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Optimization of culture conditions for *Gardnerella vaginalis* biofilm formation

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ABSTRACT

Bacterial vaginosis is the leading vaginal disorder in women in reproductive age. Although bacterial vaginosis is related with presence of a biofilm composed predominantly by *Gardnerella vaginalis*, there has not been a detailed information addressing the environmental conditions that influence the biofilm formation of this bacterial species. Here, we evaluated the influence of some common culture conditions on *G. vaginalis* biofilm formation, namely inoculum concentration, incubation period, feeding conditions and culture medium composition. Our results showed that culture conditions strongly influenced *G. vaginalis* biofilm formation and that biofilm formation was enhanced when starting the culture with a higher inoculum, using a fed-batch system and supplementing the growth medium with maltose.

1. Introduction

Bacterial vaginosis (BV) is the most common gynaecological condition in women of reproductive age and it has been associated with serious health problems including preterm birth, spontaneous abortion, pelvic inflammatory disease, postoperative gynaecologic infections and increased risk of acquisition and transmission of several sexual transmitted agents (Schwebke, 2009). This disorder is characterized by a complex imbalance of vaginal microflora which includes a loss of lactobacilli, principally hydrogen peroxide and lactic acid producing strains, and a concurrent massive overgrowth of *Gardnerella vaginalis* and other anaerobic bacteria (Verhelst et al., 2004).

Despite of its high prevalence and impact on woman health, BV aetiology remains a matter of debate (Josey and Schwebke, 2008). Importantly, it has been described that BV involves the presence of an adherent biofilm on the vaginal epithelium, being *G. vaginalis* the predominant bacterial species (Swidsinski et al., 2005). This bacterial biofilm persists after therapy with metronidazole, suggesting that *G. vaginalis* biofilm plays a key role in BV recurrence (Swidsinski et al., 2008). However, not all *G. vaginalis* causes BV and it has been recently proposed that only isolates able to form cohesive biofilms could induce BV (Swidsinski et al., 2010). Therefore, assessing the biofilm formation ability of clinical isolates of *G. vaginalis* can highlight their virulence potential. Nevertheless, very little information exists regarding *in vitro* biofilm quantification by *G. vaginalis* (Alves et al., 2014; Harwich et al., 2010; Patterson et al., 2010). It is well known that several factors can influence biofilm formation, namely growth medium composition (Kennedy and O'Gara, 2004), feeding conditions (Cerca et al., 2004), inoculum concentration (Cotter et al., 2009), incubation period (Abdallah et al., 2014), temperature (Uhlich et al., 2014), atmosphere conditions (Reuter et al., 2010), surface properties (Cerca et al., 2005) and hydrodynamics (Kim et al., 2013). Thus, our aim was to assess how *G. vaginalis* biofilms were influenced by the most common used variables in *in vitro* biofilm quantification studies, namely the bacterial inoculum concentration, incubation period, feeding conditions and culture medium composition.

2. Material and methods

2.1. Strains and growth conditions

Four strains of *G. vaginalis* recently isolated from women with BV were used (Castro et al., 2015). These strains were kept frozen in Brain Heart Infusion (BHI; Liofilchem, Roseto degli Abruzzi, Italy) with 23% (v/v) glycerol (Panreac, Castellar del Vallès, Barcelona, Spain) at −80 °C. After thawing, strains were subcultured on columbia blood agar (Liofilchem) supplemented with 5% (v/v) defibrinated horse blood (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubated anaerobically at 37 °C for 48–72 h.

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2.2. Biofilm formation

For the biofilm formation assay, pre-inoculums were prepared through inoculation of grown cultures of *G. vaginalis* in sBHI [BHI supplemented by 2% (wt/v) gelatin (Liofilchem), 1% (wt/v) yeast extract (Liofilchem), 0.1% (wt/v) soluble starch (Panreac)]. These pre-inoculums were incubated at 37 °C during 24 h with 10% CO_2 (Shel Lab, Cornelius, Oregon, USA). After incubation, bacterial density was adjusted to 10^8 or 10^6 CFU/mL in the sBHI, whenever appropriated. Then, 100 μL of each suspension was transferred to each well of a 96-well microplate (Orange Scientific, Braine-l'Alleud, Belgium) and the plates were incubated at 37 °C with 10% CO_2 during 12 h, 24 h or 48 h, whenever appropriated. Also, a negative control containing only a sterile medium was included. In order to evaluate the effect of fed-batch growth on 48 h biofilms, the culture medium was replaced by a fresh medium after 24 h of growth. To assess the influence of culture medium composition on *G. vaginalis* biofilm formation the sBHI medium was supplemented with 0.25% (wt/v) of each carbohydrate: glucose (Panreac AppliChem, Darmstadt, Germany), dextrin (Fluka Biochemika, Bucks, Switzerland), maltose (Fisher Bioreagents, Fair Lawn, New Jersey, USA) and ribose (Sigma-Aldrich, St. Louis, Missouri, USA). All assays were repeated at least three times with eight technical replicates.

2.3. Biofilm quantification

Biofilm biomass was quantified using the crystal violet (CV) staining method previously described by Peeters et al. (2008) with some minor modifications. Briefly, after biofilm formation, the spent medium was removed and the pre-formed biofilms were washed with 200 μL of phosphate buffered saline [PBS composed by 16 g/L of sodium chloride (NaCl; Liofilchem); 0.4 g/L of potassium chloride (KCl, José M. Vaz Pereira S.A., Benavente, Portugal); 1.62 g/L of disodium phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$; José M. Vaz Pereira S.A.) and 0.4 g/L of potassium dihydrogen phosphate (KH_2PO_4, José M. Vaz Pereira S.A.] per well of a 96-well microplate. Afterwards, biofilms were fixed with 100 μL of 99% (v/v) methanol (Valente e Ribeiro Lda, Belas, Portugal) per well. After 15 min, supernatants were removed and the microplates were air-dried. Then, biofilms were stained with 100 μL of 0.5% (wt/v) of CV (Acros Organics, Morris Plains, New Jersey, USA) during 20 min. Afterwards, the plates were washed twice with 200 μL of PBS to remove the excess CV. Finally, CV was solubilized by adding 150 μL of 33% (v/v) acetic acid (Fisher Scientific, Loughborough, Leicestershire, United Kingdom) per well and the microplates were gently mixed. The optical density (OD) at 590 nm was measured, using the 96-well microplate reader (Bio-Tek Synergy HT, Winooski, Vermont, USA).

2.4. Statistical analysis

Data were analysed using Wilcoxon signed rank test with statistical package for the social science 17.0 software (SPSS; Chicago, Illinois, USA) since the data did not follow a normal distribution according Kolmogorov–Smirnov's test. Statistical differences were considered significant at P values < 0.05.

3. Results and discussion

Biofilm formation enables single-cell microorganisms to assume a temporary multicellular lifestyle, in which collective behaviour facilitates microbial survival and persistency in unfavourable conditions (Donlan and Costerton, 2002). Moreover, biofilm-forming ability has been related with pathogenesis of several human infections, being one of its hallmarks the increased resistance to antimicrobials (Ciofu et al., 2015; Deva et al., 2013). Particularly, the biofilm formation by *G. vaginalis* constitutes an important virulence factor of this microorganism (Patterson et al., 2010, 2007) and it has been associated with BV occurrence (Swidsinski et al., 2010). While biofilm formation has been well studied in many bacterial pathogens (Abdallah et al., 2015; Cerca et al., 2005; Crémet et al., 2013), there has not been detailed information regarding biofilm formation by *G. vaginalis*. In this sense, we designed a series of *in vitro* assays in order to investigate the influence of some culture conditions on *G. vaginalis* biofilm formation.

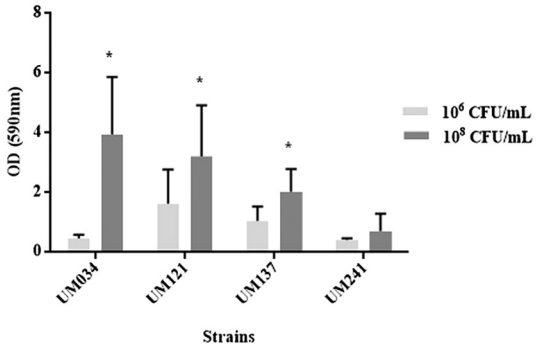

Fig. 1. Influence of inoculum concentration on *G. vaginalis* biofilm formation. Biofilms were grown in sBHI at 37 °C with 10% CO_2 during 24 h. Statistical differences in the biofilm formation using an inoculum concentration of the 10^6 CFU/mL or 10^8 CFU/mL are marked with * ($P < 0.05$).

3.1. Influence of inoculum concentration and incubation time on *G. vaginalis* biofilm formation

It has been demonstrated that inoculum concentration can considerably influence the amount of biofilm produced (Cotter et al., 2009). Commonly, an inoculum concentration of 10^6 CFU/mL (Baldoni et al., 2010; Wu et al., 2014) or 10^8 CFU/mL (Kostaki et al., 2012; Peeters et al., 2008) has been used in biofilm assays. Therefore, we started to assess the influence of inoculum concentration on biofilm formation, testing these two inoculum concentrations. As shown in Fig. 1, the majority of strains tested yielded a significant higher biofilm when we used an inoculum concentration of 10^8 CFU/mL. These results were not surprising and can be justified by the slow growth rate of the *G. vaginalis*. However, it was plausible to assume that, if given enough time, the smaller inocula could potentially reach the higher levels of biofilm formation. Generally, the density of biofilm increases with prolongation of incubation until an optimal incubation time is reached (Mathur et al., 2006). However, since mature biofilms are known to suffer shedding, by releasing cells to the surrounding environment (Boles et al., 2005; Kaplan et al., 2003), the effect of the incubation time needed to be experimentally assessed. To determine how the incubation period would

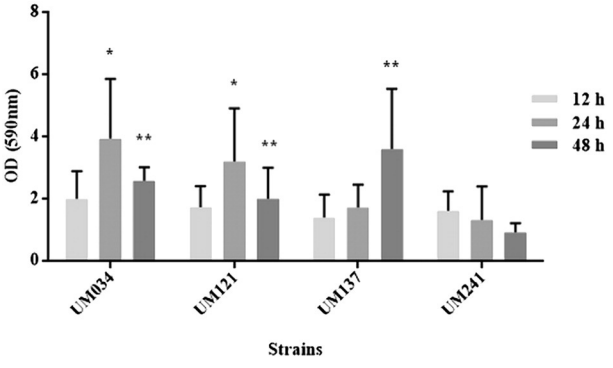

Fig. 2. Influence of incubation time on *G. vaginalis* biofilm formation. Biofilms were grown in sBHI at 37 °C with 10% CO_2 during 12 h, 24 h and 48 h. Statistical differences in the biofilm formation using an incubation time of 12 h and 24 h are marked with * ($P < 0.05$), while statistical differences between 24 h and 48 h are marked with ** ($P < 0.05$).

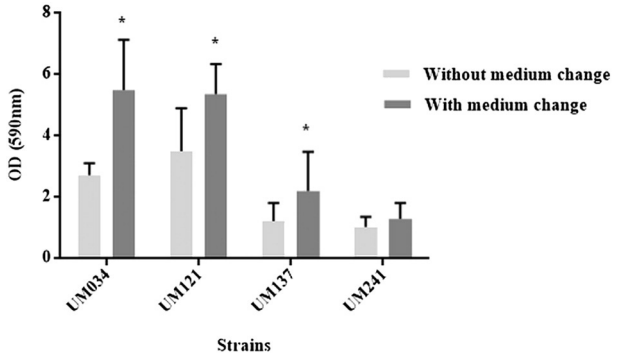

Fig. 3. Influence of feeding conditions on *G. vaginalis* biofilm formation. Biofilms were grown in sBHI at 37 °C with 10% CO_2 during 48 h. The culture medium change was performed after 24 h of incubation. Statistical differences in the biofilm formation with or without medium change are marked with * ($P < 0.05$).

influence the biofilm accumulation, we inoculated the 10^8 CFU/mL bacterial suspension in the 96-well microplates and the plates were incubated during 12 h, 24 h and 48 h. Interestingly, strain to strain variability, with opposite trends, was detected in biofilm-forming ability, over time. As can be seen in Fig. 2, strain UM 241 did not significantly changed the biofilm formation (Wilcoxon test; $P > 0.05$), contrary to strain UM 137 that showed a significant increase in biofilm formation at 48 h (Wilcoxon test, $P < 0.05$). On the other hand, strains UM034 and UM121 increased biofilm biomass from 12 h to 24 h, but then a reduction of biomass was detected, from 24 h to 48 h. This suggested that, in these strains, nutrient depletion or waste-product accumulation, over time, could be affecting biofilm accumulation (Delaquis et al., 1989; Sawyer and Hermanowicz, 2000).

3.2. Influence of feeding conditions on G. vaginalis biofilm formation

Next, we tested whether changing the culture media, after 24 h of growth, would enhance the biomass at 48 h biofilms, particularly in strains UM034 and UM121. As shown in Fig. 3, this approach allowed an increase in biofilm biomass on those strains. These results are in agreement with previous reports that demonstrated that the fed-batch growth was a favourable culture condition for biofilm formation in many other species (Cerca et al., 2004; Pongtharangkul and Demirci, 2006; Rodrigues et al., 2009).

3.3. Influence of culture medium carbohydrate source on G. vaginalis biofilm formation

Despite all the tested incubation conditions, we still observed that biofilm formation by strain UM 241 was not being affected. Probably one of the most important factors is the culture medium composition. We used sBHI as the base medium, since this was previously shown to be a good media for many BV-associated bacteria (Alves et al., 2014). However, it has been described that the biofilm formation is widely affected by the presence of certain carbon substrates in the culture medium (Kalai Chelvam et al., 2015; Rinaudi et al., 2006). In a detailed biochemical profile study using 78 *G. vaginalis* strains, more than 97% of the tested strains were able to metabolize glucose, dextrin, maltose and ribose (Greenwood and Pickett, 1979). In this sense we evaluated the effect of supplementation of sBHI with 0.25% (wt/v) of the mentioned carbohydrates on *G. vaginalis* biofilm formation. Interestingly, as can be seen in Fig. 4, maltose was the only carbohydrate that enhanced biofilm formation in all tested strains, while ribose had the opposite effect. Glucose and dextrin enhanced biofilm formation of some strains, but generally to a lesser extent than maltose. Either glucose, dextrin or maltose has been associated with increasing the amount of biofilm formed by several bacterial species, such as *Listeria monocytogenes* (Pan et al., 2010), *Salmonella enterica* (Kalai Chelvam et al., 2015) and *Enterococcus faecalis* (Creti et al., 2006). In contrast, the presence of ribose in a culture medium was related with a decrease of biofilm formation by *Streptococcus mutans*, *Streptococcus sobrinus* (Lee et al., 2015) and *Aggregatibacter actinomycetemcomitans* (Shao et al., 2007). Ribose inhibits biofilm formation through interrupting bacterial quorum sensing, due to its structural similarity with auto-inducer 2 (AI-2), since it bears a furanosyl borate diester form (Cao and Meighen, 1989). Previous data showed that ribose compete with AI-2 for binding to RbsB (ribose binding subunit B, one of the subunits of the ribose ABC transporter) and subsequently interfere AI-2-dependent phenotypes such as biofilm formation in *A. actinomycetemcomitans* (Shao et al., 2007). However, up to now, no information is available about the possible role of AI-2 in *G. vaginalis* biofilm formation.

4. Conclusion

This is the first study to quantify *in vitro* biofilm formation by clinical *G. vaginalis* strains. While previous studies used glucose as a biofilm inducer in *G. vaginalis*, our data shows that maltose is a preferable source of carbohydrates. Furthermore, a fed-batch system allows for thicker biofilms to be formed, as compared with batch.

However, it is doubtful that these optimized *in vitro* conditions can mimic the *in vivo* phenomena. Even so, the optimization of biofilm formation seems reasonable for both screening and fundamental studies, in order to better study this type of bacterial community.

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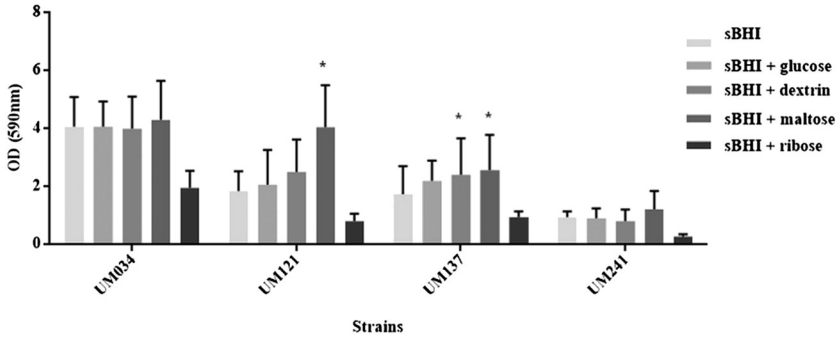

Fig. 4. Influence of culture medium composition on *G. vaginalis* biofilm formation. Biofilms were grown in sBHI and sBHI supplemented with 0.25% (wt/v) of each carbohydrate: glucose, dextrin, maltose or ribose and the microplates were incubated at 37 °C with 10% CO_2 during 24 h. Statistical differences in the biofilm formation using an sBHI medium and sBHI supplemented with each carbohydrate are marked with * ($P < 0.05$).

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