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Interaction between atypical microorganisms and *E. coli* in catheter-associated urinary tract biofilms

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Abstract

Most biofilms involved in catheter-associated urinary tract infections (CAUTIs) are polymicrobial, with disease causing (e.g. *Escherichia coli*) and atypical microorganisms (e.g. *Delftia tsuruhatensis*) frequently co-inhabiting the same catheter. Nevertheless, there is a lack of knowledge about the role of atypical microorganisms. Here, single- and dual-species biofilms consisting of *E. coli* and atypical bacteria (*D. tsuruhatensis*, *Achromobacter xylosoxidans*), were evaluated. All species were good biofilm producers (Log 5.84-7.25 CFUs cm-2 at 192 h) in artificial urine. The ability of atypical species to form biofilm appears to be hampered by the presence of *E. coli*. Additionally, when *E. coli* was added to a pre-formed biofilm of the atypical species, it seemed to take advantage of the first colonizers to accelerate adhesion, even when added at lower concentrations. Results suggest a greater ability of *E. coli* to form biofilms in conditions mimicking the CAUTIs, whatever the pre-existing microbiota and the inoculum concentration.

Running title: Catheter-associated urinary tract biofilms.

Keywords: *Escherichia coli*, atypical species, multispecies biofilms, urinary tract infections, urinary catheters.

Introduction

Hospital-acquired (nosocomial) infections are frequently related with biofilms formed in medical devices, such as prosthetic heart valves, cardiac pacemakers, urinary catheters, contact lenses and orthopedic devices (Campoccia et al. 2006; Hall-Stoodley et al. 2004; Morris & Stickler 1998; Silva et al. 2010; Tenke et al. 2006). The higher economic costs associated with these diseases is due to long hospitalization periods for infected patients (Curtis 2008; Ferrieres et al. 2007; Silva et al. 2010). The most common nosocomial infections are urinary tract infections (UTIs) (Klevens et al. 2007) and about 80% of these infections, known as catheter-associated UTIs (CAUTIs), are related to the insertion of catheters in the urinary tract (Curtis 2008; Doyle et al. 2001). These medical devices are used in hospital and nursing home settings to relieve urinary retention and incontinence (Hall-Stoodley et al. 2004). However, in patients with long-term urinary catheters, the infection is inevitable in most of the cases (Jacobsen et al. 2008).

CAUTIs originate from the colonization of the surface of catheters by microorganisms. Indeed, urinary catheters provide an attractive niche for bacterial colonization due to the intermittent flow of warm nutritious urine, leading to the formation and growth of a biofilm (Ganderton et al. 1992). Biofilms have been described as microbial communities attached to a surface and embedded in extracellular polymeric substances (EPS) (Costerton et al. 1987; Stewart & Franklin 2008). In this mode of life, microorganisms can survive in hostile environments, and are protected against external aggressive factors encountered in host tissues (e.g. antibodies, phagocytes, etc.) or other environmentally-challenging conditions (e.g. UV light, extreme temperatures, shear forces, etc.) (Donlan & Costerton 2002). In contrast to their planktonic counterparts, cells in the biofilm microenvironment are typically resistant to antibiotics (Lewis 2007). Consequently, infections on medical devices associated with biofilms are persistent and difficult to eradicate (Costerton 1999).

Recent studies involving urinary catheters have shown that CAUTIs are mostly polymicrobial (Frank et al. 2009; Hola et al. 2010; Macleod & Stickler 2007). The potential pathogens involved in initial adhesion are usually *Staphylococcus epidermidis*, *Escherichia coli* or *Enterococccus faecalis* (Matsukawa et al. 2005); but several others species (such as *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Providencia stuartii* and *Klebsiella pneumoniae)* can appear in the later stages of infection, in conjugation with initial colonizers (Jacobsen et al. 2008; Matsukawa et al. 2005). Furthermore, it was recently observed that these disease causing microorganisms can co-inhabit the catheter surface with other unusual microorganisms with unproven pathogenic potential (e.g.

Delftia tsuruhatensis, *Achromobacter xylosoxidans*) (Frank et al. 2009). While interactions of *E. coli* with other common causes of UTIs, have already been addressed (Jacobsen et al. 2008, Matsukawa et al. 2005, Cerqueira et al. 2013, Ferrieres et al. 2007); there is a lack of knowledge about the possible role that these atypical microorganisms have on the rate at which disease-causing microorganisms adhere and form biofilms and, consequently, their effect on the CAUTIs outcome. In fact, some studies have demonstrated recently, for other pathologies, that the atypical microorganisms could have some important contributions in biofilm infections (Lopes et al. 2014, Lopes et al. 2012). Both disease causing and atypical microorganisms have in common the ability to form mono or multi-species biofilms on the surface of the urinary catheter (Frank et al. 2009), which means that interactions between the different bacterial populations are possible, if not likely. For instance, some of these microorganisms are able to degrade certain components of plastics (Patil et al. 2006; Wan et al. 2007), which means that some products of their metabolism might feed other microorganisms (e.g. *Escherichia coli*) and, eventually, they could act as primary colonizers of the catheter. In opposition, it might be possible that the colonization by these atypical microorganisms can prevent the colonization by pathogenic bacteria. Hence, understanding the role that atypical microorganisms have on biofilm dynamics might be crucial to help in the development of novel strategies to prevent or minimize bacterial adhesion to catheters.

As such, in here we evaluated single-species (*E. coli*, *D. tsuruhatensis*, *A. xylosoxidans*) and dual-species (*E. coli*/*D. tsuruhatensis*, *E. coli*/*A. xylosoxidans*) biofilm formation in 96-well microtiter plates. To better mimic conditions found in urinary catheters, biofilms were formed in artificial urine medium (AUM) (Brooks & Keevil 1997) at 37°C. In order to understand which type of interactions occurs between different species, we compared dual-species biofilms with individual biofilms fitness regarding: total biomass formed, total cells counts and cultivability values. Four additional features were also explored: the growth rates of each microorganism, the siderophore production by *E. coli* and atypical microorganisms, the antimicrobial activity of biofilm supernatants and the influence of a pre-formed biofilm on the adhesion and biofilm formation of a second colonizer.

Materials and Methods

Bacterial maintenance and inoculum preparation

For each experiment, *E. coli* CECT 434, *A. xylosoxidans* B3*, D. tsuruhatensis* BM90 were streaked from a frozen stock (-80°C) on Tryptic Soy Agar (TSA) (Merk, Germany) and grown overnight at 37°C. Subsequently, colonies from each species were used to inoculate 75 ml of AUM. The cultures were incubated overnight (16-18 hours) at 37°C, under agitation (150 rpm). Cell concentration was then assessed by optical density (O.D.) at 620 nm, and the inoculum was diluted in AUM in order to obtain a final concentration of 10^8 CFUs ml^{-1} or 10^2 CFUs ml^{-1}. AUM was prepared as previously described (Brooks & Keevil 1997), using the following formulation in one litre of distilled water: peptone 1 g (Merck, Germany), yeast extract 0.05 g (Liofilchem, Italy), lactic acid 1.1 mmol l^{-1} (Fluka), citric acid 0.4 g (VWR, Belgium), sodium bicarbonate 2.1 g (Merck, Germany), urea 10 g (VWR, Belgium), uric acid 0.07 g (VWR, Belgium), creatinine 0.8 g (Merck, Germany), calcium chloride.$2H_2O$ 0.37 g (Merck, Germany), sodium chloride 5.2 g (Merck, Germany), iron II sulphate.$7H_2O$ 0.0012 g (Merck, Germany), magnesium sulphate.$7H_2O$ 0.49 g (Merck, Germany), sodium sulphate.$10H_2O$ 3.2 g (Merck, Germany), potassium dihydrogen phosphate 0.95 g (Merck, Germany), di-potassium hydrogen phosphate 1.2 g (Merck) and ammonium chloride 1.3 g (Merck, Germany) (pH was adjusted to 6.5).

Monospecies and multispecies biofilms (*E. coli* CECT 434/ *A. xylosoxidans* B3; *E. coli* CECT 434/ *D. tsuruhatensis* BM90) were formed as described below.

Biofilm formation assays

First, single-species biofilms were formed to study the biofilm-forming ability of each species. For this, 200 µl of each inoculum in AUM (10^8 CFUs ml^{-1} of initial concentration) were transferred into each well of a 96-well tissue culture plate (Orange Scientific, Braine-l'Alleud, Belgium). An additional experiment at an initial inoculum concentration of 10^6 CFUs ml^{-1} was performed in order to evaluate the influence of initial inoculation level on the biofilm formation of the three species under study (results are presented in supplemental material).

In order to understand how *E. coli* biofilm-formation is affected in the presence of the atypical microorganisms, a total of 3 species combinations (*E. coli*/ *A. xylosoxidans*; *E. coli*/ *D. tsuruhatensis*) at the same initial concentration (10^8 CFUs ml^{-1}) were also studied. For dual-species biofilms, equal volumes of each single culture (100 µl) at an initial concentration of 2 x 10^8 CFUs ml^{-1} were used.

Tissue culture plates were then placed in an incubator (FOC 225I - VELP Scientifica, Italy) at 37°C, under static conditions, during 8 days. Every 48 h the medium was carefully replaced by fresh AUM. Wells containing sterile AUM were used as a control. These assays were performed in triplicate.

In order to test how a pre-formed single-species biofilm affects the subsequent adhesion of a second colonizer, pre-colonization experiments were performed.

(i) Pre-colonization with atypical microorganisms: Wells of a 96-well tissue culture plate were pre-colonized with atypical microorganisms (initial concentration of 10^8 CFUs ml^{-1}). After 24 h, the medium was removed, biofilm was washed twice with sterile saline solution 0.85% (v/v) and 200 µl of *E. coli* suspension (initial inoculum concentration of 10^2 CFUs ml^{-1}) were added. The same assay was performed but with initial concentrations of 10^2 CFUs ml^{-1} for the atypical microorganisms and 10^8 CFUs ml^{-1} for *E. coli*.

(ii) Pre-colonization with *E. coli*: The experiments described in i) were repeated but microorganisms were added in reverse sequence.

(iii) Single-species biofilms: Single-species biofilms were developed to study biofilm-forming ability at low initial concentration (10^2 CFUs ml^{-1}). These assays were used as controls to compare the results obtained in biofilm experiments i) and ii).

At selected time points (24 h, 48 h, 96 h and 192 h), formation of single and dual-species biofilms was assessed by CV (crystal violet) staining (for quantification of biomass formed), CFUs (colony forming units) counts (for cultivable cells counts) and DAPI (4'-6-Diamidino-2-phenylindole) staining (for total cells counts), as described below.

Cultivability assessment

The number of cultivable biofilm cells was determined by CFUs. Briefly, at each time point the biofilm was washed twice in 0.85% (v/v) sterile saline to remove loosely attached cells. Subsequently, 200 µl of 0.85% (v/v) sterile saline were transferred into each well of a 96-well plate. Biofilm was sonicated during 4 min (70 W, 35 kHz, Ultrasonic Bath T420, Elma, Germany) and then resuspended by pipetting up and down three times. The sonication step was previously optimized to ensure that all cells were detached from the wells of the microtiter plate, while avoiding cell disruption (data not

shown). Subsequently, 100 µl of the disrupted biofilm were serially diluted (1:10) in saline solution, and plated in triplicate on TSA. The plates were incubated at 37°C for 12-16 h (*E. coli*), 24 h (*D. tsuruhatensis*) and 48 h (*A. xylosoxidans*). For dual-species biofilms, different selective agar media were used for a better discrimination between the two species. MacConkey agar (Liofilchem, Italy) was used to assess *E. coli* counts. MacConkey agar is a selective/differential medium, based on lactose fermentation, commonly used to discriminate *Enterobacteriaceae*. *D. tsuruhatensis* and *A. xylosoxidans* presented a slow growth in this medium, but were easily distinguished due to their non-lactose fermenting phenotype. The other media used include: Cetrimide agar (Liofilchem, Italy) for *A. xylodoxidans* and Simmons Citrate agar (ammonium dihydrogen phosphate 1 g l^{-1} [Merck, Germany]; di-potassium hydrogen phosphate 1 g l^{-1} [Merck, Germany]; sodium chloride 5 g l^{-1} [Merck, Germany]; tri-sodium citrate 2 g l^{-1} [Sigma, USA]; magnesium sulfate 0.2 g l^{-1} [Merck, Germany]; bromothymol blue 0.08 g l^{-1} [Sigma, USA]; agar 13 g l^{-1} [Merck, Germany]) for *D. tsuruhatensis* discrimination. None of these two media were able to recover *E. coli* cells. Afterwards, selective agar plates were incubated at 37°C during 12-16 h (*E. coli*), 48 h (*A. xylosoxidans*) and 72 h (*D. tsuruhatensis*). The number of cultivable bacterial cells in biofilms was determined and expressed per area of well in contact with AUM (Log CFU cm^{-2}).

As a control test, the selective medium recovery capacity for each microorganism was compared with TSA. With this purpose, one of the experiments in pure culture for each species was performed in the corresponding selective/differential medium and in TSA. No significant differences between the CFU counts in TSA and in the selective/differential media used, were found (data not shown).

Biomass quantification by the CV Assay

Biomass of single and dual-species biofilms was quantified by the CV staining method (Stepanovic et al. 2000). Briefly, the washed biofilm was fixed with 250 µl of 99% (v/v) ethanol for 15 min. Subsequently, ethanol was removed and plates were allowed to air-dry. Then, fixed biofilms were stained with 250 µl of CV (Merck, Germany) for 5 min. The wells were then washed three times with water. The plates were air dried and the dye bound to the adherent cells was resuspended by adding 200 µl of 33% (v/v) glacial acetic acid (Merck, Germany). Finally, plates were placed in agitation up to two minutes and

the O.D. measured at 570 nm using a microtiter plate reader (Spectra Max M2, Molecular Devices).

DAPI staining

To assess total bacteria cell counts in single and dual-species biofilms, 100 µl of the sonicated cell suspensions were filtered in a black Nucleopore polycarbonate membrane (Ø 25 mm) with a pore size of 0.2 µm (Whatman, Japan). Subsequently the membrane was stained with DAPI (0.2 mg ml^{-1}) (Merk, Germany) and let for 10 min in the dark. Then, the membrane was placed in a microscope slide. Finally, a drop of immersion oil (Merk, Germany) was added and the membrane covered with a coverslip. Cells were analysed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems GmbHy, Germany). The optical filter combination for optimal viewing of stained preparations (Chroma 61000-V2), consisted of a 545/30 nm excitation filter combined with a dichromatic mirror at 565 nm and suppression filter 610/75. For image capture, Leica IM50 Image Manager, was used. For each sample, a total of 15 fields with an area of 6.03×10^{-5} cm^2 were counted and the average was used to calculate the total cells per cm^2.

Determination of bacterial growth rates

The growth rate for each species at 37°C on AUM was determined. For this, cells were grown overnight (16-18 hours). Subsequently, cells were diluted in order to obtain a final O.D. (620 nm) of 0.1, incubated at 37°C, 150 rpm. O.D. at 620nm was measured at different time points until the stationary stage was reached.

Siderophores production

Siderophore production by the studied microorganisms was assessed by using the chrome azurol S (CAS) solid medium assay, prepared as described by Schwyn and Neilands (Schwyn & Neilands 1987). Following incubation at 37°C for 24 h, plates were analysed for the presence of growth and orange halos.

Antimicrobial activity of biofilm supernatants

The presence of antimicrobial activity on biofilm supernatants was assessed on lawns of *E. coli* and the two species of atypical microorganisms. In order to collect the biofilm supernatants, single- and dual-species biofilms were prepared according to the methodology described above. After 72 h, supernatants were recovered, filtered (0.22 µm filter, Frilabo, Portugal) and kept at -20°C. In order to test for possible contaminations, 10 µl of these supernatants were placed on TSA for 24 h at 37°C. Lawns of each of the microorganisms were laid onto TSA, using cotton swabs and allowed to air-dry. Then, 10 µl of each supernatant were applied onto the lawns, and left to air-dry. Afterwards, the plates were incubated for 24 h at 37°C. The formation of halos is indicative of the presence of antimicrobial activity.

Determination of the fitness and malthusian parameter

The fitness of *E. coli* relative to the atypical species ($W_{E.\ coli}$), determined for each dual-species biofilms, was estimated as the ratio of the malthusian parameters (m) of each population (Lenski et al. 1991). The malthusian parameter is defined as the average rate of increase and was calculated for both species over the time,

$$m = \ln [N (t_{final})/ N (t_{initial})] / t_{final} \quad (1)$$

where N is the value of CFUs cm^{-2} present in the biofilm at initial time and final time points. The relative fitness of *E. coli* was determined as,

$$W_{E.\ coli} = m_{E.\ coli}/m_{atypical\ species} \quad (2)$$

resulting in a fitness of 1 when competing species are equally fit.

For pre-colonization experiments, in order to understand the effect of a pre-colonized surface on the initial adhesion of a second species, the malthusian parameter of the added microorganism was calculated after 48 hours of its addition.

Statistical analysis and data accommodation

Results were compared using One-Way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software (SPSS - Statistical Package for the Social Sciences, Chicago, USA). All tests were performed with a confidence level of 95%. All raw data derived from this study are stored at the BiofOmics platform (http://biofomics.org) (Lourenco et al. 2012).

Results and discussion

Single- and dual-species biofilm experiments

It is now known that CAUTIs-associated biofilms often involve more than one microbial species, causing what can be defined as a polymicrobial disease (Frank et al. 2009; Hola et al. 2010; Macleod & Stickler 2007). As *E. coli* is one of the main pathogens involved in these infections (Hedlund et al. 2001; Niveditha et al. 2012; Ronald 2002; Svanborg & Godaly 1997), it would be expectable that the biofilm forming ability of this microorganism would surpass the ones exhibited by atypical microorganisms. Actually, the assessment of the bacteria growth rate in AUM has shown that atypical microorganisms were found to be slow-growing (values of growth rates: 0.374 h^{-1} for *D. tsuruhatensis*; 0.3107 h^{-1} for *A. xylosoxidans*) when compared to *E. coli* (0.4838 h^{-1}).

Interestingly, this behavior was not observed for single-species biofilms, either in terms of biofilm biomass (Figure 1a), cultivable cells (Figure 1b) or total cells (Supplemental material - Figure S1). In fact, higher biomass values were observed for *A. xylosoxidans*, which reached an O.D. of ~5 at 192 h, when compared with *D. tsuruhatensis* and *E. coli* (O.D. ~1.4 and ~2.4 at 192 h, respectively). Moreover, for *A. xylosoxidans* values, this biomass difference was statistically significant at 192h ($p<0.05$).

Regarding cultivability, no significant differences were found for *E. coli*, *A. xylosoxidans* or *D. tsuruhatensis*, with CFU counts ranging between Log 6.61 and Log 7.25 CFUs cm^{-2} ($p>0.05$) (Figure 1b).

All species presented similar values for total cells for the different time points (between Log 6.76 and Log 7.50 cells cm^{-2}) (Supplemental material - Figure S1) and, as expected, the CFU counts were always lower than the DAPI counts. In general, the averages of cells detected by cultivability (Figure 1c) for *E. coli* and *A. xylosoxidans* were high, but for *A. xylosoxidans*, the loss of cultivability was observed over time (77.2% at 24 h *versus* 36.6% at 192 h, $p>0.05$). For *D. tsuruhatensis*, the obtained percentages were always lower than those observed in the other two species (34.2% at 192 h, $p>0.05$).

Regarding the species interaction in dual-species biofilms, to better summarize the results (Supplemental material - Figure S2) and visualize the influence of the atypical species in *E. coli* biofilm formation, we have determined the relative fitness of *E. coli* in dual-species biofilms (Figure 2). In the presence of *D. tsuruhatensis* and *A. xylosoxidans*, the fitness of *E. coli* slightly increases over the time, reaching a value of 1.12 ($p<0.05$) and 1.07,

respectively, after 192 hours. These conclusions can be observed in more detail in the CV and cultivability graphs (Supplemental material - Figure S2). CV assays for dual-species biofilms show that when *E. coli* is co-cultured with the atypical microorganisms the total biomass profiles tend to be more similar to the one of *E. coli* single-species biofilm (Figure S2a-b). In fact, the data of cultivability assays in dual-species biofilms confirm that *E. coli* ability to form biofilms does not seem to be influenced by the presence of the other species (Figure S2e-f). In addition, dual-species biofilms present similar values for total cells for the different time points (Supplemental material - Figure S3) and, as expected, the CFU counts were always lower than the DAPI counts. However, it should be mentioned that the observations here described might be limited to the inoculum concentrations used in this study. To clarify this issue, the influence of initial inoculation level (10^6 CFUs ml^{-1} *vs*. 10^8 CFUs ml^{-1}) on the biofilm formation, was evaluated (Supplemental material - Figure S4). No significant differences were found for *E. coli* (p>0.05 for each point); which indicates that initial inoculum concentration does not seem to have influence in *E. coli* attachment and accumulation over time (Figure S4a). On the other hand, for *D. tsuruhatensis* and *A. xylosoxidans* single-species biofilm formed at an initial concentration of 10^6 CFUs ml^{-1}, lower cultivability values were observed for up to 24 h and up to 48 h, respectively. It reflects a delay on its biofilm formation. However, it does not affect the final biofilm concentration, which reached the same values (Supplemental material - Figure S4b-c).

It is well known that, in multispecies biofilms, the interactions may encourage the coexistence (synergistic interaction) or confer advantage to one species, inhibiting the growth of others (antagonistic interaction) (Elias & Banin 2012; Harrison 2007; Hibbing et al. 2010). In order to explain the possible interaction between *E. coli* and atypical species in dual-species biofilm, four additional features were analyzed: antimicrobial activity of biofilm supernatants in single- and dual-species biofilms, siderophores production, growth rate of each species and effect of a pre-formed biofilm on *E. coli* biofilm formation.

Antimicrobial activity of biofilm supernatants and siderophores production

An important factor in determining the dominant species within a mixed biofilm is the antimicrobial compounds production, which might provide an advantage to the producer species by interfering or killing the neighbor microorganisms (Hibbing et al. 2010). However, in the present work, examination of antimicrobial compounds in biofilm

supernatants, either from single- or dual-species biofilms, suggested that none of the microorganisms secreted compounds able to clearly influence the growth of the others. It might be possible that antimicrobial compounds are present in very low concentrations, as it usually happens for most part of secondary metabolites; which would also appears as a negative result. Also, some other molecules, that interfere with non-essential processes (e.g. quorum sensing molecules), are not detected in this type of assay. Nonetheless, the complete absence of any inhibitory signal suggests that the observed decrease of the atypical microorganisms when co-cultured with *E. coli,* is probably not due to the production of antimicrobial compounds by *E. coli*.

Other type of competitive interaction can be observed in mixed biofilms, in which one microorganism can sequestrate a limited and essential nutrient, facilitating its dominance over the other species (Hibbing et al. 2010). An example of this competitive behavior involves the iron sequestration by the production, release and uptake of siderophores (Griffin et al. 2004; Hibbing et al. 2010; Joshi et al. 2006; Smith et al. 2006; Weaver & Kolter 2004). Siderophores are molecules secreted under low iron availability and are used by microorganisms to sequester the iron available in the medium (Andrews et al. 2003; Hibbing et al. 2010; Ratledge & Dover 2000). The importance of iron acquisition has been reported for the survival of uropathogenic *E. coli* during CAUTIs development (Jacobsen et al. 2008; Snyder et al. 2004). Considering the low iron concentration in urine and its importance for the microorganisms growth and survival during CAUTIs (Jacobsen et al. 2008; Shand et al. 1985), this nutrient is expected to be consumed by microorganisms with high ability to produce or utilize siderophores, limiting it to the other microorganisms. CAS agar results indicated that *E. coli* produces high levels of siderophores. *A. xylosoxidans* and *D. tusuruhatensis* produced siderophores at lower levels (Figure 3). Thereby, when *E. coli* is co-cultured with these atypical species in AUM it can sequestrate, at a higher extent, iron molecules providing this microorganism with an advantage in iron-depleted conditions, such as the CAUTIs.

Pre-colonization assays

Dual-species biofilm experiments suggested that *E. coli* predominates over the co-cultured species. However, nothing is known about the ability of this bacterium to adhere to a pre-colonized surface by the two atypical species. To confirm whether *A. xylosoxidans* or *D. tusuruhatensis* biofilms affect *E. coli* colonization, 24 h biofilms of *A. xylosoxidans* or *D. tusuruhatensis* were formed and then *E. coli* was added. These

experiments were performed with different inoculum concentrations (10^8 CFU ml^{-1} and 10^2 CFU ml^{-1}) to see if the inoculation level has influence in the adhesion of a second species to the biofilm.

The addition of *E. coli* to a pre-formed biofilm does not lead to significant changes of total biomass compared to experiments with synchronized addition of species (Supplemental material - Figure S5). When *E. coli* is added to 24 h biofilms of the atypical microorganisms, the biomass profile is similar to experiments with synchronized addition of species, no matter the inoculation proportion between the two species. Also, in dual-species biofilms, the concentration of initial inoculum does not seem to have a great influence on biomass production over time (Supplemental material - Figure S5 and S6).

To better understand the possible role that a pre-colonized surface has on the rate at which species adhere and grow, the malthusian parameter of the microorganism added to a pre-formed biofilm was determined. This parameter reflects the average rate of increase of each species (Lenski et al. 1991).

Interestingly, the results show that when a low initial concentration (10^2 CFUs ml^{-1}) of any of the microorganisms is added to a pre-formed biofilm, the population of this species increases more rapidly when compared with the corresponding single-species biofilm (Figure 4 and Supplemental material - Figures S7 and S8). Several biofilms found in both environmental and clinical settings are recognized as multispecies structures (Hall-Stoodley et al. 2004), and this fact suggests that this diversity provides some advantages for these communities. In fact, it is known that diversity generally protects communities from unstable environmental conditions and, thus, it is likely that bacteria favor the development of multispecies structures (Donlan & Costerton 2002). Why the apparent induction of multispecies populations happens in the specific case of our study remains unclear. It may be, for instance, that the maintenance of atypical species, even in low densities, might be beneficial for *E. coli* if any environmentally-challenging condition occurs. Alternatively, or in addition, some of these atypical microorganisms are able to degrade certain components of plastics (Patil et al. 2006; Wan et al. 2007), which means that the products of their metabolism might be able to feed *E. coli*, explaining why *E. coli* benefit when is co-cultured with atypical microorganisms. However, despite the suitability of the 96-well microtiter plates to simulate the conditions found in catheter-associated urinary tract biofilms (Moreira et al. 2013), the results of the present work should be replicated using catheter-like materials (eg silicones, latex rubber, etc.). This

would allow to confirm if these atypical microorganisms are able to degrade certain components of catheters under conditions found in biofilms associated with CAUTIs.

Taken together, this data seems to indicate that species behavior in dual-species biofilm is also dependent on the population size and physical space available. When cellular concentrations in biofilm are low, competition was not observed; instead, species might benefit from the presence of another colonizer (Figure 4). In fact, the adhesion of a second colonizer added at low concentration was accelerated. In opposition, when cellular concentrations reach higher values, the population of atypical species slight decreases (Supplemental material - Figure S2 c and d; and Figure S7 d), which suggests that competition has taken place.

A good example of multispecies biofilm advantages is provided in the work of Lopes *et al*. (2012). They have studied the role of two novel microorganisms isolated from cystic fibrosis specimens. When *P. aeruginosa* was co-cultured with atypical microorganisms (*Inquilinus limosus* and *Dolosigranulum pigrum*), an increase in the tolerance of the dual-species biofilms to most antibiotics was observed (Oliveira et al. 2012). In another study, Sibley *et al*. (2008) reported that an avirulent species in combination with *P. aeruginosa* isolated from cystic fibrosis flora has the ability to enhance the pathogenicity of this microorganism and, consequently, to influence the outcome of the infection (Sibley et al. 2008). In addition, other studies also reported the importance of atypical pathogens (eg *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *A. xylosoxidans*) in clinical outcome of cystic fibrosis (de Vrankrijker et al. 2010; Waters 2012).

Concerning the *D. tusuruhatensis* and *A. xylosoxidans*, they have been isolated from diverse clinical sources (Amoureux et al. 2012; Ciofu et al. 2013; Duggan et al. 1996; Igra-Siegman et al. 1980; Lambiase et al. 2011; Preiswerk et al. 2011; Waters 2012), including CAUTIs (Frank et al. 2009). Thus, it is expected that these unusual species interact with the disease causing agents and have an important role on biofilm architecture and physiology.

Conclusions and future work

By combining the results obtained in this work, a schematic representation of the dual-species biofilm formation showing the main factors involved on the predominance and coexistence of *E. coli* with atypical species is proposed (Figure 5).

E. coli presented a greater ability to form biofilm in conditions mimicking catheter-associated urinary tract infections, whatever the pre-existing microbiota, which helps explain the high prevalence of *E. coli* in CAUTIs. Nonetheless, despite the probable non-pathogenic nature of the two atypical species, they were also good biofilm producers on abiotic surfaces. Additionally, *E. coli* coexistence with the two atypical species within dual-species biofilm structures was proved; and, actually, pre-colonization with these species seems to promote the pathogen adhesion.

Results also suggest that species behavior in dual-species biofilm might be dependent on the population size and space to grow. Since diversity within the biofilm population usually represents higher chances to persist in detrimental conditions, coexistence seems to be preferred. But, for mature stages of biofilm formation, competition might take place and then the higher fitness of *E. coli* in this environment becomes evident. In fact, the high *E. coli* rate growth in AUM, in association with high levels of siderophores production, helps explaining the *E. coli* ability to outcompete atypical species.

In the future, further insights into the resistance profile of these structures might provide an adequate treatment for each patient with an accurate selection of antibiotic and dosage necessary to treat a particular infection originated from a mixed biofilm (Frank et al. 2009).

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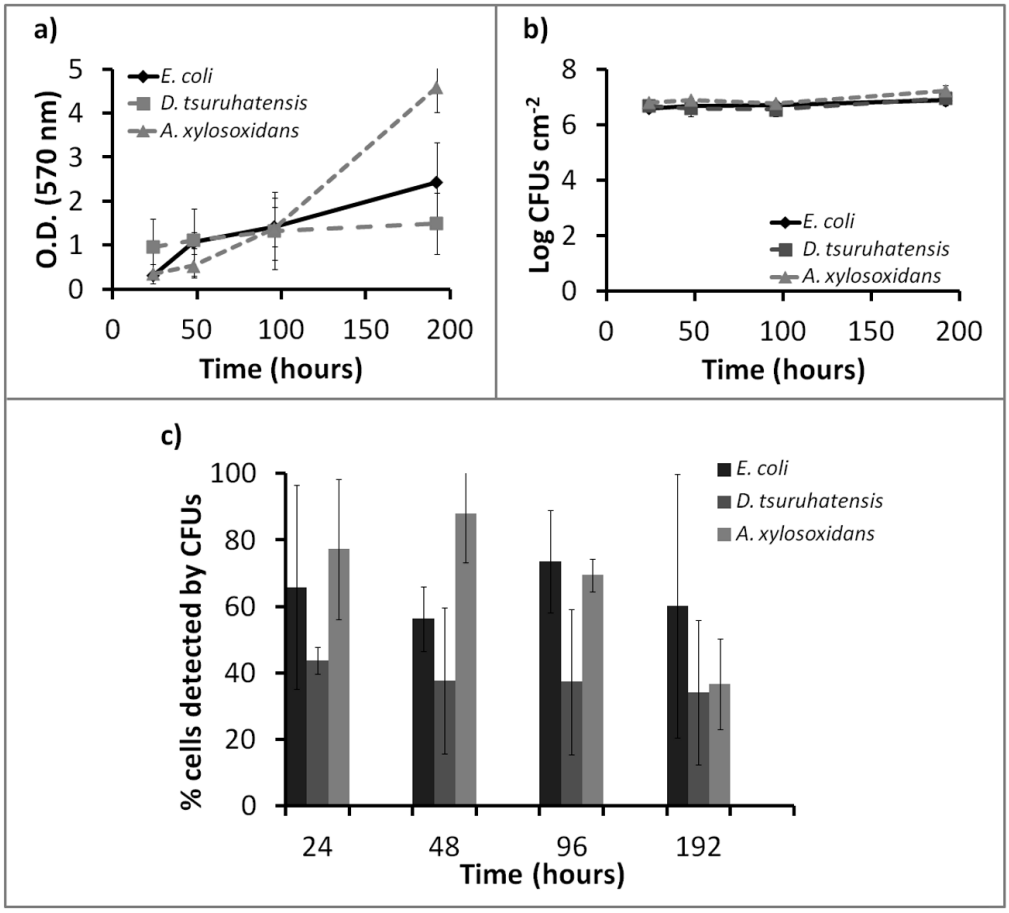

Figure 1. Biofilm formation for single-species biofilms. Values for total biomass (a), cultivability (b) and percentages of cells detected by cultivability (c) are presented for all species. Three independent experiments were performed for each condition. Error bars represent standard deviation.

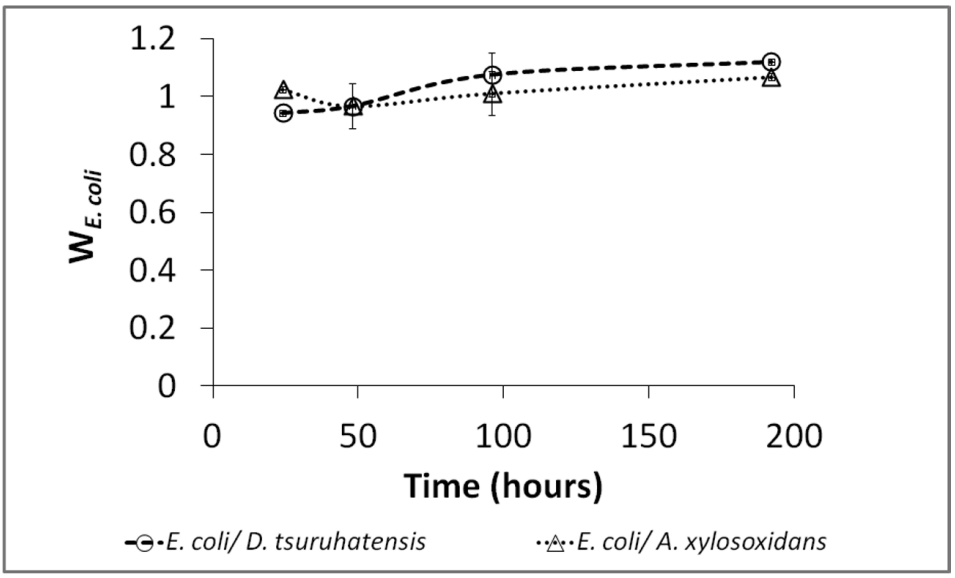

Figure 2. Relative fitness of *E. coli* in dual-species biofilms. Fitness of *E. coli* was determined in the presence of atypical species (*D. tsuruhatensis* and *A. xylosoxidans*) with simultaneous addition of the bacteria at the same initial concentration (10^8 CFUs ml^{-1}). Data are means of three independent experiments and error bars represent standard deviation.

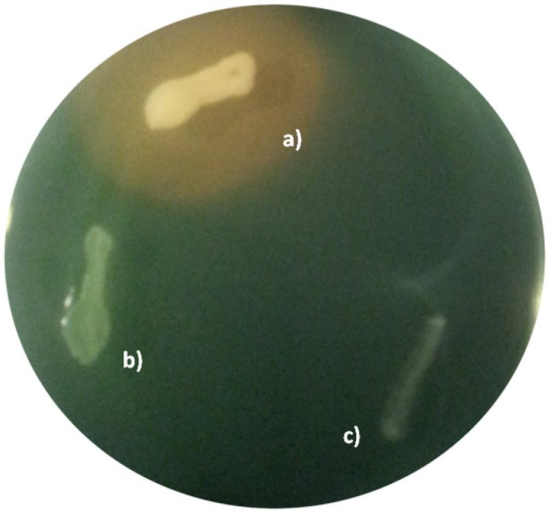

Figure 3. Screening for siderophores production using CAS agar plates. On CAS agar, an orange halo surrounding the colony indicates that *E. coli* produces high levels of siderophores (a). The presence of growth without an orange halo indicates that *D. tusuruhatensis* (b) and *A. xylosoxidans* (c) produce siderophores at less extent.

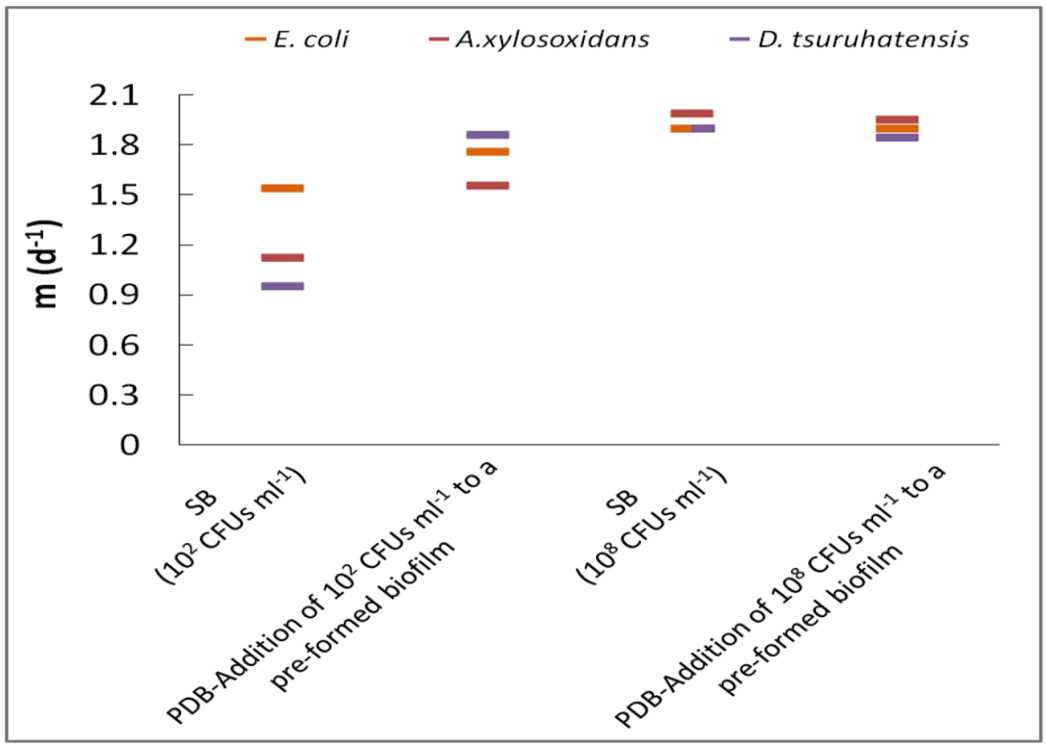

Figure 4. Values of the malthusian parameter for pre-colonization experiments. The malthusian parameter of the second microorganism added to a pre-formed biofilm was determined between time 0 and 48 hours. Values of the malthusian parameter for single-species biofilm of each microorganism was determined for comparative purpose. SB – Single-species Biofilm; PDB – Pre-colonization Dual-species Biofilm.

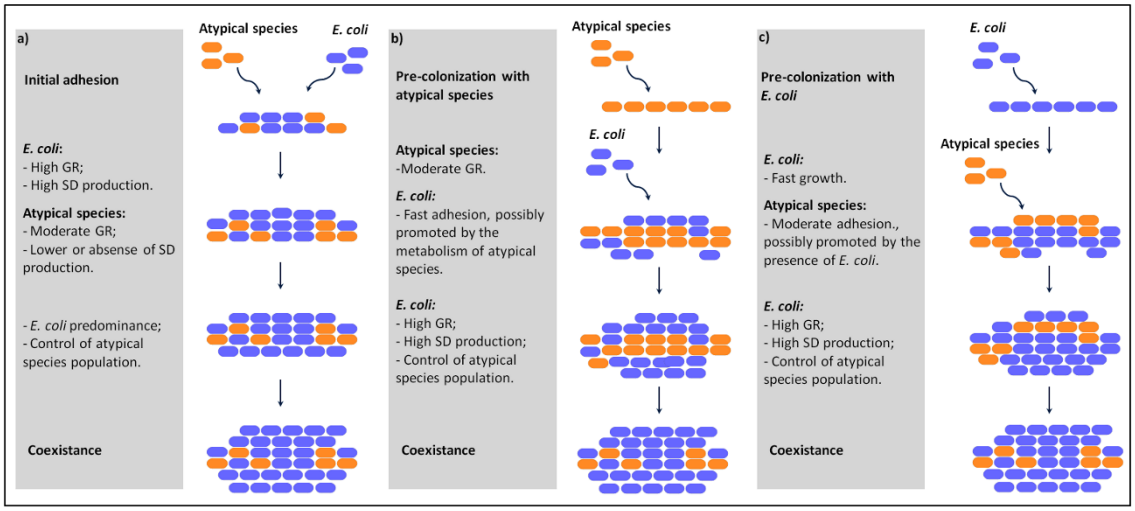

Figure 5. Schematic representation of the dual-species biofilm formation showing the main factors involved on the predominance and coexistence of *E. coli* with atypical species. Representation of the interaction between *E. coli* and atypical microorganisms in dual-species biofilm with simultaneous addition of the species (a); in dual-species biofilm subjected to a pre-colonization step with the atypical species, followed by the addition of the *E. coli* (b); and in dual-species biofilms subjected to a pre-colonization step with the *E. coli*, followed by the addition of the atypical species (c). Regardless of the initial conditions, the dual-species biofilm tends to a final state of coexistence where *E. coli* predominates over the atypical species (GR – growth rate; SD – siderophores).