

Key bacterial vaginosis-associated bacteria influence each other's growth in biofilms in rich media and media simulating vaginal tract secretions

Lúcia G.V. Sousa^a, Christina A. Muzny^b, Nuno Cerca^{a,c,*}

^a Centre of Biological Engineering (CEB), Laboratory of Research in Biofilms Rosário Oliveira (LIBRO), University of Minho, Braga, Portugal

^b Division of Infectious Diseases, University of Alabama at Birmingham, Birmingham, AL, United States

^c LABBELS – Associate Laboratory, Braga, Portugal

ARTICLE INFO

Keywords:

Bacterial vaginosis
Polymicrobial biofilms
Bacterial interactions
Growth media conditions

ABSTRACT

Bacterial vaginosis (BV) is a very common gynaecologic condition affecting women of reproductive age worldwide. BV is characterized by a depletion of lactic acid-producing *Lactobacillus* species and an increase in strict and facultative anaerobic bacteria that develop a polymicrobial biofilm on the vaginal epithelium. Despite multiple decades of research, the etiology of this infection is still not clear. However, some BV-associated bacteria (BVAB) may play a key role in the development of this infection, namely *Gardnerella* species, *Prevotella bivia*, and *Fannyhessea vaginiae*. In this work, we aimed to characterize the growth of these three species in a rich medium and in a medium simulating vaginal tract secretions (mGTS). We first assessed planktonic growth in New York City (NYCIII) medium and mGTS and observed that the three species showed distinct capacities to grow in the two media. Surprisingly, despite the ability of all three species to grow in single-species in NYCIII, in a triple-species consortium *P. bivia* was not able to increase its concentration after 48 h, as assessed by qPCR. Furthermore, when using the more restrictive mGTS media, *G. vaginalis* was the only BVAB able to grow in the triple-species consortia. Interestingly, we found that *P. bivia* growth in NYCIII was influenced by the cell-free supernatant (CFS) of *F. vaginiae* and by the CFS of *G. vaginalis* in mGTS. This antimicrobial activity appears to happen due to the acidification of the media. Single- and triple-species biofilms were then formed, and the growth of each species was further quantified by qPCR. While *G. vaginalis* had a high capacity to form biofilms in both media, *F. vaginiae* and *P. bivia* biofilm growth was favored when cultured in rich media. Differences were also found in the structure of triple-species biofilms formed in both media, as assessed by confocal laser scanning microscopy. In conclusion, while all three species were able to grow in single-species biofilms in rich media, in mGTS the growth of *G. vaginalis* was essential for incorporation of the other species in the biofilm.

1. Introduction

The most common cause of vaginal discharge in women of reproductive age worldwide is bacterial vaginosis (BV). Approximately 30% of women develop BV sometime during their life [1]. Despite the availability of multiple oral and intra-vaginal treatments for this infection, a large proportion of women experience recurrence after antibiotic treatment [2,3]. This leads to significant economic burdens due to this recurrence of infection, as well as, emotional and psychological distress among women that suffer from this condition [1,4,5]. Additionally, BV is associated with multiple adverse outcomes including preterm birth [6], pelvic inflammatory disease [7], and increased risk of acquisition of sexually transmitted infections, namely HIV [8].

BV is characterized by an imbalance in the vaginal microbiota with a decrease in lactic acid-producing *Lactobacillus* spp. and an increase in facultative and strict anaerobic bacteria [9–11]. These BV-associated bacteria (BVAB) form a polymicrobial biofilm on the surface of the vaginal epithelium that is thought to be a significant contributing factor in the setting of treatment failure [12,13]. Although the exact etiology of BV is still unclear, there are some BVAB that are highly associated with the infection, as they are commonly detected in the vaginal microbiota of women with BV. Examples of common BVAB belong to the genus *Gardnerella*, *Prevotella*, *Megasphaera*, *Sneathia*, *Mobiluncus*, and *Fannyhessea* (previously known as *Atopobium*) [14–16].

Gardnerella spp. are the most common BV-associated bacteria (BVAB), found in 95–100% of BV cases, and initially thought to be the

* Corresponding author. Centre of Biological Engineering (CEB), University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.

E-mail address: nunocerca@ceb.uminho.pt (N. Cerca).

<https://doi.org/10.1016/j.biofilm.2024.100247>

Received 30 April 2024; Received in revised form 19 November 2024; Accepted 20 December 2024

Available online 3 January 2025

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etiologic agent of the infection [17,18]. Multiple studies have shown that *Gardnerella* spp. have high ability to displace *Lactobacillus* spp. from vaginal epithelial cells [19], have a high capacity to form biofilms [20], and express multiple virulence factors, such as vaginolysin and sialidase [21,22]. The expression of virulence factors by *Gardnerella* spp. can be influenced by the presence of other BVAB when present in co-culture biofilms. For example, in the case of dual-species biofilms with *F. vaginae*, HMPREF0424_0821, a gene associated with biofilm formation, was upregulated [23].

Interestingly, *F. vaginae* is also commonly detected in cases of BV, but rarely in the absence of *G. vaginalis* [24]. This led to the hypothesis that *F. vaginae* growth was dependent on *G. vaginalis*, being demonstrated that under specific conditions *F. vaginae* required the supernatant of *G. vaginalis* culture to keep viability [25]. However, under specific growth conditions, *F. vaginae* can grow alone and also form a single-species biofilm [26]; thus, further research is necessary. Nevertheless, the role of *F. vaginae* might be very important in BV pathogenesis as some studies have reported that it can be resistant to some of the antibiotics commonly used in clinical cases of BV, namely metronidazole [27].

In a study evaluating the sequence of microbiological events preceding the development of incident BV (iBV), *P. bivia* relative abundance became significantly higher 4 days before iBV [28]. The capacity of *P. bivia* to express some virulence factors, namely the production of sialidase, may also contribute to the development of BV [29]. Furthermore, the interaction between *P. bivia* and *G. vaginalis*, where *P. bivia* produces ammonia that is used by *G. vaginalis*, which in turn produces amino acids that are used by *P. bivia*, is also a recognized synergistic relationship between the two species that can explain some developments in BV [30].

As such, further study of *G. vaginalis*, *F. vaginae*, and *P. bivia* species can contribute to a better understanding of BV biofilm development, and the consequent symptoms of BV, which can then inform future diagnostic, treatment, and prevention studies. In this work, we studied the influence of a rich growth medium and a medium simulating genital tract secretions (mGTS) on the growth of three key BVAB, namely *G. vaginalis*, *F. vaginae*, and *P. bivia*, when cultured on single- and triple-species biofilms, in order to evaluate the influence these species have on each other and on BV biofilm growth.

2. Materials and methods

2.1. Growth medium preparation

Two distinct culture media were used for the purposes of this study. New York City III (NYCIII) broth medium was prepared according to a previous study [26], and supplemented with 10% of inactivated horse serum (Biowest, Nuaille, France), although here, proteose peptone (Liofilchem, Roseto degli Abruzzi, Italy) was used instead. The mGTS media was prepared based on previous studies [31,32], with minimal modifications. The mGTS composition contained five parts: Part I: 0.35% NaCl, 0.15% KCl, 0.17% K₂HPO₄, 0.14% KH₂PO₄, 0.5% glucose, and 0.05% cysteine HCl; Part II: 0.1% glycogen, 0.03% mucin, 0.02% tween 20, 0.05% urea, 0.0005% vitamin K₁, 0.0005% hemin, 0.2% bovine serum albumin, 0.03% MgSO₄, 0.004% NaHCO₃, 0.1% sodium acetate, and 0.05% MnCl₂; Part III: 0.0005% biotin, 5.0% *myo*-inositol, 0.05% niacinamide, 0.05% pyridoxine HCl, 0.05% thiamine HCl, 0.05% D-calcium pantothenate, 0.02% folic acid, 0.001% *p*-aminobenzoic acid, 0.05% choline chloride, 0.01% riboflavin, 0.1% L-ascorbic acid, 0.0005% vitamin A (retinol), 0.0005% vitamin D (cholecalciferol), and 0.001% vitamin B₁₂; Part IV: 0.032% alanine, 0.008% arginine, 0.04% asparagine, 0.076% aspartic acid, 0.036% glutamic acid, 0.04% glutamine, 0.02% glycine, 0.016% histidine, 0.012% isoleucine, 0.02% leucine, 0.02% lysine, 0.004% methionine, 0.004% phenylalanine, 0.028% proline, 0.012% serine, 0.01% taurine, 0.012% threonine, 0.004% tryptophan, 0.02% tyrosine, and 0.068% valine; Part V (w/v):

0.05% uracil, 0.01% sodium pyruvate, and 0.02% inosine.

2.2. Bacterial culture conditions

G. vaginalis ATCC 14018, *F. vaginae* ATCC BAA-55, and *P. bivia* ATCC 29303 were used in this study. For confirmation of single-species growth, two more strains of each species were used, namely *G. vaginalis* UM121 and *G. vaginalis* UM137 [33], *F. vaginae* BVS065 and *F. vaginae* BVS067 [34], and *P. bivia* CCUG 59496 and *P. bivia* CCUG 33962. The bacteria were kept frozen at −80°C in Brain Heart Infusion (BHI) broth (Liofilchem) with 23% (v/v) of glycerol (Panreac, Darmstadt, Germany). For each experiment, the strains were cultured on Columbia Agar Base medium (Liofilchem) supplemented with 5% of defibrinated horse blood (Thermo Fisher Scientific, Lenexa, KS, USA) (CBA). For *G. vaginalis* strains, the plates were incubated for 48 h at 37°C, and 10% CO₂, and for *F. vaginae* and *P. bivia* strains, the plates were kept at anaerobic conditions (Anaerocult™ A, Merck Milipore, Taufkirchen, Germany) for 48 h at 37°C.

2.3. Single-species bacterial growth evaluation

As a pilot experiment, bacterial growth of 3 different strains of *G. vaginalis*, *F. vaginae*, and *P. bivia* was evaluated in NYCIII and mGTS by optical density (OD) measurement. Briefly, a bacterial suspension was prepared in each medium by inoculating a small amount of biomass of each species from the CBA plate in the broth media. The suspensions were then dispensed on 96-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium) and the plates were incubated at 37°C in anaerobic conditions (AnaeroGen Atmosphere Generation system, Oxoid, Basingstoke, UK). The growth was evaluated at 0 h, 24 h, 48 h, and 72 h. At each time point the content of the wells was resuspended and the OD was measured at 620 nm. The experiments were repeated at least three independent times with eight technical replicates.

2.4. Single- and triple-species total bacterial growth quantification

Total growth (biofilm plus the bulk fluid) of single- and triple-species cultures was then evaluated by colony forming units (CFUs) and quantitative PCR (qPCR), as described below. Bacterial suspensions of *G. vaginalis* ATCC 14018, *F. vaginae* ATCC BAA-55, and *P. bivia* ATCC 29303 were prepared from biomass of 48 h-CBA plates and the bacterial concentration was adjusted to 1×10^7 CFU/mL [35]. A mixed suspension with the 3 species was also prepared, with the final concentration of each species at 1×10^7 CFU/mL. The suspensions were then dispensed on 96-well plates and incubated for 48 h at 37°C and at anaerobic conditions. At 48 h the content of each well was resuspended, and the volume of wells from the same condition was combined. For CFUs, at 0 h and 48 h, serial dilutions of the suspension from each one of the conditions were performed on NaCl 0.9% (Sigma, Darmstadt, Germany), and 10 µL of each dilution was plated on CBA plates. The plates were then incubated at anaerobic conditions for at least 48 h, and CFUs were counted thereafter. Samples from 0 h and 48 h were also prepared for qPCR analysis. The experiments were performed three independent times with eight technical replicates.

2.5. Cell-free supernatants' influence on *P. bivia* growth

Inoculums of each single- and triple-species were prepared at a final concentration of 1×10^7 CFU/mL per bacterial species. The inoculums were incubated for 48 h at 37°C and at anaerobic conditions. Afterward, the suspensions were centrifuged for 30 min at 3143 g and 4°C. The supernatant was then recovered and filter-sterilized using 0.22 µm filters. Twenty microliters of cell-free supernatant (CFS) was plated on CBA plates for confirmation of sterility.

A suspension of *P. bivia* was prepared from biomass from a CBA plate and the concentration was adjusted to 9×10^7 CFU/mL. On a 96 well-

plate the suspension of *P. bivia* was inoculated together with 10% or 50% of each CFS from *G. vaginalis*, *F. vaginae*, *P. bivia*, and the triple-species suspensions. The final concentration of *P. bivia* on each well was 1×10^7 CFU/mL. A positive control containing only *P. bivia* suspension with no CFS was also included. The plate was incubated for 48 h at 37°C and at anaerobic conditions. After 48 h, the content of the wells was resuspended and combined by condition. For CFUs determination, the suspension of *P. bivia* at 0 h (1×10^7 CFU/mL), and the suspension of each condition at 48 h were used to perform serial dilutions in NaCl 0.9% and 10 µL of each dilution were plated on CBA. The plates were incubated for at least 48 h, after which time the CFUs were counted. One milliliter of *P. bivia* (1×10^7 CFU/mL) suspension at 0 h, as well as, 1 mL of each condition at 48 h were used for genomic DNA (gDNA) extraction. The experiments were repeated at least three independent times with eight technical replicates.

2.6. Effect of heat, proteinase K, and pH adjustment on the cell-free supernatants' activity

The cell-free supernatants were prepared as described above. Only the supernatants with the highest antimicrobial activity were used in these assays, namely the CFS from *F. vaginae* and triple-species in NYCIII and the CFS from *G. vaginalis* and triple-species in mGTS. After filtration, the CFS was treated by heat, proteinase K, and pH adjustment to characterize its effect on *P. bivia* growth. To test the effect of heat, 1 mL of each CFS was heated at 100°C for 30 min. After this time the CFS was centrifuged at 16000 g for 10 min, and the supernatant was used for the assay. For testing the activity of proteinase K, 1 mg/mL of proteinase K (RP102B, Blirt, Danzig, Poland) was added to the CFS, which was incubated for 1 h at 55°C. After this time, proteinase K was inactivated by incubation at 95°C for 20 min. For the supernatants formed in NYCIII, after proteinase K inactivation, the tubes were centrifuged at 16000 g for 10 min and the supernatant was used. The pH neutralization was performed by adjusting the pH of the CFS to the same pH of the growth media. A control was also used by adjusting the pH of the growth media to the same pH of the CFS. After treating the CFS by these protocols, the assay to determine the effect on *P. bivia* growth was performed, as described above, using CFUs for growth quantification. The experiments were performed with eight technical replicates at least three independent times.

2.7. Biofilm formation

Biofilms of single- and triple-species were formed using a competitive *in vitro* model previously developed [36]. For biofilms formed in mGTS, bacterial suspensions of each species were prepared in this medium by inoculating a small amount of biomass from CBA plates, and the bacterial concentration was adjusted to 1×10^7 CFU/mL. The triple-species suspension was prepared with each species in a proportion 1:1:1. The suspensions were then inoculated on 96-well plates and incubated for 48 h at 37°C and at anaerobic conditions.

For biofilms formed in NYCIII, inoculums of each species were prepared in this medium and incubated for 24 h. After 24 h, the bacterial concentration was adjusted, and single- and triple-species suspensions were prepared at 1×10^7 CFU/mL. Each suspension was dispensed on 96-well plates and incubated for 48 h at 37°C and at anaerobic conditions.

For each method, after 48 h of incubation, the medium was removed from the biofilms, and the biofilms were washed once with 0.9% NaCl. After that, the biofilm was resuspended in mGTS or NYCIII and mechanically detached from the plate.

Samples at 0 h and 48 h, were prepared for qPCR quantification, as described below. The experiments were repeated at least three independent times with eight technical replicates.

2.8. Quantification by qPCR

Samples for qPCR analysis were prepared by adding 1 mL of each suspension with 500 µL of *Escherichia coli* ATCC 25992 suspension at a final concentration of 1×10^8 CFU/mL. The *E. coli* suspension was used as an exogenous control, as demonstrated before [37], with some minor adaptations. The suspensions were then centrifuged twice at 16000 g for 10 min and kept at -20°C overnight for gDNA extraction. The commercial DNeasy Ultraclean microbial kit (Qiagen, MD, USA) was used for gDNA extraction, following the manufacturer's instructions with minor optimizations. Briefly, the cells were resuspended in PowerBead solution and lysed in a BeadBug 6 Microtube Homogenizer (Benchmark Scientific, NJ, USA) using 2×3 cycles at 4350 rpm for 30 s, and the samples were kept on ice between cycles. GDNA was then loaded on a spin column and eluted in 50 µL of DNase/RNase-free water (Grisp, Porto, Portugal). Samples were diluted 40 × for qPCR experiments. The primers used to quantify *G. vaginalis*, *F. vaginae*, and *P. bivia* were described before [38], as well as the primers for *E. coli* [39], and are listed on Supplementary Table 1.

The qPCR experiments were performed using 2 µL of 1:40 diluted gDNA and 8 µL of qPCR mix containing 5 µL of Xpert fast SYBR mix (Grisp), 1 µL of primers and 2 µL of DNase/RNase free water. Non-template controls were included to evaluate reagent contaminations. The qPCR runs were performed on CFX96™ (Bio-Rad, CA, USA) with the following cycle parameters: 95°C for 3 min and 40 cycles of 95°C for 5 s and 60°C for 20 s. Analysis of melting curves was performed to ensure the absence of non-specific products and primer-dimers. The cycle threshold (CT) results for each species at each time point were then normalized to the exogenous *E. coli* control included in the samples, using $E^{-\Delta CT}$, where E stands for the reaction efficiency and $\Delta CT = (CT_{\text{target}} - CT_{\text{control}})$. The relative quantification results were then expressed as a fold change of the values at 0 h and 48 h. These experiments were repeated at least three times.

2.9. Biofilm characterization by PNA-FISH

For peptide nucleic acid- fluorescence *in situ* hybridization (PNA-FISH) analysis the biofilms in mGTS and NYCIII were performed in the same conditions as described above, for a final volume of 400 µL. Each suspension was dispensed on 8-well chamber slides (Thermo Fisher Scientific™ Nunc™ Lab-Tek™, Rochester, NY, USA) and incubated for 48 h at 37°C and at anaerobic conditions. After this time, the medium was removed, and the biofilms were let to air-dry. Afterward, biofilm fixation was performed using 100% methanol (Thermo Fisher Scientific) for 15 min, followed by 4% paraformaldehyde (Thermo Fisher Scientific) for 10 min, and 50% ethanol (Thermo Fisher Scientific) for 15 min. When the biofilms were dried, 30 µL of hybridization solution containing 10% (w/v) dextran sulfate (Sigma), 10 mM NaCl (Sigma), 30% (v/v) formamide (Thermo Fisher Scientific), 0.1% (w/v) sodium pyrophosphate (Thermo Fisher Scientific), 0.2% (w/v) polyvinylpyrrolidone (Sigma), 0.2% (w/v) Ficoll (Sigma), 5 mM disodium EDTA (Panreac), 0.1% (v/v) Triton X-100 (Thermo Fisher Scientific), 50 mM Tris-HCl (pH 7.5; Thermo Fisher Scientific), and 200 nM of each PNA probe - Gard162 [40], FvagPNA651 [41], and PbivPNA1454 [42] - was applied and the slides were covered with a coverslip and incubated for 90 min at 60°C. After that, the slides were immersed in a washing solution for an additional 30 min at the same temperature. After the PNA-FISH procedure, the biofilms were analyzed by confocal laser scanning microscopy using an Olympus™ Fluo-View FV1000 (Olympus, Lisbon, Portugal) confocal laser scanning microscope. The lasers' wavelengths 559 nm, 635 nm, and 488 nm were used for *Gardnerella* (Alexa-Fluor 594), *F. vaginae* (Alexa-Fluor 633), and *P. bivia* (Alexa-Fluor 488) probes, respectively. The images were acquired using a magnification of 40 ×. These experiments were repeated three independent times.

2.10. Statistical analysis

Results were analyzed using GraphPad Prism version 8.2 (La Jolla, CA, USA). Statistical analysis was performed using Student T-test and One-way ANOVA with Tukey's multiple comparisons tests. Normality of the distributions was tested by the Shapiro-Wilk test ($\alpha = 0.05$). Statistical differences were considered when $p < 0.05$.

3. Results

3.1. Single-species bacterial growth in NYCIII and mGTS

As an initial assessment of bacterial growth capacity, we evaluated the growth of *G. vaginalis*, *F. vaginae*, and *P. bivia* in NYCIII and mGTS. The growth was evaluated by measuring the OD at different time points as shown in Fig. 1. We observed that all three species had distinct capacities to grow in both media, however, all of them were able to increase the OD in NYCIII media. In contrast, only *P. bivia* demonstrated a fold change above 2 in mGTS, while *F. vaginae* demonstrated a decrease in the OD values. Furthermore, the greatest increase in the OD values was observed within the first 24 h for the three bacteria. The growth in both media was further confirmed using 2 other strains from each species and the results are present on Supplementary Figure 1.

3.2. Single- and triples species bacterial growth quantification

In an attempt to investigate whether interactions between species could contribute to an increase in their growth in mGTS, we evaluated

the growth of single- and triple-species suspensions when cultured in mGTS and compared it with NYCIII media. The suspensions were left to grow for 48 h, since no further significant growth was observed at 72 h, as shown in Fig. 1.

The first qPCR analysis in NYCIII (Fig. 2A) revealed that all the species grew and increased their concentration on single-species cultures. However, when the 3 species were grown together, *P. bivia* demonstrated a fold change below one, suggesting that the concentration of *P. bivia* after 48 h of growth was below the initial concentration of the starting inoculum. In terms of culturability, the three species did not have an increase of culturable cells, compared to the starting inoculum, after 48 h of growth, in both single- and mixed-species consortia, as observed by the CFUs results (Fig. 2B).

In mGTS, when performing qPCR analysis only *G. vaginalis* had the capacity to significantly increase its bacterial concentration (Fig. 3A). However, the same amount of growth was not observed when analyzing species culturability (Fig. 3B).

3.3. Influence of cell-free supernatant on *P. bivia* growth

The observation that *P. bivia* had the ability to grow in single species in both NYCIII medium and mGTS but was significantly impaired when cultured in the triple-species consortia, led us to hypothesize that one of the other species was somehow inhibiting *P. bivia*'s growth. Thus, we evaluated the effect of CFS from each single-species inoculum, and from the triple-species consortia on the growth of *P. bivia*. In NYCIII, as described in Fig. 4A, *F. vaginae* CFS had an effect on *P. bivia* growth with a growth inhibition of approximately 50% in the presence of 10% CFS.

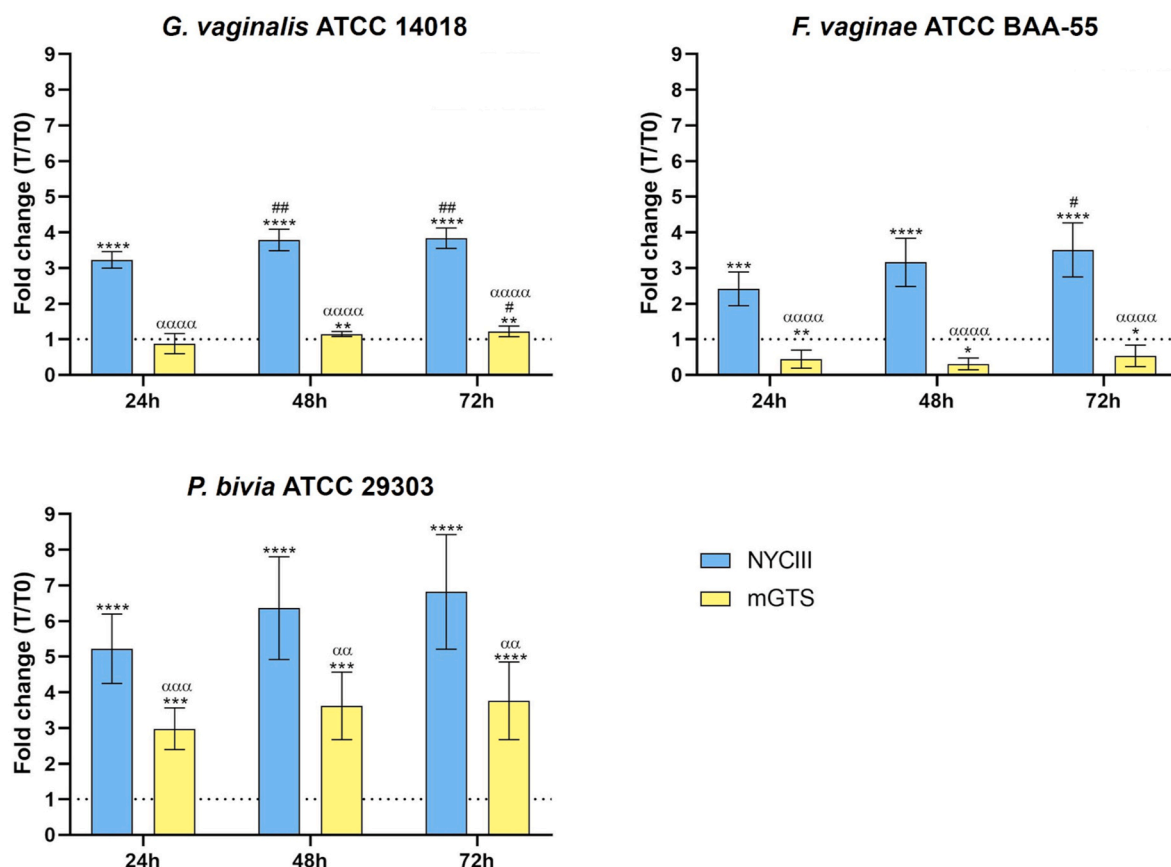


Fig. 1. Results of fold change (T/T0) of bacterial growth of *G. vaginalis*, *F. vaginae*, and *P. bivia* measured by OD readings at different time points in NYCIII and mGTS media. Results are represented by the average of OD values and error bars represent standard deviation. Statistical analysis was performed using the Student T-test. Significant differences when comparing with T0 are represented by * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$). Significant differences between time points within each media are represented by # ($p < 0.05$); ## ($p < 0.01$), only differences with 24 h were found. Significant differences when comparing with NYCIII medium at each time point are represented by α ($p < 0.01$); αα ($p < 0.001$); ααα ($p < 0.0001$).

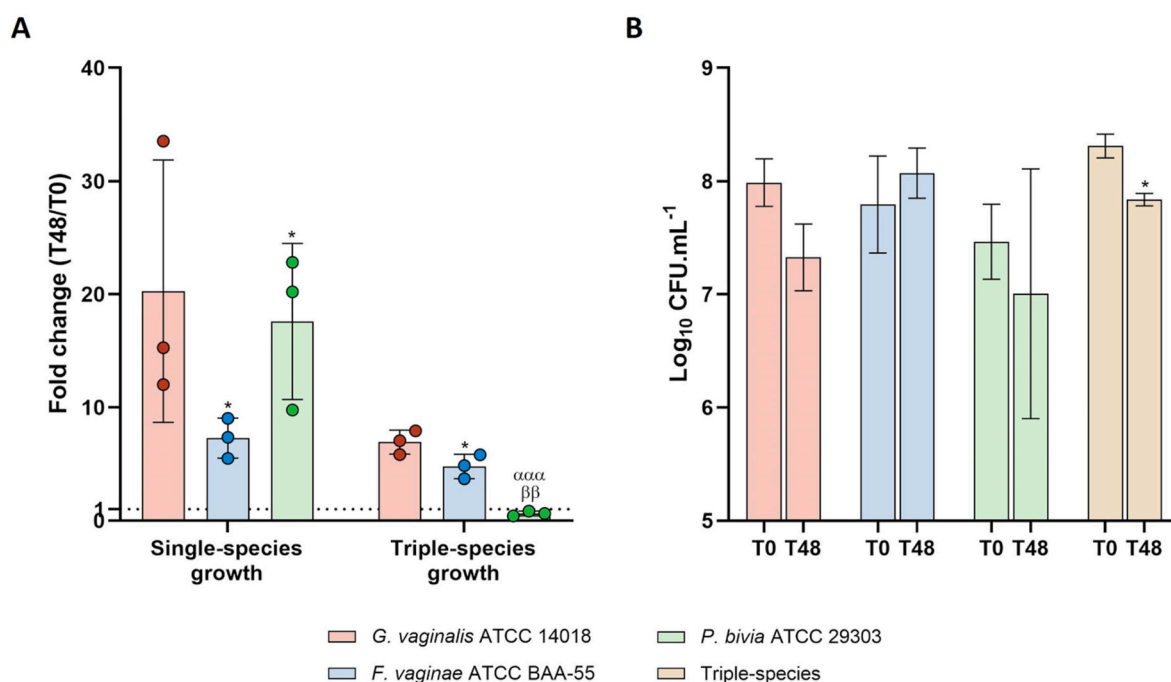


Fig. 2. Single-species and triple-species bacterial growth in NYCIII quantified by qPCR (A) and colony forming units (CFUs) (B). The results are expressed as the average of fold change (T48/T0) of the normalized expression to *E. coli* exogenous control or the average of Log₁₀ CFU/mL, with error bars representing the standard deviation. CFUs quantification experiments have a limit of detection of Log₁₀ = 2. Statistical analysis was performed using the Student T-test and One-way ANOVA. Significant differences when comparing with T0 are represented by * (* *p* < 0.05). Significant differences when comparing to *G. vaginalis* are represented by α (α *p* < 0.001), and significant differences to *F. vaginae* are represented by β (β *p* < 0.01).

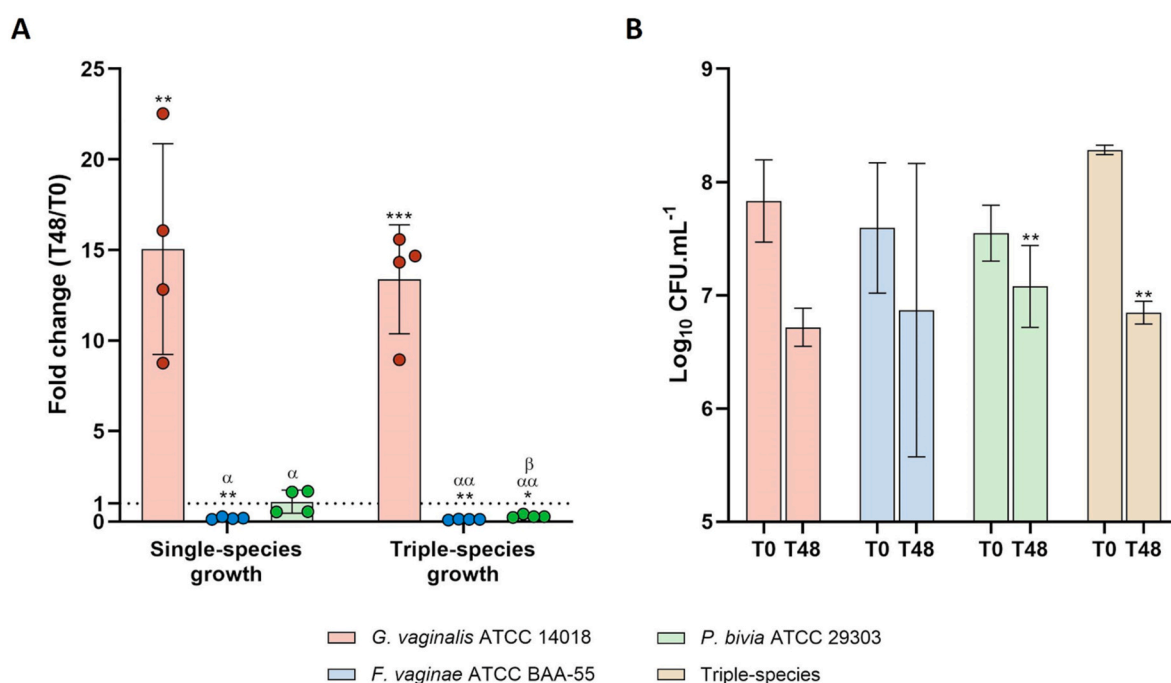


Fig. 3. Single-species and triple-species bacterial growth in mGTS quantified by qPCR (A) and colony forming units (CFUs) (B). The results are expressed as the average of fold change (T48/T0) of the normalized expression to *E. coli* exogenous control or the average of Log₁₀ CFU/mL, with error bars representing the standard deviation. CFUs quantification experiments have a limit of detection of Log₁₀ = 2. Statistical analysis was performed using the Student T-test and One-way ANOVA. Significant differences when comparing to T0 are represented by * (* *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001). Significant differences when comparing to *G. vaginalis* are represented by α (α *p* < 0.05; αα *p* < 0.01), and significant differences when comparing to *F. vaginae* are represented by β (β *p* < 0.05).

When 50% of CFS was used, the effect was more pronounced, and a reduction in *P. bivia* copy number, as compared with both the respective control (*P. bivia* grown in 50% CFS from *P. bivia*) and the starting

inoculum (at T0), was observed. When analyzing the results of CFUs (Fig. 4B), we verified that using 50% of *F. vaginae* caused a reduction on *P. bivia*'s culturability, and the effect was even higher when using triple-

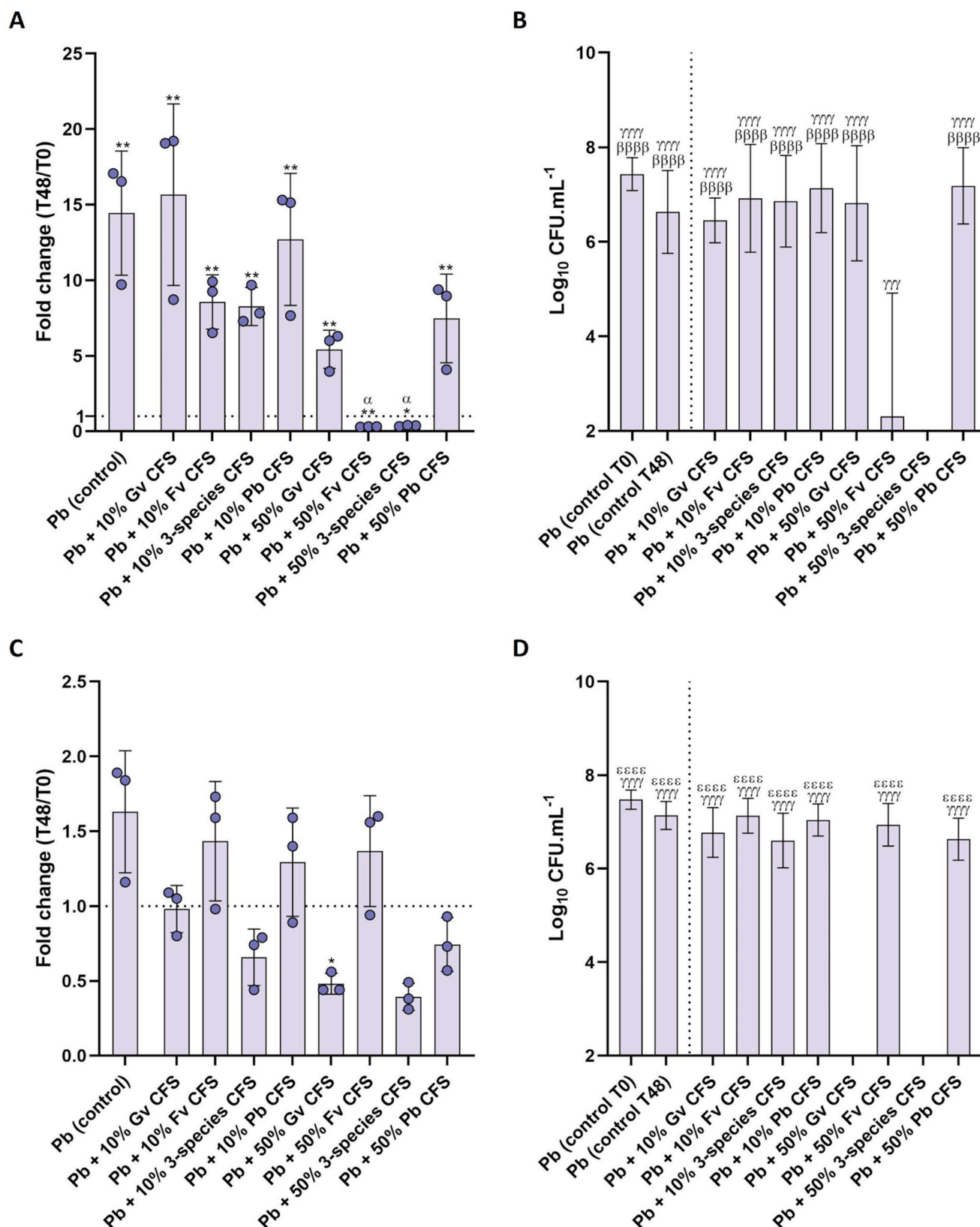


Fig. 4. Effect of single-species and triple-species cell-free supernatant (CFS) on *P. bivia* growth in NYCIII (A and B) and mGTS (C and D), quantified by qPCR (A and C) or colony forming units (CFUs) (B and D). The results are expressed as the average of fold change (T48/T0) of the normalized expression to *E. coli* exogenous control or the average of Log₁₀ CFU/mL, with error bars representing the standard deviation. CFUs quantification experiments have a limit of detection of Log₁₀ = 2. Pb: *P. bivia*, Gv: *G. vaginalis*, Fv: *F. vaginae*. Statistical analysis was performed using the Student T-test and One-way ANOVA. Significant differences when comparing to T0 are represented by * (* $p < 0.05$; ** $p < 0.01$). Significant differences when comparing to the condition Pb + 10% 3-species CFS are represented by α ($p < 0.05$), when comparing to the condition Pb + 50% Fv CFS are represented by β (ββββ $p < 0.0001$), when comparing to the condition Pb + 50% 3-species CFS are represented by γ (γγγ $p < 0.001$; γγγγ $p < 0.0001$), and when comparing to the condition Pb + 50% Gv CFS are represented by ε (εεεε $p < 0.0001$).

species CFS, since the culturability was below the limit of detection ($\text{Log}_{10} = 2$).

Curiously, in mGTS, *F. vaginalis* did not demonstrate the same phenotype, but instead a growth inhibition was detected when growing *P. bivia* in the presence of *G. vaginalis* CFS (Fig. 4C), although the effect was less pronounced. The same effect was observed on the *P. bivia* culturability with a reduction below the limit of detection in the presence of 50% of *G. vaginalis* and triple-species CFS (Fig. 4D).

3.4. Effect of heat, proteinase K, and pH on cell-free supernatant

To characterize the antimicrobial effect of CFS on *P. bivia* growth, the selected CFS were treated with either heat, proteinase K, or pH adjustment. When observing the results in NYCIII (Fig. 5A), we concluded that treatment with heat or proteinase K did not alter the CFS effect on *P. bivia*. However, a reduction in the antimicrobial activity was observed when the pH was adjusted to the same pH of the media. In mGTS, a statistically significant reduction in the antimicrobial activity was observed due to pH adjustment, while a minor reduction in antimicrobial activity when treating the CFS from 3-species with heat or proteinase K (Fig. 5B) was also detected. It is important to note that in the control pH experiment, where the pH of the media was adjusted to the same pH of the CFS, no growth was observed.

3.5. Biofilm growth characterization

We then characterized single- and triple-species biofilms on both media. Interestingly, while all the species were able to form biofilms and increase their concentrations in single-species biofilms, in NYCIII media, the concentration of *P. bivia* integrated in the triple-species biofilm was

less than the initial inoculum concentration (Fig. 6A).

Similar to what occurred with the total cultures (Fig. 3), *G. vaginalis* was the only species that was able to increase its concentration in both single-species and triple-species biofilms formed in mGTS (Fig. 6B).

3.6. Biofilm characterization by PNA-FISH

Analysis of biofilms by PNA-FISH revealed that the structure of the biofilm and spatial organization of each species seemed to be influenced by the growth medium (Fig. 7). In NYCIII (Fig. 7A), a thicker biofilm with a homogeneous distribution of species was observed. In contrast, in mGTS (Fig. 7B) the biofilm was mainly composed of clusters of species that were not homogeneously distributed on the surface of the material. Less content of *F. vaginalis* was also observed on the mGTS biofilms, which supports the previous results that showed an inability of this species to grow in this medium.

4. Discussion

The presence of a polymicrobial biofilm on the surface of the vaginal epithelium in women with BV was originally demonstrated in 2005 [43], and is considered a hallmark characteristic of BV [13,44]. However, *in vitro* and *ex vivo* studies of biofilms that mimic BV are scarce and, as such, little is known regarding bacterial interactions between BVAB, and the consequences of these interactions regarding disease progression and antibiotic therapy failure [45]. Furthermore, many studies address BV biofilm formation by *Gardnerella* species only [46,47] however, the presence of other BVAB on the biofilm and the interactions between these species are often not taken into consideration. Nevertheless, it has been shown that some, but not all, BVAB can enhance a

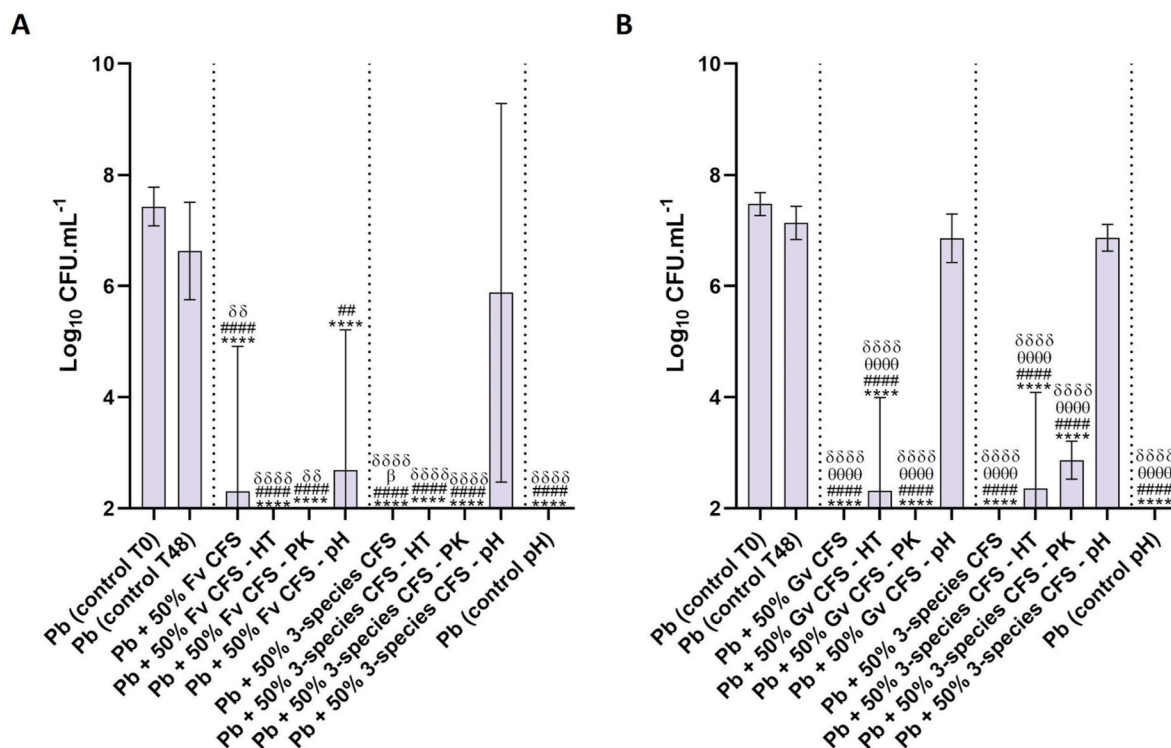


Fig. 5. Effect of heat, proteinase K, and pH adjustment on the antimicrobial activity of cell-free supernatant (CFS) on *P. bivia* growth in NYCIII (A) and mGTS (B), quantified by colony forming units (CFUs). The results are expressed as the average of Log_{10} CFU/mL, with error bars representing the standard deviation. CFUs quantification experiments have a limit of detection of $\text{Log}_{10} = 2$. Pb: *P. bivia*, Gv: *G. vaginalis*, Fv: *F. vaginalis*, HT: heat treatment, PK: proteinase K treatment, pH: pH adjustment. Significant differences when comparing to T0 are represented by * (**** $p < 0.0001$), and when comparing to T48 are represented by # (## $p < 0.01$; ### $p < 0.0001$). Significant differences when comparing to the condition Pb + 50% Fv CFS are represented by β ($\beta p < 0.05$), when comparing to the condition Pb + 50% 3-species CFS - pH are represented by δ (δδ $p < 0.01$; δδδδ $p < 0.0001$), and when comparing to the condition Pb + 50% Gv CFS - pH are represented by θ (θθθθ $p < 0.0001$).

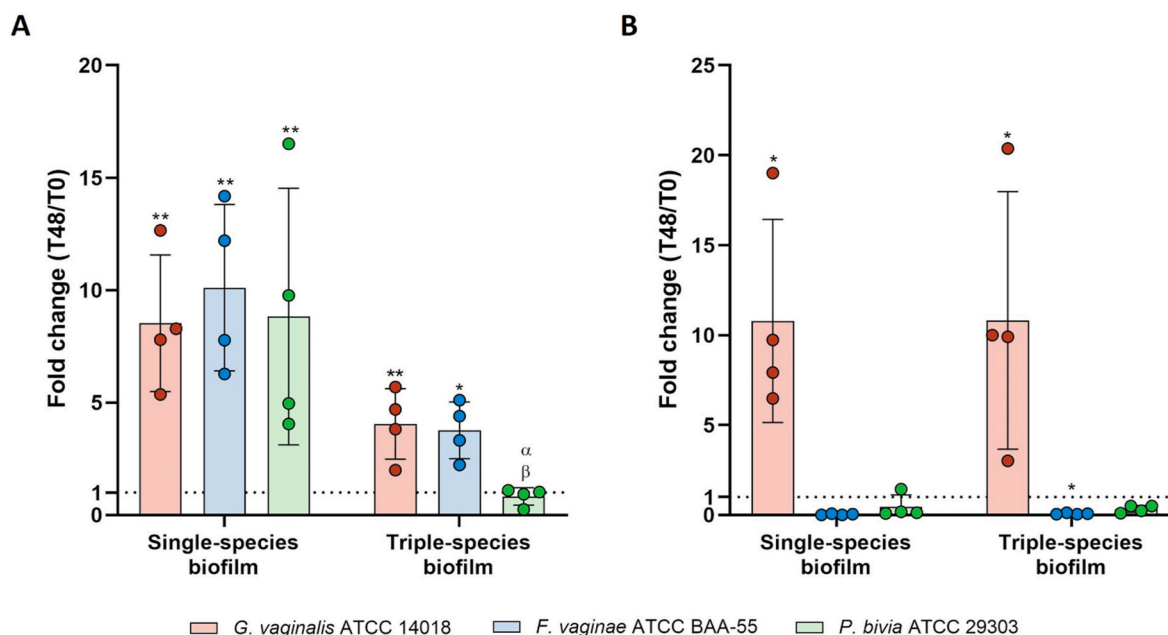


Fig. 6. Biofilm growth quantification by qPCR in NYCIII (A) and mGTS (B). The results are expressed as the average of fold change (T48/T0) of the normalized expression to *E. coli* exogenous control, with error bars representing the standard deviation. Statistical analysis was performed using the Student T-test and One-way ANOVA. Significant differences when comparing to T0 are represented by * (* $p < 0.05$; ** $p < 0.01$). Significant differences when comparing to *G. vaginalis* are represented by α ($\alpha p < 0.05$), and significant differences when comparing to *F. vaginae* are represented by β ($\beta p < 0.05$).

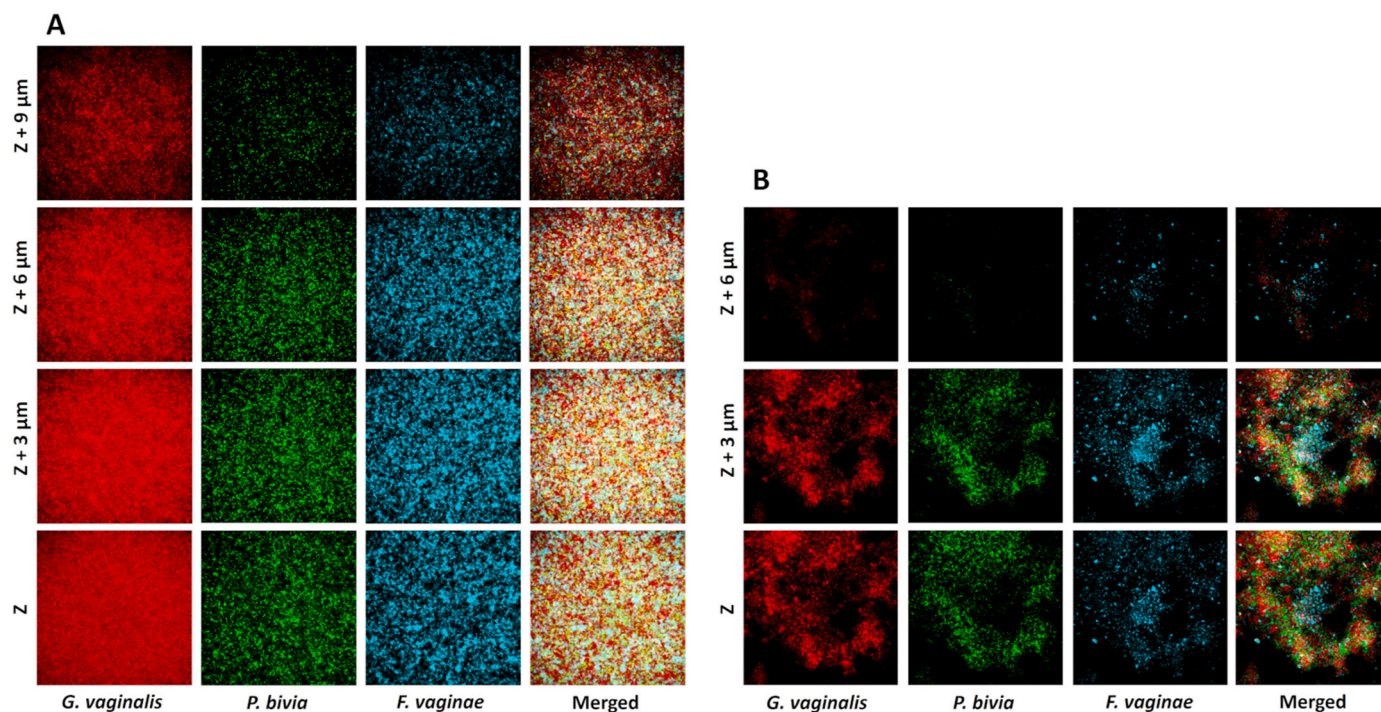


Fig. 7. Analysis of triple-species biofilms by PNA-FISH using confocal laser scanning microscopy. The biofilms were analyzed using PNA probes specific for *G. vaginalis* (Gard162 [red]), *F. vaginae* (FvagPNA651 [cyan]), and *P. bivia* (PbivPNA1454 [green]). Panel A shows biofilms in NYCIII, and panel B in mGTS. The images were acquired with a magnification of 40 $\times$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Gardnerella spp. initiated biofilm [23], and as such, more experiments focusing on polymicrobial interactions in BV-associated biofilms are warranted.

In vitro experiments with BVAB commonly use rich growth media, supplemented BHI broth [20] or NYCIII [48] that contain high amounts

of nutrients to support the fastidious mode of growth of these bacteria. These growth media fail, however, to represent the micro-environment present in the vaginal tract. Very limited studies address the use of a medium simulating vaginal secretions with these species [49,50]. The first study to use an artificial vaginal fluid was published in 1982 by Dorr

and colleagues [51]. Later in 1992, Geshnizgani and Onderdonk developed a chemically defined medium similar to genital tract secretions to study the growth of vaginal microorganisms [52]. Although a very simplified version of the mGTS, it contained glycogen and glucose, albumin, urea, mucin, Na^+ , K^+ , and Cl^- , all known to be present in the vaginal tract, as well as, several other components required for the growth of different vaginal isolates (including *Lactobacillus* spp. and many BVAB). Later, Liu et al. developed a modified genital tract secretions medium based on the previous work from Geshnizgani and Onderdonk [31], that we adapted for the purposes of this study. Herein, we aimed to observe the growth behavior of three key BVAB when growing in a rich medium commonly used in routine laboratory experiments versus a medium that simulates *in vivo* genital tract conditions.

The quantification of growth by OD measurements at different time points allowed us to investigate the growth kinetics. In NYCIII medium the three species showed an increase in OD values, while in mGTS only *P. bivia* had a noticeable increase in the OD values. Furthermore, we observed that the majority of the growth happened during the first 24 h of culture. While it has been described that mGTS was suitable for growing *F. vaginae* [26], we were not able to replicate those results. However, we noticed minor changes in the mGTS composition, not only on the brand of complex nutrients, but also with the utilization of chemicals from different manufacturers, including glucose, vitamin K1, MnCl_2 , and folic acid, that can explain the differences observed. Another possibility of the variation could be the utilization of different isolates, and the fact that the methods used in the two studies are different. With this in mind, we tested different isolates, but no significant differences were found between the different isolates used herein. It has previously been shown that the growth of different bacterial species is highly influenced by minor alterations in the growth media [53–55].

Despite observing a significant increase in the cultures' OD, in NYCIII, we did not observe an increase in CFUs. CFUs experiments have some disadvantages to fully quantify the number of anaerobic microorganisms since exposure to oxygen decreases the viability of the cells [56]. Additionally, CFUs do not quantify viable but nonculturable cells [57]. These disadvantages can be possible explanations for the fact that there was no increase in CFUs after 48 h. On the other hand, qPCR results showed an increase in the concentration after 48 h for all species when growing alone in NYCIII medium, more in line with the increase in the OD. Of note, on triple-species consortia, *P. bivia* did not increase its concentration. Results on mGTS revealed that *F. vaginae* was not able to grow in single-species suspensions and only *G. vaginalis* shows a good capacity to increase concentration on both single and triple-species growth. It seems that the growth of *P. bivia* in triple-species consortia was inhibited by the presence of the other two species on both NYCIII and mGTS, despite older studies reporting the symbiotic relationship between *Gardnerella* spp. and *P. bivia* [30]. The evaluation of CFS interaction on *P. bivia* growth was essential to understand the influence of *G. vaginalis* and *F. vaginae* on the growth of this species. While in NYCIII we observed an influence of *F. vaginae* CFS on *P. bivia* growth, in mGTS the highest inhibition was observed with CFS from *G. vaginalis*. Under our tested conditions, *F. vaginae* strain ATCC BAA-55 had a strong antagonistic effect on *P. bivia* strain ATCC 29303 when growing in a rich medium. On the other hand, the fact that *F. vaginae* did not grow in mGTS medium can explain why this species CFS does not have influence on *P. bivia* growth in mGTS. When evaluating the antimicrobial activity of the CFS on *P. bivia* culturability after treatment with heat, proteinase K, or by pH adjustment, we verified that the highest effect was observed when the pH of the CFS was adjusted to the same pH of the growth media either in NYCIII or mGTS. It is important to note that we did not detect any CFUs in the control where the pH of the media was adjusted to the same pH of the CFS, which can indicate that the major effect on *P. bivia* growth was caused by the acidification of the media, likely the result of the production of organic acids by the other two bacterial species.

In previous studies, BV-associated biofilms formed by *G. vaginalis*, *F. vaginae*, and *P. bivia* growing in rich media were characterized by

different techniques, such as PNA-FISH counts, however, none of these studies evaluated the composition of these biofilms in mGTS or the evolution of the species' concentration after incubation for a certain amount of time, showing only the final percentage of each species on the biofilm [35,36]. Nevertheless, here in this work, we quantified each species in the suspension at T0 (the suspension incubated on the well-plates for consequent biofilm formation), as well as at the final 48 h biofilm, which gives us an idea of the evolution of the concentration of each species in the biofilm. Although qPCR does not differentiate between viable and non-viable cells [58,59], considering that biofilms consist of both viable and non-viable cells [60], qPCR does provide an accurate assessment of biofilm growth. The concentration of *P. bivia* incorporated in the triple-species biofilms was less than the concentration of the initial inoculum in both NYCIII and mGTS. The same situation happened in the case of *F. vaginae* in mGTS. Still, this limitation of *P. bivia* and *F. vaginae* did not prevent both species to join the formed biofilm, mainly composed of *G. vaginalis*. This manuscript further highlights the versatility of *G. vaginalis*, being the best species with the capacity to grow and form biofilm in a rich medium or in a medium simulating vaginal tract secretions. As stated by several authors, *G. vaginalis* may have a preponderant role on the initiation of the biofilm in cases of incident BV, serving as a scaffold where other BVAB later attach, promoting the development of the infection [10,61,62]. In this study, the biofilm analysis by CLSM revealed that the structure of the biofilm and the species organization within the biofilm is dependent on the medium where the biofilm was formed but the three species were present on both biofilms.

In conclusion, the presence of some antagonistic effects between these three key BVAB, specifically the inhibition of *P. bivia* growth by *F. vaginae* in NYCIII and by *G. vaginalis* in mGTS, can contribute to a better understanding of the interactions between these species on the development of BV-associated biofilms. An ongoing RNA-sequencing study of these triple-species biofilms will further help to clarify the observed interactions.

CRediT authorship contribution statement

Lúcia G.V. Sousa: Writing – original draft, Investigation, Formal analysis. **Christina A. Muzny:** Writing – review & editing, Conceptualization. **Nuno Cerca:** Writing – original draft, Conceptualization.

Funding

This work is funded by the National Institute of Allergy and Infectious Diseases [R01AI146065-01A1 to CAM]. It was also partially funded by the Portuguese Foundation for Science and Technology (FCT), under the scope of the strategic funding of unit [UIDB/04469/2020].

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christina A. Muzny reports financial support was provided by National Institutes of Health. Nuno Cerca reports financial support was provided by the Portuguese Foundation for Science and Technology. Christina A. Muzny reports a relationship with Lupin, Gilead, Visby & Abbott that includes: funding grants. Christina A. Muzny reports a relationship with Scynexis, Cepheid, BioNTech, BioMed Diagnostics, Visby Medical, Merck Manuals, Elsevier, UpToDate, Abbott Molecular & Roche that includes: consulting or advisory. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

LS acknowledges FCT the financial support of individual Grant [2020.04912. BD].

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biofilm.2024.100247>.

Data availability

Data will be made available on request.

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