Anaerobe 36 (2015) 56–59

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

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

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ARTICLE INFO

Article history:
Received 5 October 2015
Received in revised form
19 October 2015
Accepted 21 October 2015
Available online 24 October 2015

Keywords:
Polymicrobial biofilms
Bacterial vaginosis
Gardnerella vaginalis virulence

ABSTRACT

Dual-species biofilm formation between *Gardnerella vaginalis* strains isolated from women with or without bacterial vaginosis (BV) and other 24 BV-associated microorganisms support that the key difference in virulence potential between BV-negative and BV-positive *G. vaginalis* strains seems not to be related with biofilm maturation.

Bacterial vaginosis (BV) is often characterized by a shift of the vaginal microbiota from a *Lactobacillus*-dominated community to a dense biofilm containing a complex mixture of microorganisms [1]. *Gardnerella vaginalis* is the dominant pathogen colonising BV women, often adopting the biofilm mode of growth as a survival strategy [2]. During BV, there is a complex interplay between pathogenic species, endogenous vaginal microbiota and the vaginal epithelium [1,3,4]. These interactions become more complex when microbes are adhered to the epithelium, forming biofilms, and communicate via "quorum-sensing", a cell-density dependent bacterial intercellular signalling mechanism [5]. However, *G. vaginalis* can also be a part of the vaginal microbiota in healthy women [6]. This raised the question whether there are pathogenic and commensal lineages within this species. Jayaprakash and colleagues provided genomic evidence that all *G. vaginalis* strains had the potential to form biofilm but not all strains had the potential to cause BV symptoms, namely due to absence of sialidase gene [7]. We recently also provided *in vitro* evidence that supports Jayaprakash hypothesis [8]. However, only the BV isolates demonstrated higher cytotoxicity and were able to adhere in high density clusters to a HeLa cell line [9], a condition necessary to foster *in vivo* biofilm development [5]. Another important insight providing evidence that not all *G. vaginalis* have the same virulence potential was derived from recent *in vivo* observations by Swidsinski and colleagues. They demonstrated the presence of adherent bacterial biofilms in 90% of biopsies from women with BV, while only 10% of healthy women exhibited a similar biofilms [10]. Subsequently, they proposed that the mere presence of loosely adherent *G. vaginalis* on the vaginal epithelium was of lesser clinical significance than the presence of high density clusters of *G. vaginalis* [2]. In effort to better understand the differences between virulent and non-virulent *G. vaginalis* strains, the aim of the present study was to analyze the interactions between non-BV ($n = 3$) or BV ($n = 3$) *G. vaginalis* isolates and other BV-associated pathogens ($n = 24$) using a dual-species biofilm assembly, consisting in the combination of *G. vaginalis* and secondary BV-associated species. All species used are listed in Table 1.

The dual-species biofilm formation model used was the same as described by Machado and colleagues [11], with some minor modifications. Briefly, *G. vaginalis* cultures were adjusted to 1×10^7 colony-forming units (cfu)/mL by optical density (OD) at 600 nm (Model Sunrise, Tecan). After homogenization, 100 μL of each bacterial suspension of *G. vaginalis* isolates was dispensed into each well of 96-well flat-bottom tissue culture plate (Orange Scientific). The tissue cultured plates were then placed in an incubator

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http://dx.doi.org/10.1016/j.anaerobe.2015.10.008
1075-9964/

Table 1
GenBank accession numbers of strains used in this study.

Bacteria[a,b]	Genes	Accession numbers[c]
Actinomyces neuii UM067**An**	*16S rRNA*	KT805271
Actinomyces turicensis UM066**At**	*16S rRNA*	KT805270
Aerococcus christensenii UM137**Ac**	*16S rRNA*	KT805273
Bacillus firmus UM034**Bf**	*16S rRNA*	KT805263
Brevibacterium ravenspurgense UM066**Br**	*16S rRNA*	KT805269
Corynebacterium amycolatum UM065**Ca**	*16S rRNA/rpoB*	KT805275/KT923481
Corynebacterium tuberculostearicum UM137**Ct2**	*16S rRNA/rpoB*	KT805279/KT923486
Corynebacterium tuscaniense UM137**Ct**	*16S rRNA/rpoB*	KT805278/KT923485
Enterococcus faecalis UM035	*16S rRNA*	KT614045
Escherichia coli UM056	*16S rRNA*	KT614048
Gardnerella vaginalis UM085	*16S rRNA*	KP996679
Gardnerella vaginalis UM121	*16S rRNA*	KP996681
Gardnerella vaginalis UM131	*16S rRNA*	KP996676
Gardnerella vaginalis UM137	*16S rRNA*	KP996682
Gardnerella vaginalis UM241	*16S rRNA*	KP996683
Gardnerella vaginalis UM246	*16S rRNA*	KP996677
Gemella haemolysans UM034**Gh**	*16S rRNA*	KT805264
Lactobacillus vaginalis UM062**Lv**	*16S rRNA*	KT805268
Mobiluncus mulieris ATCC 35239	whole genome	NZ_GL405260.1
Nosocomiicoccus ampullae UM121**Na**	*16S rRNA*	KT805272
Prevotella bivia ATCC 29303	*16S rRNA*	L16475.1
Propionibacterium acnes UM034**Pa**	*16S rRNA*	KT805265
Streptococcus agalactiae UM035**Sa**	*16S rRNA*	KT805266
Staphylococcus epidermidis UM066**Se**	*16S rRNA/rpoB*	KT805277/KT923483
Staphylococcus haemolyticus UM066**Sh**	*16S rRNA/rpoB*	KT805276/KT923482
Staphylococcus hominis UM224**Sh**	*rpoB*	KT923487
Staphylococcus saprophyticus UM121**Ss**	*rpoB*	KT923484
Staphylococcus simulans UM059**Ss**	*16S rRNA*	KT805267
Staphylococcus warnerii UM224**Sw**	*rpoB*	KT923488
Streptococcus anginosus UM241**b**	*16S rRNA*	KT805274

[a] Due to NCBI sequence deposition regulations, the designation of the strains previously used in Alves et al. [8], were updated (highlighted in bold).
[b] Strains were grown in supplemented brain heart infusion (sBHI) and incubated at 37 °C in 10% CO_2 for 24 h, as described by Alves et al. [8]. The exceptions were *M. mulieris* and *P. bivia* that were grown in sBHI and incubated at 37 °C, under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 48 h.
[c] The accession numbers of partial *16S ribosomal RNA* or *rpoB* gene sequence of vaginal isolates are downloadable from NCBI.

at 37 °C in 10% CO_2. Following 24 h, the culture medium covering the biofilm was carefully removed and replaced by fresh medium. A second inoculation with 1×10^7 cfu/mL of each BV-associated strain was performed and biofilms were allowed to growth for another 24 h. Quantification of biofilm was performed by the crystal violet staining, as previously described [12]. All assays were repeated at least 3 times with 8 technical replicates. The data were analyzed using the non-parametric Kruskal–Wallis test, since the data did not follow a normal distribution according Kolmogorov-Smirvon's test, with the statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL). *P*-values of less than 0.05 were considered significant.

As described in Fig. 1, our results revealed that 54% ($n = 13$) of the BV-associated species tested had a synergistic effect in most of *G. vaginalis* strains. However, only 6 species caused an increase in biofilm formation in all tested conditions: *Actinomyces neuii, Brevibacterium ravenspurgense, Corynebacterium amycolatum, Corynebacterium tuscaniense, Staphylococcus hominis* and *Staphylococcus saprohyticus*. Conversely, we observed that 42% of the tested species showed variable interactions dependent of the specific *G. vaginalis* strain used. However, no link ($P = 0.131$; Kruskal–Wallis) was found between non-BV and BV *G. vaginalis* strains, with the exception of *Mobiluncus mulieris*, which showed an antagonistic effect when added to the biofilm formed by BV strains, whereas a synergistic interaction was verified in presence biofilms formed by non-BV *G. vaginalis* isolates. Finally, our data also revealed an antagonistic interaction between all *G. vaginalis* strains tested and *Lactobacillus vaginalis*.

The most recent model for the pathogenesis of BV suggests that *G. vaginalis* adhered to vaginal epithelium might be acting as a scaffold for the attachment of a subsequent species [1,13]. However, the role of BV-associated bacteria in multi-species biofilms is still poorly understood. An early study by Machado and colleagues demonstrated that a few secondary BV-associated anaerobes, such as *Prevotella bivia*, were able to increment the concentration of cells within the biofilm, when added to a pre-formed *G. vaginalis* biofilms [11].

Herein, we were interested to determine if similar synergistic interactions occurred when using BV or non-BV *G. vaginalis* isolates. Surprisingly, with the exception of one species (*M. mulieris*), no differences were found between BV and non-BV associated *G. vaginalis* mediated dual-species biofilm augmentation. These results suggests that the key difference in BV or non-BV *G. vaginalis* virulence potential seems not to be related with biofilm maturation, at least in a dual-species model. We propose that once specific strains of *G. vaginalis* are able to outcompete the resident *Lactobacillus* species and start to growth in clusters, secondary anaerobes will easily incorporate the biofilm. This might be the key difference in virulence potential of *G. vaginalis* [9].

A particular example of synergistic interaction in dual-species biofilms is the case of *G. vaginalis* and *P. bivia*. It has been previously shown that *G. vaginalis* produces amino acids through its metabolism and *P. bivia*, a strict anaerobe, uses amino acids as its fuel source and as a result produces ammonia, which in turn is used by *G. vaginalis* [14]. Nevertheless, our data also showed that *L. vaginalis* had an antagonistic effect in the presence of all tested *G. vaginalis* biofilms. Boskey and colleagues have showed that the

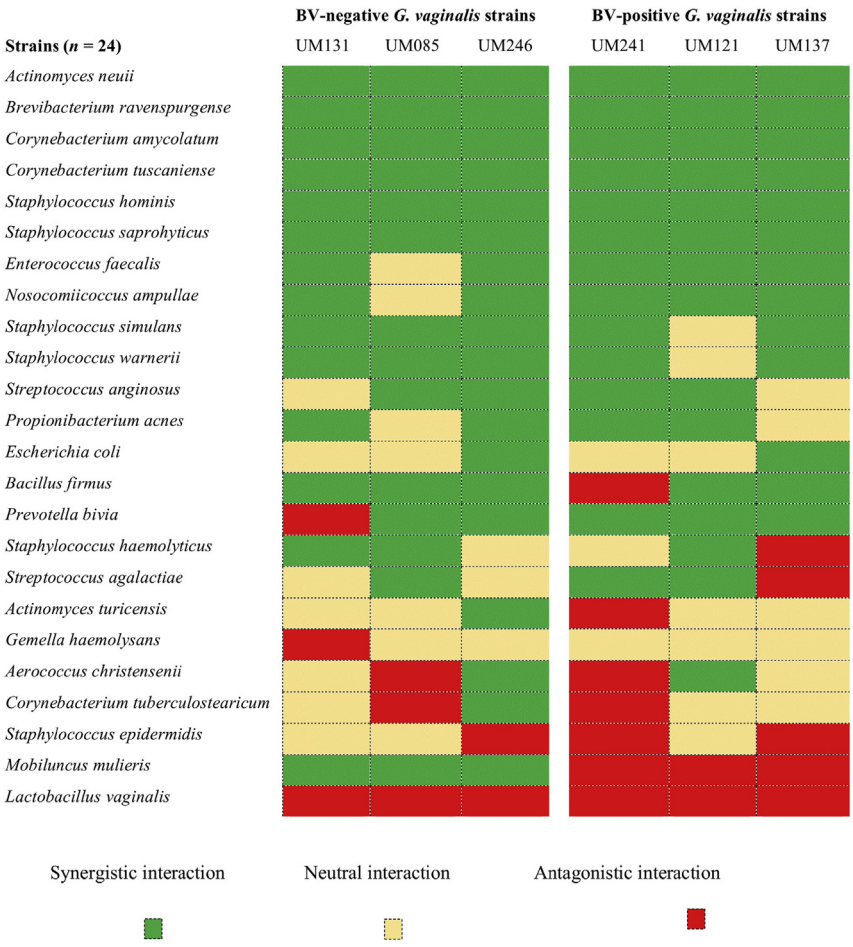

Fig. 1. Synergistic, antagonistic or neutral interactions detected in dual-species biofilms in relation to a single biofilms of non-BV or BV *G. vaginalis* isolates. The data are presented as fold change relative to the single *G. vaginalis* biofilm (fold change = 1, control). Interactions were classified as antagonistic (cut-off < 0.75-fold changes), neutral (0.75 ≤ fold changes < 1.25) and synergistic (cut-off ≥ 1.25 − fold changes). Results represents at least 3 independent experiments performed with 8 technical replicates. No significant differences between non-BV and BV *G. vaginalis* strains were found in a dual-species biofilm formation ($P = 0.131$; Kruskal–Wallis), with exception to *M. mulieris* ($P = 0.05$; Kruskal–Wallis).

growth limiting factor for *L. vaginalis* was a depletion of a metabolite or the buildup of an unspecified toxic waste product [15], that might also be toxic to *G. vaginalis* causing a disruption of the biofilm. Curiously, our findings revealed that *M. mulieris* was the only bacterial specie with opposing interactions in the presence of either non-BV or BV pre-formed *G. vaginalis* biofilms. Nevertheless, further work is required to explore the bacterial interactions between these bacterial species.

The results from our study should be interpreted in light of several limitations. First, initial adhesion by *G. vaginalis* was performed in polystyrene microtiter plate wells rather than vaginal epithelium, where the presence of host-derived factors (e.g. mucus production, specific receptors on the epithelial surface) can influence bacterial adherence and biofilm formation. This technical limitation is not easy to overcome since, as we shown before, *G. vaginalis* quickly induces cytotoxic changes and detachment of pre-adhered epithelial cultures [9]. Furthermore, the growth medium did not contain all of the factors found *in vivo*, and some *in vivo* cues may turn on expression of biofilm-related genes. However, these limitations aside, *in vitro* models can be very informative and are key to furthering our understanding on multi-species biofilms and the development of BV.

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

Acknowledgements

The authors thank the FCT Strategic Project of UID/BIO/04469/2013 unit and the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. JC was funded by the FCT individual fellowship SFRH/BD/93963/2013. NC is an Investigador FCT.

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