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Escherichia coli and *Enterococcus faecalis* are able to incorporate and enhance a pre-formed *Gardnerella vaginalis* biofilm. Pathogens and Disease (2016), 10.1093/femspd/ftw007

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Abstract

Gardnerella vaginalis is the most frequent microorganism found in bacterial vaginosis (BV), while *Escherichia coli* and *Enterococcus faecalis* are amongst the most frequent pathogens found in urinary tract infections (UTIs). This study aimed to evaluate possible interactions between UTIs pathogens and *G. vaginalis* using an *in vitro* dual-species biofilm model. Our results showed that dual-species biofilms reached significantly higher bacterial concentration than mono-species biofilms. Moreover, visualization of dual-populations species in the biofilms, using the epifluorescence microscopy, revealed that all of the urogenital pathogens co-existed with *G. vaginalis*. In conclusion, our work demonstrates that uropathogens can incorporate into mature BV biofilms.

Main text

The human vaginal microbiota plays an important role in the maintenance of the woman's health. When this community is disrupted, bacterial vaginosis (BV) may occur (Schwebke *et al.*, 2014). It has been described that as BV progresses, an highly structured polymicrobial biofilm develops in the vaginal epithelium primarily consisting of *G. vaginalis* and a variety of other bacteria (Verstraelen & Swidsinski, 2013). BV can lead to complications in pregnancies, causing premature rupture of the membranes, premature birth, or the death of the fetus or newborn (Eschenbach *et al.*, 1988). Furthermore, urogenital diseases can also result from BV, especially urinary tract infections (UTIs) (Harmanli *et al.*, 2000, Sharami *et al.*, 2007). Association between BV and UTIs probably begins with an increase in the pH of the vagina because of the reduction of vaginal lactobacilli-that are able to produce lactate and hydrogen peroxide (Sumati & Saritha, 2009). Consequently, uropathogenic microorganisms can emerge from the gut (Whiteside *et al.*, 2015) and come into contact with the vaginal microenvironment but little is known about what happens thereafter. It has been proposed that uropathogens can either bypass the microflora or successfully enter the vaginal biofilms, survive, and continue their ascension into the bladder (Reid G & Habash, 1999). Although anatomical proximity allows easy transfer of bacteria from the vagina to urinary tract and vice versa (Pedraza-Aviles *et al.*, 2001, Sharami *et al.*, 2007), to date only a few investigators have been examined the role of the uropathogens in BV. Importantly, a recent study (Amatya *et al.*, 2013) reported that in a population of 135 BV-positive women, 75% also had UTIs. The leading uropathogen isolated from women with UTIs is *Escherichia coli* (Soto *et al.*, 2007), and this organism has also been known to form biofilms of the urogenital tract (Hancock *et al.*, 2010, McMillan *et al.*, 2011). Similarly, *Enterococcus faecalis* have also been associated with UTIs and is known to form biofilms (Hola *et al.*, 2010).

This study aimed to evaluate the interactions between a *G. vaginalis* isolate from a BV-positive patient (UM241, NCBI accession number: KP996683) (Castro *et al.*, 2015) and three *E. coli* (UM056, NCBI: KT614048; UM062, NCBI: KT614049; and UM066Ec, NCBI: KT614050) or three *E. faecalis* (UM035, NCBI: KT614045; UM066Ef, NCBI: KT614046; and UM067, NCBI: KT614047) isolates using a mono- or dual-species biofilm model described previously by Machado *et al.* (2013a). The bacteria used were isolated and identified as described previously (Alves *et al.* 2014). Briefly, dual-species biofilms where initiated by inoculating a 10^7 CFU/mL bacterial suspension of *G. vaginalis* [in brain heart infusion (BHI, Liofilchem, Roseto degli Abruzzi, Italy) supplemented with 2% (w/w) gelatin (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), 0.5% (w/w) yeast extract (Liofilchem) and 0.1% (w/w) starch (Panreac, Barcelona, Spain)] in 24-well tissue culture plate (Orange Scientific, Braine L'Alleud, Belgium) for 24 h, at 37ºC under 10% CO_2. After 24 h, the planktonic cells were removed and fresh media was added to each well. Then, 10^7 CFU/mL of each uropathogen isolate was inoculated with the pre-formed *G. vaginalis* biofilm, for another 24 h. One control was performed where no secondary pathogen was added. Other mono-species biofilms controls were performed by inoculating equivalent bacterial suspension of each uropathogen in 24 well-plate for 24 h. To quantify the total cells of a mono- and dual-species biofilm we used the method suggested by Freitas *et al.* (2014). In brief, the total cells were quantified using a Neubauer chamber coupled with Olympus BX51 epifluorescence microscope equipped with a CCD camera (DP72; Olympus). Cell suspensions were stained with 4'-6-Diamidino-2-phenylindole (DAPI, 2.5 µg/mL). DAPI staining was detected in a specific filter, BP 365-370, FT 400, LP 421 present in the microscope. Next, we discriminated the bacterial population of biofilm by using the Peptide Nucleic Acid (PNA) Fluorescence *in situ* hybridization method as previously described Machado *et al.* (2013b). Briefly, after fixing the biofilm, a PNA probe specific for *G. vaginalis* (Gard 162) was added to each well of epoxy coated microscope glass slides (Thermo Fisher Scientific, Lenexa, KS, USA). An additional staining step was done at the end of the hybridization procedure, covering each glass slide with DAPI. Microscopic visualization was performed using filters capable of detecting the PNA probe (BP 530-550, FT 570, LP 591 sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe) and DAPI (as described above). Finally, biomass quantification was performed by crystal violet (CV) method as described by Peeters *et al.* (2008). In brief, after the fixation step, biofilms were stained with 100 µL of crystal violet (CV) solution 0.5% (v/v) (Acros Organics, New Jersey, USA) for 20 min. Each well was washed two times with 200 µL of phosphate buffered saline, and then the CV was removed by adding 150 µL of glacial acetic acid (v/v) (Thermo Fisher Scientifc) to each well. The plates were placed in agitation for a few minutes and finally the optical density (OD) was measured at 590 nm. To determine the extent of the coaggregation between *G. vaginalis* and uropathogens we used an experimental model suggested by Reid *et al.* (1990). In brief, 500 µL of *G. vaginalis* (10^7 CFU/mL) was combined with 500 µL of each uropathogen (10^7 CFU/mL) in 24-well plates. Then, bacteria were incubated for 4 h, at 37ºC, in 10% CO_2. The aggregates were visualized using an inverted light microscope (Leica DMI 3000B) and the score was evaluated as following: 0, no aggregation; 1, small aggregates comprising small visible clusters of bacteria; 2, aggregates comprising larger numbers of bacteria, settling to the center of the well; 3, macroscopically visible clumps comprising larger groups of bacteria which settle to the center of the well; 4, maximum score allocated to describe a large, macroscopically visible clump in the center of the well. Auto-aggregation was assessed for each bacterial isolate. All experiments were performed in triplicate with technical replicates. The data were analyzed using the t-test, with the statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL). *P*-values of less than 0.05 were considered significant.

Our results showed that all tested uropathogens isolates had a significant ability to increase the total number of cells within a pre-formed *G. vaginalis* biofilm (Fig. 1A). Remarkably, we showed that these dual-species biofilms were composed majority by uropathogens (Fig. 1B), suggesting that the tested species can live in close proximity with each other, to some extent. This was first confirmed by the coaggregation experiments, being *E. faecalis* the species with the highest ability to interact with *G. vaginalis* by coaggregation (Fig. 1C). Direct

epifluorescence microscopy further confirmed this, with clusters of uropathogens in close contact with *G. vaginalis* (Fig. 1D).

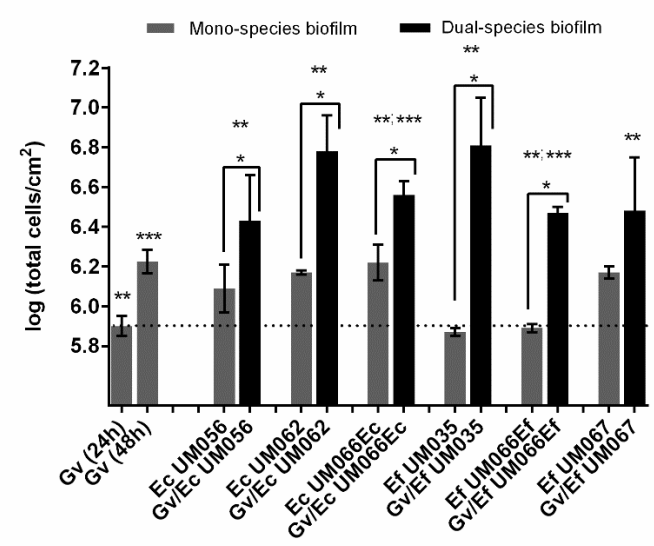

Fig. 1A | Biofilm formation profiles for each species on mono- and dual-species biofilms. Total cells counts by DAPI for mono- (controls) and dual-species biofilms. *P < 0.05 when compared the dual-species biofilms to each mono-species biofilm (uropathogen control). ** P < 0.05 when compared the 24h *G. vaginalis* biofilm (control) to dual-species biofilm. *** P < 0.05 when compared the 48h *G. vaginalis* biofilm (control) to dual-species biofilm.

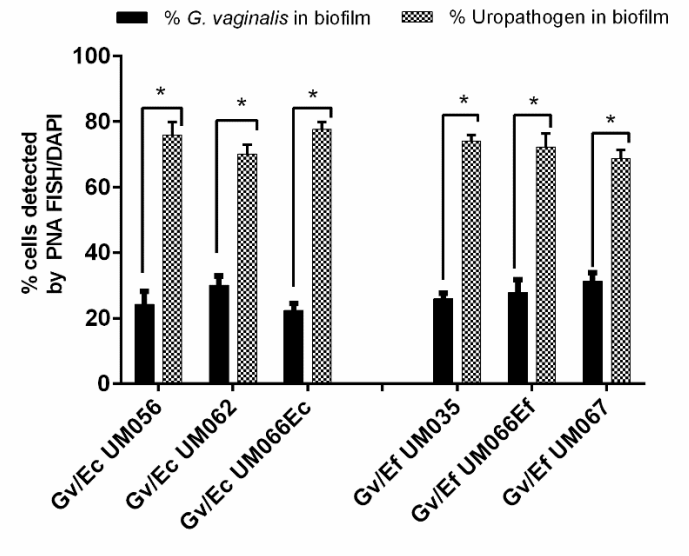

Fig. 1B | Biofilm formation profiles for each species on mono- and dual-species biofilms. Total percentage of cells detected by PNA FISH for 48h biofilms. *P < 0.05 when compared the dual-species biofilms to each mono-species biofilm (uropathogen control).

Interestingly, despite the increase in bacterial concentration, dual-species biofilm total biomass was not significantly augmented after added the uropathogens in a pre-formed *G. vaginalis* biofilm (when compared with mono-species biofilms of *G. vaginalis*), with exception to 2 *E. faecalis* isolates (UM035 and UM067). This is probably due to the relative lower biomass formation by both *E. coli* and *E. faecalis* mono-species biofilms, as compared to *G. vaginalis,* in our tested conditions (Fig. 1E). Similarly, Almeida *et al.* (2011) reported that the

total biomass profiles in dual-species biofilms tended to be more similar to mono-species biofilm.

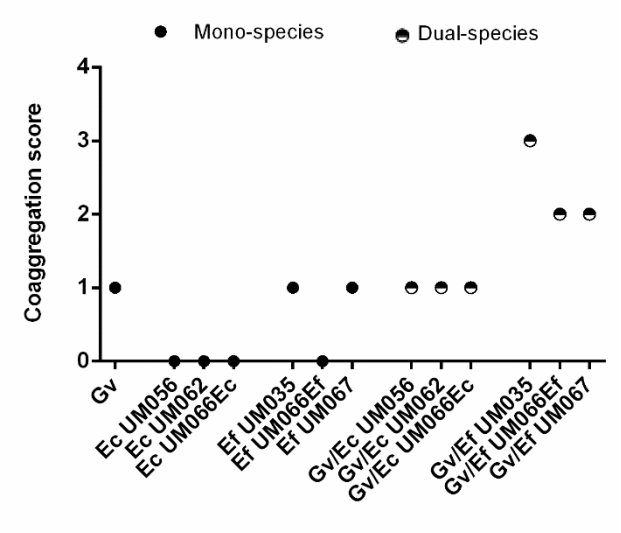

Fig. 1C| Coaggregation score of mono- and dual-bacterial species, the score was evaluated on a 0 to 4 scale.

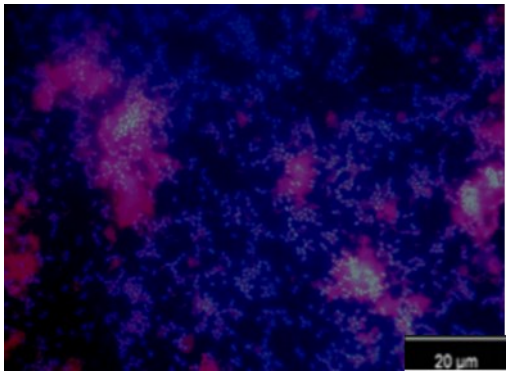

Fig. 1D | Epifluorescence image showing *E. faecalis* UM035 mixed with *G. vaginalis*, *E. faecalis* is shown blue and *G. vaginalis* in red.

Taken in consideration all the above data, we showed that both *E. coli* and *E. faecalis* had the ability to incorporate into a pre-formed *G. vaginalis* biofilm with increased cell concentrations. These findings may be explained due to virulence factors of uropathogens, namely in the coaggregation process. Importantly, Reid & Sobel (1987) showed that uropathogens have ability to adhere to each other (autoaggregation) and possibly to other microorganisms (coaggregation). *E. coli* produces antigen 43 that favours the interbacterial interactions (Danese *et al.*, 2000). Regarding *E. faecalis,* this uropathogen produce an aggregation substance, pheromone-inducible surface protein, encoded by *agg* gene that promotes aggregation during bacterial conjunction (Lopez-Salas *et al.*, 2013).

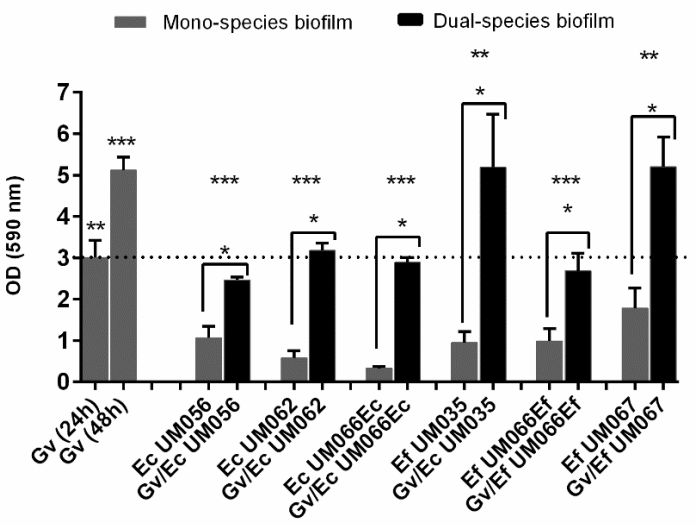

Fig. 1E | Biofilm thickness of mono- (controls) and dual-species biofilm determined by staining with crystal violet. *P < 0.05 when compared the dual-species biofilms to each mono-species biofilm (uropathogen control). ** P < 0.05 when compared the 24h *G. vaginalis* biofilm (control) to dual-species biofilm. *** P < 0.05 when compared the 48h *G. vaginalis* biofilm (control) to dual-species biofilm.

Although little is known about the underlying bacterial interactions between uropathogens and *G. vaginalis*, some studies reported that this association could lead an increase of the BV or UTIs- associated complications (Hooton *et al.*, 1989, Hillebrand *et al.*, 2002, Soto *et al.*, 2007, Lata *et al.*, 2010). In 1989, Hooton *et al.* reported that complications resulting from the use of diaphragms were higher in women with BV and UTIs. Later, some studies revealed that the incidence of poor pregnancy outcome was higher BV with UTIs (Hillebrand *et al.*, 2002, Lata *et al.*, 2010). Particularly, studies have been reported that premature rupture of the membranes can also be associated with UTIs (Soto *et al.*, 2007). On the other side, the incorporation of uropathogens in *G. vaginalis* mediated biofilms provides a protective niche for a further potential UTI infection. Interestingly, Swidsinski *et al.* (2014) showed that little clusters of *G. vaginalis* can also embed in masses of other uropathogens in a woman with UTI.

In conclusion, our novel observations revealed, for the first time, that uropathogens can associate in BV biofilm. Due to the complications associated with BV and UTIs in pregnant women, it would be important that antenatal patients should be screened for the presence of BV and UTIs. A future direction of this study will be examine gene expression in *G. vaginalis* and uropathogens, to identify biofilm genes, and examine metabolic, adhesion and coaggregation process that maintain the multi-species biofilms in order to find an effective treatment in co-occurrence of BV and UTIs.

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Conflict of Interest

The authors have declared that no competing interest exists.

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