene was detected in all isolates of clades 1 and 2, but not in clade 4 isolates.	- Clades 1 and 2 were strongly associated. - Clades 3 and 4 showed no association. - Absence of sialidase associated with healthy women.	131
Detection of the presence of sialidase gene and its activity	N=112 *Gardnerella* spp. isolates	- Sialidase gene was not detected in subgroup A. - Sialidase activity was detected in all subgroup B isolates. - Sialidase gene was detected in all subgroup C isolates, but only 9% showed activity. - Sialidase gene was detected in all subgroup D isolates.	An association between BV and *Gardnerella* spp. subgroups was not performed.	145
Detection of sialidase activity	N= 81 full genome sequences of *Gardnerella* spp.	- Absence of sialidase activity in *G. leopoldii, G. swidsinskii* and 3 *G. vaginalis* strains. - Sialidase activity detected in *G. piotii* and 1 *G. vaginalis* strain.	An association between BV and *Gardnerella* spp. was not performed.	51
Targeting clades specific genes and detecting the presence of sialidase gene.	N= 299 vaginal samples	- Sialidase gene was detected in clades 1, 2 and 3. - Sialidase gene was detected in only 5% of clade 4 strains.	- Clade 4 was strongly associated. - Clades 1 and 2 were also associated to BV. - Clade 3 showed no association.	159
Vaginolysin production				
Detecting species specific variation in vaginolysin sequence	N= 91 *Gardnerella* spp. genomes	- Type 1 vaginolysin dominated in *G. vaginalis* and in 7 genome species. - Type 2 was only detected in *G. leopoldii* and *G. swidsinskii*. - *G. piotii* had only one strain with the Type 1 vaginolysin.	- Type 1 vaginolysin was associated to BV symptoms. - Type 2 vaginolysin was associated to *Gardnerella* spp. abundance.	150

The isolates of the four *Gardnerella* spp. clades revealed biofilm-forming ability [143], in addition, no statistical differences were detected in the amount of biofilm produced by isolates from three clades (clades 1, 2 and 4) (195). Other virulence factor is antimicrobial tolerance, in this case, some studies pointed similar results between BV and non-BV isolates [126,167].

Overall, these studies point to the possibility that some *Gardnerella* spp. variants are more virulent and subsequently are more capable of inducing BV, which supports the most recent conceptual model for BV pathogenesis that proposed that virulent *Gardnerella* spp. strains are responsible for eliciting BV, by initiating BV biofilm formation, and whereby *P. bivia* and *F. vaginae* act in synergism with *Gardnerella* spp. [168].

Table 2.5.

Studies on the interactions between Gardnerella subgroups/clades.

Type of assay	Interaction result	Reference
Co-culture of *Gardnerella* subgroups/clades	Growth rate of subgroups A, B and C decreased and subgroup D increased	161
Carbon source profiling of co-cultures of *Gardnerella* subgroups/clades	Subgroup D presented a "generalist nutritional lifestyle".	162
Gardnerella subgroups C and D competition.	Bacterial concentration can affect the abundance of *Gardnerella* spp. in mixed communities.	163

Taking these findings into consideration, it is necessary to conduct further research on the virulence factors in these two groups of isolates, with the purpose of obtaining more coherent results. An important step in this direction would be the use of *in vivo* models aiming to overcome the limitations of *in vitro* assays since the growth media might not contain all the factors present *in vivo* and some *in vivo* signals can activate biofilm expression-related genes [128].

2.2.4.2 *Prevotella* spp.

Prevotella is a genus of Gram-negative anaerobic bacteria that was formally established in 1990 by Shah and Collins [169]. This classification aimed to distinguish certain oral species, previously included in the genus *Bacteroides*, that shared specific phenotypic traits. The genus was named in honor of the anaerobic microbiology pioneer A. R. Prévot. The type species, *Prevotella melaninogenica*, was originally

isolated by Oliver and Wherry in 1921 from various human anatomical sites [170]. Species of *Prevotella* are characterized as short, non-motile, non-spore-forming rods [171] with fastidious growth [172]. This genus has more than 50 species [173] and it belongs to the family *Prevotellaceae*, which also includes the closely related genera *Paraprevotella*, *Alloprevotella*, and *Hallella*. Colonies grown on blood agar typically range in diameter from very small to approximately 2.0 mm. They are generally circular with smooth, well-defined edges, and exhibit a convex, shiny, and smooth surface. Their appearance may be translucent or opaque, with coloration varying from grey to light brown or even black [169]. Their hemolytic activity differs between species, and they possess saccharolytic or moderately saccharolytic metabolic profiles [174]. Comparative genomics has revealed substantial intraspecies variability within *Prevotella*, including genetic determinants linked to antimicrobial resistance and adaptation to the vaginal environment [175,176]. This genetic diversity suggests that individual species of *Prevotella* may play distinct ecological and pathogenic roles.

2.2.4.2.1 *Prevotella* spp. in the vaginal microbiota

Although *Prevotella* spp. have been found in diverse natural habitats, this genus is most associated with humans [177], being present in different body parts, such as, the oral cavity [178], the vagina [179], the skin [180] and in the digestive tract [181]. The most common *Prevotella* spp. found in the vaginal microbiota are *P. bivia*, *Prevotella amnii*, *Prevotella timonensis* (now known as *Hoylesella timonensis* [182]), *Prevotella corporis*, *Prevotella bucalis* and *Prevotella disiens* [177]. *P. bivia* is the best-characterized and most frequently detected *Prevotella* species in the female genital tract. Notably, these species fall into distinct phylogenetic clusters and are not exclusive to the urogenital tract, as they are also present in the oral cavity and/or gastrointestinal microbiota [177]. *Prevotella* spp. are present in the female genital tract of healthy women, however, in low concentrations, their increase in the vaginal microbiota is associated with BV [183–185]. Another species from this genus had been isolated from the vagina, namely, *Prevotella denticola* [186], *Prevotella oralis* [187], *P. melaninogenica* [188], *Prevotella lascolaii* [189] and *Prevotella histicola* [177].

2.2.4.2.2 Functional analysis of virulence factors of vaginal *Prevotella* spp.

Prevotella spp. are known to have several virulence factors, such as adhesins, fimbriae, hemolysins, capacity to form biofilm and they also secrete nucleases, proteases, lipopolysaccharide, exopolysaccharides, and hydrolases [190–192]. One of the hydrolases is sialidase, that as mentioned above degrades the mucin layer of epithelial cells [193]. *P. timonensis* is the *Prevotella* spp. with the highest production of sialidase [179]. The ammonia production by *Prevotella* spp. is also considered a virulence

factor [194]. *P. timonensis* also possesses elongated microvilli and the highest number of mucin activity pathways [177,179]. The lipopolysaccharides of *Prevotella* exhibits strong pro-inflammatory activity, inducing cytokine release (IL-1β, IL-6, TNF-α) through Toll-like receptor (TLR2/TLR4) activation [195–197]. This immune modulation can disrupt mucosal integrity and increase susceptibility to sexually transmitted infections (STIs). Indeed, *Prevotella* abundance has been associated with higher risk of HIV, HPV, and *Chlamydia trachomatis* infections [198], as well as obstetric complications such as preterm birth [199]. Regarding antibiotic resistance, it was shown that *P. bivia*, *P. amnii* and *P. timonensis* have resistance to clindamycin [200] and some strains of *P. bivia* presented resistance to metronidazole [201]. Recently, a study found that women with a higher relative abundance of *Prevotella* spp. following BV treatment had an increased risk of recurrence compared to those with lower *Prevotella* spp. levels [202]. Despite these finding, the exact role of *Prevotella* spp. in BV remains unclear and further research is needed.

2.2.4.3 *Fannyhessea vaginae*

F. vaginae is a Gram-positive, anaerobic bacterium belonging to the family *Atopobiaceae* [203]. This species was first described in 1999 by Jovita and colleagues, who isolated the bacterium from vaginal samples of women diagnosed with bacterial vaginosis [203]. At that time, it was placed within the genus *Atopobium* and named *A. vaginae*, until a taxonomic reorganization in 2020 placed it within the novel genus *Fannyhessea* [204]. It is typically a small, oval to coccoid organism that thrives in anaerobic or microaerophilic environments [205]. *F. vaginae* is a fastidious organism that requires enriched media and strict anaerobic conditions for successful isolation. On solid media, such as blood agar or other enriched anaerobic agars, colonies are typically small, 0.2–0.5 mm in diameter, smooth, and opaque, with a white to grayish coloration [203]. Growth is usually slow compared with other vaginal anaerobes [206]. This species is catalase-negative, oxidase-negative and its major metabolic product is lactic acid [203,207]. In blood agar, *F. vaginae* does not show evidence of hemolysis [203]. Besides being associated to BV, this bacterium is also linked to other pathological conditions, such as tubo-ovarian abscess, salpingitis, and endometritis [208,209].

2.2.4.3.1 *Fannyhessea vaginae* in the vaginal microbiota

Although *F. vaginae* has been detected in the vaginal microbiota of healthy women [210], it occurs more frequently in BV cases [210,211]. In addition, the load of this species in the vaginal microbiota of women with BV is higher than in healthy women [212]. The presence of *F. vaginae* has been correlated with characteristic features of bacterial vaginosis, including abnormal vaginal discharge, increased vaginal pH, and the occurrence of clue cells [213]. Moreover, elevated concentrations of *F. vaginae*, particularly when

found together with *Gardnerella* spp., have been linked to adverse pregnancy outcomes such as late miscarriage and preterm delivery [214,215]. Notably, its role in BV remained underappreciated for many years due to the challenges of culturing this fastidious organism, and it was only with the advent of molecular methods that its clinical significance became clear [216].

2.2.4.3.2 Functional analysis of virulence factors of *Fannyhessea vaginae*

Fannyhessea vaginae is increasingly recognized as a relevant pathogen in the context of BV due to its multiple virulence-associated traits [213]. One of the most important is its ability to form biofilms, particularly in cooperation with *Gardnerella* spp. [217]. Within these mixed biofilms, *F. vaginae* contributes to the structural stability and persistence of the microbial community, enhancing resistance to host immune responses and antimicrobial agents [218]. Another key virulence aspect is its tolerance to metronidazole, the standard therapy for BV. It has been shown that the response of *F. vaginae* to metronidazole is highly variable among different strains [219]. Several clinical isolates exhibited marked resistance to metronidazole and secnidazole [220,221]. This has been suggested as a contributing factor to therapeutic failure [219,220]. Additionally, *F. vaginae* produces pro-inflammatory molecules that may contribute to the disruption of epithelial integrity and the induction of an inflammatory microenvironment [205]. In this case, stimulation by *F. vaginae* activates the innate immune response of epithelial cells, resulting in the local production of IL-6, IL-8, and antimicrobial β-defensin peptides through the engagement of Toll-like receptor 2 [222]. This is clinically relevant, as BV has been linked with adverse reproductive outcomes, including preterm birth and increased susceptibility to sexually transmitted infections [214,223].

2.2.5 The role of biofilm in BV

A biofilm can be defined as an aggregate of microbial cells adhered to biotic or abiotic surfaces, embedded within a self-produced hydrated polymeric matrix [224]. Its development comprises four main stages, namely, i) the attachment of planktonic cells to a surface, ii) microcolony formation, iii) biofilm maturation, and iv) dispersal of cells to the environment [225], as depicted in Figure 2.4. In contrast to planktonic cells, this mode of growth provides protection against antimicrobials [226], and the immune system [227], allowing survival in a hostile environment [228].

BV was first linked to biofilm formation in 2005 by Swidsinski and his co-workers [147]. These researchers verified the presence of a multi-species biofilm adhered to the vaginal epithelium in biopsy specimens of women with BV, using FISH. The biofilm was found to consist predominantly of *Gardnerella* spp. with the incorporation of several bacterial species [147], a polymicrobial biofilm as described in Figure

2.4 B. Since then, several *ex vivo* studies, using vaginal samples, have reported that *Gardnerella* spp. dominated the biofilm in BV cases [229–231]. An investigation performed by Patterson et al. also supports the role of biofilm in BV, by showing that *Gardnerella* spp. biofilm possesses higher tolerance to lactic acid and hydrogen peroxide than planktonic cells [232]. In fact, comparing transcriptomic analysis of *Gardnerella* spp. biofilm and planktonic cultures, allowed to infer that when *Gardnerella* spp. grow in biofilms, occur an upregulation of genes associated with antimicrobial tolerance and evasion of the immune response, which may contribute to increasing antimicrobial tolerance, and, thus, facilitate the colonization of the vaginal epithelium [233].

Regarding BV biofilm formation, the precise steps that lead to its development are unknown [50]. However, it was demonstrated that *Gardnerella* spp. isolated from women with BV have a pivotal role in the colonization of vaginal epithelium and consequent biofilm development, working as a scaffold for the incorporation of BV-associated species [234]. Studies performed more than 30 years ago had already revealed the capacity of *Gardnerella* spp. to strongly adhere to vaginal epithelial cells [235,236], a step considered essential in the establishment of BV biofilm [147]. Moreover, recent data provide evidence that *Gardnerella* spp. are involved in inducing apoptosis of vaginal epithelial cells, during BV [237]. According to the authors, this may explain the increase in exfoliated vaginal epithelial cells also known as "clue cells", present in the vaginal fluid of women with BV [237].

In order to unravel the specific role of BV-associated bacteria, research on the virulence factors of these species was carried out. Interestingly, Machado et al. reported that *Gardnerella* spp. exhibited greater ability to adhere to vaginal epithelial cells than other BV-associated bacteria, including *F. vaginae*, *M. mulieris*, *P. bivia* and *F. nucleatum*. It was also demonstrated that *Gardnerella* spp. maintained its capacity to adhere in the presence of *L. crispatus* better than the other BV-associated bacteria [238]. Furthermore, Patterson and co-workers conducted an *in vitro* study to evaluate some virulence factors of BV-associated bacteria isolated from women with BV, such as *Gardnerella* spp., *F. vaginae*, *P. bivia,* and *Veilonella* spp. The outcomes revealed that only *Gardnerella* spp. was cytotoxic to epithelial cells and presented a greater propensity to form biofilm as well as higher initial adhesion than the other BV-associated bacteria [164]. A similar study was performed by Alves et al. and it also showed that *Gardnerella* spp. isolated from women with BV possess a higher virulence compared to 29 BV-associated bacteria [239]. However, it is not known if *Gardnerella* spp. have the ability to induce BV alone or if other BV-associated bacteria may also play an important role in the infection development. Through a prospective study of women who have sex with women, Muzny et al. revealed an increase in *Gardnerella* spp. and *P. bivia*,

four and three days before the development of incident bacterial vaginosis (iBV), respectively. An increase in *F. vaginae* was verified on the day of iBV onset [240]. Based on these findings and an extensive literature review, Muzny et al. proposed a new conceptual model for BV pathogenesis. That model defines that virulent strains of *Gardnerella* spp. are responsible for the initiation of BV biofilm, first displacing lactobacilli from the epithelium and then starting biofilm formation [168]. In fact, previous findings from our research group support this model. In 2015, Castro et al., showed that *Gardnerella* spp. strains isolated from BV have a stronger capacity to displace lactobacilli than non-BV isolates [167]. Subsequently, *Gardnerella* spp. act in synergism with *P. bivia* leading to the disruption of mucin layer which promotes the adherence of other BV-associated, as *F. vaginae*, to the vaginal epithelial cells, as shown in Figure 2.5 [168]. More recently, in the segment of this conceptual model, Muzny and colleagues stated that neither *Gardnerella* spp. nor *P. bivia* generate a strong inflammatory response from vaginal epithelial cells. On the other hand, secondary colonizers such as *F. vaginae*, *Sneathia* spp., and other BV-related bacteria, are capable to produce a greater stimulus of the host immune response to BV, suggesting that these bacteria species are responsible for the symptoms of the infection under study [241]. Recently, Sousa and co-workers studied the transcriptome of *G. vaginalis*, *F. vaginae* and *P. bivia*, when grown in a *in vitro* biofilm [242]. The transcriptomic analysis revealed significant changes in gene expression across all species, with *G. vaginalis* showing the highest number of differentially expressed genes. The genes associated with metabolism, adhesion, membrane transport, and resistance were regulated, suggesting that microbial interactions strongly influence the adaptation and persistence of these bacteria [242].

Currently, the presence of polymicrobial biofilm mostly composed of *Gardnerella* spp. is recognized to have a key role in BV pathogenesis. However, there is still a lack of information about the contribution of BV-associated bacteria in biofilm formation, although they can be determinants in BV [164].

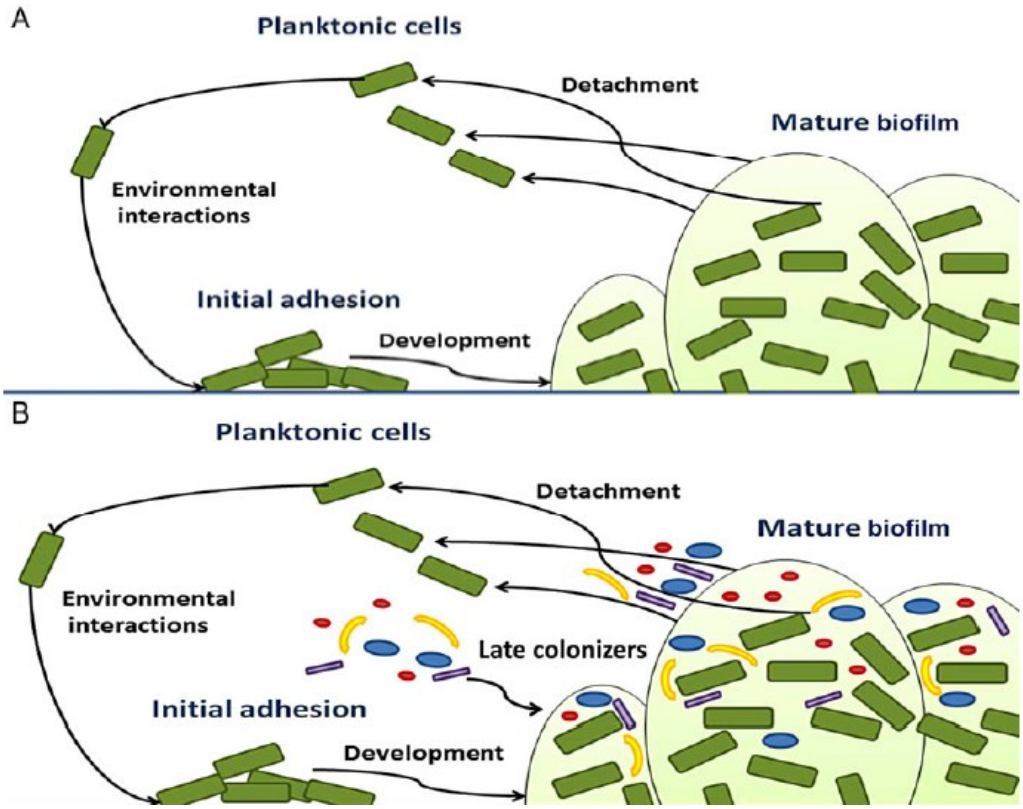


Figure 2.4.

Schematic representation of the steps that lead to monospecies (A) and polymicrobial (B) biofilms formation. From Machado et al. [243]

2.2.5.1 Interactions between BV-associated bacteria within the polymicrobial biofilm

At present, it is well accepted that most of the microorganisms live in biofilm communities, whereby synergistic and antagonistic interactions occur [244]. In the case of BV polymicrobial biofilm, it has been demonstrated that *Gardnerella* spp. exhibit different interactions with secondary anaerobic colonizers [245]. An *in vitro* study carried out by Machado et al. showed that *Gardnerella* spp. biofilm growth was enhanced by the addition of all BV-associated species tested, with a higher impact of *P. bivia* and *M. mulieris*. It was also observed that *Gardnerella* spp. promoted the growth of *P. bivia* and *F. nucleatum*, to a lesser extent in the last case [246]. According to an earlier study conducted by Pybus and Onderdonk, the symbiotic relationship between *Gardnerella* spp. and *P. bivia* may result from the amino acids produced by *Gardnerella* spp. [247] that fuel the growth of *P. bivia*, and conversely, *P. bivia* provides ammonia that promotes *Gardnerella* spp. growth [194]. Furthermore, research on *P. bivia* metabolism showed that two highly active enzymes catalyze the conversion of asparagine to the electron acceptor fumarate, essential to *P. bivia* energy production, resulting in the by-product ammonium which is extremely toxic [248]. The

authors suggested that *P. bivia* depends on ammonium-utilizing *Gardnerella* spp. to eliminate that toxic compound [248]. Hilbert et al. also demonstrated, using a mouse model, that *Gardnerella* spp. promotes the ascension and consequent infection of the uterus by *P. bivia*, after co-infection with these bacteria [249]. Thus, *Gardnerella* spp. and *P. bivia* are suggested to be "co-conspirators" in BV pathogenesis [250]. Studies about the interactions between these two microbes are shown in Table 2.6.

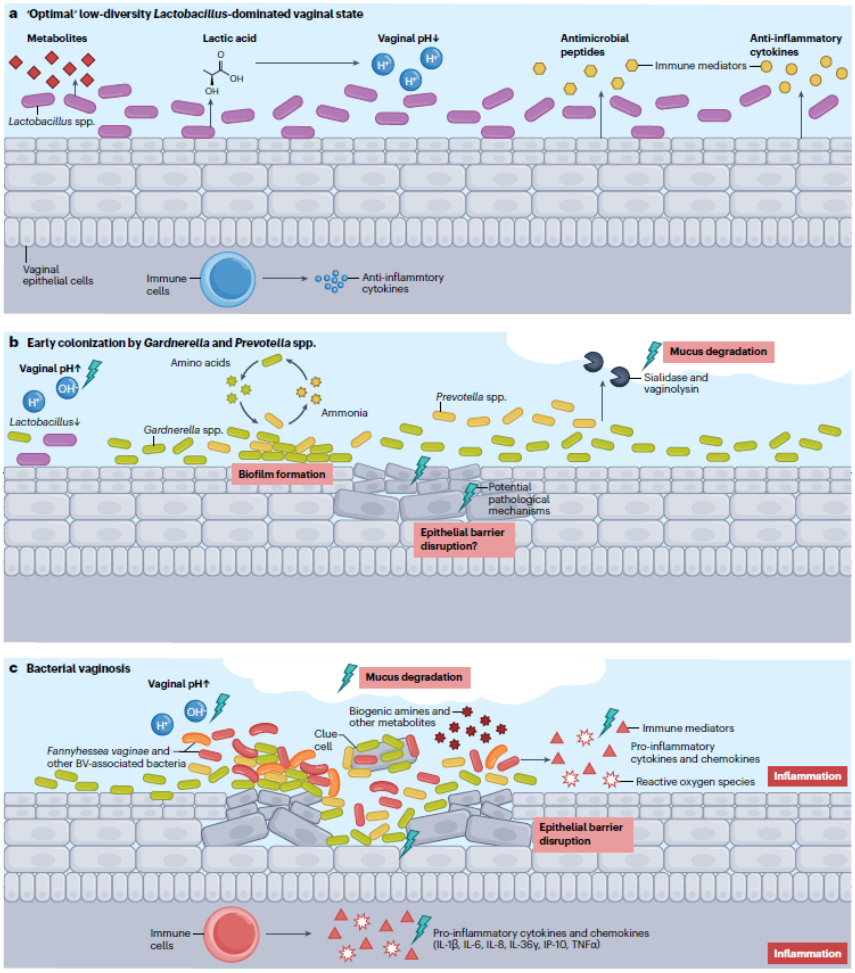


Figure 2.5.

Conceptual model for the development of polymicrobial biofilm of BV. From Muzny et [251].

Table 2.6.

Studies of Gardnerella spp. and P. bivia interactions

Type of assay	Interaction result	Reference
Dual-species biofilm growth	*Gardnerella* spp. growth was increased by the incorporation of *P. bivia*, and *P. bivia* growth was increased when co-cultured with *Gardnerella* spp.	[246]
Dual-species biofilm growth	*P. bivia* and BV-positive *Gardnerella* spp. strains exhibited a synergistic interaction in dual-species biofilm.	[252]
Coaggregation	*P. bivia* induced an increase in microbial aggregates in dual-species culture with *Gardnerella* spp.	[253]
Gene expression	*P. bivia* generated a slight but statistically significant increase in the gene expression of *Gardnerella* spp. vaginolysin.	
Gardnerella spp. and *P. bivia* vaginal co-infection in a mouse model	*Gardnerella* spp. facilitated uterine infection by *P. bivia*.	[249]

Regarding the microbial interactions between *Gardnerella* spp. and other BV-associated species, a study suggested that *Gardnerella* spp. also established a synergism relationship with *M. hominis* [109]. Moreover, Castro et al. [253] investigated the interactions of *Gardnerella* spp. BV isolates with 15 BV-associated bacteria using a dual-species biofilm model. It was observed that *F. vaginae*, *P. bivia*, *Corynebacterium tuscaniense*, *M. mulieris* and *S. anginosus* promoted microbial aggregations. From this study, it was also inferred that *Enterococcus faecalis* and *Actinomyces neuii* increased the expression of the gene that encodes for vaginolysin, a cytotoxic protein that alters vaginal cells integrity [254], as well as the expression of sialidase gene, a protein that induces the breakdown of the mucus layer [148]. *E. faecalis* was also implicated in the great increase of transcript levels of two proteins involved in antimicrobial tolerance, and the expression of the *HMPREF0424_0821* transcript, which encodes glycosyltransferases type II, was upregulated in most of the consortia tested [253]. Glycosyltransferases are enzymes that synthesize glucan polymers which contribute to the biofilm matrix [255]. More recently, Castro et al. also observed a significant upregulation of the expression of the *HMPREF0424_0821* transcript, using a novel *in vitro* triple-species biofilm model composed by a pre-established *G. vaginalis* biofilm to which *F. vaginae*

and *P. bivia* were able to incorporate, when compared to *G. vaginalis* mono-species biofilm [256]. In another study it was described that *F. vaginae* cells benefit from the presence of *G. vaginalis*, maintaining viability when co-cultured with *G. vaginalis* or cell-free supernatant of *G. vaginalis* culture. In addition, *F. vaginae* incorporated a pre-formed *G. vaginalis* biofilm, composing approximately 20% of the biofilm cells [257]. Previous studies had already shown that *F. vaginae* is not detected in vaginal samples without *Gardnerella* spp., however, *Gardnerella* spp. are detected in the absence of *F. vaginae*, indicating that *Gardnerella* spp. create a favorable environment for *F. vaginae* growth [231,258].

Further research is needed to better elucidate the molecular mechanisms of the regulatory networks between *Gardnerella* spp., the BV-associated bacteria, and their hosts, aiming to design suitable therapies to treat BV.

2.3. Treatment of BV

According to "2021 Sexually Transmitted Diseases Treatment Guidelines" established by the Centers for Disease Control and Prevention, the treatment of BV is only recommended for patients with symptoms [259]. Although there are data supporting the asymptomatic treatment, these findings resulted from trials with limitations. This, added to the fact that it is not known whether the pathogenesis of asymptomatic BV differs from the symptomatic one, generates controversy in the treatment of these cases [260]. Interestingly, the "2018 European International Union against sexually transmitted infections (IUSTI) World Health Organization (WHO) guideline on the management of vaginal discharge" established that some asymptomatic pregnant women (those with prior history of preterm birth) should be treated [261]. The therapy relieves vaginal symptoms and signs of infection in nonpregnant women, and additionally, it may decrease the risk of acquiring STIs, such as HIV and *Neisseria gonorrhoeae* [259]. Although both guidelines do not considerate the need of male partner treatment in BV cases, a very recent study conducted by Vodstrcil and coworkers showed evidence that a combination of oral and topical antibiotic treatment of male partners reduced BV recurrence [262]. Generally, BV is treated using antibiotic therapy, being the recommended first-line regimens: oral metronidazole, intravaginal metronidazole gel, and intravaginal clindamycin gel as described in Table 2.4 [259,261].

Metronidazole and clindamycin present similar cure rates, ranging from 70% to 92% [263]. Metronidazole is a synthetic antibiotic derived from nitroimidazole, and it is prescribed to treat a wide variety of medical conditions in addition to BV, such as trichomoniasis and giardiasis [264]. This antibiotic is associated with multiple side effects, such as vomiting, nausea, and headaches [265,266]. Regarding

clindamycin, it is a lincosamide antibiotic, available in different formulations, namely, vaginal (cream and ovules) and oral [267]. Although clindamycin presents fewer adverse effects than metronidazole, it is significantly more expensive [261].

As alternatives, tinidazole is the second generation of nitroimidazole, and at a lower dose, it has higher efficacy and fewer side effects than metronidazole [268]. In the "2018 IUSTI - WHO guideline on the management of vaginal discharge" dequalinium chloride was introduced as an alternative treatment [261]. Recently, Gaspar et al. demonstrated *in vitro* that dequalinium chloride has the capacity to disrupt *Gardnerella* spp. BV-associated biofilms [269]. However, its impact on polymicrobial biofilms was not assessed, and it has been shown that BV polymicrobial biofilms contribute to enhanced antimicrobial tolerance [218]. Another alternative treatment is secnidazole, which belongs to the family of 5-nitroimidazoles, and was approved by the US Food and Drug Administration in 2017 for BV therapy [270].

Although antibiotic treatment efficacy is high after a month of therapy, the recurrence rate of BV is higher than 50 % within a year [271]. The high recurrence of BV is thought to be related to its biofilm nature, like other biofilm-associated infections, in which treatment is very challenging [272]. The chronicity of BV may result from the ineffectiveness of antibiotics to completely eradicate biofilms, as shown by Swidsinski et al. [229]. After oral metronidazole treatment, all BV patients recovered, however, a dense *Gardnerella* spp. biofilm resurged within 3 weeks [229]. In a more recent study, Swidsinski and co-workers also concluded that after treating BV-positive women with moxifloxacin, *Gardnerella* spp. biofilm was not eliminated [273]. Eradication of BV biofilm must be essential to prevent BV recurrence, therefore, strategies aimed at the BV biofilm disruption have been investigated, including, plant-derived products [274], probiotics [275], antimicrobial peptides [276], DNases [277], and phages [278]. In addition, there is the need to change BV treatment approach of women with BV, by making their male partners also engaging in treatment [279].

Table 2.7.

International guidelines for BV treatment.

Antibiotic	Formulation	Dosage	Duration
Regimens for BV treatment established in 2015 Sexually Transmitted Diseases Treatment Guidelines [259]			
Recommended treatment regimens			
Metronidazole	Oral	500 mg, twice a day	7 days
Metronidazole	Intravaginal gel 0.75%	5 mg, once a day	5 days
Clindamycin	Intravaginal gel 2%	5 mg, once a day at bedtime	7 days
Alternative treatment regimens			
Tinidazole	Oral	2 g, once a day	2 days
Tinidazole	Oral	1 g, once a day	5 days
Clindamycin	Oral	300 mg, twice a day	7 days
Clindamycin	Intravaginal ovules	100 mg, once a day at bedtime	3 days
Secnidazole	Oral	2g, in a single dose	-
2018 European International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge [261]			
Recommended treatment regimens			
Metronidazole	Oral	400-500 mg, twice a day	5-7 days
Metronidazole	Intravaginal gel 0.75%	once a day	5 days
Clindamycin	Intravaginal cream 2%	once a day	7 days
Alternative treatment regimens			
Metronidazole	Oral	2 g, once a day	1 day
Tinidazole	Oral	2 g, once a day	1 day
Tinidazole	Oral	1 g, once a day	5 days
Clindamycin	Oral	300 mg, twice a day	7 days
Dequalinium chloride	Intravaginal	10 mg, once a day	6 Days

2.4 The nature of vaginal bacteria and the methods used to quantify them

One important limitation in the study of bacterial vaginosis is the fact that culture-based methods are not adequate to accurately quantify vaginal bacteria concentrations [280], which is essential for the preparation of an initial inoculum for downstream experiments as well as to perform experiments on the bacterial reduction or increase [281]. This occurs since the majority of vaginal bacteria are facultative or strictly anaerobe and present a fastidious growth, which makes them difficult to cultivate [145]. Common molecular and non-molecular, culture-independent methods that allow researchers to quantify bacteria include quantitative polymerase chain reaction (qPCR), flow cytometry (FC), and fluorescence microscopy (FM) [282].

qPCR has become a widely used technique for gene expression assessment, as well as for bacterial quantification [283]. The affordability, ease of experimental design, reproducibility, and robustness of qPCR experiments contribute to its success. Despite its technological potential, qPCR is not without its caveats, and the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) were pivotal to improve reproducibility, data analysis, interpretation, and overall transparency of qPCR experimental reports [284]. As detailed in the MIQE guidelines [284], qPCR experiments have two main issues that need to be properly addressed: those related to the extraction and purification of gDNA [285] and those related to the thermal amplification process [286]. Regarding FC, this technique is fast, robust, and reproducible for determining total and viable bacterial counts, with the potential for automation; however, it is not cost effective [287,288]. FM is more affordable than qPCR and FC, but more time consuming [289–292], and requires the use of a Neubauer chamber or ocular grids installed in the microscope [293].

The study of vaginal microbiota is essential given its significant impact on women's health. In this context, the use of adequate technology. In this context, the use of appropriate technologies, along with their correct application, is crucial. Additionally, it is important to develop new technologies in order to facilitate the study of microorganisms that are difficult to cultivate.

2.5 References

1. Smith, S. & Ravel, J. The vaginal microbiota, host defense and reproductive physiology. *Journal of Physiology* **595**, 451–463 (2017).

2. Song, S., Archarya, K., Zhu, J., Deveney, C., Whalter-Antonio, M., Tetel, M. & Chia, N. Daily Vaginal Microbiota Fluctuations Associated with Natural Hormonal Cycle, Contraceptives, Diet, and Exercise. *American Society for Microbiology* **5**, 1–14 (2020).

3. Crann, S. E., Cunningham, S., Albert, A., Money, D. M. & O'Doherty, K. C. Vaginal health and hygiene practices and product use in Canada: A national cross-sectional survey. *BMC Womens Health* **18**, 1–8 (2018).

4. Romero, R. *et al.* The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* **2**, 1–19 (2014).

5. Vodstrcil, L. A. *et al.* Incident Bacterial Vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. *Clinical Infectious Diseases* **60**, 1042–1053 (2015).

6. Zhang, X., Liao, Q., Wang, F. & Li, D. Association of gestational diabetes mellitus and abnormal vaginal flora with adverse pregnancy outcomes. *Medicine (United States)* **97**, (2018).

7. Macklaim, J., Clemente, J., Knight, R., Gloor, G. & Reid, G. Changes in vaginal microbiota following antimicrobial and probiotic therapy. *Microb Ecol Health Dis* **26**, 1–8 (2015).

8. Hickey, D. et al. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: Stratification and integration of immune protection against the transmission of sexually transmitted infections. *J Reprod Immunol* **88**, 185–194 (2011).

9. Amabebe, E. & Anumba, D. The vaginal microenvironment: The physiological role of *Lactobacilli*. *Front Med (Lausanne)* **5**, 1–11 (2018).

10. Chen, A. et al. Transient antibody-mucin interactions produce a dynamic molecular shield against viral invasion. *Biophys J* **106**, 2028–2036 (2014).

11. Chee, W., Chew, S. & Than, L. Vaginal microbiota and the potential of *Lactobacillus* derivatives in maintaining vaginal health. *Microb Cell Fact* **19**, 1–24 (2020).

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

13. Petrova, M., Lievens, E., Malik, S., Imholz, N & Lebeer, S. *Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health. *Front Physiol* **6**, 1–18 (2015).

14. Kovachev, S. Defence factors of vaginal lactobacilli. *Crit Rev Microbiol* **44**, 31–39 (2017).

15. O'Hanlon, D., Come, R. & Moench, T. Vaginal pH measured in vivo: lactobacilli determine pH and lactic acid concentration. *BMC Microbiol* **19**, 1–8 (2019).

16. Gong, Z., Luna, Y., Yu, P. & Fan, H. Lactobacilli Inactivate *Chlamydia trachomatis* through Lactic Acid but Not H2O2. *PLoS One* **9**, 1–12 (2014).

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

18. Sgibnev, A. & Kremleva, E. Vaginal protection by H2O2-producing lactobacilli. *Jundishapur J Microbiol* **8**, 4–8 (2015).

19. Stoyancheva, G., Marzotto, M., Dellaglio, F. & Torriani, S. Bacteriocin production and gene sequencing analysis from vaginal *Lactobacillus* strains. *Arch Microbiol* **196**, 645–653 (2014).

20. He, Y. *et al.* Evaluation of the inhibitory effects of *Lactobacillus* gasseri and *Lactobacillus crispatus* on the adhesion of seven Common lower genital tract infection-causing pathogens to vaginal epithelial cells. *Front Med (Lausanne)* **7**, 1–8 (2020).

21. Ravel, J. et al. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences* **108**, 4680–4687 (2011).

22. Nouioui, I., Carro, L., García-López, M., Meier-Kolthoff, J. & Woyke, T., Kyrpides, N., Pukall, R., Klenk, H., Goodfellow. M. & Göker, M. Genome-based taxonomic classification of the phylum actinobacteria. *Front Microbiol* **9**, 1–119 (2018).

23. Gajer, P., Brotman, R. & Bai, G., Sakamoto, J., Schütte, U., Zhong, X., Koenig, S., Fu, L., Ma, Z., Zhou, X., Abdo, Z., Forney, L. & Ravel, J. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* **4**, (2012).

24. De Seta, F. et al. The vaginal community state types microbiome-immune network as key factor for bacterial vaginosis and aerobic vaginitis. *Front Microbiol* **10**, 1–8 (2019).

25. Freitas, A. et al. Quantification, isolation and characterization of *Bifidobacterium* from the vaginal microbiomes of reproductive aged women. *Anaerobe* **47**, 145–156 (2017).

26. Rosca, A., Castro, J., Sousa L. & Cerca, N. *Gardnerella* and vaginal health: the truth is out there. *FEMS Microbiol Rev* **44**, 73–105 (2020).

27. Muzny, C. & Schwebke, J. Pathogenesis of Bacterial Vaginosis: Discussion of Current Hypotheses. *Journal of Infectious Diseases* **214**, S1–S5 (2016).

28. Ceccarani, C., Foschi, C., Parolin, C., D'Antuono, A., Gaspari, V., Consolandi, C., Laghi, L., Camboni, T., Vitali, B., Severgnini, M. & Marangoni, A. Diversity of vaginal microbiome and metabolome during genital infections. *Sci Rep* **9**, 1–12 (2019).

29. Ziklo, N., Vidgen, M., Taing, K., Huston, W. & Timms, P. Dysbiosis of the vaginal microbiota and higher vaginal kynurenine/tryptophan ratio reveals an association with *Chlamydia trachomatis* genital infections. *Front Cell Infect Microbiol* **8**, 1–11 (2018).

30. Zhao, H. Vaginal microbiota and susceptibility to HIV. *Physiol Behav* **176**, 139–148 (2017).

31. Bradshaw, C. & Brotman, R. Making inroads into improving treatment of bacterial vaginosis - striving for long-term cure. *BMC Infect Dis* **15**, 1–12 (2015).

32. Peebles, K., Velloza, J., Balkus, J. E., McClelland, R. S. & Barnabas, R. V. High Global Burden and Costs of Bacterial Vaginosis: A Systematic Review and Meta-Analysis. *Sex Transm Dis* **46**, 304–311 (2019).

33. Reid, G. Is bacterial vaginosis a disease? *Appl Microbiol Biotechnol* **102**, 553–558 (2018).

34. van de Wijgert, J. et al. The global health impact of vaginal dysbiosis. *Res Microbiol* **168**, 859–864 (2017).

35. Shimaoka, M., Yo, Y., Doh, K., Kotani, Y., Suzuki, A., Tsuji, I., Mandai, M. & Matsumura, N. Association between preterm delivery and bacterial vaginosis with or without treatment. *Sci Rep* **9**, 1–8 (2019).

36. Isik, G., Demirezen, Ş., Dönmez, H. & Beksaç, M. Bacterial vaginosis in association with spontaneous abortion and recurrent pregnancy losses. *J Cytol* **33**, 135–140 (2016).

37. Gupta, A., Singh, S., Chaudhary, R. & Nigam, S. Bacterial Vaginosis in Pregnancy (<28 Weeks) and its effect on pregnancy outcome: A Study from a western up city. *Indian Journal of Obstetrics and Gynecology Research* **3**, 90 (2016).

38. Ravel, J. Moreno, I. & Simón, C. Bacterial vaginosis and its association with infertility, endometritis, and pelvic inflammatory disease. *Am J Obstet Gynecol* **224**, 251–257 (2021).

39. Haggerty, C., Ness, R., Totten, P., Farooq, F., Tang, G., Ko, D., Hou, X., Fiedler, T. Srinivasan, S., Astete, S. & Fredricks, D. Presence and concentrations of select bacterial vaginosis-associated bacteria are associated with increased risk of pelvic inflammatory disease. *Sex Transm Dis* **47**, 344–346 (2020).

40. Hoang, T., Toler, E., DeLong, K., Mafunda, N., Bloom, S., Zierden, H., Moench, T., Coleman, J., Hanes, J., Kwon, D., Lai, S., Cone, R. & Ensign, L. The cervicovaginal mucus barrier to HIV-1 is diminished in bacterial vaginosis. *PLoS Pathog* **16**, 1–25 (2020).

41. Seña, A., Goldstein, L., Ramirez, G., Parish, A. & McClelland, R. Bacterial Vaginosis and its association with incident *Trichomonas vaginalis* infections: A Systematic Review and Meta-Analysis. *Sex Transm Dis* **48**, e192–e201 (2021).

42. Bautista, C. et al. Association of Bacterial Vaginosis with Chlamydia and Gonorrhea among women in the U.S. *Am J Prev Med* **52**, 632–639 (2017).

43. Adolfsson, A., Hagander, A., Mahjoubipour, F. & Larsson, P. How vaginal infections impact women ' s everyday life. *Advances in Sexual Medicine* **7**, 1–19 (2017).

44. Bradshaw, C. & Sobel, J. Current treatment of Bacterial Vaginosis-limitations and need for innovation. *Journal of Infectious Diseases* **214**, S14–S20 (2016).

45. Reiter, S. & Spadt, S. Bacterial vaginosis: a primer for clinicians. *Postgrad Med* **131**, 8–18 (2019).

46. Hay, P. Bacterial vaginosis. *Medicine (United Kingdom)* **42**, 359–363 (2014).

47. Nasioudis, D., Linhares, I., Ledger, W. & Witkin, S. Bacterial vaginosis: a critical analysis of current knowledge. *BJOG* **124**, 61–69 (2017).

48. Schwebke, J., Muzny, C. & Josey, W. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis – a conceptual model accepted. *Journal of Infectious Diseases* **210**, 338–343 (2014).

49. O'Hanlon, D. et al. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One* **8**, 1–8 (2013).

50. Jung, H. et al. Etiology of bacterial vaginosis and polymicrobial biofilm formation. *Crit Rev Microbiol* **43**, 651–667 (2017).

51. Vaneechoutte, M. *et al.* Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. Nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella. Int J Syst Evol Microbiol* **69**, 679–687 (2019).

52. Amsel, R. et al. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *AM J Med* **74**, 14–22 (1983).

53. Coleman, J. & Gaydos, C. Molecular diagnosis of bacterial vaginosis: An update. *J Clin Microbiol* **56**, 1–9 (2018).

54. Sha, B. et al. Utility of amsel criteria, nugent score, and quantitative PCR for *Gardnerella vaginalis*, Mycoplasma hominis, and Lactobacillus spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *J Clin Microbiol* **43**, 4607–4612 (2005).

55. Nugent, R. et al. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* **29**, 297–301 (1991).

56. Van De Wijgert, J. et al. The vaginal microbiota: What have we learned after a decade of molecular characterization? *PLoS One* **9**, (2014).

57. Scapaticci, M., Rin, G. & Bartolini, A. Comparison between a novel molecular tool and conventional methods for diagnostic classification of bacterial vaginosis: is integration of the two approaches necessary for a better evaluation? *New Microbiologica* **43**, 121–126 (2020).

58. Redelinghuys, M., Geldenhuys, J., Jung, H. & Kock, M. Bacterial Vaginosis: current diagnostic avenues and future opportunities. *Front Cell Infect Microbiol* **10**, 1–20 (2020).

59. Neal, C., Kus, L., Eckert, L. & Peipert, J. Noncandidal vaginitis: a comprehensive approach to diagnosis and management. *Am J Obstet Gynecol* **222**, 114–122 (2020).

60. Cartwright, C., Lembke, B., Ramachandran, K., Body, B., Nye, M., Rivers, C. & Schwebke, J. Development and validation of a semiquantitative, multitarget PCR assay for diagnosis of bacterial vaginosis. *J Clin Microbiol* **50**, 2321–2329 (2012).

61. Parolin, C., Foschi, C., Laghi, L., Zhu, C., Banzola, N., Gaspari, V., D'Antuono, A., Giordani, B., Severgnini, M., Consolandi, C., Salvo, M. & Cevenini, R., Vitali, B. & Marangoni, A. Insights into vaginal bacterial communities and metabolic profiles of *Chlamydia trachomatis* infection: Positioning between eubiosis and dysbiosis. *Front Microbiol* **9**, 1–10 (2018).

62. Cruciani, F., Biagi, E., Severgnini, M., Consolandi, C., Calanni, F., Donders, G., Brigidi, P. & Vitali, B. Development of a microarray-based tool to characterize vaginal bacterial fluctuations and application to a novel antibiotic treatment for bacterial vaginosis. *Antimicrob Agents Chemother* **59**, 2825–2834 (2015).

63. Yuan, D., Chen, W., Qin, J., Shen, D., Qiao, Y. & Kong, B. Associations between bacterial vaginosis, candida vaginitis, trichomonas vaginalis, and vaginal pathogenic community in Chinese women. *Am J Transl Res* **13**, 7148–7155 (2021).

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

65. Javed, A., Parvaiz, F. & Manzoor, S. Bacterial Vaginosis: An insight into the prevalence, alternative regimen treatments and it's associated resistance patterns. *Microb Pathog* **127**, 21–30 (2019).

66. Koumans, E. et al. The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sex Transm Dis* **34**, 864–869 (2007).

67. Borgdoff, H. et al. Women of dutch ethnic origin have lower prevalence of vaginal microbiome dysbiosis than women of other ethnic origin residing in Amsterdam. *Sex Transm Infect* **91**, 1–2 (2015).

68. Jespers, V., Crucitti, T., Menten, J., Verhelst, R., Mwaura, M., Mandaliya, K., Ndayisaba, G., Delany-Moretlwe, S., Verstraelen, H. *et al.* Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: A cross-sectional study. *PLoS One* **9**, (2014).

69. Melo, J. et al. Low prevalence of HIV and other sexually transmitted infections in young women attending a youth counselling service in Maputo, Mozambique. *Tropical Medicine and International Health* **13**, 17–20 (2008).

70. Kirakoya-Samadoulougou, F. et al. Epidemiology of herpes simplex virus type 2 infection in rural and Urban Burkina Faso. *Sex Transm Dis* **38**, 117–123 (2011).

71. Starc, M., Lučovnik, M., Eržen Vrlič, P. & Jeverica, S. The Prevalence of Bacterial Vaginosis in Pregnant Women in Slovenia, Determined via Microscopy and Semi-Quantitative Relative Culture, and Its Association with Adverse Pregnancy Outcomes. *Microorganisms* **13**, (2025).

72. Van den Munckhof, E. H. A. *et al.* Comparison of Amsel criteria, Nugent score, culture and two CE-IVD marked quantitative real-time PCRs with microbiota analysis for the diagnosis of bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases* **38**, 959–966 (2019).

73. Bhujel, R., Mishra, S. K., Yadav, S. K., Bista, K. D. & Parajuli, K. Comparative study of Amsel's criteria and Nugent scoring for diagnosis of bacterial vaginosis in a tertiary care hospital, Nepal. *BMC Infect Dis* **21**, (2021).

74. Roachford, O. S. E., Alleyne, A. T. & Nelson, K. E. Insights into the vaginal microbiome in a diverse group of women of African, Asian and European ancestries. *PeerJ* **10**, (2022).

75. Nelson, T. M. *et al.* Cigarette smoking is associated with an altered vaginal tract metabolomic profile. *Sci Rep* **8**, 1–13 (2018).

76. Shaw, T., Saleem, S., Mir, S., Masoodi, M. & Khan, M. A study of role of bacterial vaginosis in preterm labour. *Advances in Tissue Engineering & Regenerative Medicine: Open Access* **4**, 64–69 (2018).

77. Gallo, M., Macaluso, M., Warner, L., Fleenor, M., Hook, E., Brill, I. & Weaver, M. Bacterial vaginosis, Gonorrhea, and Chlamydial infection among women attending a sexually transmitted disease Clinic: A longitudinal analysis of possible causal links. *Ann Epidemiol* **22**, 213–220 (2012).

78. Fethers, K. et al. Early sexual experiences and risk factors for bacterial vaginosis. *Journal of Infectious Diseases* **200**, 1662–1670 (2009).

79. Ranjit, E., Raghubanshi, B., Maskey, S. & Parajuli, P. Prevalence of bacterial vaginosis and its association with risk factors among nonpregnant women: A hospital based study. *Int J Microbiol* **2018**, 1–10 (2018).

80. Peebles, K. *et al.* Elevated risk of bacterial vaginosis among users of the copper intrauterine device: A prospective longitudinal cohort Study. *Clinical Infectious Diseases* **73**, 513–520 (2021).

81. Bradshaw, C., Vodstrcil, L., Hocking, J., Law, M., Pirotta, M., Garland, S., Guingand, D., Morton, A. & Fairley, C. Recurrence of bacterial vaginosis is significantly associated with posttreatment sexual activities and hormonal contraceptive use. *Clinical Infectious Diseases* **56**, 777–786 (2013).

82. Yotebieng, M. *et al.* Effect of consistent condom use on six-month prevalence of bacterial vaginosis varies by baseline BV status. *Trop Med Int Health* **14**, 480–486 (2016).

83. Fethers, K. et al. Sexual risk factors and bacterial vaginosis: A systematic review and meta-analysis. *Clinical Infectious Diseases* **47**, 1426–1435 (2008).

84. Mota, A., Prieto, E., Carnall, V. & Exposto, F. Avaliação de métodos microscópicos para diagnóstico de vaginose bacteriana.

85. Henriques, A. et al. Doctor's perception on bacterial vaginosis in Portugal: prevalence, diagnostic methods and choice of treatment. *Sex Transm Infect* **88**, 421 (2012).

86. Silva, D. et al. Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in Portuguese women and association with risk factors for bacterial vaginosis. *International Journal of Gynecology and Obstetrics* **124**, 178–179 (2014).

87. Vieira-Baptista, P., Tavares, S., Lima-Silva, J., Beires, J. & Donders, G. Prevalence and epidemiology of aerobic vaginitis and bacterial vaginosis in Portuguese women. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **206**, e119–e120 (2016).

88. Machado, D., Castro, J., Martinez-de-Oliveira, J., Nogueira-Silva, C. & Cerca, N. Prevalence of bacterial vaginosis in portuguese pregnant women and vaginal colonization by *Gardnerella vaginalis*. *PeerJ* **2017**, 1–12 (2017).

89. Onderdonk, A. et al. The human microbiome during bacterial vaginosis. *Clin Microbiol Rev* **29**, 223–238 (2016).

90. Kunze, A. & Larsen, B. Current concepts of *Gardnerella vaginalis* biofilm: significance in bacterial vaginosis. *Open J Obstet Gynecol* **9**, 1569–1585 (2019).

91. Gardner, H. and Dukes, C. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. *Am J Obstet Gynecol* **69**, 962–976 (1955).

92. Evans, A. S. Limitations of the Henle - Koch postulates. *Causation and disease* 123–146 (1993).

93. Muzny, C. A. and Schwebke, J. R. *Gardnerella vaginalis*: Still a prime suspect in the pathogenesis of bacterial vaginosis. *Curr Infect Dis Rep* **15**, 130–135 (2013).

94. Criswell, B.S., Ladwig, C.L., Gardner, H.L. and Dukes, C. D. *Haemophilus vaginalis* vaginitis by inoculation from cultures. *Obstet Gynecol* **33**, 195–199 (1969).

95. Josey, E. and Schwebke, J. The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. *Int J STD AIDS* **19**, 152–154 (2008).

96. Zwittink, R. D. *et al.* The vaginal microbiota in the course of bacterial vaginosis treatment. *European Journal of Clinical Microbiology and Infectious Diseases* **40**, 651–656 (2020).

97. Coudray, M. S. & Madhivanan, P. Bacterial vaginosis—A brief synopsis of the literature. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **245**, 143–148 (2020).

98. Srinivasan, S., Morgan, M., Fiedler, T., Djukovic, D., Hoffman, N., Raftery, D., Marrazzo, J. & Fredricks, D. Metabolic signatures of bacterial vaginosis. *mBio* **6**, (2015).

99. Kaambo, E., Africa, C., Chambuso, R. & Passmore, J. Vaginal microbiomes associated with aerobic vaginitis and bacterial vaginosis. *Front Public Health* **6**, 1–6 (2018).

100. Burton, J. P., Devillard, E., Cadieux, P. A., Hammond, J. & Reid, G. Detection of *Atopobium vaginae* in postmenopausal women by cultivation-independent methods warrants further investigation. *J Clin Microbiol* **42**, 1829–1831 (2004).

101. Bradshaw, C., Tabrizi, S., Fairley, C., Morton, A., Rudland, E. and Garland, S. The association of *Atopobium vaginae* and *Gardnerella vaginalis* with bacterial vaginosis and recurrence after oral metronidazole Therapy. *J Infect Dis* **194**, 828–836 (2006).

102. Fredricks, D., Fiedler, T. Jeanne, M. and Marrazzo, L. Molecular Identification of bacteria associated with bacterial vaginosis. *N Engl J Med* **353**, 1899–1911 (2005).

103. Srinivasan, S., Hoffman, N., Morgan, M. Matsen, F., Fiedler, T., Hall, R., Ross, F., Mccoy, C., Bumgarner, R. and & Marrazzo, J., Fredricks, D. Bacterial communities in women with bacterial vaginosis: high resolution Phylogenetic analyses reveal relationships of microbiota to clinical criteria. *Obstet Gynecol* **7**, 79–84 (2012).

104. Delaney, M., and Onderdonk, A. Nugent score related to vaginal culture in pregnant women. *Obstetrics & Gynecology* **98**, 79–84 (2001).

105. Zheng, N., Guo, R., Wang, J., Zhou, W. & Ling, Z. Contribution of *Lactobacillus iners* to vaginal health and diseases: A Systematic Review. *Frontiers in Cellular and Infection Microbiology* vol. 11 Preprint at https://doi.org/10.3389/fcimb.2021.792787 (2021).

106. Petrova, M. I., Reid, G., Vaneechoutte, M. & Lebeer, S. *Lactobacillus iners*: Friend or Foe? *Trends Microbiol* **25**, 182–191 (2017).

107. Schwebke, J. and Lawing, L. Prevalence of *Mobiluncus* spp among women with and without bacterial vaginosis as detected by polymerase chain reaction. *Sex Transm Dis* **28**, 195–199 (2001).

108. Fethers, K., Twin, J., Fairley, C., Fowkes, F., Garland, S. Fehler, G., Morton, A., Hocking, J., Tabrizi, S. and Bradshaw, C. Bacterial vaginosis (BV) candidate bacteria: Associations with BV and behavioural practices in sexually-experienced and inexperienced women. *PLoS One* **7**, (2012).

109. Cox, C., Watt, A. , McKenna, J. & Coyle, P. *Mycoplasma hominis* and *Gardnerella vaginalis* display a significant synergistic relationship in bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases* **35**, 481–487 (2016).

110. Malaguti, N., Bahls, L., Uchimura, N., Gimenes, F. & Consolaro, M. Sensitive detection of thirteen bacterial vaginosis-associated agents using multiplex polymerase chain reaction. *Biomed Res Int* **2015**, 1–10 (2015).

111. Petrina, M., Cosentino, L., Rabe, L. & Hillier, S. Susceptibility of bacterial vaginosis (BV)-associated bacteria to secnidazole compared to metronidazole, tinidazole and clindamycin. *Anaerobe* **47**, 115–119 (2017).

112. Austin, M., Rabe, L., Srinivasan, S., Fredricks, D., Harold, C. & Hillier, S. *Mageeibacillus indolicus* gen. nov., sp. nov: A novel bacterium isolated from the female genital tract. *Anaerobe* **32**, 37–42 (2015).

113. Osei Sekyere, J., Oyenihi, A. B., Trama, J. & Adelson, M. E. Species-specific analysis of bacterial vaginosis-associated bacteria. *Microbiol Spectr* **11**, (2023).

114. Leopold, S. Heretofore undescribed organism isolated from the genitourinary system. *U. S. Armade Forces J* **4**, 264–266 (1953).

115. Zinnemann, K. & Turner, G. The taxonomic position of 'Haemophilus vaginalis' [Corynebacterium vaginale]. *J Pathol Bacteriol* **85**, 213–219 (1963).

116. Greenwood, J. & Pickett, M. Transfer of *Haemophilus vaginalis* Gardner and Dukes to a new genus, *Gardnerella*: *G. vaginalis* (Gardner and Dukes) comb. nov. *Int J Syst Bacteriol* **30**, 170–178 (1980).

117. Catlin, B. Gardnerella vaginalis: Characteristics, clinical considerations, and controversies. *Clin Microbiol Rev* **5**, 213–237 (1992).

118. Catlin, B. *Gardnerella vaginalis*: Characteristics, clinical considerations, and controversies. *Clin Microbiol Rev* **5**, 213–237 (1992).

119. Malone, B. Schreiber, M. Schneider, N. & Holdeman, L. Obligately anaerobic strains of *Corynebacterium vaginale* (*Haemophilus vaginalis*). *J Clin Microbiol* **2**, 272–275 (1975).

120. Turovskiy, Y., Noll, K. & Chikindas, M. The etiology of bacterial vaginosis. *J Appl Microbio* **110**, 1105–1128 (2011).

121. Woo, P., Lau, S., Teng, J., Tse, H. & Yuen, K. Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection* **14**, 908–934 (2008).

122. Hill, J. & Albert, A. Resolution and cooccurrence patterns of *Gardnerella leopoldii*, *G. swidsinskii*, *G. piotii*, and *G. vaginalis* within the vaginal microbiome. *Infect Immun* **87**, 1–12 (2019).

123. Sousa, M. *et al. Gardnerella pickettii* sp. nov. (formerly *Gardnerella* genomic species 3) and *Gardnerella greenwoodii* sp. nov. (formerly *Gardnerella* genomic species 8) isolated from female urinary microbiome. *Int J Syst Evol Microbiol* **73**, (2023).

124. Robinson, L. *et al.* Genome sequences of 15 *Gardnerella vaginalis* strains isolated from the vaginas of women with and without bacterial vaginosis. *Genome Announc* **4**, (2016).

125. Hickey, R. & Forney, L. *Gardnerella vaginalis* does not always cause bacterial vaginosis. *Journal of Infectious Diseases* **210**, 1682–1683 (2014).

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

127. Schellenberg, J., Patterson, M. & Hill, J. *Gardnerella vaginalis* diversity and ecology in relation to vaginal symptoms. *Res Microbiol* **168**, 837–844 (2017).

128. Castro, J., Jefferson, K. & Cerca, N. Genetic Heterogeneity and Taxonomic Diversity among *Gardnerella* Species. *Trends Microbiol* **28**, 202–211 (2020).

129. Piot, P., van Dyck, E., Peeters, M. Hale, J., Totten, P. & Holmes, K. Biotypes of *Gardnerella vaginalis. J Clin Microbiol* **20**, 677–679 (1984).

130. Ingianni, A., Petruzzelli, S. & Morandotti, G. Genotypic differentiation of *Gardnerella vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunol Med Microbiol* **18**, 61–66 (1997).

131. Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A. & Pleckaityte, M. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infect Dis* **17**, 1–9 (2017).

132. Pedraza-Aviles, A., Inzunza-Montiel, A., Ortiz-Zaragoza, M., Del Morales-Espinosa, M. & Ponce-Rosas, E. *Gardnerella vaginalis* biotypes: Modification of a proposed system. *Rev Latinoam Microbiol (1958)* **37**, 19–26 (1995).

133. Briselden, A. & Hillier, S. Longitudinal study of the biotypes of *Gardnerella vaginalis*. *J Clin Microbiol* **28**, 2761–2764 (1990).

134. Numanović, F., Hukić, M., Nurkić, M., Gegić, M., Delibegović, Z., Imamović, A. & Pašić, S. Importance of isolation and biotypization of *Gardnerella vaginalis* in diagnosis of bacterial vaginosis. *Bosn J Basic Med Sci* **8**, 270–276 (2008).

135. Benito, R., Vazquez, J. , Berron, S., Fenoll, A. & Saez-Neito, J. A modified scheme for biotyping *Gardnerella vaginalis*. *J Med Microbiol* **21**, 357–359 (1986).

136. Tosun, I., Alpay Karaoğlu, S., Ciftçi, H., Buruk, C., Aydin, F., Kiliç, A. & Ertürk, M. Biotypes and antibiotic resistance patterns of *Gardnerella vaginalis* strains isolated from healthy women and women with bacterial vaginosis. *Mikrobiyol Bul* **41**, 21–7 (2007).

137. Udayalaxmi, J., Bhat, G. & Kotigadde, S. Biotypes and virulence factors of *Gardnerella vaginalis* isolated from cases of bacterial vaginosis. *Indian J Med Microbiol* **29**, 165–168 (2011).

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

139. Aroutcheva, A. A., Simoes, J. A., Behbakht, K. & Faro, S. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clinical Infectious Diseases* **33**, 1022–1027 (2001).

140. Santiago, G., Deschasght, P., Nabil, A. & Kiama, T., Verstraelen, H., Jefferson, K. Temmerman, M.& Vaneechoutte, M. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. *Am J Obstet Gynecol* **204**, 450.e1-450.e7 (2011).

141. Hill, J., Swee, H., Money, D., Doyle, M. & Li, A., Crosby, W., Links, M., Leung, A., Chan, D. & Hemmingsen, S. Characterization of vaginal microflora of healthy, nonpregnant women by chaperonin-60 sequence-based methods. *Am J Obstet Gynecol* **193**, 682–692 (2005).

142. Jayaprakash, T., Schellenberg, J. & Hill, J. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PLoS One* **7**, 1–11 (2012).

143. Jayaprakash, T., Schellenberg, J. & Hill, J. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PLoS One* **7**, 1–11 (2012).

144. Ahmed, A., Earl, J., Retchless, A., Hillier, S., Rabe, L., Cherpes, T., Powell, E., Janto, B., Eutsey, R., Hiller, N., Boissy, R., Dahlgren, M., Hall, B.,, Costerton, W., Post, J., Hu, F. & Ehrlich, G. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol* **194**, 3922–3937 (2012).

145. Schellenberg, J., Jayaprakash, T., Gamage, N., Patterson, M., Vaneechoutte, M. & Hill, J. *Gardnerella vaginalis* subgroups defined by cpn60 sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PLoS One* **11**, 1–12 (2016).

146. Janulaitiene, M., Gegzna, V., Baranauskiene, L., Bulavaitė A., Simanavicius, M. & Pleckaityte, M. Phenotypic characterization of *Gardnerella vaginalis* subgroups suggests differences in their virulence potential. *PLoS One* **13**, 1–20 (2018).

147. Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S. Hale, L. and Lochs, H. Adherent Biofilms in Bacterial Vaginosis. *Obstet Gynecol* **106**, 1013–1036 (2005).

148. Hardy, L., Jespers, V., Van Bulck, M. & Buyze, J., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M. & Crucitti, T. The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PLoS One* **12**, 1–11 (2017).

149. Robinson, L., Schwebke, J., Lewis, W. & Lewis, A. Identification and characterization of NanH2 and NanH3, enzymes responsible for sialidase activity in the vaginal bacterium Gardnerella vaginalis. *Journal of Biological Chemistry* **294**, 5230–5245 (2019).

150. Garcia, E., Serrano, M., Edupuganti, L., Edwards, D., Buck, G. & Jefferson, K. Sequence comparison of vaginolysin from different *Gardnerella* species. *Pathogens* **10**, 1–15 (2021).

151. Cornejo, O., Hickey, R., Suzuki, H. & Forney, L. Focusing the diversity of *Gardnerella vaginalis* through the lens of ecotypes. *Evol Appl* **11**, 312–324 (2018).

152. Thompson, C., Chimetto, L., Edwards, R., Swings, J., Stackebrandt, E. & Thompson, F. Microbial genomic taxonomy. *BMC Genomics* **14**, 1–8 (2013).

153. Balashov, S. V., Mordechai, E., Adelson, M. E. & Gygax, S. E. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* **63**, 162–175 (2014).

154. Albert, A., Chaban, B., Wagner, E., Schellenberg, J., Links, M., Van Schalkwyk, J. & Reid, G., Hemmingsen, S., Hill, J. & Money, D. A study of the vaginal microbiome in healthy Canadian women utilizing cpn60-based molecular profiling reveals distinct *Gardnerella* subgroup community state types. *PLoS One* **10**, 1–21 (2015).

155. Hilbert, D., Schuyler, J. , Adelson, M. , Mordechai, E., Sobel, J. & Gygax, S. *Gardnerella vaginalis* population dynamics in bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases* **36**, 1269–1278 (2017).

156. Balashov, S., Mordechai, E., Adelson, M. & Gygax, S., Balashov, S. V., Mordechai, E., Adelson, M. E. & Gygax, S. E. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* **63**, 162–175 (2014).

157. Balashov, S., Mordechai, E., Adelson, M. & Gygax, S. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* **63**, 162–175 (2014).

158. Plummer, E., Vodstrcil, L., Murray, G. & Fairley, C., Danielewski, J., Garland, S., Chow, E., Bulach, D., Fethers, K., Hocking, J. & Bradshaw, C. *Gardnerella vaginalis* clade distribution is associated with behavioral practices and nugent score in women who have sex with women. *J Infect Dis* **221**, 454–463 (2020).

159. Shipitsyna, E., Krysanova, A., Khayrullina, G., Shalepo, K., Savicheva, A., Guschin, A. & Unemo, M. Quantitation of all four *Gardnerella vaginalis* clades detects abnormal vaginal microbiota characteristic of bacterial vaginosis more accurately than putative *G. vaginalis* sialidase A gene count. *Mol Diagn Ther* **23**, 139–147 (2019).

160. Tortelli, B. A., Lewis, A. L. & Fay, J. C. The structure and diversity of strain level variation in vaginal bacteria. *Microb Genom* 1–14 (2021) doi:10.1101/2020.06.26.173922.

161. Khan, S., Voordouw, M. & Hill, J. Competition among *Gardnerella* subgroups from the human vaginal microbiome. *Front Cell Infect Microbiol* **9**, 1–10 (2019).

162. Khan, S., Vancuren, S. & Hill, J. A Generalist lifestyle allows rare *Gardnerella* spp. to persist at low levels in the vaginal microbiome. *Microb Ecol* **82**, 1048–1060 (2021).

163. Khan, S. & Hill, J. Population density affects the outcome of competition in co-cultures of *Gardnerella* species isolated from the human vaginal microbiome. *Microb Ecol* 1–10 (2021) doi:10.1007/s00248-021-01745-4.

164. Patterson, J. L., Stull-Lane, A., Girerd, P. H. & Jefferson, K. K. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology (N Y)* **156**, 392–399 (2010).

165. Nisha, K., Antony, B. & Udayalaxmi, J. Comparative analysis of virulence factors & biotypes of *Gardnerella vaginalis* isolated from the genital tract of women with & without bacterial vaginosis. *Indian Journal of Medical Research* **149**, 57–61 (2019).

166. Robinson, L., Perry, J., Lek, S., Wollam, A., Sodergren, E., Weinstock, G., Lewis, W. & Lewis, A. Genome sequences of 15 *Gardnerella vaginalis* strains isolated from the vaginas of women with and without bacterial vaginosis. *Microbiol Resour Announc* **4**, 1–2 (2016).

167. Castro, J. *et al.* Using an in-vitro biofilm model to assess the virulence potential of Bacterial Vaginosis or non-Bacterial Vaginosis *Gardnerella vaginalis* isolates. *Sci Rep* **5**, 1–10 (2015).

168. Muzny, C. *et al.* An Updated Conceptual Model on the Pathogenesis of Bacterial Vaginosis. *J Infect Dis* **220**, 1399–1405 (2019).

169. Shah H. & Collins, D. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* **40**, 205–208 (1990).

170. Oliver, W. W. & Wherry, W. B. Notes on some bacterial parasites of the human mucous membranes. *The Journal of Infectious Diseases* vol. 28 https://about.jstor.org/terms (1921).

171. Shah, H. N., Chattaway, M. A., Rajakurana, L. & Gharbia, S. E. *Prevotella*. *Bergey's Manual of Systematics of Archaea and Bacteria* 1–25 (Wiley, 2015). doi:10.1002/9781118960608.gbm00249.

172. Shin, J. M. *et al.* Deciphering endodontic microbial communities by Next-generation Sequencing. *Journal of Endodontics* vol. 44 1080–1087. https://doi.org/10.1016/j.joen.2018.04.003 (2018).

173. George, S. D. *et al.* The Role of *Prevotella* species in female genital tract Infections. *Pathogens* **13** https://doi.org/10.3390/pathogens13050364 (2024).

174. Fehlner-Peach, H. *et al.* Distinct polysaccharide utilization profiles of human intestinal *Prevotella copri* isolates. *Cell Host Microbe* **26**, 680-690.e5 (2019).

175. Dos Santos, S. J., Copeland, C., Macklaim, J. M., Reid, G. & Gloor, G. B. Vaginal metatranscriptome meta-analysis reveals functional BV subgroups and novel colonization strategies. *Microbiome* **12**, (2024).

176. Segui-Perez, C. *et al. Prevotella timonensis* degrades the vaginal epithelial glycocalyx through high fucosidase and sialidase activities. *mBio* **15**, (2024).

177. Tett, A., Pasolli, E., Masetti, G., Ercolini, D. & Segata, N. *Prevotella* diversity, niches and interactions with the human host. *Nature Reviews Microbiology* **19** 585–599 https://doi.org/10.1038/s41579-021-00559-y (2021).

178. Könönen, E., Feita, D., Gursoy, U. K. & Gursoy, M. *Prevotella* species as oral residents and infectious agents with potential impact on systemic conditions. *Journal of Oral Microbiology* **14** Preprint at https://doi.org/10.1080/20002297.2022.2079814 (2022).

179. Ilhan, Z. E., Łaniewski, P., Tonachio, A. & Herbst-Kralovetz, M. M. Members of prevotella genus distinctively modulate innate immune and barrier functions in a human three-dimensional endometrial epithelial cell model. *Journal of Infectious Diseases* **222**, 2082–2092 (2020).

180. Mehmood, M., Jaffar, N. A., Nazim, M. & Khasawneh, F. A. Bacteremic skin and soft tissue infection caused by *Prevotella Loescheii*. *BMC Infect Dis* **14**, (2014).

181. Su, T. *et al.* Altered intestinal microbiota with increased abundance of *Prevotella* is associated with high risk of diarrhea-predominant irritable bowel syndrome. *Gastroenterol Res Pract* **2018**, (2018).

182. Hitch, T. C. A. *et al.* A taxonomic note on the genus Prevotella: Description of four novel genera and emended description of the genera *Hallella* and *Xylanibacter*. *Syst Appl Microbiol* **45**, (2022).

183. Muzny, C. A., Łaniewski, P., Schwebke, J. R. & Herbst-Kralovetz, M. M. Host-vaginal microbiota interactions in the pathogenesis of bacterial vaginosis. *Curr Opin Infect Dis* **33**, 59–65 (2020).

184. Sousa, L. G. V., Pereira, S. A. & Cerca, N. Fighting polymicrobial biofilms in bacterial vaginosis. *Microbial Biotechnology* vol. 16 1423–1437 Preprint at https://doi.org/10.1111/1751-7915.14261 (2023).

185. Randis, T. & Ratner, A. *Gardnerella* and *Prevotella*: Co-conspirators in the pathogenesis of bacterial vaginosis. *J Infect Dis* **220**, 1085–1088 (2019).

186. Ilhan, Z., Łaniewski, P., Tonachio, A. & Herbst-Kralovetz, M. Members of *Prevotella* genus distinctively modulate innate immune and barrier functions in a human three-dimensional endometrial epithelial cell model. *Journal of Infectious Diseases* **222**, 2082–2092 (2020).

187. Chen, P., Li, P. & Ding, D. Pelvic inflammatory disease and causative pathogens in older women in a medical center in eastern Taiwan: A retrospective cross-sectional study. *PLoS One* **16**, (2021).

188. Duerden, B. I. Black-pigmented Gram-negative anaerobes in genito-urinary tract and pelvic infections. *FEMS Immunol Med Microbiol* **6**, 223–228 (1993).

189. Diop, K. *et al.* Microbial culturomics broadens human vaginal flora diversity: genome sequence and description of *Prevotella lascolaii* sp. nov. isolated from a patient with bacterial vaginosis. *OMICS* **22**, 210–222 (2018).

190. Sharma, G., Garg, N., Hasan, S. & Shirodkar, S. *Prevotella*: An insight into its characteristics and associated virulence factors. *Microbial Pathogenesis*. **169** https://doi.org/10.1016/j.micpath.2022.105673 (2022).

191. Albaghdadi, S. Z., Altaher, J. B., Drobiova, H., Bhardwaj, R. G. & Karched, M. *In vitro* characterization of biofilm formation in *Prevotella* species. *Frontiers in Oral Health* **2**, (2021).

192. Wright, D. P., Rosendale, D. I. & Roberton, A. M. *Prevotella* enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin. *FEMS Microbiol Lett* **190**, 73–79 (2000).

193. Miyagi, T. & Yamaguchi, K. Mammalian sialidases: Physiological and pathological roles in cellular functions. *Glycobiology* vol. 22 880–896 Preprint at https://doi.org/10.1093/glycob/cws057 (2012).

194. Pybus, V. & Onderdonk, A. Evidence for a commensal, symbiotic relationship between *Gardnerella vaginalis* and *Prevotella bivia* involving ammonia: potential significance for bacterial vaginosis. *Journal of Infectious Diseases* **175**, 406–413 (1997).

195. Larsen, J. M. The immune response to *Prevotella* bacteria in chronic inflammatory disease. *Immunology* **151,** 363–374 https://doi.org/10.1111/imm.12760 (2017).

196. Dabee, S., Passmore, J.-A. S., Heffron, R. & Jaspan, H. B. The complex link between the female genital microbiota, genital infections, and inflammation. *Infect Immun* **89**. (2021)

197. Sharma, G., Garg, N., Hasan, S. & Shirodkar, S. *Prevotella*: An insight into its characteristics and associated virulence factors. *Microbial Pathogenesis* **169** (2022).

198. Gilbert, N. M. *et al.* Social, microbial, and immune factors linking bacterial vaginosis and infectious diseases. *Journal of Clinical Investigation* **135** (2025).

199. Gudnadottir, U. *et al.* The vaginal microbiome and the risk of preterm birth: a systematic review and network meta-analysis. *Sci Rep* **12**, (2022).

200. Petrina, M. A. B., Cosentino, L. A., Rabe, L. K. & Hillier, S. L. Susceptibility of bacterial vaginosis (BV)-associated bacteria to secnidazole compared to metronidazole, tinidazole and clindamycin. *Anaerobe* **47**, 115–119 (2017).

201. Petrina, M. A. B., Cosentino, L. A., Wiesenfeld, H. C., Darville, T. & Hillier, S. L. Susceptibility of endometrial isolates recovered from women with clinical pelvic inflammatory disease or histological endometritis to antimicrobial agents. *Anaerobe* **56**, 61–65 (2019).

202. Plummer, E. L. *et al. Prevotella* and *Gardnerella* are associated with treatment failure following first-line antibiotics for bacterial vaginosis. *Journal of Infectious Diseases* **228**, 646–656 (2023).

203. Rodriguez Jovita, M., Collins, M. D., Sjoden, B. & Falsen, E. Characterization of a Novel *Atopobium* Isolate from the Human Vagina: Description of *Atopobium Vaginae* Sp. Nov. *International Journal of Systematic Bacteriology* **49** (1999).

204. Parte, A. C., Carbasse, J. S., Meier-Kolthoff, J. P., Reimer, L. C. & Göker, M. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol* **70**, 5607–5612 (2020).

205. Vaseruk, A. *et al. Atopobium vaginae*: An overview of the bacteria through clinical cases. *Microbiol Res (Pavia)* **16**, (2025).

206. Polatti, F. Bacterial Vaginosis, *Atopobium vaginae* and Nifuratel. *Current Clinical Pharmacology* **7** (2012).

207. Onderdonk, A. et al. The human microbiome during bacterial vaginosis. *Clin Microbiol Rev* **29**, 223–238 (2016).

208. Knoester, M., Lashley, L., Wessels, E., Oepkes, D. & Kuijper, E. J. First report of *Atopobium vaginae* bacteremia with fetal loss after chorionic villus sampling. *J Clin Microbiol* **49**, 1684–1686 (2011).

209. Geißdörfer, W. *et al.* Tuboovarian abscess caused by *Atopobium vaginae* following transvaginal oocyte recovery. *J Clin Microbiol* **41**, 2788–2790 (2003).

210. Fredricks, D. N., Fiedler, T. L. & Marrazzo, J. M. Molecular identification of bacteria associated with bacterial vaginosis. *n engl j med* **18**, 1899–911 (2005).

211. Fredricks, D. N., Fiedler, T. L., Thomas, K., Oakley, B. B. & Marrazzo, J. M. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol* **45**, 3270–3276 (2007).

212. Wang, K.-D., Lv, Z. & Su, J.-R. Quantification of *Atopobium vaginae* loads may be a new method for the diagnosis of bacterial vaginosis. *Clin Lab* **60**, (2014).

213. Mendling, W., Palmeira-de-Oliveira, A., Biber, S. & Prasauskas, V. An update on the role of *Atopobium vaginae* in bacterial vaginosis: what to consider when choosing a treatment? A mini review. *Arch Gynecol Obstet* **300**, (2019).

214. Pierre Menard, J. *et al.* High Vaginal Concentrations of *Atopobium vaginae* and *Gardnerella vaginalis* in Women Undergoing Preterm Labor LEVEL OF EVIDENCE: III. *Obstet Gynecol* **115**, 134–174 (2010).

215. Loundou, A. High *A. vaginae* and *G. vaginalis* vaginal loads are associated with preterm birth for the GROG. *Clinical Infectious Diseases Advance Access* **12**, (2014).

216. Trama, J. P. *et al.* Rapid detection of *Atopobium vaginae* and association with organisms implicated in bacterial vaginosis. *Mol Cell Probes* **22**, 96–102 (2008).

217. Hardy, L., Jespers, V., Abdellati, S., De Baetselier, I., Mwambarangwe, L., Musengamana, V., Van De Wijgert, J. Vaneechoutte, M. & Crucitti, T. A fruitful alliance: The synergy *between Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm. *Sex Transm Infect* **92**, 487–491 (2016).

218. Rosca, A. S. *et al. In vitro* interactions within a biofilm containing three species found in bacterial vaginosis (BV) support the higher antimicrobial tolerance associated with BV recurrence. *Journal of Antimicrobial Chemotherapy* **77**, 2183–2190 (2022).

219. De Backer, E. *et al.* Antibiotic susceptibility of *Atopobium vaginae*. *BMC Infect Dis* **6**, (2006).

220. Ferris, M. J. *et al.* Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. BMC Infect Dis **4** (2004).

221. De Backer, E., Dubreuil, L., Brauman, M., Acar, J. & Vaneechoutte, M. *In vitro* activity of secnidazole against *Atopobium vaginae*, an anaerobic pathogen involved in bacterial vaginosis. *Clinical Microbiology and Infection* **16**, 470–472 (2010).

222. Libby, E. K., Pascal, K. E., Mordechai, E., Adelson, M. E. & Trama, J. P. *Atopobium vaginae* triggers an innate immune response in an in vitro model of bacterial vaginosis. *Microbes Infect* **10**, 439–446 (2008).

223. Segui-Perez, C., Van Smoorenburg, M. Y., Maranus, A. E., Geijtenbeek, T. B. H. & Strijbis, K. Impact of bacterial vaginosis on sexually transmitted viral infections: a bacterial point of view. *FEMS Microbiology Reviews* **49** (2025).

224. Gupta, P. Sarkar, S., Das, B., Bhattacharjee, S. and Tribedi, P. Biofilm, pathogenesis and prevention—a journey to break the wall: a review. *Arch Microbiol* **198**, 1–15 (2016).

225. Tolker-nielsen, T. Biofilm Development. *American Society for Microbiology* **3**, 1–12 (2015).

226. Singh, S., Singh, S. K., Chowdhury, I. & Singh, R. Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. *Open Microbiol J* **11**, 53–62 (2017).

227. Moser, C. *et al.* Immune responses to *Pseudomonas aeruginosa* biofilm infections. *Frontiers in Immunology* **12** (2021).

228. Yin, W., Wang, Y., Liu, L. & He, J. Biofilms: The microbial "protective clothing" in extreme environments. *Int J Mol Sci* **20**, (2019).

229. Swidsinski, A. *et al.* An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* **198**, 97.e1-97.e6 (2008).

230. Swidsinski, A. *et al.* Presence of a olymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS One* **8**, 4–8 (2013).

231. Hardy, L., Jespers, V., Dahchour, N., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M. & Crucitti, T. Unravelling the bacterial vaginosis-associated biofilm: A multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence in situ hybridization assay using peptide nucleic acid probes. *PLoS One* **10**, 1–16 (2015).

232. Patterson, J., Girerd, P., Karjane, N. & & Jefferson, K. Effect of biofilm phenotype on resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. *Am J Obstet Gynecol* **197**, 170.e1-170.e7 (2007).

233. Castro, J. *et al.* Comparative transcriptomic analysis of *Gardnerella vaginalis* biofilms vs. planktonic cultures using RNA-seq. *NPJ Biofilms Microbiomes* **3**, (2017).

234. Swidsinski, A. *et al.* Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). *Histol Histopathol* **29**, 567–587 (2014).

235. Scott, T. G., Curran, B. & Smyth, C. J. Electron Microscopy of Adhesive Interactions between *Gardnerella vaginalis* and epithelial cells. *J Gen Microbiol* **135**, 475–480 (1989).

236. Johnson, A. P. & Boustouller, Y. L. Extra-vaginal infection caused by *Gardnerella vaginalis*. *Epidemiol Infect* **98**, 131–137 (1987).

237. Roselletti, E. *et al.* Apoptosis of vaginal epithelial cells in clinical samples from women with diagnosed bacterial vaginosis. *Sci Rep* **10**, 1–9 (2020).

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

239. Alves, P., Castro, J., Sousa, C., Cereija, T. B. & Cerca, N. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *Journal of Infectious Diseases* **210**, 593–596 (2014).

240. Muzny, C. A. *et al.* Identification of key bacteria involved in the induction of incident bacterial vaginosis: A Prospective Study. *J Infect Dis* **218**, 966–978 (2018).

241. Muzny, C. A., Łaniewski, P., Schwebke, J. R. & Herbst-Kralovetz, M. M. Host-vaginal microbiota interactions in the pathogenesis of bacterial vaginosis. *Curr Opin Infect Dis* **33**, 59–65 (2020).

242. Sousa, L. G. V., Novak, J., França, A., Muzny, C. A. & Cerca, N. *Gardnerella vaginalis*, *Fannyhessea vaginae*, and *Prevotella bivia* strongly influence each other's transcriptome in triple-species Biofilms. *Microb Ecol* **87**, (2024).

243. Machado, A. & Cerca, N. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *Journal of Infectious Diseases* **212**, 1856–1861 (2015).

244. Giaouris, E., Heir, E., Desvaux, M., Hébraud, M., Møretrø, T., Langsrud, S., Doulgeraki, A., Nychas, J., Kacániová, M., Czaczyk, K., Ölmez, H. & Simões, M. Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. *Front Microbiol* **6**, 1–26 (2015).

245. Machado, A. & Cerca, N. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *Journal of Infectious Diseases* **212**, 1856–1861 (2015).

246. Machado, A., Jefferson, K. & Cerca, N. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* **14**, 12004–12012 (2013).

247. Chen, K., Forsyth, P., Buchanan, T. & Holmes, K. Amine content of vaginal fluid from patients with trichomoniasis and *Gardnerella* associated nonspecific vaginitis. *Sex Transm Infect* **59**, 302–305 (1983).

248. Schleicher, L., Herdan, S., Fritz, G., Trautmann, A., Seifert, J. & Steuber, J. Central carbon metabolism, sodium-motive electron transfer, and ammonium formation by the vaginal pathogen Prevotella bivia. *Int J Mol Sci* **22**, 1–18 (2021).

249. Gilbert, N., Lewis, W., Li, G., Sojka, D., Lubin, J. & Lewis, A. *Gardnerella vaginalis* and *Prevotella bivia* trigger distinct and overlapping phenotypes in a mouse model of bacterial vaginosis. *Journal of Infectious Diseases* **220**, 1099–1108 (2019).

250. Randis, T. & Ratner, A. *Gardnerella* and *Prevotella*: co-conspirators in the pathogenesis of bacterial vaginosis. *J Infect Dis* **220**, 1085–1088 (2019).

251. Bradshaw, C. S. *et al.* Bacterial vaginosis. *Nat Rev Dis Primers* **11**, (2025).

252. Castro, J. & Cerca, N. BV and non-BV associated *Gardnerella vaginalis* establish similar synergistic interactions with other BV-associated microorganisms in dual-species biofilms. *Anaerobe* **36**, 56–59 (2015).

253. Castro, J., Machado, D. & Cerca, N. Unveiling the role of *Gardnerella vaginalis* in polymicrobial bacterial vaginosis biofilms: the impact of other vaginal pathogens living as neighbors. *ISME Journal* **13**, 1306–1317 (2019).

254. Randis, T., Zaklama, J., LaRocca, T., Los, F., Lewis, E., Desai, P., Rampersaud, R., Amaral, F. and Ratner, A. Vaginolysin drives epithelial ultrastructural responses to *Gardnerella vaginalis*. *Infect Immun* **81**, 4544–4550 (2013).

255. Bowen, W., Burne, R., Wu, H. & Koo, H. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol* **26**, 229–242 (2018).

256. Castro, J., Rosca, A., Muzny, C. & Cerca, N. *Atopobium vaginae* and *Prevotella bivia* are able to incorporate and influence gene expression in a pre-formed *Gardnerella vaginalis* biofilm. *Pathogens* **10**, 1–16 (2021).

257. Castro, J., Rosca, A. S., Cools, P., Vaneechoutte, M. & Cerca, N. *Gardnerella vaginalis* enhances *Atopobium vaginae* viability in an in vitro model. *Front Cell Infect Microbiol* **10**, 1–9 (2020).

258. Hardy, L., Jespers, V., Abdellati, S., De Baetselier, I., Mwambarangwe, L., Musengamana, V., Van De Wijgert, J. Vaneechoutte, M. & Crucitti, T. A fruitful alliance: The synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm. *Sex Transm Infect* **92**, 487–491 (2016).

259. Workowski, K. *et al.* CDC Sexually Transmitted Infections Treatment Guidelines, 2021. *Morbidity and Mortality Weekly Report* **70**, 1–187 (2022).

260. Muzny, C. & Schwebke, J. Asymptomatic Bacterial Vaginosis: to Treat or Not to Treat? *Curr Infect Dis Rep* **22**, 1–9 (2020).

261. Sherrard, J., Wilson, J., Donders, G., Mendling, W. & Jensen, J. 2018 European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge. *Int J STD AIDS* **29**, 1258–1272 (2018).

262. Vodstrcil, L. A. *et al.* Male-Partner Treatment to Prevent Recurrence of Bacterial Vaginosis. *New England Journal of Medicine* **392**, 947–957 (2025).

263. Jayaram, P., Mohan, M. & Konje, J. Bacterial vaginosis in pregnancy – a storm in the cup of tea. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **253**, 220–224 (2020).

264. Ceruelos, A. Romero-Quezada, L. Ledezma, J. & Contreras, L. Therapeutic uses of metronidazole and its side effects: An update. *Eur Rev Med Pharmacol Sci* **23**, 397–401 (2019).

265. Chavoustie, S., Jacobs, M., Reisman, H., Waldbaum, A., Levy, S., Hillier, S. & Nyirjesy, P. Metronidazole vaginal gel 1.3% in the treatment of bacterial vaginosis: A dose-ranging study. *J Low Genit Tract Dis* **19**, 129–134 (2015).

266. Schwebke, J., Marrazzo, J., Beelen, A. & Sobel, J. A phase 3, multicenter, randomized, double-blind, vehicle-controlled study evaluating the safety and efficacy of metronidazole vaginal gel 1.3% in the treatment of bacterial vaginosis. *Sex Transm Dis* **42**, 376–381 (2015).

267. Leyva-g, G. Del, L. & Ortega-peña, S. Modifications in vaginal microbiota and their Influence on drug release: challenges and opportunities. *Pharmaceutics Journal* **11**, 1–18 (2019).

268. Raja, I., Basavareddy, A., Mukherjee, D. & Meher, B. Randomized, double-blind, comparative study of oral metronidazole and tinidazole in treatment of bacterial vaginosis. *Indian J Pharmacol* **48**, 654–658 (2016).

269. Gaspar, C., Rolo, J., Cerca, N., Palmeira-De-oliveira, R., Martinez-De-oliveira, J. & Palmeira-De-oliveira, A. Dequalinium chloride effectively disrupts bacterial vaginosis (Bv) *Gardnerella* spp. biofilms. *Pathogens* **10**, 1–11 (2021).

270. Elliott, W. & Chan, J. Secnidazole Oral Granules (Solosec). *Infectious Disease Alert* **37**, 733–735 (2017).

271. Vodstrcil, L., Muzny, C., Plummer, E., Sobel, J. & Bradshaw, C. Bacterial vaginosis: drivers of recurrence and challenges and opportunities in partner treatment. *BMC Med* **19**, 1–12 (2021).

272. Hardy, L., Cerca, N., Jespers, V. Vaneechoutte, M. and Crucitti, T. Bacterial biofilms in the vagina. *Res Microbiol* **168**, 865–874 (2017).

273. Swidsinski, A., Dörffel, Y., Loening-Baucke, V., Schilling, J. and Mendling, W. Response of *Gardnerella vaginalis* biofilm to 5 days of moxifloxacin treatment. *FEMS Immunol Med Microbiol* **61**, 41–46 (2011).

274. MacHado, D., Gaspar, C., Palmeira-De-Oliveira, A., Cavaleiro, C., Salgueiro, L., Martinez-De-Oliveira, J. & Cerca, N. *Thymbra capitata* essential oil as potential therapeutic agent against Gardnerella vaginalis biofilm-related infections. *Future Microbiol* **12**, 407–416 (2017).

275. Selis, N. N., Oliveira, H., Souza, C. Almeida, J. B., Andrade, Y. & Silva, L., Romano, C., Rezende, R., Yatsuda, R., Uetanabaro, A. & Marques, L. *Lactobacillus plantarum* Lp62 exerts probiotic effects against *Gardnerella vaginalis* ATCC 49154 in bacterial vaginosis. *Lett Appl Microbiol* **73**, 579–589 (2021).

276. Gottschick, C., Szafranski, S., Kunze, B., Sztajer, H., Masur, C., Abels, C. & Wagner-Döbler, I. Screening of compounds against *Gardnerella vaginalis* biofilms. *PLoS One* **11**, 1–16 (2016).

277. Hymes, S. R., Randis, T. M., Sun, T. Y. & Ratner, A. J. DNase inhibits *Gardnerella vaginalis* biofilms *in vitro* and *in vivo*. *Journal of Infectious Diseases* **207**, 1491–1497 (2013).

278. Landlinger, C., Tisakova, L., Oberbauer, V., Schwebs, T., Muhammad, A. Latka, A., Van Simaey, L., Vaneechoutte, M., Guschin, A., Resch, G., Swidsinski, S., Swidsinski, A. & Corsini, L. Engineered phage endolysin eliminates *Gardnerella* biofilm without damaging beneficial bacteria in bacterial vaginosis ex vivo. *Pathogens* **10**, 1–19 (2021).

279. Muzny, C. A. & Sobel, J. D. Bacterial Vaginosis — Time to Treat Male Partners. *New England Journal of Medicine* **392**, 1026–1027 (2025).

280. Sharma, M. *et al.* An insight into vaginal microbiome techniques. *Life* **11** (2021).

281. Carvalho, R., Candice, L., Bazana, G., Fl, M. & Fuentefria, A. M. Curve fitting and linearization of UV – Vis spectrophotometric measurements to estimate yeast in inoculum preparation. *Anal Biochem* **625**, 1–5 (2021).

282. Bogachev, M. I. *et al.* Fast and simple tool for the quantification of biofilm-embedded cells sub-populations from fluorescent microscopic images. *PLoS One* **13**, 1–24 (2018).

283. Kralik, P. & Ricchi, M. A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. *Frontiers in Microbiology* **8** (2017).

284. Bustin, S. A. *et al.* The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem* **55**, 611–622 (2009).

285. McKee, A. M., Spear, S. F. & Pierson, T. W. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biol Conserv* **183**, 70–76 (2015).

286. Ruiz-Villalba, A., van Pelt-Verkuil, E., Gunst, Q. D., Ruijter, J. M. & van den Hoff, M. J. Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). *Biomol Detect Quantif* **14**, 7–18 (2017).

287. Hammes, F. & Egli, T. Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. *Anal Bioanal Chem* **397**, 1083–1095 (2010).

288. Van Nevel, S. *et al.* Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. *Water Res* **113**, 191–206 (2017).

289. Davey, H. M. Life, death, and in-between: Meanings and methods in microbiology. *Appl Environ Microbiol* **77**, 5571–5576 (2011).

290. Overbeek, R., Olson, R., Pusch, G., Olsen, G., Davis, J., Disz, T., Edwards, R., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A., Xia, F. & Stevens, R. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* **42**, 206–214 (2014).

291. Stewart, C. & Giannini, J. Inexpensive, open-source epifluorescence microscopes. *J Chem Educ* **93**, 1310–1315 (2016).

292. Bui, S. *et al.* Finding the needle in the haystack: Comparison of methods for salmon louse enumeration in plankton samples. *Aquac Res* **52**, 3591–3604 (2021).

293. Seo, E., Ahn, T. & Zo, Y. Agreement, Precision, and Accuracy of Epifluorescence Microscopy Methods for Enumeration of Total Bacterial Numbers ↻ †. *Appl Environ Microbiol* **76**, 1981–1991 (2010).

CHAPTER 3

Development of an indirect fluorescence microscopy method to quantify vaginal lactobacilli

Summary

The absolute quantification of *Lactobacillus* species by culture-based methods is limited due to their fastidious growth. Flow cytometry can quantify the bacterial concentration of these bacteria but requires the acquisition of expensive equipment. More affordable non-culturable methods, such as fluorescence microscopy, are hampered by the small size of the bacteria. Herein, we developed an indirect fluorescence microscopy method to determine vaginal lactobacilli concentration by determining the correlation between surface area bacterial measurement and initial concentration of an easily cultivable bacterium (*Escherichia coli*) and applying it to lactobacilli fluorescence microscopy counts. In addition, vaginal lactobacilli were quantified by colony-forming units and flow cytometry in order to compare these results with the indirect method results. The colony-forming-unit values were lower than the results obtained from the other two techniques, while flow cytometry and fluorescence microscopy results agreed. Thus, our developed method was able to accurately quantify vaginal lactobacilli.

The work described in this Chapter was published in 2024, in Microorganisms, 12(1): 1-12; and presented in the Congress of Microbiology and Biotechnology 2023 (December 11-13, Covilhã, Portugal).

3.1 Contextualization of the experimental chapter

As mentioned in Chapter 2, *Lactobacillus* spp. frequently dominate the vaginal microbiota of healthy women [1]. Of note, *L. crispatus* is the most dominant vaginal *Lactobacillus* in the majority of reproductive-age women [3]. Recent reviews on the role of *L. iners* in vaginal health, have pointed out its presence in both optimal vaginal microbiota and in vaginal dysbiosis, making it unclear whether it is protective or pathogenic [4,5].

Lactobacillus spp. grows fastidiously due to gene loss that occurs during the course of their evolution, which affects their metabolism and requires a rich nutrient media [8]. Furthermore, vaginal *Lactobacillus* spp. are anaerobes that tolerate oxygen only to a certain extent [9]. Their aerobic growth may stimulate oxidative stress and cellular damage in lactic acid bacteria due to the production of reactive oxygen species [10]. These growth requirements explain why *Lactobacillus* spp. are difficult to cultivate [11]. Therefore, culture-based methods are not adequate to accurately quantify lactobacilli concentration [12]. Lactobacilli quantification can be used in the detection of BV by microbiological and molecular methods [17] and to evaluate their role as probiotics [18,19]. Two non-molecular, culture-independent methods that allow researchers to quantify bacteria are flow cytometry (FC) and fluorescence microscopy (FM) [20]. The first method is fast, robust, and reproducible to determine total and viable bacterial counts, with the potential for automation; however, it is not cost effective [21,22]. FM is more affordable but more time consuming [23–25], and requires the use of a Neubauer chamber or ocular grids installed in the microscope [26]. Smaller dimensions of bacteria present a limitation in the accuracy of this method, due to locating bacterial cells at different focal points [27]. Moreover, not all microscopes have ocular grids installed. We previously used FM to quantify vaginal lactobacilli and other vaginal bacterial species, by determining the surface colonization of these species [28,29]. However, surface colonization quantification has limited application and is unable to quantify bacterial suspension concentration, in standard measurement units, such as colony-forming units (CFU) per milliliter. Thus, we aimed to develop an indirect FM method for absolute quantification of vaginal lactobacilli in an attempt to overcome these limitations. To achieve this goal, we used an easily cultivable bacterium, *Escherichia coli*, with well-defined bacterial density for a certain optical density (OD), to perform an indirect comparison. We were able to transform a three-dimensional quantification (CFU/mL) into a two-dimensional quantification (CFU/cm^2), which allowed us to quantify vaginal lactobacilli concentrations in suspensions. The method developed here was used for the quantification of lactobacilli suspensions, used on the experiments described on Chapter 5.

3.2. Materials and Methods

3.2.1. Culture conditions and strains

L. crispatus CCUG 44128, *L. jensenii* CCUG 44492, *L. gasseri* CCUG 44075, and *L. iners* CCUG 38955 A were grown on Columbia Blood Agar Base (CBA) (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood (Oxoid), at 37 °C under 10% CO_2. *E. coli* ATCC 25922 was also grown on CBA supplemented with 5% (v/v) defibrinated horse blood, but in an aerobic atmosphere at 37°C. *Candida albicans* SC 5413 was grown on Sabouraud Dextrose Agar (SDA; Liofilchem, Waltham, MA, USA) plates and incubated for 24 h at 37°C, as described by Fernandes et al. [30].

3.2.2. Colony-forming units counting method

Each lactobacilli biomass was resuspended in 1× phosphate buffered saline (PBS) (composed by 8 g/L of NaCl, 0.2 g/L of KCl, 0.2 g/L of KH_2PO_4, and 1.15 g/L of Na_2HPO_4, with pH 7.4±0.05) and sonicated 3 times for 10 s at 40% with 10 s intervals between cycles. Each suspension OD_{620nm} was adjusted to 0.5 and serial dilutions ranging from 10^{-1} to 10^{-6} were performed in 0.9% (wt/vol) NaCl. Then, 10 µL of each dilution was spread into CBA plates and incubated for 48 h at 37 °C under 10% CO_2. For *E. coli*, biomass was resuspended in 1× PBS and sonicated 10 s at 33% on ice. This suspension was adjusted to OD_{620nm} of 0.125, 0.25, and 0.5, which were then serial diluted from 10^{-1} to 10^{-6} and plated into CBA plates. The *E. coli* plates were then incubated at 37°C for 18 h. These assays were performed with two replicates and repeated three times on separate days.

3.2.3. Flow cytometry (FC) quantification

Suspensions of the four vaginal lactobacilli of interest and *E. coli* were prepared as mentioned above, and their OD_{620nm} was adjusted to 0.5. *Lactobacillus* suspensions were stained using acridine orange (Alfa Aesar, Haverhill, MA, USA) 0.002 mg/mL for 15 min in the dark at room temperature. The *E. coli* suspension was stained using SYBR green (Invitrogen, Waltham, MA, USA) 1:10000 for 15 min. Two different dyes were used, in an attempt to obtain the higher percentage of stained bacteria. FC (Cytoflex, Beckman Coulter, Brea, CA, USA) counts were performed using the FITC filter block. The sampling rate was 10 µL/min, and the total number of events was set to 30,000 cells. Data was acquired and analyzed using CytExpert 2.4 software; multiparametric analyses were performed on Forward and Side Scatter. All assays were repeated three times on separate days.

3.2.4. Visualization of bacteria and yeast in the Neubauer chamber

A suspension of *L. crispatus* was prepared, as mentioned previously, and a suspension of *C. albicans* was obtained by resuspending its biomass in 1× PBS. The OD_{620nm} of both suspensions was adjusted to 0.5 and then diluted 10×. Next, 10 µL of acridine orange 0.02 mg/mL was added to 20 µL of each suspension. After 5 min of incubation in the dark, 10 µL of the mixture were added to a Neubauer chamber, a lamella was placed on it, and the cells were visualized under the 40× objective of an epifluorescence microscope (Olympus BX51), using filters capable of detecting acridine orange (BP 470-490, FT500, LP 516).

3.2.5. Fluorescence microscopy (FM) quantification of total counts

Suspensions in PBS 1× of the four *Lactobacillus* spp. and *E. coli* were prepared, as described above. For *Lactobacillus* spp. the OD_{620nm} was adjusted to 0.5, and for *E. coli* it was adjusted to 0.125, 0.25, and 0.5. Next, a 1:500 dilution of each suspension was prepared and 20 µL of each dilution was spread on epoxy-coated microscope glass slides (Thermo Fisher Scientific, Waltham, MA, USA), and the slides were dried at 60°C. Cells were then fixed and covered with 20 µL of acridine orange (0.02 mg/mL) for 5 min, as previously described [31]. The slides were subsequently visualized under the 40× objective of an epifluorescence microscope (Olympus BX51), using filters capable of detecting acridine orange (BP 470-490, FT500, LP 516). The number of bacterial cells were manually counted, at the adequate dilution (<100 bacteria per field). We have previously determined that at least 13 images per sample are needed to have a high correlation between bacterial counts and bacterial concentration [31]. The surface area of each image was determined using the pixel area equivalent to the Neubauer chamber. In our optical system, each image was equivalent to 3.56×10^{-4} cm^2. All assays were repeated three times on separate days.

3.2.6 Statistical analysis

The obtained results from fluorescence microscopy, FC and CFU for the four *Lactobacillus* species of interest were analyzed using the T- test paired samples with Microsoft Excel 2019. A p-value ≤0.05 was deemed statistically significant.

3.3 Results

3.3.1 Comparison of CFU and FC in the quantification of vaginal lactobacilli

To assess culturability of our vaginal *Lactobacillus* spp. of interest, we prepared suspensions of each of the 4 species, that were adjusted to obtain approximately 10^8 CFU/mL, as described above. Then, we assessed the bacterial concentration of each suspension by two independent methods. As seen in Figure 3.1, both *L. crispatus* and *L. iners* had significantly lower CFU quantification, in comparison with the FC analysis (for *L. iners* the difference was approximately 4 log).

3.3.2 Neubauer chamber to quantify bacteria vs yeast

To highlight the importance of this novel indirect method, we started by demonstrating the limitation of the Neubauer chamber to quantify *Lactobacillus* spp. As can be seen in Figure 3.2, we observed that *L. crispatus* can be found at different focal points due to its small dimensions (in comparison with the depth of the Neubauer chamber), which could lead to miscalculation on the initial bacterial concentration. For comparison purposes, we also included the yeast *C. albicans,* whose cells are significantly larger and could be observed throughout the different depths of view.

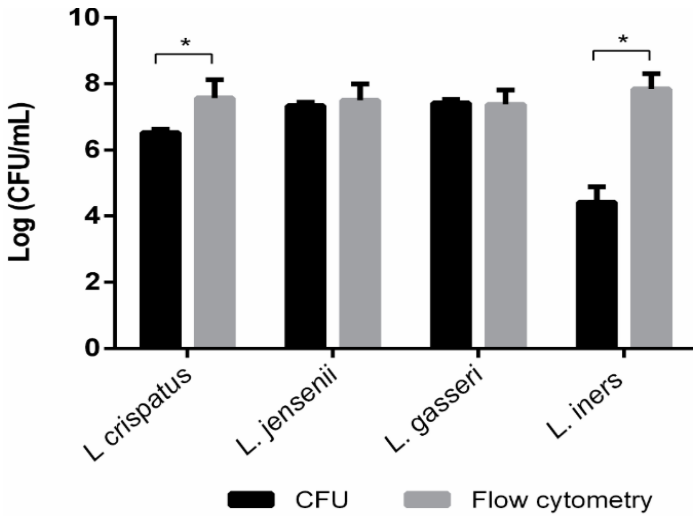


Figure 3.1.

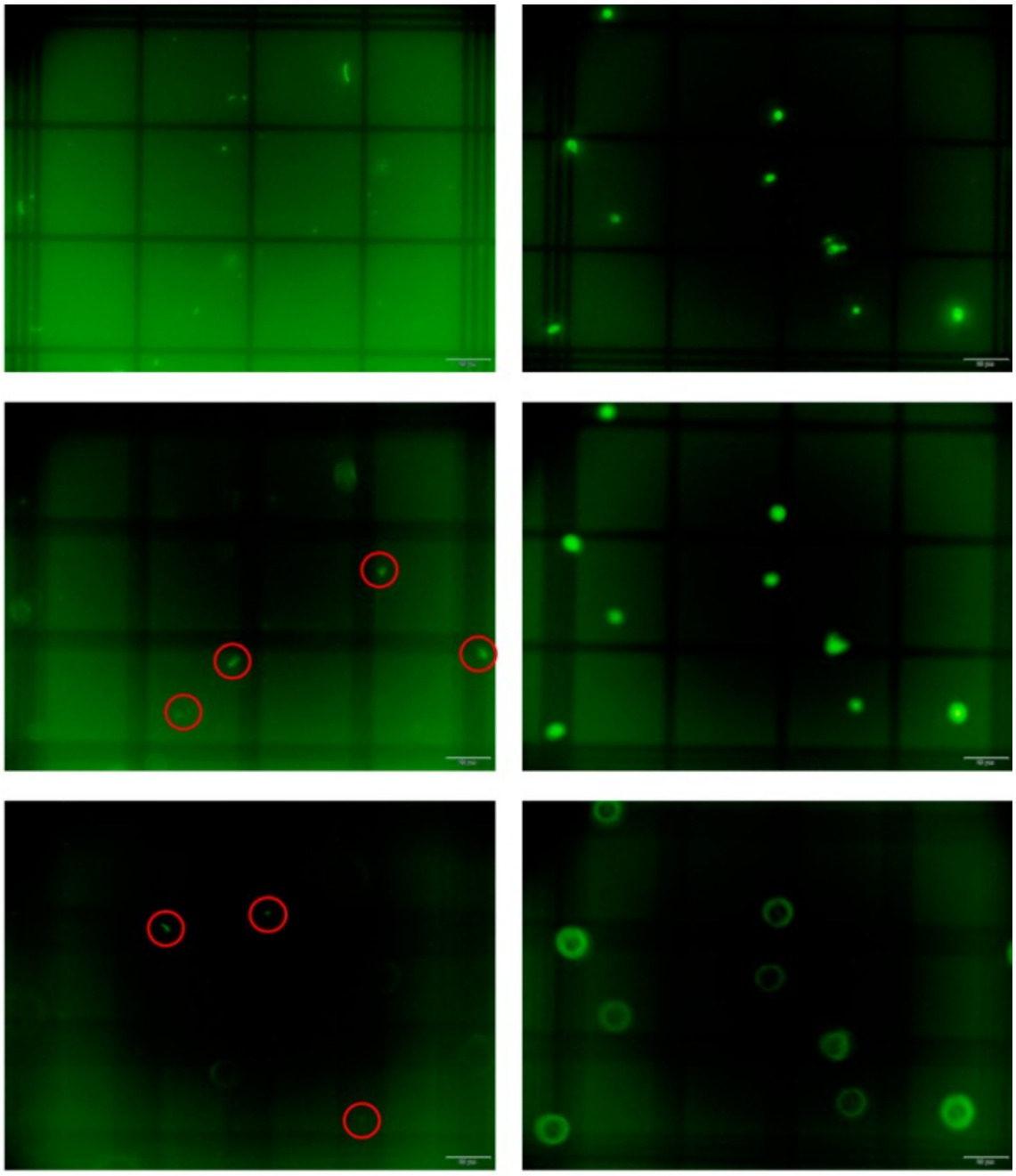
*Quantification of bacterial concentrations of L. crispatus, L. jensenii, L. gasseri, and L. iners suspensions with an OD of 0.5, by culture dependent (CFU) and independent (FC) techniques. The bars represent the mean and the standard deviation of 3 independent experiments. *Represents statistical significance (T- test paired samples, parametric, p≤0.05).*

3.3.3 Correlation of surface area bacterial measurement with initial bacterial density

In order to overcome the above focal point limitation of the Neubauer chamber, we aimed to correlate the bacterial concentration contained in an equivalent volume used on the Neubauer chamber, after fixation on a regular microscopic slide. To validate this proof of principle, we selected an easily cultivable bacterial species, *E. coli*, that is known to be accurately quantified by CFUs [32]. Three different bacterial concentrations of *E. coli*, quantified by optical density, were used in this experiment. As shown in Figure 3.3, this method allowed us to correlate the initial bacterial concentration (a 3D measurement), to a 2D bacterial measurement (surface area of the fixated suspension), thus allowing us to use FM for bacterial culture concentration determination, by eliminating the focal point limitation of the Neubauer chamber.

3.3.4 Quantification of Lactobacillus spp. concentration by an indirect microscopy method

By applying the same experimental procedure to the *Lactobacillus* suspensions, we were able to infer their initial bacterial concentrations from the analysis of the quantified adhered bacteria, as shown in Figure 3.4. After performing total counts of the lactobacilli, we applied these results to the equation in Figure 3.3, converting total counts into CFUs. To confirm the accuracy of this method, we used the same bacterial concentrations used in the FM analysis and compared them to FC quantification. As can be seen in Figure 3.5, both methods resulted in very similar bacterial concentrations, despite some significative differences for *L. jensenii,* which had a lower concentration observed for the FC measurements.



(a) (b)

Figure 3.2.

Comparison of the use of Neubauer chamber for (a) L. crispatus and (b) C. albicans using three z-stacks. The observation of these microorganisms was performed using fluorescence microscopy. For L. crispatus (a), the variation of focal points demonstrates different numbers of counts, while for C. albicans (b) the number of cells is the same for the three z-stacks. The red circles surround the bacteria that are not visible in the first focal plan.

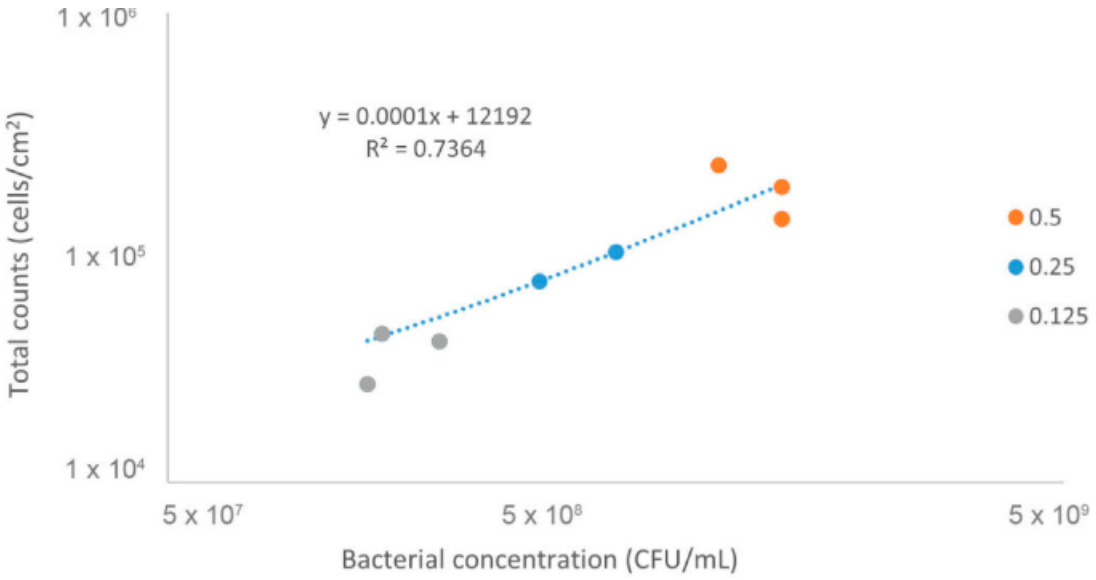


Figure 3.3.

The relationship between E. coli CFUs and total cell counts using FM for the same OD. Three bacterial suspensions initially adjusted to different OD_{620nm}, namely, 0.5, 0.25 and 0.125, were quantified by both FM and by plate counting. The dots correspond to three independent assays for each OD.

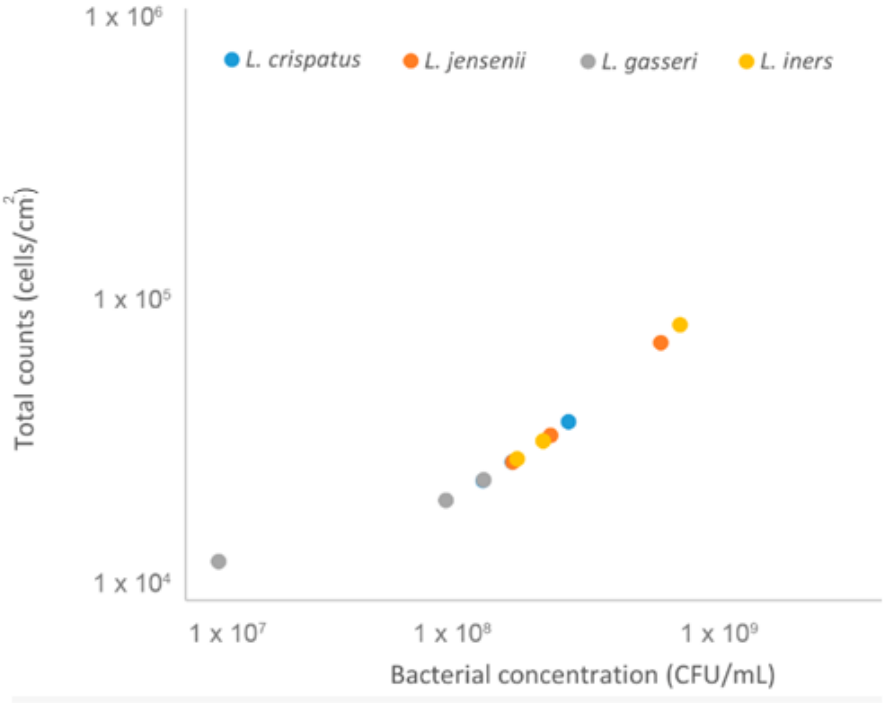


Figure 3.4.

Lactobacillus spp. concentrations obtained through total counts. Four bacterial suspensions adjusted to $OD_{620}nm$= 0.5 were quantified by FC and the results obtained were converted into CFUs, as mentioned above. The dots correspond to three independent assays for each Lactobacillus spp.

3.4 Discussion

The results of this study demonstrate a feasible and affordable indirect method to determine vaginal *Lactobacillus* spp. concentrations. With the exception of *L. iners*, these species are considered as vaginal health markers, protecting the vaginal epithelium from colonization of pathogenic microorganisms [1]. However, due to their fastidious nature, these species are difficult to cultivate [11], as demonstrated in Figure 3.1. Previously, Lahtinen et al compared four methods for the quantification of probiotic bifidobacteria and concluded that the choice of quantification method for fastidious bacteria may have a significant effect on the results of the analysis [33]. Thus, taking into consideration *Lactobacillus* growth characteristics, we considered use of culture-independent methods to enable their quantification, namely FC and FM. Our aim to develop an affordable, fast, and accurate method to quantify vaginal *Lactobacillus* spp. was motivated by the development and application of *in vitro* models to better study BV pathogenesis, in particular bacterial interactions between protective lactobacilli and key BV-associated bacteria (BVAB) [34]. We have previously proposed that some species of *Gardnerella*, the genus thought to initiate the formation of the polymicrobial BV biofilm, form a scaffold for which other BVAB can adhere, leading to a mature, polymicrobial biofilm. We have shown that *Gardnerella* spp. displace pre-adhered *L. crispatus*, which we hypothesized to be a pivotal event for BV development [35]. However, some lactobacilli have also been shown to impair *Gardnerella* spp. [36,37] and other BVAB [38,39]. As such, it is important to better study interactions between lactobacilli and BVAB, in order to better understand BV etiology, and the method developed herein can contribute to this goal.

Regarding the quantification of the four *Lactobacillus* spp. by CFUs and FC, the results of this study show that CFUs are not an appropriate method for accurate quantification of *L. crispatus* and *L. iners*, which is not surprising, as these species are considered difficult to cultivate [40]. Moreover, other studies also reported smaller bacterial concentrations determined by CFUs compared to FC and FM quantifications, suggesting that some of these bacteria may have become dormant [33,41,42].

We showed the limitation of the Neubauer chamber to quantify bacteria, which we confirmed in Figure 3.2, with bacteria appearing in certain focal points, which is corroborated by literature [27]. The reported lactobacilli and *C. albicans* dimensions found justify this limitation, with lactobacilli length as small as 2 µm [43], while *C. albicans* could have a diameter of approximately 12 µm [44]. Considering this and the high costs of FC [11], we developed a method based on FM to quantify vaginal lactobacilli, as this technique is readily available in many research labs [45]. We have shown that the relationship between total counts and plate counting for *E. coli* for three OD_{620nm} is linear, which allowed us to determine the correlation between a surface area (2D) bacterial measurement with the initial bacterial concentration

obtained with CFUs (3D). Thus, after counting total lactobacilli using FM, we converted those results to CFUs/mL.

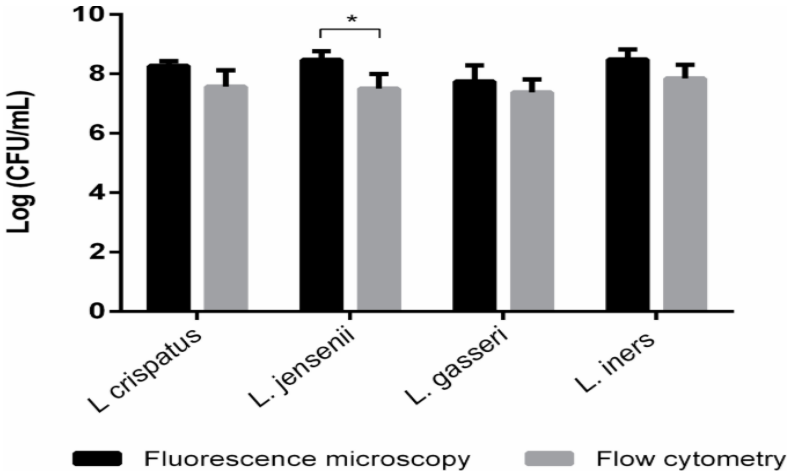


Figure 3.5.

*Quantification of bacterial concentrations of L. crispatus, L. jensenii, L. gasseri, and L. iners suspensions with an OD of 0.5, using FM and FC techniques. The bars represent the mean and the standard deviation of 3 independent experiments. *Represents statistical significance (T-test paired samples, parametric, p≤0.05).*

Comparing FC and FM results, statistical differences were verified only for *L. jensenii*, however, these differences were smaller than 1 log. Some differences between different methods are to be expected, and the subjectivity of cytometry manual gating (the process of identification of a cellular population), may have contributed to these differences [46,47]. A gate is a region in a biaxial plot of forward and side scatter, in which the cells with the desired phenotype are chosen. This first cell's gate is then visualized in another biaxial plot, being redefined. However, as this is a manual process, it becomes subjective, as the sequence of biaxial plots depends on the user's knowledge and view [48]. Nevertheless, for the other three vaginal lactobacilli there was agreement between quantifications obtained by FC and FM. Previous agreements between FC and FM measurements have been reported in studies with other microorganisms [49,50]. As such, our strategy to convert a surface area measurement in a bacterial concentration is proven successful and can be used in future studies that require accurate assessment of vaginal lactobacilli concentrations. This method also has the potential to be adapted for other fastidious and/or dormant-inducing bacterial species.

3.5 References

1. Chee, W., Chew, S. & Than, L. Vaginal microbiota and the potential of Lactobacillus derivatives in maintaining vaginal health. *Microb. Cell Fact.* **19**, 1–24 (2020).
2. Witkin, S. S. & Linhares, I. M. Why do lactobacilli dominate the human vaginal microbiota? *BJOG An Int. J. Obstet. Gynaecol.* **124**, 606–611 (2017).
3. Witkin, S. & Linhares, I. Why do lactobacilli dominate the human vaginal microbiota ? *An Int. J. Obstet. Gynaecol.* **124**, 606–611 (2016).
4. Petrova, M. I., Reid, G., Vaneechoutte, M. & Lebeer, S. Lactobacillus iners: Friend or Foe? *Trends Microbiol.* **25**, 182–191 (2017).
5. Zheng, N., Guo, R., Wang, J., Zhou, W. & Ling, Z. Contribution of Lactobacillus iners to Vaginal Health and Diseases: A Systematic Review. *Front. Cell. Infect. Microbiol.* **11**, (2021).
6. Pramanick, R. *et al.* Vaginal microbiota of asymptomatic bacterial vaginosis and vulvovaginal candidiasis: Are they different from normal microbiota? *Microb. Pathog.* **134**, 103599 (2019).
7. Bagnall, P. & Rizzolo, D. Bacterial vaginosis: A practical review. *J. Am. Acad. Physician Assist.* **30**, 15–21 (2017).
8. Duar, R. M. *et al.* Lifestyles in transition : evolution and natural history of the genus Lactobacillus. *FEMS Microbiol. Rev.* **41**, 27–48 (2017).
9. Tachedjian, G., Aldunate, M., Bradshaw, C. S. & Cone, R. A. The role of lactic acid production by probiotic Lactobacillus species in vaginal health. *Res. Microbiol.* **168**, 782–792 (2017).
10. Zotta, T., Parente, E. & Ricciardi, A. Aerobic metabolism in the genus Lactobacillus: impact on stress response and potential applications in the food industry. *J. Appl. Microbiol.* **122**, 857–869 (2017).
11. Säde, E., Johansson, P., Heinonen, T., Hultman, J. & Bjorkroth, J. Growth and metabolic characteristics of fastidious meat-derived Lactobacillus algidus strains. *Int. J. Food Microbiol.* **313**, 1–8 (2020).
12. Davis, C. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *J. Microbiol. Methods* **103**, 9–17 (2014).
13. Carvalho, R., Candice, L., Bazana, G., FI, M. & Fuentefria, A. M. Curve fitting and linearization of UV – Vis spectrophotometric measurements to estimate yeast in inoculum preparation ˆ. *Anal. Biochem.* **625**, 1–5 (2021).
14. Rhee, S. J., Lee, J. E. & Lee, C. H. Importance of lactic acid bacteria in Asian fermented foods. *Microb. Cell Fact.* **10**, 1–13 (2011).
15. Yu, J. *et al.* Molecular identification and quantification of lactic acid bacteria in traditional fermented dairy foods of Russia. *J. Dairy Sci.* **98**, 5143–5154 (2015).

16. Lhomme, E., Orain, S., Courcoux, P., Onno, B. & Dousset, X. The predominance of Lactobacillus sanfranciscensis in French organic sourdoughs and its impact on related bread characteristics. *Int. J. Food Microbiol.* **213**, 40–48 (2015).

17. Kusters, J. G., Reuland, E. A., Bouter, S., Koenig, P. & Dorigo-Zetsma, J. W. A multiplex real-time PCR assay for routine diagnosis of bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **34**, 1779–1785 (2015).

18. Gobert, G. *et al.* Droplet digital PCR improves absolute quantification of viable lactic acid bacteria in faecal samples. *J. Microbiol. Methods* **148**, 64–73 (2018).

19. Jahromi, M. F. *et al.* Dietary supplementation of a mixture of Lactobacillus strains enhances performance of broiler chickens raised under heat stress conditions. *Int. J. Biometeorol.* **60**, 1099–1110 (2016).

20. Bogachev, M. I. *et al.* Fast and simple tool for the quantification of biofilm-embedded cells sub-populations from fluorescent microscopic images. *PLoS One* **13**, 1–24 (2018).

21. Hammes, F. & Egli, T. Cytometric methods for measuring bacteria in water : advantages , pitfalls and applications. *Anal. Bioanal. Chem.* **397**, 1083–1095 (2010).

22. Van Nevel, S. *et al.* Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. *Water Res.* **113**, 191–206 (2017).

23. Davey, H. M. Life, death, and in-between: Meanings and methods in microbiology. *Appl. Environ. Microbiol.* **77**, 5571–5576 (2011).

24. Overbeek, R., Olson, R., Pusch, G., Olsen, G., Davis, J., Disz, T., Edwards, R., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A., Xia, F. & Stevens, R. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* **42**, 206–214 (2014).

25. Stewart, C. & Giannini, J. Inexpensive, open source epifluorescence microscopes. *J. Chem. Educ.* **93**, 1310–1315 (2016).

26. Seo, E., Ahn, T. & Zo, Y. Agreement , Precision , and Accuracy of Epifluorescence Microscopy Methods for Enumeration of Total Bacterial Numbers ↺ †. *Appl. Environ. Microbiol.* **76**, 1981–1991 (2010).

27. Gordeev, A. A. & Chetverin, A. B. Methods for Screening Live Cells. *Biochem.* **83**, 81–102 (2018).

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

29. Castro, J., Henriques, A., Henriques, M. & Jefferson, K. K. Reciprocal Interference between Lactobacillus spp . and Gardnerella vaginalis on Initial Adherence to Epithelial Cells. *Int. J. Med. Microbiol.* **10**, 1193–1198 (2013).

30. Fernandes, L., Costa, R., Henriques, M. & Rodrigues, M. E. Simulated vaginal fluid: Candida resistant strains' biofilm characterization and vapor phase of essential oil effect. *J. Med. Mycol.* **33**, 101329 (2023).

31. Castro, J. *et al.* Crystal Violet Staining Alone Is Not Adequate to Assess Synergism or Antagonism in Multi-Species Bio fi lms of Bacteria Associated With Bacterial Vaginosis. *Front. Cell. Infect. Microbiol.* **11**, 1–7 (2022).

32. Wohlsen, T., Bates, J., Vesey, G., Robinson, W. A. & Katouli, M. Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. *Lett. Appl. Microbiol.* **42**, 350–356 (2006).

33. Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P. & Salminen, S. J. Comparison of four methods to enumerate probiotic bifidobacteria in a fermented food product. *Food Microbiol.* **23**, 571–577 (2006).

34. Muzny, C. *et al.* An Updated Conceptual Model on the Pathogenesis of Bacterial Vaginosis. *J. Infect. Dis.* **220**, 1399–1405 (2019).

35. Castro, J. *et al.* Using an in-vitro biofilm model to assess the virulence potential of Bacterial Vaginosis or non-Bacterial Vaginosis Gardnerella vaginalis isolates. *Sci. Rep.* **5**, 1–10 (2015).

36. Castro, J., Paula, A., Elisa, M. & Cerca, N. Lactobacillus crispatus represses vaginolysin expression by BV associated Gardnerella vaginalis and reduces cell cytotoxicity. *Anaerobe* **50**, 60–63 (2018).

37. Ping, Y. *et al.* Antimicrobial substances and Mechanisms of Lactobacillus rhamnosus against Gardnerella vaginalis. *Probiotics Antimicrob. Proteins* **15**, 400–410 (2023).

38. Atassi, F., Brassart, D., Grob, P., Graf, F. & Servin, A. L. Lactobacillus strains isolated from the vaginal microbiota of healthy women inhibit Prevotella bivia and Gardnerella vaginalis in coculture and cell culture. *Pathog. Dis.* **48**, 424–432 (2006).

39. Atassi, F., Brassart, D., Grob, P., Graf, F. & Servin, A. L. In vitro antibacterial activity of Lactobacillus helveticus strain KS300 against diarrhoeagenic, uropathogenic and vaginosis-associated bacteria. *J. Appl. Microbiol.* **101**, 647–654 (2006).

40. Ma, B., Forney, L. J. & Ravel, J. Vaginal microbiome: Rethinking health and disease. *Annu. Rev. Microbiol.* **66**, 371–389 (2012).

41. Gamalero, E. *et al.* Colonization pattern of primary tomato roots by Pseudomonas fluorescens A6RI characterized by dilution plating, flow cytometry, fluorescence, confocal and scanning electron microscopy. *FEMS Microbiol. Ecol.* **48**, 79–87 (2004).

42. Bressan, M. *et al.* A rapid flow cytometry method to assess bacterial abundance in agricultural soil. *Appl. Soil Ecol.* **88**, 60–68 (2015).

43. Rajab, S., Tabandeh, F. & Khodabandeh, M. Anaerobe The effect of lactobacillus cell size on its probiotic characteristics. *Anaerobe* **62**, 102103 (2020).

44. Pereira, C., Margarida, A., Ana, C. & Sampaio, C. The effect of benzyl isothiocyanate on Candida albicans growth , cell size , morphogenesis , and ultrastructure. *World J. Microbiol. Biotechnol.* **36**, 1–12 (2020).

45. Hasan, M. M., Alam, M. W., Wahid, K. A., Miah, S. & Lukong, K. E. A Low-Cost digital microscope with real-Time fluorescent imaging capability. *PLoS One* **11**, 1–8 (2016).

46. Maecker, H. T. *et al.* Standardization of cytokine flow cytometry assays. *BMC Immunol.* **18**, 1–18 (2005).

47. Marie, D., Lopes, A., Brandini, F. P. & Vaulot, D. Estimating microbial populations by flow cytometry : Comparison between instruments. *Limnol. Oceanogr. Methods* **14**, 750–758 (2016).

48. Qiu, P. Toward exhaustive gating of flow cytometry data. in *Proceedings - IEEE International Workshop on Genomic Signal Processing and Statistics* 183–186 (IEEE, 2012). doi:10.1109/GENSIPS.2012.6507759.

49. Christaki, U. *et al.* Optimized routine flow cytometric enumeration of heterotrophic flagellates using SYBR Green I. *Limnol. Oceanogr. Methods* **9**, 329–339 (2011).

50. Vaahtovuo, J., Korkeama, M., Munukka, E., Viljanen, M. K. & Toivanen, P. Quantification of bacteria in human feces using 16S rRNA-hybridization , DNA-staining and flow cytometry. *J. Microbiol. Methods* **63**, 276–286 (2005).

CHAPTER 4

Optimizing qPCR method to accurately quantify bacteria in mixed cultures

Summary

Quantitative PCR (qPCR) has emerged as a commonly employed method to quantify bacteria in a wide range of biological samples. Despite its precision, qPCR requires careful implementation of experimental controls to minimize bias. A key concern is the loss of gDNA during extraction, which tends to be greater in samples with low bacterial loads. Exogenous DNA molecules are often added after bacterial cultures are lysed, but before DNA purification steps, to determine extraction efficiency. Enhancement of qPCR quantification methods is required to achieve reliable and reproducible outcomes. Herein, we showed that using reaction efficiency as a calibration curve for bacterial load estimation in mixed samples is inappropriate, as it fails to account for gDNA isolation efficiency, thereby significantly biasing quantification. Our findings also demonstrated that different exogenous DNA controls have different recovery rates, suggesting distinct DNA extraction efficiencies and recovery rates are also affected by the gDNA extraction method being more affected in silica-based columns than in phenol-chloroform extraction. Furthermore, we described an optimized qPCR approach for quantifying *Gardnerella vaginalis*, *Prevotella bivia*, and *Fannyhessea vaginae*, three bacteria strongly associated with BV, based on the normalization process, by adding a distinct bacterium (*Escherichia coli*) of known concentration as an exogenous control prior to gDNA extraction.

The work described in this Chapter was published in 2022, in Journal of Microbiological Methods, 194: 1-4; in 2022, in Journal of Microbiological Methods 203; and in 2024, in Journal of Microbiological Methods 219.

4.1. Contextualization of the experimental chapter

qPCR is a well-established method to quantify bacteria and is used in multiple types of biological samples [1–3]. Overall, bacterial quantification by qPCR requires nucleic acid extraction followed by the thermal amplification process. Several recent papers have neglected both the critical pitfalls of using qPCR for bacterial quantification and the standards outlined in the MIQE guidelines [4,5]. Variability in nucleic acid extraction efficiency can lead to significantly different concentrations of RNA [6] or DNA [7]extracted from similar samples [8]. To quantify this inherent technical variability, the utilization of exogenous controls has been proposed as a method to normalize total nucleic acid extraction [9,10]. This is of utmost importance when quantifying bacterial species by DNA extraction, as the lack of adequate controls hinders comparison of results between studies [11,12]. Therefore, we intended to demonstrate how the construction of calibration curves for bacterial quantification through qPCR amplification of genomic DNA (gDNA) impact the accuracy of the results, as well as the importance of using an adequate exogenous control, in this case, to quantify the absolute concentration of triple-species biofilms composed of *G. vaginalis*, *P. bivia* and *F. vaginae* for use in BV pathogenesis research. Our hypothesis is that inconsistent bacterial loss during centrifugation steps can impair the accuracy of quantification, by affecting both extraction efficiency and qPCR performance. As a corrective measure, we propose the introduction of a bacterium with a defined concentration immediately before gDNA extraction to serve as an exogenous control and normalization reference.

4.2 Methods

4.2.1 Culture conditions and strains

In the different experiments described in this chapter, different species where used, namely *G. vaginalis* ATCC 14018T, *F. vaginae* ATCC BAA-55T, *P. anaerobius* ATCC 27337T and *P. bivia* ATCC 29303T were cryopreserved at −80 °C in Brain Heart Infusion broth (Liofilchem, Italy) supplemented with 23% (v/v) glycerol (Panreac, Spain). For revival, cultures were streaked from frozen stocks onto Columbia Blood Agar Base (CBA) (Oxoid, Basingstoke, UK) enriched with 5% (v/v) defibrinated horse blood (Oxoid) and incubated anaerobically at 37 °C (AnaeroGen, Oxoid). For liquid cultures, each inoculum was subsequently cultured in New York City III broth containing 10% (v/v) heat-inactivated horse serum (Biowest, Nuaillé, France) for 24 h at 37 °C under anaerobic conditions. In Figure 4.1. is depicted the schematic organization of the experiments performed in this chapter.

4.2.2 Assessment of the accuracy of different calibration strategies

4.2.2.1 Preparation of the bacterial cultures standards

The biomass of each bacterium was resuspended in PBS 1×, separately, and each OD_{620nm} was adjusted to obtain 1 × 10^9 cells/mL, then, each bacterial culture was serially diluted to obtain concentrations between 1 × 10^9 cells/mL and 5 × 10^6 cells/mL. Following centrifugation of each suspension at 16,000 × g for 10 min at 4 °C, the supernatant was discarded and the pellet preserved at −20 °C overnight to allow for gDNA extraction.

4.2.2.2 Preparation of Mock Cultures

To validate the calibration curves, two blinded mock cultures were prepared. These cultures were prepared as described on section **4.2.2.1**. Mock culture (A) consisted of 1 × 10^8 CFU/mL of each bacterium, namely, *G. vaginalis*, *F. vaginae* and *P. anaerobius*, while mock culture (B) consisted of 3 × 10^6 CFU/mL of each mentioned bacterium.

4.2.2.3 DNA extraction

gDNA was extracted from pure and mock cultures with DNeasy Ultraclean Microbial Kit (QIAGEN), using the manufacturer's instructions with minor modifications. Cell lysis was performed in a BeadBug6 Microtube Homogenizer (Benchmark Scientific) with 2 × 3 cycles of 30 s at 4350 rpm, keeping the samples on ice between cycles. For normalization of extraction efficiency, commercially available luciferase cDNA (Promega) (10 µL of 10^8 copies of luciferase cDNA, with 67 bp) was added to each sample, before DNA isolation. Assessment of DNA concentration and purity was performed with a NanoDrop One spectrophotometer (Thermo Scientific). This assay was performed three independent times.

4.2.2.4 qPCR

To quantify the bacterial load associated with the extracted gDNA, all qPCR assays were carried out under identical amplification conditions. Reactions were run on a CFX96 thermal cycler (Bio-Rad) with the following cycling program: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Each qPCR was performed in a 10 µL reaction mixture containing 5 µL of Xpert Fast SYBR MasterMix (Grisp, Porto, Portugal), 1 µL of primer mix (10 µM), 2 µL of DNase-free water, and 2 µL of extracted gDNA diluted 1:40. Three control reactions were included in triplicate. To calibrate

fluorescence intensity across runs, *G. vaginalis* gDNA at 1 × 10^8 CFU/mL (diluted 1:40) was used as an inter-run calibrator. Melt-curve analyses were performed to verify the absence of nonspecific products and primer-dimers. The recovery rate was calculated as the difference between the qPCR cycle threshold of the target gene in the DNA samples versus the cycle threshold of the pure exogenous DNA controls, at equivalent concentrations. qPCR amplification efficiency was determined from the slope of a standard curve, developed using gDNA from one sample of each bacterium, that was serially diluted and run in the qPCR. The primers used are present in Table 4.1.

4.2.3 Assessment of the efficiency of different exogenous controls as calibrators for qPCR quantification

4.2.3.1 Preparation of the bacterial cultures standards

The biomass of *G. vaginalis* was resuspended in PBS 1×, and its OD_{620nm} was adjusted to obtain culture density of 3 × 10^8 CFU/mL. Then, this suspension was diluted to obtain culture densities ranging from 1 × 10^8cells/mL to 1 × 10^7 cells/mL. Following centrifugation of each suspension at 16,000 × g for 10 min at 4 °C, the supernatant was discarded and the pellet preserved at −20 °C overnight to allow for gDNA extraction.

4.2.3.2. DNA extraction

gDNA of each sample was extracted with either the DNeasy Ultraclean Microbial Kit (QIAGEN), using the manufacturer's instructions with minor modifications, as described above, or with the classical phenol/chloroform method [13]. A mixture containing three different exogenous controls, namely, commercially available luciferase cDNA (Promega) (10 µL of 10^8 copies of luciferase cDNA, with 67 bp), 10 µL of 1 ng/µL *Staphylococcus epidermidis* gDNA (2.6 Mb [14], extracted with DNeasy Ultraclean), and 10 µL of 1 ng/µL of a plasmid (piMAY, 5473 bp [15], extracted with GeneJET plasmid Miniprep (ThermoScientific), following manufacturer's instructions was spiked before DNA isolation to determine the % of DNA recovered, in both methods. The efficiency of extraction was assessed through measurement of DNA concentration and purity with a NanoDrop One spectrophotometer (Thermo Scientific). Three independent experiments were conducted.

4.2.3.3 qPCR

The procedure was the same as described on section **4.2.2.4**. The primers efficiency was determined by running serial-dilutions of gDNA of the target bacteria on qPCR. Three control reactions (one for each exogenous control) were performed in triplicate. The primers are described in Table 4.1.

4.2.4 Assessment of the efficiency of adding an exogenous culture as calibrator for qPCR quantification

4.2.4.1 Preparation of the bacterial cultures standards

The bacterial suspensions were prepared with densities ranging from 1×10^8 to 1×10^3 CFU/mL by adjusting the OD_{620nm}. Pure cultures of each species were suspended in PBS 1×. After reaching the desired concentrations, an exogenous control was introduced by adding 100 µL of a previously prepared suspension of *Escherichia coli* at 1×10^8 CFU/mL to 900 µL of the bacterial suspension. Each preparation was then centrifuged at 16,000 × g for 10 min at 4 °C, after which the supernatant was discarded and the pellet stored at −20 °C overnight for subsequent gDNA extraction.

4.2.4.2 Bacterial loss during centrifugation

To evaluate bacterial loss during centrifugation, the culture supernatant discarded after this initial experimental step was analyzed to quantify the remaining bacteria for each of the three tested species. As a control, a 10 µL aliquot of each suspension was plated prior to centrifugation. A starting concentration of approximately 1×10^8 CFU/mL was used, corresponding to an OD_{620nm} of 0.17 for *G. vaginalis*, 0.18 for *P. bivia*, and 0.12 for *F. vaginae*. These OD values were established based on previous work from our research group [16]. Each aliquot was serially diluted in 0.9% (w/v) NaCl, from 10^{-1} to 10^{-6}. From each dilution, 10 µL was plated onto 90 mm CBA plates and incubated anaerobically at 37 °C for 48 h. All assays were performed in at least three independent experiments, each with two technical replicates.

4.2.4.3 Preparation of Mock cultures

To validate the calibration curves, three blinded mock cultures were prepared. Mock culture (C) and (D) consisted of equal proportions of *G. vaginalis*, *P. bivia*, and *F. vaginae*, at final concentrations of 10^8 and 10^3 CFU/mL, respectively. Mock culture (E) contained 10^5 CFU/mL of *G. vaginalis*, 10^3 CFU/mL

of *P. bivia*, and 10^5 CFU/mL of *F. vaginae*. In all preparations, a constant concentration of 1×10^8 CFU/mL of *E. coli* was added as an exogenous control to normalize qPCR results.

4.2.4.4 Biofilm formation

Biofilm formation was carried out following the competitive model described by Rosca et al. [17]. Briefly, *G. vaginalis*, *P. bivia*, and *F. vaginae* cultures were prepared by inoculating biomass of each species into 4 mL of New York City broth and incubating for 24 h under anaerobic conditions at 37 °C. The cultures were then adjusted to approximately 1×10^7 CFU/mL in NYC III broth. Co-incubation of the three species was performed in 24-well tissue culture plates (Orange Scientific) for 24 h, 48 h, and 72 h at 37 °C under anaerobic conditions, as described above. Initially, the concentration of each suspension was standardized to 9×10^7 cells/mL based on OD_{620nm} measurements, in accordance with values previously reported [17]. Each bacterial suspension was then diluted in NYC III broth to reach a final concentration of 1×10^7 CFU/mL, with 1 mL of inoculum added per well. At each timepoint, the medium overlying the biofilms was gently removed and replaced after washing the biofilms with 1 mL of 0.9% (w/v) NaCl. All assays were performed in three independent experiments, with technical triplicates included in each.

4.2.4.5 DNA extraction

Extraction of genomic DNA was performed from pure cultures (used to construct calibration curves), mock cultures, and triple-species biofilms with the DNeasy Ultraclean Microbial Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions and with slight adaptations, as described before. The efficiency of extraction was assessed through measurement of DNA concentration and purity with a NanoDrop One spectrophotometer (Thermo Scientific). Three independent experiments were carried out.

4.2.4.6 qPCR

In this case, the amplification conditions used were equal to the described in the previous steps, but normalization results were done differently. The gene expression normalization was performed using the equation $\Delta\mathrm{Ct} = 2^{(CT_{target\ species} - CT_{control\ species})}$, where ΔCt represents the cycle threshold difference between the control bacterium (*E. coli*) and the target species (*G. vaginalis*, *P. bivia*, and *F. vaginae*). Calibration curves were generated with this equation, plotting ΔCt values on the y-axis against known bacterial concentrations (CFU/mL) on the x-axis.

Table 4.1.

Primer sequences.

Bacterium/target	Sequence (5'-3')	Reference
G. vaginalis	CAACGGTATCCTGACCGTCT	[18]
	CCTTGCAAAGGCAGTTAAGC	
F. vaginae	CCTCATGCAAAATGTGATGC	[18]
	CCAAAACAGAAGCACGGAAT	
P. anerobius	TGCAAAGCACGTTGATTTCT	[19]
	CCGCACATACCTACCCACTT	
Luciferase	TACAACACCCCAACATCTTCGA	[20]
	GGAAGTTCACCGGCGTCAT	
piMAY	TACATGTCAAGAATAAACTGCCAAAGC	[15]
	AATACCTGTGACGGAA GATCACTTCG	
S. epidermidis	TCAGACG ACATCATTGCGCT	[21]
	ACGTTGTCCCCTTATCTCCTC	
P. bivia	GGCTGATGAATATGCCTACG	[19]
	AAACACCATGTTGCGAATAG	
E. coli	AGCGAAACCGCCAAGACTGTTA	[22]
	GTGGATGAAGACCAGCCCTTCC	

4.2.5 Statistical analysis

Data were analyzed with GraphPad Prism version 8 (La Jolla, California, USA), using unpaired t-test or two-way ANOVA with multiple comparisons tests. Values with a p-value of < 0.05 were denoted as statistically significant.

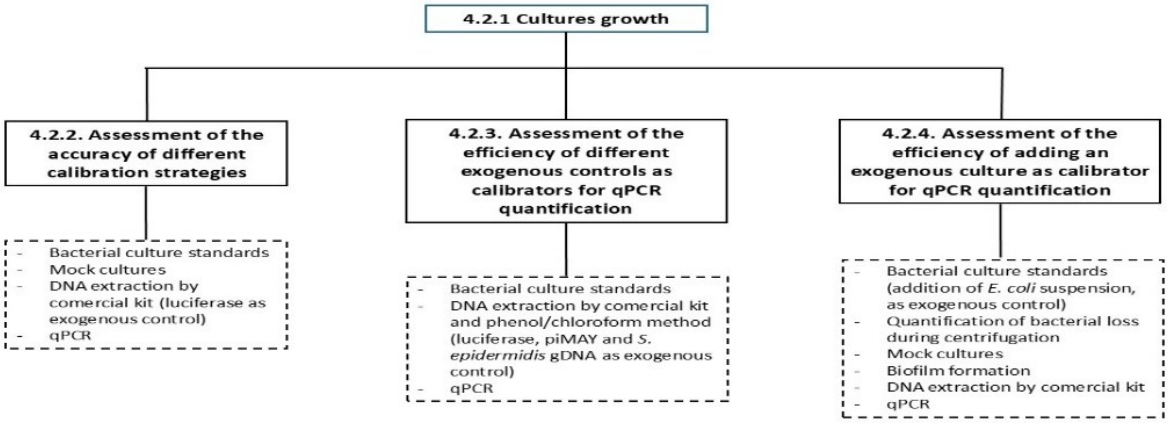


Figure 4.1.

Schematic representation of the three performed experiments.

4.3. Results

4.3.1 Assessment of the accuracy of different calibration strategies

We first explored how different calibration strategies impact bacterial quantification based on the most common strategies commonly reported [4]. First, gDNA extracted from the highest bacterial concentration was serially diluted, a common approach that does not account for potential losses during extraction. The second calibration curve utilized gDNA obtained from bacterial suspensions of varying concentrations. The third curve adjusted the cycle threshold values through the luciferase exogenous control to account for gDNA extraction losses. As illustrated in Figure 4.2, these approaches produced markedly different calibration curves. It was apparent that including samples with lower bacterial concentrations decreased curve linearity, and the use of an exogenous control was insufficient to fully correct this deviation.

When quantifying two mock mixed cultures using the three calibration curve strategies, substantial variations were observed, as shown on Table 4.2. Interestingly, the quantification accuracy decreased at lower bacterial concentrations. However, the calibration curve normalized to the concentration of the exogenous luciferase control provided the most accurate measurements across all conditions. Furthermore, the first calibration strategy exhibited the poorest quantification accuracy.

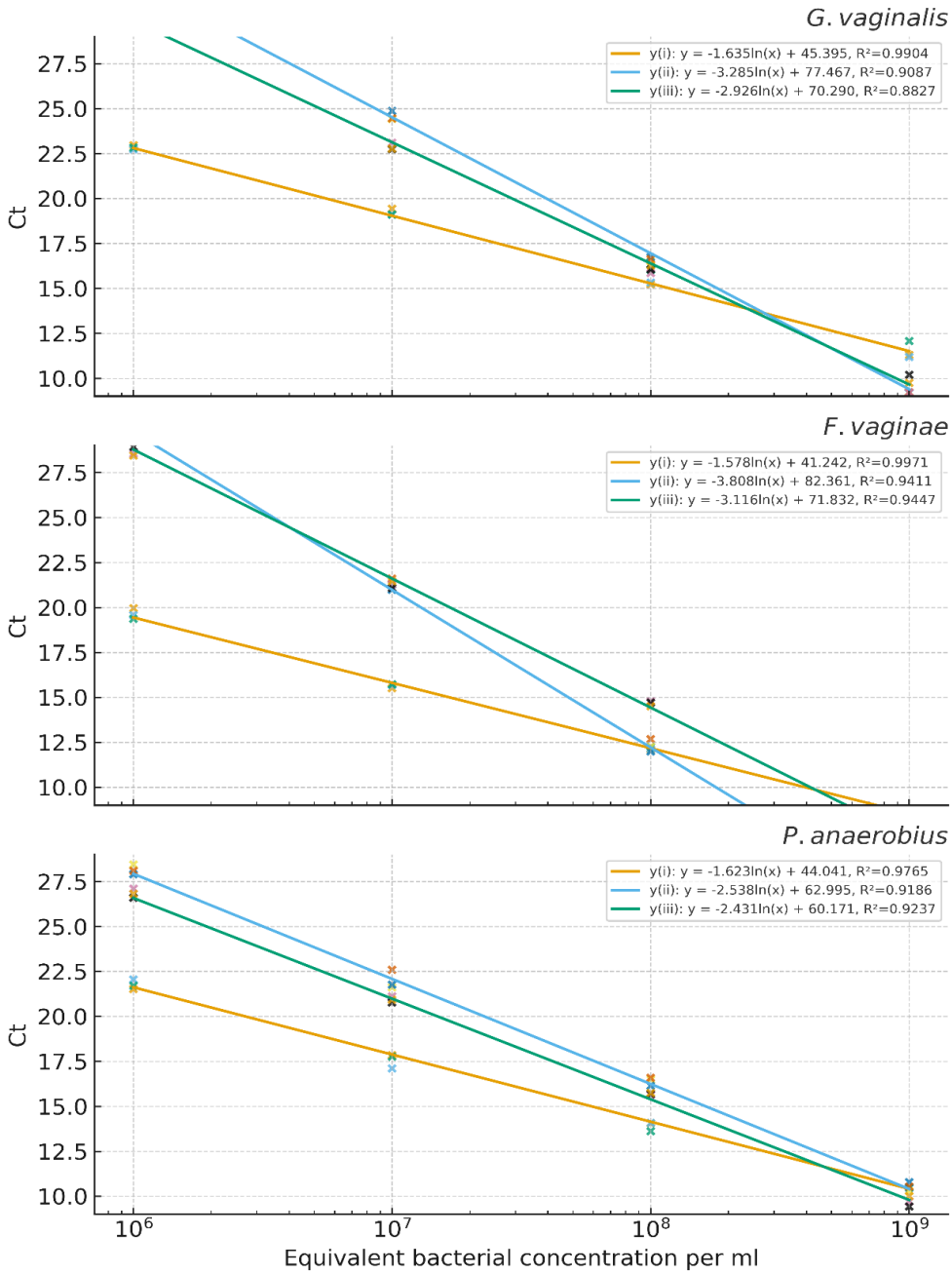


Figure 4.2.

Comparison of different calibration strategies to evaluate the relationship between cycle threshold (Ct) values and bacterial load. (i) Calibration based on serial dilution of gDNA from a known bacterial concentration (orange line); (ii) calibration using gDNA extracted from bacterial suspensions of varying known concentrations (blue line); (iii) calibration using gDNA extracted from bacterial suspensions of varying concentrations with Ct values adjusted for exogenous control (luciferase) loss (green line). Each point represents an independent biological extraction, with qPCR performed in triplicate.

Table 4.2.

Accuracy of bacterial quantification in mock mixed-species cultures containing approximately 1 × 10^8 CFU/mL (A) or 3 × 10^8 CFU/mL (B) of each tested species, evaluated using three different calibration strategies: (i) based on reaction efficiency; (ii) based on gDNA extraction; (iii) based on gDNA extraction adjusted to efficiency loss.

A			
Bacteria	(i)	(ii)	(iii)
G. vaginalis	~60 ± 10 %	~120 ± 10 %	~90 ± 10 %
F. vaginae	~20 ± 5 %	~140 ± 15 %	~100 ± 10 %
P. anaerobius	~60 ± 15 %	~170 ± 30 %	~120 ± 15 %
B			
Bacteria	(i)	(ii)	(iii)
G. vaginalis	~5 ± 2 %	~450 ± 150 %	~220 ± 70 %
F. vaginae	~5 ± 2 %	~600 ± 250 %	~250 ± 120 %
P. anaerobius	~10 ± 5 %	~300 ± 150 %	~180 ± 80 %

4.3.2 Assessment of the efficency of different exogenous controls

Taken into consideration the previous experiments, we further explored how different sizes of gDNA exogenous controls could impact quantification. We hypothesized that the low DNA mass of small exogenous controls can result in a very low recovery rate in the DNA optimized silica columns commonly used for genomic DNA (gDNA) extraction. Then, we determined the recovery rate of three different DNA molecules with different sizes, using two DNA extraction methods, namely, Phenol-chloroform extraction method and a commercially available silica based kit. The experiments performed with DNA obtained by the phenol-chroroform extraction method presented similar extraction efficiencies, suggesting that the size and conformation of the exogenous controls had little effect on the exogenous control recovery rate, as illustrated in Figure 4.3. However, for the silica column-based extraction method, recovery rates were strongly affected by the nature of the exogenous controls. At the tested bacterial concentrations, piMAY presented a significantly lower recovery rate than *S. epidermidis* gDNA. While luciferase showed a significantly lower recovery rate than *S. epidermidis* gDNA at 3 × 10^8 cells/mL.

As shown in Figure 4.4, the main differences between extraction methods were observed when using piMAY as an exogenous control, wherein, in 2 out of the 4 bacterial densities tested, the recovery rates by the two methods were statistically different.

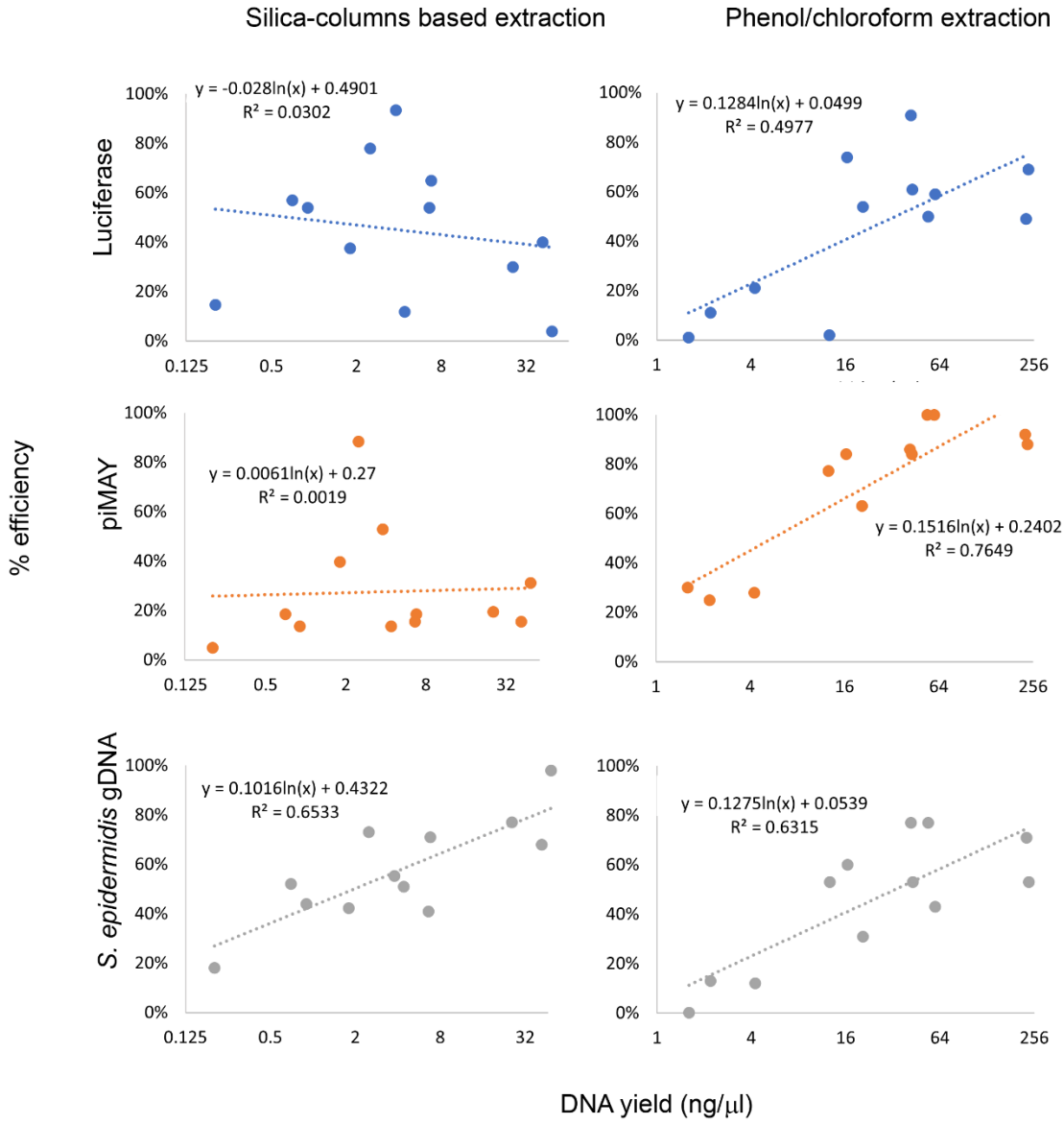


Figure 4.3.

Variability of gDNA extraction efficiency of G. vaginalis cultures with different bacterial concentrations for each exogenous control and for the two different extraction methods.

4.3.3 Assessment of the efficiency of adding an exogenous culture

Based on our previous experiments and according to the literature [4], we then aimed to develop a more robust qPCR quantification method, using an exogenous bacterial culture as normalization strategy. To better support this strategy, and to properly quantify its limitations, we started by quantifying the bacterial concentration remaining after the centrifugation step required for gDNA extraction. The results are described in Figure 4.5. Starting with ~1×10^8 CFU/mL of each culture, we found that approximately

~1 × 10^6 CFU/mL was consistently lost, i.e, remained in the supernatant. Extending the centrifugation time up to 30 min did not result in any significant improvement in recovery.

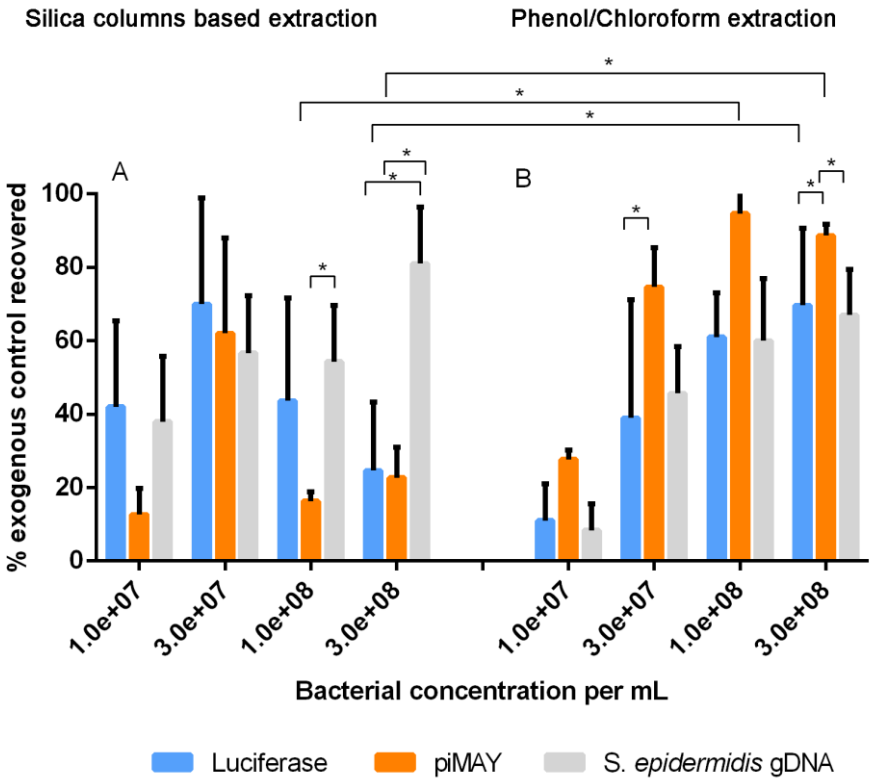


Figure 4.4.

*Quantitative differences between DNA extraction methods and the recovery rate of the exogenous controls. The bars represent the mean and the standard deviation of 3 independent assays. * represents a statistical significance (Two-way ANOVA, Turkey's multiple comparisons test, $p < 0.05$).*

To account for the described bacterial loss during centrifugation, we introduced an exogenous control consisting of a known concentration of an unrelated bacterial species (*E. coli*). While exogenous DNA controls are frequently added after bacterial lysis, this approach overlooks potential losses occurring in earlier steps. Therefore, supplementing the sample with a defined suspension of *E. coli* prior to quantification was expected to enhance the accuracy of gDNA measurement by qPCR. In this work, we used an *E. coli* suspension at a fixed concentration of 1 × 10^8 CFU/mL. The calibration curves are present in Figure 4.6.

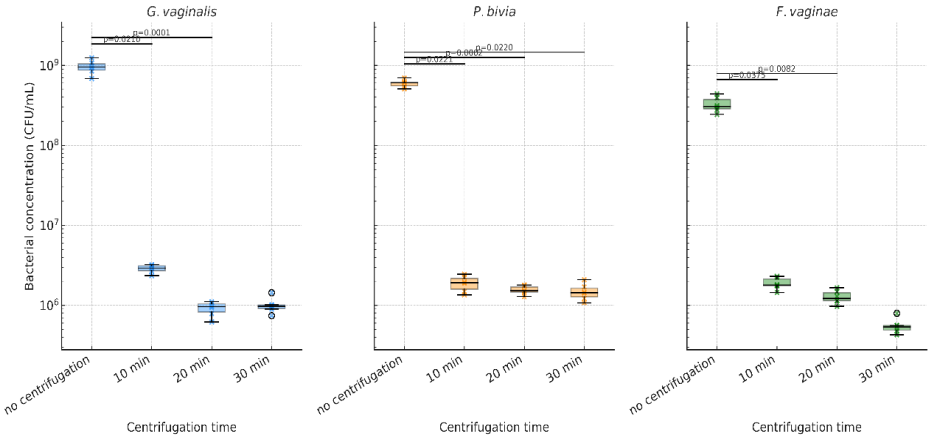


Figure 4.5.

Bacterial losses from pure cultures of G. vaginalis, P. bivia, and F. vaginae after 10, 20, and 30 min of centrifugation. The concentrations of bacteria remaining in the supernatant after centrifugation of 1 × 10⁸ CFU/mL suspensions for 10, 20, and 30 min are shown for G. vaginalis (A), P. bivia (B), and F. vaginae (C). Boxes indicate the mean ± standard deviation of at least three independent experimental replicates. Statistical comparisons were performed using a t-test.

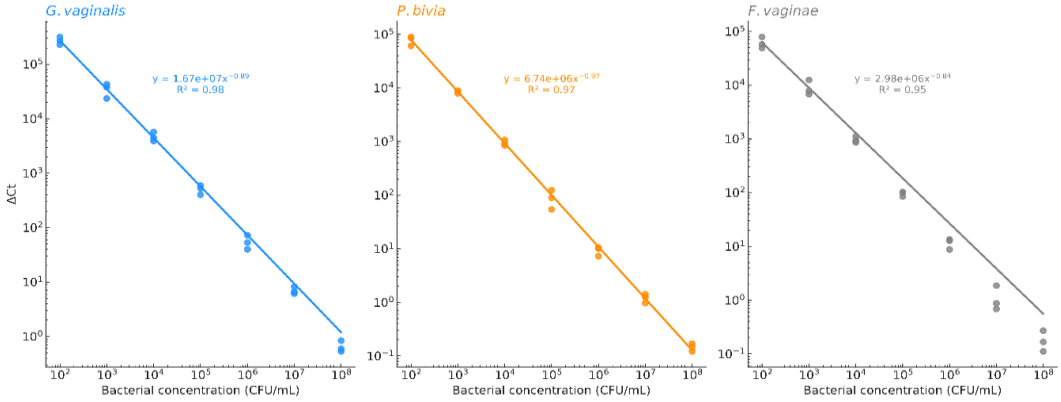


Figure 4.6.

Calibration curves illustrating the relationship between cycle threshold values (ΔCt) and bacterial load quantification. Curves were generated for G. vaginalis (A), P. bivia (B), and F. vaginae (C) by adding E. coli at 1 × 10⁸ CFU/mL as an exogenous control.

To evaluate the accuracy of the new calibration curves, three mock cultures were constructed. The three mock cultures were designed to reproduce these scenarios, based on Nugent score system, as described in Chapter 2: BV-type consortia (mock culture C), normal microbiota (mock culture D), and

intermediate microbiota (mock culture E). As illustrated in Figure 4.7, the calibration curves enabled an accurate characterization of these mixed-species cultures.

We also applied this method in biofilm cultures. An *in vitro* model simulating a polymicrobial BV biofilm [17] was employed to assess biofilm development at 24 h, 48 h, and 72 h, as shown in Figure 4.8. Consistent with the results obtained in the planktonic models, quantification of the triple-species biofilms demonstrated that *G. vaginalis* was the dominant species.

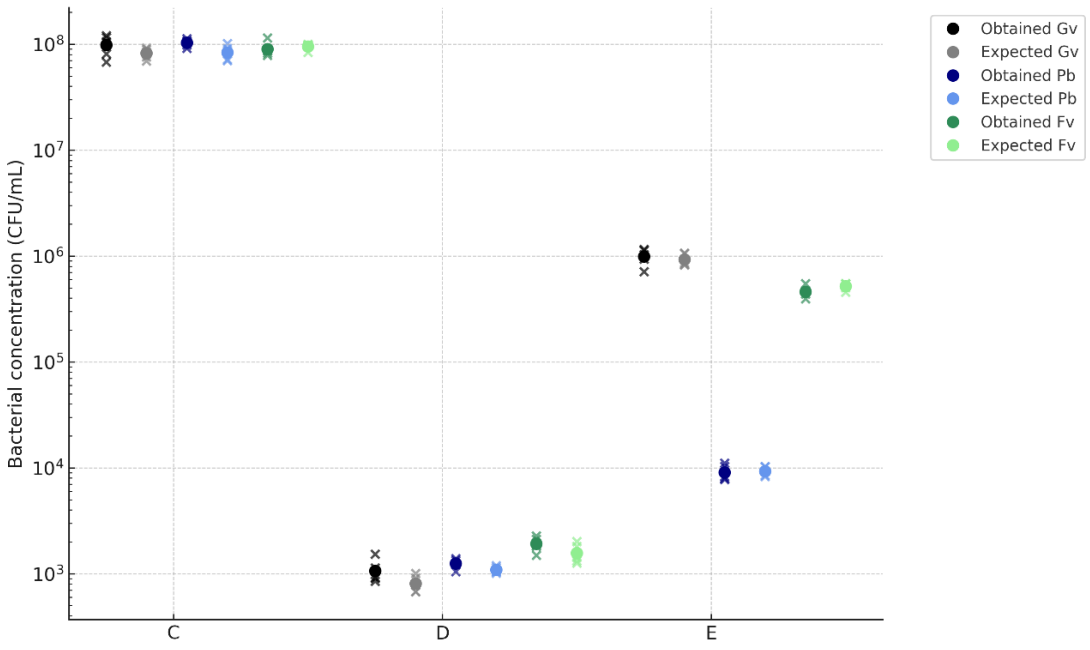


Figure 4.7.

Accuracy of bacterial quantification in mock cultures of G. vaginalis, P. bivia, and F. vaginae. Quantification of each species was performed using the three calibration curves described above. Mock culture C contained 1 × 10^8 CFU/mL of each species, mock culture D contained 1 × 10^8 CFU/mL of each species, and mock culture E contained 1 × 10^8 CFU/mL of G. vaginalis and F. vaginae together with 1 × 10^8 CFU/mL of P. bivia. In all cases, 1 × 10^8 CFU/mL of E. coli was added as an exogenous control prior to gDNA extraction. Dots represent the mean ± standard deviation of at least three independent replicates. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test; no significant differences were observed. Abbreviations: Gv, G. vaginalis; Pb, P. bivia; Fv, F. vaginae.

4.4. Discussion

Throughout this chapter, we evaluated how different forms of qPCR calibration curves impact the quantification of bacteria. Being BV, a polymicrobial infection, we intended to improve the accuracy of the quantification of BVAB, although, the described approach can be used to quantify other bacteria.

Our results revealed that incorporating samples with lower bacterial concentrations reduced the linearity of the calibration curves, which is consistent with previous findings [23]. Moreover, the addition of an exogenous control was insufficient to fully correct this deviation, when using silica column-based kits. However, the calibration curve normalized with the exogenous control provided the most accurate results across all conditions. As expected, when testing three strategies to develop calibration curves, the first strategy yielded the poorest accuracy. It is important to note, however, that this type of calibration, assessing reaction efficiency at a specific temperature and using a given primer set and reaction mix, is a standard step required in any qPCR [5]. However, despite its importance for accurate qPCR measurements, reaction efficiency calibration does not account for variable gDNA extraction efficiencies among samples and, therefore, is not suitable for reliable bacterial load quantification, as demonstrated by our results.

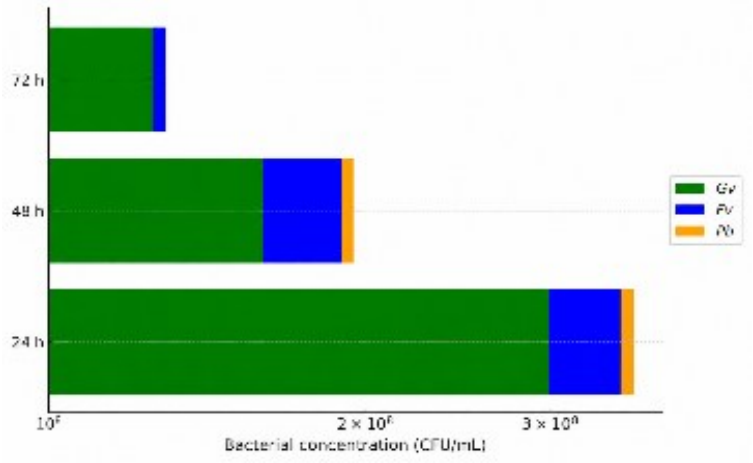


Figure 4.8.

*Triple-species quantification of 24 h, 48 h, and 72 h biofilms of G. vaginalis, P. bivia, and F. vaginae. Bacterial concentrations within the biofilms were determined by qPCR, using the calibration curves described above. Statistical analysis was performed using a t-test, *p <0.05. Abbreviations: Gv – G. vaginalis; Pb – P. bivia; Fv – F. vaginae.*

Not surprisingly, the choice of DNA extraction method had a significant impact on the experimental outcome: the phenol/chloroform method yielded more gDNA than the silica-column commercial kit which is in accordance with previous studies [24,25]. When analysing the recovery rate of the 3 exogenous controls by both methods, it became aparent that the variability observed in the gDNA yield was further aggravated. Interestingly, our results suggest that the size and conformation of the exogenous controls had little effect on the exogenous control recovery rate when using phenol/chloroform methodwhile for the silica column-based extraction method, recovery rates were strongly affected by the nature of the exogenous controls. These data support our initial hypothesis that differential affinity for the silica-columns can impact bacterial quantification by qPCR. Despite the better performance of the phenol/chloroform extraction method, researchers often use silica-based protocols, due to greater time constraints and the utilization of toxic chemicals in the classical phenol/chloroform method [26]. Importantly, we have shown that when using an exogenous control composed of gDNA molecules unrelated to the target genome, the recovery rates were similar in both DNA extraction processes. This leads us to conclude that, for silica-based columns that are optimized for gDNA extraction, exogenous DNA controls should also be composed of unrelated gDNA molecules.

Despite some improvements in qPCR gDNA quantification obtained during the first two sets of experiments, we then proceed with a distinct approach, by changing the exogenous gDNA control to a bacterial control, wherein its gDNA would undergo the same procedure as our target bacterial species. With this strategy, we were able to obtain an improved linear range, from 1×10^8 CFU/mL to 1×10^3 CFU/mL. The application of this strategy demonstrated that calibration curves incorporating *E. coli* as an exogenous control enabled accurate bacterial quantification across a wide dynamic range. Although *E. coli* may occasionally be detected in the urinary tract and contribute to urinary tract infections [27], it is generally absent in women with BV and thus unlikely to occur in significant amounts within the vaginal microbiota [28].

The method was also tested in biofilm cultures. However, the evaluation of the method efficiency was inherently more complex than with mock cultures. While the latter were prepared with defined concentrations, enabling direct verification of the method's accuracy, biofilms represent dynamic and variable systems [29] whose composition cannot be precisely predetermined. Consequently, it was not possible to confirm the absolute accuracy of quantification. Instead, our results were compared with previously published data [17], which, although informative, constitutes a limitation as ideally the same biofilm samples would be analyzed using two independent methods. Nevertheless, the primary objective

of testing the method on biofilms was to assess potential constraints related to gDNA extraction efficiency, as previous studies have reported that certain biofilm matrices can impair nucleic acid recovery [30,31]

As shown in this chapter, accurate bacterial quantification by qPCR requires the inclusion of multiple experimental controls. Overall, our results demonstrated that bacterial losses during centrifugation prior to gDNA extraction are inevitable and can compromise qPCR accuracy and quantification results. To address this issue, we optimized a method that enables reliable quantification of triple-species biofilms containing key BV-associated bacteria. This approach relies on the addition of an exogenous control at a fixed concentration prior to gDNA extraction. Although the inherent variability of biofilms prevents direct verification of absolute accuracy, our findings indicate that this approach overcomes common obstacles in nucleic acid recovery and provides a more robust framework for future studies on multispecies microbial communities.

4.5 References

1. Edslev, S. M. *et al.* Staphylococcal communities on skin are associated with atopic dermatitis and disease severity. *Microorganisms* **9**, 1–17 (2021).

2. Yin, C., Schlatter, D. C., Kroese, D. R., Paulitz, T. C. & Hagerty, C. H. Impacts of lime application on soil bacterial microbiome in dryland wheat soil in the Pacific Northwest. *Applied Soil Ecology* **168**, 104113 (2021).

3. Pacha-Herrera, D. *et al.* Vaginal microbiota evaluation and Lactobacilli quantification by qPCR in pregnant and non-pregnant women: A Pilot Study. *Front Cell Infect Microbiol* **10**, 1–13 (2020).

4. Lima, A., França, A., Muzny, C. A., Taylor, C. M. & Cerca, N. DNA extraction leads to bias in bacterial quantification by qPCR. *Applied Microbiology and Biotechnology* vol. 106 7993–8006 Preprint at https://doi.org/10.1007/s00253-022-12276-4 (2022).

5. Bustin, S. A. *et al.* The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem* **55**, 611–622 (2009).

6. Magalhães, A. P., França, Â., Pereira, M. O. & Cerca, N. RNA-based qPCR as a tool to quantify and to characterize dual-species biofilms. *Sci Rep* **9**, 1–12 (2019).

7. O'Connell, G. C., Chantler, P. D. & Barr, T. L. High interspecimen variability in nucleic acid extraction efficiency necessitates the use of spike-in control for accurate qPCR-based Measurement of Plasma Cell-Free DNA Levels. *Lab Medicine* **48**, 332–338 (2017).

8. Leigh Greathouse, K., Sinha, R. & Vogtmann, E. DNA extraction for human microbiome studies: The issue of standardization. *Genome Biol* **20**, 1–4 (2019).

9. Johnston, S., Gallaher, Z. & Czaja, K. Exogenous reference gene normalization for real-time reverse transcription-polymerase chain reaction analysis under dynamic endogenous transcription. *Neural Regen Res* **7**, 1064–1072 (2012).

10. Revilla-Fernández, S. *et al.* The use of endogenous and exogenous reference RNAs for qualitative and quantitative detection of PRRSV in porcine semen. *J Virol Methods* **126**, 21–30 (2005).

11. Ducarmon, Q. R., Hornung, B. V. H., Geelen, A. R., Kuijper, E. J. & Zwittink, R. D. Toward Standards in Clinical Microbiota Studies: Comparison of Three DNA Extraction Methods and Two Bioinformatic Pipelines. *mSystems* **5**, 1–16 (2020).

12. Schwager, E., Crabtree, J., Ma, S. & Quality, T. M. Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. *Nat Biotechnol* **35**, 1077–1086 (2018).

13. Kumar, M. & Mugunthan, M. Evaluation of three DNA extraction methods from fungal cultures. *Med J Armed Forces India* **74**, 333–336 (2018).

14. NCBI. Genome assembly ASM1192v1. https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_000011925.1/ (2005).

15. Monk, I., Shah, I., Xu, M., Tan, M. & Foster, T. *et al.* Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically Staphylococcus aureus and Staphylococcus epidermidis. *mBio* **3**, (2012).

16. Castro, J., Rosca, A., Muzny, C. & Cerca, N. *Atopobium vaginae* and *Prevotella bivia* are able to incorporate and influence gene expression in a pre-formed *Gardnerella vaginalis* biofilm. *Pathogens* **10**, 1–16 (2021).

17. Rosca, A., Castro, J., França, A., Vaneechoutte, M. & Cerca, N. *Gardnerella Vaginalis* dominates multi-species biofilms in both pre-conditioned and competitive *in vitro* biofilm formation models. *Microb Ecol* **84** (2021).

18. Sousa, L. G. V. *et al.* A new PNA-FISH probe targeting *Fannyhessea vaginae*. *Front Cell Infect Microbiol* **11**, 1–9 (2021).

19. Rosca, A. S. *et al.* Six bacterial vaginosis-associated species can form an *in vitro* and *ex vivo* polymicrobial biofilm that is susceptible to *Thymbra capitata* essential oil. *Front Cell Infect Microbiol* **12**, (2022).

20. Magalhães, A. P., França, Â., Pereira, M. O. & Cerca, N. RNA-based qPCR as a tool to quantify and to characterize dual-species biofilms. *Sci Rep* **9**, 1–12 (2019).

21. Brás, S. The adaptation of *Staphylococcus epidermidis* commensal and clinical isolates to human blood: the search for molecular diagnostics markers. (University of Minho, 2020).

22. Ribeiro, H. G. *et al.* Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model. *Sci Rep* **9**, 1–8 (2019).

23. Leigh Greathouse, K. *et al.* DNA extraction for human microbiome studies: The issue of standardization. *Genome Biol* **20**, 1–4 (2019).

24. Ibrahim, N. & Nassar, S. Comparing the Efficiency in DNA Extraction between organic phenol and magnetic beads methods. *Zagazig Journal of Forensic Medicine* **16**, 10–16 (2018).

25. Schiebelhut, L. M., Abboud, S. S., Gómez Daglio, L. E., Swift, H. F. & Dawson, M. N. A comparison of DNA extraction methods for high-throughput DNA analyses. *Mol Ecol Resour* **17**, 721–729 (2017).

26. Ali, N., Rampazzo, R. D. C. P., Costa, A. Di. T. & Krieger, M. A. Current nucleic acid extraction methods and their implications to point-of-care diagnostics. *Biomed Res Int* **2017**, 1–13 (2017).

27. Flores-Mireles, A. L., Walker, J. N., Caparon, M. & Hultgren, S. J. Urinary tract infections: Epidemiology, mechanisms of infection and treatment options. *Nature Reviews Microbiology* **13** 269–284 (2015).

28. Muzny, C. A. *et al.* Identification of Key Bacteria Involved in the Induction of Incident Bacterial Vaginosis: A Prospective Study. *J Infect Dis* **218**, 966–978 (2018).

29. Sousa, C., França, A. & Cerca, N. Assessing and reducing sources of gene expression variability in *Staphylococcus epidermidis* biofilms. *Biotechniques* **57**, 295–301 (2014).

30. Svarcova, V., Zdenkova, K., Sulakova, M., Demnerova, K. & Pazlarova, J. Contribution to determination of extracellular DNA origin in the biofilm matrix. *J Basic Microbiol* **61**, 652–661 (2021).

31. França, A., Melo, L. D. & Cerca, N. Comparison of RNA extraction methods from biofilm samples of *Staphylococcus epidermidis. BMC Res Notes* **4**, (2011).

CHAPTER 5

Evaluation of the interactions between pairwise combinations of *Gardnerella* spp. on the adhesion to HeLa cells with and without *Lactobacillus crispatus*

Summary

Recent clinical evidence suggests that women are frequently colonized by multiple Gardnerella species. Here we investigated the possible role of synergistic interactions between *G. vaginalis*, *G. swidsinskii*, *G. piotti* and *G. leopoldii* in the initial displacement of *L. crispatus*, using an in vitro HeLa cell model. Bacterial quantification was performed by qPCR. Interestingly, most of all the dual-species combinations we tested revealed an increased ability to displace *L. crispatus*, with the combination of *G. leopoldii* and *G. swidsinskii* showing the strongest displacement effect. It was also observed that the concentration of *Gardnerella* spp. was lower in the presence of *L. crispatus*. These results suggest that the presence of multiple *Gardnerella* spp. influences initial adhesion and may contribute to the development of BV, aligning with clinical observations that women with BV often harbor more than one *Gardnerella* species. Also, *L. crispatus* seems to play a protective role in the vaginal epithelium.

The work described in this Chapter was published in 2025, in PeerJ 13:e20076; and also in .ISIDOG Congress 2025 (6th International Society for infectious diseases in obstetrics and gynecology, October 23-26, Belgrade, Serbia).

5.1 Contextualization of the experimental chapter

As discussed in chapter 2, BV is the leading vaginal infection in reproductive-age women, being associated with multiple adverse health outcomes. Microbiologically, BV is a dysbiosis of the vaginal microbiota, characterized by a loss of dominance of *Lactobacillus* spp. and an increase in anaerobic bacteria, wherein *Gardnerella* spp. have a predominant role [1], thought to be pivotal in the initial formation of the BV characteristic biofilm [2].

It has been proposed that the development of BV is initiated by virulent *Gardnerella* spp. which displace *Lactobacillus* spp. from the vaginal epithelium, paving the way for the formation of the polymicrobial biofilm [3]. A previous *in vitro* study demonstrated that *Gardnerella* spp. exhibited a significantly higher ability to adhere to HeLa cells compared to 29 other BV-associated bacteria (BVAB) [4]. Additionally, it was shown that *Gardnerella* spp. isolated from BV cases had a significantly greater ability to displace lactobacilli pre-adhered to HeLa cells compared to *Gardnerella* spp. isolated from healthy women [5,6]. Importantly, multiple subgroups of *Gardnerella* spp. are frequently detected simultaneously in the vaginal microbiota [7], but there is no research on their interactions when adhering to vaginal epithelial cells. We hypothesized that frequent vaginal co-colonization by different *Gardnerella* spp. is the result of synergistic interactions that occur in the early stages of BV development, namely, during initial adhesion to the vaginal epithelium. To test our hypothesis, we devised an experimental model in which pair-wise combinations of *G. vaginalis*, *G. piotii*, *G. leopoldii* and *G. swidsinskii* challenged pre-conditioned HeLa cells colonized by *Lactobacillus crispatus*, a key indicator of vaginal health [8].

5.2. Methods

5.2.1. Growth conditions and strains

G. vaginalis UM 121, *G. leopoldii* UGent 09.48, and *G. swidsinskii* CCUG 44005 were isolated from women with BV, *G. piotii* UGent 18.01 was isolated from a woman with an intermediate microbiota (Nugent score 4-6), and *L. crispatus* CCUG 44128 was isolated from a healthy woman. *L. crispatus*, *G. vaginalis*, *G. piotii*, *G. leopoldii,* and *G. swidsinskii* were grown on Columbia blood agar base (CBA) (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood (Oxoid), at 37 °C under 10% CO_2. *Escherichia coli* ATCC 25922 was also grown on CBA, but in an aerobic atmosphere at 37 °C. The inocula were cultured in New York City III broth supplemented with 10% (v/v) inactivated horse serum (Biowest, Nuaill'e, France) for 48 h at 37 °C under 10% CO_2 in static conditions [5].

5.2.2. Adhesion assays

Human cervical HeLa cells (from the American Tissue Culture Collection, ATCC CCL-2) were cultured in Dulbecco's medium (Pan Biotech, Aidenbach, Germany) supplemented with 15 % (v/v) Fetal Bovine Serum (FBS; Pan Biotech) and 1% (v/v) antibiotic (ZellShield®, Berlin, Germany) at 37 °C and in 5% CO_2. Cells were cultured in 24-well tissue culture plates (Orange Scientific, Braine L'Alleud, Belgium). Before the displacement and adhesion assays, the epithelial cells were washed with 1 mL of 1 × PBS to remove the non-adherent cells and culture medium, as previously described [6]. The capacity of dual combinations of *Gardnerella* spp. to displace *L. crispatus* pre-adhered to epithelial cells and also to adhere to HeLa cells was evaluated using a previously developed protocol, with some modifications [6]. After washing the 24-well tissue culture plates, 1 mL of *L. crispatus* at 5×10^7 CFU/mL was added to each of the wells. After 4 h of incubation the media was removed and the wells washed with 1 mL of 1 × PBS, then *L. crispatus* was challenged with 500 µL of each of the four *Gardnerella* spp. at 1×10^8 CFU/mL, in dual combinations. These mixtures were also added to epithelial cells without *L. crispatus*, and the plates were incubated for 30 min. Two controls were included. To determine the ability of each species to adhere to HeLa cells, 1 mL of each *Gardnerella* spp. at 1×10^8 CFU/mL was added to each well containing only HeLa cells. To determine which species alone would displace the pre-adhered *L. crispatus,* 1 mL of each *Gardnerella* spp. at 1×10^8 CFU/mL was added to wells containing *L. crispatus* adhered to the HeLa cells, prepared in the same way as described for the test assays. All assays were performed three independent times, with technical duplicates each time.

5.2.3. Construction of calibration curves for quantitative Polymerase Chain Reaction (qPCR)

Calibration curves for qPCR were constructed as previously described in chapter 4. The biomass of each strain was resuspended in 1× PBS. The *L. crispatus* suspension was sonicated 3 times for 10 s at 40% with 10 s intervals, on ice, between cycles. The $OD_{620\ nm}$ of each suspension was adjusted to a concentration of 1×10^8 CFU/mL, and serial dilutions ranging from 10^8 to 10^4 were performed. 100 µL of *E. coli* at 1×10^8 CFU/mL was added to each dilution of target bacteria, as an exogenous control, to normalize extraction efficiency. In addition, 100 µL of HeLa cells suspension was also added to each dilution. The HeLa cells suspension was added to each dilution of the calibration curves to account for any potential loss of efficiency in the extraction and quantification process that may have occurred with the adhesion assay samples. Each suspension was centrifuged at 16000 *g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was frozen at -20 °C overnight for further gDNA extraction.

5.2.4. Genomic DNA (gDNA) extraction and Bacterial quantification by qPCR

gDNA was extracted from the pellets using a commercial Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, with some modifications. Briefly, the pellets were re-suspended in 300 µL of 1× PBS and 300 µL of buffer AL was added to the mixture. Bacterial quantification was performed by qPCR, as previously described [9]. All runs were performed in a CFX96 thermal cycler (Bio-Rad, Hercules, CA, USA), using the following cycle parameters: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. qPCR amplifications were conducted in 10 µL reaction mixtures containing 5 µL of Xpert Fast SYBR Master Mix (Grisp, Porto, Portugal), 1 µL of primer mixture (10 µM), 2 µL of DNase-free water, and 2 µL of a 1:40 dilution of extracted gDNA. Normalization of the qPCR results was achieved through the equation $\Delta\mathrm{Ct} = 2^{CT_{target\ species} - CT_{control\ species}}$, where ΔCt corresponds to Δcycle threshold. The target species corresponded to *G. vaginalis*, *G. piotii*, *G. leopoldii*, *G. swidsinskii*, and *L. crispatus* and the control species corresponded to *E. coli*. Calibration curves were generated using the above equation, with ΔCt values plotted on the y-axis and known bacterial concentrations (CFU/mL) on the x-axis. The bacterial concentration was determined using the generated calibration curves, with the formula $x = \left(\frac{A}{\Delta\mathrm{Ct}}\right)^{1/B}$, where x corresponds to the bacterial concentration and A and B are known. Primer efficiency was determined from the slope of a standard curve [9]. The qPCR primers used are presented in Table 1.

5.2.5 Statistical analysis

The results obtained from the displacement assays were analyzed using the multiple *t*-test (Holm-Sidak) and the results obtained from the adhesion of *Gardnerella* spp. to the HeLa cells were analyzed using the *t*-test for paired samples. GraphPad Prism 2016 was the software used. A *p*-value ≤ 0.05 was considered statistically significant.

Table 5.1.

Primers sequence.

Species	Target gene	Primer sequence (5'-3')	Reference
G. vaginalis	DNAg	TATTATAACTAAAGCTGCTG	[10]
		TCGCCACTATAGTCG	
G. piotii	DNAg	AGCTGCTTACGATTATAGT	[10]
		TTACTCATTCTAAGCTTAATAG	
G. leopoldii	DNAg	GATACTGCACTGTATCGA	[10]
		CAGTATCAATACCAGCC	
G. swidsinskii	DNAg	ATTTAGTTAGATATTTGGCAA	[10]
		ATAGTCATATATTCCGCGC	
L. crispatus	16S RNA	AGCGAGCGGAACTAACAGATTTAC	[11]
		AGCTGATCATGCGATCTGCTT	
E. coli	lacZ	AGCGAAACCGCCAAGACTGTTA	[12]
		AGCGAGCGGAACTAACAGATTTAC	

5.3.1 Calibration curves

We started by constructing qPCR calibration curves, shown in Figure 1, to be able to quantify the different *Gardnerella* spp. in our adhesion assays.

5.3.2 Adhesion of pairwise combinations of *Gardnerella* spp. on HeLa

To evaluate the ability of the selected *Gardnerella* spp. to adhere to and colonize HeLa cells, and to explore potential synergistic effects in the initial adhesion, we quantified their adhesion in single and pairwise combinations after a 30-minute challenge. As shown in Figure 2, a variable synergistic effect was observed, with most of the combinations tested resulting in significantly ($p \leq 0.05$) higher adhesion numbers in the dual-species challenges compared to single-species challenges. The combination of *G. piotii* cultured with *G. leopoldii*, resulted in a total bacterial colonization lower than *G. leopoldii* colonization alone. The combination of *G. vaginalis* and *G. leopoldii* showed the highest ability to adhere to HeLa cells, being 13 times more efficient than *G. vaginalis* and *G. piotii* pair (the least efficient dual-species combinations).

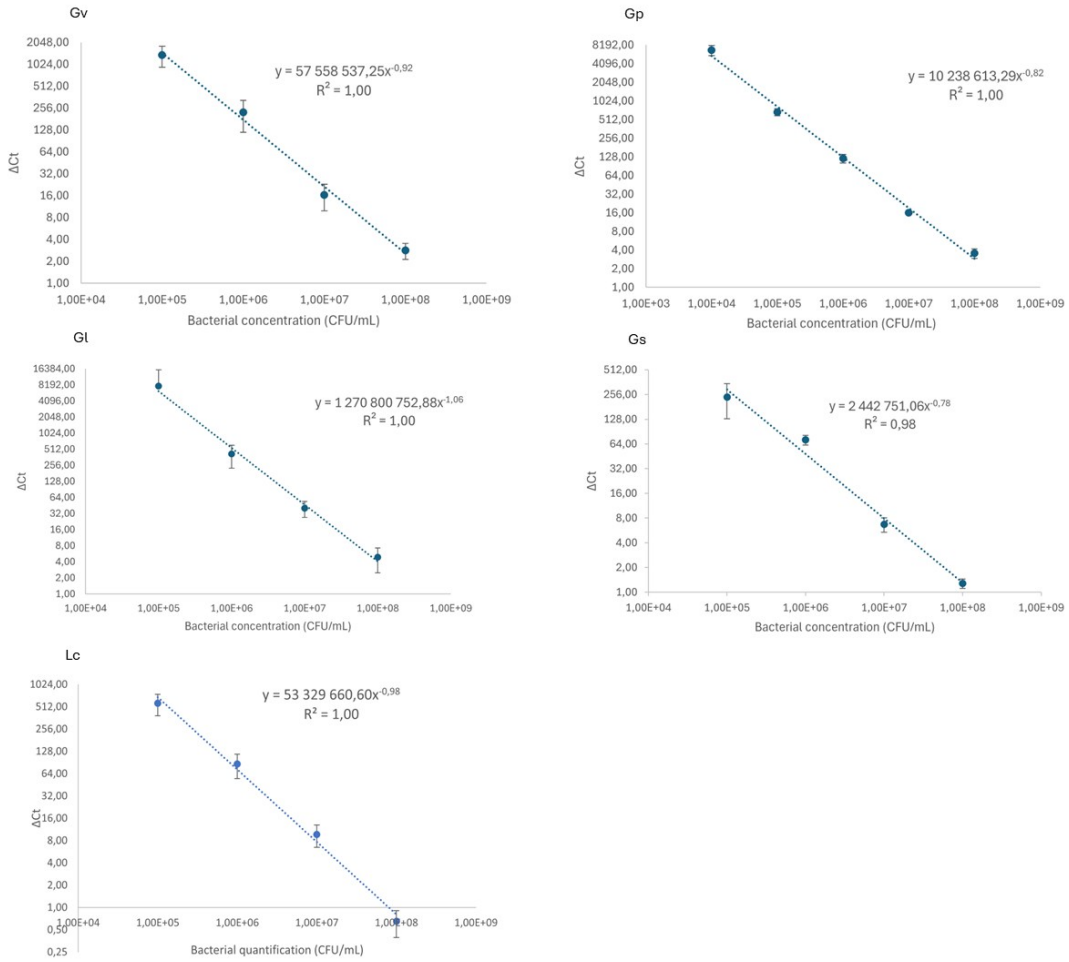


Figure 5.1.

Calibration curves to relate cycle threshold detection (ΔCt) and bacterial load quantification. Each dot represents the average of 3 independent assays and the standard deviation.

5.3.3 Displacement of *L. crispatus* by pairwise combinations of *Gardnerella* spp.

After assessing the potential synergistic effects between the different *Gardnerella* spp., we performed a second experiment, in which *L. crispatus* was first allowed to colonize HeLa cells for 4 h, after which the single and dual-species challenges were repeated. This experimental set up aimed to better simulate a possible external challenge to the vaginal epithelium of a healthy woman colonized by *L. crispatus*. The main objective here was to observe if any synergistic effects could be found when attempting to displace the pre-adhered *L. crispatus*. As shown in Figure 3, most of all the dual-species combinations of *Gardnerella* spp. tested resulted in a higher ability to displace the pre-adhered *L. crispatus*, in comparison with the single-species challenges. Also, it was observed that *G. leopoldii* was the tested species with the highest innate ability to displace *L. crispatus* from HeLa cells. Interestingly,

the observed effect was combination-dependent, with the combination of *G. leopoldii* and *G. swidsinskii* having the highest ability to reduce *L. crispatus* colonization. Compared to the less efficient pair *G. piotii* and *G. swidsinskii*, the *G. leopoldii* and *G. swidsinskii* pair was 10 times more efficient in displacing *L. crispatus*. Moreover, synergism was observed for all the pairwise combinations, excepting for the combinations involving *G. leopoldii*.

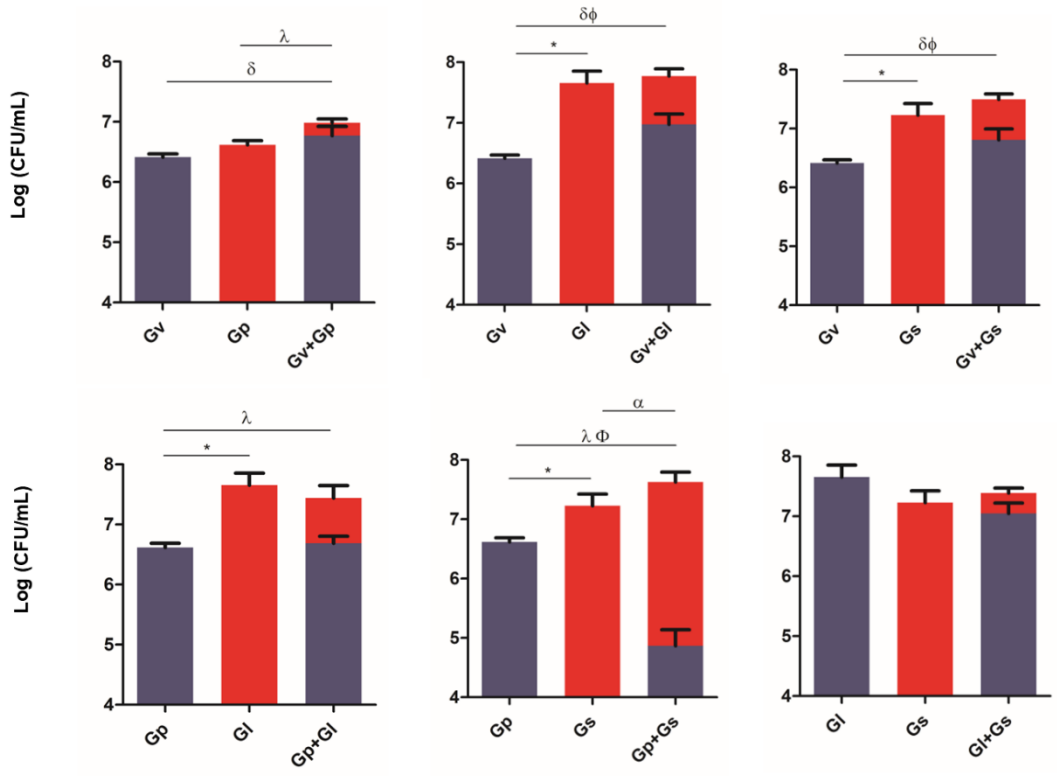
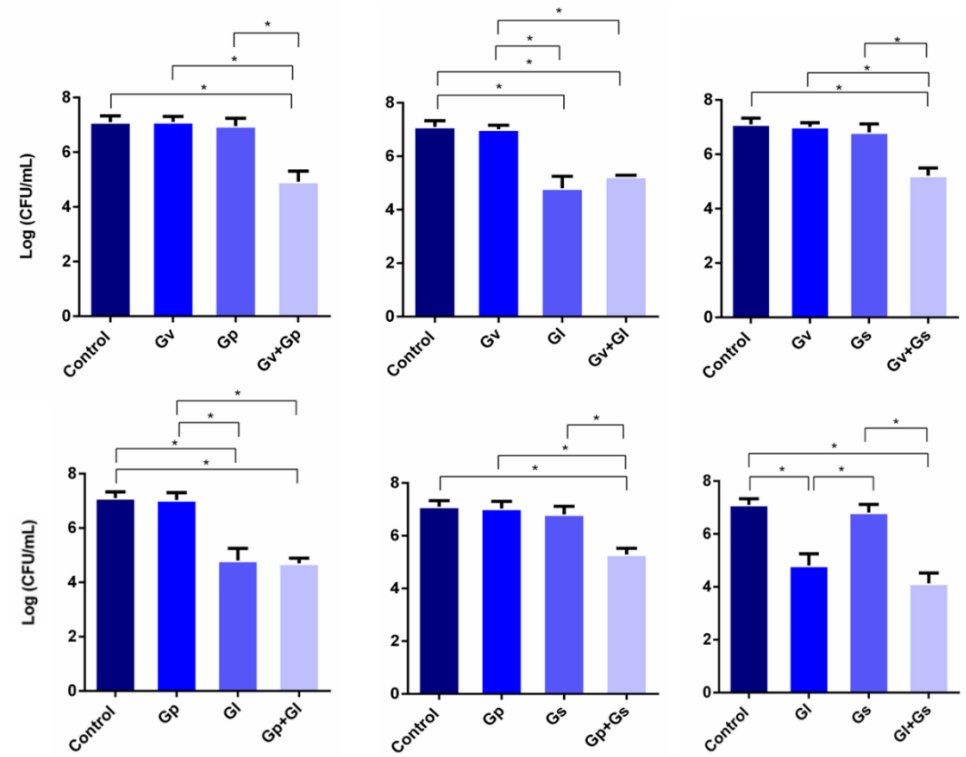


Figure 5.2.

*Adhesion of dual combinations of Gardnerella spp. to HeLa cells. The bars represent the Gardnerella spp. concentration alone or in dual combinations that adhered to HeLa cells. The bars also represent the average and the standard deviation of 3 independent experiments. * Represents statistical significance between Gardnerella spp. alone; δ represents statistical significance between G. vaginalis (Gv) alone and the mix; λ represents statistical significance between G. piotii (Gp) alone and the mix; ε represents statistical significance between G. leopoldii (Gl) alone and the mix; α represents statistical significance between G. swidsinskii (Gs) alone and the mix; Φ represents statistical significance between Gv alone and Gv in the mix and β represents statistical significance between Gs alone and Gs in the mix (t-test, paired samples, $p \leq 0.05$). Gv= G. vaginalis; Gp= G. piotii, Gl= G. leopoldii and Gs= G. swidsinskii.*

5.3.4 Adhesion of pairwise combinations of *Gardnerella* spp. on HeLa covered with *L. crispatus*

Parallel to determining *L. crispatus* quantification after the *Gardnerella* spp. challenges, we also quantified how many *Gardnerella* spp. were able to remain adhered to the HeLa cells after the challenges. Not surprisingly, when comparing Figure 4 with Figure 2, we observed that in most cases, in the presence of *L. crispatus*, the *Gardnerella* spp. pairs concentration was significantly lower than in its absence. The only exception was the dual culture of *G. vaginalis* and *G. swidsinskii,* suggesting that unique interactions between these two species may allow them to overcome *L. crispatus* defenses more effectively. Interestingly, the *G. piotii - G. leopoldii* pair adhered less than *G. leopoldii* alone, although the difference was not significant. In contrast, the combination of *G. vaginalis* and *G. swidsinskii* showed the highest adherence to HeLa cells in the presence of *L. crispatus*.



5.3.

*Displacement of L. crispatus by dual combinations of Gardnerella spp. The bars represent L. crispatus concentrations before (Control) and after being challenged with Gardnerella spp. The bars also represent the mean and the standard deviation of 3 independent assays. * Represents statistical significance (multiple t-test, $p \leq 0.05$). Gv= G. vaginalis; Gp= G. piotii, Gl= G. leopoldii and Gs= G. swidsinskii.*

5.5 Discussion

Despite many years of intensive research into the pathogenesis of BV, its etiology is not yet fully understood, hindering the development of more effective clinical treatments and contributing to its misdiagnosis [13]. As such, continued investment in the study of BV pathogenesis is essential. Here we investigated the interactions of four species of *Gardnerella* spp. (in dual combinations) on their ability to adhere to HeLa cells and also to displace pre-adhered *L. crispatus. L. crispatus* is frequently used as a model to study BV because it is the dominant vaginal lactobacilli in most women of childbearing age and has antimicrobial properties [14]. Furthermore, women with a vaginal microbiota dominated by *L. crispatus* are generally at lower risk of developing BV, and its presence during BV treatment is also associated with better outcomes [15]. Previous studies have demonstrated that *Gardnerella* spp. isolated from BV cases are able to significantly displace *L. crispatus* [5,6]. Interestingly, when we tested dual-species combinations, we observed synergism in most cases. The combination that showed the highest ability to displace *L. crispatus* was not the same pair that presented the highest capacity to adhere to HeLa cells. This suggests that the ability of *Gardnerella* spp. to displace *L. crispatus* may not be solely dependent on their capacity to adhere to epithelial cells, but also on other factors, such as competitive exclusion [16] or modulation of immune responses [17].

Our experiments support the evidence observed in recent studies that demonstrated potential synergism *in vivo*, with BV-positive participants having multiple species of *Gardnerella* detected in their vaginal swabs [18,19]. The fact that co-existence of multiple *Gardnerella* spp. may facilitate BV pathogenesis can be explained by significant differences in the accessory genome of these species, which can result in complementary virulence [20]. Nevertheless, it has been reported that antagonistic interactions can occur between different *Gardnerella* spp., wherein reduced growth rates were observed [21]. While this prior study shows different ecological interactions to the ones reported here, it should be highlighted that both studies are fundamentally different in their methodologies. The conditions we tested did not include bacterial growth in rich media, a condition very different from what occurs in the vaginal niche.

Herein, we limited our study to just pair-wise interactions and used one strain per species. While we acknowledge that strain-to-strain variation may exist, our goal was to test a specific hypothesis. Indeed, we demonstrate that synergistic interactions between different *Gardnerella* sp. provide a plausible explanation for the *in vivo* co-colonization of multiple *Gardnerella* spp., supporting our original hypothesis.

Overall, our findings support the notion that different species of *Gardnerella* contribute to successful initial colonization on the vaginal epithelium, highlighting the critical role of *Gardnerella* spp. as early colonizers in the pathogenesis of BV. Nevertheless, our results also support the notion that *L.*

crispatus has a somewhat protective effect against *Gardnerella* spp., since in most of the dual species' combinations tested, there was a decrease in *Gardnerella* spp. concentration in its presence. However, the key concept is perhaps related to the breaking point wherein the concentration of *L. crispatus* is no longer able to prevent *Gardnerella* overgrowth, leading to the onset of BV. This is of relevant importance since the adhesion to epithelial cells is a crucial step in BV development and the role of vaginal lactobacilli in the protection against pathogenic bacteria. Understanding the dynamics of this tipping point is crucial for developing preventive strategies and interventions aimed at restoring and maintaining a healthy vaginal microbiota.

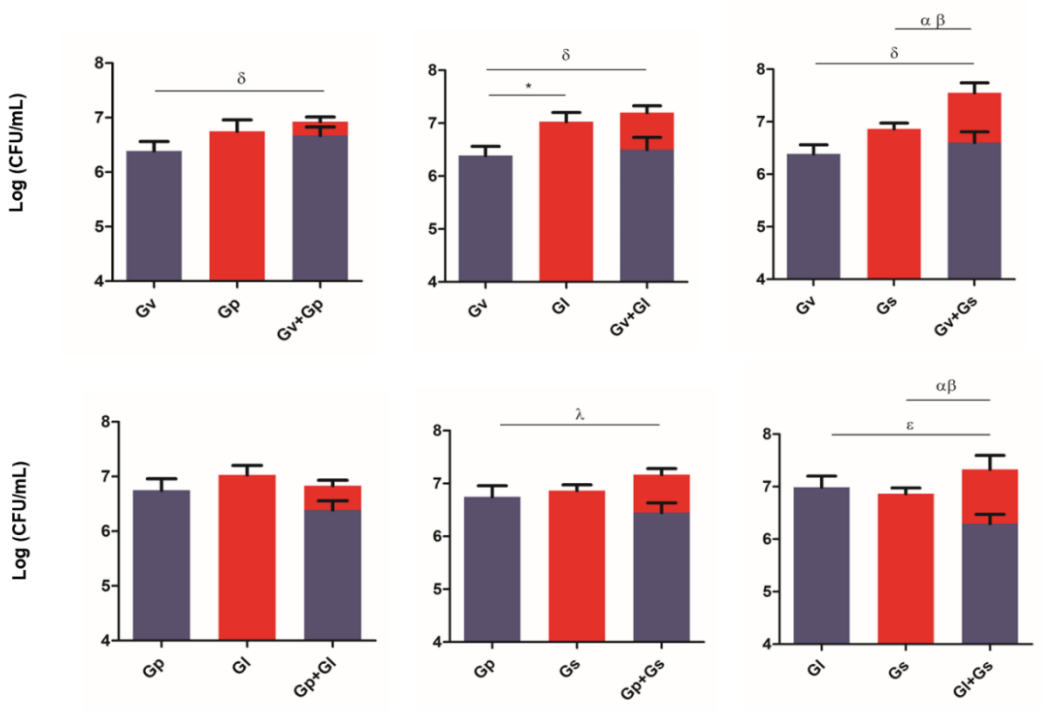


Figure 5.4.

*Gardnerella spp. dual combination adhesion to HeLa cells with pre-adhered L. crispatus. The bars represent the Gardnerella spp. concentration alone or in dual combinations that adhered to HeLa cells. The bars also represent the mean and the standard deviation of 3 independent experiments. * Represents statistical significance between Gardnerella spp. alone; δ represents statistical significance between Gv alone and the mix;; θ represents statistical significance between Gp alone and Gp in the mix; ε represents statistical significance between Gl alone and Gl in the mix; α represents statistical significance between Gs alone and Gs in the mix and β represents statistical significance between Gs alone and Gs in the mix (t-test, paired samples, $p \leq 0.05$). Gv= G. vaginalis; Gp= G. piotii, Gl= G. leopoldii and Gs= G. swidsinskii.*

5.6. References

1. Muzny, C. A. & Sobel, J. D. Understanding and preventing recurring Bacterial Vaginosis: important considerations for Clinicians. *International Journal of Women's Health* vol. 15 1317–1325 Preprint at https://doi.org/10.2147/IJWH.S383333 (2023).
2. Abou Chacra, L., Fenollar, F. & Diop, K. Bacterial Vaginosis: What Do We Currently Know? *Frontiers in Cellular and Infection Microbiology* **11** (2022).
3. Muzny, C. *et al.* An updated conceptual model on the pathogenesis of bacterial vaginosis. *J Infect Dis* **220**, 1399–1405 (2019).
4. Alves, P., Castro, J., Sousa, C., Cereija, T. B. & Cerca, N. *Gardnerella vaginalis* Outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *Journal of Infectious Diseases* **210**, 593–596 (2014).
5. Castro, J. *et al.* Using an in-vitro biofilm model to assess the virulence potential of Bacterial Vaginosis or non-Bacterial Vaginosis Gardnerella vaginalis isolates. *Sci Rep* **5**, 1–10 (2015).
6. Castro, J., Henriques, A., Henriques, M. & Jefferson, K. K. Reciprocal interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on initial adherence to epithelial cells. *International Journal of Medical Microbiology* **10**, 1193–1198 (2013).
7. Hilbert, D., Schuyler, J., Adelson, M., Mordechai, E., Sobel, J. & Gygax, S. *Gardnerella vaginalis* population dynamics in bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases* **36**, 1269–1278 (2017).
8. Huang, B., Fettweis, J. M., Brooks, J. P., Jefferson, K. K. & Buck, G. A. The changing landscape of the vaginal microbiome. *Clinics in Laboratory Medicine* **34**, 747–761 (2014).
9. Lameira, I., Pinto, A. S., Lima, Â., Muzny, C. A. & Cerca, N. Optimized bacterial absolute quantification method by qPCR using an exogenous bacterial culture as a normalization strategy in triple-species BV-like biofilms. *J Microbiol Methods* **219**, (2024).
10. Latka, A. *et al.* Optimization of Propidium Monoazide qPCR (Viability-qPCR) to quantify the killing by the *Gardnerella*-Specific Endolysin PM-477, directly in vaginal samples from women with Bacterial Vaginosis. *Antibiotics* **11**, (2022).
11. Collins, S. L. *et al.* Promising prebiotic candidate established by evaluation of lactitol, lactulose, raffinose, and oligofructose for maintenance of a *Lactobacillus*-dominated vaginal microbiota. *Appl Environ Microbiol* **84**, (2018).

12. Ribeiro, H. G. *et al.* Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model. *Sci Rep* **9**, 1–8 (2019).

13. Redelinghuys, M., Geldenhuys, J., Jung, H. & Kock, M. Bacterial Vaginosis: Current Diagnostic Avenues and Future Opportunities. *Front Cell Infect Microbiol* **10**, 1–20 (2020).

14. Witkin, S. S. & Linhares, I. M. Why do lactobacilli dominate the human vaginal microbiota? *BJOG* **124**, 606–611 (2017).

15. Chee, W. J. Y., Chew, S. Y. & Than, L. T. L. Vaginal microbiota and the potential of *Lactobacillus* derivatives in maintaining vaginal health. *Microbial Cell Factories* vol. 19 Preprint at https://doi.org/10.1186/s12934-020-01464-4 (2020).

16. Ghoul, M. & Mitri, S. The Ecology and Evolution of Microbial Competition. *Trends Microbiol* **24**, 833–845 (2016).

17. Verspecht, T. *et al.* Potential prebiotic substrates modulate composition, metabolism, virulence and inflammatory potential of an in vitro multi-species oral biofilm. *J Oral Microbiol* **13**, (2021).

18. Munch, M. M. *et al. Gardnerella* species and their association with Bacterial Vaginosis. *Journal of Infectious Diseases* **230**, e171–e181 (2024).

19. Balashov, S., Mordechai, E., Adelson, M. & Gygax, S. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* **63**, 162–175 (2014).

20. Vaneechoutte, M. *et al.* Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. Nov., *Gardnerella piotii* sp. nov. and Gardnerella swidsinskii sp. nov., with delineation of 13 genomic species within the genus *Gardnerella*. *Int J Syst Evol Microbiol* **69**, 679–687 (2019).

21. Khan, S., Voordouw, M. & Hill, J. Competition among *Gardnerella* subgroups from the human Vaginal Microbiome. *Front Cell Infect Microbiol* **9**, 1–10 (2019).

CHAPTER 6

Conclusion and Future work perspectives

Summary

This chapter presents the answer to the research questions from Chapter 1 and the main conclusions of this thesis. It also provides the future work perspectives taking into account the thesis findings.

6.1 Concluding remarks

The aim of this study was to provide new insights on BV etiology, specifically, the possible steps that lead to the development of this infection. While it is well known that different subgroups of *Gardnerella* spp. are present in the vaginal microbiota of women with BV [1–3], there are no studies on their interactions during the putative initial adhesion to vaginal epithelial cells. Furthermore, initial adhesion represents a critical step in biofilm formation [4], and BV is marked by the establishment of a polymicrobial biofilm on the vaginal epithelium [5].

Although the aim of this work was to investigate *Gardnerella* spp. interactions, the need to accurately quantify vaginal lactobacilli for use in the adhesion assays, as well as the need to optimize qPCR quantification of bacteria in mixed cultures, turned the techniques used also a focus of this study. Regarding this part of the work, we concluded that the developed indirect fluorescence microscopy was able to accurately quantify the vaginal lactobacilli and can be used to quantify other fastidious bacteria. Moreover, in the qPCR study, it was demonstrated that the best exogenous control is a bacterial suspension with a known concentration, since we noticed that during the centrifugation step before DNA extraction, there is a loss of bacteria. By using a whole bacterium, it is possible to account for that loss and perform an accurate quantification.

This work aimed to answer the research question planned in Chapter 1.

Is there synergism between *Gardnerella* spp. in the displacement of vaginal *Lactobacillus* species from the epithelial surface?

Our data demonstrated that most of the pairwise combinations of *Gardnerella* spp. presented synergism when adhering to HeLa cells without *L. crispatus* pre-adhered. Our findings also indicate that the pairwise combinations of *Gardnerella* spp. that did not involve *G. leopoldii*, displayed synergism in the displacement of *L. crispatus* from HeLa cells. While the dual combination of *G. leopoldii* and *Gardnerella swidsinskii* showed the highest capacity to outcompete *L. crispatus*, it was also observed that *G. leopoldii* alone showed a high innate ability to do it. According to our results, the bacteria concentration of single and dual species combinations of *Gardnerella* adhered to HeLa cells is lower when *L. crispatus* is previously adhered. This suggests that *L. crispatus* can have somewhat a protective effect against *Gardnerella* spp.

6.2 Study limitations

Despite the significant and novel observations and insights obtained in this study, some limitations must be acknowledged. The use of an *in vitro* model of HeLa cells is one of them since this is a tumoral cell line and for that reason, those cells are different from normal cells, for example, in microbial recognition. HeLa cell model was chosen to perform this study, as the previous studies of Dr. Nuno Cerca research group and other BV research groups used it, thereby ensuring methodological consistency and comparability of results.

Another limitation lies in the experimental design of the adhesion assays, which included only dual combinations of *Gardnerella* species. *In vivo*, however, BV cases often involve complex multispecies communities with several *Gardnerella* spp. coexisting simultaneously [1,2]. Moreover, the growth medium used in this study does not fully reproduce the vaginal environment, lacking certain host-derived factors and signaling molecules that may influence Gardnerella phenotypes and interactions.

6.3 Future work

The present work demonstrated that dual combinations of *Gardnerella* species are able to outcompete *Lactobacillus crispatus* and adhere to HeLa cells, suggesting their potential role in initiating the development of BV. However, these findings do not constitute definitive proof of such a mechanism.

Aiming to get more knowledge that sustain this hypothesis, the adhesion studies should be repeated with multiple combinations of *Gardnerella* spp., as well as the use of more than one strain. Moreover, the use of *ex vivo* models as vaginal tissue models [6] or three-dimensional organotypic human vaginal epithelial cell model [7], should be considered in future. It would also be important to study the gene expression of *Gardnerella* spp. in combinations when adhering to HeLa cells and displacing *L. crispatus*, in order to understand how enzymes related to virulence are expressed.

The elucidation of BV etiology is of utmost importance, since it will allow researchers and clinicians to create treatments and strategies to decrease BV recurrence and also to prevent this dysbiotic state.

6.4. References

1. Hilbert, D., Schuyler, J., Adelson, M., Mordechai, E., Sobel, J. & Gygax, S. Gardnerella vaginalis population dynamics in bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases* **36**, 1269–1278 (2017).

2. Munch, M. M. *et al.* Gardnerella Species and Their Association with Bacterial Vaginosis. *Journal of Infectious Diseases* **230**, e171–e181 (2024).

3. Balashov, S., Mordechai, E., Adelson, M. & Gygax, S., Balashov, S. V., Mordechai, E., Adelson, M. E. & Gygax, S. E. Identification, quantification and subtyping of Gardnerella vaginalis in noncultured clinical vaginal samples by quantitative PCR. *J Med Microbiol* **63**, 162–175 (2014).

4. Palmer, J., Flint, S. & Brooks, J. Bacterial cell attachment, the beginning of a biofilm. *J Ind Microbiol Biotechnol* **34**, 577–588 (2007).

5. Swidsinski, A., Loening-Baucke, V., Swidsinski, S. & Verstraelen, H. Polymicrobial Gardnerella biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. *Arch Gynecol Obstet* **291**, 605–609 (2015).

6. Rosca, A. S. *et al.* Six Bacterial Vaginosis-Associated Species Can Form an In Vitro and Ex Vivo Polymicrobial Biofilm That Is Susceptible to Thymbra capitata Essential Oil. *Front Cell Infect Microbiol* **12**, (2022).

7. Łaniewski, P. & Herbst-Kralovetz, M. M. Bacterial vaginosis and health-associated bacteria modulate the immunometabolic landscape in 3D model of human cervix. *NPJ Biofilms Microbiomes* **7**, (2021).